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DISSERTATION

THIOCARLIDE: A PROMISING ANTITUBERCULOSIS DRUG AND A NOVEL TOOL FOR STUDY OF FATTY ACID AND MYCOLIC ACID BIOSYNTHESIS IN *MYCOBACTERIUM* SPECIES

Submitted by
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In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
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COLORADO STATE UNIVERSITY

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BENJAWAN PHETSUKSIRI ENTITLED THIOCARLIDE: A PROMISING ANTITUBERCULOSIS DRUG AND A NOVEL TOOL FOR STUDY OF FATTY ACID AND MYCOLIC ACID BIOSYNTHESIS IN *MYCOBACTERIUM* SPECIES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF DISSERTATION

THIOCARLIDE: A PROMISING ANTITUBERCULOSIS DRUG AND A NOVEL TOOL FOR STUDY OF FATTY ACID AND MYCOLIC ACID BIOSYNTHESIS IN *MYCOBACTERIUM* SPECIES

This dissertation describes various lessons learned from a study of the effects of thiocarlide (THC) on mycobacteria: the antimycobacterial activity, the mode of action, the gene encoding the enzymatic target, and the characteristics of the target enzyme of THC. Some fundamental aspects of fatty acid and mycolic acid synthesis were unraveled through the use of THC as a study tool.

Thiocarlide (THC), a thiourea, has considerable antimycobacterial activity against various species of mycobacteria with a minimal inhibitory concentration (MIC) of 0.5-2.5 μg/ml on 7H11 agar. When used in a range of 1-10 μg/ml, THC showed effectiveness against various clinical isolates of *M. tuberculosis* which all have differences in drug resistance phenotypes and IS*6110*-based genotypes. In a murine macrophage model, THC exhibited bactericidal activity against viable intracellular *M. tuberculosis* in a dose-dependent manner (0.05-2.5 μg/ml). No acute toxicity was observed against primary macrophage cell cultures as demonstrated by diminution of redox activity in an Alamar Blue assay. A homologous series of thiourea derivatives were synthesized and in an agar proportion assay most derivatives were as effective or more effective than THC.

With the purpose of identification of drug targets, the mode of action of THC was determined. Through the use of [1,2-^{14}C]

acetate whole-cell labeling and analysis by thin layer chromatography, THC was shown to inhibit the synthesis of short-chain fatty acids and all types of mycolic acids in *M. tuberculosis*, *M. bovis* BCG and *M. aurum* A+. Gas chromatographic analysis revealed that THC specifically inhibited oleic ($C_{18:1}$ Δ9) and hence tuberculostearic acid ($brC_{19:0}$) synthesis compensated by a partial increase of stearic acid and other saturated fatty acids ($C_{20:0}$, $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$). The decreased synthesis of oleic acid concomitant with the increase of stearic acid suggests that THC acts by inhibiting Δ9 desaturase. The inhibition of the synthesis of tuberculostearic acid is a direct consequence of the inhibition of oleic acid synthesis, indicating that oleic acid is in turn metabolized to tuberculostearic acid. The selective reduction of oleic acid was dose-dependent and THC completely inhibited oleic acid synthesis at 3 μg/ml. The supplementation of 7H11 agar medium with oleic acid in the form of oleic acid-albumin-dextrose-catalase (OADC) reversed the bactericidal effect of THC, supporting the definitive effect of THC on oleic acid synthesis. Inhibition of oleic acid synthesis was not observed with isoniazid (INH) and ethionamide (ETH), which are inhibitors of mycolic acid biosynthesis, suggesting that the mode of action of THC is unique.

Attempts were made to identify the *M. tuberculosis* gene that encodes the Δ9 desaturase by the strategy of target overexpression. The *M. tuberculosis desA* genes designated *desA1*, *desA2* and *desA3* were cloned and expressed in *M. bovis* BCG. The overexpression of the *desA3* gene from a constitutive promoter and

the use of [1,2-^{14}C]acetate cell labeling revealed an obvious increase of oleic acid synthesis. Immmunoblotting of whole-cells and subcellular fractions of recombinant *M. bovis* BCG expressing His-tagged DesA3 indicates that the product of the *desA3* gene is a membrane-associated protein. By [1,2-^{14}C]acetate whole-cell labeling, it was shown that the Δ9 desaturase activity of the *desA3* gene product was completely inhibited by sterculic acid, a known Δ9 desaturase inhibitor. Unlike THC, sterculic acid did not inhibit mycolic acid synthesis suggesting that oleic and mycolic acid syntheses are not linked and that THC has a second target in the mycolic acid synthesis pathways.

An *in vitro* assay for Δ9 desaturation was developed to demonstrate the enzymatic function of the mycobacterial Δ9 desuturase. A cell lysate fraction of *M. bovis* BCG exhibited the ability to catalyze the desaturation of stearoyl-CoA to the corresponding Δ9 monounsaturated derivative, oleic acid, indicating the presence of the Δ9 desaturase in this strain. The *in vitro* assay also demonstrated that DesA3 is indeed the Δ9 desaturase. In summary, THC shows promise as an effective antituberculosis drug whose mechanism is unique, and the Δ9 desaturase has therapeutic value as a novel target for a new generation of antituberculosis drugs.

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DEDICATION

To my parents who passed on many years ago.

To my family: Paisan, my husband, and Tanachporn Phetsuksiri, my daughter, whose patience, understanding and all their support are behind the work presented in this dissertation.

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LIST OF ABBREVIATIONS

α	alpha
Δ	delta
μ	micro
μg	microgram(s)
μl	microliter(s)
aa	amino acid
A_{600}	light absorbance measured at a wavelength of 600 nm
ACP	acyl carrier protein
ADC	albumin-dextrose-catalase
AG	arabinogalactan
Ara*f*	arabinofuranose
ASM	American Society for Microbiology
bp	base pair(s)
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	Cytosine
°C	degree celcius
CFU	colony forming unit
CHAP	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CoA	Coenzyme A
cpm	counts per minute
2D PAGE	two-dimensional polyacrylamide gel electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleotide-5'-triphosphate
DOTS	direct observed therapy, short-course
EDTA	ethylenediaminetetraacetic acid
EMB	ethambutol
ETH	ethionamide
FAMEs	fatty acid methyl esters
FAS I	fatty acid synthase type I
FAS II	fatty acid synthase type II
G	guanine

g	gram
GAS	glycerol-alanine-salts
GC	gas chromatography
GlcNAC	*N*-acetylglucosamine
h	hour(s)
HEPES	*N*-2-hydroxyethylpiperine-*N'*-2-ethanesulfonic acid
His	Histidine
HIV	Human Immunodeficiency Virus
HPLC	high pressure liquid chromatography
INH	isoniazid
IPTG	isopropyl-β-D-thiogalactopyranoside
ISO	isoxyl
Kan	kanamycin
kb	kilobase(s)
kDa	kilodalton(s)
kv	kilovolt(s)
LAM	lipoarabinomannan
LB	Luria-Bertani
LM	lipomannan
l	liter(s)
M	molar
m	milli
mM	millimolar
mAG	mycolylarabinogalactan complex
mAGP	mycolylarabinogalactan-peptidoglycan complex
MAMEs	mycolic acid methyl esters
MAS	mycolic acid synthase
MDR	multidrug-resistant
mg	milligram
MIC	minimal inhibitory concentration
min	minute
Myc-PL	mycolylphospholipid
NADP	nicotinamide-adenine dinucleotide phosphate
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced form

NAM	nicotinamide
Na	sodium
nm	nanometer(s)
OADC	oleic acid-albumin-dextrose-catalase
OD_{600}	optical density measured at a wavelength of 600 nm
ORF	open reading frame
P60	60% Percoll purified enzyme preparation
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PG	peptidoglycan
PZA	pyrazinamide
Rif	rifampicin, rifampin
RT	room temperature
s	second(s)
SA	sterculic acid
SDS	sodium dodecyl sulfate
Str	streptomycin
TAE	Tris-acetate-EDTA
TB	tuberculosis
TBAH	tetrabutylammonium hydroxide
TBSA	tuberculostearic acid
TCD	thermal conductivity detector
THC	thiocarlide
THD	thiocarbanidin
TMM	trehalose monomycolate
TDM	trehalose dimycolate
TLC	thin layer chromatography
U	unit(s)
v	volt(s)
vol/vol	volume by volume
wt/vol	weight by volume
WHO	World Health Organization
WT	wild-type

Thiocarlide is the generic name of the product. However, the product we used was that generated by Continental Pharma with the trade name Isoxyl®. Both names are used throughout this dissertation.

CHAPTER 1

Literature Review Part I: Mycobacteria and Tuberculosis

1.1 *Mycobacterium spp*

Mycobacteria are Gram positive, acid and alcohol fast, aerobic, non-motile, non-capsulated, non-sporeforming and generally occur as curved or straight rods (Goodfellow and Wayne, 1982). The organisms are classified into the genus *Mycobacterium* which comprises a wide range of species. The minimal standards for including species in this genus include acid alcohol fastness, the presence of mycolic acids containing 60-90 carbon atoms, and a high guanine + cytosine (G+C) content in DNA (61-71%) (Shinnick and Rock, 1994). Currently, there are 71 recognized species of *Mycobacterium* (Table 1.1)(Shinnick and Rock, 1994) that can be divided into two subgroups based on their growth rates and 16S rRNA sequences (Jacobs *et al.*, 1991; Stahl and Urbance, 1990). The fast growers usually require 2-6 days to generate visible colonies on solid media, and have doubling times of 2-3 h. The other group consists of slow growers, which require 14-28 days for the appearance of colonies on complex media and the doubling times are usually in the range of 14-28 h (Runyon *et al.*, 1980).

Based on the life cycles, the genus *Mycobacterium* contains three categories of bacilli: saprophytes, obligate intracellular and opportunistic pathogens. Several of these are soil- or water-saphophytes such as *Mycobacterium smegmatis* and *Mycobacterium gordonae*. A few species, mostly being slow growers, are

Table 1.1 Currently recognized species of the genus *Mycobacterium* (Shinnick and Rock, 1994).

Slow grower	Rapid grower
Pathogenic	Pathogenic
Mycobacterium africanum	*Mycobacterium abscessus*
Mycobacterium asiaticum	*Mycobacterium chelonae*
Mycobacterium avium	*Mycobacterium fortuitum*
Mycobacterium bovis	*Mycobacterium peregrinum*
Mycobacterium celatum	*Mycobacterium porcinum**
*Mycobacterium farcinogenes**	*Mycobacterium senegalense**
Mycobacterium geavense	
Mycobacterium haemophilus	Nonpathogenic
Mycobacterium interjectum	
Mycobacterium intermedium	*Mycobacterium agri*
Mycobacterium intracellulare	*Mycobacterium aichiense*
Mycobacterium kansasii	*Mycobacterium alvei*
Mycobacterium leprae	*Mycobacterium aurum*
*Mycobacterium lepraemurium**	*Mycobacterium austroafricanum*
Mycobacterium malmoense	*Mycobacterium brumae*
Mycobacterium marinum	*Mycobacterium chitae*
*Mycobacterium microti**	*Mycobacterium chubuense*
Mycobacterium paratuberculosis	*Mycobacterium confluentis*
Mycobacterium scrofulaceum	*Mycobacterium diernhoferi*
Mycobacterium shimoidei	*Mycobacterium duvalli*
Mycobacterium simiae	*Mycobacterium fallax*
Mycobacterium szulgai	*Mycobacterium flaveescens*
Mycobacterium tuberculosis	*Mycobacterium gadium*
Mycobacterium ulcerans	*Mycobacterium gilvum*
Mycobacterium xenopi	*Mycobacterium komossense*
	Mycobacterium madagascariense
Nonpathogenic	*Mycobacterium methylovorum*
	Mycobacterium moriokaense
Mycobacterium cookii	*Mycobacterium neoaurum*
Mycobacterium gastri	*Mycobacterium obuense*
Mycobacterium gordonae	*Mycobacterium parafortuitum*
Mycobacterium hiberniae	*Mycobacterium phlei*
Mycobacterium nonchromogenicum	*Mycobacterium poriferae*
Mycobacterium terrae	*Mycobacterium pulveris*
Mycobacterium triviale	*Mycobacterium rhodesiae*
	Mycobacterium shanghaiense
	Mycobacterium smegmatis
	Mycobacterium sphagni
	Mycobacterium thermoresistibile
	Mycobacterium tokaiense
	Mycobacterium vaccae
	Mycobacterium yunnanense

* Pathogenic for animals

considered pathogens capable of producing a spectrum of infections ranging from localized lesions to disseminated disease in humans and animals. Some species cause only human infections but others have been isolated from a wide variety of animals. The obligate pathogenic mycobacteria include *Mycobacterium leprae* discovered in 1873 as a causative agent of leprosy (Hansen, 1880; Bloom and Godal, 1983), *Mycobacterium tuberculosis* isolated as an etiological agent of tuberculosis in 1882 (Koch, 1882) and *Mycobacterium paratuberculosis* identified as a causative agent of Johne's disease in cattle. The third group of mycobacteria is the potentially "opportunistic" pathogenic species such as *Mycobacterium kansasii* and *Mycobacterium avium*. This type of organism is usually found in the environment but occasionally produces human infections.

M. tuberculosis is an important human pathogen. It is a rod-shaped bacillus of 1-4 x 0.3-0.6 μm (Runyon *et al.*, 1974) and survives best in an environment with a pO_2 of 100 to 140 mm Hg, 5% CO_2 and physiologic pH of 7.7 (Grosset, 1980). The organism can also persist for an extended period of time in an oxygen depleted environment by shifting itself into a state of dormancy (Sever and Youmans 1957; Wayne and Diaz, 1967; Wayne and Salkin, 1956). In addition to dormancy, the distinct features of *M. tuberculosis* include slow growth, complex cell envelope, and intracellular pathogenesis (Wheeler and Ratledge, 1994).

M. tuberculosis is a facultative, intracellular bacterial pathogen (Wilson *et al.*, 1999). The tubercle bacilli are spread

from person to person exclusively by small-particle aerosols of sufficiently small size (1-5 μm) such that they are inhaled into the terminal brochioles and alveolar spaces of the lung. The bacilli colonize the walls of pulmonary cavities where oxygen tension is high and the medium is slightly alkaline (Basso and Blanchard, 1998). The inhaled tubercle bacilli are ingested by alveolar macrophages and then either directly killed or grow intracellularly (Dannenberg and Rook, 1994). Typically, the generation time of *M. tuberculosis* in infected hosts is approximately 24 h (Runyon *et al.*, 1980). The slow growth rate contributes to the chronic nature of tuberculosis, which requires a long period for treatment. The intracellular replication of bacilli results in dilation of the capillaries, followed by migration of polymorphonuclear leukocytes and macrophages to the infected areas (Dannenberg and Rook, 1994). The lymphocytes and activated microphages mediate the killing of most bacilli and the walling-off of the infection site (Bloom and Murray, 1992). As immunity wanes due to aging or immune suppression, these dormant but viable bacilli reactivate and develop into an active state which is likely to occur in about 10% of patient's lifetime after the initial infection (Styblo, 1989). In active tuberculosis, the bacilli spread from the initial site of infection throughout the lung and the regional lymph nodes causing pulmonary tuberculosis (Bloom and Murray, 1992). Extrapulmonary or disseminated tuberculosis occurs when infection develops in other parts of the body (Bloom and Murray, 1992). The typical lesion found is a

solid caseous (cheese-like) necrosis consisting of killed parenchymal cells, dead cells of infiltrating macrophages, and bacilli, which may still survive (Dannenberg and Rook, 1994). The necrotic lesions may be arrested or expand, breaking into bronchi. Cavities are then produced which allow large numbers of bacilli to spread to the outside through coughing, sneezing, or even speaking. Nevertheless, the solid necrosis may be liquefied by hydrolases from inflammatory cells, which then create a rich medium for the proliferation of bacilli. Due to the predominant mode of transmission of *M. tuberculosis* via aerosol, tuberculosis usually causes inflammatory responses and primary lesions in the lung. Tuberculosis symptoms can then be recognized by the manifestations of malaise, cachexia, fever, night sweat, coughing, chest pain, and bloody sputum (Bloom and Murray, 1992).

1.2 TUBERCULOSIS (TB)

1.2.1 Historical Background

Tuberculosis is a chronic infectious disease that has afflicted humanity for over millennia. Historians established the existence of endemic tuberculosis from Egyptian mummies dating from 2000 to 4000 BC (Bloom and Murray, 1992). Hippocrates mentioned it in 460 BC as a fatal disease of the lung (Stacher, 1999). More than 2000 years later, tuberculosis still remains a major health problem.

It is believed that the progenitor of the *M. tuberculosis* complex arose from a soil bacterium, which then became infectious

in cattle. The human bacillus, *M. tuberculosis,* is thought to have developed from the bovine form following ingestion of bovine milk contaminated with *M. bovis* (Daniel *et al.*, 1994). The discovery of *M. tuberculosis* was announced by Robert Koch on March 24th 1882 when he identified an acid fast bacterium, *M. tuberculosis,* as the causative agent of human tuberculosis (Koch, 1882; Daniel *et al.*, 1994). His criteria for proof that this organism caused tuberculosis have become known as Koch's postulates: isolation of the bacilli from the body, growth in pure culture, and reproduction of the same "morbid" condition by administering the isolated bacilli to animals. In 1891, Koch had developed a "cure" in the form of "old tuberculin" which was made from an attenuated human tubercle bacillus. However, this soon proved to be ineffective and dangerous, a severe setback for Koch (Zumla *et al.*, 1999). In 1908, the BCG (Bacille Calmette-Guerin) vaccine was first used to immunize a patient, and is currently the most widely used vaccine in the world (Basso and Blanchard, 1998). The discovery of the antibacterial and antitubercular properties of streptomycin in 1944 (Schatz and Waksman, 1944), and both isoniazid and pyrazinamide in 1952 (Kushner *et al.*, 1952; Middlebrook, 1952), led to effective chemotherapies that decreased the tuberculosis mortality rate worldwide. The later introduction of ethionamide, rifampin, ethambutol and ciprofloxacin to tuberculosis regimens provided more alternative antituberculosis agents (Basso and Blanchard, 1998). Multidrug therapeutic regimens designed to cure patients and prevent the emergence of

drug resistance were developed in 1953 at the British Medical Research Council (Zumla *et al.*, 1999). During that time, the incidence of tuberculosis declined rapidly in the United Kingdom and other coutries in economically developed world.

1.2.2 The current tuberculosis pandemic

By the 1980s, the incidence of tuberculosis was very low. Until the early 1990s the previous steady decline of tuberculosis in many developed nations began leveling off and the incidence of tuberculosis began to increase (Bloom and Murray, 1992; Reider *et al.*, 1989). Recognizing that worldwide tuberculosis is one of the most neglected health crises and that tuberculosis is out of control in many parts of the world, tuberculosis was declared a global emergency by the World Health Organization in April 1993 (World Health Organization, 1994).

M. tuberculosis, the etiological agent of tuberculosis, is recognized as the world's leading cause of death caused by a single infectious agent (Bloom and Murray, 1992; Iseman, 1994). In 1994, 3.3 million cases of tuberculosis were reported worldwide but these probably represent less than a third of all cases. The World Health Organization has estimated that nearly 90 million new cases of tuberculosis will emerge during the next decade, the majority amongst the age group 20-49, which represents men and women in their most productive years. Out of them, over 30 million people will die worldwide from tuberculosis during the next 10 years (Dolin *et al.*, 1994). It has been estimated that about one-third of the world population has been infected by the

tubercle bacilli. Most of these are infected but display no sign of disease. The bacilli may persist and give rise to active disease later, called reactivation tuberculosis (Ellner, 1994). Active tuberculosis affects 8-10 million new cases and causes about 3 million deaths annually. The annual number of deaths due to tuberculosis could rise to 4 million by the year 2004 (World Health Organization, 1998), and this number will be increasing for the foreseeable future.

Several factors have been proposed to contribute to the resurgence of tuberculosis: the HIV pandemic, homelessness, poverty, immigration, decline in health care structures, poor national surveillance and the emergence of drug-resistant strains (Brudney and Dokkin, 1991; Cohn *et al.*, 1997; Heym *et al.*, 1994; Kritski *et al.*, 1996; Ristow *et al.*, 1995). The HIV/AIDS pandemic is believed to have a central role in re-emerging tuberculosis (Barnes *et al.*, 1991). Accordingly, on March 24th 1992, the centenary of Koch's discovery, the World Health Organization decreed that the day of March 24th, and all subsequent anniversaries, would be World TB day (Zumla *et al.*, 1999).

1.2.3 The impact of the HIV/AIDS pandemic on tuberculosis

The emergence and rapid development of the AIDS epidemic has had a devastating impact on the global burden of tuberculosis and now is by far the most important of the predisposing factors for active tuberculosis (Zumla *et al.*, 1999). HIV causes the activation of tuberculosis in individuals infected by the tubercle bacilli. The risk of developing active tuberculosis in such

patients increases to 10% (Antonucci *et al.*, 1995; Zumla *et al.*, 1999). It was estimated that about 10% of all cases of tuberculosis are HIV-related and this will rise to 1.4 million cases worldwide (Dolin *et al.*, 1994). A recent report noted that tuberculosis is directly responsible for 30% of the 2.5 million deaths due to AIDS in 1998 (USAIDS, 1998).

During the late 1980s and early 1990s, there was a dramatic rise in the number of tuberculosis cases with the concurrent AIDS epidemic. However, the incidence of reported cases in the United States has steadily declined since 1992 (Centers for Disease Control and Prevention, 1999a). In 1997, a total of 19,855 tuberculosis cases was reported (Centers for Disease Control and Prevention, 1998), representing a 7% and 26% respective decrease from 1996 and 1992, the height of the TB resurgence in the United States (Centers for Disease Control and Prevention, 1998; 1993). In 1998, a total of 18,361 tuberculosis cases, a decrease of 8% from 1997 and 31% from 1992 was reported (Centers for Disease Control and Prevention, 1999b). The 1998 rate of 6.8 per 100,000 population remained above the national goal for 2000 of 3.5 and less than 1 per 100,000 population by 2010 (Centers for Disease Control and Prevention, 1993). The decline in overall number of reported tuberculosis cases reflects the apparent strengthening of tuberculosis-control programs with active cases promptly identified followed by appropriate treatment and forced compliance with DOT (direct observed therapy) (Centers for Disease Control and Prevention, 1989; 1999b).

1.2.4 Drug-resistant tuberculosis

In recent years the tuberculosis problem has been aggravated by the worldwide emergence of drug-resistant strains of *M. tuberculosis*. A multidrug-resistant strain (MDR) is defined as one resistant to isoniazid (INH) and rifampin (Rif), the two principal drugs of modern short course chemotherapy with or without resistance to other antituberculosis drugs (Kochi *et al.*, 1993). It has become apparent that resistant strains of *M. tuberculosis* develop readily before infection. Primary resistant tuberculosis occurs when patients are infected with already resistant strains. A resistant mutant of *M. tuberculosis* can occur naturally regardless of exposure to drugs (Canetti, 1965). In general, *M. tuberculosis* mutants resistant to any single antituberculosis drug are present at a frequency of approximately 1×10^{-6} (David, 1980). The frequency of mutation conferring resistance to Rif is about 1×10^{-8}, and it is about 1×10^{-6} for INH and streptomycin (Str). It was estimated that in a large extracellular population (10^{7}-10^{9}) of active tubercle bacilli in cavity lesions there are 1,000 to 10,000 organisms resistant to a single effective drug (Basso and Blanchard, 1998). Therefore, monotherapy and improperly administered two-drug therapy will select for drug-resistant mutants, which may lead to drug resistance in the entire population of tubercle bacilli. Such a circumstance creates secondary resistant tuberculosis where drug-resistant organisms are selected by treating susceptible *M. tuberculosis*. Other factors leading to the emergence of secondary

resistance (acquired resistance) include intermittent supplies of drugs, use of time-expired drugs, inappropriate prescribing, suboptimal therapy, poor supervision of treatment, and poor compliance. Inappropriate treatment and patient noncompliance are the most important factors contributing to secondary drug resistance (Huebner and Castro, 1995). In addition, the prevalence of drug resistance has been compromised by the lack of laboratories in many parts of the world able to perform standardized tests (Vareldzis *et al.*, 1994). The recent survey revealed enormous inter-regional differences in the incidence of drug-resistant tuberculosis (Zumla *et al.*, 1999). The overall mean of incidence rates of smear positive tuberculosis showing some forms of resistance is 16.7% of all tested strains, with 9.1% being resistant to one drug, 7.7% to two or more drugs and 4.3% being multidrug-resistant (Zumla *et al.*, 1999). In the United States, about 13% of new cases were resistant to at least one of five front-line antituberculosis drugs: INH, Rif, ethambutol (EMB), pyrazinamide (PZA) and Str (Bloch *et al.*, 1994).

1.2.5 Chemotherapy

Treatment of drug-resistant tuberculosis cases is difficult, expensive and often fails (Blanchard, 1996; Morris *et al.*, 1995; Neville *et al.*, 1994). The case fatality rate of multidrug-resistant tuberculosis is between 40 and 60% (Bloom and Murray, 1992) the same as the mortality rate of untreated tuberculosis. Indeed, drug sensitive tuberculosis can be successfully cured by modern short course chemotherapy consisting of the first-line

antituberculosis drugs: INH, Rif, EMB and PZA or Str (Mitchison, 1985; Stratton and Reed, 1986). During the initial intensive phase of treatment, which lasts 2 months, tuberculosis patients are treated daily with INH, Rif, PZA and EMB or Str to ensure that mutants that are resistant to a single drug cannot emerge. In the following 4 months, the continuation phase, only INH and Rif are administered to kill any persisting organisms. This modern, standard treatment for tuberculosis is referred to as short course chemotherapy, and patients who complete it produce a cure rate of > 90% (Snider *et al.*, 1984). In this regimen, bactericidal action against active metabolizing bacilli is conferred by INH, Rif, Str, and EMB (Heifets and Lindholm-Levy, 1989; Heifets *et al.*, 1990; Heifets, 1994) while PZA is effective against dormant bacilli in acidic intracellular environments (Heifets and Lindholm-Levy, 1992). The bacteriostatic second-line drugs such as ethionamide (ETH), cycloserine and *para*-aminosalicylic acid (PAS) are reserved for drug-resistant *M. tuberculosis* or in cases of allergic response or toxicity with certain first-line drugs. The newer fluoroquinolones (sparfloxacin, ciprofloxacin and ofloxacin) are bactericidal against *M. tuberculosis*. Although not indicated as first-line antituberculosis drugs, the fluoroquinolones have important roles as alternative agents in the treatment of drug-resistant *M. tuberculosis* (Basso and Blanchard, 1998). Table 1.2 summarizes the list of agents effective against *M. tuberculosis*.

Table 1.2 Antimycobacterial agents used or under investigation for the treatment of tuberculosis (Chopra and Brennan, 1998).

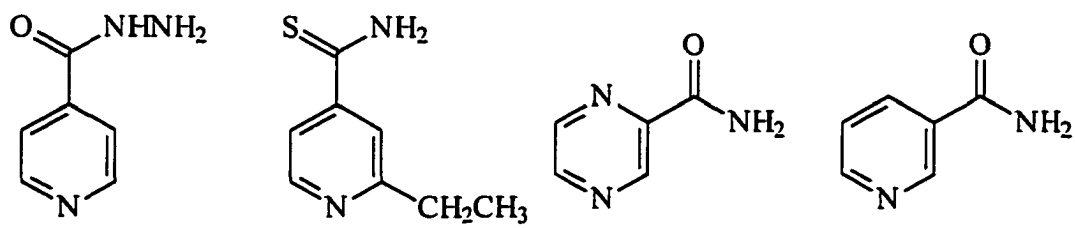
Broad-spectrum agents	Narrow-spectrum agents
Cycloserine	Capreomycin
Fluoroquinolone	Clofazimine
Macrolide	Ethambutol
Rifampicin	Ethionamide
Streptomycin	Isoniazid
Kanamycin, Amikacin, Viomycin	Isoxyl/Thiocarlide
	p-aminosalicylic acid
	Pyrazinamide
	Thiacetazone

M. tuberculosis is an intracellular pathogen. It replicates within the mammalian macrophage making it hard to be killed by microcidal agents. The pathogen also displays an intrinsic resistance to many antibacterial substances due to the impermeable nature of mycobacterial cell wall (Brennan and Nikaido, 1995; Barry and Mdluli, 1996; Jarlier and Nikaido, 1994). The intrinsic barrier consists of a covalently linked structure of the peptidoglycan, the arabinogalactan, and the mycolic acids (Brennan and Nikaido, 1995). In large part, a parallel alignment of mycolic acids in the inner leaflet of the cell wall contributes to the barrier function of this structure to the entry of hydrophobic solutes including drugs (Liu *et al.*, 1996). However, the cell wall alone cannot produce significant levels of drug resistance (Basso and Blanchard, 1998). Mostly, drug resistance is conferred by mutations of genes encoding drug target enzymes or genes

encoding enzymes involved in drug activation (Morris *et al.*, 1995). Drug resistance can also be achieved by the overproduction of enzymatic targets or through drug efflux pumps.

1.2.5.1 Mode of action and resistance to first-line antituberculosis drugs

Isoniazid (INH). INH including ETH and PZA are structural analogues of nicotinamide (NAM)(Fig. 1.1), an intermediate in the biosynthesis of pyridine derivatives which possesses an antituberculosis effect (Mckenzie *et al.*, 1948; Winder, 1982).



INH **ETH** **PZA** **NAM**

Figure 1.1 Structures of isoniazid (INH), ethionamide (ETH), pyrazinamide (PZA) and nicotinamide (NAM).

INH, an isonicotinic acid hydrazide was first described in 1912. In 1952, INH was found to exhibit powerful bactericidal activity against *M. tuberculosis* (Bernstein *et al.*, 1952), and has since been one of the principal agents in both therapeutic and prophylactic treatments of tuberculosis. INH is a very potent bactericidal drug, which can kill most of the bacillary population in the patient's lesions during the first few days of therapy. It has remarkable specificity for *M. tuberculosis* and *M. bovis* with a minimal inhibitory concentration of 0.02-0.2 μg/ml (Heifets, 1994)

while other bacteria are resistant to concentrations above 500 µg/ml (Zhang and Young, 1994).

INH exerts many effects on mycobacterial cells such as a loss of acid fastness, a decrease in the hydrophobic nature of cell wall structure and a decrease of mycolic acids. Early work demonstrated that the mode of action of INH is on the inhibition of the synthesis of mycolic acids (Takayama, 1974; Takayama *et al.*, 1972; Wang and Takayama, 1972; Winder and Collins, 1970). Subsequent studies on the biochemical effect of INH revealed that INH inhibits the desaturation of C_{24} and C_{26} acids resulting in an accumulation of saturated C_{24}-C_{26} fatty acids coincident with a depletion of mycolic acids. From this evidence, it was proposed that the site of specific inhibition of INH is at the step of tetracosanoate desaturation presumably catalyzed by $C_{24:1}$ Δ5 desaturase (Takayama *et al.*, 1975). Further evidence supporting this possibility is that in *M. tuberculosis* treated with INH there was an upregulation of a protein identified as acyl carrier protein (ACP) associated with long acyl chains. This phenomenon led to the speculation that the target of INH in *M. tuberculosis* is a tetracosanoyl-ACP requiring desaturase ($C_{24:1}$ Δ5 desaturase), and the intermediate accumulating product was the C_{24}-tetracosanoyl -ACP (Mdluli *et al.*, 1996a).

Extensive studies on the mechanism of action of INH have resulted in the definition of INH activation. INH is a prodrug which requires activation by a product of the *katG* gene known to encode an endogenous catalase-peroxidase (KatG), a 80-kDa protein

which has catalase and peroxidase activity (Middlebrook, 1952; Musser *et al.*, 1996; Zhang *et al.*, 1992; Zhang *et al.*, 1993). The activation of INH by KatG generates a number of electrophilic intermediates capable of either oxidizing or acylating amino acids in target proteins (Johnsson and Schultz, 1994; Shoeb *et al.*, 1985). The evidence that the mycobacterial catalase-peroxidase potentiated toxicity of INH by generating oxidative radicals has also been presented (Johnsson and Schultz, 1994; Johnsson *et al.*, 1995; Rouse and Morris, 1995). However, the exact metabolic intermediate(s) that exerts toxic effects on mycobacterial cells are still unknown (Musser, 1995).

Recent genetic data suggest that a major target for INH is a product of the *inhA* gene (Banerjee *et al.*, 1994) which was subsequently identified to be the NADH-dependent Δ2-*trans* enoyl-ACP reductase (Quemard *et al.*, 1995). The enzyme was shown to catalyze the NADH-specific reduction of long-chain (C_{12}-C_{24}) 2-*trans*-enoyl ACPs (Quemard *et al.*, 1995), but $C_{16} > C_{18}$ and C_{20} enoyl intermediates are preferentially substrates (Dessen *et al.*, 1995). The enoyl-ACP reductase was characterized to be part of fatty acid synthase type II (FAS II) involved in fatty acid elongation consistent with its involvement in the early stages of mycolic acid synthesis (Quemard *et al.*, 1995). The work of Johnsson *et al.* (1995) demonstrated that in *M. tuberculosis* the catalase-peroxidase-activated INH binds and inhibits the reductase in the presence of NADH. Extending this observation is the elucidation of the three-dimensional structure for the ternary complex of the

wild-type enoyl reductase, NADH and the activated INH in *M. tuberculosis* (Rozwarski *et al.*, 1998).

Although it is clear that InhA is a target for the activated INH, there is quite some controversy as to whether or not it is the primary target of INH in *M. tuberculosis*. A subsequent study demonstrated that the transformation of *M. tuberculosis* with the *inhA* gene failed to confer resistance to INH, but the same construct conferred high level-resistance to the drug in *M. smegmatis* (Mdluli *et al.*, 1996b). Accordingly, it was proposed that InhA is the primary target of the activated INH in *M. smegmatis* and the Δ5 desaturase is probably a target of the activated INH in *M. tuberculosis* (Mdluli *et al.*, 1996b) More recently, two components of FAS II composed of a 12-kDa ACP designated AcpM and β-ketoacyl ACP synthase (KasA) have been proposed as an additional target of the activated INH (Mdluli *et al.*, 1998).

The first insight into the mechanisms of INH resistance occurred when Middlebrook discovered INH-resistant organisms shortly after INH was introduced (Middlebrook, 1954). The frequency of primary resistance of *M. tuberculosis* to INH was estimated to be in the range of 10^{-5} to 10^{-7} (David and Newman, 1971) which is several orders of magnitude greater than that of Rif (Zhang and Young, 1994). Frieden *et al.* (1993) studied the outbreak of drug-resistant tuberculosis in New York City and reported that INH resistance appeared in > 20% of drug resistant tuberculosis cases. In the first report of laboratory isolates of

M. tuberculosis resistant to INH, a high correlation between INH resistance and decreased catalase-peroxidase activity was observed (Middlebrook, 1954; Winder, 1960). It was found that mutations in the *katG* gene were involved in decreased susceptibility to INH in approximately 50% of clinical isolates of *M. tuberculosis* (Cockerill *et al.*, 1995, Heym and Cole, 1992; Heym *et al.*, 1995). This observation has been confirmed by the demonstration that the transformation of selected strains resistant to INH with the wild-type *katG* gene restored INH susceptibility (Zhang *et al.*, 1993).

Further studies demonstrated that mutations in the *inhA* structural gene (Banerjee *et al.*, 1994; Kapur *et al.*, 1995) and in the *inhA* locus promoter region have been associated with INH resistance (Morris *et al.*, 1995; Musser *et al.*, 1996). Mutations in the *inhA* gene have been shown to confer resistance to INH and ETH by decreased affinity of the mutated-InhA to NADH (Dessen *et al.*, 1995).

More recently, it has been reported that the increased expression of an *aphC*-like component of the alkyl hydroperoxidase complex conferred resistance in some *M. tuberculosis* strains that lacked mutations in the *katG* or *inhA* genes. A product of *aphC*, an oxidative stress defense gene, is a protein capable of detoxifying oxygen radicals including damaging peroxides (Wilson and Collins, 1996; Wilson *et al.*, 1997). The *oxyR* gene product, OxyR, induces the expression of the *aphC* gene resulting in a significant increase of MIC to INH (Sherman *et al.*, 1995). The proposed contribution of the *oxyR* and *aphC* to INH resistance is shown in

Fig. 1.2 (Deretic *et al.*, 1997). It has been proposed that AphC, a homolog of thioredoxin-dependent alkyl hydroperoxide reductase, interacts directly with the activated INH resulting in detoxification of the INH radicals (Sherman *et al.*, 1996). However, the exact involvement of the *aphC* gene in clinical INH resistance is unclear and leaves open the issue of some other molecular mechanisms for INH resistance.

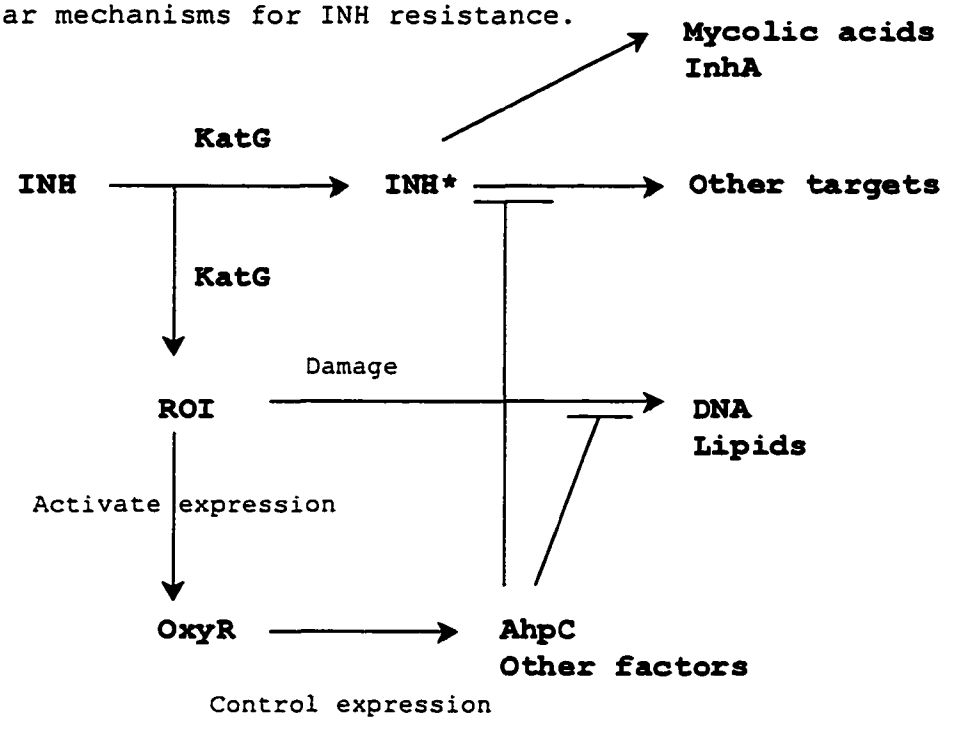


Figure 1.2 A scheme for the proposed contribution of *aphC* expression in *M. tuberculosis* to INH resistance (Deretic *et al.*, 1997).

In a recent survey, the prevalence of primary INH resistance ranged from 0 to 16.9% while the prevalence of acquired INH resistance varied in the range of 4.0 to 53.7% (World Health Organization, 1997). Mutations in the *katG* gene were associated with the majority of INH-resistance in clinical isolates (Musser *et al.*, 1996). An attempt to use higher doses of INH in treating INH resistant tuberculosis showed that it was unlikely to be more

efficacious than the standard 300-mg/day dose (Cynamon *et al.*, 1999).

Rifampin (Rif). Rifampin or rifampicin is a semi-synthetic derivative of the natural product, rifamycin B, derived from the culture filtrate of *Streptomyces mediterranei.* It was discovered in 1957 as a broad-spectrum antibiotic and was introduced for use in tuberculosis therapy in 1972 (Woodley et *al.*, 1972). Rif is another potent bactericidal drug with MIC values of 0.1-0.2 μg/ml against *M. tuberculosis* (Heifets, 1994). It possesses a strong sterilizing action that can kill the intermittently metabolizing bacilli after a short exposure to the drug (Dickinson and Mitchison, 1981). Thus, the introduction of Rif to multidrug-treatment regimens helped to shorten the course of treatment against drug-susceptible tuberculosis infections (Mitchison, 1985).

The action of Rif is due to a specific inhibition of the elongation of RNA transcripts, with lesser or no effect on transcription initiation. The molecular target of Rif *in M. smegmatis* has been identified to be DNA-dependent RNA polymerase (Levin and Hatfull, 1993). In *E. coli*, this enzyme is composed of four different subunits: α, β, β′, and σ encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively. The core part of the enzyme contains α, β, and β′ subunits. The binding of the σ subunit to the core part transforms the apoenzyme to the holoenzyme form (α, β, β′ plus σ). Rif is known to act by binding to the β subunit of

RNA polymerase resulting in an inhibition of DNA transcription (McClure and Cech, 1978). The *rpoB* gene encoding the β subunit of RNA polymerase has been sequenced in *M. tuberculosis* (Donnabella *et al.* 1994; Miller *et al.*, 1994) and *M. leprae* (Honore and Cole, 1993). It was found that resistance to Rif is mostly associated with mutations of the *rpoB* sequence (Kapur *et al.*, 1994; Ohno *et al.*, 1996; Williams *et al.*, 1994).

Rif resistance is increasing rapidly due to the widespread use of Rif in conjunction with INH (Basso and Blanchard, 1998). Resistance to Rif is determined by the proportion plate method containing 1 µg/ml of Rif (Wellstood, 1993), and isolates with greater than 1% growth on Rif containing media compared with growth on control media are considered resistant. Commonly, the MIC of Rif in resistant strains was reported to be in excess of 8.0 µg/ml (Bodmer *et al.*, 1995). The frequency of Rif resistance is 3.9% of all cases globally, but is 9% of patients previously treated for tuberculosis (Bloch *et al.*, 1994). In a recent survey of Rif resistance worldwide (Cohn *et al.*, 1997), the range of primary resistance is 0% to 3% (median, 0.2%), and acquired resistance is in the range of 0% to 14.5% (median, 2.4%). The increasing incidence of Rif-resistant *M. tuberculosis* is of significant concern and led to the development of new derivatives which appear to be more effective than Rif. These new compounds include rifabutin and rifapentine (MIC's = 0.03-0.06 and 0.01-0.06 µg/ml, respectively)(Heifets *et al.*, 1990). Rifapentine, with 10-fold greater activity than Rif against *M. tuberculosis* and

improved pharmacokinetic properties, is currently in phase II trials. Moreover, a number of new benzoaxazino-rifampicins with higher potential for the treatment of tuberculosis are in the pre-clinical or early phase of drug development (Tilotson, 1996; Luna-Herrera *et al.*, 1995). However, these compounds exhibit cross-resistance with Rif. Thus, more potent new analogs to replace Rif are in need of development.

Ethambutol (EMB). EMB was first introduced in 1961 (Thomas *et al.*, 1961). It is a specific bactericidal drug for mycobacteria and is used in modern combination therapy. However, EMB is effective only on actively multiplying mycobacteria with MIC's in the range of 0.5 to 2.0 µg/ml in 7H12, 0.95 to 3.8 µg/ml in BACTEC broth and 1.9 to 7.5 µg/ml in 7H10 agar (Heifets, 1994). In general, most strains of *M. tuberculosis* and *M. bovis* BCG are inhibited *in vitro* by 1 µg/ml and the rest by 5 µg/ml (Karlson, 1961; Thomas, 1961).

Early studies of the biochemical effects of EMB showed that upon treatment of *M. smegamatis* with EMB there was an inhibition of the transfer of mycolic acids into the cell wall (Takayama *et al.*, 1979; Winder, 1982) concurrent with an accumulation of trehalose mycolates and free mycolic acids (Kilburn and Takayama, 1981). These observations suggested that the target(s) of EMB was involved in the formation of the outer envelope of mycobacteria (Sareen and Khuller, 1990). Mikusova *et al.* (1995) demonstrated that in *M. smegmatis* the primary site(s) of EMB action is not on *de novo* synthesis of D-arabinose but on the polymerization of

arabinoses into the arabinan component of the cell wall arabinogalactan (AG). Further investigation revealed that the synthesis of the arabinan of lipoarabinomannan (LAM) was subsequently inhibited resulting in the emergence of truncated LAM with a marked accumulation of lipomannan (LM). The accumulation of a variety of free mycolic acids including trehalose mycolates was explained by the lack of the attachment site for mycolates due to the inhibitory effect of EMB on the AG arabinan biosynthesis (Mikusova *et al.*, 1995). This finding suggested the possibility that the arabinosyltransferases (I, II, III) are involved in the polymerization of the arabinans of AG and LAM and are targets of EMB. The arabinosyltransferase III which is responsible for the synthesis of arabinans of AG was proposed as a critical target of EMB (Mikusova *et al.*, 1995). Subsequent investigation of truncated structural variants of LAM in EMB resistant strain of *M. smegmatis* revealed that the arabinosyltransferases involved in the polymerization of arabinans of LAM were also sensitive to EMB and thereby probably a target of EMB (Khoo *et al.*, 1996).

A genetic approach using drug resistance, via target overexpression from a plasmid vector resulted in the identification of genes involved in EMB resistance and encoding EMB targets. The overexpression of *M. avium embA, embB and embR* in *M. smegmatis* were found to be associated with high-level resistance to EMB. It was suggested that *embA and embB* were co-transcribed and might encode an arabinosyltransferase, a primary target of EMB. The *embR* gene was shown through homology to be a

transcriptional activator, and EMB resistance decreased when this gene was missing (Belanger *et al.*, 1996). However, the *emb* region in *M. tuberculosis* containing *embCAB* exhibits difference in gene organization from that of *M. avium* (Telenti *et al.*, 1997).

The incidence of EMB resistance occurs in up to 4% of the total tuberculosis cases (Bloch *et al.*, 1994). Mutations in *embB* confer high-level resistance to EMB and are found in 61% of EMB resistant clinical isolates (Telenti *et al.*, 1997). Low-level resistance, which accounts for 30% of the remaining EMB resistant isolates is found to be associated with an increased expression of the *embCAB* gene products (Telenti *et al.*, 1997).

Pyrazinamide (PZA). PZA, a synthetic derivative of nicotinamide (NAM)(Fig. 1.1), was first recognized for its substantial antimycobacterial activity in 1952 (Yeager *et al.*, 1952). However, it was not used extensively as a front-line drug until the mid-1980s (Heifets and Lindholm-Levy, 1992; Snider *et al.*, 1982). PZA has a strong synergistic and accelerating effect when used in combination of INH and Rif (Heifets, 1994; Mitchison, 1985) and therefore, it was added to the combination therapy regimen used in the first two months of treatment. This combination regimen results in a shorter course of tuberculosis therapy from 9-12 months to 6 months (Davidson and Quoc, 1992). The MIC of PZA varies in the range of 8 to 60 µg/ml depending on assay methods and media used (Heifets, 1994). PZA does not show bactericidal activity, even when used at concentrations greater than its MIC, but it presents an impressive sterilizing effect *in*

vitro (Heifets and Lindholm-Levy, 1992) and shows marked *in vitro* activity against *M. tuberculosis* at acidic pH values (below 6).

Early work demonstrated that PZA is a prodrug, which needs to be activated by acid hydrolysis (McDermott and Tompsett, 1954). Within acidic cellular compartments, such as in inflammatory sites or macrophage phagosomes, PZA exhibits *in vivo* sterilizing activity on the semidormant *M. tuberculosis* (Mackaness, 1956). However, PZA is not effective against *M. bovis* and fast-growing mycobacteria (Konno *et al.*, 1967). Several studies reported that PZA-susceptible *M. tuberculosis* strains have a pyrazinamidase, an enzyme that metabolizes PZA to pyrazinoic acid and that PZA-resistant organisms have lost pyrazinamidase activity (Butler and Kilburn, 1983; Konno *et al.*, 1967; McClatchy *et al.*, 1981). It was suggested that upon PZA treatment, the drug induced stress and caused bacteria to produce appropriate pyrazinamidase (Mackaness, 1956). Thus, PZA exerts its action after conversion into pyrazinoic acid but the precise mechanism of action is unknown. The decrease or loss of pyrazinamidase activity appears to be associated with PZA resistance. A genetic approach resulted in the identification of *pncA* (558 bp) which encodes pyrazinamidase, and the correlation between PZA-resistance and *pncA* mutations has been demonstrated (Scorpio and Zhang, 1996). Recently, it has been proposed that a deficiency of an efflux mechanism underlies the unique susceptibility of *M. tuberculosis* to PZA (Zhang *et al.*, 1999).

Streptomycin (Str). Str is a broad-spectrum aminocyclitol glycoside antibiotic. It was first shown to be effective in tuberculosis treatment in 1944 (Schatz and Waksman, 1944). The MICs of Str against *M. tuberculosis* are in the range of 0.4-1.5 µg/ml making it one of the most effective early antituberculosis drugs (Heifets, 1994). However, Str is less potently bactericidal when compared to INH and Rif (Chan, 1994). The drug presumably binds irreversibly to 16S rRNA, a subunit of the 30S ribosome, resulting in interference with the proof-reading step in translation, misreading of the genetic code and inhibition of the initiation of mRNA translation (Moazed and Noller, 1987). Resistance to Str was found to be associated with mutations in two targets, the 16s rRNA gene (*rrs)* and the *rpsL* gene encoding ribosomal protein S12 of 16S rRNA. Most mutations occur via mutations in the *rpsL* gene and account for approximately two thirds of Str-resistance (Finken *et al.*, 1993; Nair *et al.*, 1993; Sreevatsan et *al.*, 1996). The remaining resistance has been associated with the mutations in the *rrs* gene (Finken *et al.*, 1993). Nevertheless, Str-resistance with wild-type *rrs* and *rpsL* was reported and accounted for one third of Str resistant isolates (Heym *et al.*, 1994; Kapur *et al.*, 1995). It has been suggested that this type of low-level Str resistance is due to the decreased permeability of drug into the cells (Meier *et al.*, 1996).

A recent worldwide survey reported that primary resistance to Str occurred at 3.5% while acquired resistance occurred at 4.9% of all tuberculosis cases (Cohn *et al.*, 1997). It is notable that

resistance to INH and Str is more common than resistance to Rif and EMB (Basso and Blanchard, 1998).

1.2.5.2 Mode of action and resistance to some second-line antituberculosis drugs

Second-line drugs used for tuberculosis treatment include ethionamide, fluoroquinolones, cycloserine, capreomycin, thiosemicarbazone (thiacetazone), *para*-aminosalicylic acid (PAS), kanamycin, amikacin, and viomycin (Cole, 1994). Most of these compounds have weak antitubercular activity or have high levels of adverse effects except for ethionamide (ETH) and fluoroquinolones. Only ETH and fluoroquinolones are discussed below.

Ethionamide (ETH). ETH, discovered in 1956, is a second-line antituberculosis drug that has structural features in common with INH. It is an α-ethyl derivative of isonicotinylthioamide (Winder, 1982) with potent bacteriostatic activity against *M. tuberculosis* in the range of 10-20 μg/ml (Musser, 1995). Biochemical evidence suggested that ETH inhibited mycolic acid synthesis (Quemard *et al.*, 1992; Winder *et al.*, 1971; Winder, 1982). Genetic analysis demonstrated that *inhA*-encoded enoyl reductase, a target of INH, appears to be a target for ETH, and mutations in the *inhA* gene conferred resistance to both drugs (Banerjee *et al.*, 1994). Like INH it was believed that ETH is a prodrug that needs to be activated via a different mechanism from that of INH to produce a sulfoxide activated form (Johnsson *et al.*, 1995). This proposal is derived from the evidence that INH-

resistant strains containing mutations in the *katG*-encoded catalase peroxidase remained susceptible to ETH. The enzyme that activates ETH is presumably encoded by a mycobacterial gene able to convert a prodrug into sulfoxide, a major metabolite of ETH found in humans and in a variety of animals (Prema and Gopinthan, 1976). This activated form of ETH is presumably capable of binding to an active site of the *inhA*-encoded enoyl reductase (Quemard *et al.*, 1995). It must be noted that the clinical cross resistance of ETH, thiocarlide (THC) and thiacetazone has been observed in *M. tuberculosis* (Porter and Neal, 1978; Sojkova *et al.*, 1965; Tan *et al.*, 1966; Tskamura, 1962). This cross-resistance is probably mediated by defects in genes involved in the activation of a thiocarbonyl moiety which these three drugs share in common (Barry *et al.*, 2000).

Fluoroquinolones. The quinolone antibacterials are synthetic derivatives of nalidixic acid that display broad-spectrum antibacterial activity. The incorporation of the 6-fluorine atom on to the quinolone nucleus gives rise to fluoroquinolones and resulted in an increased antibacterial activity (Bryskier and Chantot, 1995). Quinolones were first reported in 1984 to be effective against a variety of mycobacteria (Gay *et al.*, 1984). The quinolones with potent antitubercular activity include olfloxacin, ciprofloxacin, sparfloxacin and levofloxacin. These compounds are bactericidal agents and are effective against *M. tuberculosis* with a MIC less than 1 µg/ml (Heifets, 1994). In general, sparfloxacin has bactericidal effects greater than

levofloxacin and olfloxacin (Lounis *et al.*, 1997). It appears that there is no synergism between fluoroquinolones and other antituberculosis drugs. In addition, fluoroquinolones have no cross-resistance with other drugs (Marinis and Lagakis, 1985). Because of their recent discovery, fluoroquinolones are not included as a first-line antituberculosis drug, but they are mostly prescribed in cases of multidrug-resistant tuberculosis (Basso and Blanchard, 1998).

Studies of quinolones' activity have found that the mode of action of quinolones involve interaction of drugs with DNA gyrase, a type II DNA topoisomerase, and DNA topoisomerase IV (Pan *et al.*, 1996). The ATP-dependent DNA gyrase is a heterotetramer composed of two A subunits and two B subunits encoded by *gyrA* (2,517 bp) and *gyrB* (2,060 bp), respectively. The enzymes catalyze the negative supercoiling of closed circular DNA (Wang, 1985). It was demonstrated that fluoroquinolones bind to DNA gyrase inhibiting supercoiling, and subsequently inhibiting processes dependent on DNA topology such as replication and transcription. The topoisomerase IV is a *par* gene product responsible for resolution of daughter molecules after chromosomal replication. Therefore, the inhibition of enzyme activity by quinolones prevents resolution of replicated DNA (Chen *et al.*, 1996). However, the DNA gyrase is apparently the primary target of quinolones in mycobacteria (Blanchard, 1996; William *et al.*, 1996).

The mechanisms of quinolone resistance in *M. tuberculosis* are similar to those observed in non-mycobacterial species (Pan *et*

al., 1996). Mostly, quinolone resistance is conferred by mutations in the *gyrA* gene (William *et al.*, 1996). Recently, the *lfrA* gene encoding an efflux pump has been recognized in *M. smegmatis*. Genes homologous to *lfrA* have been found in *M. tuberculosis* and *M. avium*. When expressed in cells, the LfrA protein was found to confer low-level quinolone resistance (Takiff *et al.*, 1996; Lewis *et al.*, 1997). Other mutations conferring quinolone resistance were found in the *gyrB* gene (Hooper and Wolfson, 1993).

1.2.6 Future needs

Three strategies have been proposed to circumvent the problem of tuberculosis drug resistance and to meet future chemotherapy needs: 1) improve the efficiency of current therapy and develop truly short course chemotherapeutic regimens; 2) develop new drugs whose mechanisms are different from the currently available agents; and 3) develop drugs that specifically target latent organisms (Barry *et al.*, 1997). These factors led to our study on the identification of novel drug targets and the development of new antituberculosis drugs whose mechanisms of action are unique.

1.3 MYCOBACTERIAL GENETICS

Molecular genetics provide invaluable tools towards the control of tuberculosis and for the study of certain aspects of mycobacteria. Mycobacterial genetics has had a great impact in elucidating metabolic mechanisms, and thereby pinpointing new drug targets as well as virulence determinants. More recently,

mycobacterial genetics has been applied in studies of modes of drug action and drug resistance mechanisms. Therefore, the purpose of this section is to provide sufficient background to mycobacterial genetics as it has implications in certain parts of this present study.

M. tuberculosis and other mycobacterial pathogens such as *M. bovis*, *M. leprae*, and *M. avium*, pose numerous difficulties in genetic manipulations. One reason is the slow growth rates of mycobacteria (Jacobs *et al.*, 1991). Attempts were made to overcome this obstacle by using surrogate hosts such as *Streptomyces lividans* or *Escherichai coli* for cloning and expression of mycobacterial genes (Hopwood *et al.*, 1988; Kieser *et al.*, 1986; Labidi *et al.*, 1985; Thole *et al.*, 1985). However, the efficiency of gene expression in these heterologous systems is variable and the systems do not permit the expression of a majority of mycobacterial genes (Labidi *et al.*, 1985; Jacobs *et al.*, 1986; Young *et al.*, 1985; Das Kupta *et al.*, 1993). Thus, nonpathogenic mycobacterial strains such as *M. smegmatis* strain mc^2155 and *M. bovis* BCG were introduced and now are being commonly utilized as a homologous system for mycobacterial gene expression (Jacobs *et al.*, 1991). The substantial biological hazard of *M. tuberculosis* is another difficulty in genetic manipulations, making it hard to handle or neccessitating handing with extreme caution (Jacobs *et al.*, 1991).

The problem of the introduction of genes into mycobacteria was one of the early impediments to advances in mycobacterial

genetics. A variety of systems have been developed to introduce genes into mycobacterial cells (Hatfull, 1993). Successful conjugation (Greenberg and Woodley, 1984), mycobacteriophage transfection (Jacobs *et al.*, 1987; Raj and Ramakrishnan, 1970), and plasmid transformation (Jain *et al.*, 1997, Snapper *et al.*, 1988; Snapper *et al.*, 1990) have been achieved. However, transformation by electroporation produces higher efficiencies for gene transfer (Snapper *et al.*, 1990). By electroporation, plasmids can be efficiently introduced into *M. smegmatis* strain mc^2155, giving transformation frequencies in the range of 10^5 to 10^6 transformants per μg plasmid (Snapper *et al.*, 1990). Other species of mycobacteria can also be transformed such as *M. bovis* BCG (Ranes *et al.*, 1990) and *M. aurum* (Hermans *et al.*, 1990a). In addition to the efficient transfer of genes into mycobacteria, a rapid method to transfer plasmids from mycobacterial transformants into *E. coli* has been reported. The method, referred as to electroduction has facilitated the rapid analysis of mycobacterial plasmid contents in *E. coli* (Baulard *et al.*, 1992). Overall, these techniques have alleviated some previous constraints in mycobacterial genetic manipulations.

Another obstacle in manipulations of mycobacterial genetics was a lack of the repertoire of plasmid-based mycobacterial vectors. The development of vectors that replicate solely in mycobacteria is not profitable because of the slow growth and the difficulties in DNA preparation from these organisms. The identification and characterization of an autonomously replicating

plasmid provides advantages in the development of shuttle vectors which are capable of replicating in alternative hosts such as *E. coli* or *S. lividans* as well as mycobacteria. Mostly, the shuttle mycobacterial vectors originated from pAL5000, a plasmid of *M. fortuitum* (Labidi *et al.*, 1985). Mycobacterial shuttle plasmids provide convenience and ease of manipulations of recombinant DNA in alternative hosts. Subsequently, the recombinant DNA can be transferred to and stably maintained in either fast- or slow-growing mycobacteria.

In recent years, the advance genetic tools have been developed such as mycobacterial cloning vectors, mobile genetic elements and selectable genetic markers. Other advanced genetic techniques have recently been introduced: the disruption of genes on the chromosome of mycobacteria, random or site specific mutagenesis, microarray assay and others. New genetic techniques are now steadily being developed. The availability of these tools and the knowledge of the genomic sequences of varoius *Mycobacterium spp* may have a great contribution to a series of mycobacterial studies and strategies to control tuberculosis.

1.3.1 Mycobacterial genomes

M. tuberculosis will become the bacterial species of which two strains will have their entire genome sequenced. Recently, the Sanger Center, Hinxton, UK has released the complete sequence of the H37Rv laboratory strain, which has been used widely in biomedical research. The typical characteristics of this strain include the capability to retain full virulence, the

susceptibility to antituberculosis drugs and the ability to be genetically manipulated (Cole *et al.*, 1998). The organization of genomic sequences was derived from shotgun sequence analysis and ordering a set of overlapping cosmids from cosmid library clones. The assembled sequences of *M. tuberculosis* H37Rv now are available on the Worldwide Web. In addition, the genome of clinical and virulent isolate, CSU93, is being sequenced by The Institute for Genomic Research (TIGR), Rockville, Maryland, using shotgun sequencing (De Smet, 1997). Soon the genome sequencing projects of *M. tuberculosis* CSU93 including *M. leprae* and *M. avium* will be completed. The availability of vast sequence information will enable us to identify genes specific to mycobacteria by comparative genomics. It is possible to monitor different genes and gene products to understand the unusual biology and pathogenicity of mycobacteria. The completion of the *M. tuberculosis* genome sequences may prove useful in vaccine research, and towards a better understanding of physiological functions of mycobacterial genes. Target sites of drugs can be identified, and that will greatly help in drug development.

M. tuberculosis has a genome size of 4,411,529 base pairs (~4.4 Mb)(Cole *et al.*, 1998), bigger than the size of the *M. leprae* genome (~2.8 Mb) (Clark-Curtiss *et al.*, 1985), that represents the second-largest bacterial genome sequenced to date (Cole *et al.*, 1998). An estimated 4,000 encoded protein exist in *M. tuberculosis;* about 40% have known exact physiological functions, another 44% have some sequence homology to known

proteins, and about 16% have unknown functions (Cole *et al.*, 1998; Barry *et al.*, 2000). The genome contains a very high G+C content of 65.5% consisent with that of other mycobacteria containing 64-71% G+C content (Clark-Curtiss, 1990). The relative numbering of nucleotides in the genome starts at the initiation codon of the *dna* gene, a mark for the origin of replication, *oriC* (Cole *et al.*, 1998). Other typical features of the *M. tuberculosis* genome are a large number of repetitive DNA sequences, particularly insertion sequences, and numerous housekeeping genes. There are several regions that contain very high G+C content such as the polymorphic G+C rich sequences (PGRSs). The insertion sequences and the polymorphic G+C rich regions are accounted for the dynamics of the *M. tuberculosis* genome. It is noted that *M. tuberculosis* has numerous genes encoding enzymes involved in lipogenesis and lipolysis (Cole *et al.*, 1998). Some of the enzymes involved in the synthesis of fatty acids, mycolic acids and glycolipids are listed in Table 1.3.

Table 1.3 The prediction of function and subcellular location of the open reading frames that probably encode enzymes involved in fatty acid/mycolic acid and glycolipid synthesis in *M. tuberculosis* H37Rv (Salman *et al.*, 1999).

ORF	Gene	Function	Predicted Localization
(1) Synthesis of fatty and mycolic acids			
Rv3285	*accA3*	acetyl/propionyl-CoA carboxylase α subunit	cytoplasm
Rv0904c	*accD3*	acetyl/propionyl-CoA carboxylase β subunit	cytoplasm
Rv3799c	*accD4*	acetyl/propionyl-CoA carboxylase β subunit	cytoplasm
Rv3280	*accD5*	acetyl/propionyl-CoA carboxylase β subunit	cytoplasm
Rv2247	*accD6*	acetyl/propionyl-CoA carboxylase β subunit	cytoplasmic membrane(?)
Rv2243	*fabD*	malonyl-CoA-[ACP] transacylase	cytoplasm
Rv1483	*fabG1*	3-oxoacyl-[ACP] reductase	cytoplasm
Rv0649	*fabD2*	malonyl-CoA-[ACP] transacylase	cytoplasm
Rv1350	*fabG2*	3-oxoacyl-[ACP] reductase	cytoplasm
Rv2002	*fabG3*	3-oxoacyl-[ACP] reductase	cytoplasm
Rv0242c	*fabG4*	3-oxoacyl-[ACP] reductase	cytoplasm
Rv2776c	*fabG5*	3-oxoacyl-[ACP] reductase	cytoplasm
Rv0553c	*fabH*	3-oxoacyl-[ACP] synthase III	cytoplasm
Rv2524c	*fas*	fatty acid synthase	cytoplasm
Rv0033	-	possible acyl carrier protein	cytoplasm
Rv1344	-	possible acyl carrier protein	cytoplasm
Rv3472	-	possible acyl carrier protein	cytoplasm
Rv1484	*inhA*	enoyl-[ACP] reductase	cytoplasm
Rv2245	*kasA*	β-ketoacyl-ACP synthase (meromycolate extension)	cytoplasm or, cytoplasmic membrane-anchored
Rv2246	*kasB*	β-ketoacyl-ACP synthase (meromycolate extension)	cytoplasm or, cytoplasmic membrane-anchored
Rv2244	*acpM*	acyl carrier protein (meromycolate extension)	cytoplasm
(2) Modification of fatty and mycolic acids			
Rv3391	*acrA1*	fatty acyl-CoA reductase	cytoplasmic membrane (?)
Rv3392c	*cmaA1*	cyclopropane mycolic acid synthase 1	cytoplasm
Rv0503c	*cmaA2*	cyclopropane mycolic acid synthase 2	cytoplasm
Rv0824c	*desA1*	acyl-[ACP] desaturase	cytoplasm
Rv1094	*desA2*	acyl-[ACP] desaturase	cytoplasm
Rv3229c	*desA3*	acyl-[ACP] desaturase	cytoplasm
Rv0645c	*mmaA1*	methoxymycolic acid synthase 1	cytoplasm
Rv0644c	*mmaA2*	methoxymycolic acid synthase 2	cytoplasm
Rv0643c	*mmaA3*	methoxymycolic acid synthase 3	cytoplasm
Rv0642c	*mmaA4*	methoxymycolic acid synthase 4	cytoplasm
Rv0469	*umaA1*	unknown mycolic acid methyltransferase	cytoplasm
Rv0470c	*umaA2*	unknown mycolic acid methyltransferase	cytoplasm
(3) Acyltransferase and mycolyltransferase			
Rv3804c	*fbpA*	antigen 85A, mycolyltransferase (Ag85A)	cytoplasmic membrane
Rv1886c	*fbpB*	antigen 85B, mycolyltransferase (Ag85B)	cytoplasmic membrane
Rv3803c	*fbpC1*	antigen 85C, mycolyltransferase (Ag85C1)	cytoplasmic membrane
Rv0129c	*fbpC2*	antigen 85C, mycolyltransferase (Ag85C2)	cytoplasm
Rv1543	-	probable fatty acyl-CoA reductase	cytoplasm
Rv3720	-	C-terminal similar to cyclopropane fatty acid synthase	cytoplasm

1.3.2 Mycobacterial plasmids.

Several plasmids exist in mycobacteria (Crawford and Falkinham, 1990; Falkinham and Crawford, 1994; Martin *et al.*, 1990). However, most mycobacterial plasmids are commonly found in the *M. avium* complex, *M. scrofulaceum* and in *M. fortuitum* (Crawford and Falkinham, 1990, Helley *et al.*, 1991). Of all the plasmids isolated from mycobacteria, the most noted plasmid is pAL5000, a 4.8-kb plasmid originally isolated from *M. fortuitum* (Labidi *et al.*, 1985; Ranes *et al.*, 1990; Stolt and Stoker, 1996). This plasmid can replicate in both fast- and slow-growing mycobacteria (Gicquel-Sanzey *et al.*, 1989; Beggs *et al.*, 1995). It has been used as the basis for construction of numerous mycobacterial vectors including shuttle plasmid vectors (Gavigan *et al.*, 1995; Guilhot *et al.*, 1992; Stover *et al.*, 1991). For the most part, the mycobacterial-*E.coli* plasmid shuttle vectors that can replicate in both *Mycobacterium spp* and *E. coli* are the most commonly used. These plasmids were engineered to have extrachromosomal origins of replication from plasmids of *E. coli* and pAL5000 of *M. fortuitum* (Labidi *et al.*, 1985).

The first generation of mycobacterial- *E. coli* shuttle vectors was developed by Snapper and colleagues (1988). One such vector is pYUB12, which was reported later to have poor transformation efficiency because of its large size, as well as a lack of unique restriction sites. In addition, the plasmid has tetracycline resistance (Tet^R) which is not a good selective marker in mycobacteria (Snapper *et al.*, 1988; 1990). To circumvent

these problems, the second generation of extrachromosomal shuttle vectors was developed by genetic engineering using a 1.8 kb fragment of replication region (*oriM*) from pAL5000 (Rauzier *et al.*, 1988). The *oriM* which supports plasmid replication in mycobacteria was modified by PCR mutagenesis to eliminate undesired restriction sites in its sequence (Stover *et al.*, 1991). The shuttle vectors then were constructed from the modified *oriM*, the *E. coli* plasmid replicon derived from pUC19 (*oriE*), the *Tn*903-derived *aph* gene conferring kanamycin resistance (Kan^r), a mycobacterial promoter for an expression cassette, a multiple cloning site, and a transcriptional terminator. The resulting shuttle plasmids were shown to have high transformation efficiencies (10^5 to 10^6 CFU per μg DNA) when introduced into *M. bovis* BCG (Stover *et al.*, 1991). One shuttle plasmid of this generation is pMV261 (Fig 1.3) which has been used widely as an extrachromosomal mycobacterial expression vector and used extensively in the work described in this dissertation.

While extrachromosomal plasmids are quite versatile, their elevated copy numbers is undesirable for certain purpose. In addition, many extrachromosomal plasmids are not stably maintained in the absence of selection. As a consequence, an alternative vector using the site-specific integration system of a temperate mycobacteriophage (Stover *et al.*, 1991) was developed by making plasmids capable of inserting plasmid sequences into a specific chromosomal site. One of these mycobacterial integration vectors is pMV361 which shares common elements with pMV261 (Fig 1.3). The

plasmid pMV361 was constructed by replacing the *oriM* region of pMV261 with a DNA fragment carrying the attachment site (*attP*) and the integrase (*int*) gene from mycobacteriophage L5 (Stover *et al.*, 1991). The plasmids can integrate into a specific site (*attP* site) of the chromosome making it more stably maintained and present as a single copy in mycobacterial hosts. It was reported that plasmids can also integrate into non-specific chromosomal sites by recombination but this event is rare (Lee *et al.*, 1991).

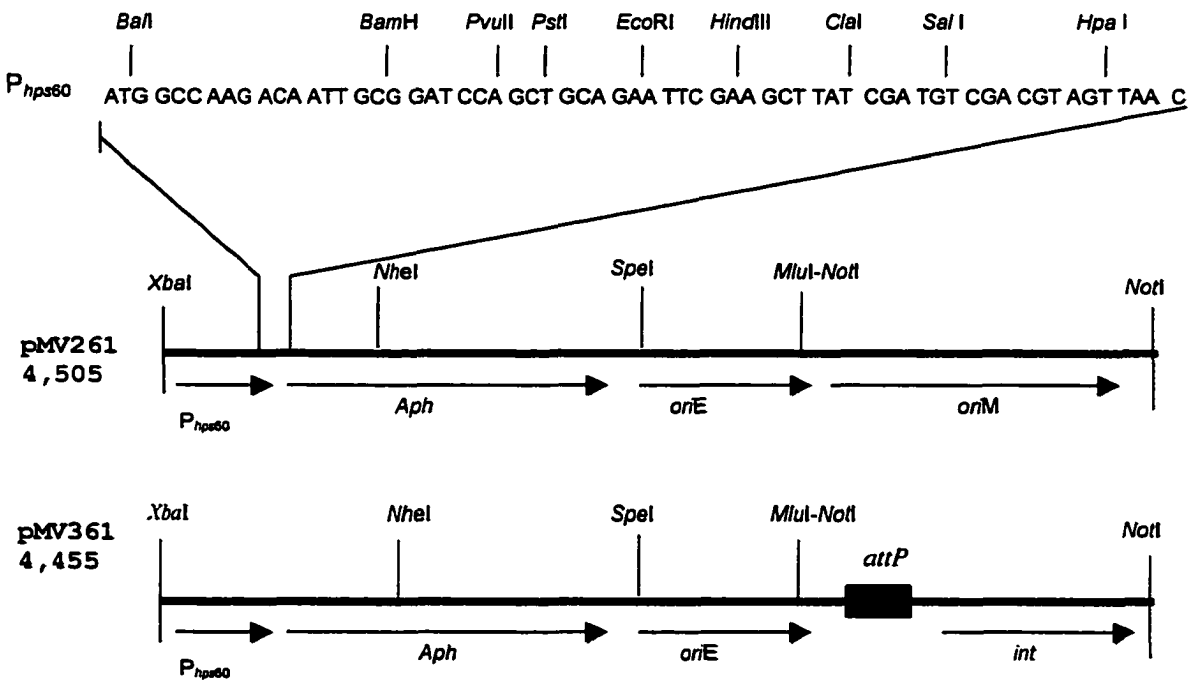


Figure 1.3 The structural organization of mycobacterial extrachromosomal (pMV261), and integrative (pMV361) expression vectors. Common elements in these two vectors include an expression cassette, the Tn*903*-derived kanamycin resistance gene, and an origin of replication functional in *E. coli* (*oriE*) derived from pUC19. The expression cassette contains the BCG *hsp60* promoter region, ribosomal binding site, part of the *hsp*60 gene containing a multiple cloning site, and the transcription terminator. The two vectors differ by inclusion of either a mycobacterial plasmid replication origin (*oriM*) or the *attP* and *int* genes of mycobacteriophage L5 (Burlein *et al.*, 1994).

The other mycobacterial expression vector that has been used in this study is pVV16, a gift from Dr. Varalakshme Vissa. The plasmid pVV16 is a pMV261 based mycobacterial expression vector, which has kanamycin and hygromycin resistance genes as selective markers. A 1.3 kbp segment encoding hygromycin (hyg) resistance was derived from the *Streptomyces* vector pIJ16 (obtained from Dr. Julia M. Inamine) by digestion with *Bam*HI and gel purification. The ends of this fragment were filled in with dNTPs using Klenow enzyme to catalyze the reaction. The resulting blunt end fragment was then ligated into the *Dra*I site of pMV261. This generated a 5.8 kb vector named pMV261.hyg. The *Nde*I cloning site was introduced into pMV261.hyg by changing the 3 bases upstream of the ATG codon of the hsp_{60} (heat shock protein) coding sequence. The obtained plasmid was designated as pJJV7. The histidine tag was introduced at the *Hind*III site of pJJV7, resulting in the final construct, namely pVV16. Plasmids pMV261 and pVV16 contain the promoter of *hsp60* which was described as a strong mycobacterial transcriptional promoter (Stover *et al.*, 1991) the same as that of the mycobacterial *hsp70* (Aldovini and Young, 1991) and the *Streptomyces groE* promoters (Winter *et al.*, 1991). The hsp60 translational initiation signals are used for expressing fusion proteins in pMV261 and its derivatives (Hatfull, 1993).

The other vector originating from pYUB12 and used in this study is pYUB18, a cosmid vector constructed by insertion of the lambda (λ) *cos* site into a *Bam*HI site of pYUB12. This cosmid can be used to clone large pieces of DNA ranging in size from 30 to 40

kb. The recombinant cosmids then can be packaged into lambda phage particles that are capable of infecting *E. coli*. The recombinant cosmids replicate in *E. coli* as a plasmid. After isolation from *E. coli* host cells, the recombinant cosmids can be used to transform mycobacterial hosts such as *M. smegmatis* or *M. bovis* BCG where they regain the ability to replicate as a plasmid (Jacobs *et al.*, 1991).

Most of the developed plasmids utilize the mycobacterial replicon from pAL5000. The pAL5000 derived plasmids include pYUB12, pRR3 (Ranes *et al.*, 1990; Snapper *et al.*, 1990), pMV261 (Stover *et al.*, 1991), and pMD31 (Donnelly-Wu *et al.*, 1993) and others. These plasmids have a copy number in mycobacteria of approximately 3-10 copies per cell (Ranes *et al.*, 1990, Stover *et al.*, 1991), and are poorly maintained in the absence of antibiotic selection (Lee *et al.*, 1991). The replication region from pAL5000 is not the only one that has been used in developing mycobacterial shuttle plasmid vectors. The replicons from the *M. scofulaceum* pMSC262 (Quin *et al.*, 1994), the *M. avium* pLR7 (Beggs *et al.*, 1995), and the *M. fortuitum* pJAZ38 (Gavigan *et al.*, 1997) were defined, sequenced and used to create other series of mycobacterial shuttle plasmids. Moreover, related plasmids possessing sequence homology to these replicons have been detected throughout mycobacteria (Falkinham and Crawford, 1994). However, little is known about the compatibility of these replicons in mycobacteria.

1.3.3 Mycobacterial insertion sequences

The insertion sequences (IS) are mobile DNA elements that are capable of transposition from one region of a DNA molecule to another on the same or a different chromosome. The insertion sequences vary in size (from 0.7 to 2.5 kb), genetic organization and behavior. In general, insertion sequences are naturally resident in host genomes. They are found in one or more copies per genome. The termini of each insertion sequence have inverted repeat sequences consisting of 16-41 base pairs (bp), the number depending on the element. The inverted repeat sequences flank a unique central region. This central region contains an open reading frame (ORF) encoding a transposase that recognizes the ends of the element, breaks the DNA at those ends and joins itself to target sequences.

Several insertion sequences have been identified in mycobacteria (McAdam *et al.*, 1994). Some insertion sequences, for example IS*900* from *M. paratuberculosis*, have specific sites of insertion (England *et al.*, 1991) while others such as IS*6110* appear to insert randomly into genomes of the *M. tuberculosis* complex. Mycobacterial insertion sequences can be used as species- or strain-specific markers. For example, IS*900* is specific for *M. paratuberculosis*, while IS*6110* has been used extensively for strain typing in the *M. tuberculosis* complex. It was noted that upon movement, insertion elements generate different types of DNA rearrangements including the integration of other genes into these elements (Hedges and Jacob, 1974).

1.3.3.1 Molecular genetics of IS*6110*

The insertion sequence designated as IS*6110* was first reported by Thierry *et al.* (1990). IS*6110* has been of great interest because of its potential for epidemiological and diagnostic applications. Two other elements, IS*986* and IS*987*, isolated from different *M. tuberculosis* and *M. bovis* strains, were found to differ in a few nucleotides (McAdam *et al.*, 1990; Hermans *et al.*, 1990b; Hermans *et al.*, 1991; Theirry *et al.*, 1990). These two insertion sequences can be considered essentially the same element and the same designation as IS*6110* except when specific copy is concerned (van Embden *et al.*, 1993).

IS*6110* is classified into the IS*3* family (van Embden *et al.*, 1993). It is present in other species of the *M. tuberculosis* complex but not in the more distantly related mycobacteria. The IS*6110* contains 1,355 base pairs (bp) and 28 bp inverted repeats at either end. Flanking the element are 3 bp direct repeats, probably resulting from target site duplication. The large open reading frame (ORF) encodes a putative transposase (Fig. 1.4). Analysis of the insertion sites of IS*6110* in strains with multiple copies of the element showed the randomness of the transposition event (Mendiola *et al.*, 1992). However, studies of *M. tuberculosis* and *M. bovis* strains, which have only one copy of IS*6110*, showed that the site of insertion is conserved (Hermans *et al.*, 1991). Therefore, the extent of random versus site-specific transposition of IS*6110* remains unclear.

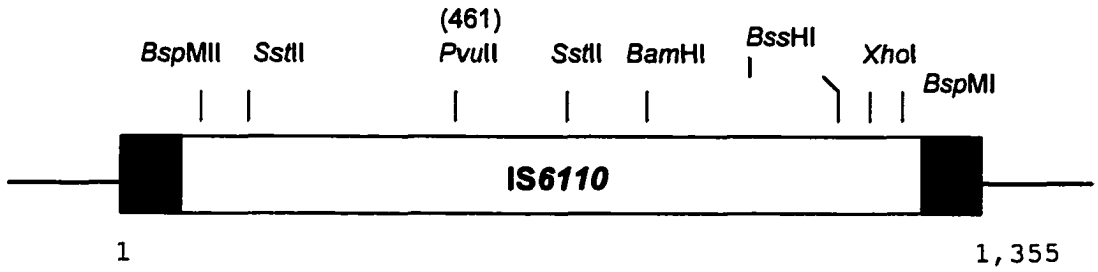


Figure 1.4 The physical map of IS*6110*. Various restriction enzymes that cleave within the 1,355 bp element are indicated. *Bam*HI, *Sst*II, *Bss*HI, and *Pvu*II have all been successfully used to generate restriction fragment polymorphisms (Mazurek *et al.*, 1991). *Pvu*II cleaves the element at base pair 461. The closed bars represent the 28 bp inverted repeat bordering IS*6110* DNA. The lines to the left and the right denote chromosomal DNA (van Embden *et al.*, 1993).

1.3.3.2 IS*6110* typing of *M. tuberculosis* complex and its application to aspects of this work

Restriction fragment length polymorphism (RFLP) in genomic DNA is commonly exploited to detect genetic diversity in the *M. tuberculosis* complex (Hermans *et al.*, 1990b). This method is based on DNA polymorphism caused by IS*6110*, which is not present in other species of mycobacteria. Different *M. tuberculosis* strains show great variability in the copy number and the locations of IS*6110* against a stable genetic background. As a result, restriction fragments carrying IS*6110* are highly polymorphic. A standardized methodology exploiting this polymorphism for discriminatory DNA fingerprints of *M. tuberculosis* strains was described (van Embden *et al.*, 1993). The standard protocol includes bacterial culturing, DNA extraction, restriction endonuclease digestion of chromosomal DNA and probing IS*6110* with its complementary sequence. The enzyme *Pvu*II is recommended for restriction enzyme digestion because it has been

used by most laboratories and it cleaves IS*6110* at a single site at base pair 461. Since *M. tuberculosis* usually contains 8-10 IS*6110* copies (van Soolingen *et al.*, 1991), the use of a DNA probe which spans both sides of the *Pvu*II site will result in 16 to 40 bands which overcrowd gel lanes or cause band overlapping. Therefore, DNA probe to the right of the *Pvu*II site on IS*6110* sequence reduces the number of IS*6110*-containing bands in the fingerprints to half of the maximum numbers. This standard method was applied as presented in detail in chapter 3 of this dissertation.

1.4 MYCOBACTERIAL CELL WALL

1.4.1 General structures

The distinctive feature of the mycobacterial cellular envelope is a thick, waxy cell wall. This structure contributes to certain characteristics of mycobacteria such as the acid fast staining properties, the low permeability of the cell wall, the resistance to harsh environments and the intrinsic resistance to many hydrophobic antibiotics (Jarlier and Nikaido, 1994; Brennan and Nikaido, 1995; Barry and Mdluli, 1996). The thick cell wall barrier is also attributed to the survival of the organism by suppressing the host immune systems (Takayama and Datta, 1991; Sibley *et al.*, 1988), preventing the access of immune factors, or acting as a direct modulator in the immunological reactions of the host to mycobacteria (Lederer *et al.*, 1975; Barry and Mdluli, 1996).

The cell wall of mycobacteria is located outside of and encompassing the bacterial plasma membrane (Fig. 1.5). It consists of the cell wall skeleton formed by a covalently linked structure of peptidoglycan, arabinogalactan, and mycolic acids (mAGP)(Daffe *et al.*, 1990; Besra *et al.*, 1995). The barrier to the influx of solutes such as nutrients or drugs is associated with a parallel alignment of mycolic acids (Liu *et al.*, 1995). As a consequence, mycobacteria develop aqueous channels formed by porin molecules in the cell wall structure. Other distinguishing cell wall components of mycobacteria include lipoarabinomannan (LAM), and outer layer lipids such as trehalose mycolates, lipooligosaccharides (LOSs), phenolic glycolipids (PGLs), glycopeptidolipids (GPLs), and sulfolipids (SLs)(Brennan and Nikaido, 1995). Recently, the roles of individual components of the cell wall have been elucidated, and much emphasis has been placed on the identification and characterization of various genes that encode enzymes involved in the synthesis of cell wall constituents. A better understanding of the cell wall structures, the functions and the biosynthesis of each cell wall component will lead to the identification of new drug targets and permit the development of new antituberculosis drugs targeting enzymes involved in the synthesis of such individual components.

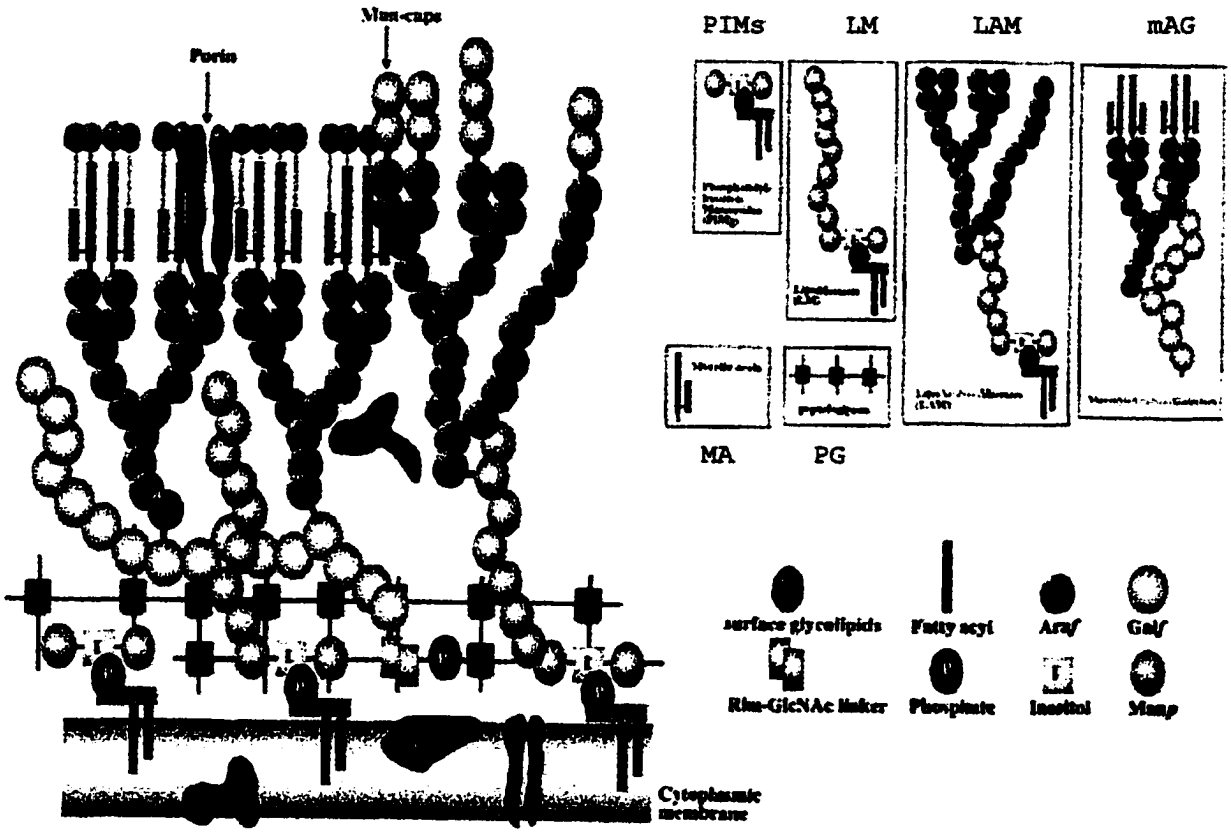


Figure 1.5 A model of mycobacterial cell envelope and associated lipids and glycolipids (adapted from Gordon *et al.*, 1999). Encompassing the cell membrane is a covalently linked structure of cell wall core consisting of peptidoglycan (PG) linked to mycolylarabinogalactan (mAG) complex. Other cell wall components noncovalently attached to mycolylarabinogalactan-peptidoglycan (mAGP) include lipoarabinomannan (LAM), lipomannan (LM), and phospholipids such as phosphatidylinositol mannosides (PIMs). Mycolic acids (MA) are esterified to the termini of arabinogalactan chains.

In the context of this review, a structural definition of some complex components of the mycobacterial cell wall will be presented, followed with a review of the syntheses of specific cell wall moieties that are related to this present study.

Cell wall core

One distinguishing characteristic of the mycobacterial cell wall is the absence of lipoteichoic acids and lipopolysaccharides (LPS), typical structures of the cell walls of Gram positive and Gram negative bacteria, respectively. Instead, the mycobacterial cell wall possesses a cell wall core which is a covalently linked skeleton of the mycolylarabinogalactan-peptidoglycan (mAGP)(Daffe *et al.*, 1990; Besra *et al.*, 1995). This structure is composed of the peptidoglycan to which is attached the linear D-galactan of arabinogalactan (AG) via a special linkage unit, -α-L-Rha*p* (1→3)-D-GlcNAc-P- (McNeil *et al.*, 1990; Mikusova *et al.*, 1996). Attached in turn to the non-reducing ends of pentaarabinose (Ara*f*) residues of AG are mycolic acids. Many lipopolysaccharides, lipoproteins and free lipids were found to be associated with the mAGP complex (Anderson and Brennan, 1994; Brennan and Nikaido, 1995). The structural units of the arabinogalactan-peptidoglycan (AGP) including linker unit are shown in Fig. 1.6.

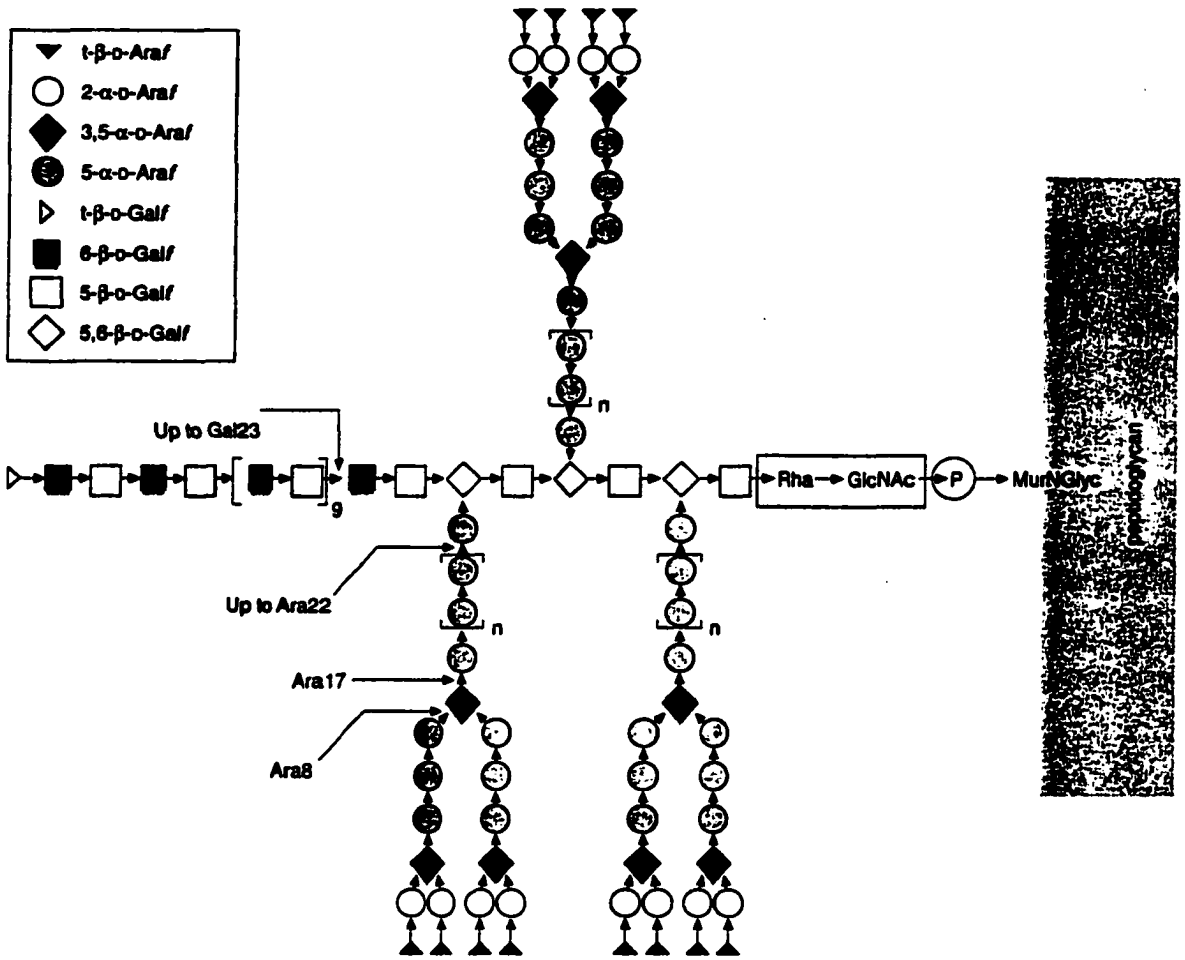


Figure 1.6 Chemical structure of the mycobacterial cell wall core, the arabinogalactan-peptidoglycan (AGP) complex. The mycolic acids in ester linkage to the terminal D-Araf units are not shown (taken from Baulard *et al.*, 1999).

Peptidoglycan (PG). The peptidoglycan (PG) is located innermost adjacent to the plasma membrane. The basic structure of PG is glycopeptides containing repeating unit of *N*-acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramic acid (Brennan and Nikaido, 1995). Attached to muramic acid is a tetrapeptide chain of L-

alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine (Schleifer and Kandler, 1972; Lederer *et al.*, 1975). This type of PG is classified as type Alaγ, which is one of the most common PG in bacteria (Schleifer and Kandler, 1972). However, the mycobacterial PG is different from PG in other bacteria in two features. The first one is the cross linkage between tetrapeptides of PG chains existing either as a linkage between two residues of diaminopimelic acid or a linkage between diaminopimelic acid and D-alanine (Ghuysen, 1968; Meitzerbin *et al.*, 1974). The second one is the muramic acid residue, which is in the form of *N*-glycolylmuramic acid instead of the usual *N*-acetylmuramic acid (Brennan and Nikaido, 1995). PG serves as a foundation structure forming the backbone of mAGP and provides shape, strength, and rigidity to the mycobacterial cell wall.

Arabinogalactan (AG). AG is a heteropolysaccharide chain of furanoid arabinose (Araf) and galactose (Galf)(McNeil *et al.*, 1987, Daffe *et al.*, 1990). The furanosyl residues are arranged into three district regions: the galactan core, the arabinan chains, and the non-reducing terminal segments of arabinan. Structural analysis of AG revealed that the linear D-galactan core of AG is composed of 5- and 6-linked β-D-galactofuranose (Galf) residues (Daffe *et al.*, 1990). The arabinan chains consist of linear 5-linked α-arabinose (Araf) residues with branching introduced by 3,5-α-Araf residues (Daffe *et al.*, 1993). The linkage of arabinan chains to the galactan core occurs at the C-5

of some of the 6-linked β-Gal*f* residues. There are approximately 2-3 arabinan chains attached to the galactan core (Brennan and Nikaido, 1995; Baulard *et al.*, 1999). Finally, the galactan core of AG, in turn, attaches to the C-6-position of muramic acids of peptidoglycan via a phosphodiester linkage of α-L-rhamnopyranose (Rha*p*)-(1→3)-D-*N*-acetylglucosamine (GlcNAc)-(1→phosphate) (Fig. 1.6) (McNeil *et al.*, 1990; Mikusova *et al.*, 1996). Clusters of four mycolic acids are then attached to the terminal arabinofuranosyl motifs of non-reducing ends of the arabinan chain via ester linkage (Fig. 1.7). Approximately two-thirds of the non-reducing ends of arabinan are mycolated at the 5 position of arabinofuranose (Ara*f*) residues (McNeil *et al.*, 1991).

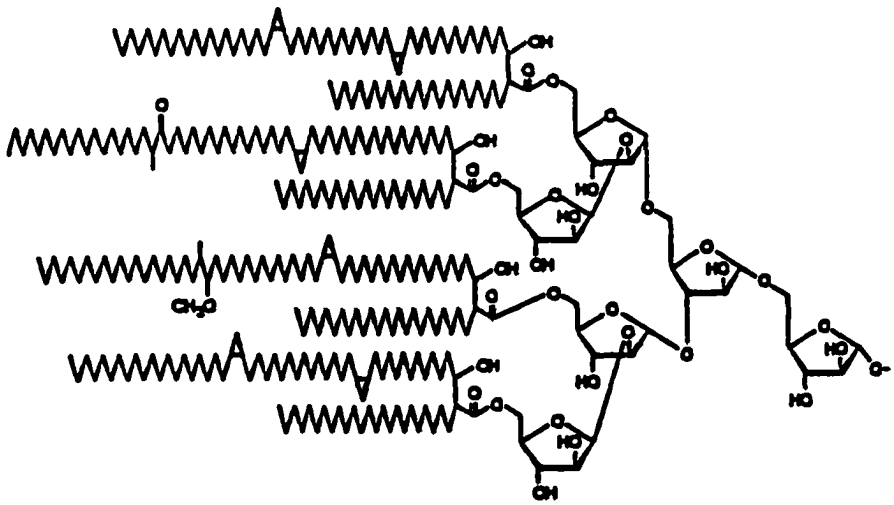


Figure 1.7 The linkage of clusters of mycolic acids to the pentaarabinosyl residues of AG (taken from Brennan and Nikaido, 1995).

Mycolic acids. Mycolic acids are large α-alkyl branched β-hydroxy fatty acids (Minnikin, 1982), constituting a major constituent of the mycobacterial cell wall (40-60% of the cell dry weight) (Brennan and Nikaido, 1995; Lee *et al.*, 1996). More commonly, mycolic acids exist in cells in two basic forms: non-extractable and extractable mycolic acids. The majority of mycolic acids are covalently bound to arabinosyl residues of AG via carboxylate ester to form a non-extractable tetramycolyl-pentaarbinosyl unit (McNeil *et al.*, 1991). Mycolic acids also occur within the fluid matrix as extractable lipids mainly in the form of trehalose 6,6'-dimycolate (TDM)(cord factor) and trehalose monomycolate (TMM) (Minnikin, 1982; Takayama and Armstrong, 1976). Small amounts of mycolates are esterified to glycerol or sugars such as glucose or fructose, usually depending on the sugar molecules present in the culture medium (Kolattukudy *et al.*, 1997).

Analysis of mycolic acid structure (R_1-CH(OH)-CH(R_2)-COOH) revealed that mycolic acids of mycobacteria range in size from ~70 to 90 carbons and frequently contain diverse functional groups in the backbone chain (R_1, the meromycolate)(Minnikin, 1982). The α-branch (R_2) is typically composed of 20-26 carbon, saturated fatty acids attached to the alpha position relative to the carboxylic group of β-meromycolic acid. It is notable that the α-branch lengths are species variable. Mostly, the fast-growing mycobacteria except *M. leprae* produce C_{20} α-branch, while slow growers produce mycolates containing C_{26} α-branched chain (Kusaka and Mori, 1986). *M. smegmatis* was found to contain mycolic acids

with 22 and 24 carbon α-branches (Kaneda *et al.*, 1995). Although α-branch chain lengths frequently generate variation in mycolic acids, the heterogeneity can be explained by the variation of the mero chain as well (Fig. 1.8). The meromycolates usually contain almost 60 carbons with a wide variety of functional groups such as unsaturates, methyl branches and cyclopropanes, or polar moieties such as ketones, methoxy groups, epoxides and esters. These modifications occur at proximal (closet to the β-hydroxy acid) and distal (closest to the ω-end) positions of meromycolates. Non polar modifications such as unsaturation and cylcopropanation occur at two parts while polar modifications are restricted to the distal position. According to these functional groups, mycolic acids can broadly be separated into two families: α-mycolates and oxygenated mycolates. The α-mycolates possess only desaturation or cyclopropanation in either *cis* or *trans* but when *trans* they also possess an adjacent methyl branch (Barry *et al.*, 1998). The α'-mycolate is exceptional because there is only one double bond existing in a short mero chain. The methoxymycolates contain a methoxy group adjacent to a methyl branch, in conjunction with a desaturation or cyclopropanation at the proximal position. In addition to a double bond or a cyclopropane, the functional group of α-methyl-branched ketone is specific for ketomycolates, while the α-methyl-branched epoxide is specific for epoxymycolates. The wax-ester mycolates contain a double bond and a cyclopropane with a typical ester group (Barry *et al.*, 1998). A variety of

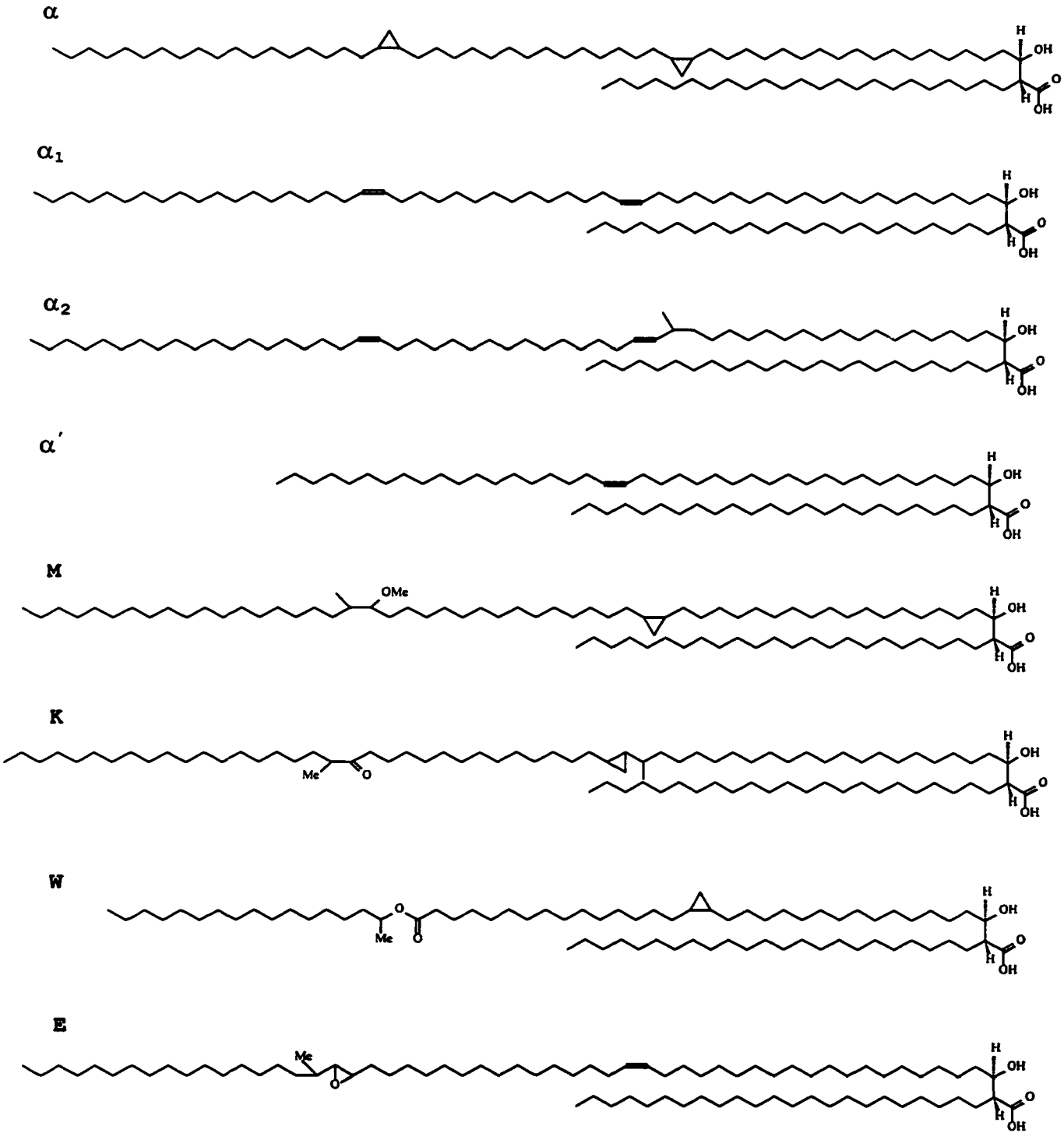


Figure 1.8 Structures of a variety of mycolic acids. M, methoxy; K, keto; W, wax-ester; E, epoxy (adapted from Barry *et al.*, 1998).

functional groups in meromycolates (Fig. 1.8) greatly contribute to mycobacterial taxonomy and can be utilized in differentiation of species of mycobacteria (Barry *et al.*, 1998; Minnikin *et al.*, 1984a; Minnikin *et al.*, 1984b).

In *M. tuberculosis*, three distinct mycolate species are present, α-mycolates, methoxy- and ketomycolates. The α-mycolates are the most common type of mycolic acids that are found in all species of mycobacteria (Barry *et al.*, 1998). However, the α-mycolates of *M. tuberculosis* as well as other pathogenic and slow-growing species contain two cyclopropane rings in *cis* configuration (Minnikin, 1982), which differ from the α-mycolates present in other mycobacterial species. Fast-growing and saprophytic mycobacteria fail to produce cyclopropanated mycolic acids, and therefore their α-mycolates contain double bonds in place of cyclopropane rings (Yuan *et al.*, 1995). Ketomycolates are the next most abundant mycolic acids present in numerous mycobacterial species (Minnikin, 1982; Barry *et al.*, 1998). It was noted that species that produce ketomycolates usually produce methoxymycolates as well. Methoxymycolates are found in *M. tuberculosis* (Minnikin and Polgar, 1967) including *M. bovis* and certain strains of *M. bovis* BCG such as strain Moreau but not strains Glaxo, Prague, or Pasteur (Minnikin *et al.*, 1983). Early work reported that methoxymycolates are also present in a few more species such as *M. microti* (Davison *et al.*, 1982), *M. marinum*, *M. ulcerans* (Daffe *et al.*, 1991), *M. asiaticum*, *M. gasti*, *M. gordonae*, *M. kansasii*, *M. szulgai*, and *M. africanum* (Kaneda *et*

al., 1986; Minnikin *et al.*, 1984b). Other widely distributed mycolates are α′ and wax-ester mycolates but they are absent from *M. tuberculosis*. Epoxymycolates are restricted to a few species such as *M. aurum* (Barry *et al.*, 1998).

1.4.2 Biosyntheses

The biosynthesis of the mycobacterial cell wall, especially the biosynthetic route to mycolic acids, is of interest and widely investigated. The distinct structure of mycolic acids plays a critical role in determining the properties of low fluidity and low permeability of an asymmetrical lipid bilayer (Liu *et al.*, 1995; 1996). Mycolic acids shield the organism from hostile environments and contribute to disease persistence as well as the refractoriness of mycobacteria to many antibiotics (Brennan and Nikaido, 1995). The success of chemotherapeutic agents such as INH and ETH that specifically inhibit mycolic acid synthesis confirms the essentiality of this structure (Winder and Collins, 1970; Quemard *et al.*, 1992). Since mycolic acids are important to mycobacterial cells, the biosynthetic pathway of mycolic acids remains a desirable target for the rational design of new antituberculosis drugs.

It must be emphasized that fatty acid biosynthesis is another area of considerable research regarding a wide range of free lipids, bound lipids and unusual fatty acids found in mycobacteria (Brennan and Nikaido, 1995; Minnikin, 1982). In addition, fatty acid synthesis also provides primer units for mycolic acid

synthesis. It is apparent that fatty acid synthesis provides potential targets for various antimicrobial agents including antituberculosis drugs (Banerjee *et al.*, 1994; Miyakawa *et al.*, 1982, Slayden *et al.*, 1996). Therefore understanding the synthesis of fatty acids is important in search of new drug targets and development of new antituberculosis drugs.

1.4.2.1 Synthesis of saturated fatty acids

Mycobacteria synthesize a variety of fatty acids such as straight-chain saturated fatty acids, straight-chain monounsaturated fatty acids, polyunsaturated fatty acids and methyl-branched fatty acids (Ratledge, 1982; Hung and Walker, 1970). Short-chain saturated fatty acids are known to be primers for the synthesis of long-chain fatty acids as well as the synthesis of α-branches and meromycolates involved in mycolic acid synthesis. A unique feature of fatty acid synthesis in mycobacteria is that all fatty acid intermediates are covalently bound to either Coenzyme A (CoA) or acyl carrier protein (ACP). The synthesis of CoA involves multiple reactions. At final, the mature CoA is used in the synthesis of ACP and the synthesis of fatty acids (Jackowski, 1996; Jackowski and Rock, 1981).

The mechanisms of short-chain saturated fatty acid synthesis proceed from acetyl-CoA and involve two stages: initiation and cyclic elongation.

Initiation of fatty acid synthesis. The first committed step of fatty acid synthesis is the conversion of acetyl-CoA (CH_2-CO-CoA)

to malonyl-CoA ($HOOC-CH_2-CO-CoA$) by acetyl-CoA carboxylase. Early studies proposed that in *E. coli* acetyl-CoA carboxylase is a multifunctional enzyme complex consisting of three separate enzymes: biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase. The acetyl-CoA carboxylase is known to catalyse the carboxylation in two steps: the carboxylation of biotin (BCCP) by biotin carboxylase and the transfer of the carboxyl group from the carboxy-biotin complex to acetyl-CoA (Fig. 1.9). The final product of the reaction is malonyl-CoA, which is utilized as a precursor in *de novo* fatty acid synthesis (Cronan and Rock, 1996).

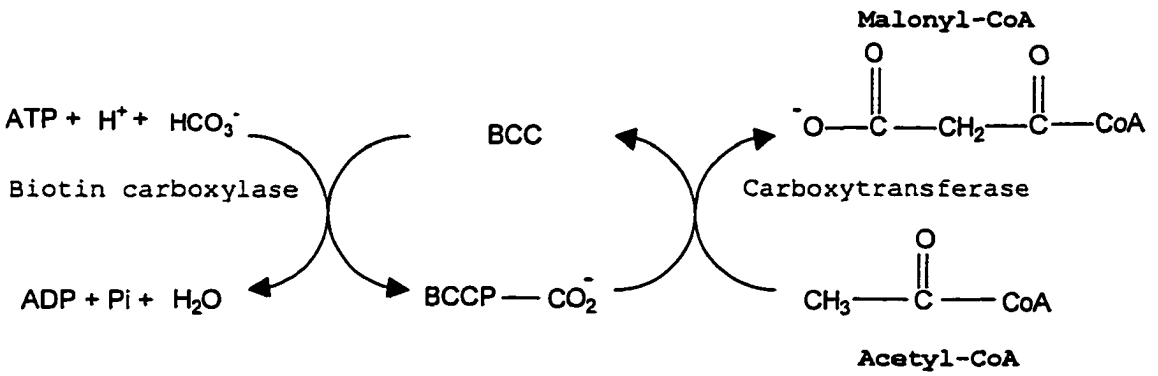


Figure 1.9 A scheme for the synthesis of acetyl-CoA catalyzed by acetyl-CoA carboxylase. The first reaction is the carboxylation of biotin (BCCP, a product of the *accB* gene) catalyzed by biotin carboxylase (a product of the *accC* gene). The second reaction is the transfer of the CO_2 moiety to acetyl-CoA by carboxytransferase, a heterodimer composed of the *accA* and *accD* gene products (Magnuson *et al.*, 1993a).

The two-step reactions of carboxylation to form malonyl-CoA also occur in mycobacteria (Khan and Venkitasubramanian, 1964). The mycobacterial acetyl-CoA carboxylase is a multienzyme complex that is capable of catalyzing the carboxylation of acetyl-CoA as well as propionyl-CoA. The product from carboxylation of

propionyl-CoA is methylmalonyl-CoA, which is utilized as a precursor for the synthesis of mycocerosic acids and phthiocerol acids rather than the conventional fatty acids (Gastambide-Odier *et al.*, 1963; Norman *et al.*, 1994; Rainwater and Kolattukudy, 1985).

Fatty acid elongation. Fatty acid elongation involves the coupling of a unit of two carbons from malonyl-CoA with the growing acyl chain. The reactions of acyl chain elongation occur through repetitive cycles of condensation, keto reduction, dehydration and enoyl reduction. The cycles are catalyzed by four key enzymes of the fatty acid synthase (FAS): β-ketoacyl synthase (KAS), β-ketoacyl reductase (KR), β-ketoacyl dehydrase (DE) and enoyl reductase (ER)(Magnuson *et al.*, 1993b; Barry *et al.*, 1998). The products of each standard cycle are straight-chains of saturated fatty acids capable of entering new rounds of elongation until a terminal extension is attained (Barry *et al.*, 1998).

The condensation of a growing acyl chain with malonate to yield a β-ketoacyl product is the first step in elongation and is catalyzed by β-ketoacyl synthase (KAS; KasA/B). The β-ketoacyl product from the synthase reaction is then reduced to the β-ketoacyl hydroxy by the NADH-dependent reductase (KR; MabA). The subsequent reaction is the dehydration by β-ketoacyl dehydrase (DE) to yield the α, β-unsaturated enoyl intermediate (Kass *et al.*, 1967). The final step is the reduction of the dehydrase product catalyzed by *trans*-enoyl reductase (ER; InhA) to form a

longer chain of saturated hydrocarbon. This in turn serves as a substrate for another round of elongation (Bergler *et al.*, 1994; Cronan and Rock, 1996). The enzymes catalyzing elongation of fatty acids are apparently effective targets for antituberculois drugs. One of these enzymes is NADH-dependent-*trans*-enoyl reductase which has been shown to be a lethal target of INH and ETH (Banerjee *et al.*, 1994; Quemard *et al.*, 1992).

Fatty acid synthase (FAS). Fatty acid synthases are cytosolic enzyme systems responsible for the elongation of hydrocarbons in fatty acid synthesis. There are two types of fatty acid synthases (FAS), FAS I and FAS II systems (Brindley *et al.*, 1969; Bloch, 1977). FAS I is a large aggregated polyfunctional enzyme, in which all of the above-mentioned enzymatic activities are encoded and found in all eukaryotes except plants (Brindley *et al.*, 1969; Kikuchi *et al.*, 1992). FAS II is comprised of multicomponent enzymes found in many bacteria and plants. It is ACP dependent and catalyzes the individual reactions by separate enzymes that function as a complete system (Cronan and Rock, 1996). Mycobacteria as well as corynebacteria are unusual because they possess both FAS I and FAS II (Bloch, 1975; 1977). The characteristics of mycobacterial FAS I and FAS II are compared in Table 1.4.

Table 1.4 Comparison of properties of mycobacterial FAS I and FAS II systems (Barry *et al.*, 1998).

Property	FAS I	FAS II
De novo synthesis	+	-
Palmitoyl-CoA elongation	+	+
Elongation products	C_{16}, C_{18}, C_{26}-C_{36}-CoA	C_{18}-C_{50}-ACP
Low ionic strength	Inactivation	No effect
Nucleotide requirement	NADPH/NADH	NADPH or NADH
Limiting step	Product dissociation	Unknown

Early studies suggested the role of the mycobacterial FAS I in *de novo* synthesis of fatty acid from acetate. However, the difference between FAS I from eukaryotes and mycobacteria is that the mycobacterial FAS I is able to extend the length of fatty acid synthesis beyond the usual C_{16} to C_{18} and up to C_{24} and C_{26} fatty acyl-CoA (Bloch, 1977; Wood *et al.*, 1977). The mycobacterial FAS II is unable to perform *de novo* synthesis, but it preferentially elongates palmitoyl-CoA to a mixture of saturated fatty acids ranging from 18 to 32 carbons (Brindley *et al.*, 1969). The work of Qureshi *et al.* (1984) demonstrated that mycobacterial FAS II is capable of catalyzing acyl elongation from malonate to primary products of 16, 18, 24 and 26 carbons. In addition, it also extends the acyl chain from palmitate to C_{26} to C_{40}-monounsaturated fatty acyl-ACPs. Attempts were made to determine the roles of FAS I and FAS II in the complete synthesis of mycolic acids. Qureshi

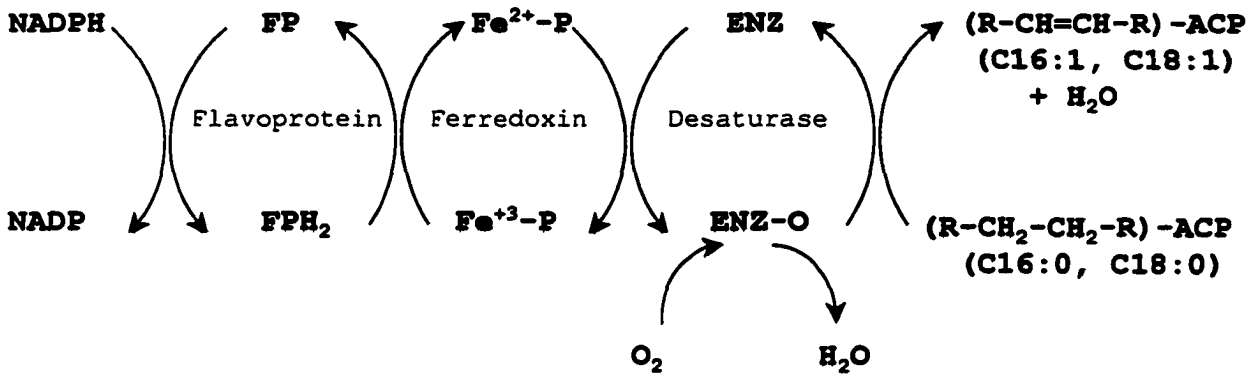
et al. (1984) also demonstrated the elongation of a C_{24} fatty acid to C_{52} meromycolate, but not to a mature mycolate, by cytosolic extract of *M. tuberculosis* H37Ra. It appears that neither cytosolic system individually nor in combination is able to produce the mycolic acids. Thus, mycolic acid synthesis requires a separate synthase system. Subsequent studies showed that a particulate fraction is capable of synthesizing complete mycolic acids from acetate (Lacave *et al.*, 1990; Slayden *et al.*, 1996) and also from a $C_{24:1}$ Δ5 fatty acid (Wheeler *et al.*, 1993). This evidence indicates the existence of a mycolic acid synthase system in the particulate fraction, and suggests that the enzymatic machinery responsible for mycolic acid synthesis is confined to the mycobacterial membrane/cell wall.

1.4.2.2 Synthesis of unsaturated and branched chain fatty acids

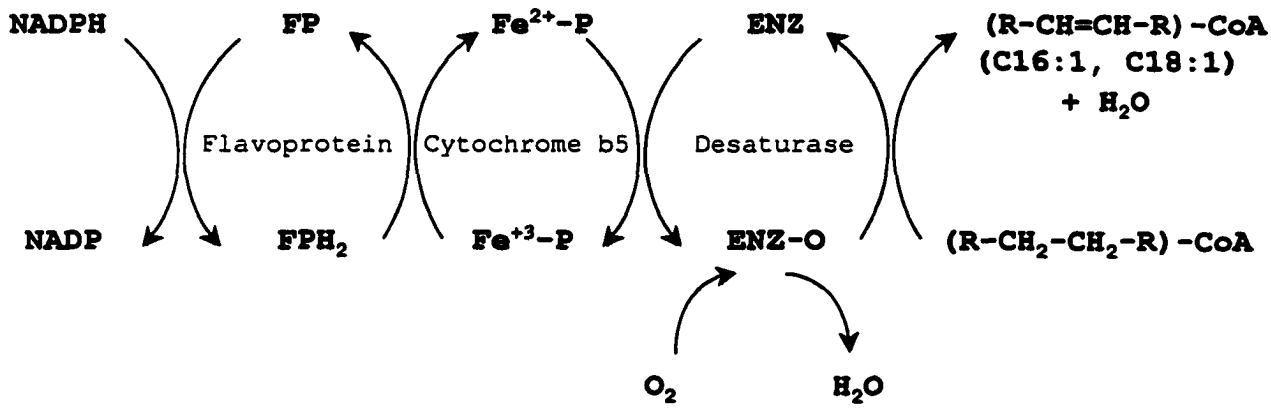
Mycobacteria have a wide variety of very long-chain saturated (C_{18}-C_{32}) and unsaturated fatty acids. The monounsaturated fatty acids exist in all mycobacteria and the most abundant is oleic acid ($C_{18:1}$ Δ9). Other monounsaturated fatty acids described in *M. smegmatis* and *M. bovis* BCG have acyl chains in the range of C_{14} to C_{26} (Hung and Walker, 1970). It was reported that the $C_{14:1}$ and $C_{16:1}$ fatty acids from *M. smegmatis* are principally Δ10, while the $C_{17:1}$, $C_{18:1}$ and $C_{19:1}$ fatty acids from both organisms are Δ9-unsaturated fatty acids. The double bond of *M. smegmatis* $C_{20:1}$, $C_{22:1}$ and $C_{24:1}$ fatty acids are principally Δ11, Δ13 and Δ15, respectively, while

the $C_{22:1}$, $C_{24:1}$ and $C_{26:1}$ fatty acids of BCG are principally Δ13, Δ15 and Δ17, respectively (Hung and Walker, 1970). The oleic acid is of interest because it is a major monounsaturated fatty acid found mainly in phospholipids, and thereby is important for membrane integrity and function. The enzyme that catalyzes the oxidative desaturation to form oleic acid is Δ9 desaturase, which converts stearoyl-CoA into oleoyl-CoA and, to a lesser extent, palmitoyl-CoA into palmitoleyl-CoA. The mycobacterial Δ9 desaturase was first identified in *M. phlei* (Fulco and Bloch, 1964). The early studies revealed that the Δ9 desaturase is sensitive to cyanide suggesting that the desaturation is associated with the electron transport system known to be FAD-requiring NADPH-cytochrome c reductase (Kashiwabara and Sato, 1973). The coupling of desaturation and electron transport in various organisms is summarized in Fig. 1.10. Further studies showed that the enzyme requires iron for its activity (Kashiwabara *et al.*, 1975), and the substrate fatty acid attaches to the enzyme via a covalent bond between the carboxyl group of the substrate and the thiol group of the enzyme. The product of Δ9 desaturation can be elongated, and therefore the presence of $C_{20:1}$ Δ11, $C_{22:1}$ Δ13, $C_{24:1}$ Δ15 and $C_{26:1}$ Δ17 acids can be explained by the C_2 elongation of oleic acids (Hung and Walker, 1970).

Stearoyl-ACP desaturase (Fulco, 1974)



Stearoyl-CoA desaturase (Holloway, 1983)



Δ9 Desaturase-CoA of *M. phlei* (Kashiwabara and Sato, 1973)

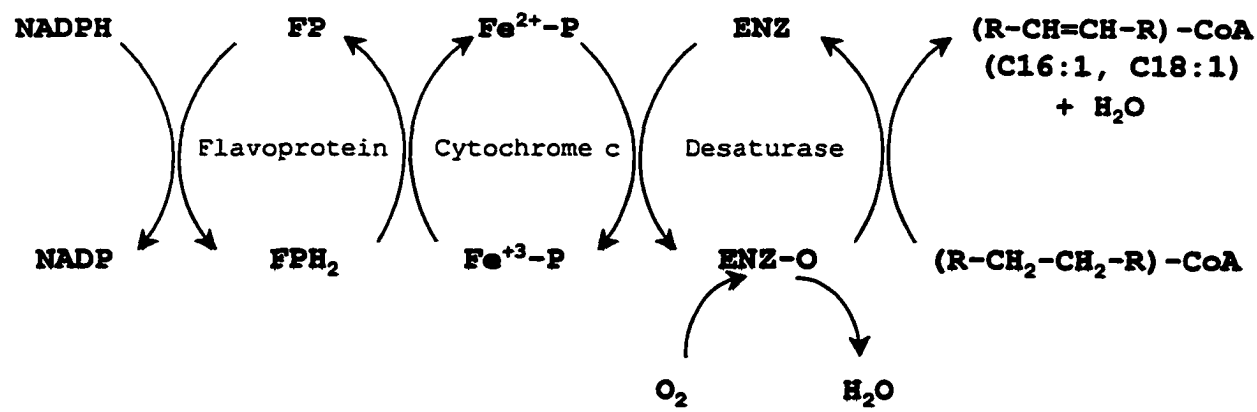
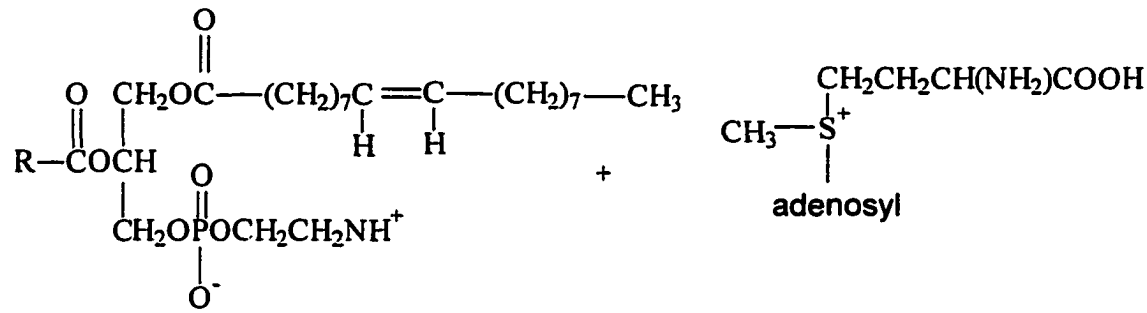


Figure 1.10 Diagrams of fatty acid desaturations coupled with electron transport. There are three types of fatty acid desaturases: acyl-ACP desaturase, acyl-CoA desaturase and lipid-desaturase (Murata and Wada, 1995). Acyl-ACP desaturases are present in plants. Acyl-CoA desaturases of animals and fungi use cytochrome b5 as electron donor. Cytochrome c is involved in the Δ9 desaturation of *M. phlei*.

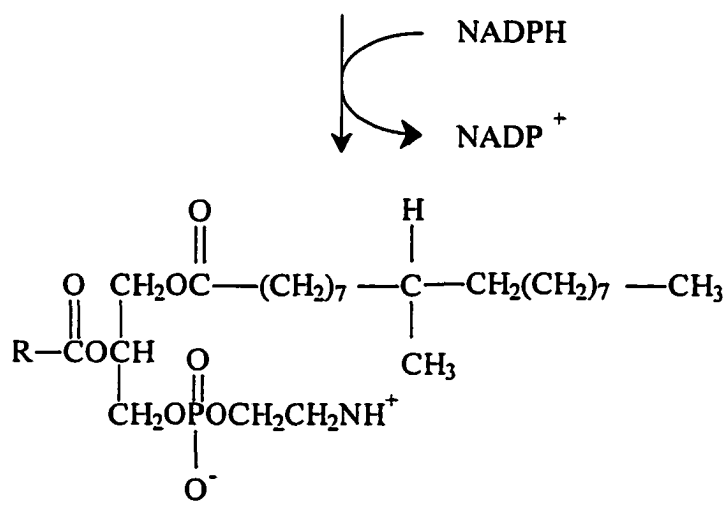
Based on the homology of amino acid sequences, there are three open reading frames in the *M. tuberculosis* genome (*desA1*, *desA2* and *desA3*) predicted to encode desaturases (Cole *et al.*, 1998). DesA1 (a product of the *desA1* gene) and DesA2 (a product of the *desA2* gene) are clear homologs to plant-stearoyl-ACP-desaturase (Jackson *et al.*, 1997; Cole *et al.*, 1998), while DesA3 (a product of the *desA3* gene) is a homolog of linoleoyl-CoA-desaturase of *Synechocystis spp.* The characterization of the enzymatic functions of proteins encoded by the *desA* genes may lead to a better understanding of fatty acid and mycolic acid synthesis in *M. tuberculosis.*

Besides monounsaturated fatty acids, polyunsaturated long-chain fatty acids were described in mycobacteria. The main type of these acids is phleic acids which have been found in *M. smegmatis* but not in *M. tuberculosis* and *M. bovis* BCG. The roles of these complex acids are unknown (Asselineau, 1972). In addition to polyunsaturated fatty acids a large number of methyl-branched fatty acids exist in mycobacteria (Campbell and Norawal, 1969). These acids fall into two main categories: iso and anteiso-acids, and acids containing one or more methyl groups in the chains. Tuberculostearic acid (D10-methyl stearic acid) is a major mono-methyl branched chain fatty acid, and is widely distributed in *M. tuberculosis* and *M. smegmatis* (Lennarz *et al.*, 1962). It is found to attach to the carbon one position of glycerolphospholipid and is found in phosphatidylinositol (PI), an anchor part of LAM. The synthesis of tuberculostearic acid has

been studied mainly in *M. phlei* where it has been shown to be synthesized by methylation of oleic acid, while oleic acid is a part of phospholipidethanolamine and *S*-adenosylmethionine (SAM) is a methyl donor (Fig. 1.11) (Lennarz *et al.*, 1962).



Oleoyl phosphatidylethanolamine S-adenosylmethionine



Tuberculostearoyl phosphatidylethanolomine

Figure 1.11 A scheme for tuberculostearic acid synthesis (Lennarz *et al.*, 1962).

Other branched chain fatty acids which have more than one methyl branched chains are pthienoic acid (trimethyl unsaturated C_{27} acid) and mycocerosic acid (tetramethyl-branched C_{28}-C_{32} fatty acid) (Polgar, 1971). These acids are confined to *M. tuberculosis* and *M. bovis*. Therefore, their roles are probably associated with

virulence and pathogenesis of virulent mycobacteria. The syntheses of pthienoic and mycocerosic acids were proposed to be entirely different from tuberculostearic aid synthesis, and probably involve the elongation of fatty acids using propionic acid as a precursor (Gastambide-Odier *et al.*, 1966).

1.4.2.3 Synthesis of mycolic acids

The biosynthesis of mycolic acids has been studied intensively. Several steps in the pathway are now understood, but knowledge of the overall metabolic process leading to this complex group of fatty acids remains obscure. However, based on structural analysis and metabolic labeling, Takayama and Qureshi (1984) proposed that mycolic acid synthesis proceeds through four main steps as described below. This early proposed pathway has been supported by the recent work of Barry and colleagues (1998). A scheme for mycolic acid synthesis is shown in Fig. 1.12.

Synthesis of α-branch and mero primer. The synthesis of the α-branch and the mero primer is analogous to the event that occurs in *de novo* fatty acid synthesis (Barry *et al.*, 1998). It was proposed that FAS I activity provides either C_{16} or C_{26} intermediates, or precursors for FAS II, which produces C_{24} and C_{26} ACP derivatives for primers of meromycolic acids. ACP is a low-molecular-weight protein responsible for carrying the growing acyl chains around the various enzymes of FAS II (Bloch, 1977; Brindley *et al.*, 1969) and capable of carrying acyl chains to the size of the full-length meromycolic acids (Barry *et al.*, 1998).

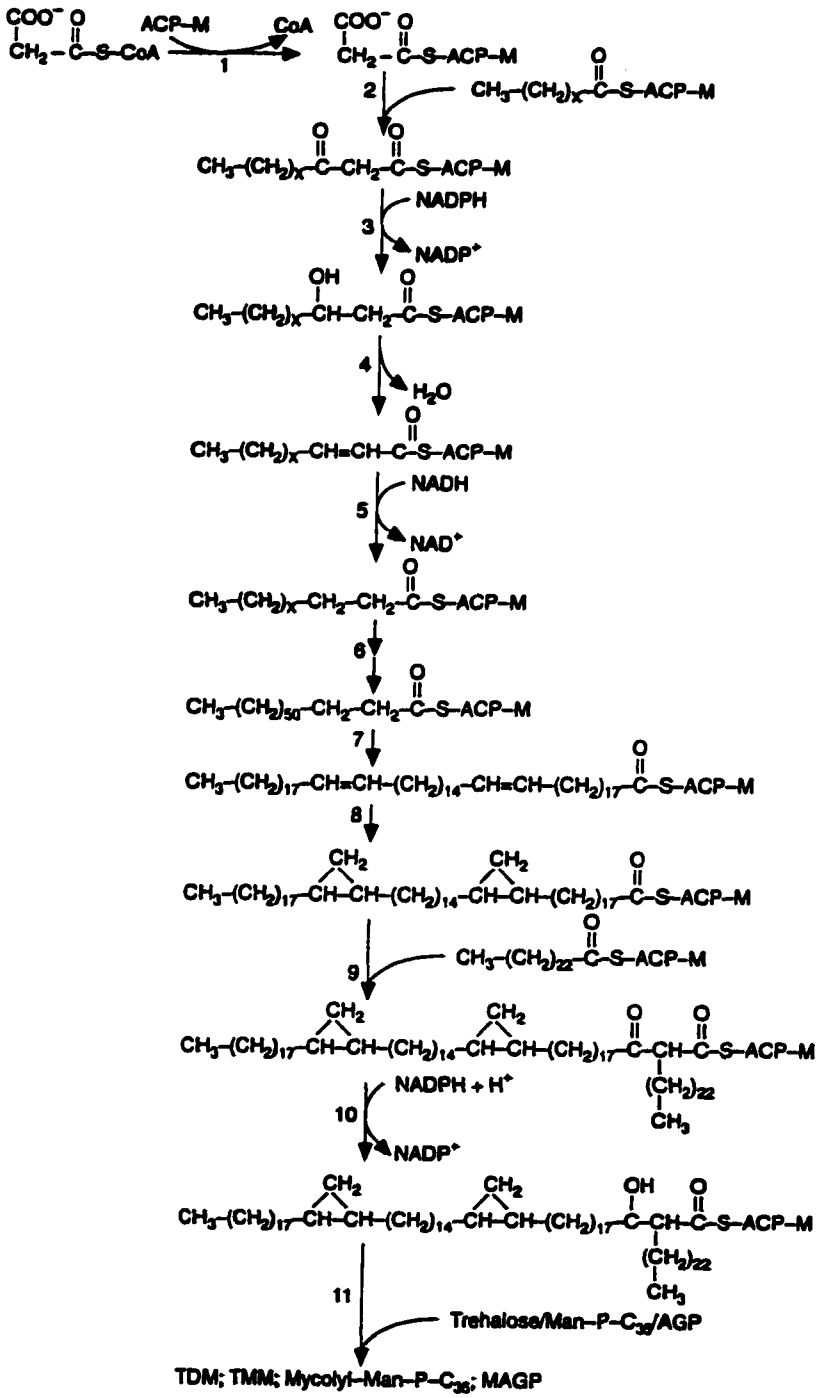


Figure 1.12 A scheme for mycolic acid synthesis (taken from Baulard *et al.*, 1999). The scheme represents the possible pathway for α-mycolic acid synthesis. Deposition of a mature mycolate in the cell wall is also demonstrated. (1) FabD, a malonyl-CoA/ACP transacylase. (2) KAS, β-ketoacyl ACP synthase. (3) KR, β-ketoacyl ACP reductase. (4) DE, β-ketoacyl ACP dehydrase. (5) EA, enoyl ACP reductase. (6) Many repetitions of step 2-5. (7) Desaturation of meromycolates. (8) Cyclopropanation. (9) Addition of the α-chain. (10) Reduction of the 3-keto group. (11) Transfer of mature mycolates from one carrier to either trehalose or a polyprenyl-P-Man or the final AGP complex.

Synthesis of long chain meromycolates. The meromycolic acids are approximately C_{40}-C_{60} acids, which form the main carbon backbone of mycolic acids. It was thought that the mero primers containing C_{16} to C_{26} carbons are derived from *de novo* synthesis of fatty acids. The primers are then extended to longer chains and intact meromycolates by the FAS II system. Recently, the enzyme machinery of FAS II was identified to consist of FabD, AcpM, KasA, KasB, AccB, InhA and MabA (Barry *et al.*, 1998). FabD contains malonyl-CoA/ACP transferase activity (Cronan and Rock, 1996). This enzyme transacylates ACP to form malonyl-ACP, which is utilized by Kas enzymes. AcpM functions by carrying growing acyl chains to the active center of each lipogenic enzyme and allowing enzymes to recognize their individual substrates. KasA and KasB (analog of *E. coli* FabF) have β-ketoacyl synthase activity presumably catalyzing the formation of primers of 26 to 30 carbons. In addition, KasA and KasB may function independently or together to produce an intact meromycolate (48-54 carbons) (Barry *et al.*, 1998). AccB, known as an acetyl-CoA carboxylase, catalyzes the carboxylation of acetyl-CoA to form malonate (Cronan and Rock, 1996). InhA (analog of *E. coli* FabI) has been well characterized to be *trans*-enoyl-ACP reductase (Quemard *et al.*, 1995; Banerjee *et al.*, 1994), while MabA contains ketoacyl reductase activity. It is possible that the synthesis of the full-length meromycolate occurs by the coupling of the stepwise elongation with desaturation. In this scenario the pathway to the synthesis of mycolic acids involves the elongation of $C_{24:1}$ Δ5

fatty acid to the meromycolate and then the condensation of this molecule with the α-branch. Another possibility has been proposed recently in which the KasA/KasB system catalyzes acyl elongation to a certain length. To attain full-length meromycolates, the mero primers are then transferred to one of many type I systems which have not yet been identified (Mdluli *et al.*, 1998).

Modifications of meromycolates. The modifications of the mero chains occur through a variety of reactions such as desaturation, cyclopropanation, as well as the formation of methoxy, keto, and epoxy derivatives. Six genes (*cmaA1, cmaA2, mmaA1, mmaA2, mmaA3,* and *mmaA4*) are now known to encode enzymes involved in the modification processes (Barry *et al.*, 1998). There are two more genes (*umaA1* and *umaA2*) whose gene products have not been identified in terms of their accurate functions but presumably they have functions involved in modifications of the meromycolates (Table 1.3) (Salman *et al.*, 1999).

Desaturation. The desaturation is thought to be the first modification reaction, which involves the insertion of double bonds into the saturated alkyl chain of meromycolates. The product of desaturation is the unsaturated mero acid containing two *cis* double bonds (one at the proximal position and the other one at distal position) and can be further modified (Barry *et al.*, 1998). Qureshi *et al.* (1984) demonstrated that the desaturation occurred on a full-length mero acid prior to condensation with the α-branch and is catalyzed by an unidentified aerobic desaturase. However, the identification of unsaturated and cyclopropanated

fatty acids shorter than the full-length mero chains suggested that desaturation occurred during elongation (Qureshi *et al*, 1980; Takayama *et al.*, 1978). In this explanation, the distal double bond was introduced by a dehydration during fatty acid synthesis and catalyzed by a specific β-hydroxyacyl-ACP dehydrase (a *fabA*-like gene product). A specialized Kas (a *fabB*-like gene product) then catalyzes the next elongation step until a full-length mero chain is attained (Barry *et al.*, 1998). Barry *et al.* (1998) further proposed that the desaturation of the meromycolate, whether coupled to elongation or not, occurs through the reaction catalyzed by a mixed function desaturase. However, a complete working model for desaturation of meromycolates is still unclear.

Modifications of meromycolates involving the methyl transfer from S-adenosylmethionine (SAM). The functional groups occurring through the addition of a methyl (or methylene) group derived from the methionine of SAM into meromycolates include the methylene of cyclopropane rings, the methyl branches adjacent to *trans*-configuration, the methoxy and the keto moieties (Daffe *et al.*, 1991, Lacave *et al.*, 1991, Daneilson and Gray 1982; Wong *et al.*, 1979). It had long been proposed that cyclopropane groups in the mero portion of mycolic acids were derived from double bonds (Grogan and Cronan, 1997). Subsequent identification of the *M. tuberculosis cmaA1* and *cmaA2* genes supported this proposal. The *cmaA1* which encodes cyclopropane mycolic acid synthase-1 (CMAS-1) introduces a cyclopropane ring into a distal *cis* double bond (Yuan *et al.*, 1995), while CMAS-2 cyclopropanates a proximal *cis* double

bond (George *et al.*, 1995). Both enzymes show a high degree of homology to other SAM-dependent methyltransferases indicating that the cyclopropanation involves the transfer of a methyl group from SAM into double bond portions (Yuan *et al.*, 1995; George *et al.*, 1995). Recent studies led to the identification of the *M. tuberculosis mmaA* gene cluster consisting of the *mmaA1, mmaA2, mmaA3,* and the *mmaA4* genes (Yuan and Barry, 1996). MMAS-4, the product of the *mmaA4* gene, catalyzes the introduction of a hydroxy group with an adjacent methyl branch at the distal position of mero acids (Yuan and Barry, 1996; Yuan *et al.*, 1998). MMAS-3 encoded by the *mma3* gene then methylates the hydroxymycolates to form methoxymycolates (Yuan and Barry, 1996; Yuan *et al.*, 1998). MMAS-2 encoded by the *mmaA2* gene is known to have similar function to the *cmaA-2* gene product, and both cyclopropanate the proximal *cis* double bond (Yuan and Barry, 1996). MMAS-1 appears to be involved in the conversion of the proximal *cis* double bond to a *trans* double bond with an adjacent methyl branch (Yuan *et al.*, 1997).

Oxidative modifications. Various reactions occur to produce oxygenated mycolic acids containing functional groups of keto-, epoxy-, wax-ester-, and methoxymycolates (Barry *et al.*, 1998). Quemard *et al.* (1992) proposed that oxygenated and unsaturated mycolic acids could be synthesized from a common unidentified precursor by parallel pathways. Yuan and Barry (1996) demonstrated that the methoxymycolates are derived from the hydroxymycolates. The conversion of the hydroxymycolates to the

methoxymycolates is catalyzed by MMAS-3 (Barry *et al.*, 1998). More recently, it has been shown that the keto- and methoxymycolates have a link by sharing a common precursor, a hydroxymycolate (Yuan and Barry, 1996; Yuan *et al.*, 1998). The epoxymycolates were identified to have an unsaturated precursor while the wax-ester mycolates were synthesized from ketomycolates (Etemadi and Casches, 1982). However, many enzymes responsible for the introduction of oxidative functional groups into mycolates have not been conclusively identified.

Condensation of α-branches and modified meromycolates. The final step in the synthesis of mycolic acids is a Claisen-type condensation step by which the α-branch (C_{20}-C_{26} fatty acids) condenses on the α position of the modified mero chain. The derived intermediate is a β-oxomycolate, which is then reduced to a mature mycolic acid (β-hydroxy acid) (Besra and Chatterjee, 1994; Lee *et al.*, 1997; Takayama and Qureshi, 1984). Most studies of this process have been performed in *Corynebacterium spp*, which has structurally simple mycolic acids. It was proposed that in this organism a β-oxocorynomycolate is a carrier in condensation (Datta and Takayama, 1993). Subsequent identification in *M. smegmatis* of a mycolylmannosyl phosphoheptaprenol (Myc-PL), which is a mannosyl-P-polyprenol attached by mycolic acid at the 6 position of mannose, suggested that the Myc-PL is the end product of the condensation and the Claisen reaction probably occurs on this polyprenol carrier (Besra *et al.*, 1994). However, Barry *et al.* (1998) proposed that the carriers for the condensation are

likely to be an ACP-type protein, CoA factor or a membrane protein. In addition to the need for carriers, it was thought that the α-carbon of the α-branch must turn into a nucleophile while the carbonyl carbon of the mero acid acts as an electrophile. The reaction of both parts results in the formation of β-oxomycolate, which is then converted to mature mycolic acids (Barry *et al.*, 1998).

At the end of mycolic acid synthesis, the newly synthesized mycolic acids are transferred to carrier molecules for deposition on trehalose or for formation the final product, mAGP. Very little is known about the transferring process of mature mycolates. As mentioned aboved, Besra *et al.* (1994) suggested that Myc-PL is the reduced end-product of the Claisen condensation reaction and it is possibly a substrate for the subsequent transfer of mycolic acids into cell wall components. More recently, Baulard *et al.* (1999) has proposed that TMM and TDM are carrier molecules for the cell wall bound mycolates. The enzymes involved in the transfer of the complete mycolates have been studied to some extent. Sathymoorthy and Takayama (1987) identified a mycolyltransferase and showed that this enzyme was involved in the synthesis of TDM from two molecules of TMM. The recent work of Belisle *et al.* (1977) showed that the antigen 85 complex (A, B, C) possess mycolyltransferase activity, and mycolyltransferase C catalyzes the reaction through the removal of a mycolate from one molecule of TMM and the addition of this mycolate to a second TMM molecule to yield TDM. This work

suggested the role for mycolyltransferases/antigen 85 complex in shuffling of such lipids among mycolic acid-associated molecules. Analysis of the complete genome of *M. tuberculosis* revealed that there are four open reading frames (*fbpA*, *fbpB*, *fbpC1*, and *fbpC2*) which may encode mycolyltransferases (Table 1.3). Interestingly, the mycolyltransferase encoded by *fbpC* was shown to be inhibited by the trehalose analog, a 6-azido-6-deoxy-α, α'-trehalose, resulting in the inhibition of cell growth as well as the inhibition of the synthesis of TMM, TDM, and non-extractable cell wall mycolates (Belisle *et al.*, 1997). These observations suggested that the mycolyltransferases are essential and thus could be a potential target for new antituberculosis drugs.

It seems clear that understanding the structures, biosyntheses, and functions of the mycobacterial cell wall is important for the rational design of new drugs and for strategies to circumvent intrinsic resistance to drugs in mycobacteria. Studies of cell wall biosynthesis should provide new insights to develop new drugs targeting in the biosynthetic pathway of cell wall components and enhance an understanding of the failures of tuberculosis treatment with the current antituberculosis drugs.

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CHAPTER 2

Literature Review Part II: Thiocarlide and Thioureas

(Part of this review is a draft of a manuscript for publication)

2.1 INTRODUCTION

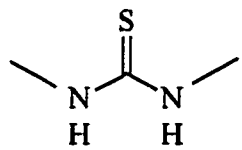
The emergence of strains of *M. tuberculosis* resistant to existing drugs has focused attention on the development of new antituberculosis drugs. Such agents must have selective targets different from the presently available agents. In the era of tuberculosis drug resistance, the re-examination of thiocarlide (THC), a thiourea derivative that was successfully used in tuberculosis treatment in past years, may prove worthwhile in the context of the development of new antituberculosis drugs or new derivatives of thiourea with greater potency and more desirable features. The aim of this part is to review known elements of THC as well as other thiourea derivatives and their usage based on standpoints presented in previous reports. This collection of scattered information will enlighten us to speculate on generally unknown aspects of THC. In addition, this review will put in perspective the problems that resulted in the disappearance of THC from current clinical application and address some of the beneficial aspects of THC that might help in its resurrection.

It had been noted in 1941 that certain sulfur containing substances had antimycobacterial activity *in vitro* (Mayer, 1941). Substituted thioureas were among these effective compounds. One such derivative of thioureas, which was effective and used in

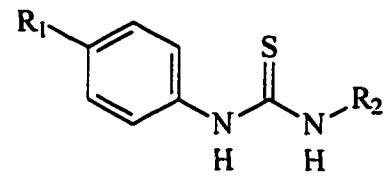
clinical tuberculosis treatment during the 1960s, was thiocarlide (THC) (4, 4'-diisoamyloxydiphenylthiourea, 4, 4'-diisoamyloxy thiocarbanilide) (Titscher, 1966; Urbancik, 1970; Gallen, 1970). Much was written on THC shortly before and after the introduction of THC to clinical use. Most information in the literature describes various studies of THC *in vitro*, in animal experiments and in clinical trials. It is notable that the antituberculous activity of THC was demonstrated extensively in both experimental and clinical studies but there was little information on molecular mechanisms of action of this drug.

2.2 STRUCTURAL RELATIONSHIP

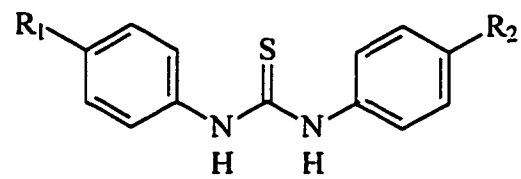
Thiourea compounds have a variety of chemical structures, but all thioureas including THC share a basic structure, a thiocarbonyl urea nucleus (Fig. 2.1). A variety of side chains attached to a common moiety generate numerous derivatives of thioureas, which elicit different antituberculous activity. Based on the differences in side chains, numerous thioureas were classified into two major subgroups: phenylalkylthioureas and diphenylthioureas (thiocarbanilides) (Fig. 2.1). Both categories possess appreciable capacity to suppress *M. tuberculosis* infection *in vitro* and in animal models. However, it was noted that diphenylthioureas (thiocarbanilides) were generally much more effective than phenylalkylthioureas (Youmans *et al.*, 1958).



Thiocarbonyl urea nucleus



Phenylalkylthiourea



Diphenylthiourea

Figure 2.1 Structures of thioureas: phenylalkylthiourea and diphenylthiourea (thiocarbanilide). R_1 and R_2 are alkyl groups.

The early studies reported the relationship between structure and antimycobacterial activity of various thioureas. The presence of alkoxy groups in the 4, 4′ position(s) of the benzene rings of diphenylthioureas was known to transform inactive unsubstituted thiocarbanilides into substances with considerable antituberculous activity (Schwartz *et al.*, 1954). Therefore, attempts to develop new active diphenylthioureas were focused on the modification of the side chains attached to the 4, 4′ positions of the benzene rings. In 1953, a number of substituted thioureas were chemically synthesized. More than 300 compounds were screened for active thioureas (Eisman *et al.*, 1954). Subsequently, only effective compounds were studied for antituberculous activity in experimental animals and in clinical trials (Konopka *et al.*, 1954; Schwartz *et al.*, 1954).

Although a large number of substituted diphenylthioureas were synthesized only a few derivatives were available in the market. One of the commercial thioureas was isoxyl® (ISO), a thiocarlide produced by Continental Pharma, Bruxelles, Belgium and Inibsa, Spain. The first marketing format of ISO was in tablet form. A powder form, believed to have better pharmacokinetic properties, was subsequently introduced. Other commercial thiocarlides are Amixyl® (Inibsa, Portugal; Leiras, Finland) and Dioxyl® (Ferrosan, Denmark).

THC was chemically described as a symmetrically substituted p,p'-dialkoxyphenylthiourea. According to its structural constitution (Fig. 2.2), THC has symmetrical side chains attached to a thiocarbonyl urea nucleus and has alkoxy substitutions at the 4,4' positions of the benzene rings. This powerful compound was first chemically synthesized in 1951 (Anonymous, 1970).

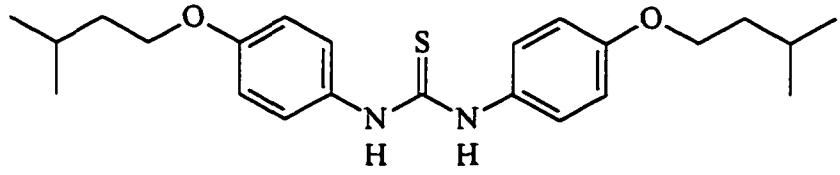


Figure 2.2 Chemical structure of thiocarlide (THC, 4,4'-diisoamyloxy-diphenylthiourea).

Thiocarbanidin (THD), 4-isobutoxy-4'-(2-pyridyl)-thiocarbanilide (Steenken *et al.*, 1958) is considered an THC related compound, and Park Davis (Park Davis; Detroit, Michigan) marketed it during the 1960s under the trade name of Thioban® (Wesley and Glazko, 1958). It is a basically substituted diphenylthiourea which contains alkoxy and basic radical

substitutions in 4,4′ positions of the benzene rings (Fig. 2.3) (Dill and Glazko, 1958; Anonymous, 1970). In general, the introduction of the alkylene bridge up to two carbon atoms between the basic radical and the benzene ring is known to increase significant tuberculostatic action as well as toxicity of basically substituted phenylthioureas. According to chemical structure, THD is a basically substituted diphenylthiourea that lacks the alkylene bridge (Anonymous, 1970).

THD was shown to be effective against *M. tuberculosis* both *in vitro* and in experimental animals (Youmans *et al.*, 1958; Steenken *et al.*, 1958). Studies of THD efficacy in tuberculosis treatment were also conducted in man (Phillips, 1958). It was noted that the treatment of patients with THD began in most hospitals in July 1958 (Larkin, 1959) while THC began to be used in clinical to treat tuberculosis in 1963 (Nickling, 1970). Although basically substituted phenylthioureas are much more effective than di-alkoxyphenylthioureas in general, THD was dropped from clinical use after THC was introduced a few years later. Based on chemical structures of THC and THD, both drugs were thought to have a similar mode of action in mycobacterial cells.

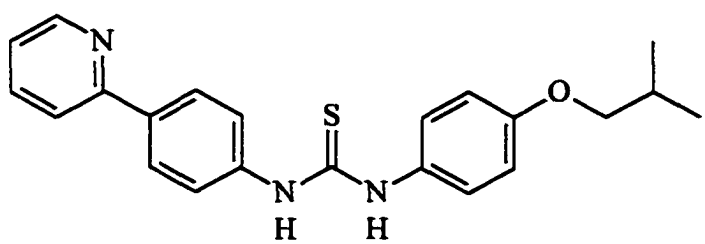


Figure 2.3 Chemical structure of thiocarbanidin (THD, Thioban®). In one side chain of THD, there is a basic radical directly attached to the benzene ring.

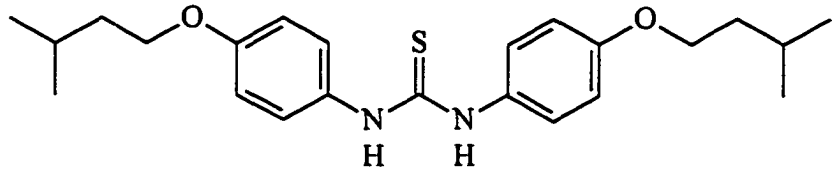
2.3 *IN VITRO* ANTIMYCOBACTERIAL ACTIVITY

Unlike other antituberculosis drugs, THC was strongly inactivated in egg media (Muzikravic, 1970). Therefore, most *in vitro* studies of THC were performed in special media such as Tarshis blood agar or non-egg based media (Virtanen, 1963). It was reported that THC exhibited *in vitro* efficacy against most clinical isolates of *M. tuberculosis* when used at 2 µg/ml or less (Anonymous, 1970). It is notable that strains resistant to ETH or thiacetazone are less susceptible to THC. This conclusion was derived from the observation that strains resistant to thiacetazone at 2 µg/ml or to ETH at least 10 µg/ml often showed cross-resistance to THC (Anonymous, 1970).

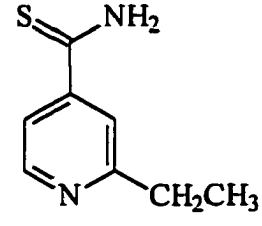
Very little work has been done on the sites of inhibition of THC *in vitro*. An early note of Winder *et al.* (1971) reported that like INH and ETH, THC markedly reduced the synthesis of mycolic acids in *M. bovis* during a 6 h exposure to 10 µg/ml of THC. These results have since been confirmed with the demonstration that all types of mycolic acids are affected: α-mycolates, methoxycolates, ketomycolates and wax-ester mycolates (chapter 4). However, the specific site of the inhibition of THC on mycolic acid synthesis appears to be different from that of INH and ETH. In the case of INH and ETH, the inhibitory effect was largely restricted to mycolic acid synthesis (Rist, 1960; Schaffer, 1960) and there was a stimulation of fatty acid synthesis, whereas THC strongly inhibited mycolic acid synthesis and partially inhibited the synthesis of fatty acids (Winder *et al.*, 1971).

Although the site of action of THC appears to be different from those of INH and ETH, the partial cross-resistance between THC and ETH and thiacetazone (4-acetylaminobenzaldehyde-thiosemicarbazone, thiosemicarbazone, Conteben ®) was observed in *M. tuberculosis* clinical isolates (Tskamura, 1962; Urbancik and Trnka, 1963; Virtanen, 1963; Sojkova *et al.*, 1965; Tan *et al.*, 1966; Porter and Neal, 1978). This observation suggested that THC, ETH and thiacetazone share a common step in their action (Winder, 1982). It is interesting to note that THC and ETH as well as thiacetazone have a thiocarbonyl moiety in common (Fig. 2.4). This point indicated that the three classes of drugs probably share a similar step(s) in their action, presumably in the drug activation process.

Thiocarlide



Ethionamide



Thiacetazone

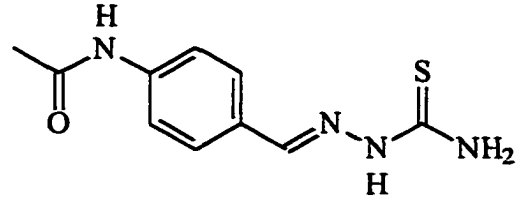


Figure 2.4 Structural relationship of thiocarlide (THC), ethionamide (ETH), and thiacetazone.

More recently, it was reported that ETH is a prodrug and is activated to a sulfoxide intermediate (Barry *et al.*, 1999). Similarly, many thiocarbonyl compounds including thioureas, thiobenzamides, and thiacetazone were thought to require metabolic activation *in vivo* via one or more enzymatic S-oxidations. The resulting sulfoxide intermediate is capable of binding to several cellular nucleophiles leading to biological injuries to mycobacterial cells (Cashman and Hanzlik, 1982). A recent genetic study revealed that cross-resistance to ETH, THC and thiacetazone is associated with the activation of the thiocarbonyl moiety. The product of a resistance-conferring gene was identified to be a putative regulator, which controls the expression of the cytochrome P450-containing oxidase activity (Barry *et al.*, 1999). The enzyme is responsible for the S-oxidations involved in the activation of thiocarbonyl moiety. Therefore, the cross-resistance to THC, ETH and thiacetazone can be explained by defects in the activation process of these thiocarbonyl compounds.

2.4 CLINICAL THERAPEUTIC EFFICACY

Much work had been done in clinical trials of thioureas. Comparative studies on the antituberculous activity in human beings indicated that, in general thiocarbanilides (diphenylthioureas) at doses of 3 to 6 g daily were less effective than Str, but more effective than *para*-aminosalicylic acid (PAS)(Schwartz *et al.*, 1954; Phillips *et al.*, 1956). Gallen (1970) demonstrated the gastrotoxicity of PAS which frequently

reduced the bioavailability of major drugs in the combination regimen for tuberculosis therapy, while THC at a dose of 6 g per day appeared to substitute quite satisfactorily for PAS. This finding suggested that thiocarbanilides, either THC or THD proved to be a substitute in place of PAS and could be used in conjunction with Str or INH in tuberculosis treatment.

ISO in monotherapy. Among THC, ISO was studied extensively for the tuberculostatic effect of THC in human tuberculosis (Freerksen, 1963; Freerksen and Rosenfeld, 1963; Titscher, 1966). The treatment with 6 g ISO daily for 6-8 weeks or for 10-18 weeks showed that ISO significantly reduced cavities in pulmonary tuberculosis, and converted sputum cultures to negative in most fresh cases. The deteriorated cases found to be associated with ISO resistance usually occurred at 6-8 weeks after treatment. Injuries of the parenchymatous organs such as liver, central nervous system or hemopoietic system were not observed (Kampelmann, 1970). The efficacy of ISO monotherapy was confirmed according to Dormer (Dormer, 1970). With an oral dose of 20 g daily, in two divided doses, ISO was effective against pulmonary tuberculosis. In a large scale trial consisting of more than 200 positive cases, the efficacy of ISO alone in conversion of sputum cultures was 30 to 40% in fresh cases and 20 to 60% in chronic multi-resistance tuberculosis (Anonymous, 1970). Urbancik (1966; 1970) and Titscher (1966) also demonstrated the therapeutic efficacy of ISO monotherapy in untreated cases of tuberculosis. The drug was able to convert bacteriological positive cultures in

chronic cases to negative by about 25% of the cases after 6 to 8 weeks of 6 g of ISO daily. When the treatment was extended longer to 10 to 18 weeks, about 50% of the patient population had their sputa converted to negative (Kampelmann, 1970). Based on the therapeutic efficacy of ISO in experiment and in monotherapy without side effects, ISO replaced PAS in the combination therapy in 1963 (Nickling, 1970).

ISO in combined therapy. ISO was considered a good compatible drug. In most cases ISO was used with INH or with INH and Str, and often also in combination with cycloserine or EMB. With the combination of INH and ISO, a mean conversion rate of 91% was found after four to six months in previously-untreated cases (Anonymous, 1970). During the 1960s, ISO had been used extensively as a tuberculostatic in routine tuberculosis therapy. Schmid (1970) reported clinical experiences in using ISO in the treatment of pulmonary tuberculosis in children. The combined therapy with INH and ISO achieved somewhat better results than monotherapy with INH, and was relatively well tolerated by treated children and infants. Toxicity or tissue injuries had never been observed (Schmid, 1970). In adults, a combination therapy consisting of 200-300 mg INH, 1 g Str twice weekly and 4 g ISO daily showed high efficacy (Nickling, 1970). With the alternative regimen of 5 mg/kg INH, 1 g Str, and 6 g ISO daily, > 80 % of sputum cultures converted to negative at the end of the fourth month of treatment without side effects (Nickling, 1970). In chronic tuberculosis, ISO proved to be slightly more effective

than pyrazinamide (PZA) but weaker than cycloserine when used in combination with INH (Boszormenyi, 1970). However, it was recommended that ISO could be used in place of cycloserine because of the many side effects of the latter (Boszormenyi, 1970). A subsequent study of the clinical efficacy of ISO was performed in chronic tuberculosis patients with resistance to other chemotherapeutic agents. ISO was given 6-8 g daily and other antituberculosis drugs were not given during the whole duration of therapy. ISO appeared to have effects on the disease by converting the bacteriological positive cultures to negative (4 out of 16 cases) after eight weeks of treatment. A good tolerance of ISO was mentioned, and no side effects were reported in difficult cases (Urbancik, 1970). In addition, ISO had been found to be effective against atypical mycobacteria (Urbancik, 1970).

In all, most clinical trials reported that ISO is effective in tuberculosis treatment particularly when used in combination with INH or INH and Str. Moreover, ISO appeared to have an effect in the prevention of emergence of INH and Str resistant strains (Gallen, 1970). However, some experimental and clinical trials yielded contradictory results and reported that ISO was a feeble agent (Boszormenyi, 1970; Tousek, 1970). The most important reason is that the antimycobacterial activity of ISO did not attain the effect of INH and Str (Freerksen and Rosenfeld, 1963). The value of ISO was equal to pyrazinamide (PZA) but weaker than cycloserine (Boszormenyi, 1970). Tousek (1970) reported that the combination of ISO and INH was significantly less effective than

PAS and INH, and ISO failed to prevent the emergence of INH resistance.

Therapeutic efficacy of thiocarbanidin (THD). Like thiocarlide (THC), THD inhibited the growth of *M. tuberculosis* H37Rv as well as a H37Rv derivative resistant to INH and PAS. It was noted that the drug was not effective against strains resistant to thiacetazone (Steenken *et al.*, 1958). This finding was not surprising because THC is closely related to ISO and shares a common moiety with thiacetazone. THC was most effective at a dose of 40 mg/kg daily when given orally to H37Rv-infected guinea pigs. When used in combination with Str or INH, it was superior to either Str or INH alone (Steenken *et al.*, 1958). Rabbits with miliary tuberculosis were susceptible to THD, whereas PAS was not effective. In all animal studies, THD showed no gross toxicity (Steenken *et al.*, 1958). Schmidt *et al.* (1959) reported poor therapeutic results against tuberculosis in monkeys with a daily dosage of THD alone at 500 mg/kg. The poor absorption, low blood level and rapid degradation of THD were reported in this animal species. These factors probably accounted for the poor results of tuberculosis therapy with THD. Similarly, low plasma levels of THC were observed in human volunteers when THC was taken orally at a dose of 2 g daily (Wesley and Glazko, 1958). Although the data showed that THD had the problems of low absorption and low blood level, its tuberculostatic activity proved sufficiently effective in experimental animals. Therefore, it had been used in the treatment of tuberculosis patients since 1958 (Phillips, 1958;

Larkin, 1959). The efficacy of THD in tuberculosis treatment appeared to be similar to PAS (Youmans *et al.*, 1958; Wesley and Glazko, 1958), while Steenken *et al.* (1958) reported that THD was ten times more active. Donohoe *et al.* (1959) showed that INH at a dosage of 100 mg three times a day combined with THD at 2 g given four times daily for 3-12 months yielded a little less than 50% of patients whose sputum cultures converted to negative. Together, it was proposed that THD might be a substitute for PAS in multiple drug regimens, particularly for patients who tolerated PAS poorly or those who were not progressing satisfactorily on current therapy. However, Larkin (1959) pointed to the unfavorable result of tuberculosis treatment when THD was given 1 g daily along with Str or INH for 2-4 months. Although a lot of controversy was found in the reports of the clinical experiences with ISO and THD, most reports mentioned a satisfactory efficacy with ISO and THD.

2.5 PHARMACOLOGICAL DATA

Available pharmacological data on THC particularly involved the intestinal absorption and the blood levels of the drug (bioavailability) studied in both animals and human volunteers. It was noted that the pharmaceutical forms of THC influenced drug absorption and blood levels and hence the efficacy of THC in tuberculosis treatment. Commercial THC (isoxyl®) was first produced in the form of a tablet and was available as old or new formula (Lambelin, 1970). The blood levels of the new formula were about 3.30, 4.11, and 2.30 µg/ml at 2, 4, and 6 h after

administration a 6 g ISO tablet indicating superior absorption to the old formula. Comparison of ISO blood levels in volunteers showed that the powder form of ISO (original formula of Continental Pharma) gave significantly higher blood levels particularly at 1 h after the first intake of 3 g of ISO. The blood levels obtained from the powder ISO were still relatively higher up to 5 h, and remained high until 24 h after intake. A similar result was reported by Lambelin *et al.* (1968) showing that ISO in a powder form gave significantly higher blood levels and accelerated the absorption of the active product from the gastro-intestinal tract. After absorption, the tuberculostatic activity of ISO could be detected in the bloodstream at 1 h. The peak of inhibitory activity was between 6 and 10 h after the first intake or 2 and 4 h after the second intake of 3 g of ISO (Lambelin, 1970). An independent study reported that the inhibitory peak of ISO in plasma was at 8 h after the first administration of an ISO tablet (Bloedner, 1967). The debate remained with the result indicating that the administration of powdered ISO had no affect on blood level concentration (Bloedner, 1967). No clinical trials between the powder and tablet forms of ISO have been performed. Therefore, it is still unknown whether the powder form shows significantly higher potency compared to the tablet.

In conjunction with the dosage form, the dosage interval also affects the blood levels of the drug. In the case of ISO, it is important to maintain a high blood level of this drug throughout for 24 h. To ensure that a high blood level was maintained,

effects of interval regimens of ISO were studied in volunteers. It was found that the regimen of 3 X 2 g was favorable and the blood level was markedly high at 1, 6 and 24 h after the first intake (Lambelin, 1970). However, in clinical tuberculosis treatment the patients were given ISO at 3 to 6 g daily with a variety of interval regimens. The period of treatment was also markedly variable usually depending on the degree of complications in individual cases. It must be noted that the prolonged treatment of tuberculosis with ISO had no effect on enzymatic induction or accumulation which might affect blood levels of drug (Lambelin, 1970).

Knowledge of the excretion of ISO was obtained from studies in rabbit and human volunteers (Lambelin, 1970). ISO was detected in bile 7 h after administration with 100 mg/kg ISO powder, suggesting that ISO was excreted mainly by the fecal route. It was thought that ISO was probably reabsorbed by the intestine when it passed through the entero-hepatic cycle (Lambelin, 1970). An early note also reported that ISO did not enter the cerebrospinal fluid, and therefore, it is not suited for the treatment of meningial tuberculosis (Anonymous, 1970).

Experimental toxicity of ISO was studied extensively in animals. The toxicological data indicated that ISO was well tolerated by mouse, rat and man (Lambelin, 1970; Lambelin and Parmentier, 1963). In monkeys, it was shown that ISO going up to up to 3 g/kg was also well tolerated in this species. This given dose was thirty times greater than the therapeutic dose applied to

human. There was no report of acute toxicity from various studies even when the dosage of 10 g/kg was given to animals (Lambelin and Parmentier, 1963). Studies of subacute (2 months) and chronic toxicity (6 months) in rats proved extremely favorable. Prolonged treatment of rats up to 18 months with the dosage of ISO ranging from 300 to 1400 mg/kg revealed no toxic effects. The only anomaly recorded in treated rats was an increase in serum alkaline phosphatase. ISO did not influence growth, food consumption, or animal behavior. Nevertheless, there were no effects of ISO on embryotoxic, teratogenic or cancerogenic toxicity (Lambelin and Parmentier, 1963). These results were similar to several observations in man, and therefore proved the absence of toxicity of ISO perfectly. The increased dosage of ISO ranging from 100 to 300 mg/kg which was up to three times the usual therapeutic dose was not toxic in humans indicating the atoxic property of ISO (Lambelin, 1970). Schmidt *et al.* (1959) suggested that poor absorption from the gastro-intestinal tract accounted for the low toxicity of this drug.

2.6 SUMMARY

Several investigations concluded that THC is an effective, well-tolerated, and atoxic antituberculosis drug. It is able to prevent the emergence of resistance to INH and Str, and possesses desirable compatibility. Various clinical trials mentioned the success of THC particularly ISO in tuberculosis treatment and the perfect tolerance in tuberculosis patients. The most worrisome

points are the poor absorption and the low blood level of THC making it fall from the current use. However, it is possible with reasonable doses of THC to achieve drug concentrations in plasma above the requirement to inhibit the growth of tubercle bacilli. The optimal form, dosage and the interval regimen of THC are still a matter to be studied and discussed. Besides THC, new derivatives of thiourea with better pharmacokinetic properties seem to be promising antituberculosis drugs.

2.7 RATIONALE FOR THE USE OF THC AND RESEARCH AIMS

The prevalence of tuberculosis and the emergence of drug-resistant *M. tuberculosis* still remain a major health problem in many countries (Kochi *et al.*, 1991; Bloch *et al.*, 1994). Drug-resistant *M. tuberculosis* is alarming as there are only a few effective drugs available, and infection with drug-resistant *M. tuberculosis* could give rise to a potentially untreatable form of disease. It must be noted that there have been no new antituberculosis drugs since the introduction of rifampin in the 1960s. The problem of drug resistance and the highly contagious nature of tuberculosis clearly demonstrate the need to develop new antituberculosis drugs. Such novel drugs must have bactericidal mechanisms different from those of presently available agents and provide superior characteristics to the current drugs. The characteristics of desirable drugs include good pharmacokinetics, no serious side effects, and high efficacy against multidrug-resistant *M. tuberculosis*. Novel drugs should also provide other

benefits such as effectiveness against dormant bacilli in addition to actively growing bacteria, enhancing the efficacy of the current chemotherapeutic agents, or reducing the duration of treatment.

It is apparent that the mycobacterial cell wall is a complex structure and offers potential targets for many existing antituberculosis drugs. Drugs that inhibit the synthesis of cell wall components may alter cellular permeability and increase the entry of toxic substances including drugs that have targets beyond cell wall biogenesis. Additionally, drugs that selectively inhibit targets present at the outer part of the cell wall may circumvent the problem of the permeability barrier. Thus, enzymes involved in the synthesis of the cell wall constituents represent potential drug targets for antituberculosis drugs (Young, 1994). Drugs targeting fatty acid and mycolic acid synthesis are of interest as demonstrated by the therapeutic efficacy of INH and ETH. Fatty acids and mycolic acids have various important roles in mycobacterial physiology (chapter 1), and the biosynthetic pathways are apparently unique to mycobacteria. These facts have been recognized and led to the considerable efforts on the identification of new drug targets in these synthetic pathways.

In attempts to develop new drugs targeting fatty acid and mycolic acid synthesis, THC/ISO, an old antituberculosis drug, has been chosen for exploring its mode of action and enzymatic targets. Little is known about the molecular mechanisms of THC action and a precise drug target has not yet been determined.

Based on previous observations, THC inhibits the synthesis of mycolic acids and free fatty acids (Winder *et al.*, 1971). It was noted that the sites of inhibition of THC in fatty acid and mycolic acid synthesis appeared to be different from those of INH and ETH (Winder *et al.*, 1971). Therefore, the targets of THC should be unique and different from those of existing drugs.

This dissertation describes the use of THC/ISO as a tool to identify its molecular targets and to reveal some fundamental aspects of the metabolic reactions involved in the synthesis of fatty acids and mycolic acids in mycobacteria. The initial hypothesis is that THC/ISO interferes with catalysis by binding to essential enzymes involved in fatty acid and mycolic acid synthesis. The identification of enzymatic targets of THC/ISO would lead to the discovery of new drug targets and eventually the development of new antituberculosis drugs. Evaluations of drug efficacy, especially of a series of newly synthesized thioureas, is an additional objective of this dissertation.

In order to accomplish the objectives of the study, several prerequisites must be fulfilled including characterization of all strains that were used in drug evaluation, generation of an ISO-resistant mutant, and construction and screening of a genomic library to identify genes involved in the mode of action of ISO. Alternative approaches to identify molecular targets of ISO include biochemical analysis of the effects of ISO on fatty acid and mycolic acid synthesis. Once a specific site of the inhibition of ISO is identified, the overexpression of the gene

presumably encoding an enzyme catalyzing that specific metabolic reaction will be performed. The procedures in this strategy involve 1) defining the specific site of inhibition of ISO on fatty acid or mycolic acid synthesis; 2) cloning of all genes that involve the specific metabolic reactions inhibited by ISO; 3) functional analysis of encoded proteins from the overexpressed genes; 4) reconstitution of the metabolic reactions inhibited by ISO *in vitro;* and 5) demonstration of specific inhibition of ISO via *in vitro* assay. The future application of this work is the integration of the *in vitro* assay into high throughput screening systems where new antituberculosis drugs targeting the same enzyme with ISO can be evaluated for their effectiveness.

The following chapters will describe experimental approaches, trouble shootings and the accomplished work on ISO research. The characterization of all mycobacterial strains used in this study and the generation of spontaneous mutants conferring resistance to ISO are presented in the first part of the study. The use of the well-defined mutant may provide information about the possible mechanisms of ISO resistance. In the second part, the efficacy and the mode of action of ISO as well as the newly synthesized thioureas are described. The last part details the identification of *M. tuberculosis* genes encoding a target enzyme of ISO. Some characteristics of the target enzyme were also studied. In turn, the data gathered unravel some fundamental aspects of fatty acid and mycolic acid synthesis in mycobacteria.

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CHAPTER 3

The Approaches to Characterize Mycobacterial Strains and Identify Genes Involved in the Mode of Action of Thiocarlide/Isoxyl®

3.1 INTRODUCTION

The primary goals of the work presented in this chapter are to characterize strains of mycobacteria used in this study and to identify genes involved in THC/ISO resistance or genes encoding targets of THC/ISO.

A panel of clinical isolates of *M. tuberculosis* and *M. bovis* BCG was used in this dissertation, several of which are resistant to one or more antimycobacterial drugs. For typing of *M. tuberculosis,* drug resistance phenotypic characteristics are of value but the discriminatory ability is confined among drug-resistant strains. The genotyping of mycobacterial strains by *Pvu*II restriction fragment length polymorphism (RFLP), based on the insertion sequence IS*6110,* is a preferred means due to its power in differentiation of both drug-susceptible and drug-resistant *M. tuberculosis* as well as other members of the *M. tuberculosis* complex (Thierry *et al.*, 1990). IS*6110* is considered to be a specific DNA marker in the *M. tuberculosis* complex (McAdam *et al.*, 1990; Thierry *et al.*, 1990) and is usually present in several copies which are well distributed throughout the mycobacterial genome (van Embden *et al.*, 1993; Zhang *et al.*, 1992). There is little discrimination in the selection of a

target sequence by IS*6110* upon transposition (Hermans *et al.*, 1990; Mendiola *et al.*, 1992; van Embden *et al.*, 1993), and the sequence of IS*6110* is apparently invariant (Dale *et al.*, 1998). These features contribute to the utilization of IS*6110* in differentiation of *M. tuberculosis* strains.

An attempt to identify targets of ISO by genetic approaches was initiated, and the details of the genetic strategies are also presented in this chapter. Typically, the target gene can be identified by cloning of the mutated gene, which usually confers a high level of resistance to the drug or by expressing the sensitive target gene on a multicopy vector. A tool for identification of a target gene was developed by generating a new strain of *M. aurum* A+ that was spontaneously resistant to ISO. The utilization of a well characterized mutant to screen a genomic library could lead to the definition of mechanisms of ISO resistance as well as the identification of genes encoding enzymatic targets or genes involved in drug activation. Although the main goal of this part of the work, the defining of ISO resistance mechanism, was not achieved, this work provides a useful reference for future applications of all characterized strains. The mutant generated in this study would be useful particularly in studies of ISO resistance mechanisms. It was decided to attempt screening of a genomic library of *M. tuberculosis* H37Rv that is readily available. However, it became apparent that the identification of the target(s) of ISO by the genomic library approach was hampered by many difficulties. As a

consequence, an alternative strategy was applied. The success of the identification of the ISO target by the alternative approach is detailed in chapters 5, 6, and 7.

3.2 MATERIALS AND METHODS

3.2.1 Differentiation of mycobacterial strains

3.2.1.1 Construction of an IS*6110* probe

A 291 bp fragment of IS*6110* was PCR amplified from the chromosomal DNA of *M. bovis* BCG using sense and antisense primers synthesized by Molecular Resource Facilities, Colorado State University. The primers are as follows:
[5′ AAGGAGCACATCAGCCGCGTCCAC 3′] (sense "IS*6110*-1") and
[5′ GAACCCTGCCCAGGTCGACACATGGGTG 3′] (antisense "IS*6110*-2"). The PCR reaction mixture contained 5 µl of 10 x PCR buffer, 2 µl of 10 mM dNTP, 10% DMSO, 0.2 µM of each primer, 30 to 70 ng of template DNA, 2 U of Vent polymerase (New England Biolabs; Beverly, MA), and distilled water to 50 µl. Thermal cycling was performed on a Perkin Elmer Geneamp PCR System 2400 (Norwalk, CT) with 5 min of an initial desaturation step at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 68°C and 30 s at 72°C, then a final elongation step at 72°C for 10 min. The PCR products were analyzed by 1.6% agarose gel electrophoresis. The 291 bp of IS*6110* was extracted from the gel by GeneCapsule (GenoTechnology; St. Louis, MO), followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction. The IS*6110* fragment was concentrated by

precipitation with 100% cold ethanol, washed with 70% ethanol and dissolved in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) buffer.

After sequencing, the 291 bp of the purified IS*6110* PCR product was cloned into the *Hinc*II restriction site of the pBluescript SK II$^-$ plasmid vector (Stratagene; La Jolla, CA). Ligation was performed in a total volume of 20 µl containing 1 U of T4 DNA ligase (Gibco-BRL; Rockville, MD), 100 ng of total DNA (vector plus insert), and 1 x ligase buffer. The mixture was incubated at 15°C for blunt end ligation. The resulting construct designated pKS-IS6110(A) (Fig. 3.1) was electroporated into *E. coli* strain XL-1 Blue (Stratagene). Numerous copies of pKS-IS6110(A) were isolated from *E. coli* host cells using Qiagen plasmid preparation kit (Qiagen; Santa Clarita, CA). Subsequently, fragments of IS*6110* were cut out from the cloning vector by the *Xho*I and *Cla*I.

The probe, which is a fragment of IS*6110*, was labeled with digoxigenin (DIG) using the Genius Random Prime Labeling Kit and following the manufacturer's instructions (Boehringer Mannheim; Indianapolis, IN).

3.2.1.2 Chromosomal DNA isolation from mycobacteria and restriction endonuclease digestions

Mycobacterial chromosomal DNA was isolated from various strains of *M. tuberculosis* including *M. bovis* BCG (Table 3.1). In brief, the organisms were grown on Lowenstein-Jensen (LJ) slant medium (Difco; Detriot, MI) and incubated at 37°C for 21 days.

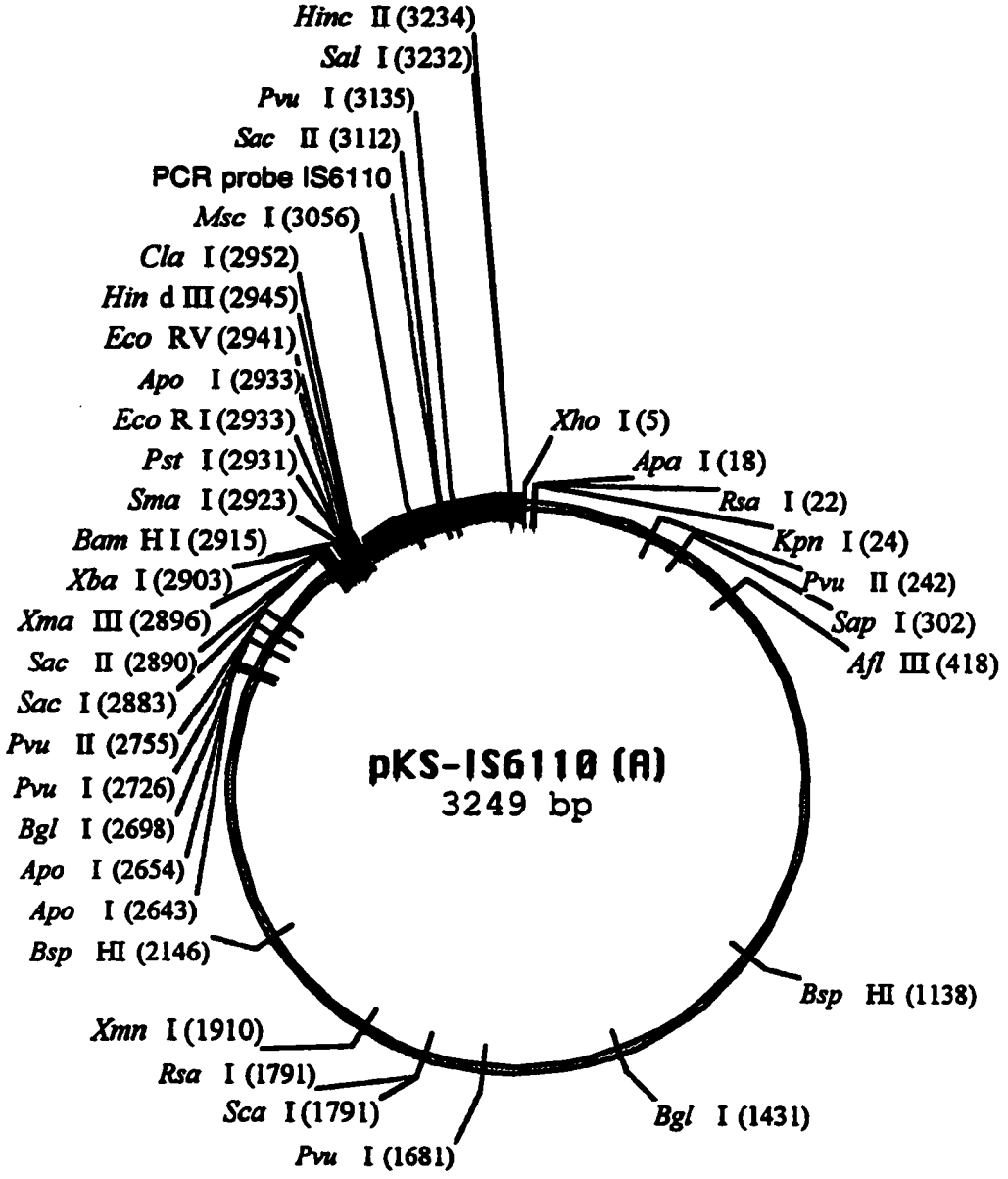

Figure 3.1 The restriction map of the construct pKS-IS6110(A). The PCR products of IS*6110* was cloned into the *Hinc*II restriction site of the pBluescript KS⁻II vector generating a new construct, pKS-IS6110(A). The cloned fragments of IS*6110* were then cut out from the cloning vector with *Xho*I and *Cla*I.

Table 3.1 Strains of the *M. tuberculosis* complex used in this study.

Strain	MDR[a]	Virulence[b]	Source[c]	Drug sensitivity/resistance									
				AMK	Cap	Cyc	EMB	ETH	INH	Kan	PZA	Rif	Str
CSU 15	No	Low	C	S	S	S	S	S	R	S	S	S	S
CSU 21	Yes	Low	K	S	S	S	R	S	R	S	S	R	R
CSU 22	Yes	High	K	S	S	S	R	S	R	S	S	R	R
CSU 32	Yes	High	N	S	S	S	R	S	R	S	S	R	R
CSU 39	Yes	High	N	R	S	S	R	R	R	S	R	R	R
CSU 44	No	High	N	R	S	S	R	R	R	S	R	S	R
W 670	Yes	ND	ND	R	S	S	R	R	R	S	R	R	R
W 3432	Yes	ND	ND	R	S	S	R	R	R	S	R	R	R
CDC 97	No	ND	C	S	S	S	S	S	R	S	S	S	S
H37Rv	No	Moderate	TMCC	S	S	S	S	S	S	S	S	S	S
H37Ra	No	Moderate	TMCC	S	S	S	S	S	S	S	S	S	S
BCG	No	ND	Pasture	S	S	S	S	S	S	S	S	S	S

[a] Drug resistance was determined by a proportional method (Ordway *et al.*, 1995; Heifets, 1991a; Heifets, 1991b). Strains were classified as multiple drug resistant (MDR) if they were resistant to the principal antituberculosis drugs, isoniazid (INH) and rifampin (Rif), or resistant to these two major drugs and one or more of the following: AMK, amikacin; Cap, capreomycin; Cyc, cycloserine; EMB, ethambutol; ETH, ethionamide; INH, isoniazid; Kan, kanamycin; PZA, pyrazinamide; Rif, rifampin; Str, streptomycin.

[b] Virulence was determined by the ability of cells to grow in the lung of C57BL/6 mice exposed to a low-dose aerosol of bacilli. Growth was classified relative to that of the type strain, Erdman (Rhoades and Orme, 1997).

[c] C, From Centers for Disease Control and Prevetion (isolates from the United States); K, from Republic of Korea (isolates from Korea and other Asian countries); N, from National Jewish Center for Immunology and Respiratory Medicine, Denver, CO (isolates from the United States); TMCC, Trudeau Mycobacterial Culture Collection (Ordway *et al.*, 1995).

Abbreviations used: ND, not determined; S, sensitive; R, resistant.

The mycobacterial colonies were then scraped, and collected into microcentrifuge tubes containing 400 µl of TE buffer. The cell suspension was heated at 80°C for 30 min to kill mycobacterial cells. After cooling at room temperature, lysozyme (Sigma; St. Louis, MO) and RNaseA (Sigma) were added to a final concentration 1.25 mg/ml and 10 mg/ml, respectively. The mixture was incubated at 37°C for 2 h before adding of 10% SDS to a final concentration of 1%, and 5 µl of proteinase K (10 mg/ml)(Sigma). The mixture was further incubated at 65°C for 30 min. Cell wall debris, denatured proteins and polysaccharides were removed by addition of 100 µl of NaCl and 100 µl of *N*-acetyl-*N,N,N*-trimethyl ammonium bromide (CTAB)(100 mg in 0.7 M NaCl)(Sigma). After 10 min of incubation at 65°C, the supernatant was collected by centrifugation. An aliquot of 750 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the supernatant, and the mixture was vortexed for 20 s. After centrifugation at 14,000 x g for 10 min, DNA was precipitated by 100% cold ethanol. Recovered DNA pellets were washed with 70% ethanol, air-dried and finally dissolved in TE buffer. The DNA concentration was determined with a spectrophotometer (Beckman; Fullerton, CA). Restriction endonuclease digestions were performed according to the manufacturer's specification. Briefly, chromosomal DNA (~5.0 µg) isolated from each organism was digested with 10 U of *Pvu*II (Gibco-BRL) in a final volume of 20 µl at 37°C for 4 h.

3.2.1.3 IS*6110* hybridization

*Pvu*II-digested chromosomal DNA was fractionated by 0.8% agarose gel electrophoresis running overnight at low voltage in 1 x TAE buffer. DNA fragments were denatured by soaking the gel in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min at RT with gentle shaking, and then in neutralization solution (1 M Tris-HCl, 1.5 M NaCl, pH 7.5) for 15 min at RT with gentle shaking. The denatured DNA fragments were blotted to a nylon membrane (Boehringer Mannheim) by capillary transfer and fixed to the membrane by cross-linking via short wavelength (310 nm) ultraviolet light in a Stratalinker (Stratagene) for 30 s. Chemiluminescent Southern blot hybridization was performed according to the protocol from the Genius™ kit (Boehringer Mannheim). In brief, after one wash in buffer 2 x SSC (0.3 M NaCl, 0.30 M Na-citrate, pH 7.0), the nylon membrane was pre-hybridized in pre-hybridization buffer for 30 min. The hybridization was carried out overnight at 55°C in 20 ml of 1 x SSC (0.75 M NaCl, 0.75 M Na-citrate) containing ~200 ng of DIG-labeled IS*6110* probe. After hybridization, the membrane was washed in 2 x SSC-0.1% SDS and 1 x SSC-0.1% SDS at 37°C for 15 min each and then exposed to anti-DIG antibody. DNA labeled by hybridization to the IS*6110* probe was visualized using LumiPhos as described by the manufacturer (Boehringer Mannheim). Light emission during chemiluminescence was detected by exposing Kodax X-Omat AR film to the membrane.

3.2.2 Selection and characterization of ISO-resistant mutants

3.2.2.1 Selection of ISO-resistant mutants

A spontaneous ISO-resistant *M. aurum* A+ was derived from *M. aurum* A+ grown in medium containing a high concentration of ISO (20 x MIC =40 µg/ml). Briefly, 1.0 ml of an overnight culture of *M. aurum* A+ was inoculated into 50 ml of nutrient broth plus 0.05% Tween 80 in a flask. The inoculated medium was incubated with agitation until the culture reached A_{600} of 0.4. The actively growing culture was then plated on 7H11 agar containing 40 µg/ml of ISO. After incubation at 37°C for 4 days, visible colonies of mutants that survived this selection appeared on plates. Several colonies were selected randomly for characterization.

3.2.2.2 Characterization of the ISO-resistant mutants

Determination of drug resistance level. Drug resistance level to ISO was determined for *M. aurum* A+ ISO-resistant strains as follows. A single colony of an ISO-resistant mutant was picked and inoculated into 5 ml of nutrient broth plus 0.05% Tween 80. The culture was grown with stirring until A_{600} was approximately 0.250. The culture was then diluted in nutrient broth with 0.05% Tween 80 to approximately 1 x 10^3 CFU/ml. Aliquots of 100 µl were plated on 7H11 agar containing ISO at concentrations of 2.5, 20, 40, and 100 µg/ml. All plates were incubated at 37°C for 4 days. The resistance level was defined as the lowest concentration of ISO where no growth occurred.

Determination of cross-resistance of ISO to INH and ETH. *M. aurum* A+ WT and ISO-resistant strains were grown in nutrient broth containing 0.05% Tween 80 to the A_{600} of 0.025. The cultures were then used to streak on 7H11 agar containing INH at 5 µg/ml, ETH at 40 µg/ml or ISO at 40 µg/ml. All plates were incubated at 37°C for 4 days. Drug resistance was defined as the ability of cells to grow on plates containing drugs at the indicated concentrations.

Determination of the effect of ISO on the incorporation of [1,2- ^{14}C]acetate into fatty acids and mycolic acids. *M aurum* A+ WT and the ISO-resistant mutant were grown in nutrient broth plus 0.05% Tween 80. The concentrations of ISO for *M. aurum* A+ WT were 0, 2 and 10 µg/ml, while 10, 20, 30, and 40 µg/ml were used for the ISO-resistant mutant. Cells were preexposed to ISO for 6 h prior to the addition of [1,2-^{14}C]acetate (110 mCi/mmol) (Dupont NEN; Boston, Mass.) to a final concentration of 1.0 µCi/ml. The cultures were further incubated with gentle shaking for 12 h. The resulting labeled cells were harvested by centrifugation at 2,500 x g and washed twice with saline. The saponification was conducted by adding 2 ml of 15% tetrabutylammonium hydroxide (TBAH), followed by incubating at 100°C overnight. Saponified fatty acids and mycolic acids were then derivatized to the corresponding methyl esters using dichloromethane and iodomethane. The resulting FAMEs and MAMEs were extracted by diethylether, and analyzed for [^{14}C]acetate incorporation by TLC.

3.2.2.3 Application of ISO-resistant mutant to identify resistance mechanisms of ISO by genomic screening

The construction of the genomic library of ISO-resistant mutant was performed according to Yuan *et al.* (1995) and Belanger *et al.* (1995).

Isolation of chromosomal DNA. Chromosomal DNA of the generated mutant was prepared from large-scale culture as described earlier. The chromosomal DNA was then partially digested with diluted *Sau*3AI. The 35-40 kb fragments were isolated by preparatory 0.6% agarose gel electrophoresis and purified by elution using GeneCapsule kit (St. Louis, MO). The recovered DNA was gel-extracted by phenol-chloroform-isoamyl alcohol (25:24:1), followed by ethanol precipitation. DNA pellets were washed with 70% ethanol, air-dried and finally solubilized in TE buffer.

Cloning procedures. A genomic library of the ISO-resistant mutant was constructed by ligating partially digested *Sau*3AI fragments of chromosomal DNA into *Bam*HI-digested, and dephosphorylated pYUB18 (Fig. 3.2) (Jacobs *et al.*, 1991). The ligation reaction was packaged into lambda (λ) phage particles using Gigapack Gold packaging extracts (Stratagene). The resulting phage particles were then transfected *E. coli* HB101 (Maniatis *et al.*, 1982). Subsequently, transfected cells were recovered in LB broth and incubated at 37°C for 1 h before being selected by plating on LB containing 25 µg/ml kanamycin. Prior to pooling transfectants, several colonies were selected and cosmids were isolated.

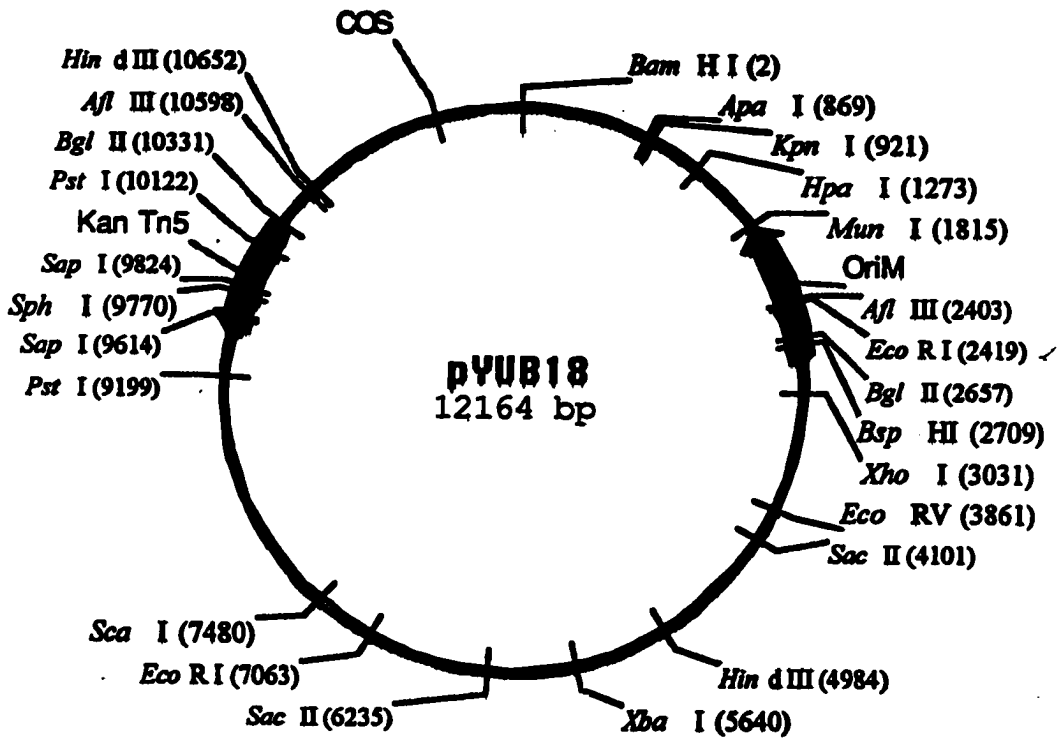

Figure 3.2 The restriction map of cosmid pYUB18 used in genomic library construction. Fragments of 35-40 kb of *Sau*3AI-digested chromosomal DNA were cloned into the *Bam*HI digested pYUB18 (Jacobs *et al.*, 1991).

Restriction mapping using *Eco*RI or *Pst*I was performed to analyze the divergence of inserts in the isolated cosmids. Transfectants were finally pooled and cosmids were extracted using Qiagen plasmid large-scale preparation kit (Qiagen). The resulting cosmid library was used to transform ISO-susceptible mycobacterial hosts, *M. bovis* BCG and *M. aurum* A+ WT strains.

Transformation of *M. bovis* BCG and *M aurum* A+. Electrocompetent cells of *M. bovis* BCG were prepared at RT as follows. Sauton's medium (50 ml) was inoculated with 1 ml of an actively growing culture of *M. bovis* BCG and incubated to A_{600} of 0.4. Cells were harvested by centrifugation at 8,000 x g for 10 min and washed once in sterile deionized water. Subsequently, cell pellets were resuspended in 50 ml of 10% sterile glycerol, and centrifuged. The resulting supernatant was discarded, and the 10% glycerol was added with reduced volume to 25, 10, and 5 ml, respectively. After a final centrifugation, the cell pellets were resuspended in 500 µl of 10% glycerol. A 200 ng quantity of *M. aurum* A+ mutant library was added to a 100 µl aliquot of cell suspension, gently mixed by pipetting up and down. Samples were then electroporated with a Bio-Rad Gene Pulser (Bio-Rad; Richmond, CA) in a 0.2 cm cuvette at 2.5 kilovolts (kv), 25 microfarads (µfad) and 1000 ohms. The electroporated cells were allowed to recover in 1 ml of Sauton's medium at 37°C overnight. An aliquot of 100 µl of cell suspension was then plated on ISO-containing (20 and 40 µg/ml) 7H11 agar plates with kanamycin 20 µg/ml. All plates were

incubated at 37°C for 21 days. In parallel, transformation of the genomic library into *M. aurum* A+ was performed in a similar manner except that electrocompetent cells were prepared at 4°C. After electroporation in pre-cooled electroporation cuvette, transformed cells were recovered in 1 ml nutrient broth plus 0.05% Tween 80 and incubated at 37°C for 4 h. Selection for the ISO-resistant phenotype was performed as described earlier but plates were incubated at 37°C for 4 days.

Analysis of mycobacterial cosmids by electroduction. To analyze cosmids that confer resistance to ISO in mycobacterial transformants, the electroduction method was applied (Baulard *et al.*, 1992). Briefly, the mycobacterial transformants were randomly selected and resuspended in 50 µl of a cell suspension of electocompetent *E. coli* XL-1 Blue (Stratagene). The mixture was allowed to sit on ice for 15 min prior to electroporation which was performed with a Bio-Rad Gene Pulser (Bio-Rad) in a 0.2 cm cuvette at 2.5 kv, 25 µfad and 200 ohms. Electroporated cells were recovered by adding 1 ml of LB broth and incubated at 37°C for 1 h. For selection, cells were plated on 25 µg/ml kanamycin and allowed to grow at 37°C overnight. Two *E. coli* transformants were picked from each transformation for further analysis of recombinant cosmids by restriction digestion.

3.2.3 Alternative approach using a genomic library of *M. tuberculosis* H37Rv

The genomic library of *M. tuberculosis* H37Rv (generated by Dr. Aimee E. Belanger) was transformed into electrocompetent *M. bovis* BCG as described earlier. The selection for ISO-resistant phenotypes was modified as follows. After overnight recovery in 1 ml Sauton's medium, electroporated cells were diluted by Sauton's medium to a low A_{600} (0.200), and selected by ISO and kanamycin at concentration 5 and 20 µg/ml, respectively. The absorbance at A_{600} was monitored for several days. Finally, 100 µl of cell suspension was plated on 7H11 medium containing 5 µg/ml ISO and 20 µg/ml kanamycin. ISO-resistant clones were randomly selected, electroduced to *E. coli* and cosmids were isolated using Qiagen column as previously described. Restriction map analysis was performed by digesting cosmids with either *Eco*RI or *Pst*I using standard procedures.

3.3 RESULTS

3.3.1 Genetic diversity of the mycobacterial strains used in this study.

By using *Pvu*II, a restriction enzyme that cleaves IS*6110* once at position 461 (van Embden *et al.*, 1993), and by probing for an IS*6110*-specific target sequence located to the right of the *Pvu*II site, we can differentiate various strains of *M. tuberculosis* including *M. bovis* BCG (Fig. 3.3). Multiple hybridizing bands were observed in DNA from *M. tuberculosis*. All 12 strains of *M. tuberculosis* contained multiple DNA fragments that hybridized with the IS*6110* probe, and each strain displayed a different banding pattern. Similar banding patterns were found in strain W 670 and W 3432 (Fig. 3.3, lane 7 and 8) suggesting that these two strains may have a close relationship. As shown in lane 14 of Fig. 3.3 only one fragment hybridized with the IS*6110* probe in *M. bovis* BCG. This result indicates that the insertion element is present as a single copy in this strain.

The analysis of IS*6110*-based restriction fragment length polymorphism (RFLP) of *M. tuberculosis* H37Rv and its avirulent mutant H37Ra was also performed using *Pvu*II restriction digestion. The IS*6110*-patterns of both strains showed difference in the copy number of IS*6110*. Most copies appear to be located at the same sites of insertion (Fig. 3.3, lane 10 and 13). As predicted, RFLP can differentiate these two strains of *M. tuberculosis*.

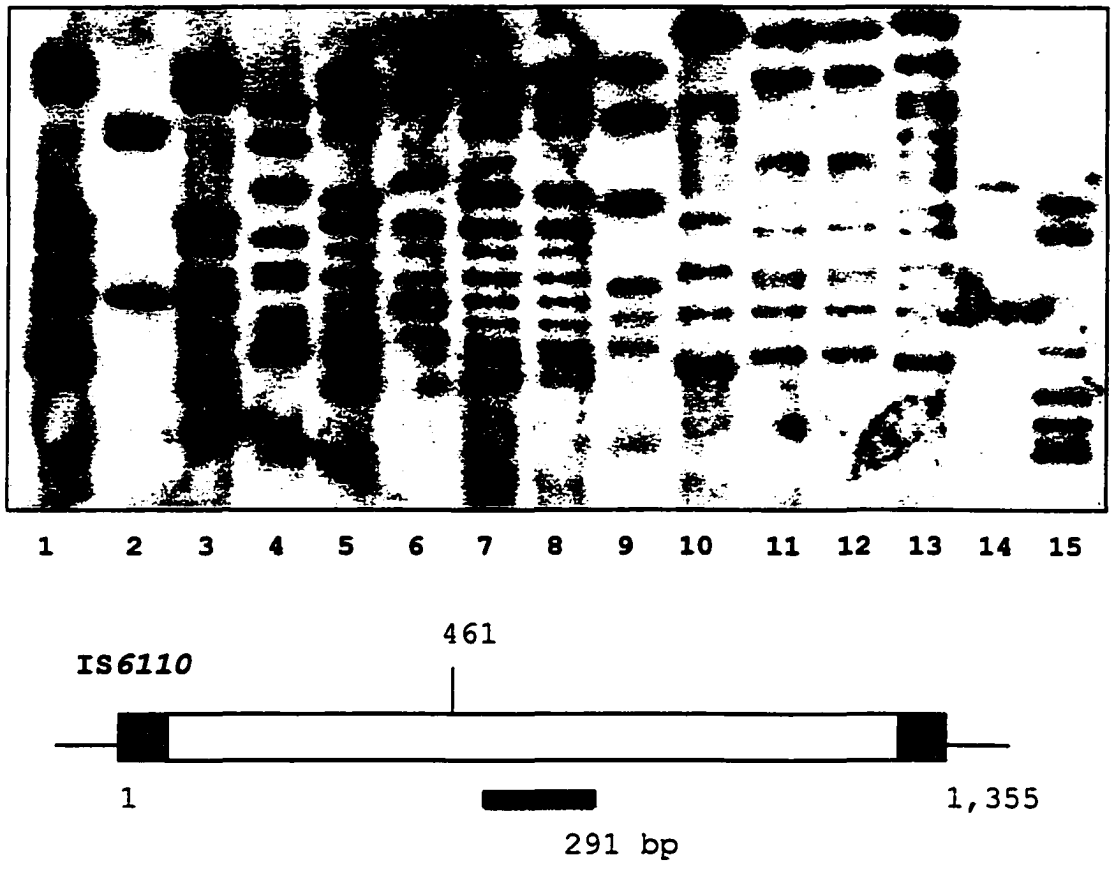

Figure 3.3 IS*6110*-based fingerprints of strains of the *M. tuberculosis* complex used in this study. The strains of *M. tuberculosis* are clinical isolates derived from different geographic areas and have different drug resistant phenotypes (Table 3.1). Genomic DNA was digested with *Pvu*II. After electrophoretic separation and blotting onto a nylon filter, the restriction fragments were probed for IS*6110*-specific sequences located to the right of the single *Pvu*II site in IS*6110*. Fingerprints of *M. tuberculosis* strains: CSU 15 (lane 1); CSU 21 (lane 2); CSU 22 (lane 3); CSU 32 (lane 4); CSU 39 (lane 5); CSU 44 (lane 6), W 670 (lane 7); W 3432 (lane 8); Erdman (lane 9); H37Rv (lane 10); CDC 97 passage 1 (lane 11); CDC 97 passage 4 (lane 12); H37Ra (lane 13); *M. bovis* BCG (lane 14); DNA markers (lane 15). The drawing at the bottom of the panel represents the physical map of the 1.35-kbp IS*6110* element showing the cleavage site of the restriction enzyme *Pvu*II at position 461 and the position of the IS*6110* probe (Black bar). Closed gray areas represent the 28-bp inverted repeats bordering IS*6110*. The lines to the left and right denote chromosomal DNA.

3.3.2 Characterization of the generated mutant

Drug resistance levels of *M. aurum* A+ WT and the generated mutant were evaluated with various concentrations of ISO (2.5 to 100 µg/ml). The results showed that the selected mutant was completely resistant to 40 and 100 µg/ml ISO. As expected, the *M. aurum* A+ WT strain could not grow on 7H11 containing 40 µg/ml and 100 µg/ml ISO. Presumably, the mutant must have a resistance level to ISO in excess of 100 µg/ml. As demonstrated, this strain has at least a 20-fold increase in MIC over the parent strain, *M. aurum* A+ (MIC of ISO, 2.0 µg/ml on 7H11 agar).

The susceptibility profile of this mutant was also determined with other mycolic acid inhibitors such as INH and ETH. The results showed that the mutant is highly resistant to ISO and ETH at drug concentrations as high as 40 µg/ml but it is susceptible to INH. Fig. 3.4 shows that the generated mutant can grow on 7H11 containing 40 µg/ml of ISO (A) and 40 µg/ml ETH (B), while the parent strain, *M. aurum* A+ WT cannot grow on the same agar plates. This observation indicates the existence of cross-resistance between ISO and ETH but not INH. The resistance level to ETH in the generated mutant was not absolutely determined in this study. According to the MIC of ETH in the *M. aurum* A+ WT (2.0 to 4.0 µg/ml)(Quemard *et al.*, 1992), this generated mutant has at least a 10-fold increase in MIC over the parent strain.

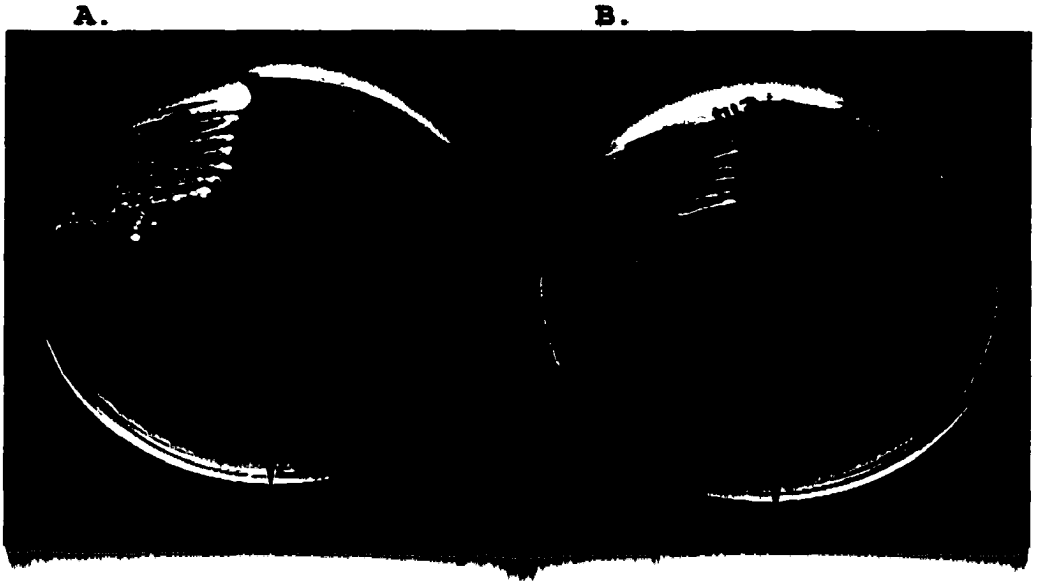

Figure 3.4 The susceptibility test of *M. aurum* A+ WT and the generated mutant resistant to ISO and ETH. The parent and the mutant strain were streaked on 7H11 agar containing 40 µg/ml of ISO (A) and 40 µg/ml of ETH (B). The left half of each plate represents the growth of *M. aurum* A+ ISO-resistant strain, while the right half of plates represents the growth of *M. aurum* A+ WT strain.

The effect of ISO on the synthesis of fatty acids and mycolic acids was determined in the *M. aurum* A+ WT and the ISO resistant mutant. TLC analysis revealed that the incorporation of radioactivity into FAMEs and MAMEs in *M. aurum* A+ WT strain decreased in the presence of ISO (Fig. 3.5, lane 1, 2, and 3). In contrast, a similar whole-cell [1,2-^{14}C]acetate labeling of the ISO-resistant mutant demonstrated that in this strain ISO did not have the inhibitory effect on the incorporation of [^{14}C]acetate into both fatty acids and mycolic acids (Fig. 3.5, lane 4, 5, 6, and 7). The comparison of the effect of ISO on the incorporation of [^{14}C]acetate into fatty acids and mycolic acids showed that the ISO-resistant mutant produced fatty acids and mycolic acids in the wild-type ratio.

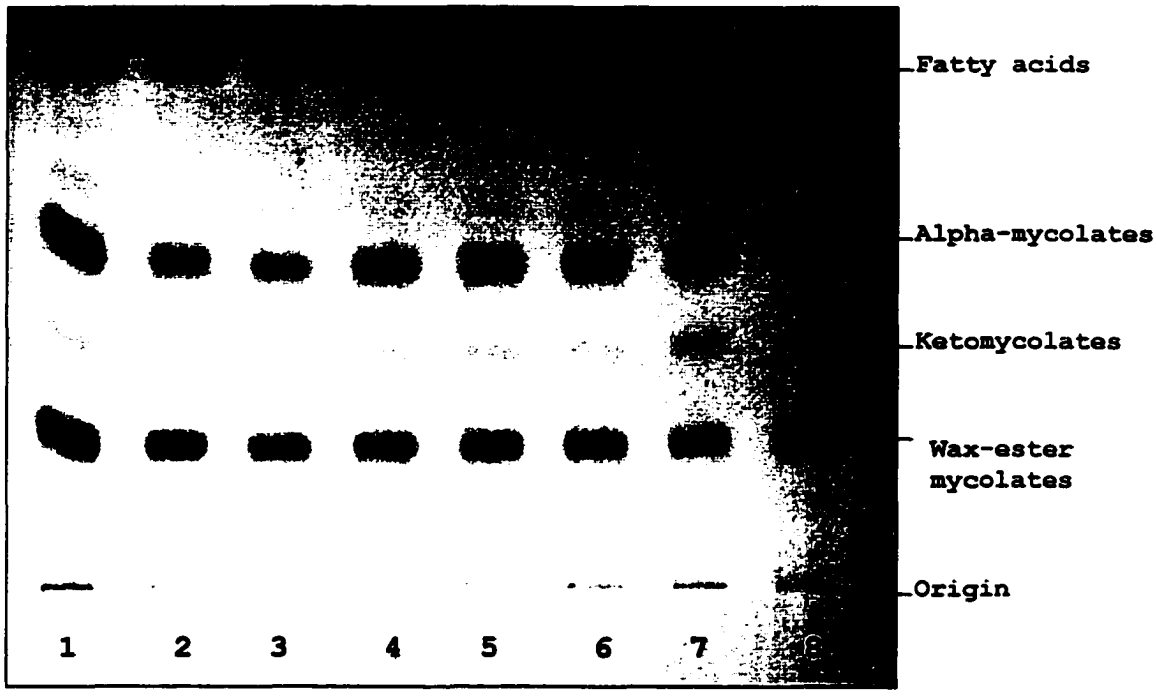

Figure 3.5 TLC analysis of the effect of ISO on [^{14}C]acetate incorporation into fatty acids and mycolic acids in *M. aurum* A+ WT and the ISO-resistant mutant. FAMEs and MAMEs from [1,2-^{14}C]acetate-labeled *M. aurum* A+ WT and ISO-resistant mutant were extracted, and 20 µl of 1 ml extracts were applied on aluminium-backed TLC plates (Silica gel 60 F_{254}). The plate was developed six times in petroleum ether and acetone (95:5). The resulting autoradiogram was obtained after exposure of the TLC plate to Kodak X-Omat AR film. FAMEs and MAMES from *M. aurum* A+ WT strain are in: lane 1, ISO 0 µg/ml; lane 2, 2 µg/ml; lane 3, 10 µg/ml. FAMEs and MAMEs from ISO-resistant mutant are in: lane 4, ISO 0 µg/ml; lane 5, 10 µg/ml; lane 6, 20 µg/ml; lane 7, 30 µg/ml; lane 8, 40 µg/ml.

3.3.2 Identification of ISO targets and mechanisms of ISO resistance by genomic library screening

The genomic library of the *M. aurum* A+ ISO-resistant mutant was constructed by cloning partially digested *Sau*3AI fragments of 35-40 kb chromosomal DNA (Fig. 3.6, panel A) into the *Bam*HI site of the mycobacterial-*E. coli* shuttle cosmid pYUB18. This particular library was used because of the high level of ISO resistance exhibited by this isolate (100 µg/ml); the MIC for the *M. aurum* A+ WT strain is 2.0 µg/ml (chapter 4). The resulting cosmid library was analyzed for the presence of chromosomal inserts by the transforming cosmid library into *E. coli* and clones were analyzed for chromosomal inserts by restriction mapping. The *Eco*RI restriction analysis showed that the cosmid library contained about 70-80% of recombinant cosmids that have chromosomal inserts (Fig. 3.6, panel B). The rest, about 20-30% of genomic library, contains empty cosmids, which were pYUB18 without inserts. *M. bovis* BCG and *M. aurum* A+ host cells were then electroporated with the genomic library of ISO-resistant mutant, followed by selection which was performed by plating transformants on 7H11 agar containing kanamycin (20 µg/ml) and ISO with respect to its resistance level (20 and 40 µg/ml). Numerous clones appeared on selective media. On the control plates, there were a lot of clones that had been transformed with vector only and could grow on agar containing 20 µg/ml kanamycin and 5 µg/ml of ISO. The result from the control indicated the high rates of chromosomal mutation resulting in the ability of empty cosmids

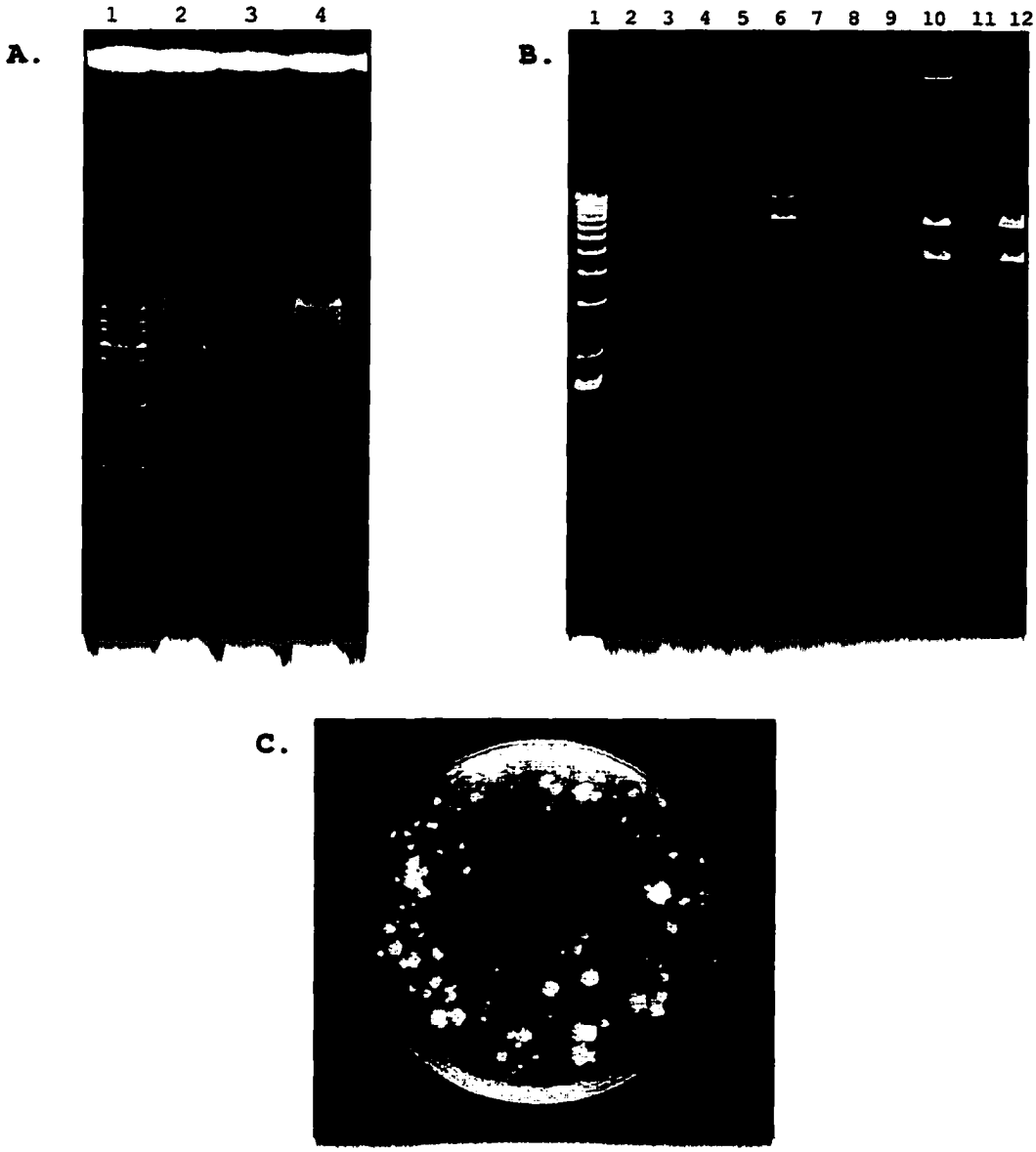

Figure 3.6 Agarose gel of *Sau*3AI-digested chromosomal DNA of *M. aurum* A+ ISO-resistant mutant (A). Agarose gel analysis of *Eco*RI digested cosmids from the genomic library of *M. aurum* A+ ISO-resistant mutant (B). Lane 1, 1 kb ladder markers; Lane 2 and 9, cosmids without inserts; Lane 3-8 and Lane 10, cosmids with chromosomal inserts; Lane 11; no sample; Lane 12, vector control cut by *Eco*RI (B). Clones of *M. bovis* BCG transformants conferring resistant to kanamycin (20 µg/ml) and ISO (5 µg/ml) (C).

to confer resistance to ISO. However, analysis of cosmids derived from transformation with the ISO-resistant library was continued and performed by electroduction (Baulard *et al.*, 1992). Restriction enzyme analysis using *Eco*RI or *Pst*I showed that all randomly picked clones (more than 40 clones) harbor the vector only. On the other hand, none of the clones conferring resistance to ISO has cosmid with chromosomal insert.

The failure to identify a chromosomal gene that may confer resistance to ISO from the genomic library of the ISO-resistant mutant can be explained as follows. 1) ISO has a low ability to select ISO-resistant mutants while it also generates mutant clones via chromosomal mutation during selection. 2) The constructed genomic library contained high numbers of empty cosmids. 3) The cosmids lost their inserts during cosmid electroduction and preparation. Alternatively, 4) the low transformation efficiency of *M. bovis* BCG and *M. aurum* A+ prevents the introduction of large pieces of DNA recombinants into the host cells.

It was decided to screen the genomic library *of M. tuberculosis* H37Rv that was readily available (a gift from Dr. Julia M. Inamine). The analysis of this cosmid library showed a high percentage of cosmids with chromosomal inserts (~90%). Although electroporation of *M. bovis* BCG with the *M. tuberculosis* cosmid library generated numerous transformants conferring resistance to ISO and kanamycin, subsequent analysis of transformants showed that all selected transformants carried empty cosmids. The strategy of selection was thought to be one of the

critical steps in generating chromosomal mutations. Therefore inoculating transformants into liquid medium containing 5 µg/ml of ISO was performed in the selection procedure. The medium and drug were changed every day to ensure that ISO was still in an active form and hence it has an ability to select the true resistant clones. After incubated with ISO in liquid medium for several days, cells were plated on 7H11 agar containing 20 µg/ml kanamycin and 5 µg/ml ISO. Several clones were observed on selection plates (Fig. 3.6, panel C). The analysis of cosmids in randomly picked transformants showed that all selected clones contained empty cosmids.

3.4 DISCUSSION

This study was divided into two separate parts. The first part is the characterization of mycobacterial strains used in this study. The most widely used method at present to differentiate strains of the *M. tuberculosis* complex, the *Pvu*II-digested IS*6110* RFLP, was applied. The genetic differentiation of mycobacterial strains is based on polymorphisms generated by IS*6110*, which is a repetitive element found in the *M. tuberculosis* complex. This particular insertion sequence consists of 1,355 bp with 28 bp repeat sequences bordering both ends (Fig. 3.3) (van Embden *et al.*, 1993). The dissimilar RFLP patterns occurred by random insertions and a variety of copy numbers of IS*6110* in the *M. tuberculosis* genome (Mazurek *et al.*, 1991; van Soolingen *et al.*, 1993; 1994). Therefore, the discriminatory power of IS*6110*-based

RFLP is a function of copy number, insertion site plus a variety of associated flanking sequences. However, if the insertion of IS*6110* occurs in preferred regions of the genome, then the variety of RFLP patterns that can be generated will be reduced (Zhigang and Forbes, 1997). In this case, alternative markers are required for differentiation (Warren *et al.*, 1996).

The standardized IS*6110*-RFLP for differentiation of *M. tuberculosis* strains has been published (van Embden *et al.*, 1993). The procedure depends on Southern blotting of the *Pvu*II restriction endonuclease fragments, which are then subjected to hybridization to a probe complementary to the 3' region of IS*6110*. The enzyme *Pvu*II cuts IS*6110* at a single site so that the probe hybridizes to one copy of the insertion sequence. IS*6110* typing is highly reproducible and the band profiles have been shown to be stable even when strains were passaged in experimental animals, or subjected to multiple passages on laboratory media (Hermann *et al.*, 1990). This previous finding was observed in this study when strain CDC 97 was passaged up to 4 times on agar medium. The IS*6110*-RFLP patterns of strain CDC 97 present in lane 11 (passage 1) and lane 12 (passage 4) are still identical (Fig. 3.3). Therefore, the repeated serial *in vitro* passage of *M. tuberculosis* does not change the position or the copy number of IS*6110* suggesting the stability of IS*6110*.

The previous study reported that a very small number of *M. tuberculosis* strains are free of IS*6110*. It was noted that the copy number of IS*6110* in *M. tuberculosis* strains varies from 1 to

21, but the majority of *M. tuberculosis* strains have 6-15 copies. The maximum number of copies that can be supported by a strain appears to be approximately 19-21 (Saunders, 1999). Strains carrying five or more copies of IS*6110* and exhibiting indistinguishable band profiles can be shown to have a close epidemiological association. The similarity of IS*6110*-RFLP patterns in the W 670 strain (lane 7) and the W 3432 strain (lane 8) (Fig. 3.3) can therefore be explained by the possibility that one strain originated from the other. Subsequently, they developed resistance to various drugs resulting in different phenotypes based on drug resistance profiles (Table 3.1).

The IS*6110*-RFLP pattern also demonstrated differences between *M. tuberculosis* H37Ra and H37Rv strains (Fig. 3.3). The virulent H37Rv strain and its avirulent derivative, H37Ra, were originally derived from the classical *M. tuberculosis* strain H37 (Steenken *et al.*, 1934; Steenken and Gardener, 1946). The serial passage of *M. tuberculosis* H37 through laboratory media allowed the dissociation of the parent strain into two forms: the virulent *M. tuberculosis* H37Rv and the attenuated *M. tuberculosis* H37Ra (Steenken *et al.*, 1934). Subsequently, the original *M. tuberculosis* H37 was lost (Brosch *et al.*, 1999). Strains of H37Ra and H37Rv can be recognized according to polymorphisms generated by IS*6110*. The IS*6110*-RFLP patterns clearly demonstrated a difference in copy number, and perhaps a difference in some of the insertion sites of IS*6110* in their genomes. However, both strains share most fragments and few more hybridizing bands were observed in H37Ra

strain (Lari *et al.*, 1999). It should be noted that the recently published sequence of the complete genome of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) reported that this strain has 16 copies of IS*6110*. This copy number is higher than those detected in this study. The discrepancy between the experimental data and the expected result is likely due to the co-migration of the probe-positive restriction fragments. Cole *et al.* (1998) proposed that in *M. tuberculosis* H37Rv most of the IS*6110* copies appear to insert in the intergenic or non-coding regions and many are clustered. These behaviors of the insertion element prevent genes from being inactivated in the H37Rv strain.

For *M. bovis* BCG (*M. bovis* Bacille Calmette Guerin), as predicted, it is quite common to see only one copy of the insertion element in its genome. *M. bovis* BCG is an attenuated strain *of M. bovis* and is a member of the *M. tuberculosis* complex comprised of *M. tuberculosis, M. bovis, M. africanum* and *M. microti*. The strain BCG was derived from an isolate of *M. bovis* which causes bovine tuberculosis and was attenuated through 230 serial passages *in vitro* over 13 years (Calmette *et al.*, 1924). The extensive subculturing of the *M. bovis* progenitor resulted in a series of mutations and evolution of the BCG sub-strains or daughter strains. It is thought that genetic deletions and additions account for the differences between *M. bovis* BCG and other members of the *M. tuberculosis* complex (Behr *et al.*, 1999). The difference between *M. bovis* BCG and *M. tuberculosis* can also be recognized by the IS*6110*-RFLP patterns.

In addition to the characterization of *M. tuberculosis* strains readily available in the stock collection, this part of the study was extended to the generation of a mutant resistant to ISO. *M. aurum* A+ was used as a selective organism because it is a fast grower and highly susceptible to ISO. The partial characterization showed that the generated mutant appeared to possess resistance to ISO and ETH. Previously, the cross-resistance of ISO, ETH and thiacetazone was observed in *M. tuberculosis* clinical isolates (Porter and Neal, 1978; Tskamura, 1962; Sojkova *et al.*, 1965; Tan *et al.*, 1966). Therefore, it is not surprising to see the cross-resistance between ISO and ETH in the *M. aurum* A+ mutant (Fig. 3.4). As reviewed early in chapter 2, ISO and ETH apparently act on different sites in the fatty acid and mycolic acid synthesis pathways, and both drugs have a thiocarbonyl moiety in common. Together, the findings suggested that ISO and ETH probably share a common step in the activation process, and it is more likely that the cross-resistance to ISO and ETH in the generated mutant strain is associated with defects in drug activation. If there is a mutation in the gene that encodes the enzyme activating ISO, it is reasonable to see no effects of ISO on fatty acid and mycolic acid synthesis in the generated mutant strain.

Attempts were made to identify mechanisms of ISO resistance by screening the genomic library of the ISO-resistant strain. The unexpected ability of the empty cosmids that could confer resistance to ISO can be explained by the spontaneous mutation of

chromosomal DNA in host cells. The high mutation rates inherent in both the *M. aurum* A+ and *M. bovis* BCG hosts enhance the problem of "brute screening". In general, screening methods are the most obvious means of isolating mutants and are one of the labor-intensive steps. Screening a large number of mutants would increase the probability of isolating the desired clones. However, the high background of clones that can confer resistance to ISO even when they contain only vector made the screening more laborious. Alternatively, double selection in liquid medium and subsequent selection on 7H11 agar was applied, but it did not yield the desired clones. The work was left waiting for some techniques that can be adapted or modified.

In conclusion, this part of the work reflects much of the learning acquired despite a major block encountered in the identification of the target gene by genomic library screening. The work provides a useful reference for future studies. Several strains of mycobacteria were characterized, and a strain cross-resistant to ISO and ETH was generated. Principles and techniques discussed here can be applied in the identification of targets of other drugs. Concerning the achievement of study goals, alternative methods were invented and applied. The identification of ISO targets was subsequently performed by defining the specific site of inhibition of ISO using biochemical approaches, followed by identifying the gene product catalyzing that specific step. The combination of biochemical and genetic means ultimately resulted in the identification of a target of ISO.

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CHAPTER 4

Antimycobacterial Activities of Thiocarlide/Isoxyl® and New Derivatives Through the Inhibition of Mycolic Acid Synthesis

(Presented in BENJAWAN PHETSUKSIRI, ALAIN R. BAULARD, ANDREA M. COOPER, DAVID E. MINNIKIN, JAMES D. DOUGLAS, GURDYAL S. BESRA, AND PATRICK J. BRENNAN. 1999. Antimicrob. Agents and Chemother. **43**:1042-1051.)

4.1 INTRODUCTION

Despite the availability of effective chemotherapies, tuberculosis, among those infectious diseases caused by a single etiology, is still a leading cause of death (Murray *et al.*, 1990; Snider *et al.*, 1994). The human immunodeficiency virus pandemic, which contributes substantially to the morbidity and mortality of tuberculosis (Barnes *et al.*, 1991; Bloom and Murray, 1992), and the emergence of multidrug-resistant strains of *M. tuberculosis* (Dooley *et al.*, 1992; Snider *et al.*, 1992) have compounded the problem. Although infections with drug-susceptible strains of *M. tuberculosis* can be successfully cured with the current combination of isoniazid, rifampin, pyrazinamide and ethambutol or streptomycin (Combs *et al.*, 1990), the problem of drug resistance and the continuing rise in disease incidence have prompted research on new drug development, particularly the search for new drug targets and the definition of mechanisms of drug resistance.

Isoniazid (INH), which is one of the most efficient and the most widely used antituberculosis drugs (Youatt, 1969), has been

the subject of intensive research on its modes of action and mechanisms of resistance. Both *M. tuberculosis* and *M. bovis* BCG are extremely susceptible to INH when used in the range of 0.02-0.2 μg/ml (Bernstein *et al.*, 1952), and much early evidence suggested that INH specifically inhibited the synthesis of mycolic acids in *M. tuberculosis* (Takayama, 1974; Takayama *et al.*, 1972; Wang and Takayama, 1972; Winder and Collins, 1970). INH is a prodrug which requires activation by the endogenous mycobacterial enzyme catalase-peroxidase (KatG) (Middlebrook, 1952; Zhang *et al.*, 1992) to form an electrophilic species (Johnsson and Schultz, 1994; Winder, 1960; Winder, 1982) before reacting with targets such as InhA (Banerjee *et al.*, 1994). Other targets of the activated INH have been suggested to include two component of the type II fatty acid synthase system, a 12-kDa acyl carrier protein (ACP) designated AcpM and β-ketoacyl ACP synthase (KasA)(Mdluli *et al.*, 1998).

Ethionamide (ETH), a structural analog of INH, is a useful second-line antituberculosis drug (Winder, 1982), and both drugs have almost identical effects in strongly inhibiting the synthesis of mycolic acids, slightly decreasing the synthesis of bound non-mycolic acids, and stimulating the synthesis of soluble lipids in susceptible species of mycobacteria (Quemard *et al.*, 1992; Winder *et al.*, 1971). ETH is inhibitory for *M. tuberculosis* in liquid medium at about 1 μg/ml and is more active against INH-resistant strains (Winder, 1982). The work of Banerjee and colleagues (1994) demonstrated that a single mutation in the *inhA* gene, now

known to encode a NADH-specific, 2-*trans*-enoyl ACP (acyl carrier protein) reductase, conferred resistance to both INH and ETH, leading to the impression that the mode of action of both drugs was identical. The one notable distinction between the actions of ETH and INH is in the absence of complete accord in resistance patterns; strains resistant to ETH are still susceptible to INH, while strains resistant to INH show slightly increased susceptibility to ETH (Rist, 1960; Winder, 1982).

Isoxyl (ISO), a 4,4'-diisoamyloxydiphenylthiourea (4,4' diisoamyloxythiocarbanilide; thiocarlide)(Winder, 1982) is an old drug used for the clinical treatment of tuberculosis in the 1960s. Urbancik (1966; 1970) and Titscher (1966) demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary tuberculosis of various degrees of difficulty. The drug was able to convert about 25% of bacteriologically chronically positive cases to negative after 6 to 8 weeks of 6 g of ISO daily. However, when the treatment was extended to 10 to 18 weeks, about 50% of the patient population had their sputa converted to negative (Kampelmann, 1970). Schmid (1970) concluded that combined INH and ISO was more effective than monotherapy with either drug. It had been noted in the early 1950s that ISO exhibited strong antimycobacterial activity *in vitro* (Winder, 1982). A note from Winder *et al.* (1971) showed that like INH and ETH, ISO strongly inhibited mycolic acid synthesis in *M. bovis* during 6 h of exposure to 10 µg/ml. ISO also partially inhibited the synthesis of the fatty acids of free lipids, which were

stimulated by INH and ETH. This is the extent of published work conducted on the mechanisms of action of ISO. Consequently, we examined the efficacy of ISO in an attempt to decipher its mode of action.

4.2 MATERIALS AND METHODS

4.1.1 Growth and maintenance of mycobacterial strains

M. tuberculosis H37Ra (TMCC 25711), *M. bovis* BCG strain 1173P2, and *M. avium* strain 724 were grown in 250-ml tissue culture flasks containing 50 ml of liquid Sauton's medium, and were incubated without agitation. Cells were grown to mid-exponential phase (for *M. tuberculosis* ~21 days; *M. bovis BCG* ~14 days; *M. avium* ~10 days), and harvested, and sterile glycerol was added to a final concentration of 10%. Cell suspensions were dispensed into tubes and stored at -70°C until required. Thawed suspensions were added to 50 ml of Sauton's medium to yield identical cultures for further studies. The fast-growing mycobacteria, *M. aurum* A+ (from GlaxoWelcome; Stevenage, United Kingdom), which is susceptible to INH, was grown in nutrient broth (Difco; Detroit, Mich.) containing 0.05% Tween 80. Cells were incubated to mid-exponential phase (~5 days) at 37°C with shaking, as previously described (Quemard *et al.*, 1991). *M. smegmatis* mc^2155 was grown in 250-ml Erlenmeyer flasks containing 100 ml of Sauton's medium. Cells were incubated at 37°C with shaking for 4 days, and growth was monitored by measuring A_{600}. Virulent *M. tuberculosis* type strain H37Rv (TMCC 102) and Erdman (TMCC 107)

were grown in 250-ml Erlenmeyer flasks containing 100 ml of Sauton's medium and incubated to mid-exponential phase at 37°C with shaking. A variety of human clinical isolates of *M. tuberculosis* had been stored in 2-ml aliquots and frozen at -70°C until used. The frozen stocks were counted by serial dilution in saline and plating onto 7H11 agar. The varied drug resistance patterns of these strains is shown in Table 4.1. Drug resistance profiles were identified at the time of collection, as described elsewhere (Ordway *et al.*, 1995; Rhoades and Orme, 1997).

Table 4.1 Antimycobacterial activity of ISO against clinical isolates of *M. tuberculosis*.

Strains Designation	MDR[a]	Growth inhibition with the following concentration of ISO (μg/ml)			
		1.0	2.0	5.0	10.0
CSU 15	no	no	no	yes	yes
CSU 21	yes	no	no	yes	yes
CSU 22	yes	no	no	no	yes
CSU 31	yes	no	no	no	yes
CSU 32	yes	no	no	no	yes
CSU 37	no	no	no	yes	yes
CSU 39	yes	no	no	no	yes
CSU 44	yes	no	no	no	yes
W 670	yes	no	no	yes	yes
W 3432	yes	no	no	yes	yes
BB	no	yes	yes	yes	yes
LL	yes	yes	yes	yes	yes

[a] Drug resistant profiles of these strains were as follows:
CSU 15, INH^R, Rif^S, EMB^S, Str^S, Kan^S, Cyc^S, Cap^S, ETH^S, PZA^S
CSU 21, INH^R, Rif^R, EMB^R, Str^R, Rfb^R, Kan^S, Cyc^S, Cap^S
CSU 22, INH^R, Rif^R, EMB^R, Str^R, Kan^R, Cyc^S, Cap^R, PAS^S, AMK^R
CSU 31, INH^R, Rif^R, EMB^R, Str^S, Kan^S, Cyc^S, Cap^S, PAS^S, AMK^S, ETH^S
CSU 32, INH^R, Rif^R, EMB^R, Str^S, Kan^S, Cyc^R, Cap^S, ETH^R, PZA^R
CSU 37, INH^S, Rif^S, EMB^S, Str^S, Kan^S, Cyc^S, Cap^S, PAS^S, AMK^S, ETH^S
CSU 39, INH^R, Rif^R, EMB^S, Str^R, Kan^R, Cyc^S, Cap^S, PAS^S, AMK^R, ETH^R, PZA^S
CSU 44, INH^R, Rif^R, EMB^R, Str^R, Kan^R, Cyc^S, Cap^S, PAS^S, AMK^R, ETH^R, PZA^S
W 670, INH^R, Rif^R, EMB^R, Str^R, Kan^R, Cyc^S, Cap^S, ETH^S, PZA^S
W 3432, INH^R, Rif^R, EMB^R, Str^R, Kan^R, Cyc^S, Cap^S, AMK^R, ETH^R, Cip^R
BB, INH^S, Rif^S, EMB^S, Str^S, Kan^S, Cyc^S, Cap^S, PAS^S, AMK^S, ETH^S, PZA^S, Cip^S
LL, INH^R, Rif^R, EMB^S, Str^R, Kan^R, Cyc^S, Cap^S, PAS^S, AMK^R, ETH^S, PZA^R

Profiles were determined by a conventional proportion method (Heifets, 1991a; 1991b). Strains were classified as multiple-drug resistant if they were resistant to the major antituberculosis drugs, INH and rifampin, or were resistant to these major drugs and one or more of the following: ethambutol (EMB), streptomycin (Str), kanamycin (Kan), cycloserine (Cyc), *para*-aminosalicylic acid (PAS), rifabutin (Rfb), ethionamide (ETH), amikacin (AMK), capreomycin (Cap), ciprofloxaxin (Cip) and pyrazinamide (PZA). The following responses were not determined: Rfb for CSU 15, CSU 22, CSU 31, CSU 32, CSU 37, CSU 39, CSU 44, W 670, W 3432, BB, and LL; PAS for CSU 15, CSU 21, CSU 32, W 670, and W 3432; PZA for CSU 21, CSU 22, CSU 31, CSU 37 and W 3432; and AMK for CSU 15, CSU 21, CSU 32, and W 670.

4.2.2 Determination of the MICs of ISO and new derivatives

ISO, a derivative of thiourea was a gift from M.J. Colston and P. Draper, National Institute of Medical Research, London, United Kingdom. New derivatives of thioureas included alkyl, *para*-alkoxy, *para*-alkyl and *para*-thioalkyl thioureas (Table 4.2); the synthesis of ISO and new derivatives will be documented separately. The MICs of ISO and new derivatives on solid medium were determined by the microdrop agar proportion test which was modified from the method of McClatchy (McClatchy, 1986). Briefly, a series of ten-fold dilutions of cultures of *M. tuberculosis* H37Ra, H37Rv, *M. tuberculosis* Erdman, *M. bovis* BCG, and *M. aurum* A+ were prepared by using phosphate buffered saline (PBS: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 1000 ml of distilled H_2O, adjusted pH to 7.4) as a diluent. An aliquot (5 µl) of each dilution was spotted on plates of 7H11 agar (Difco) containing oleic acid-albumin-dextrose-catalase (OADC) as a supplement (Cohn *et al.*, 1968), and 0.1, 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, and 20.0 µg of each tested drug per ml. The plates were incubated at 37°C (~4 days for *M. smegmatis* mc^2155 and *M. aurum* A+; ~12 days for *M. avium*; ~14 days for *M. bovis BCG*; ~21 days for *M. tuberculosis*), and the number of viable bacteria was scored by counting colonies. The MIC was defined as the lowest concentration of ISO or new derivatives resulting in a 99% reduction in the number of colonies on that plate compared to those on plate free of the drug at the same suspension of the culture dilution.

Table 4.2 MICs of ISO and new thiourea derivatives against slow-growing (*M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. avium*) and fast-growing (*M. aurum* A+) mycobacteria.

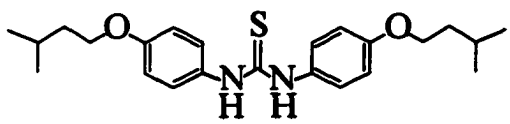
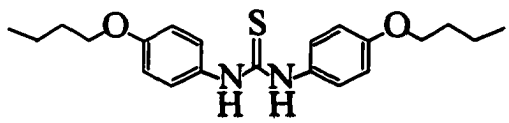
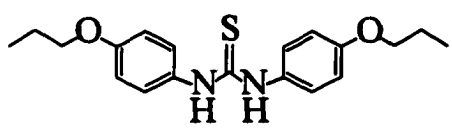
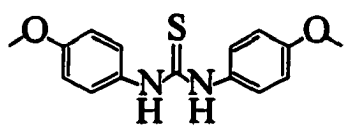
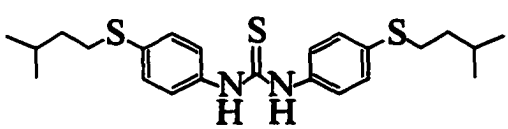
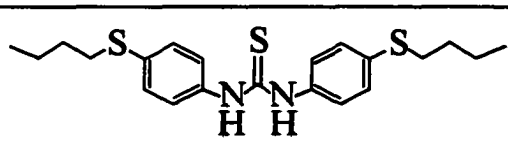
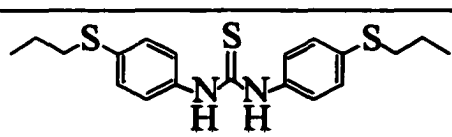
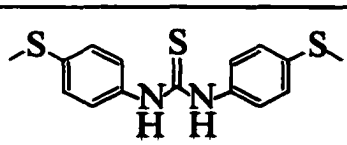
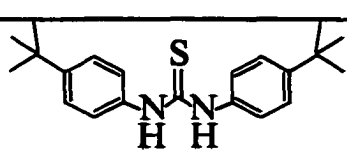
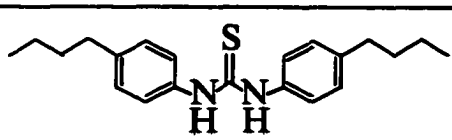
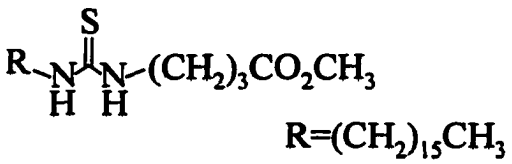
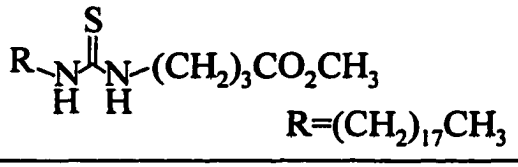
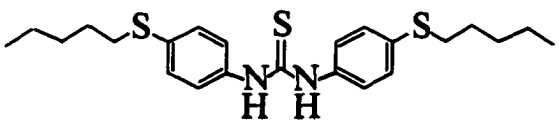
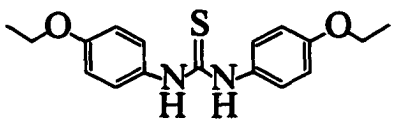
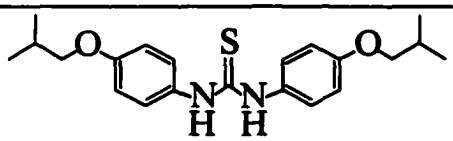
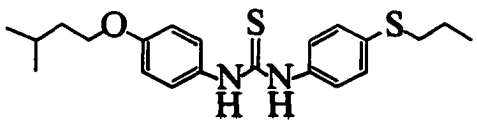
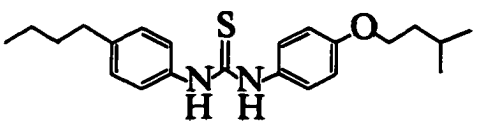
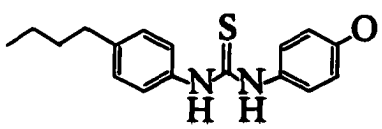
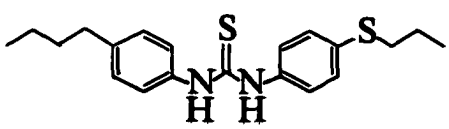
Thiourea derivatives			MIC (μg/ml) Organisms			
No.	Code	Structure	*M. tuberculosis*	*M. bovis BCG*	*M. avium*	*M. aurum A+*
1	B27		2.0	0.5	2.0	ND
2	B59		0.1-0.5	0.1-0.5	>20.0	2.5
3	B57		0.1-0.5	0.1-0.5	>20.0	2.5
4	C06		1.0	>20.0	>20.0	ND
5	C26		2.5	>20.0	2.5	10.0
6	C33		<0.1	2.5	5.0	ND
7	C25		<0.1	0.5	>20.0	ND
8	C32		0.5	0.5	>20.0	ND
9	C30		>20.0	>20.0	>20.0	>20.0
10	B01		0.1-0.5	0.1-0.5	ND	ND

Table 4.2 -continued.

Thiourea derivatives			MIC (μg/ml) Organisms			
No.	Code	Structure	*M. tuberculosis*	*M. bovis BCG*	*M. avium*	*M. aurum A+*
11	B86	 R=$(CH_2)_{15}CH_3$	>20.0	>20.0	20.0	5.0
12	B77	 R=$(CH_2)_{17}CH_3$	>20.0	10.0	>20.0	>20.0
13	C90		ND	ND	ND	ND
14	JDDD18		1.0	2.5-5.0	ND	ND
15	JDDD23		0.5	1.0	5.0	5.0
16	JDDD38		0.1	2.5-5.0	20.0	5.0-10.0
17	JDDD44		0.5	1.0	5.0	1.0-2.5
18	JDDD45		0.5	1.0	10.0	5.0-10.0
19	JDDD46		0.5	0.5	5.0	10.0-20.0
20	D14	not described	2.5-5.0	10.0	20.0	10.0-20.0

The analysis of growth curves and the estimation of broth MICs of ISO for both *M. tuberculosis* H37Ra and *M. bovis* BCG was performed in Sauton's medium. Subcultures from master cell stocks were incubated to mid-exponential phase (A_{600} ~0.600), and 500 µl of culture was inoculated in triplicate into 13 by 100-mm culture tubes containing 4.5 ml of fresh Sauton's medium. The cultures were incubated at 37°C with gentle agitation until early exponential phase (A_{600} ~0.250), at which time ISO was added to cultures for final concentrations of 1.0-8.0 µg/ml in 1% DMSO. The cultures were then subjected to gentle agitation with small magnetic stirrer bars, incubated at 37°C and growth was monitored by measuring the A_{600} with a Bausch & Lomb Spectronic 1001 Spectrophotometer once a day. The broth MIC was defined as the lowest concentration of ISO able to stop cell growth definitely after one doubling time (Quemard *et al.*, 1992). In all of the experiments, the stock of ISO and new derivatives were prepared in DMSO, and it was verified that at concentrations of DMSO up to 2% (vol/vol), the solvent had no effect on growth of these bacteria.

4.2.3 Drug susceptibility testing of drug-resistant clinical isolates of *M. tuberculosis*

A panel of clinical isolates of drug-resistant strains of *M. tuberculosis* from different geographical areas with various drug resistance patterns were diluted in microplates with phosphate-buffered saline to a final concentration of 10^6 CFU/ml for each strain. A sample (5 µl) of the 10^6-CFU/ml dilution was

then spotted on 7H11 supplemented with OADC and containing ISO at different concentrations (1.0, 2.0, 5.0, and 10.0 µg/ml and no ISO in a control plate). After inoculation of drug-resistant strains, the plates were incubated at 37°C for 21 days. Susceptibility of strains to ISO was defined as the absence of colonies on plates after the exposure of organisms to ISO for 21 days.

4.2.4 Incorporation of [1,2-^{14}C]acetate into fatty acids and mycolic acids

M. bovis BCG and *M. tuberculosis* H37Rv were grown in 5 ml of Sauton's medium in a set of culture tubes to early exponential phase, at which point ISO (other drugs tested included INH, ETH, and the butyl derivative of thiourea) was added. The cells were then incubated with gentle shaking for 10 h prior to adding [1,2-^{14}C]acetate (110 mCi/mmol) (Dupont NEN; Boston, Mass.) at 1 µCi/ml to both control and drug-treated cultures which were further incubated at 37°C with gentle agitation for an additional 24 h. The resulting [^{14}C]-labeled cells were harvested by centrifugation at 2500 x g, and washed twice with saline and once with sterile water (Slayden *et al.*, 1996). In parallel experiments, *M. aurum* A+ was grown in nutrient broth to early exponential phase, preincubated with ISO for 6 h and exposed to [1,2-^{14}C]acetate for 12 h.

4.2.5 Determination of the effects of ISO, INH and ETH on mycolic acid biosynthesis

The [^{14}C]-labeled control and drug-treated cells were resuspended in 2 ml of 15% tetrabutylammonium hydroxide (Sigma; St. Louis, MO) and saponified at 100°C overnight. After cooling, 2 ml of water, 3 ml of dichloromethane and 300 µl of iodomethane (Aldrich Chemical Co.; Milwaukee, Wis.) were added to the entire reaction mixture which was then shaken on a rolling shaker for 1 h. After centrifugation, the upper layer was discarded and the lower organic phase was washed three times with 3 ml of water. The washed lower phase was dried by a nitrogen flow, extracted with 4 ml of diethylether, sonicated for 5 min, and centrifuged at 2500 × g (Beckman GPR Desktop Centrifuge). The ethereal extract was transferred into new 13 x 100-ml glass tubes, dried, and resuspended in 1.0 ml of dichloromethane for counting of radioactivity. Scintillation counting was conducted in vials containing 10 ml of EcoLume™ (ICN; Costa Mesa, Calif.) by using a Delta 300 scintillation system (Tracor Analytic, Elk Grove, Ill.). Equal volumes of this extract, which contained fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs), were applied to preparative TLC plates of silica gel (silica gel 60 F_{254}, Merck; Darmstadt, Germany), and developed six times in petroleum ether-acetone (95:5) (Slayden *et al.*, 1996). The radioactive bands on the plate were also located and scanned by Bioscan System 200 Imaging Scanner. Each band was read stepwise for 10 min. Autoradiograms were produced by overnight exposure at

-70°C to Kodak X-Omat AR film to reveal the [^{14}C]-labeled FAME and MAME products. Separate bands of FAMEs and MAMEs were marked, cut from the TLC plates, and placed directly in 10 ml of EcoLume™, and radioactivity was counted to estimate the degree of inhibition of the synthesis of FAMEs and individual populations of MAMEs.

A more complete resolution of individual mycolate populations of *M. bovis* BCG, *M. tuberculosis* H37Rv, and *M. aurum* A+ was obtained by two-dimensional silver ion argentation-TLC (2D Ag+ TLC). To prepare TLC plates, 90% of a 10-by 10-cm silica gel plate was immersed in 5% (wt/vol) aqueous silver nitrate solution, air dried, and activated at 100°C for 1 h prior to use. A known aliquot (ca.~80,000 cpm) of the [^{14}C]-labeled FAME-MAME mixture was then applied to the Ag^{+} TLC plate and developed in the first direction in hexane-ethyl acetate (95:5) two times. The plate was air dried and run in the second direction three times in petroleum ether-diethyl ether (85:15). The TLC plate was then exposed to Kodak X-Omat AR firm at -70°C overnight, and individual FAMEs and MAMEs were marked.

4.2.6 *In vitro* murine macrophage model

Six-to 8-week-old female specific-pathogen-free C57BL/6 mice were purchased from Charles River Laboratories (Walmington, Mass.) and were sacrificed by cervical dislocation and femurs aseptically removed. The marrow was flushed out of the femurs with ice cold Dulbecco's minimal essential media (DMEM) supplemented with 10 mM HEPES, 2 mM L-glutamine, 0.05 mM 2-

mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 250 ng amphotericin B per ml, and 10% L-929 fibroblast conditioned medium (supplemented DMEM [sDMEM]) and with 10% heated-inactivated, low-endotoxin fetal calf serum (Summit Biotechnologies; Fort Collins, Colo.). All other tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The marrow plugs were disrupted by gentle pipetting, washed twice and plated at 10^6 cells per well in 24-well tissue culture microplates (Falcon 3047; Becton Dickinson, Lincoln Park, NJ). After 48 h of incubation at 37°C in a 5% CO_2 and 96% humid environment, the non-adherent cells were removed and new sDMEM was added every two days. The sDMEM was changed every two days, and two days prior to infection, medium free from antibiotic (incomplete sDMEM) was added. Eight days after plating, macrophage cells were infected with 10^6 CFU of *M. tuberculosis* strain Erdman in 200 μl of medium for 2 h. Macrophages were then extensively washed to remove extracellular bacteria and incubated in incomplete sDMEM containing various concentrations of ISO or the butyl derivative at concentrations given in Fig. 4.6A. The series of concentrations of the butyl derivative used are given in Fig. 4.6B. Macrophage cells were lysed in 1 ml of distilled water with 0.05% Tween 80 after 6 days of incubation. Three 10-fold dilutions were made, and 0.1 ml from each dilution was plated on 7H11 medium (Difco) and incubated in a 37°C dry-air incubator. The number of viable bacteria in each well was scored by counting the number of colonies resulting from each dilution on 7H11

plates. As a control, several wells were lysed immediately after the initial infection to determine the number of bacteria phagocytosed and to assess the extent of growth over time.

4.2.7 Determination of possible *in vitro* toxicities of ISO and the butyl derivative by microplate Alamar Blue assay

Murine macrophages were prepared from C57BL/6 mice as described above. On day 6 after plating, the sDMEM was changed and a fresh 2-ml aliquot of incomplete DMEM medium was added into each well. On day 8, the old medium was removed, and 1.8 ml of incomplete medium containing ISO or the butyl derivative at concentrations 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.032, 0.017 µg/ml was added with 200 µl Alamar Blue reagent (AccuMed International; Cleveland, Ohio), and plates were incubated at 37°C in the controlled-humidity and -CO_2 environment. The color of the Alamar Blue dye mixed with incomplete DMEM, and cell morphology were observed periodically within 3 days.

4.3 RESULTS

4.3.1 Determination of MICs of ISO and new derivatives

It is impossible to find accord in the literature on a standardized means of measuring MICs applicable to the different physical properties of drugs and different mycobacteria. Accordingly, MICs were evaluated under the defined conditions described in Materials and Methods, with species selected to

reflect most of the known mycolic acid molecular types. ISO and a series of new derivatives with various substituted side chains were subjected to a preliminary evaluation of their *in vitro* activity against various species of mycobacteria by applying our defined quantitative agar plate proportion test involving use of 7H11 solid medium containing OADC supplement. The MIC of ISO for *M. tuberculosis* was in the range of <1.0 to 2.5 μg/ml compared to published values of 0.02 to 0.2 μg/ml for INH (Bernstein, 1952) and 5 to 10 μg/ml for ETH (Table 4.3). The fast-growing mycobacterium, *M. aurum* A+, was also susceptible to ISO with a MIC of 2.0 μg/ml. In contrast, *M. smegmatis* mc^2155 was resistant to ISO as demonstrated by the presence of a number of colonies on 7H11 plates containing concentrations of ISO as high as 200 μg/ml (Table 4.3). Thus, *M. smegmatis* mc^2155 was excluded from further studies. The growth kinetics and the broth MIC of ISO for *M. tuberculosis* H37Ra were determined in Sauton's medium. ISO at 3.0 μg/ml substantially reduced the growth rate of *M. tuberculosis* and *M. bovis* BCG and inhibited growth entirely at a concentration of 4.0 μg/ml, leading to the conclusion that the broth MIC of ISO for this strain of *M. tuberculosis* and *M. bovis* BCG is 4 μg/ml (Fig. 4.1). The MICs of most of the newly synthesized derivatives of thiourea (Table 4.2) against *M. tuberculosis* were in the range of <0.1 to 2.5 μg/ml (Table 4.2). Most of the new derivatives were as effective or more effective than ISO. We also examined the efficiency of ISO against a collection of drug-resistant

Table 4.3 MIC values of ISO against slow- and fast- growing species of mycobacteria on 7H11 medium [a].

Mycobacteria	MIC (μg/ml)
Slow-growing mycobacteria	
M. tuberculosis Erdman	<1.0
M. tuberculosis H37Rv	2.5
M. tuberculosis H37Ra	2.0
M. bovis BCG	0.5
M. avium	2.0
Fast-growing mycobacteria	
M. aurum A+	2.0
M. smegmatis mc^2155	>200.0

[a] MIC was determined on solid medium under the defined conditions as described in Materials and Methods, i.e., the MIC of ISO was defined as the lowest concentration of ISO that completely inhibited 99% of the growth of the organisms (Stone *et al.*, 1983). Different mycobacterial species reflecting the various types of mycolic acids were included in the assay. The strains most susceptible to ISO then were chosen for further studies on its mode of action.

clinical isolates of *M. tuberculosis* (Table 4.1). Under the test conditions, ISO exhibited potent antimycobacterial activity against all evaluated strains. In particular, the strains resistant to the major first-line drugs (INH and rifampin) were susceptible to ISO in the range of 1 to 10 μg/ml. No colonies of any of the drug-resistant strains were observed on plates containing 10 μg of ISO per ml (Table 4.1).

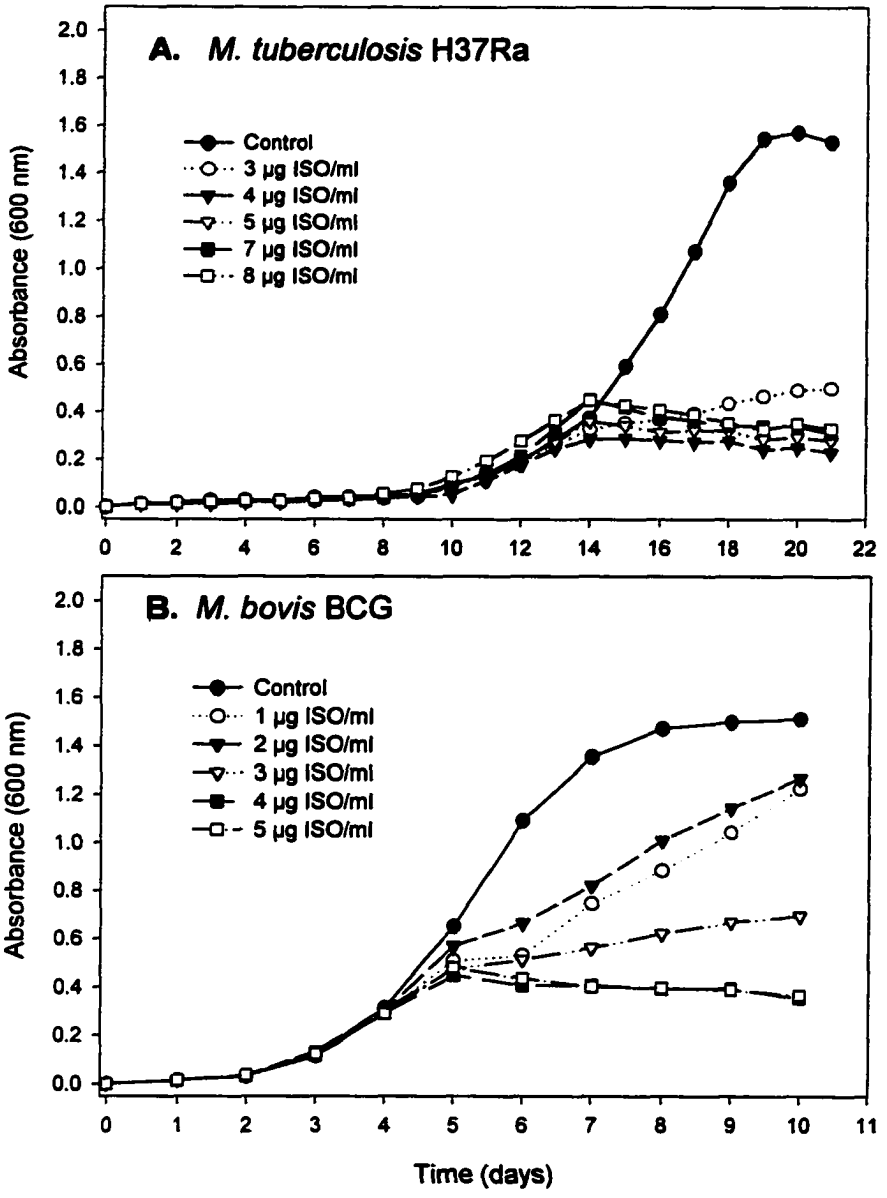


Figure 4.1 Growth characteristics of *M. tuberculosis* H37Ra (A) and *M. bovis* BCG (B) and effects of ISO on growth rates. Cells were grown in Sauton's medium to an approximate A_{600} of 0.250 in a set of culture tubes. ISO in DMSO was added at the indicated concentrations; an equivalent amount of 1% DMSO was added to control cultures. Cells were further incubated and monitored for growth rates once a day. In the presence of 3 µg/ml ISO, the growth rate of *M. tuberculosis* H37Ra was reduced, and at 4 µg/ml, ISO completely inhibited growth. Thus, the broth MIC of ISO against *M. tuberculosis* H37Ra was estimated to be 4 µg/ml. For *M. bovis* BCG in broth culture, the MIC of ISO was also 4 µg/ml. Each data point represents the mean of triplicate readings of A_{600}.

4.3.2 Selective effects of ISO on inhibition of fatty acid and mycolic acid synthesis

M. bovis BCG was grown in the presence and absence of ISO at various concentrations, following which cultures were labeled with [1,2-^{14}C]acetate. Combined MAMEs and FAMEs were extracted, resolved and fractionated on TLC plates. The results demonstrated a decrease in the incorporation of radioactivity into FAMEs and MAMEs in the presence of ISO (Fig. 4.2).

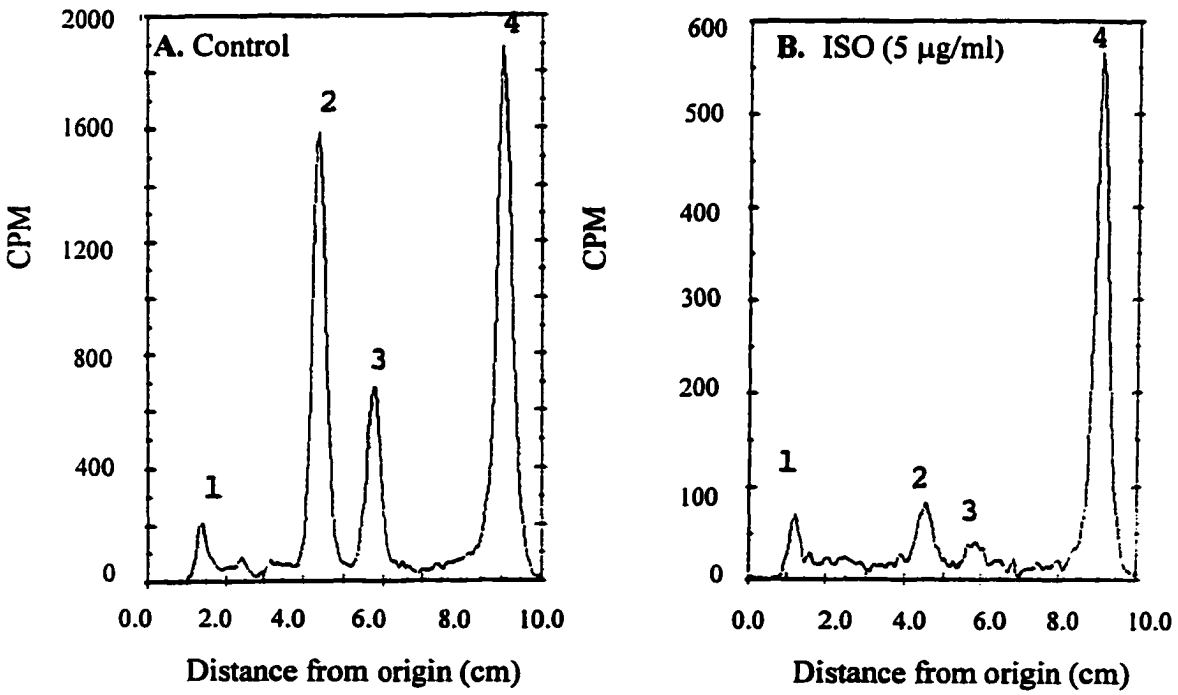


Figure 4.2 Radioactive scan of a TLC of the FAMEs and MAMEs synthesized by *M. bovis* BCG under conditions of ISO exposure. The samples applied were a mixture of esters of [^{14}C]-labeled fatty acids and mycolic acids from equal amounts of control cultures (A) and cultures of *M. bovis* BCG treated with ISO (5 µg/ml) (B). The time of preexposure of cells to ISO was 10 h prior to the labeling of cells, which lasted for 24 h. Families of FAMEs and MAMEs are indicated. Peak 1, sample origin; Peak 2, ketomycolate; Peak 3, α-mycolate; Peak 4, fatty acid.

Thus, the general effects of ISO were in accordance with those reported by Winder *et al.* (1971), i.e., a generalized inhibition of fatty acid and mycolic acid synthesis. The approach was extended to an examination of the effects of ISO on the individual classes of mycolates. Initial two-dimensional TLC demonstrated that the mycolic acid composition of *M. bovis* BCG consisted primarily of α-mycolates and ketomycolates (Fig. 4.3), and one-dimensional TLC revealed that, at the appropriate broth MIC of ISO for *M. bovis* BCG (4.0 μg/ml), the syntheses of both α-mycolates and ketomycolates were inhibited by 87.20% and 88.49%, respectively (Table 4.4). A similar whole-cell [1,2-^{14}C]acetate labeling approach was extended to *M. aurum* A+, which has the advantage of containing α-mycolates, ketomycolates and wax-ester mycolates, and to *M. tuberculosis* H37Rv, which contains α-mycolates, methoxymycolates, and ketomycolates (Fig. 4.3). ISO inhibited the synthesis of all of these classes of mycolic acids and also fatty acids of both *M. tuberculosis* H37Rv and *M. aurum* A+ (Fig. 4.4 and Fig. 4.5).

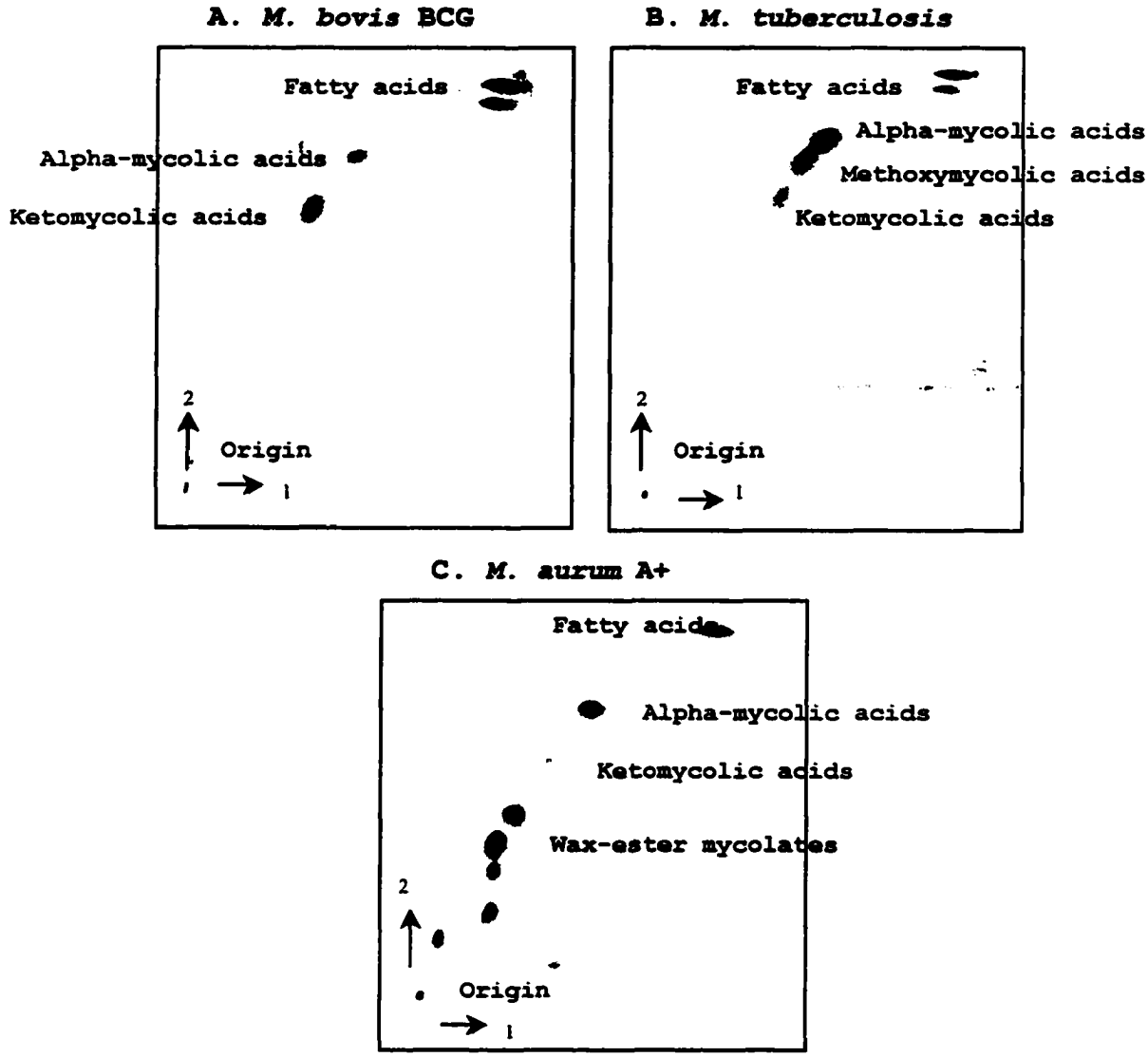


Figure 4.3 Two-dimensional silver ion argentation autoradiographic TLC of [1,2-^{14}C]acetate-labeled cells of mycobacteria to resolve and identify FAMEs and the different types of MAMEs. (A), *M. bovis* BCG; (B), *M. tuberculosis* H37Ra; (C), *M. aurum* A+. Cells were grown in a set of cultured tubes; [1,2-^{14}C]acetate was added to 5 ml of each culture for a final concentration of [^{14}C]acetate of 1 µCi/ml, and cultures were further incubated with gentle agitation at 37°C. Labeled FAMEs and MAMEs were extracted as described in Materials and Methods. The labeling time was 12 h for *M. aurum* A+, and 24 h for *M. tuberculosis* H37Ra and *M. bovis* BCG. About 80,000 cpm of each extract was applied to two-dimensional silver ion argentation TLC plates, which were developed twice in one direction in hexane-ethyl acetate (95:5) and three times in a second direction in petroleum ether-diethylether (85:15). Autoradiograms were obtained after exposure to Kodak X-Omat AR film at -70°C for 24 h.

Table 4.4 Effects of INH, ETH, ISO, and the butyl derivative on the incorporation of [1,2-^{14}C]acetate into FAMEs and MAMEs of *M. bovis* BCG.

Agents and concn. (μg/ml)	% inhibition		
	Fatty acids	Mycolic acids	
		Alpha	Keto
INH			
0.01	-12.23	14.72	16.75
0.02	-25.60	34.43	39.91
0.10	-70.00	84.36	90.54
1.00	-46.88	97.83	97.42
ETH			
1.00	-34.62	38.15	58.95
2.00	-47.82	63.84	99.08
5.00	-48.82	75.70	85.82
10.00	-66.88	87.76	92.51
ISO			
1.00	35.94	53.27	59.81
2.00	64.90	83.07	83.09
4.00	60.43	87.20	88.49
5.00	72.75	89.07	91.65
Butyl derivative			
0.50	23.50	45.57	60.94
1.00	25.57	62.44	75.92
5.00	40.94	72.78	91.12

[a] Percent inhibition was determined based on the percent difference in incorporation of [1,2-^{14}C]acetate into the free fatty acids and mycolic acids in the presence and absence of drugs (INH, ETH, ISO, and the butyl derivative). A negative value indicates stimulation of activity. All four drugs inhibited the synthesis of alpha- and ketomycolates. The different effect on fatty acid synthesis is that INH and ETH stimulate the synthesis of short-chain fatty acids, whereas ISO and the new derivative inhibit this activity.

According to one-dimensional TLC plates, ISO at its respective MICs inhibited the synthesis of α-mycolate by 87.10%, ketomycolate by 87.20% and wax-ester mycolate by 86.48% in the case of *M. aurum* A+ and α-mycolate by 91.61%, methoxymycolate by 94.29%, and ketomycolate by 91.12% in the case of *M. tuberculosis* H37Rv (Fig. 4.4; Fig. 4.5).

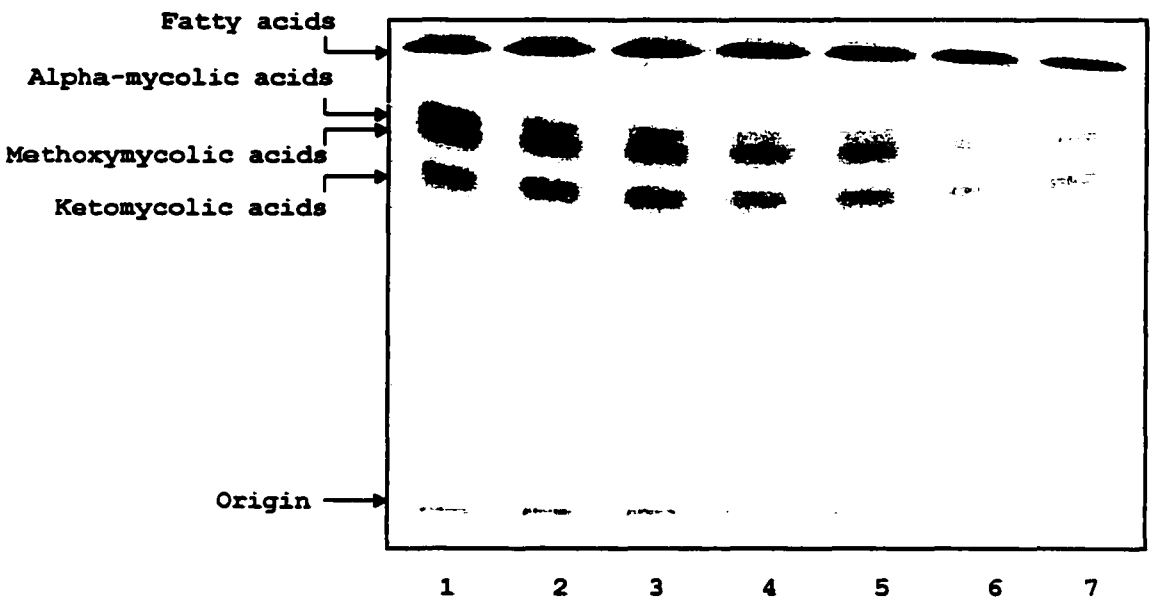


Figure 4.4 One-dimensional autoradiographic TLC of FAMEs and MAMEs from [1,2-^{14}C]acetate-labeled *M. tuberculosis* H37Rv in the presence and absence of ISO. A mixture of FAMEs and MAMEs were isolated, and equal volumes of the extract (20 µl of 1 ml) were spotted on aluminum-backed TLC plates, which were developed six times in petroleum ether-acetone (95:5) in one direction. The resulting radiograms were obtained after exposure to Kodak X-Omat AR film at -70°C for 24 h. Separated bands of FAMEs and MAMEs were cut out and placed directly into scintillation fluid for radioactive counting. Degrees of inhibition of FAMEs and each type of MAMEs were determined. Lane 1, control; Lane 2, 0.5 µg/ml; Lane 3, 1.0 µg/ml; Lane 4, 2.0 µg/ml; Lane 5, 3.0 µg/ml; Lane 6, 4.0 µg/ml; Lane 7, 5.0 µg/ml.

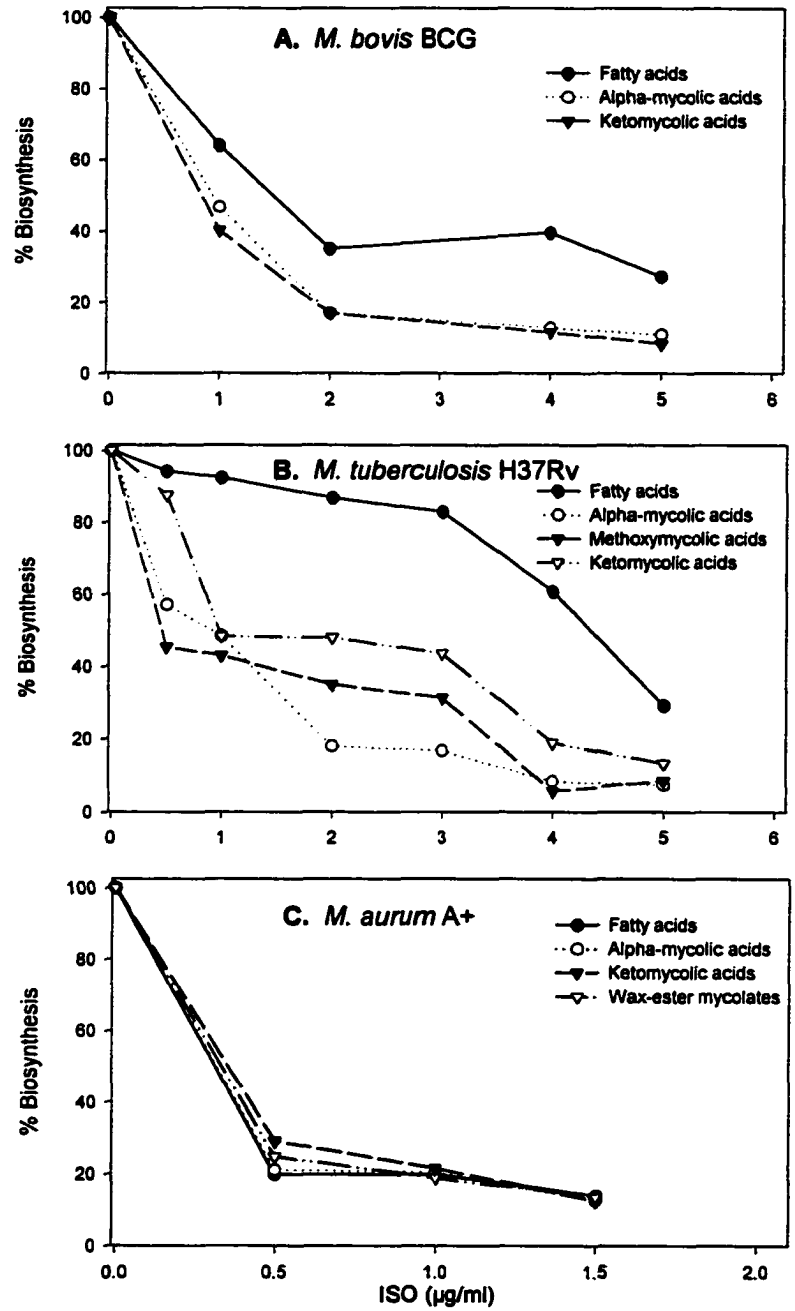


Figure 4.5 Dose-response effects of ISO on fatty acid and mycolic acid synthesis in *M. bovis* BCG (A), *M. tuberculosis* H37Rv (B), and *M. aurum* A+ (C). Labeling of cultures was performed in triplicate and terminated by the addition of 15% TBAH at 100°C, overnight. The FAME and MAME bands arising from each organism were isolated and counted.

4.3.3 Comparisons of the effects of ISO and the butyl derivative with those of INH and ETH

Comparison of the effects of ISO and the butyl derivative to those of INH and ETH on mycolic acid biosynthesis was studied through cell labeling with [1,2-^{14}C]acetate. The effects of all these drugs were similar in that the synthesis of mycolic acids was inhibited. However, as reported previously (Winder *et al.*, 1971), both INH and ETH slightly stimulated fatty acid synthesis upon treatment of mycobacteria with concentrations of 0.01, 0.02, 0.1, 1.0 µg/ml of INH and with ETH at 1.0, 2.0, 5.0, 10 µg/ml, whereas ISO and the butyl derivative inhibited the synthesis of these types of lipids (Table 4.4).

4.3.4 The effect of ISO and the butyl derivative on viable *M. tuberculosis* within an *in vitro* bone marrow macrophage assay: absence of cytotoxicity

The addition of ISO to macrophage cultures containing *M. tuberculosis* Erdman resulted in bacterial killing in a dose-dependent manner (Fig. 4.6). In the absence of ISO, viable bacteria grew to log 3.79 within 6 days, while in its presence, not only was growth inhibited but there was a reduction in the initial inoculum, indicating some bactericidal activity. The butyl derivative showed greater bactericidal activity in that it completely reduced the number of viable intracellular bacteria in macrophage cells at a concentration of 1.5 µg/ml.

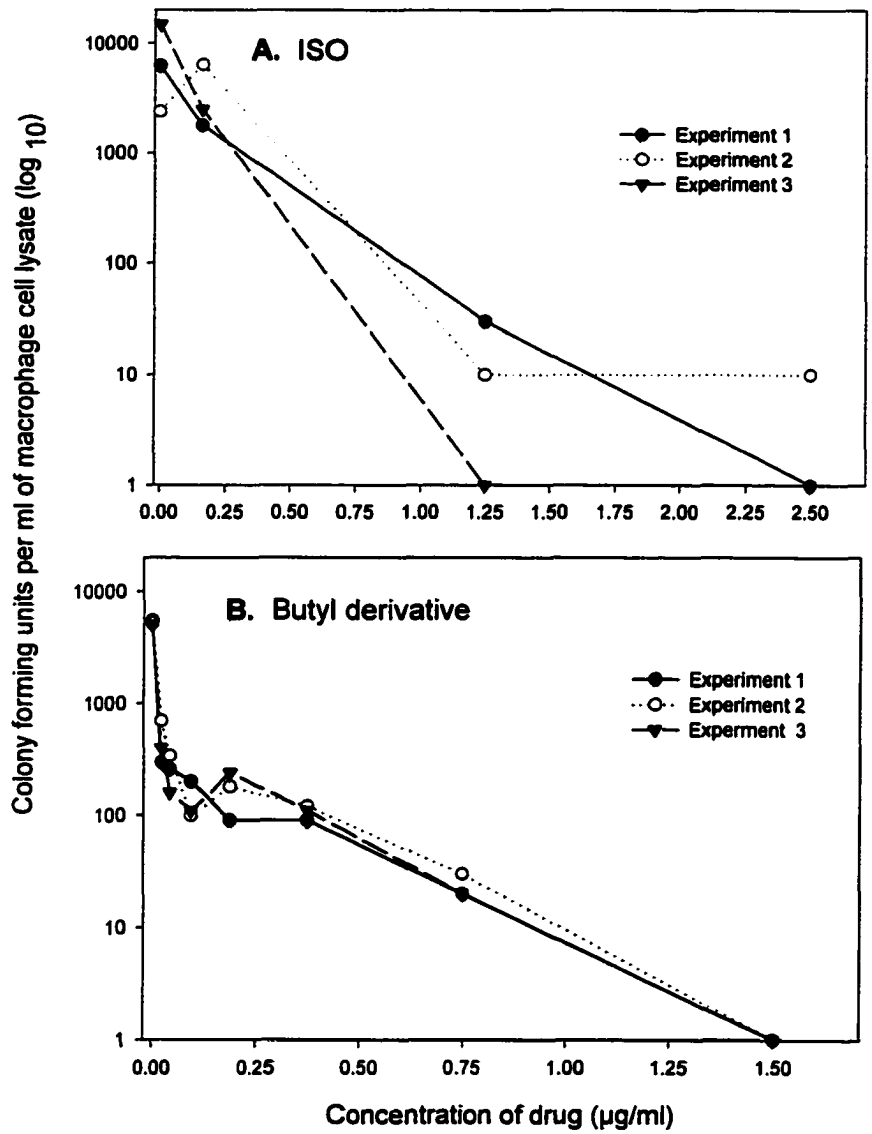


Figure 4.6 Bactericidal activity of ISO and the butyl derivative against *M. tuberculosis* Erdman in infected macrophage cell cultures. Murine bone marrow-derived macrophages were infected with 10^6 bacteria for 2 h, extracellular bacteria were removed, and infected macrophages were incubated with various concentrations of ISO or the butyl derivative for 6 days. Macrophage lysates were then individually plated on 7H11 agar to score the number of viable bacteria. (A), Bactericidal activity of ISO inside murine macrophage cells. ISO at a concentration of 2.5 µg/ml resulted in a 4-log reduction in viable bacteria. (B), Bactericidal activity of the butyl derivative. At a concentration of 1.5 µg/ml, the butyl derivative completely killed intracellular mycobacteria, resulting in a 3.72-log reduction in viable bacteria.

The Alamar Blue oxidation-reduction dye was applied as an indicator of the effects of ISO and the butyl derivative on macrophage cell viability. In this assay, the blue, oxidized form becomes red due to the normal redox reactions within macrophage cells and thus the red color represents cell viability. Mouse macrophage cells were grown in tissue microplates and treated with ISO or the butyl derivative. When the Alamar Blue dye was mixed with incomplete DMEM, the color was purple. All of the dilutions that contained ISO up to the highest tested concentration (2µg/ml) exhibited the red color. Likewise, cells treated with the butyl derivative maintained their viability. Accordingly, this thiourea is apparently not acutely toxic for mouse macrophages.

4.4 DISCUSSION

The slow-growing mycobacteria used in this study, i.e., *M. tuberculosis, M. bovis* BCG and *M. avium,* doubled about every 18 to 26 h and thus yielded colonies from single cells in 14 to 21 days. In contrast, the fast-growing mycobacteria, *M. aurum* A+ and *M. smegmatis* mc^2155, have doubling times of 2 to 3 h and yielded colonies from a single cell in 3 to 4 days. The act of growing cells in tissue culture flasks without agitation in the case of *M. tuberculosis, M. bovis* BCG and *M. avium* yielded high levels of reasonably unclumped viable cells. These species of *Mycobacterium* were included in preliminary experiments in order to identify ISO-susceptible and -resistant strains and to identify the set of suitable organisms for subsequent biochemical and genetic studies.

The results of the MIC studies show that ISO is capable of inhibiting the growth of various mycobacteria within a narrow range of low concentrations. Importantly, a panel of virulent clinical isolates of *M. tuberculosis* also exhibited susceptibility to ISO when exposed to ISO in the range of 1.0 to 10.0 μg/ml. Some virulent strains appeared to be less susceptible, while others showed evidence of high susceptibility to ISO even used at the very low concentration of 1.0 μg/ml. It is clear, however, that all of the clinical isolates of *M. tuberculosis,* which varied in patterns of resistance to other drugs (Table 4.1), were consistently susceptible to ISO at a concentration of 10 μg/ml. The suggestion is that ISO may be suitable for the treatment of tuberculosis, particularly the multidrug-resistant kind.

ISO (compound B27 in Table 4.2) is a substituted diacyl thiourea, and previous studies had demonstrated that the thiourea nucleus is required for antimycobacterial activity. In the hope of generating more-effective variants, random substitutions were made in the side chains attached to the key structure. This strategy resulted in an array of new derivatives of thiourea with variations in the symmetry and asymmetry of the side chains (Table 4.2) attached to the key structure. Some thioureas substituted in the *para* and *para'* positions by alkyl, alkoxy, or sulfur functional groups were transformed from inactive thiocarbanilides into substances with considerable antimycobacterial activity. For instance, the butyl derivative of thiourea (compound B01 in Table 4.2), the first synthesized thiourea derivative, possessed low

MICs (0.1 to 0.5 µg/ml) and was chosen for further evaluation of its effects on mycobacteria. Replacement of the oxygen with sulfur in the side chain(s) provided extremely high antibacterial activity against *M. tuberculosis*, as demonstrated by low MICs of <0.1 for C25 and C33 and 1.0 µg/ml for JDDD38. Thus, several of thiourea derivatives with various side chains of alkyl, alkoxy, and alkylthio units were superior to ISO in their activities against *M. tuberculosis*. Other slow growers, including *M. bovis* BCG and *M. avium*, were also susceptible to ISO and new derivatives within a narrow range of low MICs. The present results suggest that a concerted approach to chemical modification of the basic thiourea nucleus would lead to even more-powerful inhibitors of *M. tuberculosis* and *M. avium*.

The range of mycobacteria selected for the present study was based on their susceptibility to ISO and range of constituent mycolic acids (Minnikin *et al.*, 1984). The most-susceptible species, *M. bovis* BCG, *M. tuberculosis* H37Rv, and *M. aurum* A+, which also presented a representative spectrum of mycolic acids, were chosen to analyze the effects of ISO on mycolic acid synthesis through whole-cell labeling with [1,2-^{14}C]acetate. Previous reports had indicated that prolonged exposure of mycobacteria to a low concentration of drugs, rather than the short exposure to higher concentrations, provides a better gauge of its effects on bacterial metabolism (Rist, 1960; Winder, 1982). Thus, the effects of ISO on mycolic acid synthesis could be clearly seen when the drug exposure times were 34 h for *M. bovis*

BCG and *M. tuberculosis* H37Rv and 18 h for *M. aurum* A+. This approach allowed us to confirm that the mode of action of ISO is through the specific inhibition of mycolic acid synthesis, and that the inhibitory effect of ISO on mycolic acid synthesis is dose dependent (Fig. 4.5; 4.6). Based on the effects of ISO on the different species of mycobacteria, it can be concluded that ISO inhibited the synthesis of all types of mycolic acids, consistent with earlier observations (Winder, 1982). The naturally high resistance of *M. smegmatis* to ISO is striking and cannot be explained at this time.

The use of an *in vitro* macrophage model allowed an assessment of the ability of ISO and the butyl derivative to cross membranes and target viable bacteria within the confines of the macrophage and phagosome. The drugs also demonstrated strong intracellular bactericidal activity by reducing the initial inoculum of virulent *M. tuberculosis,* suggesting cidal rather than static action.

INH and ETH are specific antituberculosis drugs which clearly affect mycolic acid synthesis (Mdluli *et al.*, 1996; Quemard *et al.*, 1992; Winder, 1982). INH, and apparently ETH, first requires conversion to an activated form, either an isonicotinic acyl anion (Shoeb *et al.*, 1985) or an isonicotinic acyl radical (Johnsson and Shultz, 1994) by the mycobacterial catalase-peroxidase enzyme (KatG) (Johnsson and Shultz, 1994; Mdluli *et al.*, 1998; Zhang *et al.*, 1992) before it exerts its lethal effect on mycolic acid biosynthesis. The activated form of INH is capable of attaching

to NAD(H) as it is bound to the active site of InhA to generate a covalent INH-NAD adduct (Sacchettini and Blanchard, 1996). InhA is a long-chain (C_{12} to C_{24}) enoyl-ACP-dependent reductase (Quemard *et al.*, 1995; Sacchettini and Blanchard, 1996) which catalyzes the NADH-dependent reduction of a double bond at position 2 of a growing fatty acid chain linked to ACP. However, it had previously been observed (Takayama, 1974; Takayama *et al.*, 1975; Takayama *et al.*, 1972) and was recently reconfirmed (Mdluli *et al.*, 1998) that *M. tuberculosis*, in response to INH treatment, accumulates saturated hexacosanoic acid ($C_{26:0}$). It is now known that this acyl group is attached to a 12-kDa ACP (AcpM) (Mdluli *et al.*, 1998), and, according to this latest work, the β-ketoacyl ACP synthase in association with AcpM is the target of INH (Mdluli *et al.*, 1998). Thus, at this point, the exact sites and mechanisms of action of INH on mycolic acid synthesis are varied and may be species-dependent.

Unlike INH and ETH, ISO is not a nicotinamide derivative but is of higher molecular weight and is a thiourea modified by long hydrophobic side chains. It is difficult to envisage ISO as a prodrug capable of being converted into an electrophile. Hence, it is unlikely to form a complex with NAD+ at InhA or any other active site. Moreover, the utilization of [1,2-^{14}C]acetate as a precursor of fatty acid and mycolic acid synthesis demonstrated that ISO is distinct from INH and ETH in that it inhibits the synthesis of short-chain fatty acids (Table 4.4), a result that is

consistent with the previous report on the mode of action of ISO (Winder, 1982) and suggesting that the targets of ISO may lie at the points shared in the synthesis by both short-chain fatty acids and mycolic acids. The mycobacterial multifunctional FAS I, the monofunctional FAS II, and the largely undefined mycolic acid synthase are responsible for fatty acid and mycolic acid synthesis. FAS I is a single polypeptide with multiple catalytic activities that generates several shorter coenzyme A (CoA) esters from the acetyl-CoA primer (Jackowski *et al.*, 1991; Kolattukudy *et al.*, 1997; Noto *et al.*, 1982). The primary products of the *de novo* FAS I system are C_{16} to C_{18} and C_{24} to C_{26} fatty acyl-CoA derivatives (Bloch, 1975; 1977). Therefore, FAS I creates the precursor for further elongation. FAS II consists of dissociable enzyme components which act on a substrate bound to ACP. FAS II is incapable of *de novo* fatty acid synthesis, but instead elongates a C_{16} fatty acid primer (palmitoyl-ACP) to fatty acids ranging from C_{24} to C_{56} carbon in length (Ratledge, 1982). Several different components of FAS II were reported to be targets of isoniazid, including the enoyl-ACP reductase (InhA) (Banerjee *et al.*, 1994) and the ketoacyl-ACP synthase (KasA) in association with ACP (AcpM) (Mdluli *et al.*, 1998). It seems likely that ISO acts on other components of FAS II, resulting in the inhibition of short-chain fatty acid synthesis, a distinctive effect from that of INH. The mode of action of ISO remains elusive. An understanding of the specific mode of action of ISO is important

in the search for new antimycobacterial drug targets and for the development of more effective chemotherapy. Furthermore, effects on fatty acid synthesis which differed from those of INH and ETH provide the prospects of identifying new fatty acid biosynthesis genes in addition to mycolic acid biosynthesis genes.

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CHAPTER 5

A Unique Mechanism of Action of Thiocarlide/Isoxyl® on the Inhibition of Oleic Acid and Tuberculostearic Acid Synthesis in *Mycobacterium tuberculosis*

(Some parts of the work presented in this chapter were done in collaboration with the laboratory of Dr. Clifton E. Barry III.)

5.1 INTRODUCTION

There are few publications of the mode of action of ISO. An early note reported that like isoniazid (INH) and ethionamide (ETH), ISO strongly inhibited mycolic acid synthesis (Winder, 1982; Winder *et al.*, 1971). It also inhibited the synthesis of shorter-chain fatty acids of free lipids, which, however, were stimulated by INH and ETH (Winder, 1982; Winder *et al.*, 1971). Together, these results indicated that ISO probably possesses a unique mode of action, which is likely involved in the synthesis of short-chain fatty acids. Consequential effects of ISO on fatty acids synthesis may exist since fatty acids have been implicated in various constituents of mycobacterial structures.

Understanding the mode of action of ISO is important in the development of new effective drugs that exhibit selective action on a specific target similar to ISO. In the search for new drug targets, attempts were made to define the specific site of the inhibition of ISO on fatty acid synthesis. This chapter describes the unique effects of ISO on fatty acid synthesis and pleiotropic effects of ISO on mycobacterial cells. The exposure of

mycobacterial cells to inhibitory levels of ISO resulted in an accumulation of fatty acid precursor concomitant with the depletion of the biosynthetic product. This approach led to the discovery that ISO inhibits oleic and tuberculostearic acid synthesis, which is not observed for INH and ETH.

5.2 MATERIALS AND METHODS

5.2.1. Chemicals and radioactive compounds

Fatty acids or fatty acid methyl esters used as standards were purchased from Sigma Chemical Co. (St. Louis, MO) and Alltech Chromatography (Deerfield, IL). [1,2-^{14}C]acetate (110 mCi/mmol) and [^{35}S]methionine (1175.0 mCi/mmol) were purchased from Dupont NEN (Boston, Mass.). Kieselgel 60 F_{254} silica TLC plates were from EM Science (Gibbstown, NJ) and LKC_{18} reverse-phase TLC plates were from Whatman (Maidstone, England). Acrylamide/bis-acrylamide, Bio-Lyte ampholytes and Coomassie Brilliant Blue R-250 were from Bio-Rad (Richmond, CA). Other chemicals were purchased from common commercial sources (Sigma Chemical Co., Aldrich Chemical Co., and Fisher Scientific).

5.2.2 Growth conditions

M. tuberculosis H37Ra (ATCC 25711) was obtained from the American Type Culture Collection (Rockville, MD). Initially, this strain was inoculated from 1-ml frozen stocks of approximately 10^6 CFU/ml into 10 ml of Sauton's medium (chapter 4). After incubation at 37°C for 14 days, 1 ml of culture was transferred to 50 ml of Sauton's medium for lipid labeling. For protein

labeling, *M. tuberculosis* were grown in glycerol-alanine-salts (GAS) medium (Takayama *et al.*, 1975).

To determine if the addition of an oleic acid supplement could over-ride the effects of ISO, a series of 10-fold dilutions of *M. tuberculosis* was prepared from stock culture in GAS medium using phosphate buffered saline (PBS) as diluent. An aliquot (5 µl) of each dilution was spotted on two types of agar plates, those with 7H11 agar medium supplemented with OADC (oleic acid-albumin-dextrose-catalase) or those with non-oleic acid-containing ADC (albumin-dextrose-catalase) prepared with the incorporation of ISO to final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/ml. After inoculation, the plates were then incubated at 37°C for 21 days. The growth rates and bactericidal effects were scored by comparing colony sizes and number of colonies.

5.2.3 [1,2-^{14}C]acetate labeling, fatty acid and mycolic acid extraction and derivatization procedures

The whole-cell labeling, the extraction and derivatization of labeled-fatty acids and mycolic acids were performed as described earlier (chapter 4). Briefly, *M. tuberculosis* was grown in Sauton's medium to the early exponential phase, at which point different concentrations of ISO were added, followed by further incubation at 37°C for 8 h prior to the addition of [1,2-^{14}C]acetate at 1 µCi/ml. Cells were labeled for 24 h, harvested and washed. Saponification was performed with 15% tetrabutylammonium hydroxide at 100°C overnight. After cooling,

dichloromethane and iodomethane were added to generate fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) which then were extracted as previously described (chapter 4).

5.2.4 Chromatography

TLC. Silica gel TLC plates were impregnated with 10% silver nitrate and heat activated as described (Morris, 1966). Saturated and unsaturated fatty acids and mycolic acids in extracts were resolved in a mixture of petroleum ether-acetone (90:10). Autoradiograms were produced by overnight exposure of TLC plates to Kodax X-Omat AR film at -70°C. Separated bands of FAMEs and MAMEs were marked, cut from the TLC plates, placed directly in 10 ml of scintillation fluid (EcoLume®) and counted with a Beckman 6000 LSC (Beckman; Fullerton, CA).

GC. The labeled fatty acid/mycolic acid ester mixtures were also analyzed by GC. Firstly, they were silated with Trisil® (Pierce; Rockford, IL) to convert the hydroxyl of the mycolates into trimethylsilyl derivatives. A Hewlett Packard HP 5890 Series II GC was used with a thermal conductivity detector (TCD) coupled to a "GC-Ram™ radio-detector" (Inus Systems; Tampa, FL). Samples (2 µl) in hexane were injected onto a capillary HP-1 column 5 m long with an interior diameter of 0.53 mm and a film thickness of 2.65 µm (Supelco Inc.; Bellefonte, PA). The temperatures of the injector port and detector were maintained at 290°C and 280°C, respectively. Helium was the carrier gas for TCD and P-10 (Argon and 10% methane) for radioactivity detection. The initial column

temperature was 80°C, which was increased to 185°C at a rate of 30°C/min. Then, the temperature was increased at the rate of 5°C/min to a final temperature of 345°C. The range of attenuation was -2 to 0. Peaks were integrated with a HP software package. FAMEs were identified by comparison of the retention times of radioactive peaks, detected with the radio-detector, with those of commercially available methyl esters of $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$ $\Delta 9$, $C_{19:0}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$, $C_{26:0}$, as detected with the TCD. The $brC_{19:0}$ (tuberculostearic acid), a gift from Dr. J.T. Belisle, Colorado State University, was also included as a standard.

HPLC. The methyl ester derivatives of mycolic acids were analyzed by HPLC model 332 (Beckman) on a reverse-phase C_{18}-bonded silica cartridge with a linear gradient of 40-100% tetrahydrofuran in acetonitrile at a flow rate of 2.0 ml/min over 90 min. A "Beta-Ram™ radio-detector" (Inus System; Tampa, FL) was applied to detect and quantitate labeled mycolic acid derivatives.

5.2.5 2D PAGE and native PAGE to analyze the effects of ISO on acyl carrier protein (ACP)

M. tuberculosis control and ISO-treated cells were labeled with either [^{35}S]methionine or [^{14}C]acetate for 4 h. To prepare whole cell lysate, 50 ml of labeled cultures were harvested by centrifugation and resuspended in phosphate buffer containing 1 mM of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF). Suspensions were transferred to 1.5 ml tubes containing 0.1-mm glass beads, which were placed in a Mini Beadbeater-8 (BioSpec

Products; Bartlesville, Okla.) and lysed at maximum speed for 3 min. After brief centrifugation at 5,000 × g, the cell lysate was recovered in the supernatant.

[^{35}S]methionine labeled samples were separated by 2-dimensional PAGE (2D PAGE) as described previously (O'Farrel, 1975). Briefly, approximately 100 µg of lysate protein was resuspended in isoelectric focusing sample buffer (IEF-SB) containing 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte (pH 5 to 7 and 0.4% Bio-Lyte (pH 3 to 10) and incubated for 30 min at 20°C. An aliquot (20 µl) was applied to a 4% polyacrylamide isoelectric focusing tube gel (1.5 mm by 6.5 cm) containing 1.6% Bio-Lyte (pH 5 to 7) and 0.4% Bio-Lyte (pH 3 to 10). The proteins were focused for 4 h at 750 v with 10 mM H_3PO_4 and 20 mM NaOH as the catholyte and anolyte, respectively. After running the electrofocusing gel, the tube gels were subsequently placed on a preparative SDS-polyacrylamide gel (7.5 cm by 10 cm by 1.0 mm) containing a 6% stack over 18% resolving gel. Electrophoresis in the second dimension was carried out at 150 v for 1 h. Gels were dried on Whatman filter membrane using a gel drier (Sambrook *et al.*, 1989a). The separated proteins were visualized by exposure dried gels to PhosphoImager plates (Molecular Dynamics; Sunnvale, CA).

[^{14}C]-Labeled proteins were loaded and applied to native polyacrylamide gels according to the method described by Rock and Cronan with minor modifications (Rock and Cronan, 1992). Gels (7.5 by 10 cm by 0.75 mm) containing 6% stack over a 18% resolving

gel were applied and run at 150 volt for 1 h. Proteins were visualized by either staining with Coomassie Brilliant Blue R-250 (Chen *et al.*, 1993) or dried and exposed to a PhosphorImager plate.

Analytical procedures. For N-terminal sequencing, the resolved proteins from native PAGE gels were transferred to a polyvinylidene difluoride membrane (Novex; San Diego, CA) by electroblotting at 50 v for 1 h with transfer buffer containing 10% methanol (Sambrook *et al.*, 1989b). The membrane was stained with 0.1% Coomassie Brilliant Blue R-250 in methanol and destained with 50% methanol solution. Immobilized proteins were marked and sequenced at the Macromolecular Resources Facilities (Colorado State University) using automated Edman degradation on a gas phase sequencer (Hawke *et al.*, 1985) equipped with a continuous-flow reactor (Shively *et al.*, 1987). The resulting N-terminal sequences were searched for amino acid sequence homology using the BLAST program (National Center for Biotechnology Information).

To analyze acyl chains associated with increased ACPs, purified ACPs from ISO-treated cells were saponified, and the methyl esters of the attached lipids were analyzed by LKC_{18} reverse-phase TLC as described by Mdluli *et al.* (1998). The methyl esters of fatty acids on the reverse-phase TLC plate were visualized with a PhosphorImager plate (Molecular Dynamics).

5.3. RESULTS

5.3.1 ISO selectively inhibits the synthesis of unsaturated fatty acids and mycolic acids

Previously, it had been shown that ISO inhibited the synthesis of both mycolic acids and short-chain fatty acids; in this latter respect, it differed markedly from INH and ETH (chapter 4). Therefore, this present work was focused on defining the effect of ISO on fatty acid synthesis. Argentation-TLC provides a simple means of differentiating among saturated, unsaturated fatty acids, and mycolic acids. The extracts containing a mixture of FAMEs and MAMEs from [1,2-^{14}C]acetate-labeled control and ISO-treated *M. tuberculosis* were applied to the $AgNO_3$-impregnated TLC plates and chromatographed; the silver ion reacts with double bond(s) resulting in retardation of unsaturated fatty acid esters. In the presence of ISO at concentrations as low as 1.0 µg/ml, there was a significant decrease in the synthesis of an unsaturated fatty acid that was identified to be oleic acid ($C_{18:1}$ Δ9), compensated by a partial increase in stearic acid ($C_{18:0}$). Counting of the relevant bands established that ISO at 1.0 µg/ml inhibited oleic acid synthesis. The effect on mycolic acid synthesis can also be distinguished by this simple analysis. Clearly, ISO also inhibited the incorporation of [1,2-^{14}C]acetate into all types of resolved mycolic acids (Fig. 5.1).

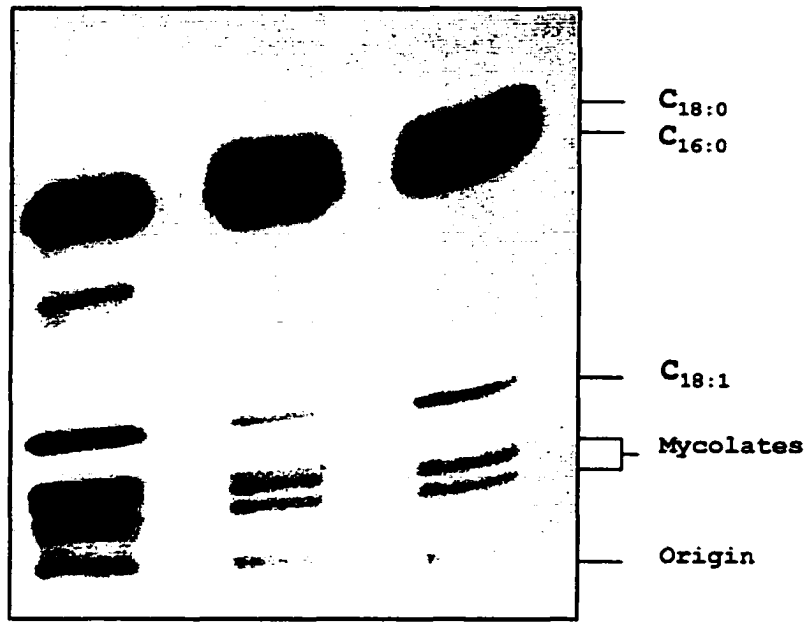

Figure 5.1 TLC autoradiography of effects of ISO on the incorporation of [^{14}C]acetate into fatty acids and mycolic acids of *M. tuberculosis*. An equal number of counts (Ca. 80,000 cpm) from whole-cell labeling with [1,2-^{14}C]acetate were spotted on the argentation-TLC plates which were developed in petroleum ether-acetone (90:10). During the experiment cell growth was also monitored and was not affected by the presence ISO at a concentration of 1.0 or 2.0 μg/ml. Lane 1, ISO 0 μg/ml (control); Lane 2, ISO 1.0 μg/ml; Lane 3, ISO 2.0 μg/ml.

5.3.2 Evidence that ISO specifically inhibits the synthesis of oleic acid and tuberculostearic acid

Radio-GC showed that the major fatty acids of *M. tuberculosis* synthesized under present conditions are palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$ Δ9), and tuberculostearic acid ($brC_{19:0}$) (Fig. 5.2, panel A), in accord with previous publications (Chou *et al.*, 1996; Chou *et al.*, 1998). The relative incorporation of [1,2-^{14}C]acetate into oleic and tuberculostearic acids in control cells was 13.4% and 14.8% of total cellular fatty acids, respectively. In cultures treated with ISO at 1.0 μg/ml, [1,2-^{14}C]acetate incorporation was decreased to 4.4% for oleic acid

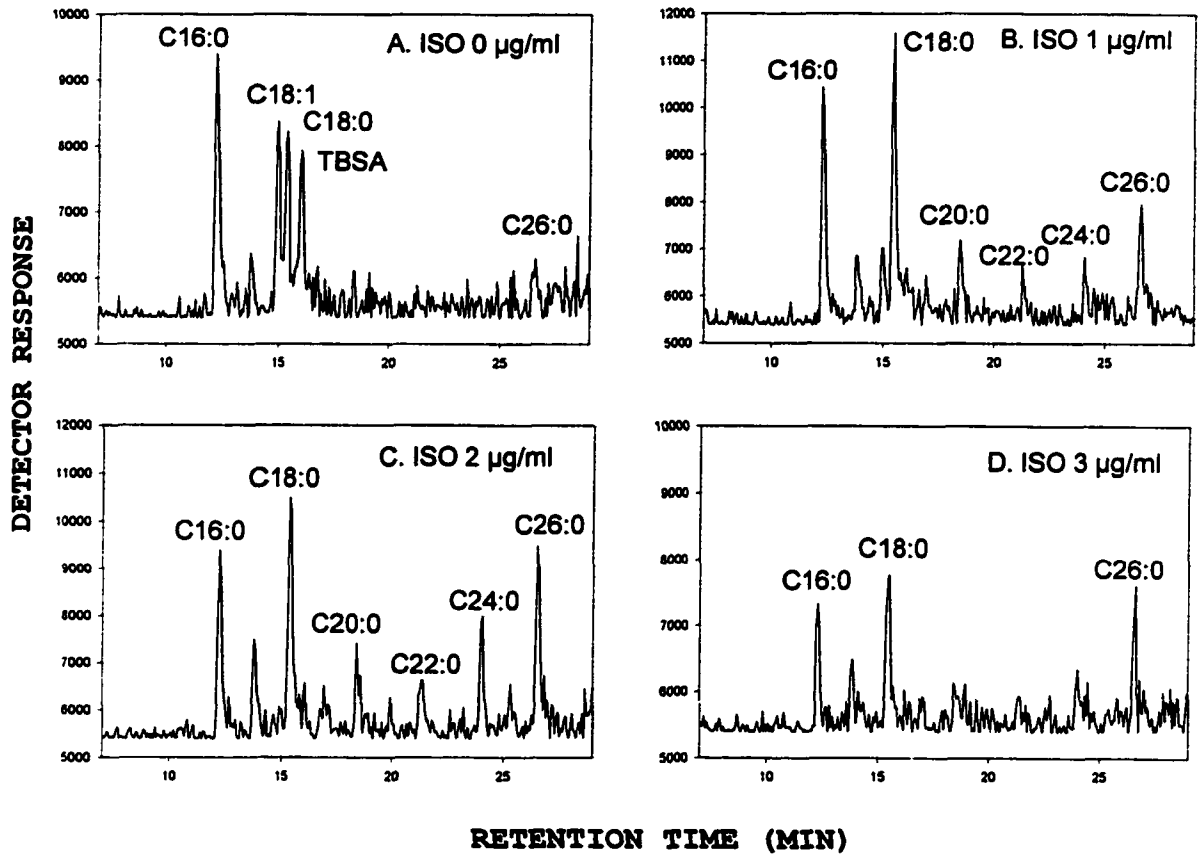

Figure 5.2 Radio-gas-chromatograms of fatty acid methyl esters (FAMEs) of *M. tuberculosis* in the absence (panel A) and presence of ISO at various concentrations (panel B, C, D). The relative abundance of the individual fatty acids versus their respective retention time is shown.

and 6.2% in the case of tuberculostearic acid (Table 5.1). At higher concentrations of ISO (2, 3 and 4 μg/ml), [1,2-^{14}C]acetate incorporation into oleic and tuberculostearic acid was further reduced. For instance, synthesis of oleic acid was completely shut down at a concentration 3 μg/ml of ISO (Fig. 5.2, panel D; Table 5.1). It should be noted that ISO at high concentrations decreased the synthesis of all fatty acids except for $C_{26:0}$ which increased in the response to ISO treatment (Fig. 5.2, panel D).

Table 5.1 Fatty acid synthesis in response to ISO treatment in *M. tuberculosis*.

ISO conc.	% Fatty acids [a]				
(μg/ml)	$C_{16:0}$	$C_{18:1}$ Δ9	$C_{18:0}$	TBSA	$C_{26:0}$
0	17.3	13.4	16.1	14.8	6.2
1.0	15.2	4.4	21.7	6.2	8.7
2.0	10.7	2.3	18.1	4.4	11.5
3.0	9.0	0	13.3	0	14.9
4.0	12.5	0	14.2	0	26.5

[a] Values are % fatty acids calculated from area under peaks of GC chromatograms. The % fatty acids were determined based on the percent of the incorporation of [1,2-^{14}C]acetate into the cellular fatty acids in the presence or absence of ISO.

5.3.3 The concomitant effects of ISO on mycolic acid synthesis

Previously (chapter 4), and in Fig. 5.1, it was shown that ISO inhibited mycolic acid synthesis, of all types that could be differentiated by TLC, namely α-mycolates, methoxymycolates, and ketomycolates. On this occasion, HPLC, which is capable of resolving mycolate esters on the basis of chain lengths and functional groups including degree of unsaturation was applied. In overall profile, the pattern of mycolate esters from an untreated-culture of *M. tuberculosis* (Fig. 5.3) was similar to patterns as described by Butler *et al.* (1991). The results (Fig. 5.3) confirm that the synthesis of all classes of mycolates was inhibited in the presence of a low concentration (1 μg/ml) of ISO. It must be noted that the incorporation of acetate into all classes was reduced by at least 80%. However, in addition, certain mycolates were much more affected. These are currently being identified with the specific notion that they may be unsaturated mycolates.

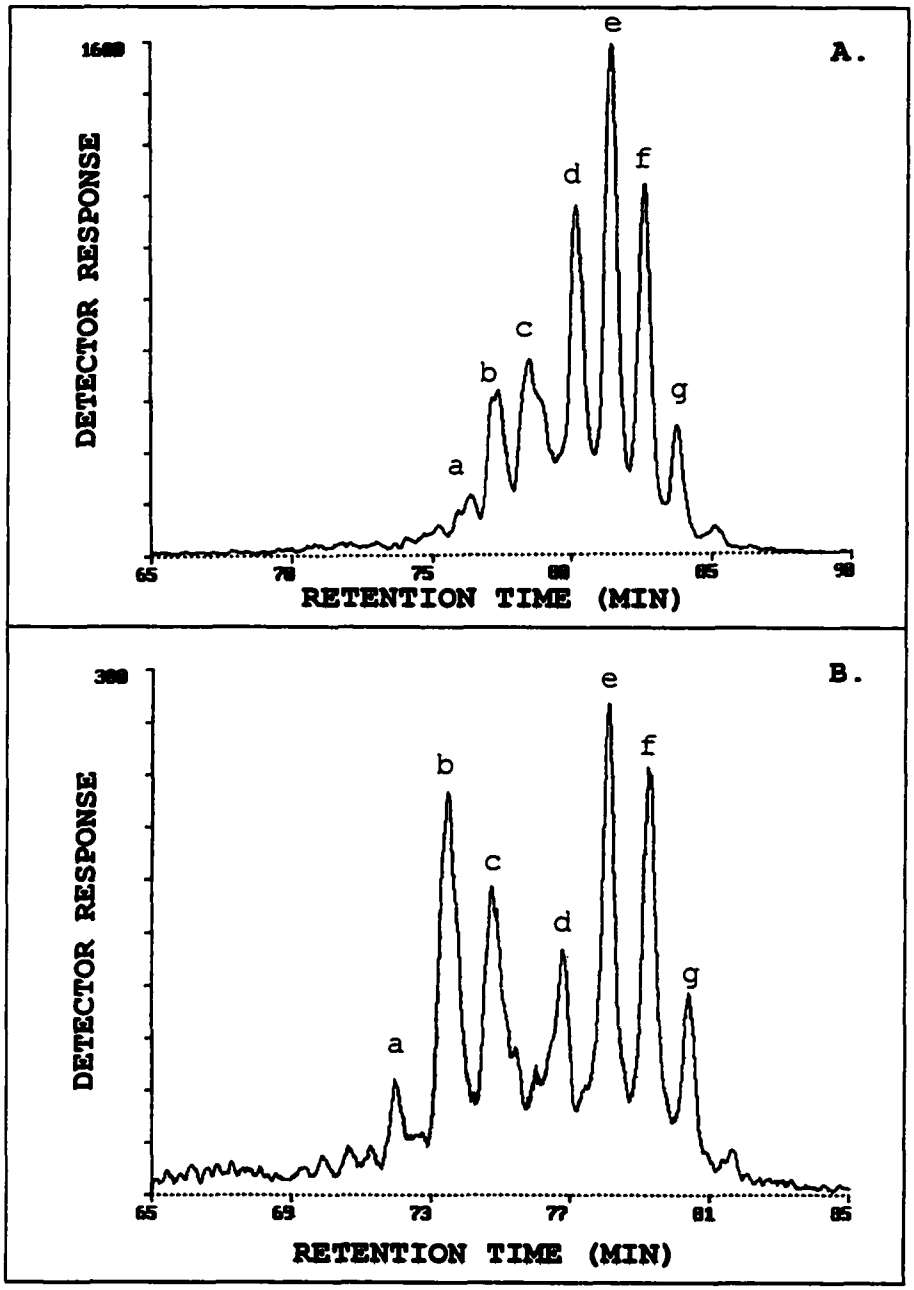

Figure 5.3 HPLC-chromatograms of mycolic acid methyl esters (MAMEs) from saponified cells of *M. tuberculosis*. The mycolic acid peaks are designated by letters corresponding to the order of their elution. Panel A, mycolic acid pattern from untreated cells; Panel B, pattern from a culture treated with 1.0 µg/ml of ISO.

5.3.4 Effect of ISO on fatty acyl-ACPs

The immediate precursors of the bound fatty acids and mycolic acids examined in the present work are expected to be the corresponding acyl-ACPs. To examine the effect of ISO on the synthesis of these, whole cells of *M. tuberculosis* were treated with ISO (10.0 µg/ml; 2 h) while being labeled with either [^{35}S]methionine or [^{14}C]acetate. 2D PAGE of [^{35}S]methionine labeled proteins demonstrated a dramatic increase of apparent ACPs with a molecular mass value of ~12 kDa (Fig. 5.4).

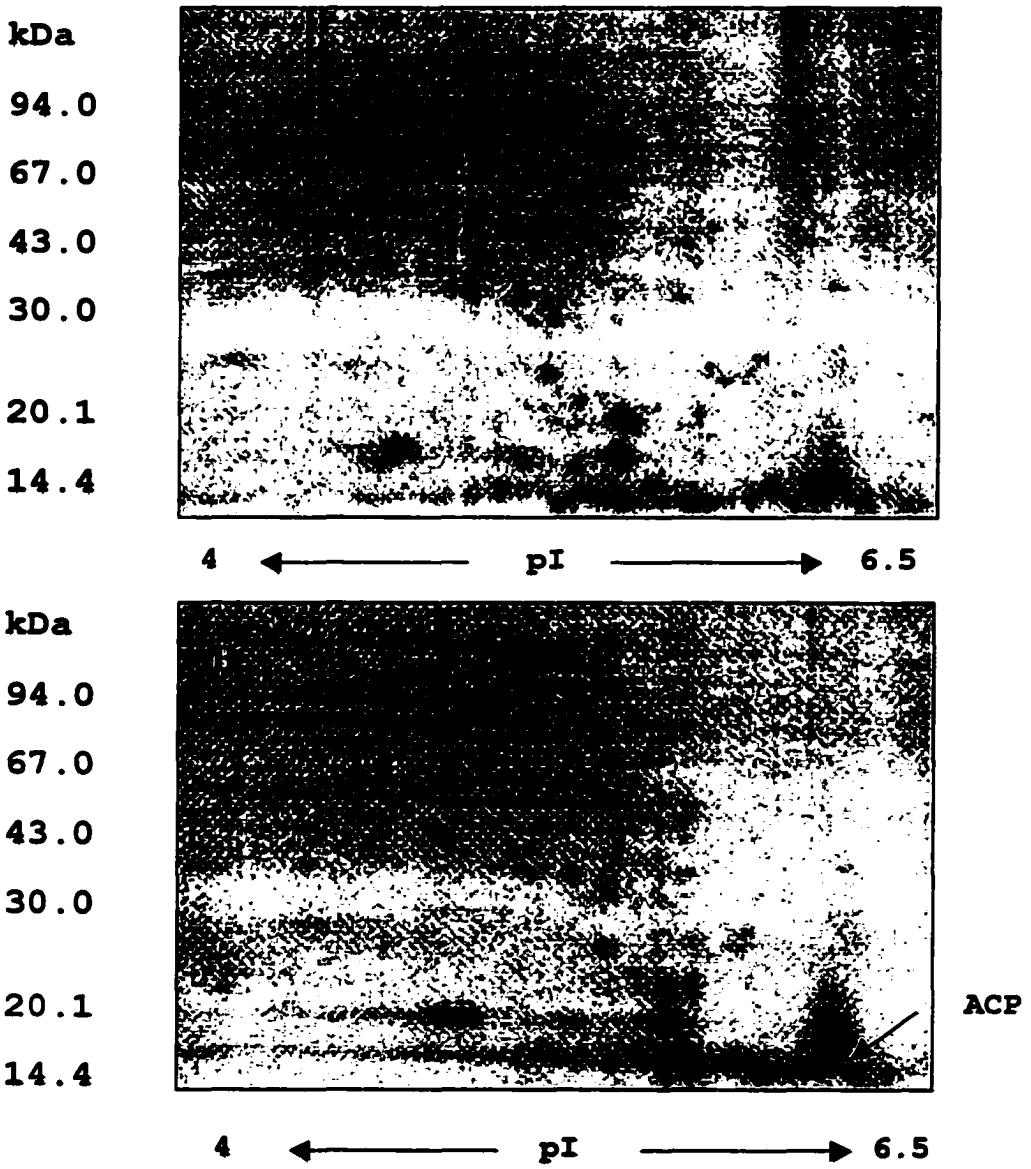

Figure 5.4 Comparative 2D PAGE of the effect of ISO on ACP. *M. tuberculosis* was treated with ISO at a concentration of 10 µg/ml for 2 h prior to labeling with [^{35}S]methionine for 4 h. Crude cell lysate was prepared as described in Materials and Methods. The soluble extracts were loaded on gels, developed in two dimensions, and analyzed by autoradiography. The isoelectric focusing is the horizontal dimension while SDS-PAGE is the vertical dimension. Panel A, untreated culture; Panel B, 10 µg/ml ISO-treated culture.

Native PAGE is also an effective tool to identify ACPs in their native forms which are holo-ACPs bound to acyl chains (Rock and Cronan, 1992). Native 18% polyacrylamide gels were then used to analyze the effect of ISO on acyl-ACPs. Cells were labeled with [^{14}C]acetate and protein samples were loaded on native polyacrylamide gels, developed and subjected to autoradiography (Fig. 5.5, panel A). Treatment with ISO resulted in the increase of fatty acyl-ACPs, which were resolved by native PAGE into at least three differentiated bands (Fig. 5.5, panel A).

The N-terminal sequences of these differentiated proteins were determined to be PVTQEEIIAGIAEII. Searching of the recently completed mycobacterial genomic database (Cole *et al.*, 1998) revealed that this sequence is present in *M. tuberculosis* only once, where it corresponds to the NH_2-terminus of a 12,492-dalton acyl carrier protein named AcpM (Mdluli *et al.*, 1998). Analysis of the fatty acyl chains associated with AcpM by LKC_{18} reverse-phase TLC revealed the presence of $C_{16:0}$, $C_{18:0}$ and $C_{26:0}$ saturated fatty acids (Fig. 5.5, panel B). Significantly, there was no oleic acid ($C_{18:1}$ Δ9) among the products; $C_{18:0}$-ACP is the expected precursor of $C_{18:1}$ Δ9.

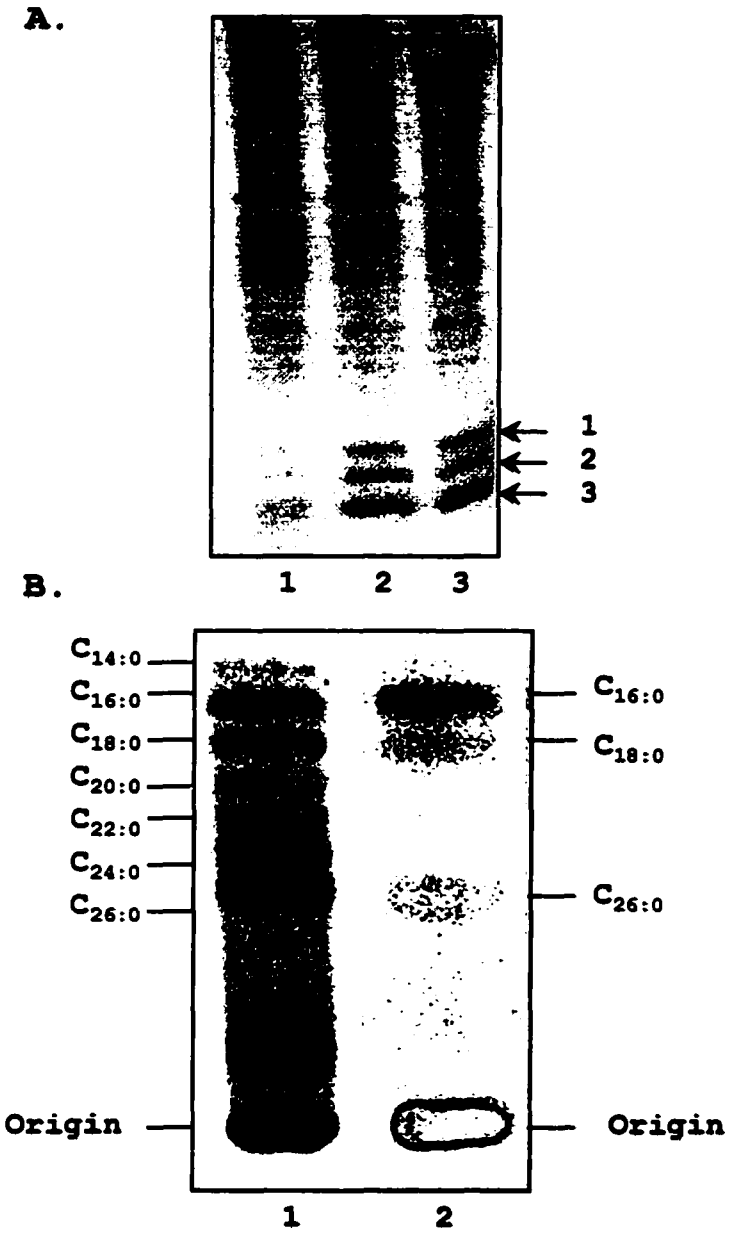

Figure 5.5 Analysis of the effect of ISO on acyl-ACPs. Panel A, native PAGE to identify the effect of ISO on the increase of acyl-ACPs. 18% native PAGE was applied to analyze the increased acyl-ACPs upon treatment in *M. tuberculosis* with ISO. Cells were exposed to ISO for 2 h, then labeled with [1,2-^{14}C]acetate for 4 h. Lane 1, untreated cells; Lane 2, treated with ISO at 1 µg/ml; Lane 3, treated with ISO at 10 µg/ml. Arrows (1, 2, 3) indicate bands of the increased acyl-ACPs. The increased acyl-ACPs were then extracted from gels and hydrolyzed. Panel B, a reverse-phaseTLC to identify ACP-bound lipids. Lane 1, acyl fatty acids from whole cell control; Lane 2, acyl fatty acids bound to the increased ACP upon ISO treatment.

5.3.5 The effects of an oleic acid supplement in protecting cells from ISO

The basis behind this experiment was that if ISO inhibits oleic acid synthesis, then supplement of the growth medium with oleic acid should rescue cells from the killing effect of ISO. As shown in Fig. 5.6, the addition of oleic acid to the 7H11 agar medium in the form of OADC partially reversed the bactericidal effect of ISO; for instance, at 0.5 µg/ml of ISO. There were no colonies on 7H11 agar supplemented with ADC. However, in the case of the same medium containing OADC, cells did survive the killing effect of ISO.

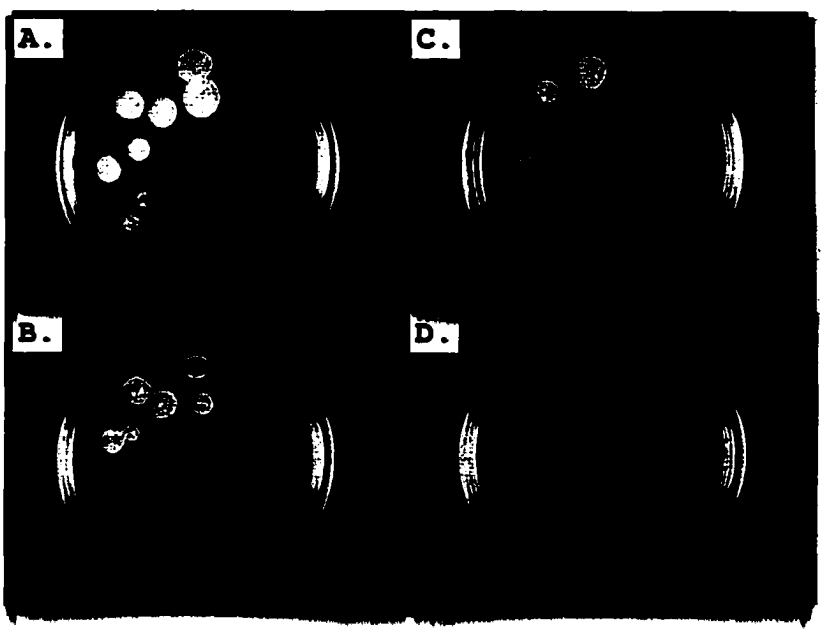

Figure 5.6 Effects of the oleic acid supplement in reversing the bactericidal effect of ISO. Panel A, 7H11 medium + OADC supplement; Panel B, 7H11 + OADC + ISO 0.5 µg/ml; Panel C, 7H11 medium + ADC supplement; Panel D, 7H11 + ADC + ISO 0.5 µg/ml. A series of 10-fold dilutions of *M. tuberculosis* were prepared in GAS medium. An aliquot (5 µl) of each dilution was spotted on 7H11 agar containing OADC or ADC as the supplement (Duplicates of dilution droplets were made on 7H11 + OADC plates).

5.4 DISCUSSION

In this present study, efforts have been placed on the elucidation of specific effects of ISO on fatty acid and mycolic acid synthesis. It is clear that the hallmark of ISO is its unique ability to inhibit the synthesis of oleic acid (Fig. 5.1; 5.2) and tuberculostearic acid (Fig. 5.2). GC analysis showed that the inhibitory effect on oleic acid is dose dependent (Fig 5.2; Table 5.1). At 1 µg/ml, ISO inhibited 67.2% of oleic acid synthesis in *M. tuberculosis* and ISO completely inhibited the synthesis of oleic acid at a concentration of 3 µg/ml. Thus, the IC_{50} of ISO on oleic acid synthesis is lower than 1.0 µg/ml. The evidence of the effect of ISO on oleic acid synthesis suggested that ISO acts by inhibiting stearoyl-desaturase, the enzyme responsible for the insertion of a double bond at carbon position 9 of stearic acid to form oleic acid (Fulco and Bloch, 1964; Kashiwabara and Sato, 1973; Kashiwabara *et al.*, 1975). The site of action of ISO as suggested by the present work is shown in Fig. 5.7.

Oleic acid is the most abundant monounsaturated fatty acid in *Mycobacterium spp* (Ratledge, 1982) and is a vital constituent of mycobacterial membrane phospholipids (Okuyama *et al.*, 1967; Walker *et al.*, 1970) where it presumably plays an essential role in membrane physiology as in other organisms (Los and Murata, 1998). At physiological temperatures, phospholipids containing only saturated fatty acids cannot form a lipid bilayer (Stubbs and Smith, 1984). The introduction of an appropriate number of

unsaturated fatty acids into phospholipids decreases the temperature for transition from the gel to the liquid-crystalline phase and provides membranes with the necessary fluidity for physiological function (Russell, 1984; Hazel, 1995) such as the activation of certain membrane-bound enzymes (Houslay and Gordon, 1984). In similar fashion, it can be assumed that oleic acid in mycobacterial membrane plays a crucial role in the proper functions of such membrane-associated enzymes. Furthermore, the presence of unsaturated fatty acids in membrane phospholipids is known to decrease membrane rigidity which markedly influences permeability and stability of biological membranes (Cook, 1996).

ISO also had a dramatic effect on the synthesis of tuberculostearic acid, a D10-methyl-branched stearic acid. Typically, mycobacteria contain a large number of methyl-branched fatty acids classified into two main categories: iso- and ante-iso acids (ω-, and ω-1-methyl acids), and acids containing one or more methyl groups (Kaneda, 1963; Campbell and Nowaral, 1969), mainly tuberculostearic acid and the multi-methyl-branched acids such as phthienoic acid (2,4,6 trimethyltetracos-Δ2-enoic acid) (Anderson, 1929; Carson and Sumrell, 1950) and mycocerosic acid (2,4,6,8-tetramethyloctacosanoic acids)(Ginger and Anderson, 1945). The latter are confined to *M. tuberculosis* and *M. bovis* but tuberculostearic acid is more widely distributed, being found in *M. tuberculosis, M. phlei and M. smegmatis* (Lennarz *et al.*, 1962). Our contention that the inhibition of tuberculostearic acid by ISO is secondary to the effect on oleic acid synthesis arises from the

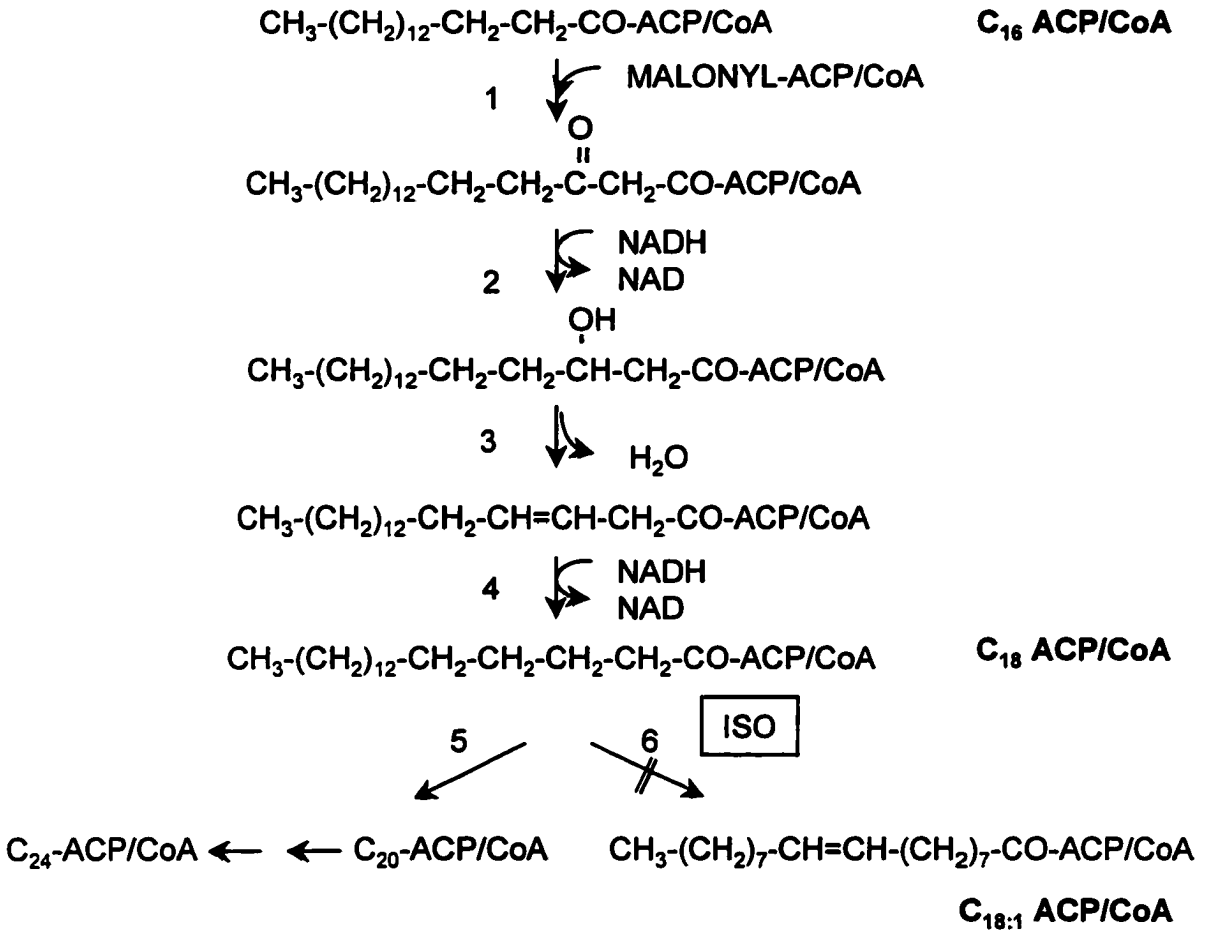

Figure 5.7 A scheme for fatty acid synthesis in mycobacteria and the proposed site of action of ISO. Fatty acids synthesis occurs through repetitive cycles of condensation, keto reduction, dehydration, and enoyl reduction (Barry *et al.*, 1998). The first reaction is initiated by β-ketoacyl-ACP/CoA synthase (KAS)(1), which condenses malonyl-ACP/CoA onto acyl-ACP/CoA. The β-ketoacyl-ACP/CoA from the synthase reaction is reduced to β-hydroxyacyl-ACP/CoA by β-hydroxyacyl reductase (KR)(2). The subsequent reaction is dehydration catalyzed by β-hydroxyacyl-ACP/CoA dehydrase (DE)(3). The dehydrase product is then reduced to longer chain acyl-ACP/CoA. This step is catalyzed by *trans*-enoyl reductase (ER)(4), a target of INH action (Banerjee *et al.*, 1994). A product of *trans*-enoyl reductase (ER) will enter new rounds of acyl chain elongation to produce longer acyl chain fatty acids (5) until a termination extension is attained. Desaturase is an enzyme that inserts one double bond into stearic acid ($C_{18:0}$) to form oleic acid ($C_{18:1}$ Δ9)(6) (Fulco and Bloch, 1962; Kashiwabara and Sato, 1973; Kashiwabara *et al.*, 1975). The inhibition of desaturase is the proposed mode of action of ISO.

demonstration that tuberculostearic acid is formed by methylation of oleic acid (Akamatsu and Law, 1970) using S-adenosylmethionine as the active donor of methyl group (Lennarz *et al.*, 1962). Prior to the conversion oleic acid must be first esterified to a phospholipid in the form of oleyl-phosphatidylethanolamine (Akamatsu and Law, 1970). It was noted that tuberculostearic acid is the only methyl-branched chain acid esterified to the 1-position of the glycerol unit of phosphoglycerides (Okuyama *et al.*, 1967; Walker *et al.*, 1970) and is therefore likely involved in membrane fluidity and integrity. The action of ISO on tuberculostearic acid synthesis should lead to the inhibition of phospholipid synthesis resulting in the disintegration of plasma membrane and cell death. The effect of the inhibition of tuberculostearic acid is thought to extend to the synthesis of PIMs and eventually LAM because tuberculostearic acid is a part of the PI anchor portion of PIMs, LM and LAM (Besra *et al.*, 1997).

Other apparently secondary effects of ISO are the increase of saturated fatty acids, $C_{18:0}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ (Fig. 5.2). This phenomenon can be explained by an increased flow of precursors into saturated fatty acid synthesis arising from the defect in unsaturated fatty acid synthesis (Fig. 5.7). Otherwise, this event is probably a consequential effect from the inhibition of ISO on mycolic acid synthesis, which resulted in the accumulation of short-chain fatty acid precursors.

It is apparent that ISO exerts many effects on mycobacterial cells; ISO-treated cells lose their acid-fast staining character,

and show the decreased synthesis of mycolic acids (Winder *et al.*, 1971; chapter 4) and short-chain fatty acids (Winder, 1982; chapter 4). In order to study further the effects on mycolic acid synthesis a simple one step HPLC system to separate different types of mycolic acids was developed. HPLC chromatography clearly confirmed the inhibitory effect of ISO on mycolic acid synthesis consistent with the previous report that like INH and ETH, ISO inhibited mycolic acid synthesis (Winder *et al.*, 1971). It seems that certain mycolic acids were much more affected by ISO. However, the specific site of the inhibition of ISO on mycolic acid synthesis has not been identified and awaits further investigation.

ISO also has an effect on ACPs which function to carry the growing fatty acyl chain between component enzymes of the type II fatty acid synthase in fatty acid synthesis (Prescott and Vagelos, 1972). The ACPs in mycobacteria appear to be different from other bacterial ACPs in that there are four open reading frames (ORFs) that may encode mycobacterial ACPs: Rv2244, Rv0033, Rv1344 and Rv3472 (Cole *et al.*, 1998). Mycobacterial AcpM was known to be encoded by the ORF of Rv2244 and was proposed to function as acyl carrier for the synthesis of fatty acids ranging in size from 18 to 50+ carbons (Barry *et al.*, 1998). Recently, it has been reported that upon treatment of *M. tuberculosis* with INH for a short interval, INH caused a dramatic upregulation of ACP which was identified by N-terminal amino acid sequencing to be AcpM (Mdluli *et al.*, 1998). Similarly, there was an obvious increase

of AcpM in the response of ISO treatment (Fig. 5.5, panel A). This effect of ISO on the increase of AcpM is thought to be another toxic effect of the drug since it was reported that AcpM was toxic for *M. smegmatis* when overexpressed at high levels (Mdluli *et al.*, 1998). In addition, overexpression of ACP in other systems was reported to be lethal to cells (Keating *et al.*, 1995).

In accordance with the proposed locus of ISO action, stearic acid ($C_{18:0}$) was found to be one of the major fatty acids attached to the increased AcpM and other increased products are palmitoyl-ACP ($C_{16:0}$-ACP) and hexadecanoyl-ACP ($C_{26:0}$-ACP). This evidence suggests that the substrate of the desaturase of mycobacteria is stearic acid probably in the form of stearoyl-ACP. However, the substrate of the desaturase may be in the form of stearoyl-CoA and the partial increase of C_{16}-, C_{18}- and C_{26}-ACPs can then be explained by the consequential effect of ISO on mycolic acid synthesis, an argument that needs to be resolved by an *in vitro* assay for desaturase activity.

According to the pleiotropic effects of ISO on mycobacterial cells, it is possible that the concert of effects of ISO is secondary to the effect on the inhibition of oleic acid synthesis. Alternatively, ISO may have many potential enzymatic targets resulting in an ability of ISO to exert many effects on mycobacterial cells. In this scenario, the effect of ISO on mycolic acid results from the interaction of ISO on a second target located on the mycolic acid synthesis pathway.

In summary, this study provides evidence for a novel mechanism of drug action on *M. tuberculosis* through the inhibition of oleic and tuberculostearic acid synthesis. It was decided to develop an *in vitro* desaturase assay, which will be used to characterize the Δ9 desaturase and screen derivatives of newly synthesized thioureas. Cloning and characterization of the *M. tuberculosis desA* genes is an approach to identify the gene that encodes the Δ9 desaturase. The compliance between antimycobacterial properties, low MIC and the inhibition of oleic and tuberculostearic acid synthesis provides evidence that the Δ9 desaturase is a promising target for a new generation of antituberculosis drugs, and ISO has possible therapeutic value in the treatment of tuberculosis.

5.5 FOOTNOTES

Nomenclature of fatty acids and abbreviations. The nomenclature of fatty acids in this paper was standardized as described by Ratledge (1982). In brief, the first number given indicates the total number of carbon atoms in the molecules, and the second number following the colon is the number of double bonds; thus $C_{16:0}$ = palmitic acid; $C_{18:1}$ = a monounsaturated C_{18} acid--usually this is oleic acid, and can be designed as $C_{18:1}$ Δ9. Branched-chain acids are indicated by "br"; thus $brC_{19:0}$ is a D10-methyl-branched stearic acid--usually this is tuberculostearic acid, $brC_{19:10}Me$.

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CHAPTER 6

Mycobacterial Desaturation and Identification of the *M. tuberculsosis desA* Gene that Encodes the Δ9 Desaturase Involved in Oleic Acid Synthesis and a Target of Thiocarlide/Isoxyl®

(Most of the work presented in chapter 6 and some parts from chapter 5 and 7 are parts of a manuscript for J. Biol. Chem.)

6.1 INTRODUCTION

Identifying and understanding the functions of specific genes is a fundamental and essential step to find and validate new targets for drug design and development. Elucidation of such genes can be approached by using molecular genetics, biochemical analysis and enzyme inhibitors that affect the function of gene products. Such compounds or drugs may selectively inhibit specific targeted enzymes and thereby cause an accumulation of precursor(s) and depletion of product(s) leading to an identification of the mode of action of drugs and the specific gene that encodes the targeted enzyme.

Recently, it has been shown that exposure of *M. tuberculosis* to an inhibitory level of ISO caused an accumulation of stearic acid concomitant with the depletion of oleic acid (chapter 5). This evidence suggested that ISO acts by inhibiting Δ9 desaturase, an enzyme that introduces one double bond at carbon 9 of stearic acid to form oleic acid (Fulco and Bloch, 1964; Kashiwabara and Sato, 1973; Kashiwabara *et al.*, 1975). In turn, esterified oleic

acid is a direct precursor of tuberculostearic acid (Lennarz *et al.*, 1962). Therefore, ISO also inhibits tuberculostearic acid synthesis. However, little information is available on the *M. tuberculosis* desaturases. With the advent of the complete sequence of the *M. tuberculosis* genome, searching the genomic database indicates that the *M. tuberculosis desA* gene family consists of three open reading frames, *desA1*, *desA2* and *desA3* (Cole *et al.*, 1998). The *desA1* encodes DesA1 protein, which has 30% identity at amino acid level to soluble-ACP desaturase from plant (Jackson *et al.*, 1997). DesA2 protein encoded by the *desA2* gene is weakly similar to plant stearoyl-ACP desaturase but very similar to the *M. tuberculosis desA1*-encoded protein. The *desA3* gene encodes DesA3 protein that has an amino acid sequence homologous to the membrane desaturase from *Synechocystis spp* (24% identity and 44% similarity). For the purpose of identifying the *M. tuberculosis* gene that encodes the Δ9 desaturase, the *M. tuberculosis desA* genes were cloned and expressed in *M. bovis* BCG, and fatty acids resulting from whole-cell labeling were analyzed by chromatography. This present work demonstrates that the *M. tuberculosis desA3* gene encodes the Δ9 desaturase and the enzyme activity can be inhibited by sterculic acid, a specific Δ9 desaturase inhibitor (Johnson *et al.*, 1967; Quintana *et al.*, 1998). A comparison of the effects of sterculic acid and ISO on fatty acid and mycolic acid synthesis demonstrates that, like sterculic acid, ISO is a Δ9 desaturase inhibitor which has an additional effect on mycolic acid synthesis. The Δ9 desaturase

was shown in this work to be a target of ISO and a membrane-associated enzyme.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains and growth conditions

E. coli strain XL-1 Blue (Stratagene; La Jolla, CA) was used as a bacterial host for general manipulation of recombinants. The mycobacterial expression plasmid vectors pMV261 and pVV16 were gifts from Dr. William R. Jacobs Jr., Albert Einstein College of Medicine, NY, and from Dr. Varalakshmi Vissa, Colorado State University, respectively. *E. coli* was cultured on Luria-Bertani (LB) agar plates or in LB broth (Gibco-BRL; Rockville, MD). Kanamycin (25 µg/ml) (Sigma; St Louis, MO) was added to the LB medium for growth of recombinant clones of *E. coli*. *M. bovis* BCG Pasteur 1173P2 was used as the mycobacterial expression host in the expression of *desA* genes, in whole-cell labeling and in MIC determinations. Cells were grown in Sauton's medium containing 0.025% Tyroxapol (Sigma) as a detergent (chapter 4). Media for growth of *M. bovis* BCG recombinant strains contained kanamycin (20 µg/ml). All bacterial strains were cultured at 37°C.

6.2.2 Construction of expression clones; cloning and overexpression of *M. tuberculosis desA* gene family

DNA manipulations in *E. coli* were performed according to standard protocols (Sambrook *et al.*, 1989) and manufacturer's instruction manuals (Qiagen; Santa Clarita, CA). Standard PCR strategies (Asubel *et al.*, 1991) with Vent DNA polymerase (New

England Biolabs; Beverly, MA) and a Perkin Elmer GeneAmp PCR System 2400 were used to amplify *desA* genes from genomic DNA of *M. tuberculosis* H37Rv. Two primers (sense and antisense) were designed to correspond to the 5′ and 3′ ends of each open reading frame (ORF) previously identified as *M. tuberculosis desA1* and *desA2* (ORF no. Rv0824c and Rv1094, respectively)(Cole *et al.*, 1998). A 1,034-base pair *Eco*RI fragment of *desA*1 was amplified with primers (sense) [5′ CTCTGAATTCTCACCGGGCTAACGAC 3′] and [5′ CAGCCAAACTGACCGACCTGCA 3′] (antisense) using a PCR program consisting of an initial 5 min denaturation step (94°C), then 35 cycles at 94°C for 1 min, followed by 68°C for 1 min, and then a final elongation step at 72°C for 10 min. A 895-base pair *Eco*RI fragment of *desA*2 was amplified with the following primers; [5′ CACAAAACCTGTTGCTGAAGCCGCTGACCC 3′] (sense) and [5′ GCTAGAATTCGAGTGCGACGCTACTCCG 3′] (antisense). The underlined sequences indicate the *Eco*RI restriction site that was introduced into primers to facilitate gene cloning. Thermal cycles for PCR were 5 min initial desaturation at 94°C, 35 cycles at 94°c for 1 min, 70°C for 2 min, and a final elongation at 72°c for 10 min. The PCR products were gel purified, cut by *Eco*RI and then cloned into *Bal*1-*Eco*RI sites of pMV261 which has the heat shock protein60 (hsp_{60}) promoter and kanamycin resistance (Kan^r) as a marker (Stover *et al.*, 1991). The ligation of the *Eco*RI-digested PCR product into the *Bal*I-*Eco*RI site of pMV261 allowed the use of the hsp_{60} promoter and the start codon (ATG) of the hsp_{60} gene fragment of pMV261 for the generation of in-frame fusion products. The

desA3 was overexpressed in pVV16, a derivative of pMV261, which has a His-tag at the C-terminal sequence. The *des*A3 ORF (Rv3229c) was amplified using primers: [5′ GTCGAGCAAAGCTTGGCTGCCAGATCGTC 3′] (sense) and [5′ AGGGAGAAGCATATGGCGATCACTGACGTC 3′] (antisense). The underlined sequences correspond to *Hind*III and *Nde*I restriction sites, respectively. The thermal conditions for PCR were 5 min initial desaturation at 94°C, followed by 35 cycles at 94°C for 1 min, and 72°C for 2 min, then, a final elongation step at 72°C for 10 min. The 1,360-base pair PCR product was digested with *Nde*I and *Hind*III and cloned into the similarly digested pVV16. This strategy fused the *desA3* coding sequence in-frame to a pVV16 encoding hexa-His domain. The resulting constructs, pMV261desA1, pMV261desA2 and pVV16desA3 were then transformed into *E. coli*, and selected on LB-agar plates supplemented with kanamycin. The transformants were confirmed to contain the desired plasmid recombinants by restriction analysis. To express Des proteins, the DNA recombinants were electroporated into *M. bovis* BCG electrocompetent cells. Transformants of *M. bovis* BCG were then selected on 7H11 plates supplemented with OADC and kanamycin. Again, the BCG transformants were analyzed for proper constructs by restriction analysis of plasmid DNA and direct colony PCR. Plasmids from *M. bovis* BCG were prepared by rapid-freeze-thawing lysis in TE buffer with 100 μg of RNAseA per ml.

6.2.3 DNA and amino acid sequence analysis

For all constructs, the insert orientations and identities

were confirmed by partial DNA sequencing performed by Macromolecular Resource Facilities (Colorado State University). BLAST analysis (National Biotechnology Information) (Altschul *et al.*, 1997) was applied to analyze DNA and amino acid sequences. Alignments of amino acid sequences were performed using the Multalign (http://www.toulouse.inra.fr/multalin.html). Hydropathy analysis for transmembrane domains were predicted with a Kyte-Doolittle plot in a DNA-Strider program.

6.2.4 Analytical procedures

6.2.4.1 Analysis of effects of ISO on *M. bovis* BCG

Conditions for cell labeling, saponification and derivatization of wild-type (WT) *M. bovis* BCG fatty acids and mycolic acids were similar to the procedures performed with *M. tuberculosis* (chapter 5). Finally, an aliquot of the extracts containing FAMEs and MAMEs (100 µl) was counted in 10 ml of liquid scintillation (Ecolume ™; Costa Mesa, CA), and then equal counts of radiolabel from the control and ISO-treated samples were applied to impregnated TLC plates (Morris, 1966). After the plates were developed two times in petroleum ether-acetone (90:10), autoradiograms were produced.

6.2.4.2 Analysis of the expression of Des proteins

SDS-PAGE and immunoblotting were applied to analyze the overproduced *M. tuberculosis* Des proteins. A single colony of a *M. bovis* WT strain and a *M. bovis* BCG transformant harboring the recombinant plasmid pMV261desA1 were grown in Sauton's medium to

A_{600} of 0.600. Cells were harvested by centrifugation and washed once with deionized water prior to resuspension in 20 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM dithiothreitol. Disruption of cells was performed using an internal probe sonicator (Sanyo Soniprep 150) for 20 cycles of 40 sec on and 90 sec off. Whole cells and cell debris were then removed by centrifugation. The resulting crude cell lysate was quantitated for protein concentration by the bicinchoninic acid method (BCA)(Smith *et al.*, 1985) using bovine serum albumin (BSA) (Pierce; Rockford, IL) as standard protein. Appropriate amounts of protein (100 µg) were loaded and separated on 0.1% SDS-12% polyacrylamide gels as described by Laemmli (1970). Gels were then either stained with Coomassie Brilliant Blue R-250 (Bio-Rad; Richmond, CA)(Chen *et al.*, 1993) or transferred by electroblotting to 0.2 µM pore-size nitrocellulose (Schleicher & Schuell; Keene, NH). The electroblotting was performed using 100 v at 4°C for 1 h in buffer containing 25 mM Tris (pH 8.0), 192 mM glycine and 20% methanol as described by Towbin *et al.* (1979). After blotting, the membranes were blocked with 3% BSA in Tris-buffered saline containing Tween 20 (TBS/T) (10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.1% Tween 20), incubated with mouse anti-DesA1 polyclonal antibodies (a gift from Dr. Mary Jackson) diluted 1:1000 in TBS/T, washed three times in TBS/T and incubated with alkaline phosphatase-conjugated antibodies specific for mouse antibodies. Proteins were visualized with Nitro Blue

Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega; Madison WI).

6.2.4.3 Analysis of the effect of overexpression of the *M. tuberculosis desA* gene family

The enzyme activity of mycobacterial Δ9 desaturase was determined by measuring the formation of radiolabeled-oleic acid in whole-cell labeling. Labeling of fatty acids in the WT and recombinant strains, and argentation-TLC analysis were conducted as described previously (chapter 5). The bands corresponding to methyl ester oleates were counted in 10 ml of scintillation fluid.

FAMEs were also analyzed for the effect of the overexpression of *desA* genes by GC as described previously (chapter 5). The eluted peaks of labeled-FAMEs were identified by comparison of the retention times with those available methyl ester standards of $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $Cl_{16:1}$, $C_{18:0}$, $C_{18:1}$ Δ9, $brC_{19:0}$ (TBSA), $C_{20:0}$, $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$ as detected with TCD.

6.2.5 Immunoblotting to locate DesA3 in cellular fractions

Cell pellets of the *desA3* overexpression strain were disrupted as described above. After spinning at 27,000 x g for 30 min, unbroken cells and debris were discarded. The resulting crude cell lysate was further centrifuged at 200,000 x g for 1 h with a Beckman Ultracentrifuge and a Ti70.1 Beckman rotor (Beckman; Fullerton, CA). The cytosol was recovered as the supernatant, and the pellets represented the cell membrane fraction. 0.25 M sucrose was used to resuspended membrane

pellets. The protein concentrations of the prepared fractions were determined using a BCA protein assay kit (Pierce). Aliquots of 100 µg of proteins from each fraction were loaded and separated on 0.1% SDS-12% polyacrylamide gels and transferred to nitrocellulose for immunoblotting as described above. The fusion protein of DesA3 and hexa-His was probed with primary mouse anti-His-tag antibodies (Qiagen) and alkaline phosphatase-conjugated secondary mouse antibodies. Color detection was performed as described previously.

6.2.6 Determination that *desA3* gene encodes a target of ISO

The microplate Alamar Blue assay modified from Yajko *et al.* (1995), was used to determine the MIC of ISO for the *desA3* overexpression strain. Inocula were prepared from *M. bovis* BCG/pVV16 and the recombinant strain harboring the pVV16desA3 construct. Cells were grown in Sauton's medium to a turbidity equal to that of a No. 1 McFarland standard (~2.0 x 10^7 CFU/ml). Serial dilutions of ISO were prepared from DMSO stock in Sauton's medium. From each dilution 10 µl was then inoculated into 170 µl of cultures that had been dispensed into clear-bottomed, 96-well microplates prior to adding the drug. Duplicate treatments with different concentrations of ISO were performed. Four controls were included which contained medium only, culture only, medium plus DMSO, and culture plus DMSO. At the outset, 10 x Alamar Blue solution (Accumed; OH) was added to the controls. After overnight incubation at 37°C in a moisture-controlled chamber, Alamar Blue

was added to the all treated wells. Plates were further incubated, and the color in each treated culture was recorded until the color in the culture control turned pink. The MIC was determined as the lowest concentration of ISO in which the blue dye in the treated culture did not turn pink.

6.2.7 Analysis of the effect of sterculic acid (SA)

The effect of sterculic acid on the growth rate of *M. bovis* BCG was determined by the microplate Alamar Blue assay and the broth dilution technique as described previously. The comparative effects of sterculic acid and ISO on oleic acid synthesis were then determined by whole-cell labeling and analysis of the incorporation of isotope from [1,2-^{14}C]acetate into oleic acid by GC. TLC was applied to analyze the incorporation of [^{14}C]acetate into mycolic acids.

6.3 RESULTS

6.3.1 ISO inhibits *in vivo* synthesis of oleic acid and mycolic acid synthesis

The effects of ISO on *M. bovis* BCG were assessed prior to using it as a mycobacterial expression host to characterize *M. tuberculosis* Des proteins and to identify the *M. tuberculosis desA* gene that encodes the Δ9 desaturase. Similar to the situation in *M. tuberculosis*, ISO inhibited the synthesis of oleic acid and all types of *M. bovis* BCG mycolic acids, identified as α- and ketomycolates (Fig. 6.1). Moreover, a concomitant accumulation of

stearic acid which was an immediate effect of the inhibition of oleic acid was also observed (Fig. 6.1). The effects of ISO on *M. bovis* BCG emulated what happened in *M. tuberculosis* and led to the use of *M. bovis* BCG as a host for the expression of the *M. tuberculosis desA* genes.

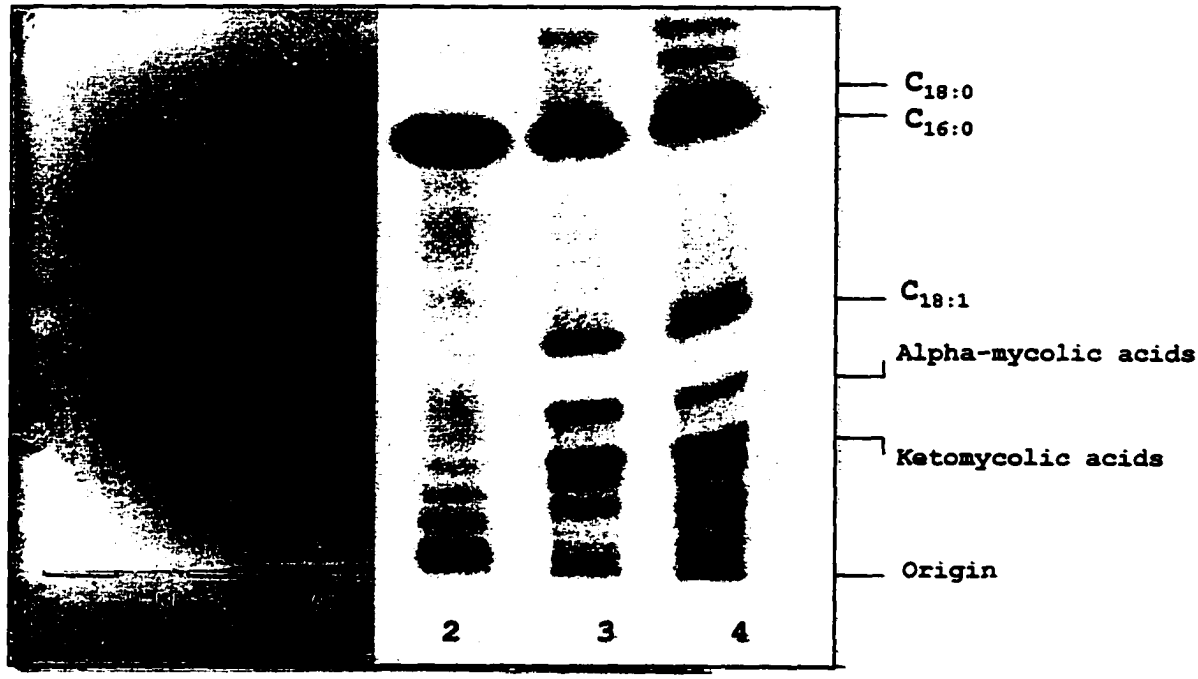


Figure 6.1 Argentation autoradiographic TLC of the effect of ISO on the incorporation of [^{14}C]acetate into FAMEs and MAMEs of *M. bovis* BCG. A mixture of FAMEs and MAMEs was extracted from labeled-cells of *M. bovis* BCG. The extracts from control and ISO-treated cultures containing equal CPM (~80,000 cpm) were spotted on the aluminum-backed TLC plate, which was impregnated with 10% silver nitrate solution. The plate was developed twice in petroleum ether-acetone (90:10) and exposed to Kodax X-Omat AR film at -70°C for 48 h. Anisaldehyde and 10% sulfulic acid were used to stain the $C_{18:1}$ Δ9 standard. Lane 1, $C_{18:1}$ Δ9 standard; Lane 2, [^{14}C]$C_{16:0}$ standard; Lane 3, untreated culture; Lane 4, 1.0 μg/ml ISO-treated culture.

6.3.2 Expression of Des proteins in *M. bovis* BCG

To identify the *M. tuberculosis desA* gene that encodes the Δ9 desaturase, the ORFs of the *desA* genes were successfully amplified by PCR from the *M. tuberculosis* H37Rv genome and cloned in-frame into mycobacterial expression vectors. An approach using *M. bovis* BCG as an expression host and cloning *desA* genes downstream from the hsp_{60} constitutive promoter in pMV261 or pVV16 made possible the high-level production of *M. tuberculosis* Des proteins.

The level of expression of DesA1 (the gene product of the *desA1* gene) was determined by means of immmunoblotting using mouse polyclonal antibodies specific to DesA1. Fig. 6.2 shows the degree of expression of DesA1 in a crude a cell lysate prepared from the recombinant *M. bovis* BCG. The predicted molecular mass of the *M. tuberculsois* DesA1 is about 37 kDa (338 amino acids (aa) in length, which corresponds to the relative migration distance observed by electrophoresis. The overproduced DesA2 and DesA3 (the gene products of the *desA2* and *desA3* genes, respectively) in the recombinant strains of *M. bovis* BCG were hardly distinguished by SDS-PAGE analysis.

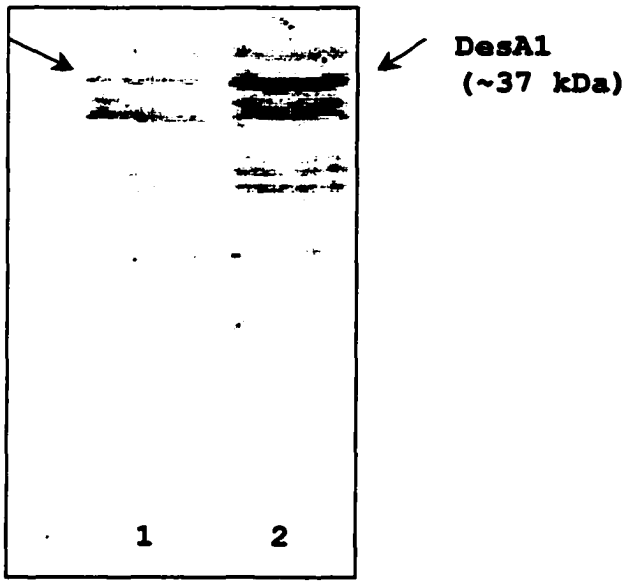


Figure 6.2 Immunoblot analysis of the expression of DesA1 protein. Equal amounts of crude protein extract were loaded on the gel and then transferred to nitrocellulose. Mouse anti-DesA1 IgG was used as the primary antibody, and alkaline phosphatase-conjugated anti-mouse antibodies were used as a secondary antibody. The positions of DesA1 in the wild-type strain (lane 1) and overproduced DesA1 in the recombinant strain of *M. bovis* BCG (lane 2) are marked.

6.3.3 Overexpression of the *desA3* gene resulted in an increase of the synthesis of oleic acid *in vivo*

To assess the correlation between the Δ9 desaturase and the cloned ORFs, the FAMEs from *M. bovis* BCG WT and three overexpression strains were analyzed by argentation-TLC. The results revealed no obvious increase in oleic acid synthesis in the *desA1*- and *desA2*-overexpression strains (Fig. 6.3). Quantitation by scraping the bands corresponding to oleic acids and counting them in scintillation fluid indicated that the *desA1*- and the *desA2*-overexpression strains synthesized oleic acid approximately 0.2 fold less than that of a WT ratio. In contrast, oleic acid was

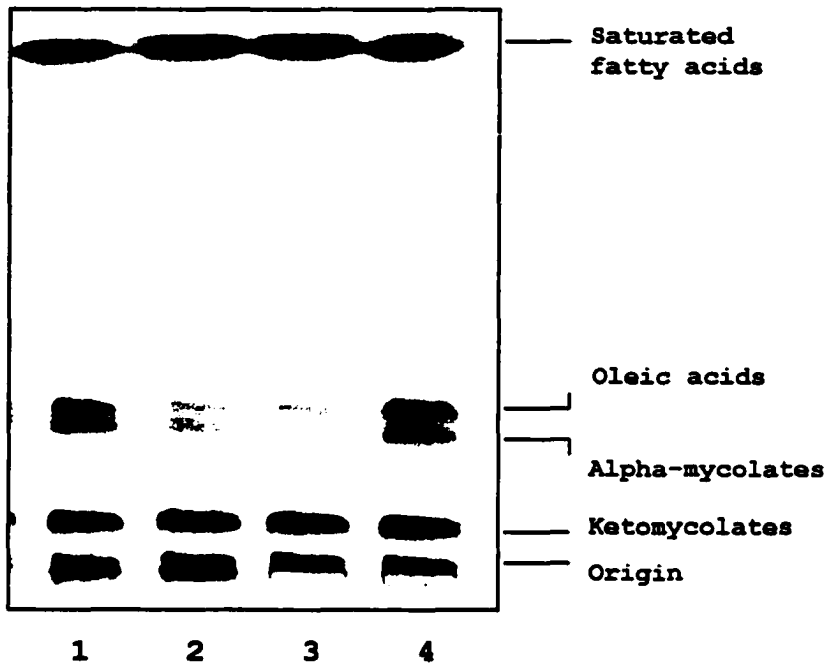


Figure 6.3 TLC autoradiogram of effects of the overexpression of the *M. tuberculosis desA* genes on fatty acid and mycolic acid compositions. FAMEs and MAMEs were extracted from wild-type and *desA*-recombinants of *M. bovis* BCG. Equal counts of FAMEs and MAMEs from each strain were applied to the silver impregnated TLC plate, which was developed in petroleum ether-acetone (90:10). The autoradiogram was produced by exposure of the TLC plate to Kodax X-Omat AR film. Lane 1, *M. bovis* BCG WT/pVV16; Lane 2, *M. bovis* BCG/pMV261desA1; Lane 3, *M. bovis* BCG/pMV261desA2; Lane 4, *M. bovis* BCG/pVV16desA3.

overproduced in the *desA3*-overexpression strain. There was an obvious increase in the amount of oleic acid synthesis as shown by autoradiography (Fig. 6.3). Direct counting showed that the *desA3*-overexpression strain had an increase of the incorporation of isotope from [1,2-^{14}C]acetate into oleic acid from 2,404 cpm to 4,848 cpm or had approximately a 2.0-fold of an increase in oleic acid synthesis when compared to that of WT. This initial analysis indicated that the *desA3* gene but not *desA1* or *desA2* encoded the Δ9 desaturase responsible for the synthesis of oleic acid.

Analysis of the radiolabeled FAMEs by radio-GC showed that the majority of fatty acids in *M. bovis* BCG consisted of palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$ Δ9), and tuberculostearic acid (Fig. 6.4, panel A), similar to the composition of principal fatty acids existing in *M. tuberculosis* (Chou *et al.*, 1996; Chou *et al.*, 1998). The relative incorporation of [^{14}C] from [1,2-^{14}C]acetate into oleic acid in the *desA3*-overexpression strain was obviously increased when compared to the oleic acid content in the *M. bovis* BCG WT strain (Fig. 6.4, panel B). The results of the whole-cell labeling and analysis of labeled FAMEs by radio-GC are consistent with the previous results obtained by argentation-TLC showing that the *desA3*-overexpression strain had an increased capacity to synthesize oleic acid compared to the wild-type strain (Fig. 6.4). In all, these results provided strong evidences that the *desA3* gene encoded the Δ9 desaturase required for the formation of oleic acid in *M. tuberculosis*.

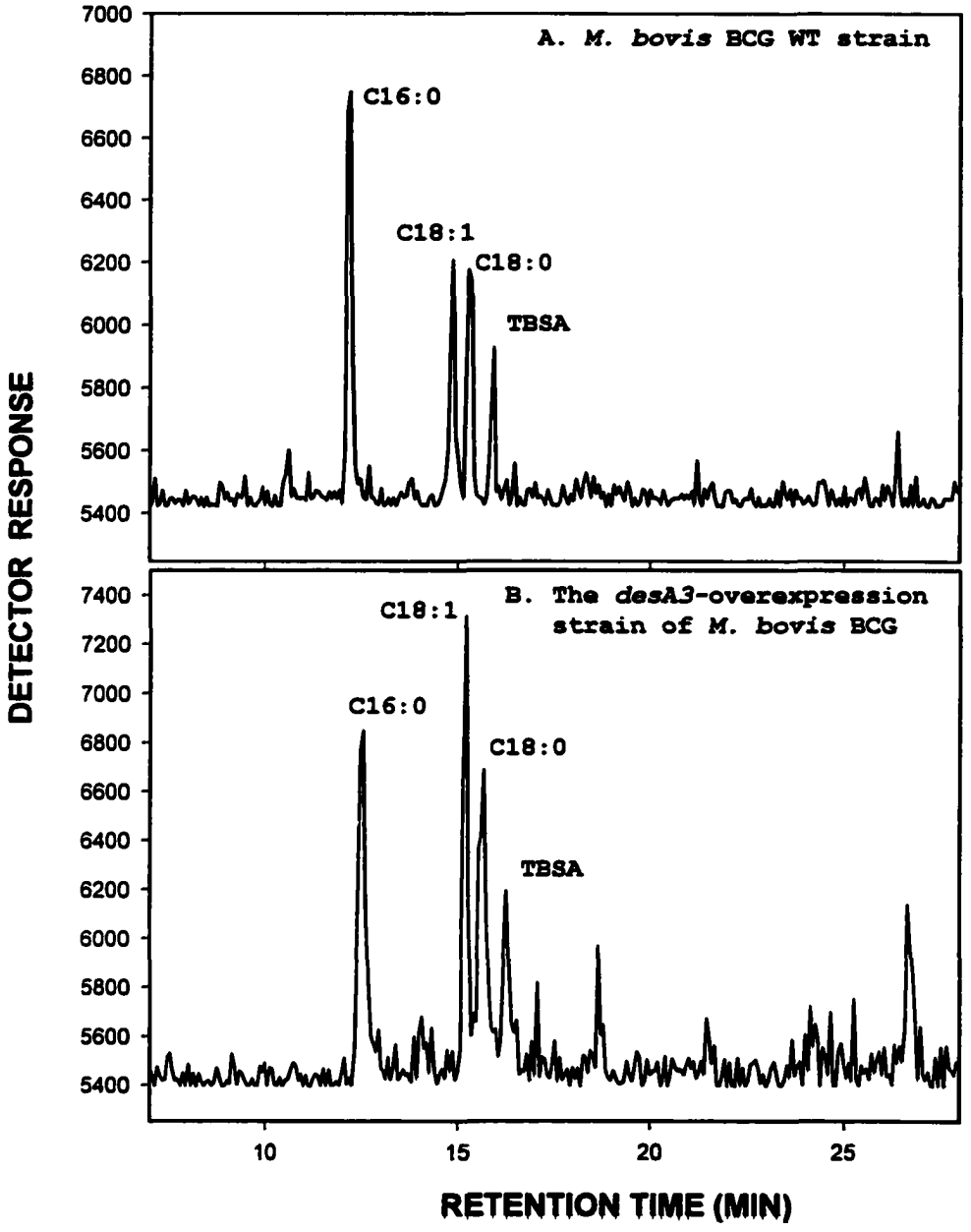


Figure 6.4 Comparative radio-GC chromatograms of fatty acid methyl esters from the *M. bovis* BCG WT (A) and the *desA3*-overexpression strain (B). The mixture of labeled FAMEs and MAMEs were silated and applied to a Hewlett Packard HP 5890 GC equipped with a capillary HP-1 column and the Lablogic GC-RAM detector counter. The temperature program used for resolution of all FAMEs was started at 80°C, which was increased to 185°C at a rate of 30°C per min. Then, the temperature was increased at a rate of 5°C per min to a final temperature of 345 °C. Eluted peaks were identified by comparing the retention times with those of FAME standards.

6.3.4 DesA3 is a membrane-associated desaturase

A comparison of the amino acid sequence of DesA1 (MTV043.16c; 338 aa), and DesA2 (MTV017.47; 275 aa) is shown in Fig. 6.5A. As predicted (Cole *et al.*, 1998), the alignment showed that the *M. tuberculosis* DesA2 sequence is closely related to *M. tuberculosis* DesA1 (32.2% identity in 270 aa overlap). The EX_2H sequences previously identified as iron ligands of soluble desaturases (Shanklin *et al.*, 1994; Fox *et al.*, 1994) were found in two repeats spanning amino acids 106 to 110 and 196 to 200 in both DesA1 and DesA2. The alignment of DesA3 (MTCY20B11.04c; 427 aa) with DesA1 and DesA2 resulted in few areas of homology (Fig. 6.5B).

The analysis of some membrane desaturases and the mycobacterial DesA3 revealed that DesA3 has three regions of primary sequences conserved in membrane desaturases which are characterized by $HX_{(3\ or\ 4)}H$, $HX_{(2\ or\ 3)}HH$, and $HX_{(2\ or\ 3)}HH$ (Shanklin *et al.*, 1994) (Fig. 6.6). Thus, there are eight His residues in these highly conserved regions among membrane desaturases. BLAST analysis demonstrated the homology of DesA3 to several membrane desaturases, for instance Δ5, Δ6, and Δ8 desaturases (Table 6.1). However, the Δ6 desaturase from *Synechocystis spp* is the most similar to the mycobacterial DesA3. It also has conserved regions of the His-rich motifs similar to that of DesA3 (Fig. 6.6). Moreover, Δ9 desaturases from rat, mouse and yeast are regarded as membrane desaturases (Shanklin *et al.*, 1994), and they also possess the His-rich conserved motifs of membrane desaturases

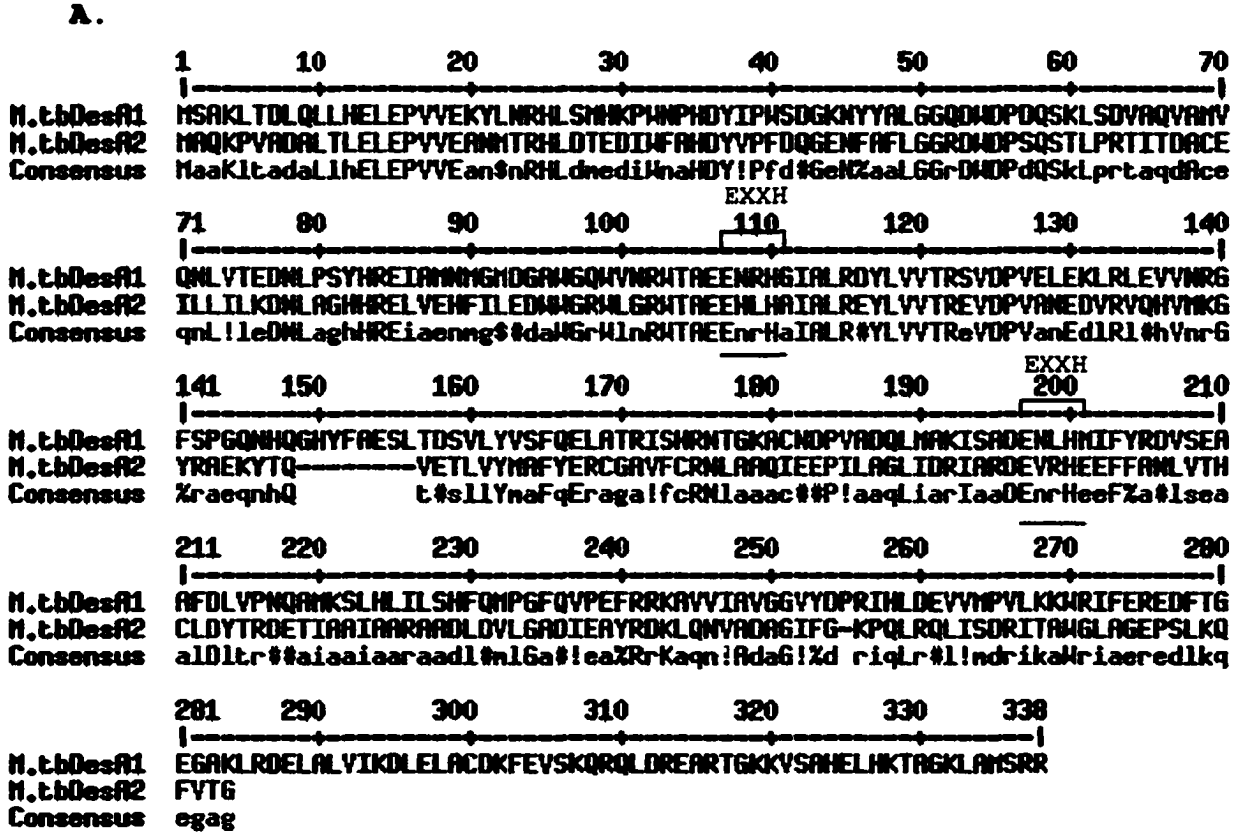


Figure 6.5 Alignment of amino acid sequences *of M. tuberculosis* Des proteins. (A), The alignment of DesA1 and DesA2. Both have two repeats of consensus sequences of the soluble desaturase, which is EXXH as indicated by underlined sequences. Red letters represent the identical amino acids; Blue letters represent the similar amino acids.

B.

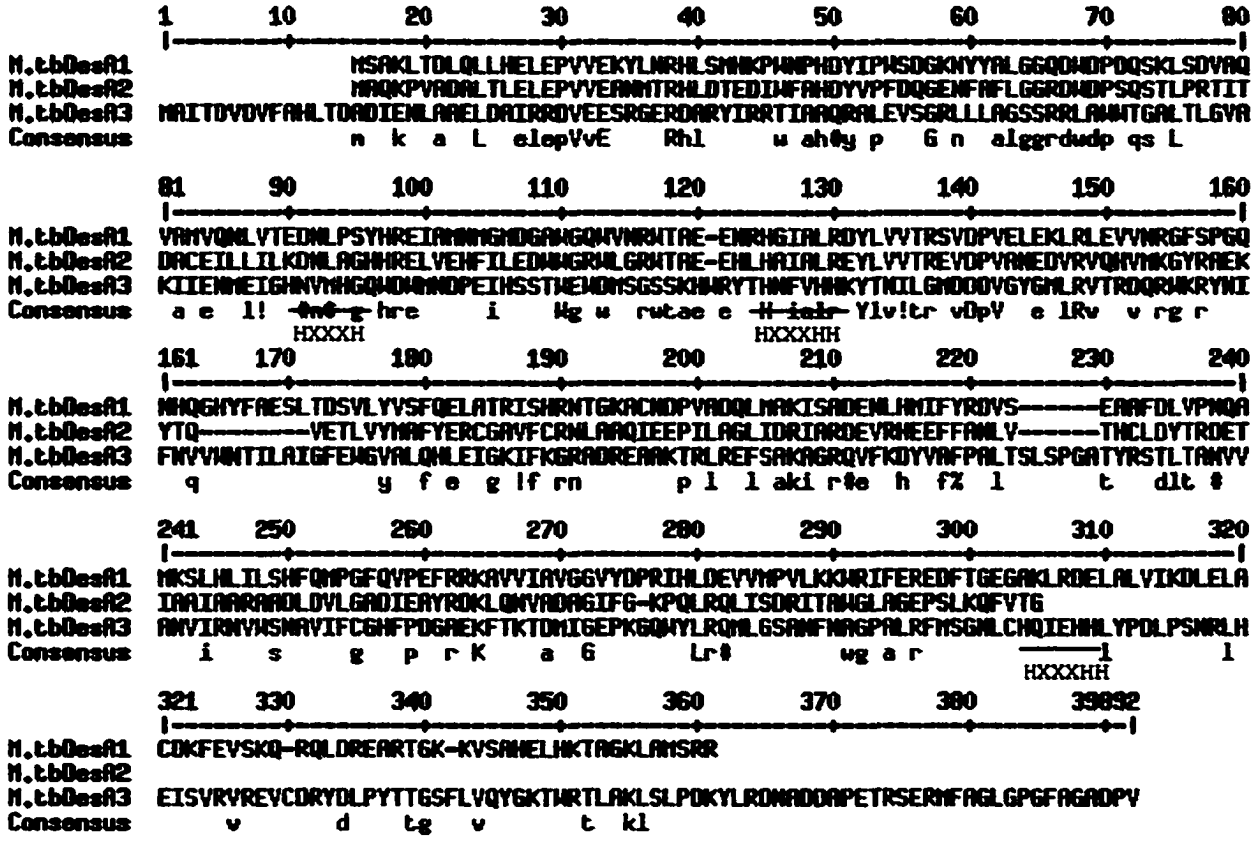


Figure 6.5 Alignment of amino acid sequences *of M. tuberculosis* Des proteins. (B), The alignment of DesA1, Des2 and DesA3. The two repeats of consensus sequence of the soluble desaturase which is EXXH present in DesA1 and DesA2 but not in DesA3. Instead, DesA3 has the consensus sequences of the membrane desaturase, which are HXXXH, and the two repeats of HXXXHH as indicated by underlined sequences. Red letters represent the identical amino acids, while blue letters represent the similar amino acids.

A.

Membrane desaturases

Δ9 position

Region Ia — Region Ib

```
Rat     114  ITAGA HRLWSHR TYKARLPLRIFLIIANTMAFQNDVYEWARD HRAHH KFSETHADPHNSR  173
Yeast   156  ITAGY HRLWSHR SYSAHWPLRLFYAIFGCASVEGSAKWWGHS HRIHH RYTDTLRDPYDAR  215
Mouse   114  ITAGA HRLWSHR TYKARLPLRLFLIIANTMAFQNDVYEWARD HRAHH KFSETHADPHNSR  173
```

Δ6 position

```
Synd6    83  SFNVG HAD NHN AYSSNPHINRVLGMTYDFGLSSF  LWRYR HNYLHHTYTNILGHDVEIH  141
```

***M. tuberculosis* DesA3**

```
M. tb    85  NMEIG HNV MHG QWDWMNDPEIHSSTWEWDMSGSSK HWRYT HNFVHH KYTNILGMDDDVG  143
DesA3
```

B.

Membrane desaturases

Δ9 position

Region II

```
Rat     257  LNATWLVNSAAHLYGYRPYDKNIQSRENILVSLGSVGE GF HNYHH AFPYDYSASEYRWH  315
Yeast   295  QQATFCINSMAHYIGTQPFDDRRTPRDNWITAIVTFGE GY HNFHH EFPTDYRNAIKWYQ  353
Mouse   257  LNATWLVNSAAHLYGYRPYDKNIQSRENILVSMGAVGE RF HNYHH AFPYDYSASEYRWH  315
```

Δ6 position

```
Synd6   262  LTPDGESGAIDDEWAICQIRTTANFATNNPFWNWFCGG LN HQVTHHLFPNICHIHYPQLE  320
```

***M. tuberculosis* DesA3**

```
M. tb   264  KFTKTDMIGEPKGQWYLRQMLGSANFNAGPALRFMSGN LC HQIEHHLYPDLPSNRLHEIS  322
DesA3
```

Figure 6.6 Multiple alignment of putative "membrane desaturase" regions of the *M. tuberculosis* Δ9 desaturase (DesA3) and related desaturases. (A), Comparative amino acid sequence analysis of the region Ia and Ib of the various membrane desaturases and DesA3 of *M. tuberculosis*. The five conserved His residues are indicated by shading (two in region Ia, and three in region Ib). Positions containing identically conserved residues are in bold letters. Gaps are introduced to facilitate the sequence alignment. (B), Comparison of the region II amino acid sequences of the membrane desaturases. Three conserved His residues are indicated by shading. Representatives of membrane Δ9 desaturases are from *Rattus norvigicus* (Rat), *Saccharomyces cerevisiae* (Yeast), and *Mus muscarus* (Mouse). The putative region from the Δ6 desaturase of Cyanobacterium *Synechocystis spp* is included.

Table 6.1 BLAST results obtained with DesA3

Accession no.	Sequences producing significant pair alignment	Score bits	E Value
S35157	linoleoyl-CoA desaturase(delta6)[*Synecho.*	83	3e-15
AF007561	delta6-desaturase[*Borago officialis*]	68	2e-10
U79010	Delta6-desaturase[*Borago officialis*]	68	2e-10
S54809	linoleoyl-CoA desaturase[*Spir..*	67	2e-10
S68358	hypothetical protein-common sulflower	64	2e-09
AJ224160	delta8 sphingolipid desaturase[*B..*	60	4e-08
AF005096	desaturase/cytochrome b5 protein[*Ric..*	59	7e-08
AB029311	fatty acid desaturase[*Dictyoste..*	59	7e-08
AF139720	delta8 fatty acid desaturase	56	5e-07
AC005397	putative cytochrome b5[*Arabidopis* t..	56	6e-07
AJ224161	delta8 sphingolipid desaturase[*A..*	55	8e-07
AF067654	delta5 fatty acid desaturase[*Mortie..*	55	1e-06
AF054824	delta5 microsomal desaturase[*Mortie..*	54	2e-06
AF078796	delta5 fatty acid desaturase[*caenor..*	54	2e-06
AF134404	delta6 fatty acid desa..	54	2e-06
Z81122	similarity with *Helianthus annus..*	54	5e-06
AL078610	putative delta fatty acid desatu..	53	5e-06
AF001394	fatty acid desaturase/cytochrome b5 f..	49	8e-05
AF126798	delta6 fatty acid desaturase[*Mus..*	49	8e-05
AB021980	delta6 fatty acid desaturase[*R..*	49	8e-05
AL0505118	hypothetical protein[*Homo sapiens*]	49	8e-05
AF031477	delta6 fatty acid desaturase[*Caenorh..*	48	2e-04
AC004770	BC269730_2[*Homo sapiens*]	46	5e-04
AB022097	delta5 fatty acid desaturase[*Dic..*	44	0.003

(Fig. 6.6). Thus, the similarity in amino acid sequences as well as the presence of His-rich motifs characteristic of membrane desaturases predict that DesA3 is a membrane-associated protein.

Hydropathy analysis of the secondary structure of DesA3 indicated that DesA3 contains up to three hydrophobic domains, which would be long enough to span the membrane bilayer twice (Fig. 6.7A). This finding supported the result of the amino acid sequence analysis indicating that DesA3 is a membrane-associated enzyme. Immunoblotting to identify the intracellular location of DesA3, using antibody specific to the hexa-His-tag fused with

DesA3 at the C-terminus, confirmed that DesA3 is a membrane-associated enzyme (Fig. 6.7B).

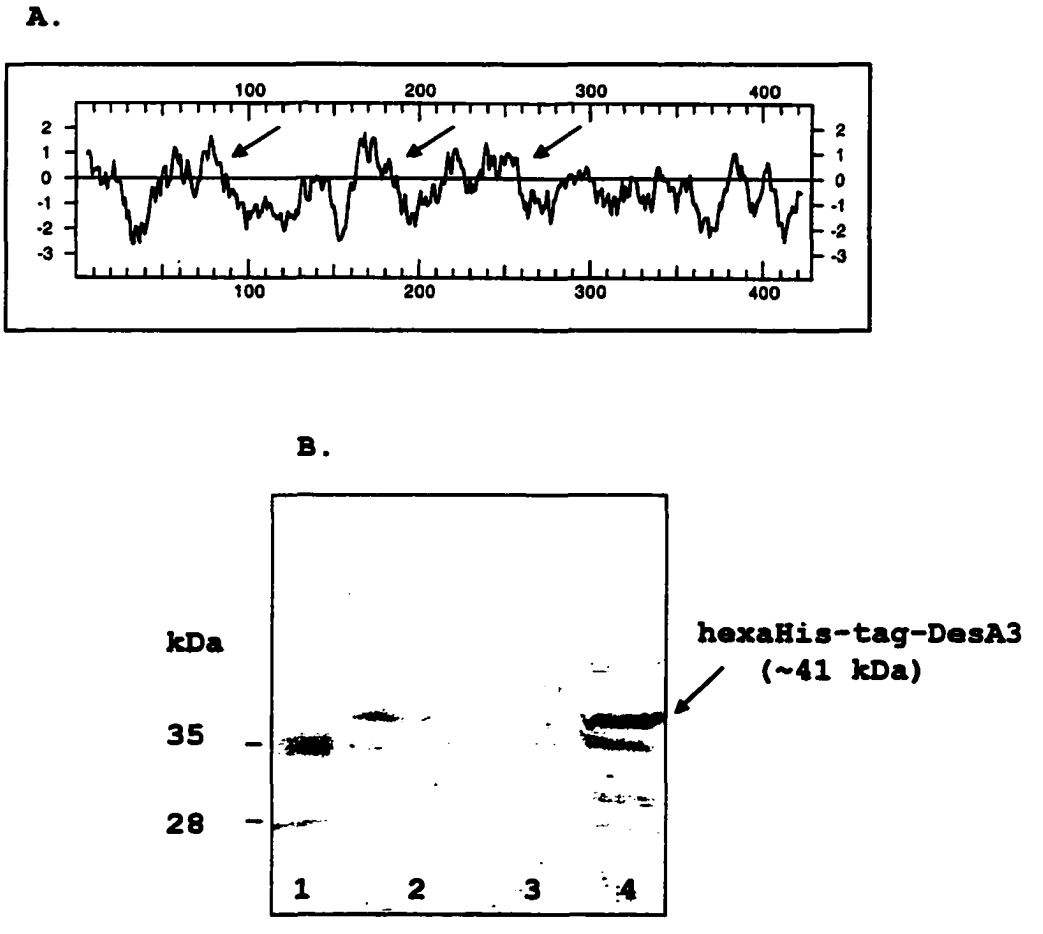


Figure 6.7 Hydropathy analysis of transmembrane domains of mycobacterial DesA3 (A) and immunoblot of the *M. tuberculosis* membrane desaturase (B). The three transmembrane domains of DesA3 allowing three spans of the membrane are shown on the Kyte-Doolittle plot (A). Immunoblot to locate the DesA3 (B). Antibody against hexaHis-tag was used as a probe to detect the DesA3-His-tagged fusion protein. Proteins prepared from various subcellular fractions of the recombinant strain ovrexpressing *desA3* were loaded onto the polyacrylamide gel in equal amounts. Lane 1, protein markers; Lane 2, whole cell proteins; Lane 3, cytosolic proteins; Lane 4, membrane proteins.

6.3.5 Effects of the overexpression of the *desA3* gene on the MIC of ISO

The overexpression of the ISO target on a multiple copy plasmid should impart the resistant phenotype as seen by an increase of MIC to ISO. To assess the contribution of the overproduced DesA3 to the activity of ISO, the MICs of ISO against WT and *M. bovis* BCG overexpressing the *desA3* gene were determined by microplate Alamar Blue assay. Alamar Blue is a dye indicator which changes color from blue to pink during cell growth (Jenkins *et al.*, 1991; Pfaller *et al.*, 1994; Pital *et al.*, 1958). In *M. bovis* BCG, colorimetric MICs by microplate Alamar Blue assay were interpretable on the third day of incubation. The MIC of ISO on the WT strain was 3.0 µg/ml, whereas in the case of the *desA3*-overexpression strain the MIC was in excess 6.0 µg/ml (Fig. 6.8).

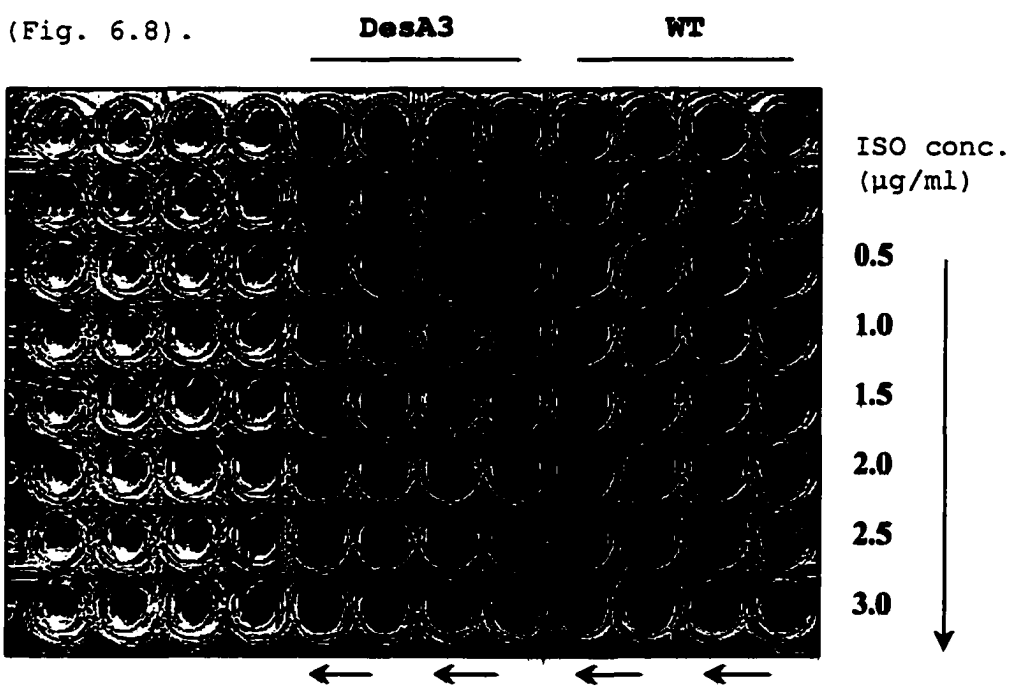


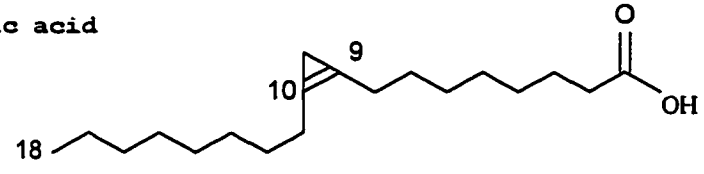
Figure 6.8 Analysis of the effect of the overexpression of the *desA3* gene on the MIC of ISO. The MIC of ISO was determined by colorimetric method using microplate Alarmar Blue assay (See text how to interprete MIC from tests).

It should be noted that the MIC from this test is the minimal concentration of ISO that completely inhibits the metabolic activities in 3.4 x 10^6 CFU/ml of mycobacterial cells. The increase of MIC to ISO in *M. bovis* BCG overexpressing the *desA3* gene suggests that the *desA3* gene encodes a target of ISO.

6.3.6 Inhibition of mycobacterial Δ9 desaturase by sterculic acid and ISO

Sterculic acid is a naturally occurring cyclopropene fatty acid and is a potent inhibitor of the Δ9 desaturation of stearic to oleic acid (Johnson *et al.*, 1967). Sterculic acid has no overt structural relationship to ISO (Fig. 6.9). Hence sterculic acid was used as a tool to further examine whether DesA3 is a target of ISO.

Sterculic acid



Isoxyl (ISO)

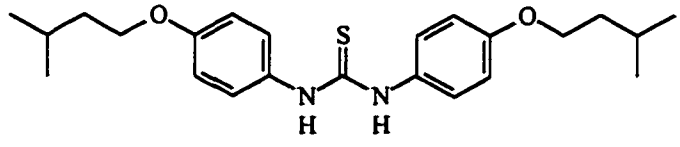


Figure 6.9 Chemical structures of sterculic acid (Andrianaivo-Rafehivola *et al.*, 1994) and ISO.

Firstly, the effects of sterculic acid on the growth of *M. bovis* BCG was determined by the microplate Alamar Blue assay with three concentrations of ISO (1, 10 and 100 μg/ml) and approximately 1.0 x 10^6 CFU/ml of the WT strain was used. This

experiment clearly showed that sterculic acid could inhibit the growth of *M. bovis* BCG when used at 100 µg/ml. Moreover, the broth dilution technique confirmed that sterculic acid at 50 µg/ml was sufficient to inhibit the growth of the cells and that complete inhibition was obtained at a concentration of 100 µg/ml (Fig. 6.10).

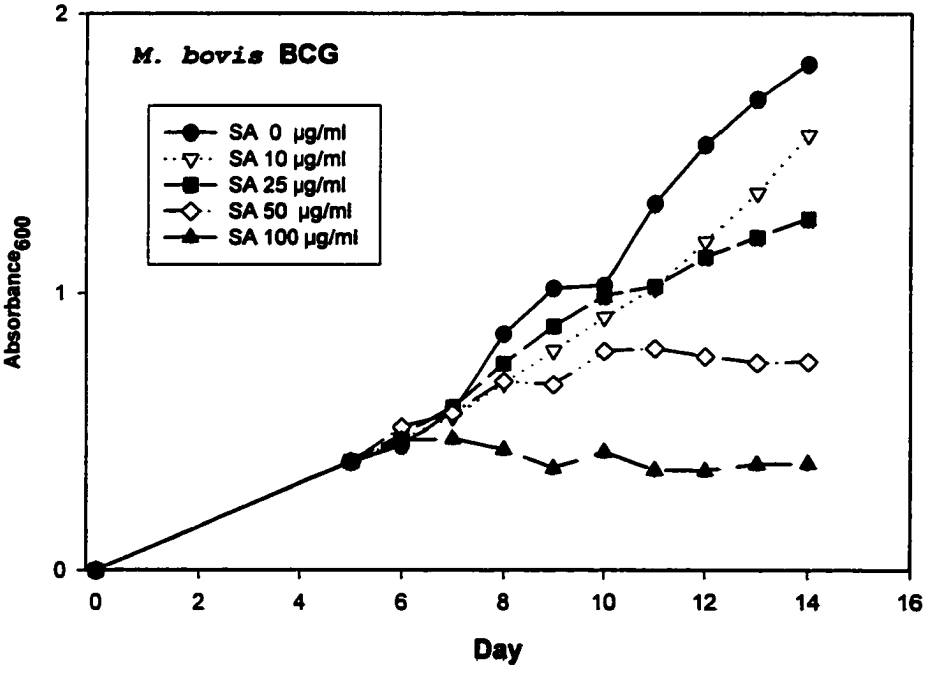


Figure 6.10 Inhibitory effects of sterculic acid on the growth rate of *M. bovis* BCG. The effects of sterculic acid on the growth rate of *M. bovis* BCG was determined by broth dilution technique.

To assess if sterculic acid was bacteriostatic or cidal, the treated cultures were plated on 7H11 agar at the end of the test. After incubation, there were no colonies on plates, which had been inoculated with the sterculic acid-treated cultures at concentrations of 50 or 100 µg/ml. This analysis demonstrated the bactericidal effect of sterculic acid and indicated that the Δ9 desaturase is a lethal target.

The inhibitory effect of sterculic acid on the Δ9 desaturase activity was further evaluated in intact cells of *M. bovis* BCG WT by GC analysis of the incorporation of [^{14}C]acetate into oleic acid. The GC chromatograms revealed a marked diminution in the synthesis of oleic acid in the presence of sterculic acid of 50 µg/ml (Fig. 6.11). Comparing FAME chromatograms of the sterculic acid-treated culture to that of ISO, it is notable that both sterculic acid and ISO exhibited the similarly inhibitory effect on the incorporation of [^{14}C]acetate into oleic acid. This analysis further demonstrated that ISO is a Δ9 desaturase inhibitor.

The effect of sterculic acid on oleic acid synthesis was further analyzed by argentation-TLC and compared to the effect of ISO (Fig. 6.12). In the presence of ISO at 1.0 µg/ml, the incorporation of [^{14}C]acetate into oleic acid decreased from 21.6% to 13.3% while oleic acid synthesis in sterculic acid-treated cells was completely shut down at 50 µg/ml of sterculic acid. This finding confirmed that ISO has a mechanism of action similar to that of sterculic acid by inhibiting Δ9 desaturase.

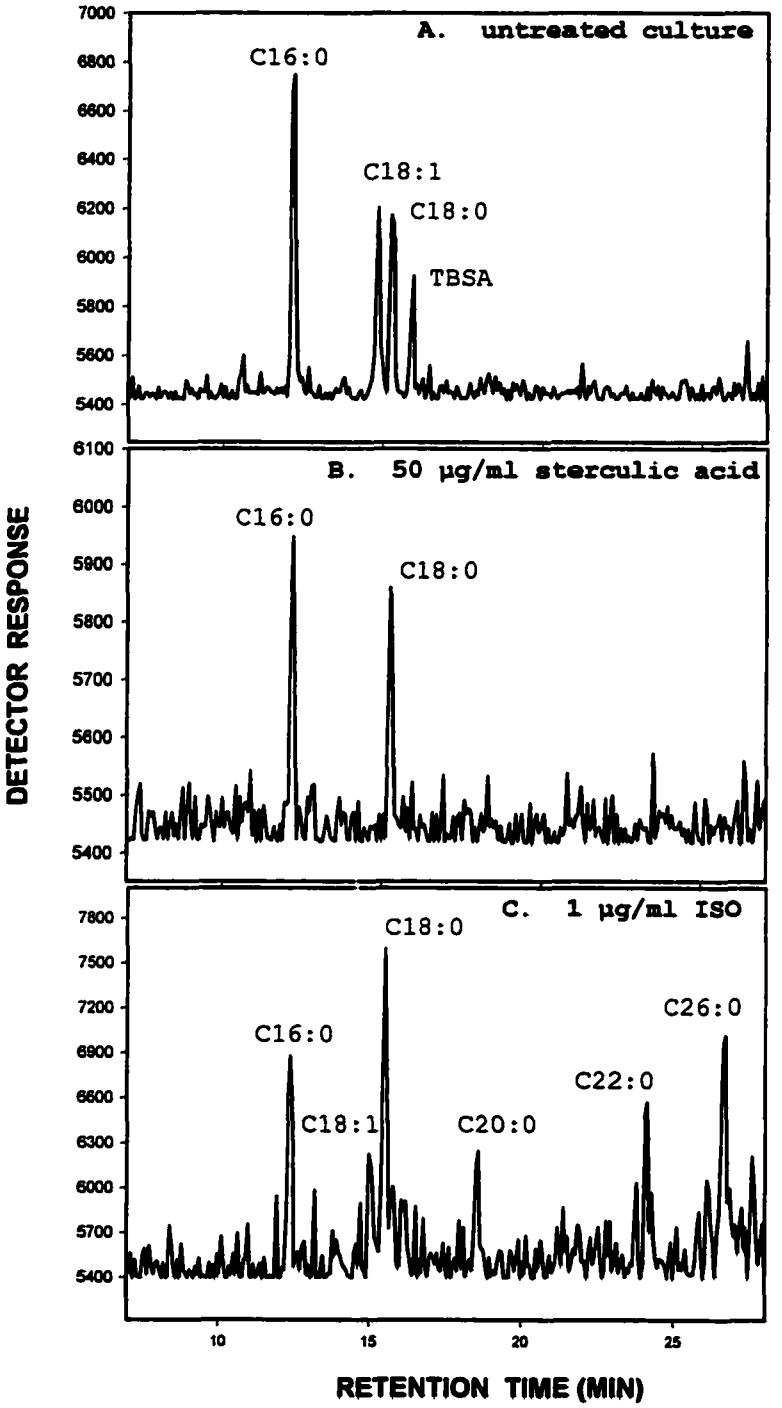


Figure 6.11 Radio-GC chromatograms of the effects of sterculic acid and ISO on the synthesis of oleic and tuberculostearic acids in *M. bovis* BCG. (A), untreated culture; (B), sterculic acid-treated culture (50.0 µg/ml); (C), ISO-treated culture (1.0 µg/ml).

6.3.7 Comparison of the effects of sterculic acid and ISO on mycolic acid synthesis

Fig. 6.12 shows the comparative effects of sterculic acid and ISO on the incorporation of [^{14}C]acetate into mycolic acids. The level of inhibition was measured through argentation-TLC and scintillation counting of the corresponding bands of α- and keto mycolates. Sterculic acid at a concentration of 50 µg/ml exhibited strong inhibition on oleic acid synthesis but had no effect on the synthesis of any kind of mycolic acids. In contrast, at 1 µg /ml ISO inhibited the synthesis of α- and ketomycolates by 64.0% and 27.15%, respectively.

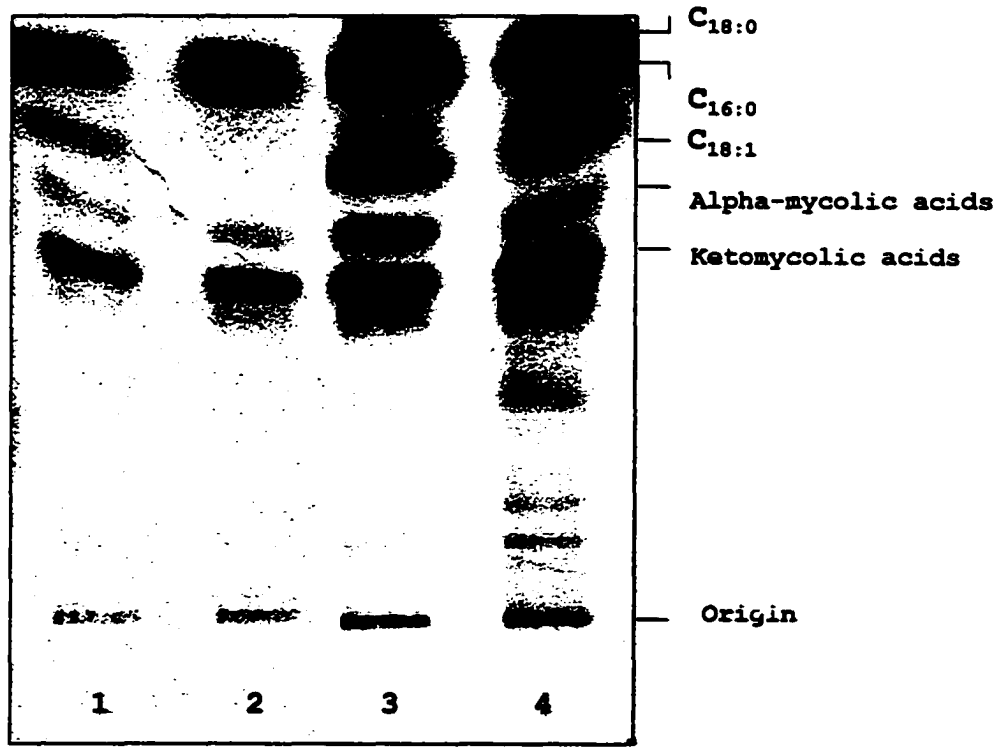


Figure 6.12 TLC autoradiographic comparison of the effects of sterculic acid and ISO on mycolic acid synthesis in *M. bovis* BCG. Lane 1, sterculic acid-untreated culture; Lane 2, sterculic acid-treated culture (50 µg/ml); Lane 3, ISO-untreated culture; Lane 4, ISO-treated culture (1.0 µg/ml).

6.4 DISCUSSION

Recent studies have shown that ISO has pleiotropic effects on mycobacterial cells, making it difficult to define its specific target(s) (Chapter 4; 5). However, ISO as well as sterculic acid have proved to be invaluable in identifying the unique mechanism, the inhibition of the synthesis of oleic acid and tuberculosteric acid, leading to the identification of the *M. tuberculosis* gene encoding the Δ9 desaturase.

ISO effectively inhibits the growth of drug-susceptible *M. tuberculosis* including selective drug-resistant strains of *M. tuberculosis*. However, a very high concentration (200 μg/ml) of ISO showed no effect on the growth of *M. smegmatis* mc^2155 (chapter 4). Therefore, the genetic approach to identify the molecular target(s) of ISO could not practically be performed in a rapidly growing, nonpathogenic host like *M. smegmatis* mc^2155. However, ISO possesses inhibitory effects on the growth rate and on the synthesis of oleic acid in *M. bovis* BCG similar to that of in *M. tuberculosis*. Therefore *M. bovis* BCG was used as a surrogate for *M. tuberculosis* in this study.

The availability of the complete sequence of the *M. tuberculosis* genome (Cole *et al.*, 1998) greatly facilitated the identification of the *M. tuberculosis* desaturase gene that specifically encodes Δ9 desaturase. There are three coding sequences that were predicted to encode desaturases, *desA1* (Rv0824c, 1,024 bp), *desA2* (Rv1094, 827 bp) and *desA3* (Rv3229c, 1,283 bp) in the H37Rv strain (Cole *et al.*, 1998). DesA1 has the

amino acid sequence highly homologous to that of DesA2, and the alignment (Fig. 6.5A) revealed a consensus iron binding motif containing two repeats of EX_2H separated by approximately 100 amino acids present in both DesA1 and DesA2 as in all other soluble desaturases (Fox *et al.*, 1994). DesA3 does not have this conserved motif and the alignment of the amino acid sequence of DesA3 with DesA1 and DesA2 resulted in a lesser degree of homology (Fig. 6.5B), suggesting at once that DesA3 may be of a class of desaturase unrelated to DesA1 and DesA2.

Overall, the membrane desaturases are distinguished by the presence of three conserved His tracks which are presumed to comprise the iron-binding active centers of the enzymes (Los and Murata, 1998). Examination of deduced amino acid sequences for the membrane desaturase from animals, fungi, insects, higher plants, and cyanobacteria has revealed three regions of conserved primary sequence containing $HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$, and $HX_{(2\text{ or }3)}HH$ (Shanklin *et al.*, 1994). Fig. 6.6 shows a comparison of three regions of His conserved motifs of membrane desaturases in *Saccharomyces cervisiae, Mus musculus, Rattus norvegicus,* Cyanobacteria and *M. tuberculosis*. These three conserved regions were designated as Ia, Ib and II according to Shanklin *et al.* (1994). In region Ia (Fig. 6.6), all Δ9 desaturases contain the sequence Hx_4H, while Cyanobacterial membrane desaturase and *M. tuberculosis* DesA3 contain the sequence HX_3H. In region Ib, the representatives of Δ9 membrane desaturases contain the sequence HX_2HH, while the Δ6 desaturase from the cyanobacterium

Synechocystis spp and *M. tuberculosis* DesA3 have HX_3HH. In region II, the same representative Δ9 desaturases have the second occurrence of HX_2HH, while the *Synechocystis spp* Δ6 desaturase has a second occurrence of HX_3HH which is the same as that of DesA3 from *M. tuberculosis* (Fig 6.6). Accordingly, DesA3 has the amino acid sequence highly homologous to the Δ6 desaturase of *Synechocystis spp* (Table 6.1). The presence of the His conserved motifs suggests that DesA3 is a membrane desaturase. This conclusion is consistent with the result from immunoblotting (Fig. 6.7A) and hydropathy analysis (Fig 6.7B) demonstrating that DesA3 is a membrane-associated protein.

To assess that the *desA3* gene encodes a target of ISO, the microplate Alamar Blue assay was used to determine the MIC of ISO for the *M. bovis* BCG WT and *M. bovis* BCG overexpressing the *desA3* gene. Alamar Blue is an indicator of oxidation-reduction capability (Yajko *et al.*, 1995) in which the color change from blue to pink is indicative of sufficient bacterial metabolism and growth after a period of incubation. For *M. bovis* BCG, the length of incubation that is needed for sufficient metabolic activity is 3 days. At this point, the blue color in the culture controls turned pink indicating that sufficient metabolic activity had occurred to allow the tests to be read. This colorimetric method is rapid, quantitative and shows good agreement with the results obtained with the broth dilution technique (Yaiko *et al.*, 1995). By Alamar Blue assay, it was demonstrated that the increased copy number of the ISO target obtained by overproducing DesA3 led to an

increased MIC of ISO. This result indicates that the *desA3* encodes a target of ISO.

The results with sterculic acid confirmed that the mode of action of ISO is to inhibit the Δ9 desaturase, and thus ISO is an inhibitor of Δ9 desaturation of stearic to oleic acid. This work again demonstrates that ISO not only inhibits oleic acid synthesis but also mycolic acid synthesis (Fig. 6.12). However, sterculic acid singularly affects only oleic acid synthesis. Thus the effect of ISO on mycolic acid synthesis is not a consequence of the inhibition of oleic acid synthesis. Although sterculic acid depleted the oleic acid pool in sterculic acid-treated culture, cells still synthesized all types of mycolic acids (Fig. 6.12). This result suggests that the process of mycolic acid synthesis is not linked to the synthesis of oleic acid, and therefore ISO has more drug targets, presumably enzymes involved in mycolic acid synthesis.

In summary, this is the first report demonstrating that the *M. tuberculosis desA3* gene encodes the Δ9 desaturase, a membrane-associated enzyme catalyzing oleic acid synthesis. The Δ9 desaturase activity can be inhibited by ISO and sterculic acid. Comparative effects of ISO and sterculic acid leads to the conclusion that the synthesis of oleic and mycolic acid synthesis is not linked, and ISO has more than one enzymatic target.

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CHAPTER 7

Development of *In Vitro* Assays for the Target Enzymes of Thiocarlide/Isoxyl®: Δ9 Desaturase, Fatty Acid Synthase, and Mycolic Acid Synthase

7.1 INTRODUCTION

Previous analysis provided the evidence that isoxyl (ISO) like sterculic acid acts as an inhibitor of Δ9 desaturase and therefore blocking the synthesis of oleic acid results in the accumulation of stearic acid in *M. tuberculosis* and *M. bovis* BCG.

Fatty acid desaturases are enzymes that introduce double bonds into saturated fatty acid chains. They are present in many groups of organisms such as bacteria, fungi, plants and animals, and play key roles in the maintenance of the proper structure and function of biological membrane (Los and Murata, 1998). The desaturation of stearic acid to oleic acid (the Δ9 derivative of octadecanoic acid) by a particulate enzyme has been well-characterized in yeast (Bloomfield and Bloch, 1960) and rat liver (Enoch *et al.*, 1976). The Δ9 desaturase system of eukaryotes requires molecular oxygen and a reduced pyridine nucleotide for its activity (Bloomfield and Bloch, 1960). The study in *M. phlei* revealed that stearic acid is directly converted to oleic acid under aerobic conditions by Δ9 desaturase, a particulate enzyme that introduces a double bond between carbon 9 and 10 of stearic acid (Fulco and Bloch, 1964). A Δ9 desaturation product, oleic acid is important for membrane integrity and function, and is a

precursor of tuberculostearic acid (D10-methyl-branched stearic acid) biosynthesis (Lennarz *et al.*, 1962). Previous studies reported that the Δ9 desaturase in a cell free preparation from *M. phlei* requires ferrous ion (Fe^{++}) and a flavin, in addition to a reduced nicotinamide nucleotide and molecular oxygen as the electron donor and oxidant, respectively (Fulco and Bloch, 1962; 1964; Kashiwabara *et al.*, 1975). The enzyme also has an ability to convert palmitoyl-CoA into its Δ9 derivative (Δ9-hexadecanoic acid) but to a lesser extent (Fulco and Bloch, 1964). Subsequent investigation demonstrated that in *M. phlei* the Δ9 desaturase is sensitive to cyanide indicating a link of Δ9 desaturation to NADPH via an FAD-requiring NADPH-cytochrome *c* reductase (Kashiwabara and Sato, 1973). Up to the present, there has been no literature addressing the formation of Δ9-unsaturated fatty acid and the enzymatic activity of the Δ9 desaturase in *M. tuberculosis* or *M. bovis* BCG. It was decided to define the Δ9 desaturation system in *M. tuberculosis* and *M. bovis* BCG, and apply the cell free system to demonstrate the mode of action of ISO and the function of the *M. tuberculosis* DesA3. In addition, it was previously shown that ISO probably has more than one enzymatic target. Therefore, in parallel, *in vitro* assays for fatty acid synthase (FAS) and mycolic acid synthase (MAS) were conducted to determine the effects of ISO on the activities of those enzymes.

7.2 MATERIALS AND METHODS

7.2.1 Development of the *in vitro* Δ9 desaturase assay

Bacterial strains and growth conditions. *M. bovis* BCG WT and the recombinant overexpressing the *desA3* gene were maintained and aerobically grown in Sauton's medium. Incubation was carried out at 37°C with gentle shaking to an A_{600} of 0.400. For the recombinant strain, the recombinant DNA (pVV16desA3) was maintained in an expression host (*M. bovis* BCG) by adding 20 µg/ml kanamycin into Sauton's medium. Ferrous sulfate ($FeSO_4$) was added to supplement the medium at a final concentration of 20 mg/L (Kashiwabara *et al.*, 1975).

Cell fraction preparation. Cell fraction was prepared according to Fulco and Bloch (1964) with minor modifications. In brief, cells were harvested by centrifugation and washed once with 0.9% sodium saline at 4°C. The whole cell pellets then were resuspended (200 mg per ml) in 0.25 M sucrose (100 mg/ml wet weight/ml). This suspension was pulsed-disrupted by Sanyo ultra-sonication (Soniprep 150; Sussex, UK) for a total time of 20 min in 60-s on, 90-s off, 20 times on ice. The whole sonicate was centrifuged at 27,000 x g for 30 min at 4°C to remove unbroken cells, cell wall and cell debris. The resulting supernatant fraction containing crude cell lysate was quantitated for protein concentration by a bicinchoninic acid (BCA) protein kit (Pierce; Rockford, IL) (Smith *et al.*, 1985) prior to being stored at -70°C until used. Similarly, the sonicated extract of *M. bovis* BCG expressing DesA3 was prepared as described above.

Reconstitution of the *in vitro* Δ9 desaturation reaction. The reconstitution of the *in vitro* Δ9 desaturation was adapted from Fulco and Bloch (1964). All aerobic incubations were performed in reaction tubes with continuous shaking. The Δ9 desaturase was assayed for activity in a standard mixture (final volume, 1 ml) containing 1 µmole NADPH (Sigma; St. Louis, MO) in 0.1 M potassium phosphate buffer (pH 7.2)(795 µl), and crude cell lysate (2.0 mg protein; 200 µl) of *M. bovis* BCG WT strain. The mixtures were preincubated at 37°C for 10 min prior to the addition of 25 nmole (0.25 µCi, 5 µl) of [1-^{14}C]stearoyl-CoA (10 µCi/µmol in 0.01 M 1:1 NaOAc:EtOH)(American Radiolabeled Chemicals, Inc.; St. Louis, MO). In one reaction the NADPH was omitted to determine the effect of NADPH on Δ9 desaturase activity. The mixtures were further incubated at 37° for 15, 30 or 60 min. After incubation, 2 ml of 15% tetrabutylammonium hydroxide was added to terminate the reactions. The saponification was performed at 100°C overnight followed by methylation of saponified fatty acids as previously described (chapter 4). FAMEs were extracted by diethylether and dried under a stream of nitrogen. The dried materials containing FAMEs were finally dissolved in 200 µl of dichloromethane.

Analysis of the *in vitro* Δ9 desaturase activity. The Δ9 desaturase activity was determined by measuring the incorporation of radiolabel from [1-^{14}C]stearoyl-CoA into the oleic acid derivative. Separation and quantitation of the labeled substrate and product from the Δ9 desaturation reaction were conducted

either by argentation-TLC or GC. For TLC analysis, an aliquot of 20 µl from the final extracts was applied on argentation TLC plates previously described (Morris, 1966; chapter 5). The TLC plates were developed to fractionate FAMEs in the solvent containing diethylether and acetone (90:10). Separated radioactive spots on TLC plates were located by either a Bioscan System 200 Imaging Scanner (Bioscan Inc; Washington, D.C.) or autoradiography. The product of Δ9 desaturation which is [^{14}C]-labeled oleic acid was identified by a commercial standard, methyl ester of oleic acid. The incorporation of [^{14}C] into individual bands of FAMEs was determined by scintillation counting (Beckman; Fullerton, CA). The activity of Δ9 desaturase was expressed as the percent calculated from the ratio of CPM in the newly formed oleic acid to the total CPM recovered from the entire fatty acids. The analysis of desaturase activity by GC was performed as previously described.

7.2.2 Inhibitory effect of ISO on the Δ9 desaturase activity *in vitro*.

ISO was prepared in a stock solution of 50% DMSO and 1% Tween 20 (polyoxyethylene-sorbitol monolaurate). The reaction mixture contained the reconstituted components as described earlier. In ISO treated reactions an aliquot of 20 µl from each stock dilution was added into the reaction mixtures to achieve a final concentration of ISO at 0.1 and 1.0 µg/ml. In the control reaction 20 µl of 50% DMSO and 1% Tween 20 was added to obtain

final concentrations of DMSO and Tween 20 at 0.1 and 1.0%, respectively. ISO was preincubated with the reaction mixtures at 37°C for 20 min with continuous shaking, and then the Δ9 desaturation reaction was started by the addition of 0.25 μCi of [1-^{14}C]stearoyl-CoA. The reaction was further incubated at 37°C for 30 min before being stopped by the addition of 15% tetrabutylammonium hydroxide. Fatty acid extraction, derivatization and analysis of methyl ester stearoate and oleate were conducted as previously described.

7.2.3 Substrate specificity of Δ9 desaturation

7.2.3.1 Acyl chain length specificity

[1-^{14}C]Palmitoyl-CoA (850 μCi/μmol in ethanol) (Dupont NEN; Boston, Mass.) was added in amount of 0.25 μCi into the reconstituted mixture instead of [1-^{14}C]stearoyl-CoA. The incubation was performed under standard conditions as described above. The reaction was stopped, and fatty acids were saponified, methylated and extracted. Argentation-TLC was applied to analyze the Δ9-monounsatuarated derivative produced from the desaturation reaction.

7.2.3.2 Purification of ACP for stearoyl-ACP preparation

Bacterial strains, plasmids and growth conditions. The *E. coli* expression plasmid used in this study was pET26b (*E. coli* expression vector, T7 promoter, Kan^r)(Novagen; Madison, WI). *E. coli* strain XL-1 Blue (Stratagene; La Jolla, CA) was used as a

standard cloning host, and strain BL21 (λDE3)(Novagen) which contains the gene encoding T7 polymerase under the control of the IPTG-inducible lac promoter (Studier, 1990) was used as an expression host in T7 expression experiments. The *E. coli* transformants were grown in Luria-Bertani (LB)(Gibco-BRL; Rockville, MD) broth and agar media at 37°C. The antibiotic kanamycin (Kan) (Sigma; St. Louis, MO) was added to the medium at a concentration of 25 μg/ml.

Polymerase chain reaction (PCR) amplification. PCR amplification was performed with *M. tuberculosis* H37Rv as template DNA in a final volume of 50 μl containing dNTPs (200 μM each), primers (1.0 μg each), 2 U of Vent DNA polymerase (New England Biolabs; Beverly, MA), and 10% DMSO. Amplification was carried out in a Perkin Elmer GeneAmp PCR system 2400 thermal cycle (Norwalk, CT) under thermal cycle temperature of 94°C 5 min for denaturation, 35 cycles of 94°C 1 min, 60°C 20 s for annealing, 72°C 30 s for extension, and the last final extension at 72°C for 10 min.

General cloning procedures. Standard molecular and recombinant DNA techniques were performed according to Sambrook *et al.* (1989) and manufacturer's recommendations (Qiagen; Santa Clarita, CA). Small quantities of plasmid DNA were isolated from *E. coli* stain XL-1 Blue with a spin prep kit (Qiagen) while larger scale preparations were purified with a large scale plasmid kit (Qiagen). DNA was digested with restriction enzymes (New England Biolabs) according to the manufacturer's instructions. Ligation was performed using T4 DNA ligase (Gibco-BRL). DNA fragments were

purified from agarose gels with a Qiagen gel extraction kit (Qiagen). *E. coli* strains were transformed by the standard $CaCl_2$ method.

Construction of a recombinant plasmid for *acpM* expression in *E. coli*. The *acpM* gene was amplified by PCR using the primers: [5′ GAAGCATCATATGCCAGTCACTCAGGAAGA 3′] (sense) and [5′ GGCTGAAGCTTTTGGACTCGGCCTCAAG 3′] (antisense). The underlined sequences indicate the *Nde*I and *Hind*III restriction sites introduced into the sense and antisense primers, respectively. The 350-bp PCR product encoding AcpM was digested by *NdeI* and *Hind*III prior to cloning into *NdeI-Hind*III-digested pET26b, an *E. coli*-His-tag cloning vector. The resulting plasmid recombinant that has a hexa-His-tag domain fused in-frame with AcpM at its C-terminal was designated pETAcpM (pET26b carrying a 350-bp fragment of *acpM*) and allowed the overproduction of a C-terminal His-tagged AcpM.

DNA sequence analysis. Double-stranded plasmid constructs were sequenced by Macromolecular Resource Facilities (Colorado State University). Primers for sequencing were standard forward and reverse primers specific for pET26b.

Expression and purification of ACP. The recombinant plasmid extracted from *E. coli* XL-1 Blue (a cloning host) was transformed into *E. coli* strain BL21 (λDE3) for expression. A 3 ml inoculum in LB-Kan media was prepared from plates with fresh colonies of *E. coli* BL21 (DE3) cells transformed with pETAcpM. One-liter cultures were inoculated with 1 ml of the overnight culture and

then grown at 37°C to an A_{600} of 0.6. The induction of expression was conducted by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. The cells were incubated for an additional 12 h at 30°C after which they were harvested by centrifugation (8,000 x g for 20 min). The harvested cells were lysed by sonication in 50 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The sonicate was then spun at 27,000 x g for 30 min. The AcpM protein was fractionated and recovered in the supernatant. This soluble fraction was then applied directly to a metal (Ni^{2+}) chelation agarose column (1 x 5 ml) (Novagen Hisbind Resin) previously equilibrated with 20 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole (van Dyke *et al.*, 1992). The column was washed with 10 volumes of equilibration buffer, followed by 10 volumes of washing buffer (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 60 mM imidazole). Finally, the protein was eluted in 20 mM Tris-HCl (pH 7.9) containing 0.25 M NaCl and 500 mM imidazole (Novagen buffer kit). The purified protein was dialysed against 50 mM Tris-HCl (pH 7.5). The dialysis fraction was resolved by polyacrylamide electrophoresis on a 0.75 mm thick gel containing a 6% stacking over a 18% resolving SDS- and native polyacrylamide gels (Rock and Cronan, 1981). Gels were run with a constant current at 150 volt for 1 h. The proteins were visualized by Coomassie Brillant Blue R-250 staining (Bio-Rad)(Chen *et al.*, 1993). The purified protein was stored at -20°C until it will be coupled to stearic acid to form stearoyl-ACP,

which will then be used to further characterize substrate specificity for the Δ9 desaturase.

7.2.4 Effect of ISO on the *in vitro* activities of fatty acid synthase (FAS) and mycolic acid synthase (MAS)

Mycobacterial strain and growth condition. *M. aurum* A+ (from GlaxoWellcome, Stevenage, UK) was grown in 4-liter Fernbach flasks containing 2 liters of nutrient broth plus 0.05% Tween 80. Cells were grown to the mid exponential phase, then harvested by centrifugation, washed with 0.9% normal saline, and stored at -70°C until used. Each flask yielded about 2 g (wet weight) of cell pellets.

Preparation of soluble cytosolic and particulate cell wall fraction. *M. aurum* A+ (30 g wet weight) was resuspended in buffer (30 ml) containing 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$ and 2 mM phenylmethyl-sulfonyl fluoride at 4°C. This suspension was subjected to probe sonication on ice by Sanyo ultra-sonication (Soniprep 150) for 20 min of 60-s pulses with 90-s cooling interval between pulses. Unbroken cells, cell wall fractions and cells debris were removed by centrifugation at 27,000 x g for 30 min at 4°C. The resulting crude cell lysate was centrifuged at 200,000 x g for 1 h with a Beckman Ultracentrifuge and a Ti70.1 Beckman rotor (Beckman). The soluble pale yellowish cytosolic fraction was recovered and quantitated for protein concentration by the bicinchoninic acid method (BCA) (Pierce; Rockford, IL) using bovine serum albumin

(BSA) (Pierce) as standard protein. The resulting cytosol containing FAS I and FAS II was immediately used in assay for FAS activities.

The particulate (P60) cell wall enzyme fraction with mycolate-synthesizing (MAS) activity was prepared as described previously (Wheeler *et al.*, 1993; Mikusova *et al.*, 1996). Briefly, the disrupted cells derived from probe sonication of 10 g wet weight cell pellets were centrifuged at 27,000 x g for 12 min at 4°C. The resulting cell wall-containing pellets were then resuspended in buffer A containing 50 mM MOPS (adjusted to pH 8.0 with KOH), 5 mM 2-mercaptoethanol, and 10 mM $MgCl_2$. A final volume of 20 ml of suspension was divided between two centrifuge tubes. Percoll solution (Pharmacia, Sweden) was added to each tube to achieve 60% suspension, and the mixture was centrifuged at 27,000 x g for 60 min at 4°C. The particulate, upper, diffuse, cell wall-containing band was collected and washed three times in buffer A. Finally, the cell wall fraction was resuspended in 5 ml of buffer A. Protein concentration was determined by BCA prior to using the Percoll-60, enzymatically-active cell wall fraction in a mycolic acid synthesizing activity assay.

Reconstitution of the *in vitro* FAS I and FAS II reactions. The standard reaction mixtures for the incorporation of [^{14}C] from [2-^{14}C]malonyl-CoA into C_{16} to C_{24} fatty acids catalyzed by FAS I were performed as previously described (Bloch, 1975; 1977; Slayden *et al.*, 1996). The reaction mixtures contained 100 mM potassium phosphate, 5 mM EDTA, 5 mM dithiothreitol, 300 µM acetyl-CoA, 100

μM NADPH, 1 μM flavin mononucleotide, 500 μM α-cyclodextrin, 20 μM malonyl-CoA, 100,000 cpm of [2-^{14}C]malonyl-CoA, and 100 μl of the cytosolic enzyme preparation (1 to 2 mg of protein) in a total volume of 500 μl. Similarly, the standard reaction mixtures for the activity of FAS II which catalyzes the incorporation of isotope from [2-^{14}C]malonyl-CoA into C_{24} to C_{30} fatty acids were as follows: 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM dithiothreitol, 100 mM palmitoyl-CoA, 140 μM NADPH, 140 μM NADH, 180 μg of ACP, 40 μM malonyl-CoA, 200,000 cpm of [2-^{14}C]malonyl-CoA, and 100 μl of cytosolic preparation (1 to 2 mg of protein) in a total volume of 500 μl. In ISO-treated reactions of both FAS I and FAS II activities, ISO at a final concentration of 500 μg/ml was preincubated with other components in the reaction mixtures prior to the addition of [2-^{14}C]malonyl-CoA. The reactions of FAS I and FAS II with and without ISO were performed in triplicate and incubated at 37°C for 30 min. To stop the reactions, a 500 μl of 20% potassium hydroxide in 50% methanol was added and the mixture was then incubated at 100°C for 30 min, followed by the acidification with 300 μl of 6 M HCl. The resulting [^{14}C]-labeled acids were extracted three times with diethylether. The organic extracts were washed once with an equal volume of water, then dried under airflow in glass tubes. The recovered residues of FAMEs were resuspended in 500 μl of dichrolomethane prior to scintillation counting.

Reconstitution of the *in vitro* MAS reaction. The reaction for MAS activity was reconstituted using the mycolate-synthesizing P60

cell wall fraction as previously described (Wheeler *et al.*, 1993). The reaction mixture was as follows: 50 mM potassium phosphate (pH 6.0), 10 mM $KHCO_3$, 1 µCi of [1,2-^{14}C]acetate (110 mCi/mmol), and 440 µl of the P60 preparation (2.0 to 3.5 mg of protein) in a total volume of 1 ml. ISO was added to the reaction mixture to achieve a final concentration of 2, 20, 50, and 100 µg/ml prior to the addition of [1,2-^{14}C]acetate. Reaction mixtures, which were prepared in duplicate at each concentration of ISO, were incubated at 37°C for 1 h. Following the addition of 2 ml 15% tetrabutylammonium hydroxide, fatty acids and mycolic acids were saponified, and derivatized. The resulting FAMEs and MAMEs were separated by TLC and counted by scintillation counting as described earlier.

7.3. RESULTS

7.3.1 Cell free system for the Δ9 desaturase activity

The supernatant after 27,000 x g centrifugation of sonicated *M. bovis* BCG was shown to have the ability to convert stearoyl-CoA to a monounsaturated fatty acid in the presence of atmospheric oxygen and NADPH. The reaction conditions were optimized to obtain a sole product identified as oleic acid (Fig. 7.1; 7.2) demonstrating the existence of Δ9 desaturation in *M. bovis* BCG.

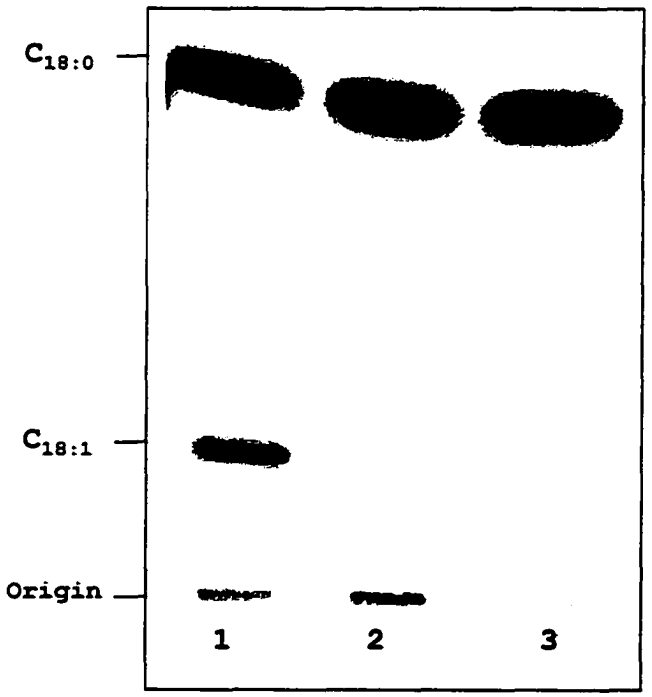


Figure 7.1 TLC autoradiogram of fatty acids extracted from the *in vitro* Δ9 desaturation reactions. FAMEs derived from saponification and methylation of fatty acids were resolved on argentation-TLC using petroleum ether-acetone (90:10). Autoradiogram was produced by exposing TLC plate to Kodax X-Omat AR film. Lane 1, FAMEs from a reaction containing active enzyme; Lane 2, from a reaction containing inactive enzyme; Lane 3, from the reaction with no NADPH.

It is known that this type of desaturase in *M. phlei* requires exogenous ferrous ions and NADPH as enzyme cofactors (Fulco and Bloch, 1964, Kashiwabara *et al.*, 1975). The specific requirement for NADPH for the Δ9 desaturase activity reported previously was confirmed in this present study. As can be seen, a reconstituted mixture of the Δ9 desaturase system with a depletion of NADPH showed no detectable level of labeled oleic acid (Fig. 7.1).

In order to define the optimal incubation time for Δ9 desaturation, the time course of the incorporation of isotope from [1-^{14}C]stearoyl-CoA into oleic acid was studied. Crude cell lysate of *M. bovis* BCG was incubated with 1 μmole NADPH in 0.1 M potassium phosphate buffer in the presence of [1-^{14}C]stearoyl-CoA. The reaction mixtures were incubated at 37°C with shaking for 15, 30 and 60 min. TLC analysis showed that the incorporation of [^{14}C] into the oleic acid increased over the increment of the incubation time. The incorporation of [^{14}C] into oleic acid at 15, 30 and 60 min of incubation was 1,483, 2,085, and 2,176 CPM, respectively. Based on this result, the incubation time for the *in vitro* mycobacterial Δ9 desaturase was set at 30 min and applied to all *in vitro* assays. It must be noted that the enzyme activity is stable as previously reported (Fulco and Bloch, 1964), and therefore the enzyme extract prepared from *M. bovis* BCG could be stored in the frozen state until used.

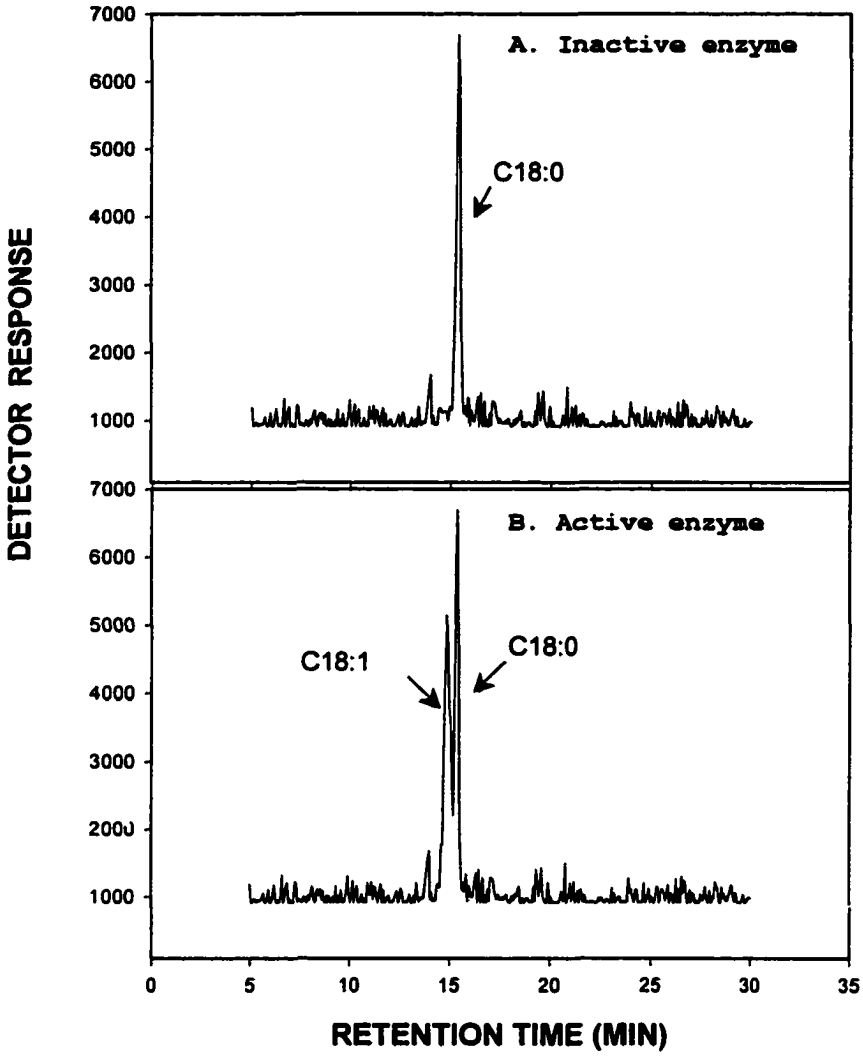


Figure 7.2 Radio-GC chromatograms of fatty acid methyl esters from the *in vitro* desaturation reactions. The resulting FAMES were analyzed by GC conducted on a capillary HP-1 column coupled to the Lablogic GC-RAM radiodetector. Radio-GC chromatograms of fatty acids recovered from the reaction with alkaline inactivated enzyme (A); fatty acids from the desaturation reaction containing an active enzyme fraction (B). Identities of peaks were determined by comparing their retention times with those of authentic standards.

7.3.2 Selectivity of ISO inhibition on the Δ9 desaturase activity

The decreased synthesis of oleic acid by ISO in the whole-cell labeling experiment was further assessed by measuring the

formation of [^{14}C]-labeled oleic acid in the cell free system. Crude cell lysate of *M. bovis* BCG WT strain was used as an enzyme source for Δ9 desaturase activity. The entire fatty acids produced from the reactions were saponified, and derivatized to FAMEs which were then analyzed by argentation-TLC. The radiolabeled-oleic acid was determined by radioscanning as well as scintillation counting. TLC analysis revealed that ISO inhibited the incorporation of isotope from [1-^{14}C]stearoyl-CoA into oleic acid in the cell free system assay (Fig. 7.3). This result suggested that the observed inhibition of oleic acid synthesis by ISO during the whole-cell labeling with [1,2-^{14}C]acetate is due to the selective inhibition of ISO on Δ9 desaturase activity. The enzyme activity was very sensitive to ISO even when the concentration of ISO was as low as 0.1 μg/ml. At this low concentration, ISO inhibited the incorporation of isotope into oleic acid through its effect on Δ9 desaturase by about 7%. There was a 61% decrease in the formation of oleic acid at a concentration of 1.0 μg/ml (Fig. 7.3). In another independent experiment, ISO at 1.0 μg/ml and 0.1 μg/ml showed about 60% and 1% reduction of oleic acid synthesis, respectively (Table 7.1). Efforts have been made to increase the concentration of ISO in the reaction mixture to more than 1.0 μg/ml but the insolubility problem of the drug limited this trial. However, the cell free system of Δ9 desaturase activity clearly showed that the inhibitory effect of ISO on oleic acid synthesis is through the inhibition of Δ9 desaturase activity.

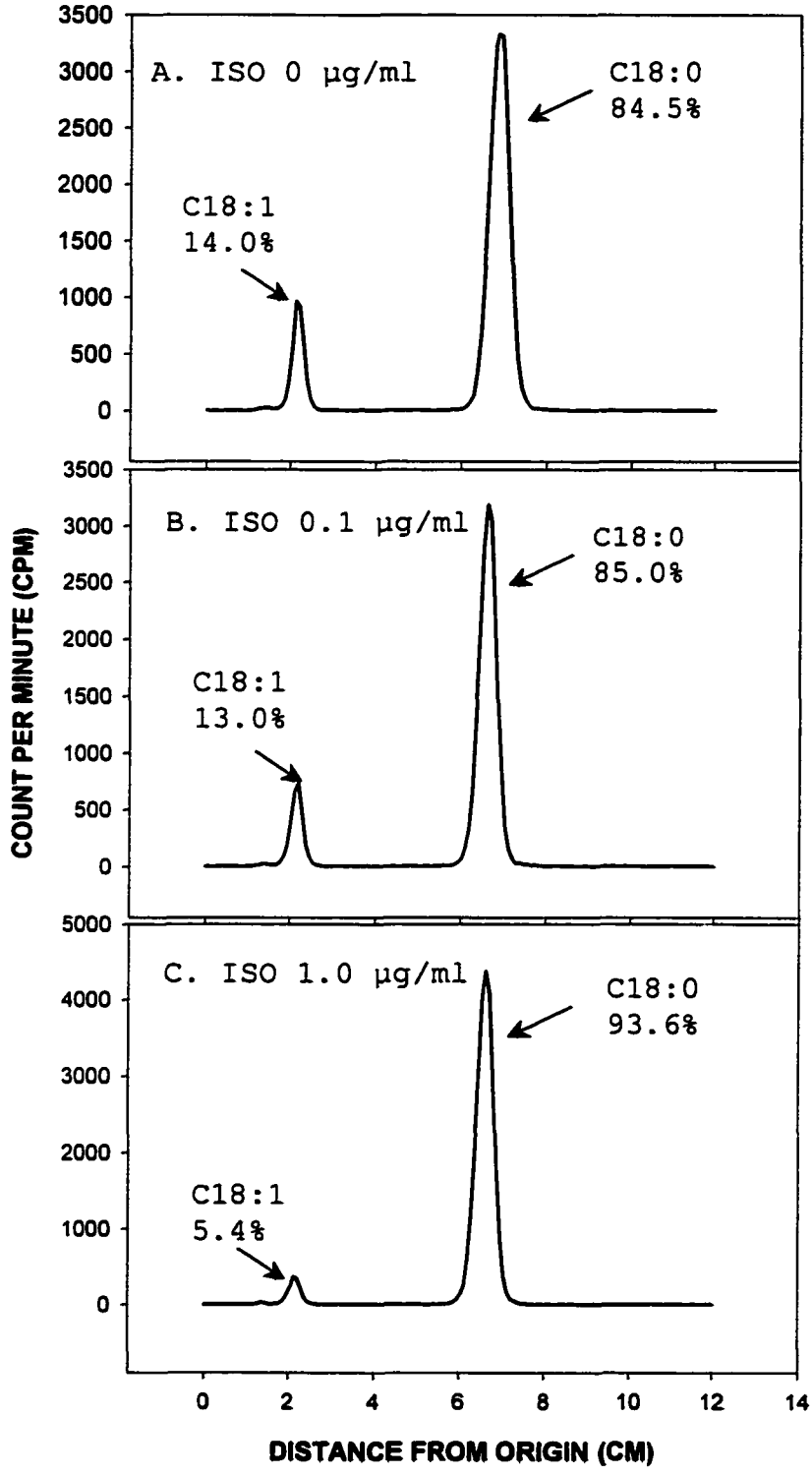


Figure 7.3 Inhibitory effect of ISO on the *in vitro* Δ9 desaturase activity. Extracted FAMEs were separated by argentation-TLC and then scanned by BioScan. Scanning profiles of FAMEs extracted from ISO-untreated reaction (A); 0.1 µg/ml ISO-treated reaction (B); 1.0 µg/ml ISO-treated reaction (C). The percent inhibition of oleic acid synthesis by ISO was determined from the ratio of the radiolabel of methyl ester of oleic acid and total FAMEs in each reaction.

Table 7.1 Analysis of the effect of ISO on the incorporation of isotope from [1-^{14}C]stearoyl-CoA into oleic acid of *M. bovis* BCG in a cell free system.

ISO (µg/ml)	Count per minute (CPM)[a]					
	$C_{18:0}$	$C_{18:1}$	Total	$C_{18:1}$/Total	%synthesis	%inhibition
0	47526.9	10534.8	58061.7	0.18	100	0
0.1	49918.9	10076.7	59995.6	0.16	98.89	1.11
1.0	51492.9	3825.6	53318.5	0.06	40.71	59.28

[a] The inhibitory effect of ISO on the Δ9 desaturase was determined based on the decreased incorporation of isotope from $C_{18:0}$ into $C_{18:1}$ in the *in vitro* desaturation assay. The reaction mixture for the formation of $C_{18:1}$ Δ9 was the standard one described in "Materials and Methods" and contained 200 µl of crude cell lysate (2.0 µg protein) from *M. bovis* BCG; 0.25 µCi of [1-^{14}C]stearoyl-CoA, 1 µmole NADPH in 0.1 M potassium phosphate to a final volume of 1 ml in the presence and absence of ISO at the indicated concentrations. Quantitation of Δ9 desaturase activity was determined by scintillation counting of the bands corresponding to labeled derivatives of stearic and oleic acids.

7.3.3 *In vitro* effect of the overexpression of the *M. tuberculosis desA3* gene on oleic acid synthesis

Crude cell lysate from *M. bovis* BCG overexpressing the *desA3* gene was assayed in the cell free system for Δ9 desaturase activity to determine whether the overexpression of the *desA3* gene affects the oleic acid synthesis. The control reaction contained the same amounts of protein lysate (2 µg) from the *M. bovis* BCG containing pVV16 (control vector). Radiolabeled-oleic acid produced from the *in vitro* Δ9 desaturation reaction was analyzed by argentation-TLC. The autoradiogaphic TLC of the extracted FAMEs showed an increase in labeled-oleic acid in the reaction

mixture containing protein lysate from the *desA3*-overexpression strain (Fig. 7.4) suggesting a higher level of the Δ9 desaturase activity where the *desA3* is overexpressed. The percent of the radiolabel of oleic acid increased from 14.33 to 18.13% in this sample. This evidence indicated that the *M. tuberculosis desA3* gene encodes the Δ9 desaturase involved in the synthesis of oleic acid from stearoyl-CoA.

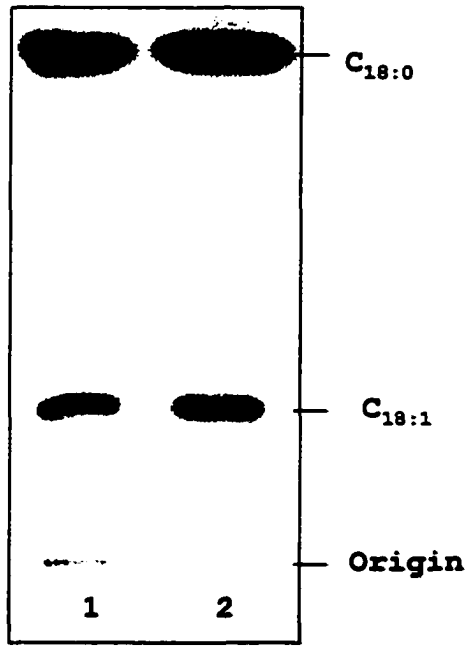


Figure 7.4 TLC-autoradiogram of the effect of the overexpression of the *M. tuberculosis desA3* gene on the activity of the Δ9 desaturase. The Δ9 desaturase activity from *M. bovis* BCG transformed with pVV16 (vector control) (lane 1) was assayed and compared with that of the recombinant *M. bovis* BCG/pVV16desA3 (pVV16 containing the *M. tuberculosis desA3* gene expressed from the hsp_{60} promoter) (lane 2) by cell free reactions. The assay conditions were given in the text. The Δ9 desaturase activity was determined from the product of the Δ9 desaturation analyzed by argentation-TLC and quantitated by scintillation counting.

7.3.4 Substrate specificity of the Δ9 desaturase

The activity of the Δ9 desaturation for other acyl-CoAs such as palmitoyl-CoA was examined. The Δ9 desaturase activity towards palmitoyl-CoA was obvious as observed by argentation-TLC (Fig. 7.5). From this result, both stearoyl-CoA and palmitoyl-CoA were desaturated by the cell free supernatant fraction of *M. bovis* BCG in the presence of NADPH and atmospheric oxygen indicating the non-specific chain length of acyl chain as a substrate for the Δ9 desaturase.

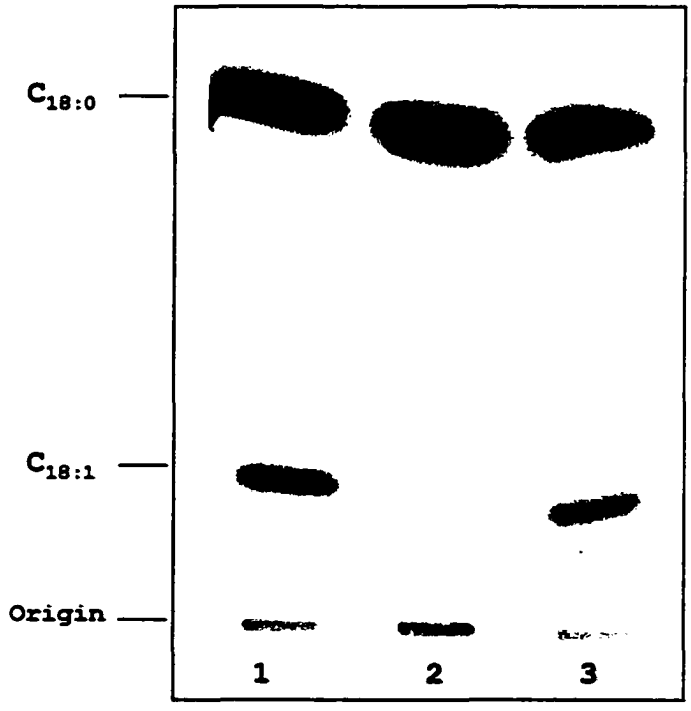


Figure 7.5 Argentation-TLC of fatty acids recovered from the desaturation reaction with [1-^{14}C]palmitoyl-CoA as a substrate. The standard reaction mixture for Δ9 desaturation was reconstituted as described. Fatty acids from the reaction were saponified and methylated prior to TLC chromatographic analysis. The TLC plates were developed twice in petroleum ether-acetone (90:10). TLC autoradiogram of fatty acids recovered from the reaction using [1-^{14}C]stearoyl-CoA (lane 1); fatty acids from the desaturation reaction without [1-^{14}C] stearoyl-CoA (lane 2); fatty acids from the desaturation reaction with [1-^{14}C]palmitoyl-CoA (lane 3).

An attempt to determine whether the stearoyl-ACP is an alternative substrate of mycobacterial Δ9 desaturase was initiated. This part of work has not been completed but the success in the purification of AcpM indicates some promises sufficient to further investigate this possibility. SDS-PAGE analysis of purified AcpM via Ni^{2+} affinity chromatography revealed that AcpM had been purified to near homogeneity (Fig. 7.6). On this gel, AcpM migrated at the position of a 28 kDa protein, significantly larger than its calculated molecular mass of 12 kDa based on deduced protein from the *acpM* gene. The anomalous migration of *M. tuberculosis* AcpM on SDS-PAGE is consistent with observations for ACPs from other bacteria (Morbidoni *et al.*, 1996; Rawlings and Cronan, 1992; Shen *et al.*, 1992; Kutchma *et al.*, 1999). This characteristic is attributed to the protein's high charge-to-mass-ratio (with ACP being highly acidic; calculated pI 3.8) as well as its low hydrophobic amino acid content. These two factors contribute to the considerable influence on SDS binding (Rock and Cronan, 1979; Kutchma *et al.*, 1999). The native gel was also applied to characterize the modified and unmodified ACP forms (Rock and Cronan, 1981). The stearoyl-ACP will be prepared by coupling stearic acid to this purified AcpM using the procedure described by Cronan and Klages (1981).

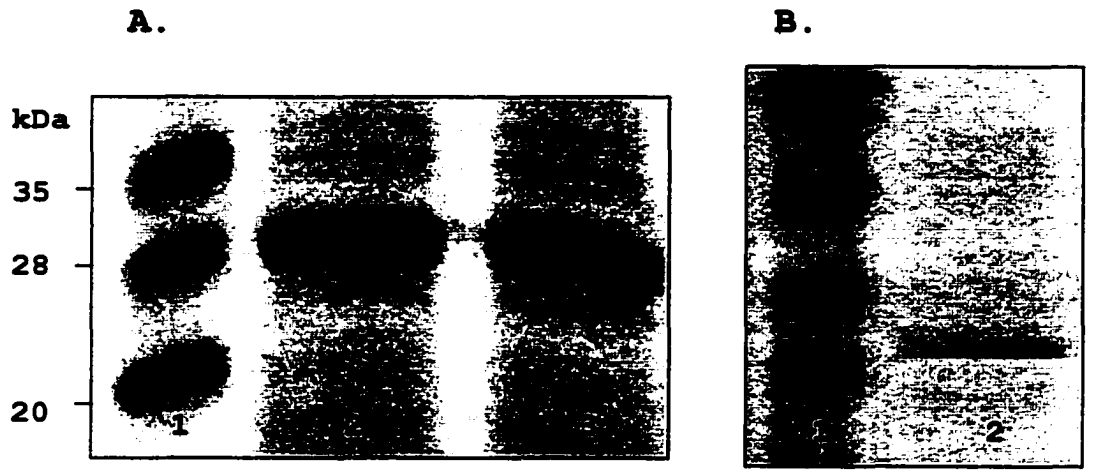


Figure 7.6 A Coomassie Brilliant Blue stained SDS-PAGE (18%) of the purified AcpM (A) and a 18% native PAGE of the purified AcpM (B). Lane 1, molecular mass markers (Bio-Rad); Lane 2 and 3, affinity purified AcpM from cell lysate with pETAcpM.

7.3.5 The effects of ISO on saturated fatty acid and mycolic acid synthesis *in vitro*

Mycobacteria are unusual in that they possess both multifunctional FAS I and dissociated FAS II (Bloch, 1975; 1977). Recently, whole cell labeling with [1,2-^{14}C]acetate in *M. aurum* A+, *M. tuberculosis* and *M. bovis* BCG demonstrated that ISO inhibited fatty acid and mycolic acid synthesis (chapter 4). Therefore the assays for FAS I, FAS II and MAS were anticipated to determine whether ISO selectively inhibited mycobacterial FAS I, FAS II or MAS activities. At 500 µg/ml of ISO, the FAS I activity in a cell extract from *M. aurum* A+ slightly increased by 14.77%, while FAS II system demonstrated that 9.04% of FAS II activity was inhibited by ISO. This finding indicated that the multifunctional mycobacterial FAS I system was completely insensitive to ISO, while the dissociated FAS II was relatively insensitive.

For examination of the effects of ISO on mycolic acid synthesis, cell free particulate cell wall enzyme (P60) extracts, which preferentially catalyzes the synthesis of mycolic acids were analyzed in a dose-response manner against ISO. As predicted, the synthesis of the *M. aurum* A+ mycolic acids was strongly inhibited, resulting in approximately 53.22% inhibition at 50 µg/ml of ISO. The dose-response effect of ISO on the mycolate-synthesizing activity is shown in Fig. 7.7. It should be noted that the mycolate formation in the *in vitro* mycolate assay was less sensitive to ISO than in the *in vivo* labeling experiment described earlier (chapter 4).

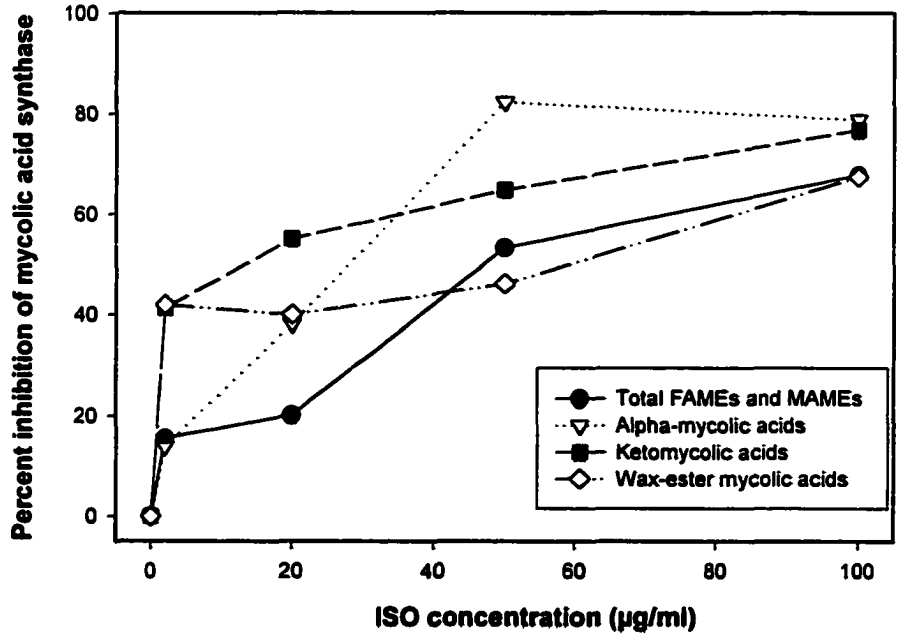


Figure 7.7 Effects of ISO on P60 mycolate-synthesizing activity *in vitro*. Cell wall enzymatic fractions (P60) were prepared from *M. aurum* A+ and assayed for mycolic acid synthase activity in the presence of ISO at 2, 20, 50, 100 µg/ml. The enzyme reactions were performed in duplicate. After saponification, methylation and extraction, the resulting mixtures containing FAMEs and MAMEs were counted for radiolabel and analyzed by TLC. The incorporation of [^{14}C]isotope into individual types of mycolic acids was determined by scintillation counting.

7.4 DISCUSSION

Fatty acid desaturases are enzymes that convert a single bond between two carbon atoms (C-C) to a double bond (C=C) in fatty acyl chains (Stumpf, 1980). The resultant fatty acids are often referred as unsaturated fatty acids, and the reactions catalyzed by these enzymes are known as desaturation reactions. The distribution of fatty acid desaturases is almost universal. The enzyme has been found in all organisms examined (Los and Murata, 1998), with the exception of some bacteria such as *E. coli* that has fatty acid synthase capable of introducing a double bond during the elongation of fatty acids (Heath and Rock, 1996).

Three types of fatty acid desaturases have been described: acyl-CoA, acyl-ACP, and acyl-lipid desaturase (Murata and Wada, 1995). Acyl-lipid desaturases catalyze most desaturations in plants and cyanobacteria. The enzymes introduce unsaturated bonds into fatty acids that are in a lipid-bound form (Stubbs and Smith, 1984). Acyl-desaturases exist in plastids of plant cells and introduce the double bond into fatty acids that are bound to ACP (Murata *et al.*, 1992; Holloway, 1983). Acyl-CoA desaturases which introduce unsaturated bonds into fatty acids bound to CoA are present in animal, yeast and fungal cells (Macartney *et al.*, 1994). Typically, each fatty acid desaturase introduces an unsaturated bond at a specific position in a fatty acyl chain. The specific site of desaturation by desaturases is defined by reference to the carboxyl terminus (Δ position) of fatty acids;

for example, desaurations at the Δ6, Δ9 or Δ12 position. The Δ9 desaturase is relevant to this study.

Up to the present, two fatty acid desaturation systems have been described in mycobacteria. The particulate system that desaturates stearoyl-CoA to form Δ9-octadecanoate (oleic acid) was reported in *M. phlei* (Fulco and Bloch, 1964; Kashiwabara and Sato, 1973; Kashiwabara *et al.*, 1975). The soluble system that desaturates lignoceroyl-CoA to form Δ15 tetracosenoate has been recently characterized in *M. smegmatis* (Kikuchi and Kusaka, 1986). There has been no report on the Δ9 desaturation in *M. tuberculosis* and *M. bovis* BCG. More recently, the re-examination of effects of ISO in mycobacteria by whole-cell labeling revealed an unexpected result: ISO decreased oleic acid synthesis and caused an accumulation of stearic acid in *M. tuberculosis* as well as in *M. bovis* BCG. This finding suggested that ISO acts by the inhibition of Δ9 desaturase. Therefore, it was of interest to examine the Δ9 desaturation in *M. tuberculosis* and *M. bovis* BCG. Furthermore, the issue that is most relevant to ISO in this present context concerns the mode of action of ISO on the Δ9 desaturase activity.

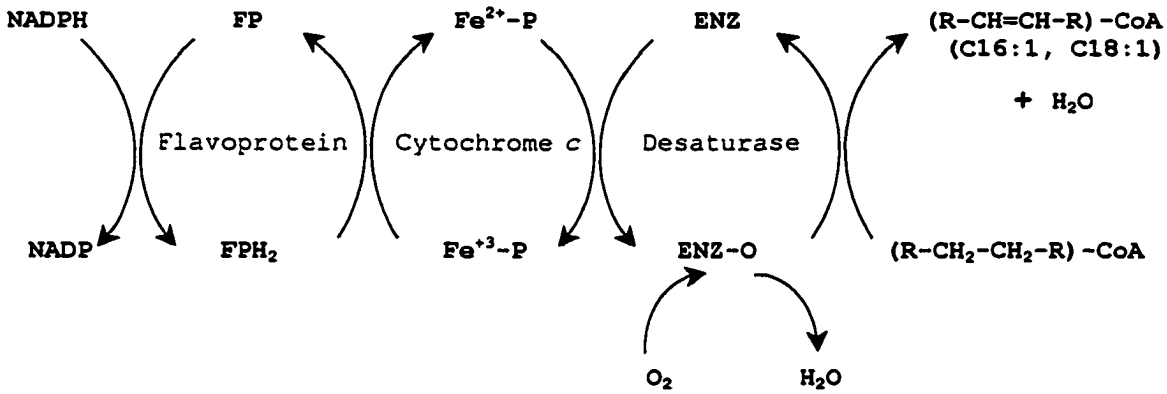
It was already known that the fatty acid Δ9 desaturase system of *M. phlei* is a membrane bound enzyme, requires the fatty acid-CoA as the substrate, and utilizes NADPH as electron donor and oxygen as electron acceptor (Fulco and Bloch, 1962; 1964; Kashiwabara and Sato, 1975). These characteristics make the *M. phlei* Δ9 desaturase resemble the Δ9 desaturase of rat hepatic

microsomal system rather than that of *Euglena gracilis* (Kashiwabara and Sato, 1973). The microsomal desaturase of eukaryotes requires fatty acyl-CoA as a substrate and is firmly bound to a membrane structure, while the *Euglena* system specifically attacks fatty-acyl-ACP derivatives and is recovered in the cytosolic fraction of cell homogenate (Nagai and Bloch, 1968). In addition, in the microsomal system, the transfer of reducing equivalents from NADPH to the terminal system is mediated by flavoproteins and cytochrome b_5 (Holloway, 1983). The *M. phlei* Δ9 desaturase was described as a terminal oxidase of an electron transport chain requiring FAD-dependent NADPH-cytochrome *c* reductase and oxygen for activity (Kashiwabara and Sato, 1973).

The previous study reported that a particulate fraction prepared from *M. phlei* grown in a iron-deficient medium exhibited a greatly reduced activity of stearoyl-CoA desaturase compared to that from normally grown cells (Kashiwabara *et al.*, 1975). It is known that iron has no effect on FAD-dependent NADPH-cytochrome *c* reductase activity but it binds to the Δ9 desaturase at the active sites involved in electron transport as shown in the following diagram. Iron plays an important role for the Δ9 desaturase activity and the assembly of the terminal portion of the desaturase. Thus, it was recommended that the culture be supplemented the culture medium by iron to enhance the desaturase activity.

The *M. bovis* BCG Δ9 desaturase system reported here is similar to the *M. phlei* system (Fulco and Bloch, 1964) in terms of

the requirement of $C_{18:0}$-CoA as a substrate, the intracellular location as a membrane associated enzyme and the requirements of cofactors for its activity. The *M. bovis* BCG and perhaps *M. tuberculosis* desaturases require NADPH as a reducing agent involved electron flow from the NADPH to oxygen molecule. On the basis of the above discussion, the following scheme may be written for the Δ9 desaturase system of *M. bovis* BCG.



The increased activity of the Δ9 desaturase in the *desA3*-overexpression strain suggested that the *desA3* gene encodes the Δ9 desaturase *in M. tuberculosis.* The enzyme activity and cofactor requirements are presumably similar to that of *M. bovis* BCG.

At present, we have no experimental evidence to indicate that the CoA derivative is the sole substrate form of the mycobacterial Δ9 desaturase, even though the Δ9 desaturase can use stearoyl-CoA as a substrate as described earlier. The question arises from the previous work using native, 2D PAGE and reverse phase-TLC demonstrating that the $C_{18:0}$ was found to be associated with an accumulated AcpM upon treatment with ISO. It is unclear whether

the Δ9 desaturase can use fatty-acyl-ACP as its substrate. For further investigation, the purified AcpM will be coupled with stearic acid to form stearoyl-ACP, which will then be used in the *in vitro* desaturase assay to resolve this question.

The present study using a cell free system confirms and extends the early observation with *in vivo* labeling demonstrating that ISO acts by inhibiting Δ9 desaturase activity. ISO strongly inhibited Δ9 desaturase activity at very low concentrations suggesting that the Δ9 desaturase is probably the primary site of action of ISO in *M. tuberculosis* and *M. bovis* BCG. A recent report by Barry *et al.* (1999) proposed that ISO as well as ETH, which both have a thiocarbonyl group in their structures, is a prodrug that needs to be activated before exerting its effects. Therefore, it is necessary to preincubate ISO with the cell extract prior to the start of the desaturation reaction. The presence of the activated form of ISO in the reaction mixture is thought to affect the degree of inhibition of ISO in the *in vitro* desaturation reaction.

Through precedence with the FAS I and FAS II system of *M. aurum* A+ which was previously presumed to be a target of ISO, the FAS I and FAS II cell free system clearly demonstrated that the activities of FAS I and FAS II are apparently insensitive to ISO. This is not surprising, since this study has defined the primary target of ISO as the Δ9 desaturase in mycobacteria. The inhibitory effect of ISO on MAS activity confirmed that a second

enzymatic target of ISO exists in the mycolic acid synthesis pathway.

In conclusion, the *in vitro* data provides the obvious evidence that the Δ9 desaturase exists in *M. tuberculosis*, and *M. bovis* BCG, and the *M. tuberculosis* Δ9 desaturase is indeed the product of the *desA3* gene. It is clear that ISO has more than one drug target and exerts many effects in mycobacterial cells. The inhibition of Δ9 desaturase by ISO in the cell free system provided the definitive evidence that the Δ9 desaturase has the potential to be a novel target for a new generation of antituberculosis drugs and ISO is a promising antituberculosis agents whose mechanism of action is unique.

7.5 REFERENCES

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CHAPTER 8

Final Conclusions and Future Directions

8.1 CONCLUSIONS

Many lipid components as well as numerous genes involved in lipogenesis and lipolysis exist in mycobacteria. Mycobacterial lipids are found in free form and bound to various cell wall structures and cellular membrane. Destruction of lipid synthesis would then be expected to impair structural integrity and increase the entry of toxic solutes including drugs into the cells. The distinct characteristics and the important role of lipids to mycobacterial cells make the biosynthesis of fatty acids and mycolic acids attractive as targets for antituberculosis drugs.

The work details the use of THC (isoxyl®), a thiourea derivative that had been known to inhibit the synthesis of mycolic and fatty acids, to identify and characterize its target enzymes. An approach to identify drug targets, described in chapter 3, involves the screening of the genomic library of a mutant highly resistant to THC as well as the screening of the genomic library of the *M. tuberculosis* wild-type strain. Although the major goal could not be achieved by this approach, the study provided a reference mutant that confers resistance to both THC and ETH. Based on the evidence of cross-resistance as discussed in chapter 2, the mechanism of THC resistance is presumably mediated by mutation in the gene involving in the activation of THC into its active form. With modifications in techniques and using this generated strain, the activation process of THC could be defined.

The chapter 3 also describes a study of genetic diversity of mycobacterial strains that were used in this work and all have different drug resistance profiles.

In chapter 4, an examination of the efficacy of THC against a variety of reference mycobacterial strains revealed that THC is a novel antituberculosis drug. It is effective against both drug-susceptible and -resistant *M. tuberculosis* when used in the range of 1-10 μg/ml. Moreover, some newly synthesized derivatives of thiourea exhibited a greater potency than THC. Further analyses of biochemical effects showed that fatty acids and all types of mycolic acids were affected by THC: α-mycolates, methoxymycolates, ketomycolates and wax-ester mycolates. The inhibitory effect on fatty acid synthesis was not observed for INH and ETH. Instead, INH and ETH stimulate this activity.

The work in chapter 5 detailed pleiotropic effects of THC on mycobacterial cells, which in turn make THC a difficult tool to use to identify its molecular targets. THC exerts its effects in mycobacterial cells by inhibiting the synthesis of free fatty acids, mycolic acids, oleic acid, tuberculostearic acid and increasing the amount of AcpM inside the cells. It may have other lethal or consequential effects but those have not been identified. The *in vivo* labeling with [1,2-^{14}C]acetate and analysis by TLC and GC clearly demonstrated that THC inhibits the synthesis of oleic acid and hence tuberculostearic acid. On the other hand, it affirmed that oleic acid is in turn metabolized to tuberculostearic acid. The unexpected result also indicates that

THC is an invaluable tool in identifying a unique mode of action for antituberculosis drugs that has never been associated with any currently available agent. Furthermore, upon treatment of *M. tuberculosis* and *M. bovis* BCG with THC there was an obvious increase in stearic acid concomitant with a dramatic decrease of oleic acid. This finding suggested that ISO acts by inhibiting the Δ9 desaturase.

With the advent of the complete genome of *M. tuberculosis*, a search of the genomic database indicates that there are three open reading frames probably involved in desaturation. The functional analysis by whole-cell labeling with [1,2-^{14}C]acetate detailed in chapter 6 showed that the *M. tuberculosis des*A3 gene product is indeed the Δ9 desaturase involved in oleic acid synthesis. Elucidation of gene function by the over-expression of the *M. tuberculosis des*A3 gene resulted in an increase of the Δ9 desaturase activity and hence an increased amount of oleic acid as well as an increased MIC of THC. The Δ9 desaturase that catalyzes the synthesis of oleic acid appears to be an attractive target for a new generation of antituberculosis drugs. Although the postulated importance of oleic acid in retaining the membrane integrity and physiology is not advanced further in this study, the fact that oleic acid plays a key role in the maintenance of the proper structure and functioning of biological membranes suggests such an essential role (Los and Murata, 1999). Drugs that inhibit oleic acid synthesis presumably have drastic effects on the ability of organisms to control membrane permeability and

integrity. It is possible that the singular inhibition of oleic acid synthesis by drugs is sufficient to make cells die. As evidenced by the effect of sterculic acid in specifically inhibiting the synthesis of oleic acid, this mode of action apparently has the capacity to cause cell death.

THC appears to have multiple targets. By comparing the effects of ISO and that of sterculic acid it can be concluded that the inhibitory effect of THC on mycolic acid synthesis is not a consequence of the inhibition of oleic acid synthesis. Clearly, the synthesis of mycolic acids and oleic acid are not linked. The inhibitory effect of THC on mycolic acid synthesis then can be explained as a result of drug interaction with a second target in the mycolic acid synthesis pathway. This finding again pointed out that THC is an invaluable tool that can reveal some fundamental aspects of fatty acid and mycolic acid metabolism of mycobacteria.

Apparently the Δ9 desaturase is an effective target for antituberculosis drugs. Efforts were made to characterize Δ9 desaturation particularly in *M. tuberculosis* and *M. bovis* BCG. The *M. tuberculosis* Δ9 desaturase is characterized by the presence of three conserved histidine tracks that are presumed to form the Fe-binding active center. The expression of recombinant *M. tuberculosis* Δ9 desaturase fused to the vector-derived His-tagged domain followed by immunoblotting provides the first evidence that the *M. tuberculosis* Δ9 desaturase is a membrane-associated protein. The secondary structure of the enzyme was demonstrated

to contain up to three hydrophobic domains that would be long enough to span the membrane bilayer twice.

The *in vitro* assay for the Δ9 desaturase activity was developed as described in chapter 7. The *in vitro* desaturation showed that stearoyl-CoA is used by Δ9 desaturase to synthesize oleic acid in the presence of oxygen, NADPH and presumably other cofactors (Fulco and Bloch, 1964; Kashiwabara *et al.*, 1973; Kashiwabara and Sato; 1975). The cell free assay for Δ9 desaturase activity also showed that THC does inhibit oleic acid synthesis and that the *M. tuberculosis des*A3 gene actually encodes the Δ9 desaturase.

A culminating point of this work was the identification of the *desA3* gene function and demonstration of the potential of the *desA3* gene product as a novel target for a new generation of antituberculosis drugs. Subsequent studies may lead to the discovery of novel Δ9 desaturase inhibitors.

8.2 FUTURE DIRECTIONS

Much work is waiting to be done regarding the drug, THC and its target, Δ9 desaturase.

The Δ9 desaturase was demonstrated to be a therapeutic target for tuberculosis. Thus, there is interest in characterizing the enzyme and its activity. The availability of large amounts of purified enzyme will facilitate studies of the molecular aspects of the Δ9 desaturase as well as approaches toward the design of

novel Δ9 desaturase inhibitors. Purification of the *M. tuberculosis* Δ9 desaturase using the *des*A3-histidine tag construct and Ni^{2+} affinity chromatography was not successful. The suspected problem that made the initial attempts to purify the protein unsuccessful was probably due to the insolubility of this membrane protein. An effort was made by trying to dissolve the membrane protein using several non-denaturing detergents such as Tween 20, Triton X-100, and CHAPS. However, it appeared that the Δ9 desaturase is firmly bound to the cellular membrane and as a result it could not be dissolved by such detergents. With modifications, the protein may be purified on Ni^{2+} column after the protein is denatured with urea. The denatured protein will then be refolded after purification. Alternatively, cloning of the *des*A3 fused to special tags such as solubilized maltose should allow the DesA3 fusion protein to be dissolved well in solution and then could be purified by affinity chromatography.

Subsequently, the purified Δ9 desaturase will be used to define the characteristics of Δ9 desaturation using the recently developed Δ9 desaturase cell free assay. A variety of substrates of Δ9 desaturase such as stearoyl-ACP or palmitoyl-ACP can be explored with the purified enzyme in the *in vitro* assay as well. The assay could then be utilized in high throughput screening to identify other agents including newly synthesized ISO derivatives as new potent inhibitors of Δ9 desaturase.

The crystallographic structure of the Δ9 desaturase can be achieved from the purified enzyme. The defined structure obtained will be used to identify the active site of the enzyme presumed to contain the histidine rich motifs. Moreover, the crystallography structure will give insight in order to design new specific compounds to block the active site of the protein and then elicit various degrees of antimycobacterial activity. The purified protein will also be used in the *in vitro* assay to determine whether ISO actually binds to the Δ9 desaturase.

If the Δ9 desaturase has therapeutic value in tuberculosis treatment, it is necessary to determine the essentiality of the *desA3* gene in mycobacterial viability. One approach to demonstrate the essentiality of the *desA3* gene involves the construction of the *desA3* knock out strain of *M. bovis* BCG by homologous recombination. The *M. bovis* BCG *desA3* homolog will be cloned since the homology between the *M. tuberculosis* and *M. bovis* BCG *desA3* genes is unknown. The isolation of a mutation in a cloned wild-type *desA3* gene with the subsequent reintroduction of that altered gene back into its chromosomal wild-type locus to generate a chromosomal mutation constitutes an allelic exchange. The hypothesis to be tested is that the disruption of the *desA3* gene will abolish the function of the Δ9 desaturase and hence the production of oleic acid resulting in an oleic acid auxotroph. If the *desA3* gene is essential, a knock out strain by double homologous recombination (allelic exchange) will not be obtained. Alternatively, supplementation of oleic acid in the selected

medium would yield the oleic acid auxotroph, which would be a valuable tool to elucidate the critical function of oleic acid. This approach also greatly helps in demonstrating the essential function of the *desA3* gene in *M. bovis* BCG. It is plausible to apply this technique to virulent strains of *M. tuberculosis* such as *M. tuberculosis* type strain H37Rv or Erdman. Although the procedure is the same as described above, the Biosafety Level 3 containment is absolutely required and intensive caution must be taken to carry it out. In addition, low efficiency of the allelic exchange is expected to occur with *M. tuberculosis*.

The complementation can also be applied to demonstrate the essential function of the *desA3* gene. In such a test, a plasmid containing the *desA3* gene will be transformed into the mycobacterial oleic acid auxotroph. The assumption is that the plasmid construct can confer complementary activity to the *desA3* mutant resulting in the ability of cells to grow on medium without oleic acid supplement.

The Δ9 desaturases are well characterized in other organisms such as in animal, yeast and fungal cells (Nakamishi *et al.*, 1996, Stukey *et al.*, 1989; Meesters and Eggink, 1996; Tiku *et al.*, 1996; Mihara, 1990; Tebby and Buttke, 1994). All Δ9 desaturases characterized to date have the three histidine clusters, which are localized at strongly conserved positions in the amino acid sequence of each protein (Murata and Wada, 1995; Shanklin et al., 1994). These three conserved histidine tracks are presumed to be an active center of the Δ9 desaturases where the iron binds

(Shanklin *et al.*, 1994; Fox *et al.*, 1994). Site-directed mutagenesis of the stearoyl-CoA desaturase of rat (Shanklin *et al.*, 1994) revealed that the substitution by another amino acid of any of the conserved histidine residues leads to the loss of enzymatic activity. Such loss of activity is probably due to the inability of iron to bind to the enzyme (Avelenge-Macherel *et al.*, 1995; Schneider *et al.*, 1992). With this in mind, the mutagenesis of the histidine rich regions in *M. tuberculosis desA3* gene would result in the inactivation of the enzyme as well. There is interest to prove that the histidine rich regions represent the active center of the *M. tuberculosis* Δ9 desaturases. Therefore, the histidine residues will be replaced by other amino acids using site-specific mutagenesis. The resulting mutagenized enzyme will then be assayed for the Δ9 desaturase activity. The site-specific mutagenesis of the *M. tuberculosis* Δ9 desaturase is important since it will provide the evidence for the active site of this enzyme.

An innovative approach to accomplish the need to develop new drugs is the identification of new potential drug targets and design effective enzyme inhibitors. THC has been proved in this study to be a valuable tool to identify a new drug target. Its mechanism of action is unique in the fact that it inhibits oleic acid and tuberculostearic acid synthesis, and its target is the Δ9 desaturase responsible for the synthesis of oleic acid from stearic acid. THC is not only a good tool, but by itself it is promising as a clinical antimycobacterial agent. THC is effective

against both drug-susceptible and a variety of drug-resistant *M. tuberculosis*. Therefore, in light of drug resistance, it is worthwhile to re-examine this old antituberculosis agent formerly deemed effective in tuberculosis treatments. It is feasible that THC or new related compounds could be an addition to current tuberculosis treatments. However, many questions remain concerning various aspects of THC. The re-visiting of THC will help to answer such questions, as for instance, the mechanisms of THC resistance; the mechanisms of drug activation; the target of THC on mycolic acid synthesis; the therapeutic value of in the context of the current situation of tuberculosis; the toxicity and efficiency of THC in both experimental animals and clinical trials; the pharmacokinetics of THC; and the potential of the new candidates among the newly synthesized derivatives of thioureas. Encouragingly, some of the new derivatives of thioureas showed a greater potency than THC. As a whole, the extension of this work in trying tc answer such questions will enlighten the development and the discovery of new inhibitors targeting Δ9 desaturase and effective against both drug-susceptible and drug-resistant *M. tuberculosis*.

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