RECOMBINANT *SALMONELLA* TYPHI TY21A AS VACCINE CANDIDATE AGAINST TUBERCULOSIS

by

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DEDICATIONS

This thesis is especially dedicated to:

My beloved wife, Agustine Nengsih bt. Said @ Fauzi
My beloved children, Amni Batrisyia and Akhil Hazim
My parents, Mat Zainuddin b. Mat Sohor and Jumrah bt. Hj. Mohd. Yasin

Thanks a lot for your co-operation, love, patience and support in finishing this thesis.

May Allah bless you all...

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LIST OF ABBREVIATIONS

AFB	Acid-fast bacilli
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BCG	*Mycobacterium bovis* bacille Calmette-Guerin
CD	Cluster of differentiation
CDC	Communicable Disease Centre
CTL	Cytotoxic T lymphocyte
DOTS	Directly observed treatment short-course
DOTS-PPM	Private-Public Mix DOTS
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
LAM	Lipoarabinomannan
LTBI	Latent TB infection
MDR	Multi drug resistant
MDR-TB	Multi drug resistant *Mycobacterium tuberculosis*
MHC	Major histocompatibility complex
MVA	Modified Vaccinia Virus Ankara
MTB	*Mycobacterium tuberculosis*
NTP	National TB Control Programme, Malaysia
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PPD	Purified Protein Derivative (Tuberculin)

RE	Restriction enzyme
ROI	Reactive oxygen intermediate
RNI	Reactive nitrogen intermediate
SDM	Site-directed mutagenesis
TB	Tuberculosis
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TST	Tuberculin skin test
WHO	World Health Organization

SALMONELLA TYPHI TY21A REKOMBINAN SEBAGAI CALON VAKSIN TERHADAP TUBERKULOSIS

ABSTRAK

Vaksin rekombinan *Salmonella* Typhi Ty21a [rSTvacIII(M2)] telah dibangunkan yang mempamerkan antigen mikobakteria pelbagai epitop iaitu VacIII(M2) (mengandungi ubiquitin dan 4 epitop mikobakteria imunogenik iaitu ESAT-6, PhoS1, Hsp 16.3, dan MTB 8.4) di atas permukaan *Salmonella* Typhi Ty21a (Ty21a) menggunakan protin PgsA sebagai protin pembawa. Pengekspresan protin VacIII(M2) pada permukaan Ty21a telah disahkan melalui kaedah pemblotan Western. Vaksin DNA yang mengkodkan gen VacIII (pVaxVacIII) juga telah dibangunkan sebelum ini. Pengimunisasian ke atas tikus BALB/c menggunakan pVaxVacIII dan rSTvacIII(M2) sama ada secara homologus atau heterologus telah merangsang gerak balas imun Th1 yang tinggi berdasarkan kepada penghasilan IFN-γ *in vitro* yang banyak dan perembesan IL-4 yang rendah. Walau bagaimanapun, pengimunisasian secara '*prime-boost*' yang menggunakan pVaxVacIII diikuti dengan rSTvacIII(M2) telah menghasilkan IFN-γ yang kurang berbanding pengimunisasian secara homologus menggunakan rSTvacIII(M2) atau pVaxVacIII. Oleh sebab imuniti perlindungan terhadap tuberkulosis dihasilkan melalui gerak balas imun Th1, maka rSTvacIII(M2) dan pVaxVacIII mempunyai potensi sebagai calon vaksin yang efektif terhadap tuberkulosis.

RECOMBINANT *SALMONELLA* TYPHI TY21A AS VACCINE CANDIDATE AGAINST TUBERCULOSIS

ABSTRACT

A recombinant *Salmonella* Typhi Ty21a vaccine was developed where the candidate [rSTvacIII(M2)] displaying a multi-epitopes mycobacterial antigen called the VacIII(M2) (containing ubiquitin and 4 immunogenic mycobacterial epitopes of ESAT-6, PhoS1, Hsp 16.3, and MTB 8.4 genes) on the surface of *Salmonella* Typhi Ty21a (Ty21a) using PgsA protein as a carrier. The expression of VacIII(M2) protein on the surface of Ty21a was verified by Western blot. A DNA vaccine was constructed namely as pVaxVacIII, encoding the VacIII gene. Immunization in BALB/c mice with pVaxVacIII and rSTvacIII(M2) either as homologous or heterologous vaccination, induced strong Th1-type responses based on high levels of *in vitro* IFN-γ but low levels of IL-4 secretion. However, the heterologous prime-boost vaccination using pVaxVacIII followed by rSTvacIII(M2) induced lower IFN-γ production compared to homologous rSTvacIII(M2) or pVaxVacIII vaccination. Since the protective immunity against tuberculosis required Th1-type response, these rSTvacIII(M2) and pVaxVacIII have potential as effective vaccine candidates against tuberculosis.

CHAPTER 1

INTRODUCTION

1.1. Introduction

Tuberculosis (TB) is a chronic or acute infectious disease caused by *Mycobacterium tuberculosis* (MTB), which can infect any organ in the body but usually affecting the lungs (Nevel, 2002). MTB is a very dangerous pathogen since it can survive and multiply within macrophages. This pathogen can also be transmitted easily from an active TB patient to a normal person via the respiratory route (Kanai, 1990). The World Health Organization (WHO) has estimated that there are 9 million new TB cases worldwide with approximately 2 million deaths annually (WHO Report, 2006a).

1.2. History of Human Tuberculosis (TB)

Since early civilization, human beings have suffered from TB (Ratledge & Stanford, 1982). TB has infected humans since 2050 to 1650 BC based on the presence of MTB complex DNA in the skeletons of Egypt mummies (Zink *et al.*, 2003; Smith, 2003). The disease was also mentioned in ancient Greek literature and Hippocrates (459-377 BC) described TB as the most widespread disease which always caused deaths (Online at http://www.umdnj.edu/~ntbcweb/history.htm).

The first suggestion that TB can be transmitted via air-borne particles ("contagium vivium") was stated by Frascatorious (1483-1553). However, most did not agree with his hypothesis and it was generally believed that heredity was the main causative factor for TB (Kanai, 1990). In 1868, Jean-Antoine Villemin conducted a few experiments to that successfully prove that TB was caused by a specific agent which was transmissible, from man to cattle and from cattle to rabbits (Kanai, 1990)

The specific agent for TB was clearly identified by Robert Koch in 1882. He had created a guideline to determine the causative agents of several specific infectious diseases. This guideline was named as "four Koch's Postulates". According to this guideline, bacteria suspected as the causative factor of a disease must exist in the infected tissue (such as blood) of an infected person or animal. When the infected tissues were innoculated into solid media, it must produce pure colonies and the pure colonies must be infectious to experimental animals. Then, the bacteria must be retrieved as pure colonies from the second infected animal tissues (Koch, 1884; Kanai, 1990). Before Robert Koch identified the MTB, he had successfully determined the causative factor of anthrax (called anthrax bacilli) by his "four Koch's Postulates" (Koch, 1884).

When the causative factor of TB had been identified and proven transmissible, efforts were taken to minimize the spread of TB in human populations; such as to improve living conditions as well as to search for TB vaccine or anti-TB drugs (Online at http://www.umdnj.edu/~ntbcweb/history.htm). In 1908, Léon Charles Albert Calmette found that virulent bovine tubercle bacilli can be attenuated when cultured in a medium containing bile. He worked together with Camille Guerin to generate live attenuated vaccine against TB by sub-culturing virulent bacilli in a medium containing bile. After 13 years (1908-1921) of sub-culturing the bacilli over and over, a new bovine tubercle bacilli strain which was not infectious to animals was found. The strain was named as Bacillus Calmette Guerin (BCG). BCG was proven safe to humans and the strain was successfully given to newborn infants (Online at http://www.whonamedit.com/doctor.cfm/2413.html).

In 1944, Waksman discovered the first anti-TB drug from *Streptomyces griceus* and named it as streptomycin (Comroe, 1978; Kanai, 1990). Since then, other anti-TB drugs begin to appear such as *p*-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampicin (1963) (Online at http://www.umdnj.edu/~ntbcweb/history.htm).

Following the discoveries of BCG and anti-TB drugs as well as the improvement of living conditions, TB cases began to decrease. However, in the 1980s TB cases started to re-emerge in the United States and the rest of the world leading to the WHO finally declaring TB as a global public health emergency in 1993. (Online at http://www3.niaid.nih.gov/news/newsreleases/1996/tbtip.htm).

The re-emergence of TB is partly related to the increase of HIV infections which can then lead to TB co-infection and the appearance of a multidrug resistant (MDR) strain of MTB (Kauffmann, 2004). The MDR strain of MTB is defined as a MTB strain which is resistant to more than two generally used drugs such as Isoniazid and Rifampicin. Other factors such as increased migration, overcrowding and reduced public health services may also contribute to the resurgence of TB (Nevel, 2002).

1.3. Epidemiology of TB

Tuberculosis is one of the leading causes of death among some of the most infectious diseases in various parts of the world. Even though the BCG vaccine and anti-TB drugs have been used for the past 50 years, the mortality rate caused by TB is still considerably high (Zumla *et al.*, 1999). There were 8.9 million new TB cases worldwide in 2004, which caused 1.7 million deaths (WHO Report, 2006a).

By the end of 2004, 200 (95%) out of 211 countries worldwide have reported TB cases to WHO (Figure 1.1). WHO has classified these 200 countries into nine epidemiologically different regions namely the Central Europe Region, Established Market Economies Region, Latin America Region, South-East Asia Region, Western Pacific Region, Eastern Mediterranean Region, and African countries with low HIV cases, African countries with high HIV cases and the Eastern Europe Region. During 1990 to 2004, the incidence rate of TB decreased or stabilized in six regions (Central Europe Region, Established Market Economies Region, Latin America Region, South-East Asia Region, Western Pacific Region and Eastern Mediterranean Region). In contrast, three other regions (African countries with low HIV cases, African countries with high HIV cases and Eastern Europe Region) had an increased incidence rate. In general, the incidence rate of TB worldwide was increasing at an annual rate of 0.6% (WHO Report, 2006a) up until 2005 when it stabilized to about 150 per 100,000 populations (Online at http://www.scidev.net/en/news/who-report-identifies-challenges-to-tb-control.html). Nevertheless because the world population is increasing, actual numbers of infected people continue to grow.

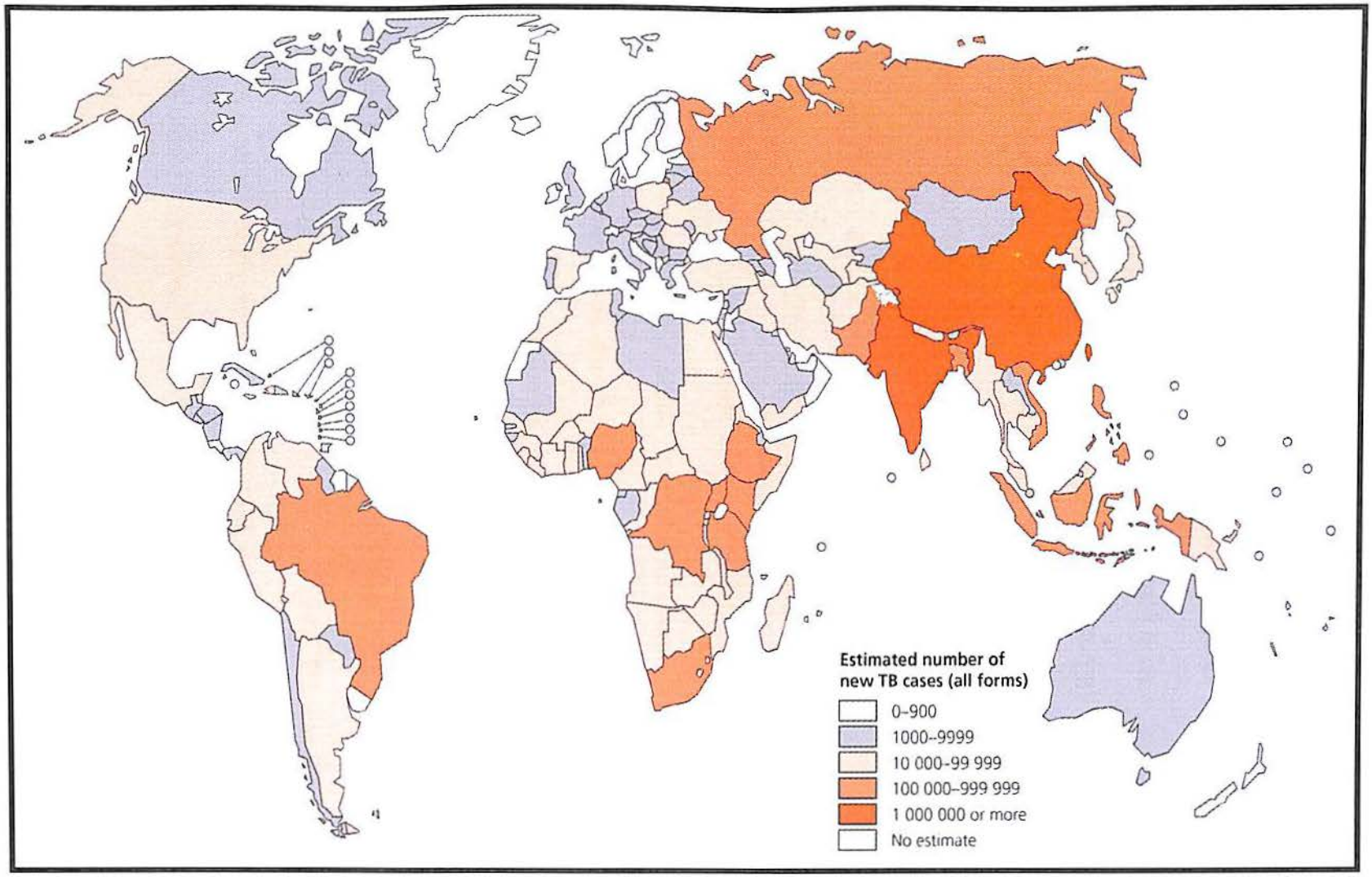

Figure 1.1. Estimated number of new TB cases (all forms) 2004 worldwide (Adapted from WHO Report, 2006a).

There are many reasons contributing to the increase of the TB in the three mentioned regions as well as worldwide. In African countries, the increase of TB cases was associated with the high incidence of HIV infections. High rates of HIV positive-TB patients were found in countries of eastern and southern Africa (WHO Report, 2006a). In the Eastern Europe Region, the increasing TB cases were associated with the high prevalence of multidrug resistant TB (MDR-TB) cases as well as the increase of HIV infections especially in Estonia, Lithuania and Latvia (Freiden *et al.*, 2003; Kauffmann, 2004).

TB is also known as the disease of poverty (Grange, *et al.* 1999; Editorial The Lancet, 2006). In 2004, 83% of the new and relapsed cases were detected in three regions namely the African Region (24%), South-East Asian Region (35%) and Western Pacific Region (24%) which consist of developing countries. In contrast, a low TB incidence rate was found in the Established Market Economies Region which consists of developed countries (WHO Report, 2006a).

In the early 1940s and 1950s, TB was the leading cause of death in Malaysia. However, since the National TB Control Programme (NTP) was launched in 1961, morbidity and mortality rate decreased rapidly. In 1988, TB was the 11th among all causes of deaths in Malaysia (Iyawoo, 2004). Even though TB is not the number one cause of deaths anymore, the mortality rate is still considerably high compared to other infectious diseases including HIV. For example, during 1990-2002, TB has caused the highest number of deaths compared to other infectious diseases. In 2001, a total of 14,830 cases of all forms of TB were reported which caused 1,326 deaths. However during 2002-2004, the mortality caused by TB decreased, even lower

compared to HIV infection. In 2004, a total of 13,942 cases (all forms of TB) were reported which caused 310 deaths (Online at http://dph.gov.my/dcd/survelans/).

The high prevalence of TB during the year 2000-2001 is probably due to the migration of foreign workers into Malaysia. Approximately 10% of TB cases reported in Malaysia involved the immigrant population. 90% out of the above 10% involves foreigners from the Philippines and Indonesia. These two countries have been classified as high burden countries of TB by WHO (Iyawoo, 2004).

1.4. Clinical Manifestations of TB

The clinical manifestation of TB is relatively inconsistent and depends on several factors such as age, immune status and virulence of the organism, site of involvement and severity of the disease. The most frequent type of TB cases is pulmonary TB which represents ≈ 85% of the total cases and ≈15% are related to extrapulmonary or both pulmonary and extrapulmonary (American Thoracic Society, Centers for Disease Control and Prevention, 2000; Iyawoo, 2004). This ratio however, is significantly different for TB patients with HIV infection in which 30% had only extrapulmonary, 38% had only pulmonary, 32% had both pulmonary and extrapulmonary TB (Small *et al.*, 1991).

The clinical symptoms of TB can be divided into systemic, pulmonary and extrapulmonary (American Thoracic Society, Centers for Disease Control and Prevention, 2000). Systemic effects of TB involve any sites of the body and are exclusively not related to any specific organ or tissue. Systemic symptoms include fever, loss of appetite, weight loss, weakness, night sweats and malaise. Besides that, hematologic symptoms such as an increase of peripheral blood leucocyte count and

anemia can also occur (American Thoracic Society, Centers for Disease Control and Prevention, 2000).

Cough is the most common symptom of pulmonary TB. It may be nonproductive during the early phase, but after inflammation and tissue necrosis develop, sputum is generally produced. Sometimes hemoptysis (blood in cough) might be present, but it is not a good indicator for pulmonary TB since it may result from other bacterial or fungal infection such as *Aspergillus* infection. A radiographic finding is a useful diagnostic tool, since it indicates abnormalities in chest x-ray of pulmonary TB patients. However, a chest x-ray is unable to detect an important indicator which is endobronchial lesion. Sometimes, a normal chest x-ray result is commonly found in patients with pulmonary TB and HIV infection (American Thoracic Society, Centers for Disease Control and Prevention, 2000).

Extra-pulmonary TB usually causes more difficulties in diagnosis compared to pulmonary TB. This type of TB, commonly involves inaccessible sites that always require invasive procedures. In addition, extrapulmonary TB also involves low numbers of bacilli which affects the diagnosis. Examples of extra-pulmonary TB are namely genitourinary TB, pleural TB, central nervous system TB, abdominal TB, TB lymphadenitis, bone and joint TB, miliary TB and pericardial TB (American Thoracic Society, Centers for Disease Control and Prevention, 2000). In Malaysia, the most common forms of extra-pulmonary TB include TB lymphadenitis, bone and joint TB, and miliary TB (Iyawoo, 2004).

1.5. The Aetiological Agent of TB: *Mycobacterium tuberculosis* (MTB)

1.5.1. General Features of MTB

TB is caused by a bacterium named *Mycobacterium tuberculosis* (MTB). This non-motile organism has a slender or slightly curved rod shape of 1-4 μm in length and 0.3-0.6 μm in breadth (Kanai, 1990). The mycobacterium genus has a lipid containing cell wall. This lipid containing cell wall is responsible for acid-fastness, namely when stained with carbol fuchsin dye, it is resistant to decolorization by acid-alcohol. The lipid containing cell wall is also associated with the resistance of MTB to environmental stresses such as drying and chemical antimicrobials (Tortora *et al.*, 1997).

MTB has a slow growth (20 hours or longer generation time) and forms fungus-like pellicles on the surface of broth which gave the genus its name (*myco* is the Greek word for fungus) (Tortora *et al.*, 1997). Meanwhile, on oleic acid-albumin agar, the colonies of MTB are identified by its rough, nonpigmented and corded colonies (American Thoracic Society, Centers for Disease Control and Prevention, 2000; Kanai, 1990). MTB is an aerobic bacterium but is able to survive within a microaerophilic environment by changing its metabolic machinery. Therefore, MTB is able to survive within the phagosome of macrophages (Kanai, 1990).

The cell structure of mycobacteria consists of plasma membrane, pili, plasmid, mesosome, ribosome, lipoidal droplet, metachromatic granule, chromosome and cell wall (Kanai, 1990). The mycobacterial cell wall consists of peptidoglycan, peptides side-chains, glycolipid and mycolic acids (Figure 1.2). The peptidoglycan forms the innermost layer of MTB cell wall, which acts as the backbone of the cell wall skeleton (Ratledge & Stanford, 1982). The peptidoglycan layer is linked to the

arabinogalactan layer and also to mycolic acids. The cell wall of MTB consists of three classes of mycolic acids namely α-, keto- and metoxymycolates (Riley, 2006).

α-Mycolic acid has two cyclopropane rings (in the *cis* configuration) whereas keto- and metoxymycolates have one ring each (either in the *cis* or *trans* configuration). These mycolic acids are associated with glycolipid trehalose dimycolate (TDM) on the cell wall of MTB. The differences in the relative composition of mycolic acids can affect the host immune response. For instance, MTB which has an alteration in the *trans*-cyclopropane rings of mycolic acids are found to be hypervirulent in mice whereas the absence of keto- and metoxymycolates result in the attenuation of MTB in mice. Besides mycolic acids, other molecules such as lipoarabinomannan, lipomannan and 19 kDa lipoprotein are also associated with the cell wall of MTB. These molecules are able to stimulate host immune responses (Karakousis *et al.*, 2004; Riley, 2006).

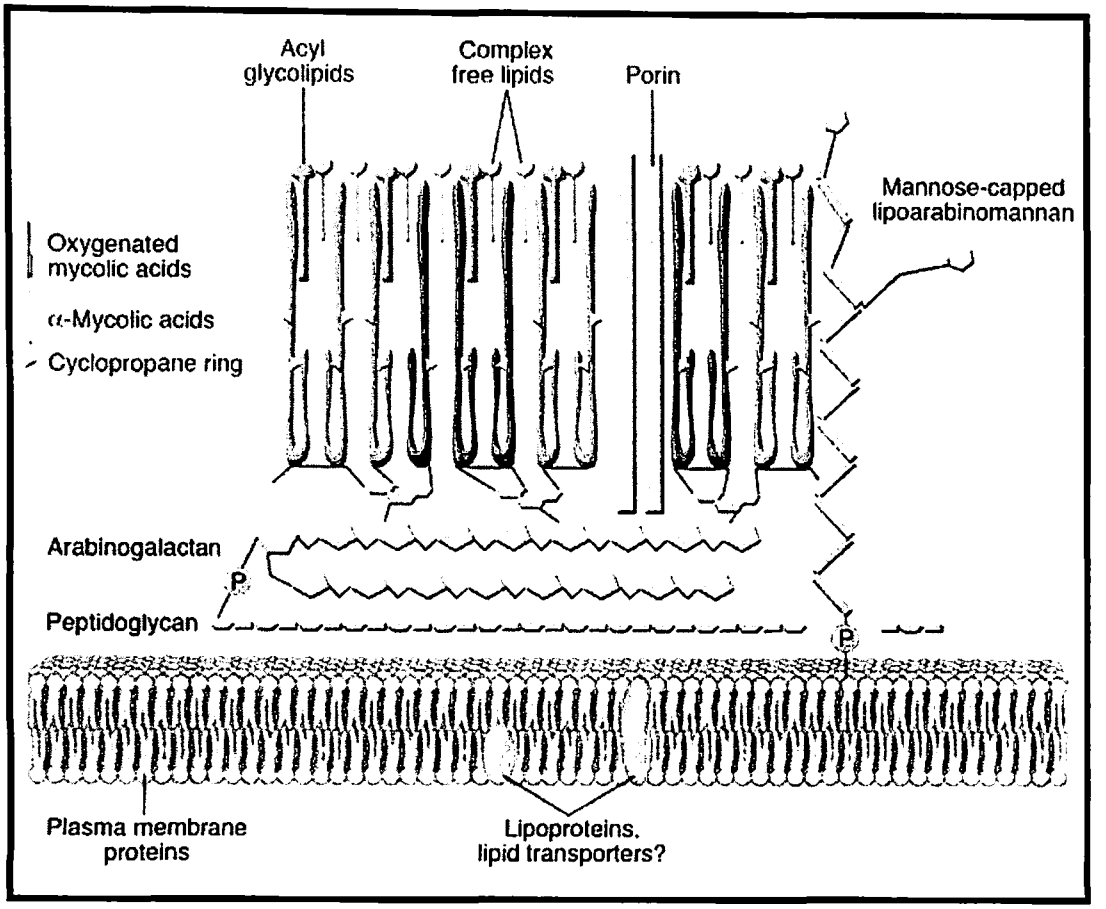

Figure 1.2: Composition of *Mycobacterium tuberculosis* cell envelope (Adapted from Riley, 2006).

1.5.2. Genetics of MTB

For many years, the genetics of MTB was a neglected topic due the difficulties in working with this organism as well as the lack of appropriate tools (Stanford & Ratledge, 1982; Smith, 2003). With the development of many genetic manipulation methods generally and for MTB specifically and in particular the complete DNA sequencing and annotation of the MTB H37Rv genome (Cole *et al.*, 1998) the genetics of MTB has been studied extensively (Smith, 2003).

The genome is defined as the whole set of genes within a cell (Ratledge & Stanford, 1982). The size of a MTB H37Rv genome is 4.4 X 10^6 bp which consists of ≈4000 genes. The genome of MTB has several unique characteristics compared with other bacterium. MTB has more than 200 genes involved in the metabolism of fatty acids.