CHAPTER 1

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1.1. Genus *Mycobacterium*

1.1.1. Classification. Mycobacteria belong to the Order *Actinomycetales*, Family *Mycobacteriaceae*. Robert Koch identified *Mycobacterium tuberculosis* as the causative organism of tuberculosis in 1882. It was referred to as Koch’s bacillus till Lehmann and Neumann gave the generic name *Mycobacterium* (meaning fungus bacterium) due to the mould-like growth of the bacillus in liquid medium (Lehmann & Neumann, 1896).

Some mycobacteria are pigmented and based on the production of pigments, they can be classified as scotochromogens (produce yellow pigment in the dark) or photochromogens (produce an orange pigment in the light) or achromogens (do not produce any pigment). Based on the growth characteristics mycobacteria are grouped into fast growers and slow growers, the latter comprising mostly of the pathogenic mycobacteria. *Mycobacterium tuberculosis* and *M. leprae* are two important human pathogens that cause tuberculosis and leprosy respectively.

1.1.2. Features of mycobacteria

Bacteria of the genus *Mycobacterium* are aerobic, non-motile and non-sporulated rods. Their genome show high G + C content (61-71 %) and their cell wall shows unique features with notably high lipid content in their cell wall. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium*, *Gordona*, *Nocardia*, *Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance. The mycobacterial lipid-rich cell wall can be stained with basic dyes such as carbol fuchsin and cannot be decolourised with acid-alcohol. This unique property is termed “acid-fastness” and is the basis of the Ziehl-Neelsen staining technique for the identification of mycobacteria.

1.1.3. Unique cell envelope of mycobacteria

The cell envelope is composed of three major constituents; the plasma membrane, the cell-wall core, and the extractable, non-covalently linked glycans, lipids and proteins. The structure of the cell envelope is illustrated in Fig.1 (Brennan & Crick, 2007). External to the membrane is peptidoglycan in covalent attachment to arabinogalactan, which in turn is attached to the mycolic acids with their long meromycolate and shorter a-chains. This portion is termed the cell-wall core (the mycolyl arabinogalactan-peptidoglycan complex (MAPc). Very long-chain fatty
acids, the mycolic acids, are covalently bound to the arabinogalactan-peptidoglycan co-polymer and were proposed to form the inner layer of an asymmetric outer membrane while other lipids constitute the outer leaflet (Brennan & Crick, 2007, Brennan & Nikaido, 1995) (Fig. 1). The mycolic acids extend perpendicular to the arabinogalactan / peptidoglycan, and other cell wall-associated glycolipids intercalate into the mycolic acid layer to form a ‘pseudo’ lipid bilayer. The cell wall of mycobacteria consists of large amount of lipids. The free lipids comprise the extractable material, which include the phthiocerol-containing lipids, the phosphatidylinositol mannosides, lipomannan, lipoarabinomannan, trehalose dimycolate (cord factor), trehalose monomycolate, and the diacyl- and polyacyl-trehaloses presumably intercalating with the a-and meromycolate chains of the mycolic acids (Russell, 2007). When the cell wall is subjected to treatment with various solvents, the free lipids and proteins are solubilised and the MAPc remains as an insoluble residue. Hence it was considered that these lipids, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell (Deres et al., 1989).

1.1.4. Proteome of the cell membrane of M. tuberculosis

The cell membrane of M. tuberculosis is likely to harbor proteins functioning as enzymes, receptors, transporters or signal transducers that could be of vital importance to the microbe (Nigou et al., 2003; Asselineau et al., 2002). Bioinformatic analysis of the M. tuberculosis genome predicts >65 lipoproteins of ‘cell envelope’ origin, some of which were identified previously as ‘secreted’ proteins, or enzymes involved in cell wall biogenesis. There are about 600 ‘putative’ membrane proteins. They harbor a number of transmembrane hydrophobic segments, and include proteins belonging to the major facilitator and ATP-binding cassette (ABC) superfamilies (Tekaia et al., 1999). These proteins play a role in the uptake and effects of various metabolites, peptides, drugs and antibiotics. Proteome analysis of the plasma membrane of M. tuberculosis by Sinha et al. (2002) demonstrates the presence of important proteins such as heat shock proteins four HSPs (Rv2031c, α-crystallin; Rv0251c, HSP20; Rv3417c, groEL1; and Rv0440c, groEL2), the iron storage protein the bacterioferritin (bfRA, Rv1876) and ATP synthase (Rv1308, Rv1309, Rv1310). ESAT-6, a well characterised culture filtrate protein, which belongs to the family of early secretory antigenic target, was found to be expressed in the membrane of M.
tuberculosis (Sinha et al., 2005). Along with ESAT-6, some of the components of its secretory machinery such as the products of the genes Rv3870, and Rv3877 (possible conserved transmembrane proteins) are present in the inner membrane of the cell (Abdallah et al., 2007).

1.2. Mycobacterium tuberculosis and tuberculosis

1.2.1. Epidemiology of tuberculosis

Tuberculosis is a disease of great public concern as it is a leading cause of death among the infectious diseases. Despite extensive efforts to control the causative organism M. tuberculosis since its discovery by Robert Koch in 1882, it still remains an enigma and the disease remains a major health problem worldwide.
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Each year, 55 million people become newly infected with the pathogen worldwide (WHO report, 2007 & 2006). Fig. 2 illustrates the estimated tuberculosis incidence rate worldwide. It has been estimated that one-third of the world’s population is infected with *M. tuberculosis* and roughly 10% of these individuals will develop active tuberculosis within their lifetime. With the rise in HIV infections, tuberculosis has been on the rise and death due to tuberculosis in HIV infected people increase at least twice that of the person infected only with HIV. This alarming rise led the WHO to declare tuberculosis ‘a global emergency’ in 1993 (WHO report, 1993). In addition, about one third of human population is estimated to suffer from latent tuberculosis, which can be reactivated even after several decades (Glassroth, 2005). It is estimated that, between 2000 and 2020 nearly one billion people will be newly infected, the active disease will affect 200 million, and 35 million will die of TB, if control measures are not significantly improved (WHO report, 2007).

Fig. 2 - Estimated tuberculosis incidence rate. (WHO report, 2007)
1.2.2. Pathogenesis

1.2.2.1. Infection and granuloma formation

The first stage of mycobacterial infection begins with the inhalation of tubercle bacilli as droplets, released into the atmosphere from an infected individual. Alveolar resident macrophages are the primary cells involved in the initial uptake of *M. tuberculosis*. Dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (Henderson *et al.*, 1997). The bacilli are taken up by receptor-mediated phagocytosis using a variety of macrophage receptors including CR3, CR4 and mannose receptors.

The outcome of an infection in the new host depends on the balance between (i) host immune response and effective killing of the invading pathogen (ii) the extent of tissue necrosis, fibrosis, and regeneration (Van Crevel *et al.*, 2002). In the lungs, the bacteria are phagocytosed by alveolar macrophages and induce a localized proinflammatory response that leads to the recruitment of mononuclear cells from neighbouring blood vessels. These cells are the building blocks for the granuloma, or tubercle, which is the signature of tuberculosis. The granuloma consists of a kernel of infected macrophages, surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the structure (Russell, 2007) (Fig. 3). The interactions of the pathogen with the macrophage are dominated by the ability of the pathogen to prevent phago-lysosome biogenesis (Vergne *et al.*, 2004, Armstrong & Hart, 1971), by modulating the phagosomal compartment and preventing its fusion with acidic lysosomal compartments and actively excluding vesicular proton ATP-ases, resulting in an elevated pH of 6.3–6.5 (compared to the normal lysosomal pH of 4.5).

The granuloma formation typifies the ‘containment’ phase of the infection in which there are no overt signs of disease and the host does not transmit the infection to others. In the later stages, the granuloma develops a marked fibrous sheath and the number of blood vessels penetrating the structure diminishes markedly. Containment usually fails when the immune status of the host changes, which could be associated with old age, malnutrition or co-infection with HIV; essentially any condition that reduces the number, or impairs the function, of CD4⁺ T cells. Following such a change in the immune status, the granuloma caseates (decays into a structure-less mass of cellular debris), ruptures and spills thousands of viable, infectious bacilli into
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the airways (Fig. 3). This results in the development of a productive cough that facilitates aerosol spread of infectious bacilli. (Russell, 2007).

1.2.2.2. Immune response and the outcome of infection

As *M. tuberculosis* is an intracellular pathogen, T-cell effector mechanisms play a role in the elimination of the pathogen. The proteins from the pathogen mediate the interactions between the pathogen and the host immune system; they are degraded in the phago-lysosomal compartment and the resulting peptides are complexed with the MHC class II and presented to CD4 cells (Andersen, 1997). To survive in macrophages, *M. tuberculosis* has evolved mechanisms to block immune responses. These include modulation of phagosomes, neutralization of macrophage effector molecules, stimulating the secretion of inhibitory cytokines, and interfering with processing of antigens for T-cells (Cooper & Flynn, 1995). The balance of the host–pathogen interaction in *M. tuberculosis* infection is determined by the interaction of
T-cells and infected macrophages. The outcome of this interaction results either in control of the infection or development of active disease.

The CD4 subset comprises the regulatory Th1 and Th2 cells each producing the regulatory and characteristic panel of cytokines. The Th1 cells are important players in the control of TB infection due to the production of IFN-γ and TNF-α, with both the cytokines playing an important role in macrophage activation. In addition to CD4+ T-cells, other T-cell subsets such as, γβ, CD8+ and CD1-restricted T-cells have roles in the immune response to *M. tuberculosis* (Schaible & Kaufmann, 2000). A diverse T-cell response allows the host to recognize a wider range of mycobacterial antigens presented by different families of antigen-presenting molecules, and thus greater ability to detect the pathogen. The slow growth and chronic nature of *M. tuberculosis* infection results in prolonged exposure to a large diversity of antigens (Boom *et al.*, 2003). This is likely an important factor in generating this T-cell diversity. CD8+ cells are also primed in response to TB infection but the pathway leading to MHC class I restricted antigen presentation is still not clear.

CD4+ T cells recognize mycobacterial peptide fragments presented to them by MHC class II molecules on antigen-presenting cells such as macrophages. The loss of CD4+ T cell number and function results in progressive primary infection, reactivation of endogenous *M. tuberculosis* and enhanced susceptibility to re-infection (Branes *et al.*, 1991). Mice with deleted genes for CD4+ or MHC class II molecules are susceptible to *M. tuberculosis*, firmly establishing a central role for CD4+ T cells in protection (Caruso *et al.*, 1999). A number of proteins, recognized by a majority of healthy tuberculin skin-test positive persons have been identified; they include the three 30–32 kDa 85 complex proteins, ESAT-6 and CFP-10, the 19 and 38 kDa lipoproteins. Recognition of the mycobacterial antigens by phagocytic cells results in a complex process of regulation and cross-regulation by the different cytokines. This cytokine network plays a crucial role in the inflammatory and the outcome of mycobacterial infections (Fig. 4) (Fenton & Vermeulen, 1996). Some of the pro-inflammatory cytokines are TNF-α, IL-1β, IL-6, IL-12, IFN-γ, IL-18 and IL-15. The anti-inflammatory cytokines are IL-10, TGF-β and IL-4 (Flynn & Chan, 2001).

IFN-γ is a key cytokine in control of *M. tuberculosis* infection (Cooper *et al.*, 1993). Mycobacterial antigen specific IFN-γ production *in vitro* could be used as marker of infection with *M. tuberculosis*. IFN-γ is produced by healthy subjects as well as those with active tuberculosis (Sahirratmadja *et al.*, 2007). Though IFN-γ
production varies among subjects, some studies suggest that IFN-\(\gamma\) levels are depressed in patients with active tuberculosis (Zhang et al., 1995 & Lin et al., 1996). IFN-\(\gamma\) has also been shown to influence the cellular iron status of the macrophage (Boelaert et al., 2007). IFN-\(\gamma\) activation of human monocytes down-regulates transferrin receptors on the cell surface (Byrd & Horwitz, 1989) and the rate of macrophage iron acquisition from holo transferrin (Olakanmi et al., 2002), thereby decreasing the availability of iron to the intracellular pathogen.

1.2.3. Control measures

Control measures for tuberculosis include timely diagnosis and chemotherapy. Tuberculosis can be cured in 95% of patients with active, drug sensitive pulmonary TB (Spigelman & Gillespie, 2006). Multi-drug therapy with a combination of three frontline drugs, isoniazid, rifampin and pyrazinamide and one or more of the second-tier antibiotics including ethambutol, ethionamide, \(p\)-aminosalicylic acid, D-cycloserine, streptomycin, capreomycin, kanamycin and thiacetazone considerably
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reduced the incidence of TB, especially in developed countries. Nevertheless, this
decline has been reversed in the past two decades due to several factors including
poverty, overcrowding, travel and the synergy between HIV and TB (Corbett et al.,
2003). WHO initiated directly observed therapy short-course (DOTS), which is
currently being adopted by 119 countries including all 22 high burden countries that
contain 80% of all estimated cases (Collins & Kaufmann, 2001). Despite the efforts
like the DOTS, numbers of drug resistant cases are on the rise, thereby necessitating
newer and better drugs (Reece & Kaufmann, 2008).

Existing diagnostic methods can detect up to 60% of tuberculosis cases but
tuberculosis management in developing countries is difficult as the existing diagnostic
methods are non-specific and time-consuming. The present day diagnosis for human
tuberculosis include clinical examination followed by radiological and laboratory
testing comprising of chest X-ray, skin testing by Mantoux test, AFB testing of
sputum smears and biopsies followed by culture confirmation. The latter is the gold
standard but it is time consuming. Many patients in endemic areas are never
diagnosed and in several cases, death due to disease progression results (Brodie &
Schulger, 2005). Modern diagnostic methods like PCR, ELISA, and more rapid ways
to detect the positive cultures are being developed.

Preventive measures like vaccination and early diagnosis will help in the
control of this disease. Vaccination with *M. bovis* BCG (see section 1.2.4 below),
however has not been promising. The variable efficacy of the BCG vaccine and the
 genetic heterogeneity between strains demands that a better vaccine be developed for
the prevention of this disease (Agger & Andersen, 2001).

1.2.4. Vaccines: BCG as a vaccine

The BCG vaccine, derived from the virulent *M. bovis* is the only vaccine
available for tuberculosis. The efficacy of this vaccine is however controversial. It is
given to infants soon after birth in countries where tuberculosis is endemic. The
efficacy of this vaccine in preventing adult pulmonary tuberculosis is low, as
concluded from the extensive 10-year follow-up trial by Tuberculosis Research
Centre (ICMR), Chennai of BCG vaccination in Chingleput (Tamil Nadu, India)
involving 360,000 individuals (Tuberculosis Research Centre, 1999). Several
researchers are making efforts to increasing the efficacy of BCG, by the cloning of
specific genes into BCG (Olsen & Andersen, 2003).
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1.2.5. Diagnosis of tuberculosis

1.2.5.1 AFB staining

AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because culture results become available only after 4-6 weeks. Over 90% of the tuberculosis cases occur in the developing countries, where clinical diagnosis of tuberculosis is based primarily on microscopic examination of smears for acid-fast bacilli and radiological testing. Table 1 shows the recommended grading of the smear testing.

**Table 1**: Quantitation scale recommended by the WHO and the International Union against Tuberculosis and Lung Disease

<table>
<thead>
<tr>
<th>Count</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No AFB observed</td>
</tr>
<tr>
<td>1-9/100 fields</td>
<td>Exact count</td>
</tr>
<tr>
<td>10-99/100 fields</td>
<td>1+</td>
</tr>
<tr>
<td>1-10/field</td>
<td>2+</td>
</tr>
<tr>
<td>&gt; 10/field</td>
<td>3+</td>
</tr>
</tbody>
</table>

A high bacterial load is needed in the specimen to render an AFB microscopy result positive. Smear-positive cases constitute only about 50% of pulmonary tuberculosis cases and the sensitivity of the test ranges from 22 to 78% of culture-proven cases in different studies (Kim et al., 2008). In addition, it is non-specific and does not confirm the disease diagnosis because of the detection of environmental mycobacteria other than *M. tuberculosis*.

1.2.5.2. Culture

Culture confirmation is the “gold standard” for tuberculosis diagnosis as it is specific and sensitive. However, this is time-consuming as *M. tuberculosis* has a long generation time; growth and subsequent biochemical analysis for species identification requires up to 6–8 weeks. Culture techniques, using conventional egg-
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based Lowenstein Jensen media have been estimated to detect as many as 10–1,000 viable mycobacteria per mL of specimen. Middlebrook media are also used extensively for culture in several labs.

The BACTEC TB-460 system was the first semi-automated system used. This is based on the liquid media for mycobacteria culturing and allows for culture confirmation within 7-10 days. It is however expensive and is not economical for screening all samples. Other commercial systems include the Mycobacteria Growth Indicator Tube (MGIT), Bact / Alert, ESP Mice, MB Redox and KRD "Niche B", biphasic Septic-Check AFB and Mice-Acid, and BACTEC MGIT960 systems.

1.2.5.3. Mantoux test-tuberculin skin testing

Tuberculin skin testing (TST), first introduced in 1890 is the oldest diagnostic test in use (Lee et al., 2002, Huebner et al., 1993). 0.1 mL of 5 TU (tuberculin unit) purified protein derivative (PPD) is injected intra-dermally on the forearm and the induration is measured after 48-72 hours. A positive reaction is indicated by erythema and induration of > 10 mm in size.

The TST attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to PPD, which is an unFractionated mixture of the culture filtrate proteins from the spent growth medium of *M. tuberculosis*. Infection with *M. tuberculosis* results in a cell mediated immune response giving rise to sensitized *T* lymphocytes (both CD4+ and CD8+) targeted to *M. tuberculosis* antigens. Stimulation by *M. tuberculosis* antigens causes these T-cells to release IFN-γ. The TST functions by eliciting this response in previously sensitized individuals. In such individuals, an intra-dermal injection of PPD evokes a delayed-type hypersensitivity response mediated by sensitized T-cells and results in cutaneous induration. The amount of PPD administered and the erythema produced varies from endemic region to a non-endemic region.

The antigens in PPD are shared among *M. tuberculosis*, *M. bovis* BCG, and several non-tuberculosis mycobacteria (NTM). As a result, the TST has lower specificity in populations with high BCG coverage and NTM exposure. The sensitivity may be low in individuals with depressed immunity (eg, AIDS and other immunosuppressive conditions, advanced tuberculosis, malnutrition). Despite these limitations, the test is still widely used because of its ability to predict active disease
in latently infected individuals. A major advantage of this test is its low material cost and it does not require any laboratory infrastructure.

1.2.5.4. Molecular methods

Using polymerase chain reaction, amplification of the insertion element IS6110 was first developed as an important molecular tool for the diagnosis of tuberculosis (Eisanech, 1994). This was fairly successful in developed countries, where the IS6110 element was present in multiple copies (from four to 20) in more than 95% of M. tuberculosis strains (Coros et al., 2008). However in India, this has not been successful as many of the clinical isolates either harbor single copy of IS6110 gene or it is absent (Krishnan et al., 2007). Other successfully used DNA regions include the 65 kDa hsp gene, the gene encoding the 126 kDa fusion protein, and the gene encoding the β-subunit of ribonucleic acid (RNA) polymerase; all of them are present in single copies in M. tuberculosis complex genomes (Baba et al., 2008, Negi et al., 2007).

The two commercially available molecular techniques approved by U.S. FDA are the Amplified Mycobacterium tuberculosis direct test (MTD test; Gen-Probe, San Diego, CA, USA) and the Cobas Amplicor M. tuberculosis assay (Roche Diagnostics, Mannheim, Germany). They show a sensitivity >95% and 100% specificity when sputum positive samples were used. The sensitivity was lower (83–85%) when the test was used for testing smear-negative specimens, though the specificity was high (99%) (Reischl et al., 1998). The use of radiometric systems in conjunction with nucleic acid probes has considerably reduced the detection time, as these procedures require a minimum of 1 week before a definitive laboratory diagnosis can be made. However, these techniques are too expensive and technologically complex for widespread application in laboratories in developing countries.

1.2.5.5. Tests based on secretory proteins

Antibody based tests

Assays based on the detection of antibody response to tuberculosis are alternatives to current methods for diagnosing active tuberculosis because they are simple, rapid, inexpensive and relatively non-invasive (Gennaro, 2000). Conventional immunoassays such as ELISA and Western blot employing CFPs have limits for
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widespread diagnostic use. Immuno-chromatographic assays, also called lateral-flow tests (or strip tests), which are extension of latex agglutination tests use secretory proteins of *M. tuberculosis* as antigens to detect antibodies in tuberculosis infected individuals. The benefits of immuno-chromatographic tests include, they are less time consuming, have long-term stability over a wide range of climates and are relatively inexpensive to make, so that they can be used in developing countries (Houghton *et al.*, 2002). A rapid immunochromatographic assay, the VetTB STAT-PAK test, was recently developed by Chembio Diagnostic Systems, Inc., Medford, New York, to detect antibodies of three isotypes (IgM, IgG, and IgA) against mycobacterial antigens in various host species. The test employs a unique cocktail of *M. tuberculosis* or *M. bovis* antigens and a blue latex bead-based signal detection system. The ready-to-use disposable device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with test antigen (Waters *et al.*, 2006). The important antigens employed in these assays include the 38 kDa protein (Bartoloni *et al.*, 2003), ESAT-6/CFP-10 complex and MPB 83 antigens (Lyashchenko *et al.*, 2007).

**Cell mediated immune response assays**

Different types of blood tests based on cell-mediated immune response have been suggested for diagnosis of tuberculosis. Most *M. tuberculosis* infected individuals mount a relatively strong CD4⁺ T-cell response to a broad spectrum of *M. tuberculosis* antigens. Assays were developed based on CD4⁺ T-cell response for use in diagnosis. Such assays were performed using the whole blood, purified PBMCs and stimulated *in vitro* with PPD, culture filtrate protein or defined *M. tuberculosis* antigens (Andersen *et al.*, 2000). The responses are monitored by measurement of the stimulation indices and / or production of IFN-γ. The important CFPs explored in T-cell responses include the Mtb 8.4, Mtb 9.8 (coller *et al.*, 1998), ESAT-6, CFP-10, (Arend *et al.*, 2000) Ag85 B (Nagai *et al.*, 1991) and MPT-64 (Johnson *et al.*, 1999). The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells specific for *M. tuberculosis* antigens. IFN-γ released *ex vivo* from these cells can be detected by ELISPOT (Lalvani *et al.*, 1998) (see section 1.3.6.1).
1.3. Culture filtrate proteins (CFPs)

1.3.1. Excretory - secretory antigens

Culture filtrate proteins (CFPs) are proteins released into the immediate environment by growing mycobacteria. Andersen and his group have done extensive work and divided CFPs of mycobacteria into three groups based on the antigens that are released (Andersen *et al.*, 1991). They are the secretory proteins, the excretory proteins and the cytoplasmic antigens. The excreted proteins are produced in large quantities into the medium by *M. tuberculosis* during the first few days of the culture. They accumulate in large quantities in the media and are present in minor quantities in intact bacilli. The secreted proteins are from the outer cell wall and they are gradually released during the growth of the bacilli. Short-term culture filtrate (ST-CF) is enriched in secreted antigens from *M. tuberculosis* (Andersen *et al.*, 1991). The concentration of the antigens increases steadily during the culture period. Cytoplasmic antigens are released from dead bacteria and they appear in high concentrations during the late logarithmic phase of the bacterial growth. The appearance of these proteins is related to the release of iso-citrate dehydrogenase (ICD), which is the indicator of autolysis.

1.3.2. Secretory proteome of *M. tuberculosis*

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the CFPs of *M. tuberculosis* followed by the identification of the separated proteins by protein sequencing has led to the creation of 2D-PAGE mycobacterial databases such as the Berlin database and Copenhagen database both displaying 2D images of both cellular and culture filtrate proteins. Table 2 lists the important CFPs identified in 2D-PAGE and their role.

**Table 2** – Secretory proteome of *M. tuberculosis* (modified from Okkels *et al.*, 2003)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Theoretical Mass (kDa)</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESAT-6 *</td>
<td>9.9</td>
<td>DTH</td>
</tr>
<tr>
<td>Ag 85A</td>
<td>35.7</td>
<td>Fibronectin binding surface protein</td>
</tr>
<tr>
<td>Ag 85B</td>
<td>34.6</td>
<td>Mycolyl-transferase surface protein</td>
</tr>
<tr>
<td>MPT 51</td>
<td>27</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>45-47 complex</td>
<td>45-47</td>
<td>DTH</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass (kDa)</th>
<th>Function</th>
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<tbody>
<tr>
<td>MPT 32</td>
<td>32</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>MPT 64</td>
<td>24.8</td>
<td>Vaccine</td>
</tr>
<tr>
<td>CFP 10*</td>
<td>10.8</td>
<td>Dimer with ESAT-6</td>
</tr>
<tr>
<td>TB 10.4*</td>
<td>10.4</td>
<td>DTH</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>38</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>TB 9.8*</td>
<td>9.8</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>Mtb 8.4*</td>
<td>10.9</td>
<td>Unknown</td>
</tr>
<tr>
<td>CFP 6*</td>
<td>12.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>PstS-1</td>
<td>38.2</td>
<td>Phosphate uptake Surface protein</td>
</tr>
<tr>
<td>SOD</td>
<td>23</td>
<td>Removal of free radicals</td>
</tr>
<tr>
<td>MPT 63</td>
<td>16.5</td>
<td>Unknown</td>
</tr>
<tr>
<td>Mtb 41</td>
<td>41.4</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* indicates proteins unique to *Mtb* complex, while others are also seen in other mycobacteria.

#### 1.3.3. CFPs as vaccine candidates

CFPs prepared from *M. tuberculosis* have been shown to be highly stimulatory to T cells of human tuberculosis patients (Demisse *et al*., 1999), mice (Olsen & Andersen, 2003) and cattle (Wedlock *et al*., 2005). Immunization of mice and guinea pigs with CFP from *M. tuberculosis* gave high levels of protection against aerogenic challenge with *M. tuberculosis* (McMurry *et al*., 2005). The potential of the CFPs led to their separation and characterisation, especially the short term culture filtrate (ST-CF) proteins (Okkels *et al*., 2003, Skjot *et al*., 2000). For eg., two narrow molecular mass fractions of ST-CF, containing molecules of low mass (<14 kDa) and medium mass (26–34 kDa) were particularly strongly recognized by cells from infected mice and patients with minimal tuberculosis (Boesen *et al*., 1995, Andersen *et al*., 1992). The immuno-dominant antigens in these pools were identified as ESAT-6 and the Antigen 85 complex respectively. The vaccine potential of these secretory antigens is studied either singly or as antigen pools (Dietrich *et al*., 2006).

Horwitz and coworkers (Horwitz *et al*., 1995) had proposed the following three hypotheses regarding the role of extracellular proteins of intracellular pathogens in protective immunity and the potential for their use in a subunit vaccine.

(i) Extracellular proteins play a key role in inducing cell-mediated immune responses that provide immuno-protection against these pathogens during natural infection.
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Such proteins, by virtue of their release by live organisms into their intracellular compartment in the host cell, are available for proteolytic processing and subsequent presentation on the surface of the infected host cell as MHC-bound peptide fragments. These surface-exposed fragments would allow the host immune system to recognize live pathogens sequestered within a host cell and to exert an antimicrobial effect against them. In particular, T-cells may activate the host macrophage, allowing it to inhibit multiplication of intracellular organisms, or they may lyse the host cell, thereby denying the pathogens an intracellular milieu in which to multiply.

(ii) Immunization of a naive host with extracellular proteins of intracellular pathogens—particularly in the case of pathogens such as *L. pneumophila* and *M. tuberculosis*, which reside within a phagosome rather than free in the cytoplasm in host cells would induce a population of lymphocytes capable of later recognizing and exerting an immune response against infected host cells. These lymphocytes would recognize infected host cells by identifying MHC-bound fragments of extracellular proteins displayed on the host cell surface consequent to the release of the proteins by the intracellular pathogen.

(iii) Among the extracellular proteins of intracellular pathogens, the ones released in greatest abundance will be among the most effective in inducing immuno protection. Such proteins, due to their abundance in the phagosome, would be processed and presented most frequently and therefore induce a particularly strong cell-mediated immune response.

1.3.4. Mechanism of secretion of mycobacterial CFPs

Bacterial protein secretion involves either of the two types of signal peptide-dependent translocations: the essential Sec-dependent pathway (Pugsley, 1993) and the Sec-independent pathway (Berks et al., 2000). The Sec Pathway is evolutionarily conserved and is thus a primary pathway for protein transport in most bacteria, including mycobacteria. Secreted proteins are produced as precursors containing signal sequences. Proteins secreted via the Sec pathway are accompanied to the membrane by SecB, a chaperone molecule. SecA, an ATPase required for translocation at the multisubunit translocase SecY-SecE-SecG, is found associated with both the signal sequence of secretable proteins, and also with the membrane at SecE-SecY-SecG. During translocation ATP is hydrolyzed, the protein is inserted across the membrane, the signal sequence is cleaved, and the protein is released.
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*Mycobacterium tuberculosis* has a functional Sec pathway. The genome sequence includes homologues of all Sec pathway components with the exception of SecB a protein not found in all Sec systems (Braunstein & Belisle, 2000). There are exported or secreted proteins synthesized with consensus Sec signal sequences (Lim et al., 1995). *M. tuberculosis* contains two homologues of SecA, namely the SecA1 and the secA2 (Fig. 5) (Braunstein & Belisle, 2000). SecA1 is a house-keeping gene and SecA2 is a non-essential gene. SodA of *M. tuberculosis* is secreted by the SecA2 mechanism (Braunstein et al., 2003).

![The Mycobacterial Sec Pathway: Two SecA Proteins](image)

**Fig. 5. SecA2-dependent export pathway in mycobacteria** (Braunstein & Belisle, 2000). The SecA protein plays a central role in this export pathway.

Sec-independent system was named TAT for twin arginine translocation because the precursor proteins engaged in this mechanism of export contain a conserved motif, S/T-R-R-X-F-L-K, with two contiguous arginine residues near the N terminus of the leader peptide. It was shown that in certain organisms, the TAT system was responsible for the export of a wide variety of substrates and, in particular, virulence factors (Dilks et al., 2003). The Tat pathway of *E. coli* includes at least four components: TatA, TatB, and TatE, predicted to be anchored to the cytoplasmic membrane via an N-terminal hydrophobic alpha-helix, and TatC, with six predicted transmembrane helices. In some prokaryotes such as *Bacillus subtilis*, only
tatA and tatC are present, suggesting that the minimal translocation system comprises TatA and TatC. The genome sequences of *M. tuberculosis* and *Mycobacterium leprae* revealed that both mycobacteria contained clearly identifiable *tatA, tatB, tatC*, and *tatD* genes which form functional Tat system. However, the *tatABCD* gene cluster identified in *E. coli* was not found in *M. tuberculosis*. It resembles *B. subtilis* by having a *tatAC* operon, with *tatB* and *tatD* located elsewhere (Saint-Joanis et al., 2006). *Mycobacterium tuberculosis* genes encoding homologues of TatA (Rv 2094c), TatC (Rv 2093c) and TatB (Rv 1224) are present in the genome. Substrates for the TAT pathway are synthesized as precursor proteins containing N-terminal signal sequences (Braunstein & Belisle, 2000) (Fig. 6). Phospholipase C (Rv2439c), localized in the cell wall is among the several substrates for TAT system (Raynaud et al., 2002).

1.3.5. CFPs of vaccine/diagnostic potential
1.3.5.1. Heat shock proteins and Ag85 complex

The heat shock proteins, Hsp10, Hsp60 and Hsp70 are strongly immunogenic and serve as important T cell antigens (Young & Garbe, 1991). The proteins of the antigen 85 complex (85A, 85B, and 85C) represent a major part of the secreted
proteins of *M. tuberculosis* and are most well studied (Wiker & Harboe, 1992). The three proteins in this complex are encoded by three genes located at different sites in the mycobacterial genome and show extensive cross-reactivity as well as homology at amino acid and gene levels (Wiker *et al*., 1990). The antigen 85 complex is the major secreted protein constituent of mycobacterial culture fluids, but it is also found in association with the bacterial surface. The antigens in this complex have been demonstrated to be associated with different functions like fibronectin binding and mycolyl transferase activity (Abou-Zeid *et al*., 1991, Ronning *et al*., 2000).

### 1.3.5.2. ESAT family

ESAT-6 and CFP-10 are two major targets of the immune response in infected individuals (McLaughlin *et al*., 2007). Now, it is known that several proteins sequence similarities are present with the identification of 23 ESAT-6 family members in *M. tuberculosis* strain H37Rv, which are located in 11 genomic loci and 25 in strain CDC1551, and their genes have been given genetic names *esxA*-W, with *esxA* encoding ESAT-6 and *esxB* encoding CFP-10.

The ESAT-6 gene is seen in the members of the *M. tuberculosis* complex (Harboe *et al*., 1996) and is absent in *M. bovis* BCG strains. The latter, by virtue of the loss of RD1 region encompassing the *esat-6 gene* does not elaborate the protein (Mahairas *et al*., 1996). The ESAT-6 and CFP-10 have been shown to form a 1:1 hetero-dimeric complex and adopt a stable fully folded structure, which is biologically active (Renshaw *et al*., 2002).

As ESAT-6 expression is restricted to the *M. tuberculosis* complex and not the vaccine strain *M. bovis* BCG and the majority of environmental mycobacteria, it is used as a diagnostic marker to differentiate between the BCG vaccinated healthy individuals and individuals with tuberculosis (Andersen *et al*., 2000). ESAT-6 stimulates T-cells from tuberculosis patients to proliferate and produce IFN-γ. The antigenicity of ESAT-6-CFP-10 has been utilized to develop diagnostic T-cell assays such as the skin test (Weldingh & Andersen, 2008). The combination of ESAT-6 and CFP-10 was found to be highly sensitive and specific for both *in vivo* and *in vitro* diagnosis. In humans, the combination had a high sensitivity (73%) and a much higher specificity (93%) than PPD (7%) (Van Pinxteren *et al*., 2000).

Mori *et al*., (2004) have demonstrated the use of two of these antigens, CFP-10 and ESAT-6, in a whole blood IFN-γ assay as a diagnostic test for tuberculosis in
BCG-vaccinated individuals. The results demonstrate that the whole blood IFN-γ assay using CFP-10 and ESAT-6 was highly specific and sensitive for *M. tuberculosis* infection and was unaffected by BCG vaccination status.

Humoral response to ESAT-6 has been demonstrated to have a diagnostic potential in tuberculosis patients, cattle and non-human primates. The data by Kanaujia et al. (2004) have demonstrated that use of synthetic peptides in lieu of the full-length ESAT-6 protein in diagnostic antibody detection assays.

### 1.3.6. Commercial tests based on CFPs

#### 1.3.6.1. Interferon-gamma (IFN-γ) tests

One of the current tests includes the assay of interferon-gamma (IFN-γ) released by blood cells in response to mycobacterial antigens. The first generation assay utilises PPD as antigen to stimulate the lymphocytes for IFN-γ production. The second generation assay uses *M. tuberculosis* specific antigens ESAT-6 and CFP-10. The third generation is also similar to second one with the inclusion of TB7.7 (Rv2654) to the earlier antigens. IFN-γ assays are used as an alternative to the TST in the form of a new type of *in-vitro* T-cell-based assay (Ewer et al., 2003). They are based on the principle that T-cells of individuals sensitised with tuberculosis antigens produce IFN-γ when they re-encounter mycobacterial antigens. IFN-γ assays are useful in various applications, such as diagnosis of active tuberculosis, distinguishing between non-tuberculous mycobacterial and *M. tuberculosis* infection, differentiating between *M. tuberculosis* infection and previous BCG vaccination (Pai et al., 2004). IFN-γ assay serves to assess vaccine efficacy, prediction of reactivation of the disease and monitoring treatment response (Barnes et al., 2004).

The commercial IFN-γ assay kits include the QuantiFERON-TB assay (Cellestis Limited, Carnegie, Victoria, Australia) and the T SPOT-TB assay (Oxford Immunotec, Oxford, UK). Both tests measure cell-mediated immunity by measuring IFN-γ released from T cells in response to tuberculosis antigens, using methods such as ELISA and enzyme-linked immunospot (ELISPOT) assay (Pai, 2005). The first generation QuantiFERON-TB is a whole-blood assay that measures IFN-γ response to PPD with ELISA. This test is approved by the US Food and Drug Administration (FDA), and is commercially available in many countries. The enhanced QuantiFERON-TB Gold assay utilises ESAT-6 and CFP-10. The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells
specific for *M. tuberculosis* antigens. IFN-γ released *ex vivo* from these cells can be detected by the sensitive ELISPOT (Lalvani *et al.*, 1998). Each such T cell gives rise to a dark spot and the read-out is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen *in vivo* and can rapidly release IFN-γ when re exposed to the antigen (Kaech *et al.*, 2002). The T-SPOT-TB assay employs ESAT-6 and CFP-10 as antigens and detects (by use of ELISPOT) the number of T-cells producing IFN-γ. Studies that used antigen cocktails with ESAT-6 and CFP-10 yielded sensitivity estimates higher than PPD-based assays (Brock *et al.*, 2004). The best combination of sensitivity and specificity was seen in studies with cocktails of RD1 antigens (QuantiFERON TB Gold and T SPOT-TB assays). Studies among individuals with suspected latent tuberculosis show that IFN-γ assays detect about 80% of this population (Adetifa *et al.*, 2007, Brock *et al.*, 2004.). IFN-γ assays were less affected by BCG vaccination than is the TST. Lalvani *et al.* (2001) studied the correlation between ELISPOT (using PPD and ESAT6) and BCG status in a contact investigation study and showed that the ESAT-6 based ELISPOT showed no correlation with BCG vaccination status whereas TST results were positive in BCG-vaccinated contacts.

1.3.6.2. Tests based on the detection of antibodies against CFPs

A 38 kDa protein antigen was demonstrated in the majority of the sera of the pulmonary tuberculosis individuals but absent in the normal controls (Espitia *et al.* 1989). This antigen is also included as a component in three different commercial immunochromatographic test kits (ICT Tuberculosis AMRAD-ICT (Amrad, Sydney, Australia), RAPID test TB, and PATHOZYME-MYCO), which detect TB in from 25 to 64% of patients with smear-negative and smear-positive TB (Perkins *et al.*, 2003). However the 38 kDa antigen was poorly recognized in by HIV- infected Tb patients. Weldingh *et al.*, (2005) identified immuno-dominant serological antigens using 35 recombinant antigens. TB9.7, TB15.3, TB16.3, and TB51 are four potential candidate antigens, which were recognized with recognition frequencies ranging from 31 to 93% with a specificity of 97%.
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1.4. Iron and mycobacteria
1.4.1. Role of iron in bacteria

Iron is essential as a co-factor for many enzymes involved in vital cellular functions ranging from respiration to DNA replication (Sritharan, 2000). It exists in two oxidation states, ferric Fe$^{3+}$ and ferrous Fe$^{2+}$ forms, with the oxidation reduction potential for the Fe$^{2+}$/Fe$^{3+}$ couple varying between +300 mV to −500 mV, which enables it to serve as a carrier molecule in the electron transport chain. Iron is biologically unavailable due to the inherent insolubility of the ferric iron. Though it was earlier thought that iron existed as insoluble Fe(OH)$_3$, it has been shown that it exists as Fe(OH)$_2^+$ at pH 7, with a solubility of approximately $1 \times 10^{-9}$ M (Chipperfield & Ratledge, 2000). At physiological pH free (Fe$^{3+}$) is limited to $10^{-18}$ M, whereas virtually all-living microorganisms require a minimum effective concentration of $10^{-8}$ M for growth. In mammals, 99.9% of iron is held as protein bound iron. Free iron is not readily available in the host as it is bound to high affinity iron binding proteins like the lactoferrin, transferrin and ferritin. The host limits the availability of iron as part of its innate defense mechanism, which is called nutritional immunity (Kohan, 1976).

1.4.2. Adaptation of bacteria to conditions of iron limitation

Bacteria have adapted to conditions of iron limitation by the elaboration of novel iron acquisition machinery (Ratledge, 2004; Sritharan, 2000). They elaborate two types of iron acquisition machinery, namely

1. Direct removal of the protein-bound iron by specific transferrin and lactoferrin receptors as seen in Neisseria (Genco & Desai, 1996).
2. Siderophore-mediated uptake: Siderophores are low molecular weight ligands with a high specificity for ferric iron. The ferric siderophores are then taken up by a receptor-mediated membrane bound protein.

1.4.3. Iron acquisition in mycobacteria

Iron is an obligate cofactor for at least 40 different enzymes encoded in the Mycobacterium tuberculosis genome (Cole et al., 1998). Mycobacteria are unique in that they produce two kinds of siderophores namely the intracellular mycobactins and the extracellular carboxymycobactins / exochelins. Two forms of mycobactin are produced, which differ in the length of an alkyl substitution and in polarity and
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solubility. The more polar form (carboxymycobactin) is released into the medium, whereas the less polar form (mycobactin) remains cell-associated (Ratledge, 1999). Carboxymycobactin has the capacity to remove iron that is bound to iron-binding proteins of the host (Gobin & Horwitz, 1996). Non-virulent mycobacteria such as *M. smegmatis* produce mycobactin as the intracellular siderophore and a peptidic siderophore known as exochelin (Ratledge & Ewing, 1996). The essential nature of siderophores for iron acquisition was demonstrated by the generation of an *mbtB* mutant *M. tuberculosis* strain, which is unable to produce either carboxymycobactin or mycobactin. This mutation impairs the ability of *M. tuberculosis* to replicate in low-iron media and in infected macrophages (De Voss *et al.*, 2000). Based upon the type of siderophore(s) expressed, mycobacteria can be classified into four groups, namely

1) Those producing mycobactin and both the types of extracellular siderophores such as *M. smegmatis*.

2) The strains producing mycobactin and carboxymycobactin, such as *M. tuberculosis*.

3) Those producing only the exochelins and no mycobactin, e.g. *M. vaccae*.

4) Those that not produce any siderophores e.g. *M. paratuberculosis*.

1.4.3.1. Mycobactins

These are found in the cell wall of mycobacteria and are extracted using organic solvents. Ferric ion in the mycobactin is reduced to ferrous ion and thus release of iron by mycobactin reductase in the presence of NAD(P)H. Ferrous ion has little affinity towards the mycobactin and then is inserted into the carrier molecule. Mycobactin belong to the mixed ligand type wherein they have two hydroxamate groups and the third pair being provided by an oxygen atom on the aromatic residue and nitrogen in the oxazoline ring. Snow (1970) elucidated the structures of several mycobactins isolated from different mycobacterial species. The structure of mycobactin is represented in Fig. 7.

Mycobactin from different mycobacterial species have the core nucleus that consists of a 2-hydroxyphenyloxazoline moiety linked by an amide bond to an acylated ε-N-hydroxylysine residue. The second ε-N-hydroxylysine is cyclised to form the seven membered lactam and is attached to β-hydroxyacid via an amide bond.
This, in turn is connected to the $\alpha$-carboxyl of the first lysine residue. Within this core, a methyl group may or may not be present at the $6^{th}$ position of phenolic ring and the $5'$ position of the oxazoline (Dover & Ratledge, 2000). Mycobactin chelates iron forming very stable complexes, the coordinating ligands being the two hydroxamate groups, the third pair being provided by the nitrogen atom of the oxazoline group and the hydroxyl group of salicylic acid. The long alkyl / alkenyl chain at R1 accounts for its hydrophobicity.

**Fig. 7. General structure of mycobactin.** (Snow, 1970). The side chains at R1 are usually long chain alkyl or alkenyl groups with a cis double bond at C2. Substituents at R2, R3 and R5 are usually H or CH$_3$ and at R4 could be simple CH$_3$, C$_2$H$_5$ or long chain alkyl groups.

*Mycobacterium tuberculosis* genome codes for a cluster of 10 genes (*mbt A-J*) called the *mbt* operon which is involved in the biosynthesis of mycobactin. They encode two polyketide synthetases (MbtC and MbtD), three peptide synthetases (MbtB, MbtE and MbtF), and isochorismate synthase (MbtI) (Quadri *et al*., 1998).

1.4.3.2. Carboxymycobactins

Carboxymycobactins were previously referred to as chloroform-soluble exochelins (due to their extractability into chloroform in their ferric form). Their structure has been elucidated in *M. avium* (Fig. 8) (Lane *et al*., 1995), *M. tuberculosis*.
(Gobin et al., 1995; Wong et al., 1996), *M. bovis* and *M. bovis* BCG (Gobin et al., 1999). They are expressed as a family of related molecules. They differ from the mycobactin in the side chain R1, terminating in either -COOH or -CO.CH3 that renders them more polar and thus water-soluble. They can solubilise iron from transferrin, lactoferrin and ferritin. The synthesis of carboxymycobactin is similar to mycobactin and differs from mycobactin after the formation of the common precursor. The final step in the synthesis of carboxymycobactin differs as the alkyl chain terminates in a carboxylic acid (Ratledge, 2004).

### 1.4.3.3. Exochelins

Exochelins are water-soluble, ornithine derived siderophores produced by non-pathogenic mycobacteria and well-characterised in *M. smegmatis* and *M. neoaurum* (Sharman et al., 1995a and 1995b) (Fig. 9 a, b). The exochelins are peptidyl in nature but involve D-amino acids and thus there are no conventional peptide bonds present (Ratledge & Dover, 2000). The coordination center with Fe$^{3+}$ is hexadendate in an octahedral structure involving the three-hydroxamic acid groups, which are from ornithine. The exochelin MS from *M. smegmatis* is a formylated pentapeptide derived from three molecules of δ-N-hydroxyornithine, β-alanine and a threonine (Fig. 9 a). Exochelin MN from *M. neoaurum* is a hexapeptide with two δ-N-hydroxyhistidines, providing the coordination center for iron chelation and two β-alanine and an ornithine (Fig. 9 b) (Sharman et al., 1995 a and 1995 b).
Identification of IREPs in mycobacteria

Several bacteria express iron regulated envelope proteins (IREPs) / Iron regulated membrane proteins (IRMPs) that function as receptors for ferric siderophores. Mycobacteria also express IREPs as part of the iron acquisition machinery (table 3) (Sritharan, 2000). Hall et al. (1987) first demonstrated the expression of IREPs in *M. smegmatis*. The 29 kDa IREP was found to be a potential receptor for the siderophore exochelin MS by specifically blocking its uptake with monospecific antibodies. This specificity was subsequently used to purify the 29 kDa protein using affinity chromatography (Dover & Ratledge 1996). Subsequently, IREPs were identified in other mycobacterial species not only under defined lab conditions of established iron status but also under *in vivo* conditions. *Mycobacterium avium* isolated from infected tissues of C57 black mice showed the expression of IREPs of 180, 29, 21, 14 kDa. *Mycobacterium leprae*, isolated from infected armadillo liver tissue showed the 21 kDa protein in the cell wall fraction. The expression of the IREPs in *in vivo* grown mycobacteria strongly suggests that an iron-
deficient environment exists under *in vivo* condition (Sritharan, 2000). Table 3 lists the IREPs identified in mycobacteria (Sritharan 2000).

**Table 3.** Iron-regulated envelope proteins in mycobacteria grown *in vitro* and *in vivo*. (Sritharan, 2000)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Expression of IREPs of size (kDa) with reference to iron status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low iron</td>
</tr>
<tr>
<td></td>
<td>180</td>
</tr>
<tr>
<td><strong>Defined iron status (in vitro-derived mycobacteria)</strong></td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>+</td>
</tr>
<tr>
<td><em>M. neoaurum</em></td>
<td>+</td>
</tr>
<tr>
<td>ADM 8563</td>
<td>-</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>+</td>
</tr>
<tr>
<td><strong>Undefined iron status (in vivo-derived mycobacteria)</strong></td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>+</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>-</td>
</tr>
<tr>
<td>+ and - denote the presence or absence of the protein</td>
<td></td>
</tr>
<tr>
<td>? denotes a very faint band.</td>
<td></td>
</tr>
</tbody>
</table>

**1.4.4. Global response of *M. tuberculosis* to iron availability**

In addition to expressing iron-uptake systems during iron deficiency, *M. tuberculosis* displays several changes in response to iron availability detected at the level of protein and mRNA. Differences in mRNA levels in bacteria subjected to iron deprivation versus those replicating in iron-rich medium were examined by Rodriguez *et al.* (2002) revealed 155 iron regulated genes. Iron deficiency induced about two-thirds of those genes, whereas the remainders were induced in iron-rich medium. Half of the genes induced under low iron conditions are of unknown function. The other half includes iron acquisition genes such as *mbtA–J*, the mbt-2 cluster and the irtAB operon in addition to genes encoding membrane proteins, members of the glycine-rich PE/PPE protein family, putative transporters and several genes encoding proteins involved in basic metabolism (Rodriguez *et al.*, 2002). High iron levels in the culture medium results in induction genes including *bfrA* and *bfrB*,
which encode putative iron-storage proteins (i.e. bacterioferritin and ferritin, respectively) and \( katG \), which encodes a catalase–peroxidase.

1.4.5. Iron-dependent regulation at molecular level

The adaptive changes that occur in response to iron availability are controlled in bacteria by regulating the expression of genes, whose regulation is controlled at the level of transcription. The control is mediated by iron-dependent transcriptional regulators (Bagg & Neilands, 1987). The ferric uptake regulator (Fur) and the diphtheria toxin repressor (DtxR) are two families of transcriptional regulators that control iron homeostasis in bacteria. Proteins of these two families are widely distributed in many bacteria and they are functional homologs. *Mycobacterium tuberculosis* contains two Fur-like proteins (FurA and FurB) and two members of the DtxR family (IdeR and SirR) (Cole *et al.*, 1998). FurA negatively regulates the expression of \( katG \) (the gene immediately downstream of furA), thereby modulating the response against oxidative stress (Pym *et al.*, 2001). The furB promoter is induced by Zn\(^{2+}\).

*Mycobacterium tuberculosis* relies on the iron-dependent regulator (IdeR) to maintain iron homeostasis. IdeR is a 230-amino acid protein that is closely related to the DtxR protein (Schmitt *et al.*, 1995). It functions as a homodimer and each monomer has three functional domains with two metal-binding sites. The N-terminal region contains the DNA binding domain, which has a helix-turn-helix motif, and the dimerization domain, which includes most of the metal-binding residues. The third domain, located in the C-terminal region, folds like a SH3 (Src homology domain 3) domain. This domain interacts with the two first domains and contributes two ligands for metal binding, which are required for stable dimer formation and full DNA binding activity. IdeR binds to the DNA as a double dimer with each dimer on opposite sides of the DNA (Rodriguez, 2006). IdeR can be activated *in vitro* by several metals but iron (the natural cofactor) is the optimal metal for IdeR function. The direct effect of IdeR on the transcription of iron sensitive genes correlates with its ability to bind to a 19 bp inverted-repeat consensus sequence (TTAGGTTAGGCTAACCTAA) which is present in the promoters of IdeR regulated genes (Gold *et al.*, 2001). In the presence of Fe\(^{2+}\), IdeR binds to the promoters of siderophore synthesis and iron-storage genes but it affects their expression in opposite ways. It represses transcription of the \( mbt \) genes, whereas it activates that of \( bfrA \) and
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*bfrB* (Fig. 10). IdeR combines with Fe$^{2+}$ and binds to specific sequences (iron boxes) in the promoter region of iron-regulated genes modulating their transcription (Fig. 10) (Rodriguez & Smith, 2003). In these conditions, IdeR–Fe$^{2+}$ down regulates iron uptake by repressing siderophore production and increases iron storage by activating transcription of *bfrA* and *bfrB* encoding bacterioferritin and ferritin.

In an indirect manner, IdeR also positively modulates protection against oxidative stress. In low-iron conditions, the IdeR–Fe$^{2+}$ complex is not formed, and IdeR-repressed genes are transcribed while iron storage genes are not expressed (Rodriguez & Smith, 2003).

1.4.6. Iron and virulence factors

Iron levels and expression of virulence factors have been demonstrated in many bacterial systems like *E. coli, C. diphtheriae, P. aeruginosa, V. cholerae*. (Salyers & Whitt 1994). Diphtheria toxin is encoded by the *tox* gene, carried only in those strains of *C. diphtheriae* lysogenized by beta and omega phages. The DtxR

![Fig. 10. Iron-dependent regulatory function of IdeR.](Rodriguez & Smith, 2003). When intracellular iron levels increase, IdeR combines with Fe$^{2+}$ and binds to specific sequences (iron boxes) in the promoter region of iron-regulated genes modulating their transcription.
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repressor, as the DtxR-Fe complex binds to the ± 10 region of the promoter and the transcription start site of the tox gene and blocks its transcription. Under low iron conditions, the repressor no longer binds and toxin production is increased. The toxin lyses the host cells and releases the iron from within these cells. It is thus clear that toxin production is part of the iron acquisition machinery of this pathogen. In *V. cholerae*, virulence is associated with the expression of two genes, the irgA and the hemolysin gene, whose expression is controlled by iron (Sritharan, 2000).

In *M. tuberculosis*, mycobactin is recognized as a virulence determinant (Quadri *et al.*, 1998; De Voss *et al.*, 1999, 2000). De Voss *et al.* (1999) by generation of mutants defective in the biosynthesis of mycobactin T, highlighted the importance of this siderophore not only for normal growth *in vitro* but also within the macrophages. Dussurget *et al.* (1996) showed an increase in sensitivity to hydrogen peroxide in IdeR mutants due to the decreased activity of catalase-peroxidase (KatG) and superoxide dismutase (SodA) activity, implying that IdeR is most likely to play a central role in oxidative stress. Manabe *et al.* (1999) by the introduction of a dominant Dtx (E175K) iron-dependent repressor into *M. tuberculosis* proved that the IdeR repressor controlled events influenced the virulence in a murine model of infection. Iron and *ideR* were reported to play a role in the expression of ESAT-6 proteins (Rodriguez *et al.*, 2002).
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Objectives of the study

In this study, the major objectives are to

I. Study the influence of iron levels on the protein profile of the secretory / excretory proteins of *Mycobacterium tuberculosis*: identification of iron-regulated culture filtrate proteins.

II. Assess the cell-mediated immune response of peripheral blood mononuclear cells from tuberculosis patients to defined culture filtrate antigen (CF-Ag) pools.

III. Screen serum samples of tuberculosis patients for antibodies against CF-Ags.