CHAPTER 1

REVIEW OF LITERATURE
1.1. Introduction

Tuberculosis is a disease of great public concern globally as it is one of the leading causes of death. There are 2-3 million deaths every year and latent tuberculosis persist in over a billion individuals worldwide (WHO report 2009). In addition, the emergence of multi drug-resistant tuberculosis (MDRTB) is of great concern. This can be attributed to the human immunodeficiency virus (HIV) epidemics as well as demographic and socio-economic factors such as poverty and malnutrition, which have served to maintain the reservoir of potential infections (Bloom & Murray, 1992). This alarming rise led the WHO to declare TB ‘a global emergency’ in 1993.

Robert Koch first identified *Mycobacterium tuberculosis* as the causative organism of tuberculosis in 1882; it was however 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). *Mycobacterium tuberculosis* is thus the etiologic agent of tuberculosis in humans and the closely related *Mycobacterium bovis* causes disease in cattle and livestock. These two species, along with the *M. marinum*, *M. canettii* and *M. africanum* comprise the *M. tuberculosis* complex. Other mycobacterial species, including *M. avium-intracellulare* complex (Girard et al., 2005) and *M. kansasii* (Canueto-Quintero et al., 2003) that are not normally the causative organisms in human tuberculosis, have been demonstrated to cause disease in immune-compromised individuals, as seen in HIV-infected people.

1.2 Classification of mycobacteria

Mycobacteria belongs to

- **Kingdom**: Bacteria
- **Phylum**: Actinobacteria
- **Order**: Actinomycetales
- **Family**: Mycobacteriaceae
- **Genus**: *Mycobacterium*

Mycobacteria are classified based on the production of pigments, they can be classified as scotochromogens (produce yellow pigment in the dark) for example *M.*
scrofulaceum, M. gordonae; photochromogens (produce an orange pigment in the light) for example M. kansasii, M. marinum and achromogens (do not produce any pigment) for example M. avium, M. intracellulareae and M. ulcerans. The cultivable members of the genus can be divided into two main groups on the basis of growth rate, they can be grouped into fast growers, which include M. fortuitum, M. kansasii, M. smegmatis and slow growers, comprising mostly the pathogenic mycobacteria, including M. tuberculosis, M. bovis and M. leprae.

1.3. 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. Mycobacterium and other closely related genera (i.e. Corynebacterium, Nocardia and Rhodococcus) have similar cell wall compounds and structure, and hence show some phenotypic resemblance (Gordon, 1966). Unique property of mycobacteria is the presence of lipid-rich cell wall. It 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.

The lipid rich cell envelope of mycobacteria 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. 1.1 (Kaiser, 2008). External to the membrane is peptidoglycan in covalent attachment to arabinogalactan, which in turn is attached to the mycolic acids with their long mero-mycolate and shorter alkyl-chains. This portion is termed the cell-wall core, the mycolyl arabinogalactan-peptidoglycan complex (MAPc). The mycolic acids unique to mycobacteria are long-chain fatty acids that are covalently bound to the arabinogalactan-peptidoglycan co-polymer; they are implicated in the formation of the inner layer of an asymmetric outer membrane while other lipids constitute the outer leaflet (Brennan & Crick, 2007, Brennan & Nikaido, 1995) (Fig. 1.1). The mycolic acids extend perpendicular to the arabinogalactan / peptidoglycan while other cell wall-associated glycolipids intercalate into the mycolic acid layer to form a ‘pseudo’ lipid bilayer.
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 alkyl-branches and mero-mycolate 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).

Fig 1.1. Schematic representation of the cell envelope of *Mycobacterium tuberculosis*. Peptidoglycan, arabinogalactan and mycolic acids are covalently linked together and form 60% of cell wall

1.4. Epidemiology of tuberculosis

Globally, there were an estimated 9.27 million cases of tuberculosis in the year 2007. This is an increase from 9.24 million cases in 2006, 8.3 million cases in 2000 and 6.6 million cases in 1990 (WHO report, 2009). Fig. 1.2 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 is two-fold higher in individuals with only HIV infection.

![Estimated TB incidence rates, by country, 2007](image)

Fig. 1.2. Estimated tuberculosis incidence rate (WHO report 2009)

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 new cases will be identified and the active disease will affect 200 million, with about 35 million deaths, if control measures are not significantly improved (WHO report, 2009). India is classified along with the sub-Saharan African countries with high burden tuberculosis. It ranks first in the five most tuberculosis prevalent countries from the estimated numbers of cases in the year 2007. India accounts for one-third of the global TB burden, with 1.8 million developing the disease each year and nearly 0.4 million dying due to TB annually (Chouhan, 2003).

Tuberculosis is not only a disease of humans, but also has a devastating effect on cattle and livestock. Bovine tuberculosis caused by *M. bovis* is a significant public health
problem and it causes great economic losses in countries with infected livestock. Despite the control measures, the incidence of bovine tuberculosis in some countries for example in New Zealand, United Kingdom and Republic of Ireland has remained the same or increased due to the presence of endemic wildlife reservoirs (Olsen & Anderson, 2003). Also, bovine tuberculosis remains a significant problem in developing countries; indeed more than 94% of the world population live in countries in which the control of bovine tuberculosis is either limited or completely absent (Vordermeier et al., 2006).

1.5. Pathogenesis of pulmonary tuberculosis

The development of pulmonary tuberculosis from its onset on its various clinical manifestations can be viewed as a series of battles between the host and the invading pathogen. The mode of infection of *M. tuberculosis* involves a sequence of events (Fig. 1.3). It begins with the inhalation of tubercle bacilli as droplets, released into the atmosphere from an infected individual / animal. 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 inhaled bacilli may multiply or it may be estimated by alveolar macrophages before any lesion is produced.

In lungs, the infected macrophages 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. Small caceous lesions may progress or may heal or stabilize before they are detectable by radiograph. 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. 1.3). Larger caceous lesions may grow locally and shed bacilli into the blood and lymph. 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).

Fig. 1.3. Infection with *Mycobacterium tuberculosis* follows a relatively well-defined sequence of events.

The host-pathogen interactions are dominated by the ability of the pathogen to prevent phago-lysosome biogenesis (Vergne et al., 2004), 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. Containment usually fails when the immune status of the host changes, which could be associated with essentially any condition that reduces the number, or impairs the function, of CD4+ T cells as seen in old age, malnutrition or co-infection with HIV. Following such a change in the immune status, the granuloma caseates (decays into a structure-less mass of cellular debris), ruptures and spread within
Review of Literature

The uncontrolled growth of *M. tuberculosis* inside the human host leads to infection. Human tuberculosis is divided into pulmonary and extra pulmonary tuberculosis based on the site of infection. Pulmonary tuberculosis is caused by infection of lungs and may also spread to other organs. The symptoms include cough, breathlessness, fatigue, fever and unintentional weight loss. Extra pulmonary tuberculosis is a disseminated infection occurs after the primary infection due to the immune status and nutritional deficiency of the individual. The granuloma cæcates, ruptures and bacilli infects different parts of the body like lymphatic system through blood stream causing military tuberculosis and to the brain causing tubercular meningitis.

1.6. Control measures

Control measures for tuberculosis include timely diagnosis, chemotherapy and preventive measures by vaccination.

1.6.1. Diagnosis

Existing diagnostic methods can detect up to 60% of tuberculosis cases but tuberculosis management is difficult as the existing diagnostic methods are time consuming. Clinical examination by radiological testing, chest X-ray, AFB, tuberculin skin test (Mantoux test) and biopsies are routinely done in several labs globally. AFB testing of sputum samples taken on three consecutive days are taken as confirmatory evidence. Culture confirmation is the “gold standard” for tuberculosis diagnosis as it is specific and sensitive. However, it is time-consuming and can take even 4-6 weeks as *M. tuberculosis* has a long generation time. Today, culture confirmation by radiometric BACTEC system MB / Bact Alert system take only a week and are superior to the conventional culture methods; however the cost and the lack of economic viability does not allow its use in all hospitals in developing countries.

Other diagnostic methods are being developed that offer the hope of fast and more accurate testing. These include PCR amplification of the insertion element IS6110, and QuantiFERON TB Gold and T SPOT-TB assays based on cell-mediated immune
response monitored by measurement of the stimulation indices and/or production of IFN-γ in response to mycobacterial proteins such as ESAT-6. These tests are more expensive and not affected by immunization or environmental mycobacteria, in turn generate fewer false positive results. The development of a specific, sensitive, rapid and inexpensive diagnostic test would be particularly valuable in the developing world.

1.6.2. Chemotherapy

Tuberculosis can be cured by chemotherapy in 95% of patients with active, drug sensitive pulmonary TB (Spigelman & Gillespie, 2006). It involves multi-drug therapy with a combination of three frontline drugs, isoniazid, rifampicin and pyrazinamide and one or more of the second-tier antibiotics including streptomycin, aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin. The treatment strategy for the complete elimination of active and dormant bacilli involves two phases; in the initial intensive phase, three or more drugs (isoniazid, rifampicin, pyrazinamide and streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria and the continuation phase, in which fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any residual bacilli to prevent, not only the recurrence of the disease but also prevent the development of drug resistant organisms.

1.6.2.1. Mode of action of Front line drugs

(a) Isoniazid

INH or isonicotinic acid hydrazide, was synthesized in the early 1900s but its anti-tubercular action was first detected in 1952 (Middlebrook, 1952; Bernstein et al., 1952). Both *M. tuberculosis* and *M. bovis* are susceptible to isoniazid in the range of 0.02 / 0.05 µg / mL (Heifets, 1994; Youatt, 1969). Isoniazid is bactericidal anti-tubercular drug and the most commonly prescribed for active infection and prophylaxis. INH enters the pathogen as a pro-drug and is activated by the catalase-peroxidase expressed by the pathogen. The peroxidase activity of the enzyme is necessary to activate INH to the active drug in the bacterial cell (Zhang et al., 1992) that blocks mycolic acid
biosynthesis, thereby disrupting the cell wall synthesis (discussed in detail in section 1.13.2).

(b) Rifampicin

Rifampicin, one of the front line drugs obtained from culture filtrates of *Streptomyces mediterranei*, was introduced in 1972 as an anti-tubercular drug (Woodley *et al*., 1972). Rifampicin is extremely effective against *M. tuberculosis*, (MIC 0.1-0.2 pg / mL) and its rapid bactericidal activity (Mitchison, 1985; Heifets, 1994) in combination with the other front line drugs helped to shorten the course of treatment against drug-susceptible infections. Rifampicin binds to the β-subunit of DNA-dependent RNA polymerase and blocks transcription, thereby killing the organism.

(C) Pyrazinamide

Pyrazinamide, a nicotinamide analog, was first discovered to have anti-tubercular activity in 1952 (Kushner *et al*., 1952). The MIC for pyrazinamide varies from 8 to 60 pg / mL depending on the assay method and media, and the drug is most active against cultures of *M. tuberculosis* at pH values below 6. It targets an enzyme involved in fatty-acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy (Somoskovi *et al*., 2001). However, during the first two days of treatment, it has no bactericidal activity against rapidly growing bacilli (Zhang & Mitchison, 2003). Pyrazinamide is a pro-drug, which is converted to its active form, pyrazinoic acid by the pyrazinamidase elaborated by the pathogen. The activity of PZA is highly specific for *M. tuberculosis*, as it has no effect on other mycobacteria.

(d) Ethambutol

Ethambutol is a front line drug used in combination with other drugs and is specific to mycobacteria. It inhibits arabinosyl transferase used for the synthesis of arabinogalactan involved in cell wall biosynthesis (Takayama & Kilburn, 1989). The inhibition of arabinogalactan biosynthesis by ethambutol could account for the accumulation of mycolic acids and their trehalose esters and affects the permeability of cell wall.
(e) Streptomycin

Streptomycin, an aminocyclitol glycoside, is an alternative first line anti-tubercular drug recommended by the WHO (Cooksey et al., 1996). It interacts with the 16S rRNA and S12 ribosomal protein (Escalante et al., 1998 & Finken et al., 1993), resulting in the misreading of the mRNA and inhibition of protein synthesis.

Due to the emergence of drug resistant organisms attributed mainly due to the inconsistency in the administration of the drugs, WHO initiated “directly observed therapy short-course” (DOTS). This is currently being adopted in 119 countries including all 22 high burden countries that contain 80% of all estimated cases (Collins & Kaufmann, 2001). India has the second largest DOTS programme in the world in population coverage. However, India's DOTS programme is the fastest expanding programme, and the largest in the world in terms of patients initiated on treatment, placing more than 100,000 patients on treatment every month and about 70% case detection is achieved (RNTCP report, 2009).

Subsequently, DOTS-Plus program included second tier anti-tubercular drugs in the treatment strategy (WHO report, 2006). The recent aim of the Stop TB Partnership’s Global Plan to Stop TB program (WHO report, 2009) includes six major components, pursue high-quality DOTS expansion and enhancement; address TB / HIV, MDR-TB and the needs of poor and vulnerable populations; contribute to health system strengthening based on primary health care; engage all care providers; empower people with TB and communities through partnership; and enable and promote research.

1.6.3. Vaccines: BCG as a vaccine

*Mycobacterium bovis* BCG derived, as an attenuated organism from the virulent *M. bovis* is the only vaccine available for tuberculosis. The efficacy of this vaccine is controversial as there are varied reports on the protection afforded by BCG. It is given to infants soon after birth in countries where tuberculosis is endemic. The efficacy of BCG vaccination in preventing adult pulmonary tuberculosis was found to be low, as concluded from the extensive 10-year follow-up trial in Chingleput (Tamil Nadu, India).
In the developed countries like USA and UK, mass immunization with BCG is not implemented, as it is believed to interfere with the interpretation of the tuberculin test.

1.7. Advances in mycobacterial research: Development of genetic tools to manipulate mycobacteria and Whole genome sequencing

Until recently, the progress in mycobacterial metabolism was slow as it was difficult to genetically manipulate the bacteria. However, significant advances have been made in the genetic manipulation of mycobacteria. Also, whole genome sequencing of several mycobacterial genomes has been done after the first genome sequencing of the pathogenic *M. tuberculosis* H37Rv (Cole et al., 1998). They include the sequencing of *M. bovis* (Garnier et al., 2003), *M. bovis* BCG (not published), *M. leprae* (Cole et al., 2001), the comparative genomics study, may provide an insight into their virulence.

The genome of *M. tuberculosis* (Fig. 1.4) comprises of 4,411,529 bp, contains 3924 genes (Cole et al., 1998). The general classification of *M. tuberculosis* annotated genes is presented in Table 1.1. The *M. tuberculosis* genome has some unusual features like the number of genes involved in fatty acid metabolism and the presence of unrelated PE and PPE families of acidic, glycine rich proteins.

![Circular map of the chromosome of M. tuberculosis H37Rv](Cole et al., 1998)

**Fig. 1.4. Circular map of the chromosome of M. tuberculosis H37Rv.** The outer circle shows the scale in mega bases, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others
are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red.

### Table 1.1. General classification of annotated *M. tuberculosis* genes

<table>
<thead>
<tr>
<th>Function</th>
<th>No. of genes annotated</th>
<th>% of total genes</th>
<th>% of total coding capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid metabolism</td>
<td>225</td>
<td>5.7</td>
<td>9.3</td>
</tr>
<tr>
<td>PE &amp; PPE proteins</td>
<td>167</td>
<td>4.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Cell wall &amp; cell process</td>
<td>517</td>
<td>13</td>
<td>15.5</td>
</tr>
<tr>
<td>Information pathways</td>
<td>877</td>
<td>22</td>
<td>24.6</td>
</tr>
<tr>
<td>Regulatory proteins</td>
<td>188</td>
<td>4.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Virulence, detoxification and adaptation</td>
<td>91</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>IS elements and bacteriophages</td>
<td>137</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Conserved hypothetical function</td>
<td>911</td>
<td>22.9</td>
<td>18.4</td>
</tr>
<tr>
<td>Unknown function</td>
<td>607</td>
<td>15.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Stable RNAs</td>
<td>50</td>
<td>1.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Comparative sequence analysis of orthologous genes (genes that perform the same function) from different bacteria is the basis of evolutionary relatedness. However, species’ phylogenies based on the comparison of single genes are often inconsistent. This is due to the high rate of horizontal gene transfer in bacteria (Sassetti & Rubin, 2002). Comparative genomics presents an attractive tool for evolutionary analysis of strain relatedness, as whole genomes can be examined rather than just individual genes (Gordon *et al.*, 1999).

### 1.8. Comparative genomics of the *M. tuberculosis* complex
The *M. tuberculosis* complex contains 5 pathogenic species that share identical 16S rRNA sequences and over 99.9% nucleotide identity (Sreevatsan et al., 1997; Garnier et al., 2003). They include *M. tuberculosis*, *M. africanum*, *M. microti*, *M. canetti* and *M. bovis*. The members of *M. tuberculosis* complex differ in terms of their host range, phenotype and virulence for humans (Brosch et al., 2000a).

Comparative genomics has identified at least 18 variable regions ranging from 0.3 kb to 12.7 kb, which are present in *M. tuberculosis* and not in BCG (Fig. 1.5). RD1 is the only region that is absent from all BCG strains but present in virulent *M. bovis* and *M. tuberculosis* strains (Brosch et al., 2000; Gordon et al., 1999; Mahairas et al., 1996). RD2, another variable region (Rv1978- Rv1988c) is a recent deletion restricted to BCG strains derived since 1927, and includes genes coding for a variety of functions including methyl transferases, permeases, ribonucleotide reductase, a regulatory protein and a secreted protein, namely MPT64. The RD3 locus is a prophage (phiRv1) and RD4 encodes enzymes involved in the biosynthesis of lipopolysaccharides and both are absent from *M. bovis* and *M. bovis* BCG strains. RD5 contains eight ORFs, three of them encode phospholipase C enzymes (*plcA*, *plcB*, *plcC*), and the remaining five encode proteins, belonging to the Esat-6 and PPE families respectively (Gordon et al., 1999). The RD6 region varies with the *M. tuberculosis* complex members and essentially consists of PPE proteins and IS1532. The RD7 region contains one of the 4 *mce* operons that encode invasin-like proteins required for *M. tuberculosis* (Arruda et al., 1993). The effect of the loss of *mce3* on virulence is not known, but it was suggested that the remaining three *mce* operons could balance for any lost activity (Gordon et al., 1999). The RD8 region contains genes belonging to the ESAT-6 family, PE and PPE families and an *ephA* gene that encodes epoxide hydrolase (Brosch et al., 2000; Gordon et al., 1999). The RD9 contains genes encoding for an export protein, oxidoreductase, and a pre-corrin methyltransferase that is involved in cobalamin biosynthesis (Gordon et al., 1999).

The RD10 encompasses the genes encoding for an enoyl CoA hydratase and an aldehyde dehydrogenase. RD 11, 12 and 13 were absent in both *M. bovis* and *M. bovis* BCG, while the RD 14, 15 and 16 were restricted to few members of BCG (Brosch et al., 2002). These regions can be accessed for the potential to differentiate between *M.*
**tuberculosis** and *M. bovis* / *M. bovis* BCG. Though the role of these deletions in strain differentiation is unclear, they can be applied to propose a new evolutionary scenario for the members of the *M. tuberculosis* complex (Brosch et al., 2002). The authors analysed the distribution of the 20 variable regions in a total of 100 strains belonging to *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. microti*, *M. bovis* and *M. bovis* BCG. Their study showed that *M. bovis* had undergone several deletions compared to *M. tuberculosis* (Fig. 1.5) (Brosch et al., 2001). The characteristic deletions among the BCG strains are represented on Table 1.2.

![Diagram of the proposed evolutionary pathway of the *M. tuberculosis* complex](image)

(Brosch et al., 2002)

**Fig. 1.5.** Scheme of the proposed evolutionary pathway of the *M. tuberculosis*: illustrating successive loss of DNA in certain lineages.
Table 1.2. Characteristics of deletions from BCG (Mostowy et al., 2003)

<table>
<thead>
<tr>
<th>BCG strain</th>
<th>Deleted regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Russia</td>
<td>RD1, RD Russia</td>
</tr>
<tr>
<td>Moreau</td>
<td>RD1, RD1</td>
</tr>
<tr>
<td>Japan</td>
<td>RD1</td>
</tr>
<tr>
<td>Sweden</td>
<td>RD1</td>
</tr>
<tr>
<td>Birkhaug</td>
<td>RD1</td>
</tr>
<tr>
<td>Prague</td>
<td>RD1, RD2</td>
</tr>
<tr>
<td>Glaxo</td>
<td>RD1, RD2, RD Denmark / Glaxo</td>
</tr>
<tr>
<td>Denmark</td>
<td>RD1, RD2, RD Denmark/ Glaxo</td>
</tr>
<tr>
<td>Tice</td>
<td>RD1, RD2, nRD18</td>
</tr>
<tr>
<td>Connaught</td>
<td>RD1, RD2, nRD18, RD8</td>
</tr>
<tr>
<td>Frappier</td>
<td>RD1, RD2, nRD18, RD8, RD Frappier</td>
</tr>
<tr>
<td>Phipps</td>
<td>RD1, RD2, nRD18</td>
</tr>
<tr>
<td>Pasteur</td>
<td>RD1, RD2, nRD18, RD14</td>
</tr>
</tbody>
</table>

1.8.2. Classification of BCG strains

Genetic variations among the BCG strains were due to the changes occurred during the continuous passages of pathogenic strain *M. bovis* (Behr & Small, 1997) and also lack of standardized growth storage procedures that gave rise to several BCG strains, found today in several geographical locations worldwide (Osborn, 1983). Based on the genetic variations among the BCG strains, they are classified into two major groups. BCG Japan, Moreau, Russia, and Sweden secrete large amounts of the MPB70 gene, have two copies of the insertion sequence IS6110, and contain methoxymycolate and MPB64 genes. In contrast, BCG Pasteur, Copenhagen, Glaxo and Tice secrete little MPB70, have a single copy of the insertion sequence IS6110, and do not contain the methoxymycolate and MPB64 genes (Ohara, 2001) (Fig. 1.6).
Fig. 1.6. Genealogy of BCG vaccine strains based on historical data (Behr MA, 2002). The results of analysis for the \textit{mpt64} gene and the number of IS6110 copies are shown at the bottom. This analysis could suggest that the original BCG had \textit{mpt64} and 2 copies of IS6110; one copy of IS6110 was lost around 1925, and \textit{mpt64} was lost between 1927 and 1931 (Behr, 2002).

1.9. Host-pathogen interactions with specific reference to iron acquisition

Iron is the second most abundant metal after aluminum and the fourth most abundant element in the earth’s crust. It is an important micronutrient for all bacteria except lactobacilli, is a co-factor for several enzymes involved in vital cellular functions ranging from respiration to DNA replication (Sritharan, 2000). It exists in the two oxidation states, \( \text{Fe}^{3+} \) and \( \text{Fe}^{2+} \), with the oxidation-reduction potential for the \( \text{Fe}^{2+} / \text{Fe}^{3+} \) couple varying between +300 mV to –500 mV, which enables it to serve as a carrier molecule in the electron transport chain. However, it is insoluble at biological pH and exists as insoluble ferric hydroxides and oxyhydroxides. At physiological pH 7.0, the major form of iron is \( \text{Fe(OH)}_2^+ \) (and not \( \text{Fe(OH)}_3 \) as thought earlier) with a solubility of approximately 1.4 \( \times 10^{-9} \) M (Chipperfield & Ratledge, 2000) that is too low to support the growth of microorganisms (requiring \( 10^{-7} \) M iron).

Nature has perhaps made iron highly insoluble, as excess iron is toxic, due to its catalytic role in the Fenton reaction, resulting in the formation of free radicals (Sritharan, 2000). Pathogenic bacteria face additional iron deprivation as 99.9% iron is held as protein-bound iron within the mammalian host; it is held by transferrin and lactoferrin (extracellular fluids) and by ferritin (storage) (Bullen & Griffiths, 1999). The ability of a
pathogen to acquire iron from the mammalian host determines the outcome of an infection; the balance between the ability of a mammalian host to withhold iron from the invading microorganisms and the ease with which the latter can acquire this iron from the host is critical. Limitation of this essential nutrient is one of the innate immune defense mechanisms of the mammalian host and is referred to as ‘nutritional immunity’ by Kochan (1976).

1.9.1. Bacterial adaptations to iron-limitation

Microorganisms, including mycobacteria have adapted to conditions of iron-limitation by the elaboration of novel iron acquisition machineries (Ratledge and Dover 2000; Sritharan, 2000; Ratledge, 1999; De Voss et al., 1999). They are well studied in E. coli and several gram-negative organisms. Two common mechanisms of iron acquisition include (a) siderophore-mediated acquisition and (b) direct acquisition via specific receptors from host iron-containing molecules like hemin, transferrin and lactoferrin etc.

a. Siderophore mediated iron acquisition machinery

Siderophores are low molecular weight (500-1000 Da) Fe$^{3+}$-specific high affinity molecules with binding affinity constant $K_s$ ranging from $10^{-22}$ to $10^{-50}$ and can remove iron from the insoluble Fe(OH)$_3$ and from host-iron binding compounds, but not from heme proteins. As the Fe$^{3+}$-siderophore complex is greater than 600 Da, uptake of these molecules is a receptor-mediated process. Many of these iron transport receptors are multi-functional and mediate the transport of other molecules that include vitamin B12 and certain colicins. A vast majority of bacteria elaborate this mechanism of iron acquisition and mycobacteria also employ siderophores to acquire iron.

b. Direct acquisition

Bacteria can acquire protein-bound iron by elaborating specific cell surface receptor proteins for transferrin, lactoferrin, heme and haemoglobin (Braun & Killmann, 1999). Lactoferrin and transferrin receptors have been demonstrated in pathogenic Neisseria (Genco & Desai, 1996), Pasteurella spp. (Gray-Owen & Schryvers, 1996), Haemophilus influenzae (Herrington & Sparling, 1985), Pseudomonas aeruginosa (Sriyosachati et al., 1986), Bordetella spp. (Redhead et al., 1987), Helicobacter pylori
Review of Literature

(Husson et al., 1993), *Staphylococcus aureus* (Park et al., 2005), and *Candida albicans* (Knight et al., 2005).

1.9.2. Iron-regulated membrane proteins (IRMPs)

Siderophores and their receptors, the iron-regulated membrane proteins (IRMPs) are extensively studied in *E. coli* (Griffiths & Chart, 1999). Six siderophore-mediated iron-transport systems have been demonstrated; FhuA, FepA and FecA functioning as receptors for ferrichrome, ferric enterobactin and ferric citrate respectively have been crystallized and uptake of Fe$^{3+}$ via these receptors are well studied. Some of the IRMPs demonstrated in other bacterial system are represented in Table 1.3.

Table 1.3. Bacterial siderophores and their receptors, the iron-regulated membrane proteins (IRMPs)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Siderophore</th>
<th>Iron-regulated membrane proteins</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Ferrichrome</td>
<td>FhuA (Coulton et al., 1983)</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Enterobactin</td>
<td>FepA (McIntosh &amp; Earhart, 1977)</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Ferricitrate</td>
<td>FecA (Wagegg &amp; Braun, 1981)</td>
<td>80.5</td>
</tr>
<tr>
<td></td>
<td>Aerobactin</td>
<td>CirA (Curtis et al., 1988)</td>
<td>74</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>Yersiniabactin</td>
<td>FyuA (Rakin et al., 1994)</td>
<td>71.4</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pyochelin</td>
<td>Ferri-pyochelin receptor (Sokol &amp; Woods, 1983)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Pyoverdin</td>
<td>Ferri-pyoverdin receptor (Meyer et al., 1990)</td>
<td>80</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Vibriobactin</td>
<td>ViuA (Butterton et al., 1992)</td>
<td>74</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>Ornibactin</td>
<td>OrbA (Sokol et al., 2000)</td>
<td>81</td>
</tr>
<tr>
<td><em>Bordetella spp.</em></td>
<td>Alcaligin</td>
<td>FauA (Brickman &amp; Armstrong, 1999)</td>
<td>79</td>
</tr>
</tbody>
</table>
1.9.3. Regulation by iron at the molecular level

Intracellular iron regulates the expression of the components of the iron-acquisition machinery, as demonstrated in *E. coli* (Griffiths & Chart, 1999). A 17 kDa regulator molecule Fur (Ferric Uptake Regulator encoded by ‘fur’ gene) and the Fur – Fe$^{2+}$ complex binds to a region called the ‘Fur’ or iron box (consensus sequence 5’-GATAATGATAATCATTATC -3’) located upstream of the start point of the genes encoding the iron acquisition machinery (Braun et al., 1998). When iron is limiting, the repressor molecule, on its own does not bind to the iron box, thereby resulting in the induction of expression of components of the iron acquisition machinery (Fig. 1.7). The Fur repressor has been identified as a member of Gram-negative bacteria, including *Bordetella spp.*, *Haemophilus influenza*, *Legionella*, *Neisseria spp.*, *Pseudomonas spp.*, *Vibrio spp.*, etc. The corresponding homologue of Fur in Gram-positive bacteria is DtxR and was first identified in *C. diphtheriae* (Boyd et al., 1990). DtxR is slightly larger than Fur, approximately 25 kDa to 17 kDa and they have very less amino acid homology. DtxR and Fur also differ in the specificities of interaction with different of operators. The DtxR homologue identified in mycobacteria is IdeR and has been demonstrated in *M. smegmatis* and *M. tuberculosis*.

**Fig. 1.7. Iron as a regulatory molecule.** When iron is plentiful, the inactive repressor binds to the co-repressor Fe$^{2+}$ and the resulting complex binds as a dimer, thereby blocking transcription.
1.9.4. Siderophore-mediated iron acquisition machinery in mycobacteria

Mycobacteria are unique in that they produce two kinds of siderophores, namely the intracellular mycobactins and the extracellular carboxymycobactins / exochelins. Pathogenic mycobacteria express mycobactin and carboxymycobactin while the saprophytic mycobacteria express mycobactin and exochelin predominantly though carboxymycobactin have been identified in small concentrations in *M. smegmatis* (Ratledge & Ewing, 1996). Mycobactin is hydrophobic and is located in the cell wall, while the more polar carboxymycobactin is released into the medium (Ratledge, 1999), Based upon the type of siderophore(s) expressed, mycobacteria can be classified into four groups, namely those

1. expressing mycobactin and carboxymycobactin, eg. *M. tuberculosis*.
2. expressing mycobactin, carboxymycobactin and exochelin, eg. *M. smegmatis*.
3. expressing only the exochelins and no mycobactin, e.g. *M. vaccae*.
4. that do not produce any siderophores and require the addition of exogenous mycobactin for *in vitro* growth e.g. *M. paratuberculosis*.

1.9.4.1. Mycobactins

Mycobactins are intracellular hydrophobic siderophores localized in the lipid-rich cell wall. They have high affinity for Fe$^{3+}$ (Ks of ca. 10$^{36}$) with low binding to Fe$^{2+}$. They 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 moiety. Snow (1970) elucidated the structure of mycobactin and performed extensive analyses of mycobactin from different mycobacterial species and showed that they can be used as chemotaxonomic markers. The yield of mycobactin varies among the species, with *M. smegmatis* expressing up to 10% of the cell dry weight while *M. kansasii* produces only about 0.05% of the cell dry weight.

All mycobactins have the same core nucleus that consists of a 2-hydroxyphenyloxazoline moiety linked by an amide bond to an acylated ε-N-hydroxylysine residue (Fig. 1.8). 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 α-carboxyl of the first lysine residue. Within this core, a methyl
group may or may not be present at the 6th position of phenolic ring and the 5’ position of the oxazoline (Gobin et al., 1995). The variation in the structure occurs in the alkyl substituents of the hydroxyacids (R3 and R4) and the acyl moiety R5. In general, the R5 group is a long chain fatty acid that is unsaturated and with an unusual cis double bond conjugated to the carbonyl group. Variation in R groups of mycobactins from different mycobacterial species is represented in the Table 1.4.

![General structure of mycobactins](image)

Fig. 1.8. General structure of mycobactins. Three pairs of iron chelating sites are represented in red colour. Variations in the R groups among the mycobacterial species are represented in the table below.

Table 1.4. Variations among the mycobactins produced by different mycobacterial species

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mycobactin</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. aurum</td>
<td>A</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>C₁₃</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>F</td>
<td>CH₃ / H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>C₉-₁₇</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>C₁₇-₁₉</td>
</tr>
<tr>
<td>M. marinum</td>
<td>M</td>
<td>H</td>
<td>CH₃</td>
<td>C₁₅-₁₈</td>
<td>CH₃</td>
<td>C₁</td>
</tr>
<tr>
<td>M. marinum</td>
<td>N</td>
<td>H</td>
<td>CH₃</td>
<td>C₁₅-₁₈</td>
<td>CH₃</td>
<td>C₂</td>
</tr>
<tr>
<td>M. phlei</td>
<td>P</td>
<td>CH₃</td>
<td>H</td>
<td>CH₂CH₂</td>
<td>CH₃</td>
<td>C₁₅-₁₉</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>C₉-₁₉</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>T</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>C₁₇-₂₀</td>
</tr>
<tr>
<td>M. avium</td>
<td>Av</td>
<td>H</td>
<td>CH₃</td>
<td>CH₂CH₂</td>
<td>CH₃</td>
<td>C₁₁-₁₄,₁₈</td>
</tr>
</tbody>
</table>
1.9.4.2. Carboxymycobactins

The carboxymycobactins are structurally related to mycobactins. The lipophilicity of the mycobactins due to the long chain acyl group at R5 is replaced either by –COOH / -COOCH₃ thus rendering it water-soluble. These are produced mainly by the pathogenic mycobacterial species (Gobin et al., 1999), but they also have been detected in small quantities in the non-pathogenic *M. smegmatis* (Ratledge & Ewing, 1996).

![Structure of carboxymycobactin of *M. tuberculosis*](image1)

Fig. 1.9. (a). Structure of carboxymycobactin of *M. tuberculosis* (Gobin et al., 1995). (b) HPLC purification of carboxymycobactins from culture supernatant of *M. tuberculosis* (Gobin & Horwitz, 1995).

Their structures have been elucidated in *M. avium* (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 differ from the mycobactin in the side chain at the R positions (R positions as represented in Table 1.4. Mycobactins (Ratledge & Ewing, 1978) and carboxymycobactins (Lane et al., 1995) are usually a mixture of closely related molecules that differ in the length of the acyl groups at R5; for example, 17 different mycobactins from *M. smegmatis* was demonstrated by HPLC analysis. They are expressed as a family of related molecules (Fig. 1.9b) and differ marginally from each other, due to varying levels of esterification of the COOH group, as evident by HPLC analysis (Barclay et al., 1986).

### 1.9.4.3. Exochelins

Exochelins are water-soluble, peptide siderophores produced by non-pathogenic mycobacteria and well characterized in *M. smegmatis* and *M. neoaurum* (Sharman et al., 1995a & 1995b). They are small peptides (5-10 a.a) consisting of D-amino acids, predominantly ornithine and do not contain conventional peptide bonds (Ratledge & Dover, 2000).

![Structure of exochelins MS and MN](Sharman et al., 1995a & b)

**Fig. 1.10. Structure of exochelins MS and MN.** Exochelin MS (a), from *M. smegmatis* is formyl-D-ornithine 1 β-alanine-D-ornithine 2-D-allo threonine-L-ornithine-3 and exochelin MN (b) from *M. neoaurum* is L-threonine-β-hydroxy histidine-β alanine-β alanine-L-α methyl ornithine-L-ornithine-L-(cyclo)ornithine.
The coordination center with Fe$^{3+}$ is hexa-dendate; it is held in an octahedral structure involving the three-hydroxamic acid groups donated by ornithine. The exochelin MS from *M. smegmatis* is a formylated pentapeptide derived from three molecules of δ-N-hydroxyornithine, β-alanine and threonine (Fig. 1.10a). Exochelin MN from *M. neoaurum* is a hexapeptide with two δ-N-hydroxyhistidines (providing the coordination center for iron chelation), two β-alanine residues and an ornithine (Fig. 1.10 b). Till date, there are no reports on the expression of exochelins by pathogenic bacteria.

1.9.4.4. Biosynthesis of mycobactin / carboxymycobactin

Mycobactins are synthesised by the polyketide synthase / non-ribosomal peptide synthetases (NRPs) strategy. The different enzymes involved in mycobactin and carboxymycobactin synthesis are encoded with the genes in the *mbt* operon in the *M. tuberculosis* genome (Cole *et al*., 1998). The *mbt* operon (Fig. 1.11) includes a cluster of 10 genes (*mbt A-J*) referred to as *mbt*-1 cluster (Quadri *et al*., 1998) involved in the synthesis of mycobactin core and the *mbt*-2 cluster which includes 4 genes *mbt K-N* shown to be Rv1347c, Rv1344, *fadD3*, and *fadE14* respectively (Krithika *et al*., 2006), which is involved in the incorporation of lipophilic aliphatic chain. However most of the aspects of the biosyntheses of mycobactin and carboxymycobactin outlined remain to be experimentally explored and validated.

![Fig. 1.11. Organisation of *mbt* operon in *M. tuberculosis* H37Rv genome.](Krithika *et al*., 2005)

Fig. 1.11. Organisation of *mbt* operon in *M. tuberculosis* H37Rv genome. Contains *mbt*-1 cluster and *mbt*-2 cluster involved in the biosynthesis of mycobactin and carboxymycobactin.
The proposed biosynthetic sequence followed the predicted pathway of assembly of mycobactin starting with the synthesis of salicylic acid. The \textit{mbt-1} cluster includes the genes for the enzymes that synthesize didehydroxymycobactin, the salicyl-capped non-ribosomal peptide-polyketide core scaffold of mycobactin and carboxymycobactin from the building blocks salicylic acid, serine or threonine, lysine, acetyl CoA and malonyl CoA. The salicylate moiety in \textit{M. smegmatis} is synthesised by the shikimate pathway, while the 6-methyl salicylate in \textit{M. tuberculosis}, is a polyketide metabolite synthesized by the condensation of four acetate units by the enzyme isochorismate synthase recently shown to be salicylate synthase (MbtI) (Hudson \textit{et al.}, 1970). MtbB, a NRPs is believed to activate serine, condense it with the salicylate moiety, and cyclize this product to a hydroxyphenyloxazoline. There are two other NRPSs, encoded by \textit{mbtE} and \textit{mbtF} that have the appropriate activation, condensation, and peptide carrier domains for donation of the two lysine-derived moieties of MB. Also in the gene cluster are \textit{mbtC} and \textit{mbtD}, which encode proteins that are homologous to polyketide synthases. The encoded proteins appear to contain the appropriate modules to produce the required \(\beta\)-hydroxybutyrate. Additionally, \textit{mbtG} encodes a protein that is homologous to known ornithine and lysine oxygenases, performs the N6-hydroxylation step to generate functionally important hydroxamate moieties. MbtF has a terminal domain that was assigned a role as either an epimerization domain or as a thioesterase responsible for releasing the MB from the enzyme by lactamization of the terminal hydroxy-lysine residue.

Thus, these seven genes, \textit{mbtA} to \textit{mbtG}, appear to encode sufficient activities for the biosynthesis of the core of the MBs (Fig. 1.12). There are two other proposed gene products, MbtH and MbtJ, to which no clear biochemical role has been assigned. The loaded building blocks are bound to their corresponding enzymes by a thioester linkage to the phosphopantetheinyl group, which would be added to the carrier protein domains by PptT (Quadri \textit{et al.}, 1998, Chalut \textit{et al.}, 2006). MbtK protein of \textit{mbt-2} cluster showed exclusive specificity to acylate at the \(\varepsilon\)-amino position, and the amino group at \(\alpha\)-position was not readily modified. Interestingly, this protein was able to acylate the \(\delta\)-position of ornithine amino acid and also catalyzed transfer of acyl chains on to two ornithine
residues (Krithika et al., 2006, Card et al., 2006) to yield mycobactin and carboxymycobactin. The unsaturation of the lipidic chain is produced by acyl-acyl carrier protein (ACP) dehydrogenase (MbtN) (Krithika et al., 2006). MbtG has a five-fold preference for acetylated lysine over lysine (Krithika et al., 2006) and didehydroxymycobactin has recently been isolated from *M. tuberculosis* (Moody et al., 2006) suggesting that hydroxylation takes place after didehydroxymycobactin assembly and release.

![Proposed scheme for mycobactin and carboxymycobactin biosynthesis](image)

**Fig. 1.12. Proposed scheme for mycobactin and carboxymycobactin biosynthesis.** Proposed biosynthetic cascade for mycobactin and carboxymycobactin catalyzed by Mbt locus.

De Voss and coworkers (2000) made a knock out mutant of *mbtB* that was unable to produce either mycobactin or carboxymycobactin. Failure to synthesize the siderophores resulted in drastic decrease in growth both under iron deficient medium and in macrophages giving the relevance of both the siderophores involved in the bacterial multiplication in iron limiting conditions and also intracellularly. It is also proposed that the structure of MbtI, a salicylate synthase is used to design the inhibitors of mycobactin
biosynthesis, which may be useful in the production of anti-tuberculosis drugs (Harrison et al., 2006).

1.9.4.5. Biosynthesis of Exochelin

The genes involved in the production of exochelin MS were first identified by Fiss et al. (1994). An exochelin-deficient strain of M. smegmatis was obtained by UV mutagenesis. The gene FxbA, encodes a protein with homology to formyl transferases was obtained by compliment analysis. The complementing fragment contains additional ORFs fxuA, fxuB and fxuC bearing homology to FepG, FepC and FepD of E. coli that are ferric-enterobactin permeases; the mycobacterial proteins are presumed to be associated with transport of exochelin. Yu and his group (1998) identified a 30 kb complementing fragment with a total of nine ORFs along with fxbA. The locus composed of (i) orf1 and orf2, encoding proteins homology to ABC transporters; (ii) fxbB and fxbC encoding proteins homology to nonribosomal peptide synthetases; (iii) fxuD, encoding a protein homology to periplasmic siderophore receptors, (iv) orf3, encodes proteins with an ATP-binding domain and (v) orf4 and orf5, encoding proteins with multiple transmembrane-spanning segments. Presumptively, all the genes would be responsible for the complete assemblage of the exochelin molecule by sequential attachment of ornithine to β-alanine, then to ornithine and on to the attachments of threonine and the final ornithine molecule. Based on the structural information available it was proposed that the exochelin MS was synthesised non-ribosomally via the multiple-carrier thiotemplate mechanism. Zhu et al. (1998) also reported the genetic organization of exochelin MS locus of M. smegmatis and identified exiT, encoding the protein proposed to be involved in secretion of exochelin. In addition fxbB and fxbC were identified. Much remains to be understood about exochelin biosynthesis.

1.9.4.6. Uptake of ferri siderophore in mycobacteria

A. Uptake of ferri-exochelin

Uptake of ferri-exochelin has been well studied in M. smegmatis (Ratledge, 2004). It is thought to be an active transport process, inhibited by energy poisons and uncouplers of oxidative phosphorylation (Macham et al., 1975). Uptake involves the
complete transfer of the molecule along with the metal ligand (Stephenson and Ratledge, 1979). Several proteins are involved in the uptake process that includes a 29 kDa ferri-exochelin receptor (Hall et al., 1987). After recognition by the receptor, the ferri-siderophore complex is taken up by the FxuD protein and then transferred through the cytoplasmic membrane proteins FxuA, FxuB and FxuC (Fig. 1.13), which share amino acid sequence homology with FepG, FepC and FepD, which are involved in the uptake of ferri-enterochelin in E. coli (Ratledge, 2004). The release of iron involve reduction of the ferric iron to ferrous iron involving an appropriate reductase (ferri-mycobactin reductase may represent a non-specific NAD(P)H-dependent siderophore reductase). The exochelin, after releasing its iron into the cytoplasm, is transferred back into the extracellular environment of the cells using a specific exiting protein, ExiT (Zhu et al., 1998) operating in conjunction with other proteins and involving the input of energy (Pavelka, 2000).

Fig. 1.13. Proposed mechanism for uptake of iron by M. smegmatis via ferri-exochelin.

B. Uptake of ferri-carboxymycobactin

The mechanism of uptake of carboxymycobactin is not known completely. It is thought that it might traverse the cell envelope either by diffusion, by virtue of its hydrophobicity (Rodriguez & Smith, 2006), or it is transported via a porin-like molecule (Fig. 1.14). The hypothesis involving porins is supported by the size of the inner diameter
of the porin molecule, which is about 2.2 nm, and the diameter of the carboxymycobactin may be equivalent to the mycobactin *i.e.* 1.1-1.4 nm (Trias & Benz, 1992). Calder and Horwitz, (1998) identified two iron-regulated proteins Irp10 and Mta72 from *M. tuberculosis*, which is hypothesized to be involved in the uptake of ferri-carboxymycobactin across the envelope and directly to a ferri-reductase. These two proteins, by their close homology to metal-transporting P-type ATPases and may function as a two-component metal transport system. Iron would be released from mycobactin by the same reductase. This is contradictory to the previous reports of Stephenson and Ratledge (1980), who showed that it does not require energy.

Recent studies have suggested the involvement of two IdeR - regulated ABC transporter proteins Rv1348 and Rv1349, also known as IrtA and IrtB respectively, in carboxymycobactin mediated iron acquisition (Rodriguez & Smith, 2006).

![Proposed mechanism for uptake of iron as mediated by carboxymycobactin.](Ratledge, 2004)

**Fig. 1.14. Proposed mechanism for uptake of iron as mediated by carboxymycobactin.**

### 1.9.4.7. Iron-regulated envelope proteins (IREPs) in mycobacteria

Hall *et al.*, (1987) first demonstrated the expression of IREPs in *M. smegmatis*. The authors compared the protein profile of cell wall and membrane of *M. smegmatis* grown in the presence of 4 µg Fe / mL (high iron) and 0.02 µg Fe / mL (low iron) and demonstrated several iron regulated envelope proteins. Among them, a 29 kDa IREP was
Review of Literature

shown as a potential receptor for the exochelin MS by specifically blocking its uptake using monospecific antibodies against the 29 kDa protein. This was further substantiated by the specificity of this protein as a receptor by Dover & Ratledge (1996). In *M. neoaurum* (Sritharan & Ratledge, 1989), a 21 kDa IREP was shown to be coordinately regulated with the siderophores mycobactin and exochelin MN. IREPs were identified in other mycobacterial species not only under defined lab conditions of established iron status but also under *in vivo* conditions (Sritharan & Ratledge, 1990). IREPs of 180, 29, 21, 14 kDa were identified in *M. avium* isolated from infected C57 black mice, while the 21 kDa IREP was demonstrated in the cell wall fraction of *M. leprae* obtained from infected armadillo liver. It is worth mentioning here that *M. leprae* was able to acquire iron from ferri-exochelin MN and not from other ferri-siderophores, whether this reflects the presence of the 21 kDa IREP in both the organisms is a possibility. Table 1.5 lists the IREPs identified in mycobacteria.

Studies on the effect of iron limitation in *M. tuberculosis* include the identification of several proteins influenced by iron levels as analysed by two-dimensional gel electrophoresis combined with mass spectrometry (Wong *et al.*, 1999). A putative cation transporting ATPase, a mycobacterial homologue of PEPCK (phosphoenolpyruvate carboxykinase) and an NADP-dependent dehydrogenase were identified in iron-limited organism. On the other hand, FurA (homologous to Fur protein), a homologue of a translational factor EF-Tu and aconitase were synthesized in higher amounts in bacteria grown in iron-rich medium.

The expression of 153 proteins was altered upon transcriptional profiling of *M. tuberculosis* grown under iron-regulated conditions (Rodriguez *et al.*, 2002). About one third of these proteins were IdeR dependant, while iron levels alone regulated the IdeR-independent proteins. Among the proteins identified, two-thirds of these were up regulated by iron limitation, half of which are of unknown function. The other half includes iron acquisition genes such as *mbt-1* cluster, 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 of genes including *bfrA* and *bfrB*, which encode putative iron-storage
proteins (i.e. bacterioferritin and ferritin, respectively) and *katG*, which encodes a catalase-peroxidase.

### Table 1.5. 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>
<th>Low iron</th>
<th>High iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>180</td>
<td>84</td>
</tr>
<tr>
<td>Defined iron status (<em>in vitro</em>-derived mycobacteria)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. neoaurum</em></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ADM 8563</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Undefined iron status (<em>in vivo</em>-derived mycobacteria)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ and - denote the presence or absence of the protein

? denotes a very faint band.

### 1.10. Iron regulation at molecular level in mycobacteria

Intracellular iron levels operate at molecular level and regulate the expression of the components of iron acquisition machinery. The well-characterized iron regulator in mycobacteria is IdeR (iron dependant regulator) homologous to DtxR of gram-positive bacteria. Iron-regulation is probably more complex in mycobacteria, as additional iron regulators have been identified from whole genome sequencing, namely the FurA and FurB (Fur family) and SirR (DtxR family).

#### 1.10.1. IdeR

IdeR is present in both pathogenic as well as non-pathogenic mycobacteria. It is a 230-amino acid protein sharing 90% homology with DtxR proteins in the first 180 amino acids (Schmitt *et al.*, 1995). It functions as a homodimer and each monomer has three functional domains with two metal-binding sites. In addition to iron, IdeR can also bind
other divalent metal ions such as Mn, Zn, Co, Ni, and Mg (Pohl et al., 1999). From the crystal structure of IdeR (Feese et al., 2001), four IdeR monomers form two functional dimers, as observed previously in DtxR (Qiu et al., 1995). Metal binding activates the protein’s DNA-binding ability by causing a conformational change in the DNA-binding domains. This change is mediated by amino acids at the amino-terminal that also participate in metal binding and therefore link the DNA- and metal-binding domains.

Fig. 1.15. Regulation of iron metabolism in *Mycobacterium tuberculosis*. (a) Under low iron condition, IdeR present in the cytoplasm lacks iron and is inactive for binding to the promoters of iron-regulated genes. Consequently, genes that are negatively regulated by IdeR like the *mbt* clusters and the *irtAB* operon required for iron uptake are actively transcribed, whereas the iron-storage genes *bfrA* and *bfrB* that are positively regulated by IdeR are not transcribed. (b) 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.
IdeR can be activated *in vitro* by several metals but iron (the natural cofactor) is the optimal metal for IdeR function. In the presence of iron, IdeR binds to a 19 bp (5'-TTAGGTTAGGCTAACCTAA-3') sequence called the IdeR box in the vicinity of promoter region of iron-regulated genes. It is a dual function regulator; under high iron conditions it represses siderophore biosynthesis and induces iron-storage proteins bacterioferritin and ferritin (BfrA and BfrB) (Gold *et al.*, 2001). In the presence of Fe$^{2+}$, IdeR-Fe$^{2+}$ complex binds to the iron box, upstream of *mbt* genes and *bfrA* and *bfrB* genes but it affects their expression in opposite ways (Fig. 1.15). It represses transcription of the former genes and activates the latter. 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 and Smith, 2003). Transcriptional profiling of organisms grown under iron-defined conditions showed that IdeR controls genes encoding putative transporters, transcriptional regulators, proteins involved in general metabolism, members of the PE / PPE family of conserved mycobacterial proteins and the virulence determinant MmpL4 (Camacho *et al*., 1999).

The essential nature of *M. tuberculosis* ideR contrasted with the dispensability of ideR reported in *M. smegmatis* (Dussurget *et al*., 1996). In *M. smegmatis* the inactivation of ideR resulted in iron-independent production of siderophores (Dussurget *et al*., 1996) and salicylic acid (Adilakshmi *et al*., 2000). A direct role for IdeR as a repressor of siderophore production was supported by the presence of putative IdeR binding sites in the promoter regions of exochelin synthesis and transport genes (Yu *et al*., 1998; Dussurget *et al*., 1999).

In *M. smegmatis*, the ideR mutations resulted in the decreased levels of catalase and the major superoxide dismutase, SodA (Dussurget *et al*., 1996), as IdeR is required for the full expression of *katG* and *sodA*. However, this effect is not direct, as there are no IdeR binding sites in the promoters of these genes (Dussurget *et al*., 1998). Whereas in *M. tuberculosis*, the inactivation of ideR results in increased sensitivity to oxidative stress is not well understood and no difference was found in the expression of genes involved in oxidative stress protection between the ideR mutant and the wild-type strains (Rodriguez *et al*., 2002). It is possible that there is an increase in a redox-reactive iron pool in the...
ideR mutant, and this, combined with decreased expression of bacterioferritin and ferritin, may result in increased sensitivity to oxidative stress.

1.10.2. FurA (Ferric uptake regulator)

FurA regulates the expression of catalase-peroxidase encoded by KatG (Pym et al., 2001). The *furA-katG* is expressed as an operon (Fig. 1.16), with FurA auto-regulating its own expression by binding to a unique sequence upstream of the *furA* gene (Sala et al., 2003) called *pfurA* and has been reported in *M. tuberculosis*, *M. smegmatis* and *M. bovis* BCG (Milano et al., 2001). *pfurA* is negatively controlled by the mycobacterial FurA protein, which binds upstream of the *furA* gene and in turn auto-regulates its own expression.

![Fig. 1.16. Schematic representation of the regulatory functions of *M. tuberculosis* FurA and FurB.](image)

In *M. tuberculosis* (Pym et al., 2001) and *M. smegmatis* (Zahrt et al., 2001), FurA negatively regulates the expression of *katG*, thereby modulating the response to oxidative stress. This effect, however, is iron-independent in *M. smegmatis* (Pym et al., 2001). It was proved by Pym et al. (2001) that FurA is not the principle regulator for siderophore production. Where as FurB acts as a Zinc uptake regulator (Zur) in *M. tuberculosis* and it is co-transcribed with its upstream gene (Rv2358), which encodes another zinc-dependent regulator. SirR present *Staphylococcus epidermidis* is an additional iron-
dependant regulator belonging to the DtxR family. The function of SirR homologue in *M. tuberculosis* is yet to be determined.

**1.11. Iron-regulated expression of virulence determinants**

The intracellular iron levels regulate not only the iron acquisition machinery but also the expression of virulence factors/toxins in several bacterial systems (Salyers & Whitt, 1994). This was first demonstrated in *C. diphtheriae* in which iron levels control the expression of ‘tox’ gene (Boyd *et al.*, 1990). When Fe\(^{2+}\) binds to DtxR as a corepressor molecule, the DtxR - Fe binds to the –10 region upstream of transcription start site of the *tox* gene encoding diphtheria toxin, thus blocking its transcription by RNA polymerase. Thus under low iron conditions, the toxin production is increased. Iron regulates the expression of Shiga toxin in *Shigella* spp., exotoxinA in *P. aeruginosa*, haemolytic toxin of *V. cholerae*, vero-cytotoxin of enterohaemorrhagic *E. coli* and α-hemolysin in *E. coli* (Litwin & Calderwood, 1993; Sritharan, 2000). The relationship between iron and bacterial virulence has also been demonstrated in experimental animals using *S. aureus* (Gladstone & Walton, 1970), *V. cholerae* (Ford & Hayhoe, 1976) and *Y. enterocolitica* (Robins-Brown & Prpic, 1985). The virulence of the organisms and their multiplication increased significantly upon injection of exogenous iron into these animals, while reducing the iron availability helped to control the growth of the pathogen and thus the infection.

Pathogenic mycobacteria are facultative intracellular bacteria with the ability to survive and proliferate inside the phagolysosomes of macrophages. One of the bactericidal mechanisms of macrophages is the production of reactive oxidative intermediates (ROI) such as superoxide anion (O\(_2\)\(^{-}\)), hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radical (•OH) and singlet oxygen (\(^{1}\)O\(_2\)). These oxygen species are extremely toxic to microorganisms (Edwards *et al.*, 2001). Mycobacteria produce enzymes like catalase-peroxidase (KatG), superoxide dismutase (SOD), alkyl hydroperoxidase (AhpC) that provide defence against the ROI.
DeVoss and his coworkers (2000) reported that the deletion of the peptide synthetases gene mbtB of the mbt cluster of *M. tuberculosis* resulted in a mutant unable to produce either mycobactin or carboxymycobactin. Further the mutant showed a decrease in growth both in low iron conditions and inside macrophages. Manabe *et al.* (1999) established the relevance of proper IdeR-dependant regulation for the virulence of *M. tuberculosis*, by the construction of *M. tuberculosis* strain expressing an iron-independent positive dominant corynebacterial dtxR and proved that the wild type IdeR controlled events influenced the virulence in a murine model of infection. In *M. smegmatis* Dussurget *et al.* (1996) demonstrated the role of IdeR in oxidative stress response in addition to iron metabolism and 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.

### 1.12. Mycobacterial catalase-peroxidases

Within the genus *Mycobacterium* three different types of catalase-peroxidases have been identified: they include the T, M and A catalases. The heat-labile, H$_2$O$_2$-inducible KatG catalase-peroxidase (T-catalase) is a member of the HPI group of catalases. The heat-stable, non-inducible KatE catalase-peroxidase (M-catalase) belongs to the HPII group. The HPI KatG catalase-peroxidase is resistant to aminotriazole, while the HPII KatE catalase-peroxidase is sensitive. The third type of catalase (A-catalase) was identified and described in strains of *M. avium* and *M. intracellulare*. It is similar to the mycobacterial KatE HPII catalase, but has greater resistance to high temperature, has a different charge, is more hydrophobic, and fails to react with antibody to KatE (Wayne and Diaz, 1988). *M. tuberculosis* expresses only a single catalase, the KatG, heat-labile, H$_2$O$_2$-inducible, HPI type catalase- peroxidase (Wayne & Diaz, 1982). Different types of catalase-peroxidases identified in different mycobacterial species are represented in the Table. 1.6.
Table 1.6. Catalase-peroxidases in different mycobacterial species (Bartos et al., 2004).

<table>
<thead>
<tr>
<th>Mycobacterial species</th>
<th>Catalase-peroxidase</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPI-type</td>
<td>HPII-type</td>
</tr>
<tr>
<td><em>M. asiaticum</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1986</td>
</tr>
<tr>
<td><em>M. aurum</em></td>
<td>Nd</td>
<td>Kat E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quemard et al., 1991</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1986, Mayer &amp; Falkinham, 1986; Milano et al., 1996</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Kat G</td>
<td>Wilson et al., 1995</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1982; 1986; 1988; Mayer &amp; Falkinham, 1986; Sherman et al., 1996</td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1986</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1986</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>Nd</td>
<td>Wheeler &amp; Gregory, 1980</td>
</tr>
<tr>
<td><em>M. lepraemurium</em></td>
<td>Nd</td>
<td>Ichihara et al., 1977</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>Nd</td>
<td>Chikata et al., 1975</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>Kat G</td>
<td>Kusunose et al., 1976b; Marcinkeviciene et al., 1995</td>
</tr>
<tr>
<td><em>M. terrae</em></td>
<td>Nd</td>
<td>Wayne &amp; Diaz, 1986</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Kat G</td>
<td>Middlebrook &amp; Cohn, 1953; Kusunose et al., 1976b; Wayne &amp; Diaz, 1986; Zhang et al., 1993</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>Kat G</td>
<td>Wayne &amp; Diaz, 1982</td>
</tr>
</tbody>
</table>

Nd = not detected – identification of the enzyme in organism was not described so far

1.13. Structure of catalase-peroxidase KatG of *M. tuberculosis*

The *M. tuberculosis* catalase-peroxidase is a multifunctional heme-dependent enzyme. Bertrand et al. (2004) crystallized the enzyme and reported its crystal structure refined to 2.4-Å resolution. The study reveals the dimeric assembly of the protein (Fig. 1.17a) of about 80 kDa, with each subunit of ~ 40 kDa. Each monomer (Fig. 1.17b) is composed of two domains that are mainly α-helical and display a common core structure shared by members of the bacterial and plant peroxidase families including yeast.
cytochrome c peroxidase. The N-terminal domain contains the active site of the enzyme, which includes the heme b prosthetic group. The fold of the C-terminal domain of the enzyme is similar to that of the N-terminal domain, consistent with the proposal that the enzyme arose from a gene duplication event. No heme is observed in this domain.

Fig. 1.17. Structure of *Mycobacterium tuberculosis* catalase- peroxidase. (a). Schematic representation of the homodimer. N-terminal residues of each monomer subunit form interlocking hooks via hydrophobic interactions. (b). A monomer. The N-terminal domain is shown in light pink, and C-terminal domain is shown in dark pink. The heme is shown in gray. N-terminal residues 24–30 are highlighted in green. Residues 278–312 are highlighted in red.

Fig. 1.18. Heme environment of *M. tuberculosis* KatG. Active site residues (R418, Y229, R104, H108, and S315) are displayed in green.
KatG from *M. tuberculosis* is particularly important due to its role in the activation of the drug INH. Considerable interest lies in understanding how the catalytic site has the enzyme that can bind and the drug. Studies have focused on the determination of those residues that are involved in this interaction. Among the several observations on mutations in the katG gene, resulting in a non-functional molecule, Ser-315 is found to be the most commonly occurring (Bertrand *et al.*, 2004), resulting in up to a 200-fold increase in the minimum inhibitory concentration for the drug. Ser-315 has reported to be mutated to asparagine, isoleucine, arginine, and glycine, although the most frequently occurring mutation is to threonine. It has been postulated that Ser-315 forms hydrogen bonds to one of the heme propionate groups and that mutation to threonine would, therefore, modify the heme pocket, altering INH binding. Asp-137 is another amino acid residue that is thought to play a key role in the binding and activation of INH, as this residue appears to be a catalase-peroxidase specific proton donor in the enzyme-catalyzed activation pathway.

1.14. KatG and activation of isoniazid

INH is active against growing tubercle bacilli but not resting bacilli. Oxygen plays an important role in INH activation since INH has no activity against *M. tuberculosis* under anaerobic conditions. The work of several researchers (Lei *et al.*, 2000; Nguyen *et al.*, 2002) has shed light on the mechanism of action of INH. INH is activated by KatG to generate a range of reactive (oxygen and organic) species, which then attack multiple targets in the tubercle bacilli. One of these species, the isonicotinic acyl radical attacks the nicotinamide group of NAD⁺ to form an INH-NAD adduct (Fig. 1.19). These adducts have been shown to occur in different isomeric forms including the open isomeric forms and it is not clear which of them is the active species. Adduct inhibits the InhA which is NADH-specific enoyl acyl carrier protein (NAD reductase) which is encoded by *inhA*. InhA is part of the fatty acid synthase type-II (FAS-II). This explains the earlier observation by Takayama *et al.* (1973) who showed that inhibition of mycolic acid led to the characteristic distribution of membrane and the cell death. The blocking of the FAS-II pathway leads the accumulation of the FAS I products that blocks the synthesis of the cell wall. The KatG mediated INH activation can also achieved with
manganese, which enhances the production of the INH-NAD adduct formation (Nguyen et al., 2002). Mdluli et al., (1993a) demonstrated the involvement of β-ketoacyl ACP synthase KasA, also a part of the FAS-II system was a target of INH.

![Schematic representation of KatG mediated INH activation and its mechanism of action (Zhang et al., 2005)](image)

**Fig. 1.19.** Schematic representation of KatG mediated INH activation and its mechanism of action (Zhang et al., 2005)

### 1.15. INH resistance: Alterations in *katG* as a contributing factor

Middlebrook (1952) first demonstrated catalase-peroxidase as a virulence determinant. He reported spontaneous INH resistant mutations of *M. tuberculosis* in *in vitro* culture and showed that these mutants lacked catalase activity and became attenuated in guinea pigs. Based on several reports world wide on INH resistant strains it is now evident that *katG* deletions and more commonly the *katG* point mutations are encountered. Lack of KatG due to alterations in the *katG* gene, reduces the ability KatG to activate INH thus leading to INH resistance. Between 20-80% of INH resistant *M. tuberculosis* showed a mutation in the *katG* gene depending upon the geographical location. Among these mutations the Ser 315 Thr is the most common and occurs in 50-93% of INH resistant isolates. These mutations reduces the catalase and peroxidase
activity by 50% and increases the MIC of these organisms to 5-10 µg / mL. This mutation affects the binding of INH to KatG.

Other genes, which are potentially involved in INH resistance, include *kasA*, *inhA*, *mdh* (NADH encoding type-II enzyme). Another candidate is the efflux protein EfpA that is induced by INH (Colangeli *et al*., 2005). It is also a possibility that aryl amine N-acetyl transferase (Nat), which is present in humans as two isoforms NatI and NatII and acetylate aryl amine and hydrazine and can inactivate INH could be a candidate for INH resistance. The Nat homologues are seen in *M. tuberculosis* and *M. smegmatis* on the purified enzyme to convert INH to N-acetyl INH.

**1.16. Relevance of studying the role of iron in pathogenic mycobacteria**

There are several reports stating that the iron levels contribute to the pathogenesis of *M. tuberculosis*. Anemia is frequently encountered in tuberculosis patients, indicating that this is one of the mechanisms of the mammalian host to lower the bioavailability of this essential nutrient. The iron-withholding ability of the host, as manifested through the transferrin and lactoferrin proteins is vital as a defense mechanism against infection and, if this is compromised by the administration of iron, then the consequences can indeed be dire for the patient as the iron will preferentially feed the pathogen and not the patient (Ratledge, 2004). Though there is increasing evidence that pathogenic mycobacteria, specifically *M. tuberculosis* grows under conditions of iron limitation *in vivo*, the influence of iron levels on the host-pathogen interactions is yet to be understood fully. This will help in the identification of candidate antigens that can be exploited as potential drug targets.

**Objectives of the study**

I. Studies on the iron acquisition machinery in *Mycobacterium tuberculosis*

1. Establishment of conditions of iron-limitation for the growth of mycobacteria.
2. Analysis of IREPs in *M. tuberculosis*.
3. Studies on the characterization of the iron-regulated HupB protein.

II. Effect of iron deprivation on mycobacterial catalase-peroxidases and the implications on the efficacy of the anti-tubercular drug isoniazid on *Mycobacterium tuberculosis*.