BIOCHEMICAL STUDIES ON REACTIVE OXYGEN SPECIES GENERATION AND ITS PHYSIOLOGICAL ROLE IN *MYCOBACTERIUM SP.*

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For the degree of

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IN

BIOTECHNOLOGY

By

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The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

- **Albert Einstein**

Dedicated to……..

World's one third population infected with latent tuberculosis

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biochemical studies on reactive oxygen species generation and its physiological role in Mycobacterium sp.**" submitted by **Abhishek Mishra** was carried out under my supervision at Combichem Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune – 411008, Maharashtra, India. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. Dhiman Sarkar

(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biochemical studies on reactive oxygen species generation and its physiological role in Mycobacterium sp..**", submitted for the Degree of **Doctor of Philosophy** to the University of Pune, has been carried out by me at Combichem Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. Dhiman Sarkar (Research supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Abhishek Mishra

(Research Scholar)

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ABSTRACT

Nearly two billion of the world's population is latently infected with tuberculosis, and a drug effective against this latent form of the disease has been unavailable for the past 50 years. The etiologic agent, *Mycobacterium tuberculosis* (Mtb), has the ability to survive in a latent stage for years before reactivation and subsequent induction of full-fledged tuberculosis in humans. The major bottleneck in controlling tuberculosis is a lack of understanding of the mechanisms involved in the development of latency and resuscitation of the causative agent Mtb within the host. Therefore, there is an urgent need to understand the underlying mechanisms responsible for intracellular survival and persistence of the bacilli. The intracellular environment exposes Mtb to multiple stresses, including hypoxia, nutrient limitations, oxidative stress, nitrosative stress, and acidic conditions. The true regulators involved at the molecular level in achieving dormancy and reactivation of the bacilli are still unknown.

First part of this study effectively demonstrates the significant presence of Reactive Oxygen Species (ROS) in *M. smegmatis.* We critically evaluated production of superoxide by using dihydroethidium (DHE) and nitro blue tetrazolium (NBT). Similarly, we detected Hydrogen peroxide (H_2O_2) production by 2', 7'-dichlorofluorescin–diacetate (DCFH-DA) and Amplex red. A rare biological molecule, hydroxyl ion was also detected in *M. smegmatis* by 2-deoxy- D-ribose assay.

Due to lower E'_0 there is scarcity of biological molecules which can transfer reduced electron to oxygen, flavins and quinones are considered as preferred molecules. After number of combinations of substrate and inhibitors, NADH oxidase has been identified as the major source of superoxide production in the bacilli. Mostly, H_2O_2 is produced by dismutation of superoxide. We could also identify Fenton's reaction *in vivo* for generation of highly reactive hydroxyl radical.

A decreased level of superoxide and H_2O_2 in the presence of antioxidants, such as ascorbic acid, caffeic acid, and p-coumaric acid induces non-cultivability in *Mycobacterium smegmatis* cells. This is confirmed by Colony Forming Unit (CFU) counts, morphological appearance and induction of the DosRS regulon genes. Involvement of ROS is confirmed by reactivation of antioxidant induced dormant bacilli upon the addition of menadione, pyrogallol, a

superoxide generators and 1-Hydroxypyridine -2- thione (HOPT), a hydroxyl radical generator. Thus, these results clearly implicate ROS, particularly hydroxyl radical in the transformation of actively growing bacilli into the dormant form and vice versa. HOPT was identified as most potential molecule by carrying out kinetic study of reactivation. Further, global expression of proteins is studied in different conditions such as actively growing, hypoxia induced dormant and HOPT induced resuscitated dormant cells. We identified a stepwise metabolic shift in above mentioned condition with induction of stage specific stress proteins. The shift in energy metabolism, alternative carbohydrate metabolism, fatty acid metabolism and amino acid synthesis was identified in dormant stage was in many aspects different in HOPT induced resuscitated cells than even the actively growing cells.

As NADH oxidase was identified as major source of superoxide generation its role was further studied in the fourth chapter. There are four different kind of NADH oxidase present in *Mycobacterium smegmatis* as suggested by bioinformatics databases. We attempted to find out role of different NADH oxidase in actively growing, dormant and resuscitated bacilli by transcript analysis. It was found that NADH oxidase IV may have potential role in resuscitation. So, NADH oxidase I was cloned, purified and partially characterized.

Altogether, this study provides an insight in molecular mechanism of latency and resuscitation of *Mycobacterium smegmatis.* It claims the importance of ROS in development of dormancy and commencement of resuscitation. Furthermore, this study finds the link between independently proposed earlier models to study latency. Finally it provides a new strategy to combat most deadly pathogen, *Mycobacterium*.

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List of Abbreviations

ADASA:	Active Dormant Antitubercular Screening Assay
ADP:	Adenosine diphosphate
ADAS:	Active Dormant Antitubercular Screening
AFB:	Acid Fast Bacilli
ATCC:	American Type Culture Collection
ATP:	Adenosine triphosphate
BCG:	Bacillus Calmette Guerin
Bio-SiV:	Bioluminescent Screening In vitro
CDC:	Centre for Disease Control
CFP-10:	Culture Filtrate Protein 10
CFU:	Colony Forming Unit
DMSO:	Dimethyl sulfoxide
DOTS:	Directly observed therapy shortcourse
E. coli:	*Escherichia coli*
EMB:	Ethambutol
ESAT-6:	Early Secretary Antigen Target-6
ESI-MS	Electron Spray Ionization Mass Spectrometery
ETH:	Ethionamide
FAD:	flavin adenine dinucleotide
FDA:	Food and Drug Administration
GAN:	Gene Accession Number
GS:	Glutamine Synthetase
GTH:	Glutathione

GTHH	Glutathione reduced
HAART:	Highly Active Antiretroviral therapy
HIV:	Human Immunodeficiency Virus
HSR:	Head Space Ratio
HTS:	High throughput screening
H_2O_2	Hydrogen Peroxide
IFN-γ:	Interferon-γ
INH:	Isoniazid
IUATLD:	International Union Against Tuberculosis and Lung Disease
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LAM:	Lipoarabinomannan
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MBC:	Minimum Bactericidal Concentration
M. bovis:	*Mycobacterium bovis*
MDR:	Multidrug resistance
MIC:	Minimum Inhibitory Concentration
M. smegmatis:	*Mycobacterium smegmatis*
M. tuberculosis:	*Mycobacterium tuberculosis*
MW:	Molecular Weight
NAD:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide reduced
NADP:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Nicotinamide adenine dinucleotide phosphate reduced
NO_3:	Nitrate
NO_2:	Nitrite

NR:	Nitrate Reductase
$O_2^{-\cdot}$	Superoxide
$^{\cdot}OH$	Hydroxyl Radical
PAS:	Para amino salicylic acid
PCR:	Polymerase Chain Reaction
PK/PD:	Pharmacokinetics Pharmacodynamics
PPD:	Purified Protein Derivative
PZA:	Pyrazinamide
RIF:	Rifampicin
RNI	Reactive Nitrogen Intermediates
RNS	Reactive Nitrogen species
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen species
SAR:	Structure Activity Relationship
SDS-PAGE:	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SEM:	Scanning Electron Microscopy
STM:	Streptomycin
TB:	Tuberculosis
TEM:	Transmission Electron Microscopy
THP-1:	Human acute monocytic leukemia cell line
WHO:	World Health Organization
XDR:	Extremely Drug resistant

CHAPTER 1

Introduction and Overview of Tuberculosis

1.1. Classification and characteristics of *Mycobacterium tuberculosis*

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteridae

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

Species: *Mycobacterium tuberculosis*

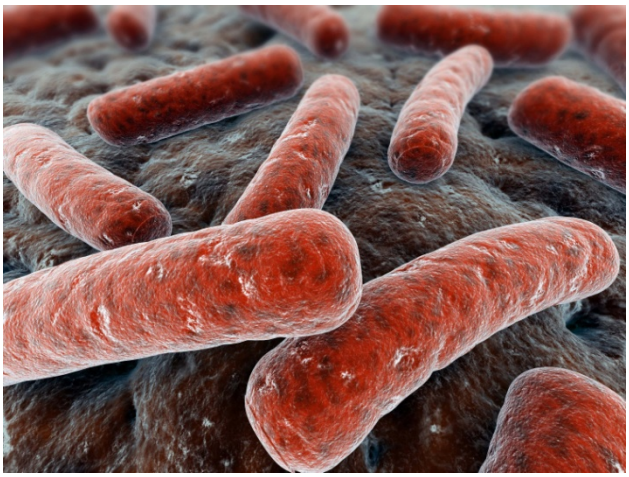

Fig. 1.1 Scanning electron micrograph of M. tuberculosis (extracted from http://www.bioquellus.com/technology/microbiology/mycobacterium-tuberculosis/).

Mycobacterium is extensively studied bacterial taxa because of its clinical importance as a group of most successful pathogen known to mankind. They bear a taxonomical relationship to actinomycetes such as the antibiotic producing *Streptomycetes* because of the high guanine cytosine (GC) content in their DNA (1). 16S rRNA analysis also identifies similarities to

other families such as Corynebacteriaceae and Nocardiaceae establishing the CMN branch within the Actinomycete genera (2, 3). Within this group of organisms many structural and biosynthetically related complex systems are present. Mycobacteria bear several notable distinctions like; resistance to decolorization by acid alcohol following basic fuchsin staining ("acid fast" bacilli) (4); production of two different siderophores (iron scavangers) as exochelins and mycobactins (5, 6); complexity of cell wall components as mycolates (3,7); and extremely slow rate of division in comparision to other bacteria (2).

Mycobacteria are typically rod-shaped aerobic bacteria as illustrated (Fig.1.1) but variable morphology like shorter cocci-bacilli or curved rods can be observed when grown on different artificial media (8). Although mycobacteria are considered as obligate aerobes, metabolic pathways exist to support adaptation in environment with reduced to no oxygen (9, 10). The ability to adapt extends to numerous environmental stresses, a likely survival tool for their natural habitat mainly soil, water and host cells (in the case of facultative pathogenic species). There are more than 70 distinct *Mycobacterium* species (2). With the exception of *M. leprae*, which cannot be cultivated in vitro, mycobacteria are assigned to two groups based primarily on the relative growth rates of the individual species.

A unique and complex cell envelope is one of the most characteristic properties of *M. tuberculosis* (Fig. 1.2). This complex cell wall confers protection against toxic environment within the host and effectively limits the influx of antibiotics. Almost 60% of the weight of this envelope is lipids (11). *M. tuberculosis* dedicates approximately 250 genes towards lipid biosynthesis that is fairly high in comparision to *Escherichia coli* which has only about 50 genes (12). The cell envelope of *M. tuberculosis* can be divided into three main components (Fig. 1.2); plasma membrane, mycolic acid-arabinogalactan-peptidoglycan complex and surface capsule. The plasma membrane (PM) forms the innermost layer of this cell envelope and is a typical lipid-bilayer, structurally and functionally similar to the PM of other eubacteria. On contrary, external to the PM is the multiple layers of peptidoglycan (PG) that is unusual compared to other eubacteria in two main aspects: the location and number of cross-links between its layer and the presence of N-glycollyl muramic acid in place of N-acetylmuramic acid. The tetra-peptide side chain of most eubacterial PG consists of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine with cross-links occurring between *meso*-diaminopimalic acid (*meso*-DAP) residues of one chain and D-glutamic acid residues of the

other. However, in mycobacteria additional cross-links occur such as those between two *meso*-DAP residues and those between *meso*-DAP and D-alanine (13). The PG layer is covalently linked to a layer of sugar residues known as arabinogalactan.

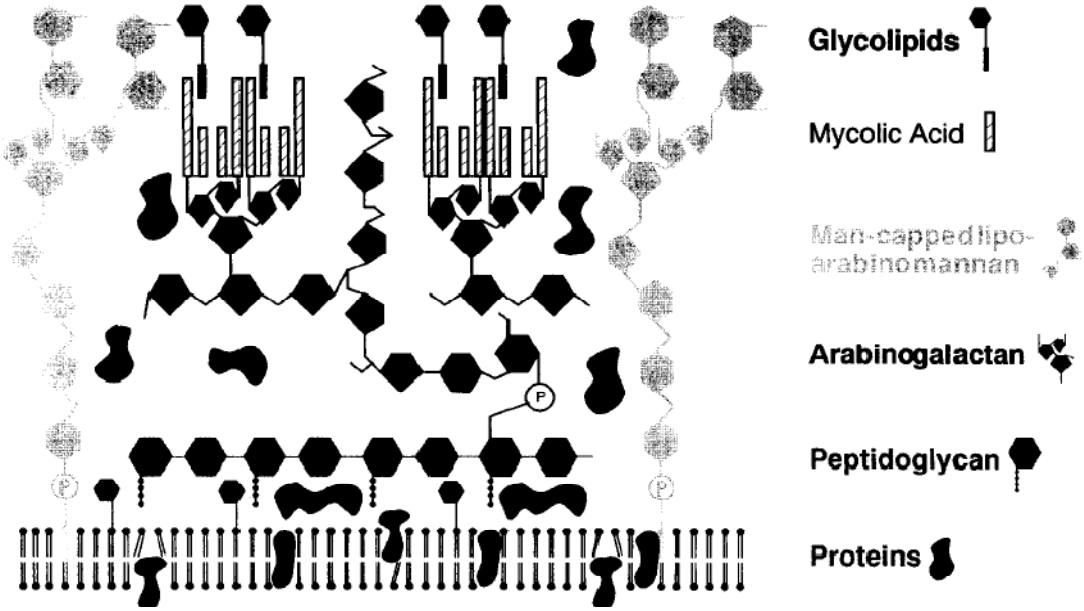

Fig. 1.2 Overview of the structure of the cell wall of *M. tuberculosis* (Extracted from *Infect. Immun.* 1996, 64(3):683.).

At the distal end, arabinogalactan is attached to a special class of lipids found in mycobacteria known as Mycolic acid (MA) through ester-linkage with the arabinans. Mycolic acids are long, branched 2-alkyl-3-hydroxy fatty acids that may be as many as 90 carbon atoms in length (14, 15). The mycolic acid chains are perpendicular to the bacterial cell surface and form a monolayer. MAs are often species-specific and are thought to be responsible for the impermeability of mycobacterial cell wall (15). Special classes of MAs are found in *M. tuberculosis* include tehalose-6,6'-dimycolate (TDM) that is commonly known as 'cord factor', sulpholipids (SLs), phthicerol dimycocerosates (PDIMs), and phenolic glycolipids (PGLs). TDM is a potent modulator of the immune response and is also thought to be responsible for the characteristic cording phenotype of *M. tuberculosis* (16). The PG-AG-MA complex forms the core cell wall.

The cell envelope also contains a variety of loosely attached polar lipids and lipoglycans that are unique to mycobacteria. These are thought to be interspersed between the MAs. While the types of lipid present vary from one mycobacterial species to another, some commonly occurring ones include phosphatidylinositol mannosides (PIMs), lipoarabinomannans (LAMs) (13). PIM is the precursor of LM, which in turn is the precursor of LAM (16). PIMs have been shown to have a role in natural killer cell recruitment as well as adhesion to non-phagocytic host cells (17). The type of LAM present in the species correlates with its pathogenic ability. For example, Mannose caps, present on LAM (ManLAM) in *M. tuberculosis* make ManLAM a much stronger immunosuppressor as compared to PILAM of *M. smegmatis*, which contains phosphatidylinositol caps on the LAM (18).

The outermost layer of the mycobacterial cell envelope is considered to be a loosely attached capsule composed primarily of glucans, arabinomannans and mannans. Mycobacterial glucan is a branched polymer of D-glucosyl residues with branch points consisting of monoglucosyl to pentaglucosyl substitutions. The arabinomannan consists of a heteropolymer of D-arabino-D-mannan. Structurally the arabinan found in the mycobacterial capsule is similar to the arabinan found in the arabinogalactan portion of the cell wall. The capsular mannan is a branched polymer of D-mannosyl subunits with branch points consisting of single D-mannosyl units (13). Lipids and proteins are also known to ornament the capsule, albeit in smaller amounts. Capsular proteins fall in two categories: the 'secreted' proteins and 'resident' capsular proteins. Both types of protein are found to be present in culture filtrate indicating that the 'resident' capsular proteins are likely shed from the cell surface into the culture medium (13). Lipids comprise only 2-3% of the capsular material. Since mycobacteria are cultured in media containing dispersing agents, such as Tween-80, this capsule is frequently lost. Capsular lipids are most commonly glycopeptidolipids (GPLs).

1.2. History and epidemiology of tuberculosis

Robert Koch famously identified *Mycobacterium tuberculosis* as the organism that causes TB in 1882. The world press hailed an imminent cure, but an effective treatment remained

elusive for over 70 years. While the disease could be diagnosed with accuracy by 1905, largely due to Wilhelm Konrad Rontgen's discovery of X-rays in 1895 and Koch's stain for microscopy, nothing could be done to cure the disease. The standard care of TB was quarantine with a sanitarium pneumothorax, or "lung collapse" therapy twice per week (19). The first effective treatment of TB was discovered by Selman Waxman at Rutgers University in 1939. Rutgers observed that certain soil dwelling organisms had an inhibitory effect on mycobacterial growth and coined the term "antibiotic" (20). His laboratory subsequently isolated streptomycin from *Streptomyces griseus*, and Waxman was awarded the 1945 Nobel Prize in medicine. Other compounds rapidly followed the discovery of streptomycin, including p-amino salicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin (1963) (21). The advent of multidrug therapy dropped the rate of TB infection precipitously in the latter half of the 20th century.

In 2006, around 14.6 million people had active TB disease with 9 million new cases (Table 1.1). The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the Americas. TB has also now become the world's greatest infectious killer of women of reproductive age and the leading cause of death among people with HIV/AIDS.

The emergence of HIV infection, especially within incarcerated populations such as prisons, hospitals and homeless shelters lead to outbreak of infection (22). The rise in HIV infections and the neglect of TB control programs have enabled a resurgence of tuberculosis. The emergence of drug-resistant strains has also contributed to this new epidemic with, from 2000 to 2004, 20% of TB cases being resistant to standard treatments and 2% resistant to second-line drugs. The rate at which new TB cases occur varies widely, even in neighboring countries, apparently because of differences in health care systems.

There are some other known factors that make people more susceptible to TB infection. Smoking more than 20 cigarettes a day also increases the risk of TB by two to four times (23). Diabetes mellitus is also an important risk factor that is growing in importance in developing countries (24).

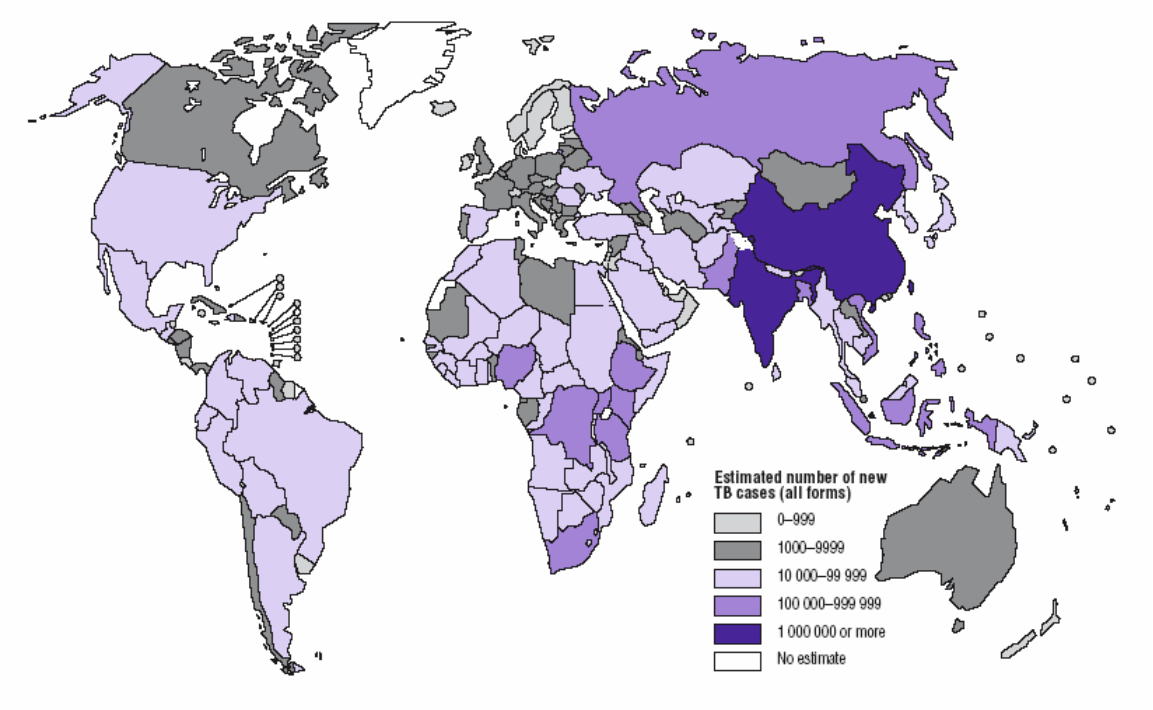

Fig. 1.3 Estimated number of TB cases by country 2006 (Extracted from WHO report 2008, Global Tuberculosis Control: Surveillance, Planning and Financing).

1.3. Pathogenesis of *Mycobacterium tuberculosis*

TB is the example of the interaction between an exogenous agent and the host immune defense system. It may be estimated that while 1900 million people throughout the world are infected with *M. tuberculosis* (representing an enormous reservoir that contributes to perpetuation of this disease), only 8 million actually suffer from the disease each year. This situation is explained by the fact that the human defense mechanism is highly effective and can overcome the disease in most cases.

Primary infection. Primary infection refers to the general biological phenomena that take place when an individual comes into contact with the tubercle bacillus for the first time (Fig. 1.5) (25). During primary infection, 95% of all affected individuals remain asymptomatic or present with only minimal clinical manifestations similar to those seen with the common cold. Only 5% develop manifest disease. The preferential zone of arrival is the best-ventilated part of the lungs, corresponding to the subpleural region of the inferior lobes. Upon arrival in the alveolar region,

the bacteria encounter three types of cells that potentially oppose infection: the alveolar macrophages within the alveolar lumen, the natural killer cells, and the T lymphocytes.

In humans, alveolar macrophages are considered to be the key type of cells involved in the initial interaction with the tubercle bacillus (26). These cells originate in the bone marrow and reach the alveoli after coming into contact with the systemic circulation. As a result, different systemic and local factors can influence their functional characteristics. For example, HIV is able to infect these alveolar macrophages and thereby increase host sensitivity to tuberculous infection. Second, the antigen presenting capacity of alveolar macrophages is low in humans, as compared with other animal species. Third, alveolar macrophages are cells that live in an oxygen-rich environment, as a result of which their free oxygen radical production potential is theoretically great. However and probably to avoid toxicity due to these radicals, alveolar macrophages lack myeloperoxidase (although they do generate superoxide radicals). Fourth, alveolar macrophages contain abundant lysosomal enzymes.

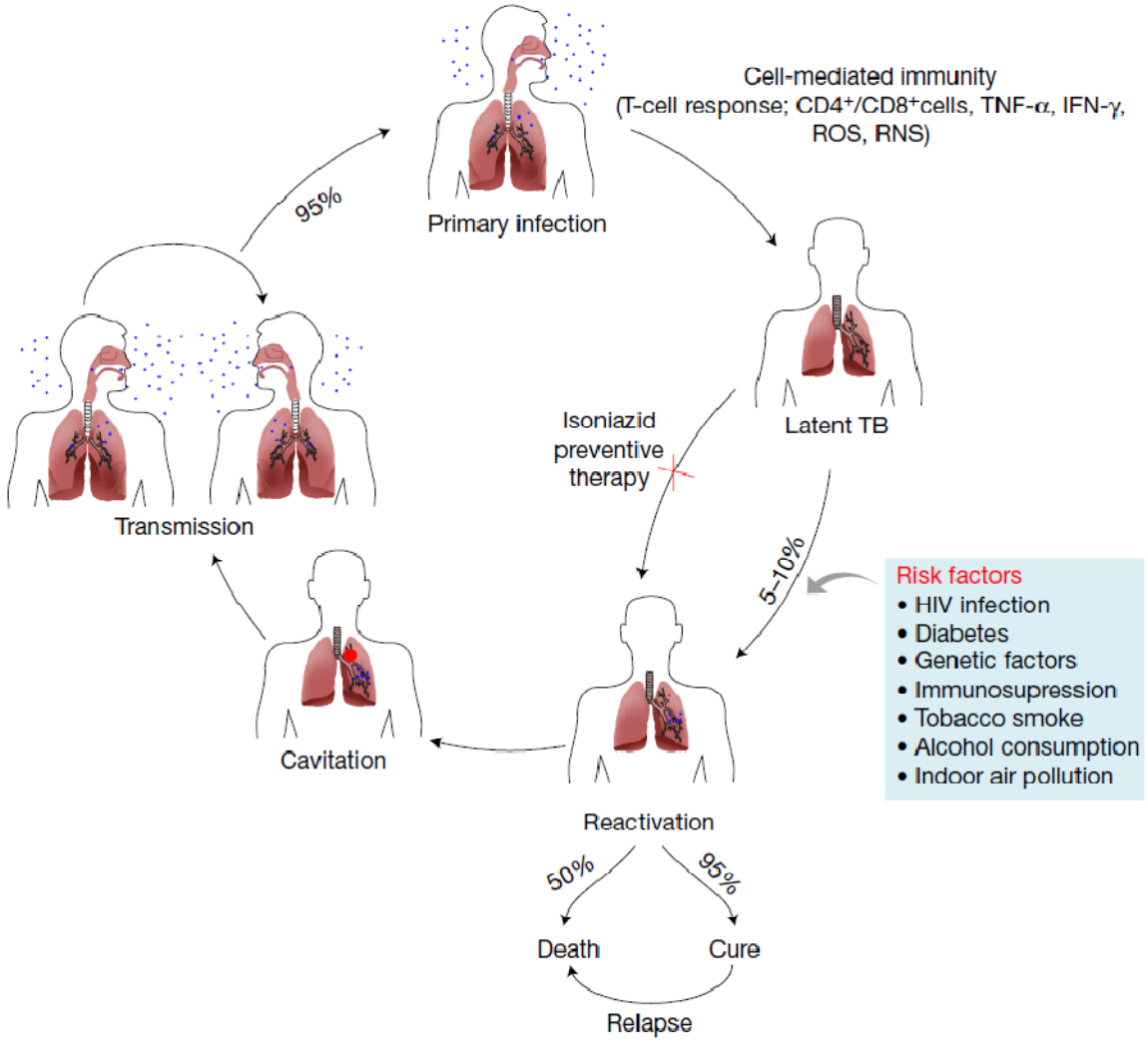

Fig. 1.4 Virulence life cycle of Mycobacterium tuberculosis and progression of TB. (extracted from experts review in molocular medicine, 2011) Mtb is transmitted by aerosol, and in 95% of cases, wherein the tubercle bacilli are inhaled, a primary infection is established. This is either cleared by the surge of the cell-mediated immunity or contained inside the granuloma in the form of latent TB, defined by no visible symptom of disease, but persistent, yet dormant, live bacilli within the host. The progress of TB can be stalled at this stage in some cases by isoniazid preventive therapy. This state might last for the lifespan of the infected individual, or progress to active TB by reactivation of the existing infection, with a lifetime risk of 5–10%. This risk of progression is exacerbated by immunecompromising factors such as HIV-AIDS, diabetes, indoor air pollution and tobacco smoke. Reactivation of TB is shown to occur at the upper and more oxygenated lobe of the lung, which can be cured by compliance with drug therapy. However, untreated or poorly treated TB might lead to the formation of tuberculous lesions in the lung. The development of cavities close to airway spaces allows shedding (e.g. coughing) of the bacilli through

the airway, a stage of transmission. Subsequently, in a cyclic manner, the TB bacilli are transmitted to other individuals to establish primary infection.

The initial interaction between *M. tuberculosis* and alveolar macrophages involves non-specific phagocytosis of the bacilli and their inclusion within phagocytic vacuoles. Considering that these alveolar macrophages have not been primed by lymphocytic cytokines and that various mycobacterial components inhibit the bactericidal systems of these cells, it is reasonable that bacterial growth predominates in this initial stage. Practically all bactericidal macrophage systems are inhibited by products derived from the mycobacteria. Thus, glycolipids inhibit phagosome-lysosome fusion, while other less well-known components alter lysosomal acid pH, thereby complicating enzyme action. Catalase, in turn, destroys hydrogen peroxide, and different mycobacterial components inhibit superoxide production. This phase concludes with destruction of the alveolar macrophages by proliferating intracellular bacilli. Natural resistance to the infection fundamentally occurs during this phase. Tubercle bacilli products such as cord factor and the activation of other chemokine factors exert a potent chemical effect, attracting blood monocytes that ingest the released bacilli. At this point, a symbiotic relation is established in which the bacteria and young macrophages do not destroy each other. The monocytes have not been activated, and the bacteria are not toxic, at least on an acute basis. The tubercle bacilli increase exponentially in a similar manner, killing host cells and spreading locally. In the lung, intense alveolitis takes place at the expense of the young cells of the mononuclear phagocyte system.

The third essential phenomenon in this phase of the disease is mycobacterial spread systemically via the lymphatics towards the regional lymph nodes (27). In this region, the host immune response to tuberculous infection takes place. In some instances, this immune response is sufficient to arrest the progression of infection, although often times the bacilli escape towards the lymphatic duct and penetrate the pulmonary bloodstream, from where there is hematogenous spreading of the bacilli to the other organs. The main metastatic or target zones of such bacterial dissemination are the highly irrigated organs and tissues—the central nervous system, spongy bone, liver, kidneys, and genitals. In each of these zones, the arriving bacilli are phagocytosed by the local cells of the mononuclear phagocyte system. In most cases, this period implies

immunologic control of the infection as a result of two mechanisms: cell-mediated immunity and delayed hypersensitivity. From the bacteriological perspective, the consequence of this situation is an abrupt interruption of the bacterial growth curve in both resistant and susceptible individuals. Cellular immunity is not responsible for this growth arrest, since susceptible individuals have only a weak cell mediated immune response and resistant subjects have not yet developed an effective immune reaction. Delayed hypersensitivity is the phenomenon responsible for the destruction of macrophages that contain intracytoplasmic bacteria, thereby forming a characteristic focus of caseous necrosis (28). Although the bacteria may survive within this necrotic focus for years, they are unable to reproduce due to the prevalent acidosis, the lack of oxygen, and the presence of inhibitory fatty acids. The principal factors influencing delayed hypersensitivity reactions are the cytotoxic T lymphocytes, although other factors such as cytokines (tumor necrosis factor), oxygen reactive species, and nitrous oxide may also be involved. Such initial necrosis is therefore beneficial for control of the infection. However, delayed hypersensitivity must be "reinforced" by cell-mediated immunity, since susceptible hosts with weak immune responses are not only unable to control the infection but also produce granulomas with an increased caseous presence, probably due to the intervention of mycobacterial proteins.

Tuberculous reactivation. Tuberculous reactivation is defined as the development of tuberculous disease in a patient who had already been infected with the tubercle bacillus in the past. Although there are well-documented cases of exogenous reinfection, it is currently accepted that most cases of infection are attributable to endogenous reactivation (29). However, this assumption may change in the coming years, in view of recent information appearing in the literature. It has been calculated that only a minority of people infected with *M. tuberculosis* actually progress to active disease. In general terms, 90% of infected individuals can be expected to keep the tubercle bacilli in a latent state for life, owing to the intervention of the host immune defenses. Five percent will develop progressive primary TB, and the remaining 5% will develop the disease in later stages of life, a condition known as reactivation or post-primary TB (Fig 1.4). This situation changes drastically in patients with HIV infection, of whom an estimated 50% to 60% who are also infected with *M. tuberculosis* will develop active TB in the course of their lifetime. The number of patients at risk of TB has increased in the course of history, and

presently includes some populations that do not demonstrate immunodeficiency but that have other risk factors for TB.

Based on the above considerations, all TB-infected individuals should be considered patients, and the greatest protection against *M. tuberculosis* corresponds to a subject who has never been infected. The great problem with TB facing us today is that there are a very large number of infected individuals who are never free from the risk of the disease. These individuals, while potentially capable of developing the disease at any time in life, particularly if they develop some form of immunodeficiency, are expected to present with alerted memory lymphocytes in the event of past exposure to tubercle bacilli. Theoretically, this would confer relative protection against such possible exogenous reactivation. In this sense, there has been speculation as to whether the ideal situation is instead represented by the infected subject, who would be more protected against future exogenous reinfections. However, such reasoning is mistaken, since the development of TB due to endogenous reactivation in infected individuals is much more likely than the development of the disease due to exogenous infection in non-infected subject.

1.4. Reactive Nitrogen and Oxygen Intermediates and Bacterial Defenses: Unusual Adaptations in *Mycobacterium tuberculosis*

Specialized mammalian cells of the immune system utilize a variety of mechanisms to control infection by bacterial pathogens. A subset of these mechanisms includes the production of reactive oxygen Species (ROS) and reactive nitrogen intermediates (RNI). These agents, generated under physiological conditions, are capable of damaging DNA bases and lipids, and disrupting the activity of important cellular proteins containing Fe-S clusters, transition metals, hemes, thiols, sulfhydryl, or tyrosyl groups.

There are three categories of oxidants generated by the Phagocyte oxidase (Phox) and Nitric Oxide Synthase (NOS) (Fig. 3). First, NADPH oxidase generates ROS through the reduction of molecular oxygen. The primary products generated from this reaction are superoxide ($O_2^{-\cdot}$) and its various dismutation and decomposition products, including hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), an especially strong oxidant in biological systems

(32). Second, *i*NOS uses L-arginine as a substrate to generate nitric oxide ($NO^{\cdot}$). A stepwise oxidation of nitric oxide with oxygen to nitrate (NO_3^-) results in production of various intermediates including nitrite (NO_2^-) and nitrogen dioxide ($^{\cdot}NO_2$). Following interaction of nitric oxide with cysteine residues, sulfhydryls and glutathione or its equivalents can be converted into nitrosothiols or sulfenic acid (33). A third category of reactive intermediates occurs when the products of NADPH oxidase and *i*NOS interact to form highly potent antibacterial compounds. For example, peroxynitrite ($^{\cdot}OONO_2$) is generated following the reaction of superoxide with nitric oxide, and is one of the most potent natural oxidants in biological systems (34). Apart from NADPH oxidase and nitric oxide synthase (NOS), other leukocyte enzymes contribute to the generation of ROS. Production of HOCl and HOBr is mediated by eukaryotic myeloperoxidases in a reaction dependent on the peroxidation of halide ions in the presence of H_2O_2 (35, 36). HOCl and HOBr are highly destructive and also act on a variety of cellular components, including enzymes (37) and DNA (38, 39). HOCl generates hydroxyl radicals in the presence of Fe^{2+} or Fe^{3+} or superoxide anions (40), and oxygen singlet radicals upon acidification (41, 42) or interaction with H_2O_2 (43, 44).

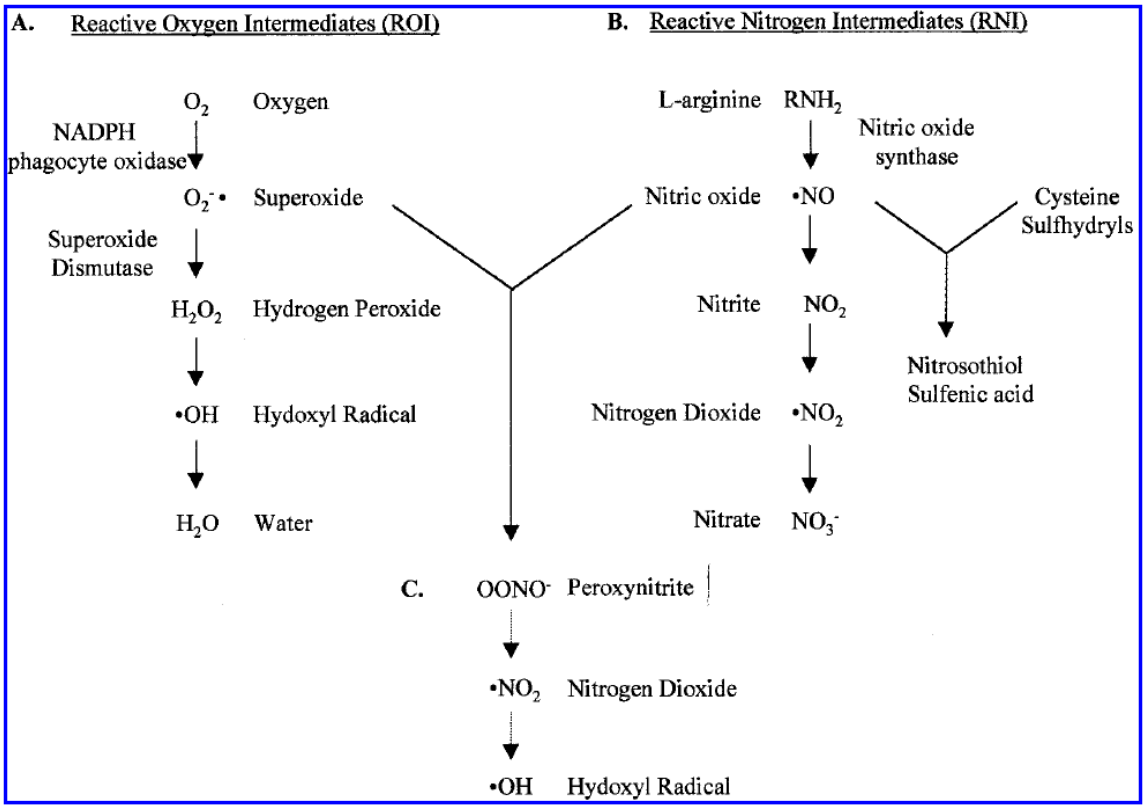

Fig. 1.5 Pathways of ROI and RNI generation. (**A**) ROI including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxy radical ($^{\cdot}OH$) are generated by NADPH Phox in the presence of iron and upon superoxide dismutation. (**B**) The inducible nitric oxide synthase (*i*NOS) generates RNI including nitric oxide ($NO^{\cdot}$) and its various oxidative products including nitrite (NO_2^-), nitrogen dioxide ($^{\cdot}NO_2$) and nitrate (NO_3^-) in the presence of substrate L-arginine. The reaction of nitric oxide with cysteine sulfhydryls results in the generation of nitrosothiols and sulfenic acids. (**C**) The interaction of O_2^- generated by Phox and $NO^{\cdot}$ generated by *i*NOS combine to form the toxic intermediate peroxynitrite ($^{\cdot}OONO_2$) and its various decomposition products. (Extracted from ANTIOXIDANTS & REDOX SIGNALING Volume 4, Number 1, 2002)

Pathogenic mycobacteria are facultative intracellular bacteria with the ability to survive and proliferate inside the phagolysosomes of macrophages. Intracellular survival and proliferation of mycobacteria is required for their virulence (45) and consequently, there has been a focus on discovery of mechanisms of intracellular survival. Inhibition of phagosome-lysosome fusion (46) and pH reduction in phagosomes (47) are two of the ways *Mycobacterium tuberculosis* and *Mycobacterium avium* overcome the bactericidal activities of human macrophages. The interaction of mycobacterial cells with its host leads to their replication, yet

the macrophages must remain viable (48). In addition, oxidative response gene products, including catalase, peroxidase and superoxide dismutase, have also been shown to be agents of survival of mycobacteria in macrophages (49, 50, 51).

One of the bactericidal mechanisms of macrophages is the production of reactive oxidative intermediates (ROI) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and single oxygen ($•O_2$). These oxygen species are extremely toxic to microorganisms (52). Therefore, in order to survive within a macrophage it is necessary that any microbial cell is intrinsically resistant to these agents (52). Mycobacteria have the capacity to inhibit production of ROI and produce enzymes that degrade these microbicidal host metabolites (53, 54). Presence of mycobacterial catalase-peroxidases and superoxide dismutases provides defence against ROI and consequently affects virulence. In addition, the role of catalases, peroxidases, and superoxide dismutases in susceptibility to the antibiotic isonicotinic acid hydrazide (INH or isoniazid) is covered because of the long known interrelationship between virulence and susceptibility to isoniazid (55, 56).

1.4.1. ROS and mechanisms of maintenance of "redox homeostasis"

The process of redox signalling is adopted by various organisms including bacteria to induce protective responses against oxidative stress and to restore the original state of "redox homeostasis" after temporary exposure to ROS/RNS. Prokaryotes have several different signalling pathways for responding to ROS or to alterations in the intracellular redox state. Studies on *Escherichia coli* explored that low levels of ROS activate expression of several gene products involved in antioxidant defense including Mn- SOD, catalase, glutathione reductase, and others. Several proteins that are synthesised in *E. coli* after exposure to hydrogen peroxide are under the control of the OxyR locus. The OxyR protein controls protective responses against lethal doses of hydrogen peroxide or against killing by heat (57). Hydrogen peroxide or an oxidative shift in the intracellular thiol/disulphide redox state converts the reduced form of OxyR (containing –SH groups) into its oxidised and regulatory active form containing –S–S– groups. The formation of disulphide bonds can be reversed by glutaredoxin and by thioredoxin.

1.4.2. Mycobacterial catalases and peroxidases

Catalases (hydroxyperoxidases) degrade H_2O_2 to water and oxygen in a single reaction. Peroxidases also degrade H_2O_2, but use H_2O_2 to oxidize a variety of substrates. Co-factors for peroxidases include the red and green hemes. Mycobacteria produce a catalase-peroxidase, so-called because the enzyme has both these activities.

Within the genus *Mycobacterium* three different types of catalase-peroxidases have been described. The heat-labile, H_2O_2-inducible Kat*G* catalase- peroxidase (T-catalase) is a member of the HPI group of catalases. The heat-stable, non-inducible Kat*E* catalase-peroxidase (M-catalase), belongs to the HPII group. The HPI Kat*G* catalase-peroxidase is resistant to aminotriazole, while the HPII Kat*E* 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 Kat*E* HPII catalase, but has greater resistance to high temperature, has a different charge, is more hydrophobic, and fails to react with antibody to Kat*E* (58). *M. tuberculosis* expresses only a single catalase, the Kat*G*, heat-labile, H_2O_2-inducible, HPI type catalase- peroxidase (58). The first cloning of *M. tuberculosis* catalase-peroxidase (Kat*G*) and demonstration of its role in INH resistance published by Zhang et al. (1992). The enzyme consists of two similar domains, like cytochrome-c-peroxidase of the yeast. The catalase-peroxidase domain and activity is located in the NH2-terminus. The function of C-terminal end has not been described so far, but could be involved in the binding of the enzyme to substrate (59). The *katE* gene of *M. avium*, encoding an HPII-type, heat-stable, M-type catalase-peroxidase has been cloned (60) as has the *katG* gene of *M. intracellulare*, encoding a HPI-type, heat-labile and T-type catalase (61).

Another antioxidant protein is alkylhydroperoxide reductase (AhpC). This H2O2-inducible enzyme may act to protect mycobacteria from peroxides, especially in the absence of Kat*G* (62, 63).

1.4.3. Catalase-peroxidases as mycobacterial virulence factors

The Kat*G* catalase-peroxidase of *M. tuberculosis* appears to promote the persistence in infected tissue. Kat*G*–deletion mutants of *M. tuberculosis* had lower levels of survival in spleens of mice and guinea pigs (64, 65). Introduction of a cloned *katG* catalase peroxidase gene into the *katG*–deletion strain led to persistence in spleens of mice and guinea pigs (65). The same appears not

to be the case for *M. intracellulare*. An isogenic *katG*+ and *katG*– pair of *M. intracellulare* strains had the same growth kinetics in mouse tissue (66).

Catalases and peroxidases may also have a role in mycobacterial resistance to reactive nitrogen intermediates (RNI). RNI, which include nitric oxide (NO•), are bactericidal. It has been shown that RNI are responsible for killing mycobacteria in alveolar macrophages (67). Recently it has been shown, that the *M. tuberculosis* Kat*G* catalase-peroxidase has peroxynitratase activity (68), suggesting that it could be involved in resistance to reactive nitrogen intermediates. Possibly, RNI interact with peroxide, generated by mycobacterial cell metabolism, to produce other bactericidal products such as singlet oxygen (67). Alternatively, the iron within catalase (i.e., Kat*G* and Kat*E*) may interact with, and thus detoxify, NO•. Results of studies using different, non-isogenic strains of *M. tuberculosis* have provided conflicting data. *M. tuberculosis* strains that lacked catalase activity (i.e., strains B1453 and H37RaHR) were the most sensitive to RNI, but other *M. tuberculosis* strains that were catalase-positive (i.e. strains 79112 and H37a) were also sensitive to RNI (69). Those results show the weakness of comparing non-isogenic strains. It is quite likely that other RNI detoxification systems exist. A similar result was seen when hydrogen peroxide susceptibility of *M. tuberculosis* was investigated. H_2O_2 susceptibility was correlated with the presence of low levels of catalase activity in INH-resistant isolates, but not in low-virulence, INH-sensitive, catalase-positive isolates (69).

1.4.4. Mycobacterial superoxide dismutases

Superoxide dismutases (SOD) catalyse the dismutation of the superoxide radical to H_2O_2 and molecular oxygen. Superoxide dismutases have been distinguished on the basis of their associated metals, namely iron, copper-zinc or manganese (70).

SOD activity has been detected in a variety of mycobacteria. The first report about cloning of *M. tuberculosis* SOD and demonstration of a secreted feature of the SOD was described in Zhang et al. (1991). *M. tuberculosis* produces two SODs: one (Sod*A*) that employs an iron-cofactor (71) and a second (Sod*C*) thatcontains a copper-zinc co-factor (70). *M. leprae* (72) and *M. smegmatis* strain TAKEO (73) have been reported to produce a manganese-containing SOD. The pattern of inhibition of SOD activity of strains of *M. avium*, *M. intracellulare*, and *M. scrofulaceum*, suggested that these species produced SODs containing

both iron and manganese (74). However, those studies were performed on crude extracts, not purified enzymes. The *sodA* gene of *M. avium* was cloned and shown to encode a 23 kDa protein consisting of 207 amino acids that shares a high degree of similarity with SodA superoxide dismutases of *M. tuberculosis* and *M. leprae* (75, 76). Unlike the Sod*A* of *M. tuberculosis*, the *M. avium* enzyme was described as a Mn SOD enzyme (76). Both the iron-SOD of *M. tuberculosis* and the Mn SOD of *M. avium* are exported from cells and large amounts are found in the extracellular medium (52, 76, 77). The iron-SOD of *M. tuberculosis* is secreted via a *secA2*-catalyzed export pathway (78).

1.4.5. Superoxide dismutases as mycobacterial virulence factors

Like catalase-peroxidase, mycobacterial superoxide dismutase may be involved in macrophage survival and consequently, the virulence of mycobacteria. However, like the data on catalase-peroxidase there are conflicting reports. In one report, a null mutation in the *M. tuberculosis* copper-zinc superoxide dismutase gene (*sodC*) did not reduce survival in activated and inactivated murine bone marrow-derived macrophages. There was also no difference in the survival of the two isogenic strains in guinea pig tissues (70). In contrast, a second report demonstrated that a *sodC*-null mutant was more susceptible to killing by gamma interferon-activated murine peritoneal macrophages (79). In both reports, the *sodC*-null mutants were more susceptible to superoxides compared to their *sodC*-wild type parents (70, 79). *M. tuberculosis* mutants with reduced levels of the exported iron-SOD were more susceptible to H_2O_2 and were almost avirulent (52).

1.5. Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (79). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (KatG) and Alkyhydroxy peroxidase (AhpC). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants.

This balance is essential for the survival of organisms and their health. Mycothiol (MSH) is the principal low-molecular-weight thiol, unique to mycobacteria and other actinomycetes, that performs a role analogous to glutathione found in other organisms. MSH plays a key role in oxidative stress management and is oxidized to the dimeric mycothiol disulfide (MSSM) in the process. NADPH-dependent Mycothiol disulfide reductase (Mtr) helps to maintain an intracellular reducing environment by reducing MSSM back to MSH. Mtr inhibition studies are currently impaired by limited availability of MSSM.

The main protective roles of glutathione against oxidative stress are (80): (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionetransferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase; (iv) glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, *via* reduction of semidehydroascorbate to ascorbate. The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) (81).

Ascorbic acid is a well-known antioxidant; also it is known as an effector of gene expression and a modulator of ROS-mediated cell signaling. It is been reported that M. tuberculosis produces a novel, highly specific L-golono-1, 4-lactone dehydrogenase (Rv 1771) and has capacity to synthesize Vitamin C (82). The presence of this system in mycobacterium also indicates the requirement of maintaining ROS balance and cell signaling in the organism. Recently, it is reported that ascorbic acid is involved in developing dormancy phenotype in ***M. smegmatis*** (83). Also earlier it is reported that *Allicin*, an antioxidant from *Allium sativum*, reduced tuberculosis survival in macrophages. It is found that *Allicin* suppressed M. tuberculosis induced ROS and TNF-α mRNA expression in human monocytes. Hence it is suggested as a valuable natural antioxidant for combating tuberculosis (84) here, we like to suggest that a wide range of natural antioxidants can be equally important to profligate tuberculosis.

1.6 Persistent and dormant tubercle bacilli and latent tuberculosis

There is a great deal of confusion concerning the term dormancy, persistence, latency, and latent infection.

1.6.1. Definition of Dormancy Dormancy can be defined as a reversible state of bacterial metabolic shutdown (85, 86). The term "dormant bacteria" refers to bacteria in a state of dormancy, where the bacteria with low metabolic activity remain viable but do not form colonies directly or immediately on solid medium but can be resuscitated to form colonies on plate under appropriate conditions (85, 86). Dormancy phenomenon is not unique to M. tuberculosis, and various bacterial species also form dormant bacteria under appropriate conditions such as starvation, aging, low oxygen or low temperature (85, 86, 87). Bacteria undergo morphological changes, i.e., become smaller and coccoid or ovoid, upon extended starvation in old cultures (85, 87, 88).

1.6.2. Definition of persistence According to Walsh McDermott, a pioneer and expert in microbial persistence, the term persistence refers to "the phenomenon whereby otherwise drug-susceptible microorganisms have the capacity to survive indefinitely within mammalian tissues despite continued exposure to the appropriate drug or drugs" (90). McDermott and his colleagues tested 10 antituberculosis drugs singly or in combination as pairs of 2-3 drugs, which all failed to alter the persistence phenomenon. They went on, "Even the administration of pyrazinamide and a companion drug, a chemotherapy which rendered the tuberculous infection truly latent, i.e., hidden beyond the limits of diagnostic reach, did not abolish the phenomenon of microbial persistence" (90).

1.6.3. Definition of latency. To avoid confusion with the term dormancy, which is used to describe a bacterial property, the term latency should refer to *in vivo* situation where bacteria and the host have established a balanced state without causing apparent symptoms in the host, as in latent infection (91). Latent infection is commonly detected by tuberculin skin test (TST) and more recently also by detecting interferon-γ. (QuantiFERON-TB test) produced by blood lymphocytes in response to specific M. tuberculosis antigens (92, 93). It is worth noting that latent infection does not say anything about the metabolic or growth status of the tubercle bacilli in the host. It simply indicates the host is infected but has not developed symptoms. It could be that a small number of bacteria are actively multiplying but are controlled by the host so that an equilibrium is established between the bacteria and the host.

1.6.4. Models of Dormancy and Persistence

1.6.4.1. The mouse model of mycobacterial persistence

Although various circumstantial evidence suggests that M. tuberculosis can persist or remain dormant *in vivo* in humans for long periods of time (94), the most convincing evidence of dormant M. tuberculosis was demonstrated by McDermott and McCune and colleagues at Cornell University in New York in the 1950's and 1960's in a mouse model, called the Cornell model (95, 95). In this model, mice were infected with virulent M. tuberculosis and the infection was allowed to establish for two weeks, followed by treatment with a combination of INH and PZA for 3 months. No bacilli could be demonstrated in the mouse spleen as assessed by plating tissue homogenates on agar medium after 3-month treatment with INH and PZA. However, one third of the mice relapsed with culture-positive tubercle bacilli when the treatment was discontinued for 3 months (95). Almost all mice relapsed with TB when immunosuppressive steroids were given (96). Clearly, dormant tubercle bacilli persisted in the tissue and were insensitive to antibiotic treatment. This indifference of the dormant bacilli to the drug treatment was not due to development of stable drug resistance, as these bacilli were still susceptible to the drugs upon subculture. This phenomenon of mycobacterial persistence is thought to be the cause for the lengthy TB chemotherapy. There are variations of the Cornell model that basically show the same disappearance and reappearance phenomenon of tubercle bacilli in mice with different infectious dose, route of infection (aerosol or intravenous), different drug combinations (97, 98), and different agents other than steroids used in inducing the reactivation of the disease.

1.6.4.2 The Wayne model of TB "dormancy"

Because tubercle bacilli *in vivo* are thought to be located in low oxygen environment such as inside macrophages, granulomas or caseous lesions, Wayne established an "*in vitro* model of dormancy" where *in vitro* grown TB cultures are subjected to gradual oxygen depletion to mimic tubercle bacilli *in vivo* (99, 100, 101). Using this model of low oxygen tension, Wayne proposed a two stage nonreplicating persistence for M. tuberculosis *in vitro* (101). The first stage designated NRP 1 (nonreplicating persistence stage 1), occurred when the declining oxygen level reached 1% saturation (equivalent to microaerophilic conditions) and this stage is characterized by increased production of glycine dehydrogenase and steady ATP generation. The second stage,

NRP 2, happened when the oxygen level reached 0.06% saturation (equivalent to anaerobic conditions) and this stage is characterized by a marked decline of glycine dehydrogenase and susceptibility to metronidazole (101). Nitrate reduction was increased in hypoxic shiftdown in non-replication persistence and was proposed as a marker for monitoring the shiftdown (102). The protein synthesis in the bacilli was shutdown in the Wayne model but remained responsive to heat shock and the bacterial viability remained unchanged (103, 104). However, the bacilli in NRP 1 or NRP2 stage of the Wayne model were fully viable and gave about 108 cfu/ml (101), presumably because the relatively short time 10-14 days employed is not sufficient to convert most bacilli to dormant stage yet. (101). According to the definition of dormancy (which refers to bacteria unable to form colonies on direct plating but can be resuscitated under appropriate conditions), the Wayne TB "dormancy" model does not fit this criterion and appears to be more like a model of low oxygen adaptation. Nevertheless, the Wayne model has led to the identification of several factors such as isocitrate lyase (ICL) and glycine dehydrogenase that are related to persistence *in vivo* (105, 106). Thus the Wayne model may represent a stage on the way towards dormancy and would still be a useful model to study dormancy as low oxygen may trigger dormancy upon extended incubation. Indeed, a recent study showed that longer incubation time of several months in the Wayne model appears to produce significant numbers of dormant bacilli (89). Despite the susceptibility to metronidazole by the bacilli under anaerobic condition in the Wayne model (101), metronidazole had little or no activity in the mouse model of persistent TB (107, 108), indicating that the persistent bacilli *in vivo* may not be the same as those in the Wayne persistence model.

1.6.4.3. The rifampin “persister” model

This model was recently developed by Hu *et al.* (104), where a 100 day old stationary phase M. tuberculosis culture was subjected to incubation with high dose of rifampin (RIF) at 100 μg/ml for 5 days. The bacilli exposed to RIF treatment failed to form colonies on 7H11 agar plates but were able to grow in fresh 7H9 liquid medium. These authors went on to show the persistent bacilli still had the ability to metabolize C14-palmitate and produce mRNA for sigB, rpoB and hspX (104) The inability of the rifampin persisters to form colonies on plates but still grew in liquid medium is analogous to the dormant bacilli found in the Cornell model. A modification of the rifampin persister model is to add PZA along with RIF to old cultures, eliminating a further

bacterial population and the residual bacilli more resemble dormant bacilli (104). The nonculturable orpersistent cells from RIF or PZA treated bacilli still had mRNA transcripts and incorporated radioactive uridine into their RNA, indicating that persistent bacilli still had transcriptional activity (104).

1.6.4.4. The "non-culturable" resuscitation model

Cultivation of Mycobacterium smegmatis cells in a nitrogen-limited minimal medium followed by prolonged storage at room temperature without shaking resulted in the gradual accumulation of morphologically distinct ovoid forms characterized by (i) low metabolic activity; (ii) elevated resistance to antibiotics and to heat treatment; and (iii) inability to produce colonies on standard agar plates (non-platable cells). Detailed microscopic examination confirmed that ovoid cells possessed an intact cell envelope, specific fine structure and large electron-transparent bodies in the cytoplasm (105). Non Culturable cells of wild-type *M. smegmatis* resume growth when transferred to a suitable resuscitation medium. Significantly, resuscitation was observed when either recombinant Resuscitation Promoting Factor (Rpf) protein or supernatant derived from a growing bacterial culture was incorporated into the resuscitation medium. Moreover, co-culture with *Micrococcus luteus* cells (producing and secreting Rpf) also permitted resuscitation (89). The mechanism of this RPF mediated resuscitation is still unknown.

1.7 Latency: The root cause of Tuberculosis problems

In the 21st century, we face the problems of billions of people with latent tuberculosis infection. Latency has been defined as the "presence of any tuberculosis lesion which fails to produce symptoms of its presence" (110). Despite the immune system's ability to clear much of the bacilli and arrest an infection, the lungs may still contain small caseous foci. The first evidence of latent TB in the caseous foci was the reoccurrence of an infection with non drug resistant tubercle bacilli after treatment with a regimen of antibiotics (111). The nature of latency was further elucidated by chemoprophylaxis treatment, which showed that the longer the period of treatment, the lower the chances of reactivation (112). Since susceptibility to antibiotics required some level of growth and metabolism, it was suggested that there was some degree of growth and metabolism of *M. tuberculosis* during latent state. Therefore, persistent bacilli in a

lesion are likely to be in a steady state in which intermittent replication is balanced by immune system destruction. The balance of this steady state will determine active disease versus latent infection.

Latency is achieved by cell-mediated immune response which restricts the growth of *M. tuberculosis* bacilli. The restriction, however, does not eliminate the pathogen, leaving the bacilli as a present danger to reactivate years later. Secondary infection occurs as the reactivation of an old lesion with latent bacilli at the apical zone of the lungs (113). Individuals infected with *M. tuberculosis* have a 10% chance during their life time to develop active tuberculosis from a latent infection. 5% of the infected population will develop the disease after 5 years and the others will suffer from it at some point during their lives (114). Often, the precipitating factor for latent tuberculosis reactivation is a waning immunity, which takes place mostly in the elderly at an estimated rate of 5% per year untills complete disappearance of immunity (115). Factors such as corticosteroids, immunosuppressant, HIV and other factors that lower resistance are a danger for reactivation as well.

The contribution of exogenous reinfection to the incidence of secondary tuberculosis has been largely ignored because it was assumed that the primary infection would provide protection against secondary infection. However, there is skepticism about the idea of dormant bacilli waiting to reactivate due to immunosuppression. Often, TB primary complex is sterile within five years, suggesting that secondary infection is an infection with exogenous bacteria (116). It has also been documented that reinfection of some immunocompetent individuals occur with new strains of *M. tuberculosis*. This indicates that immunity to tuberculosis can be incomplete, and that reinfection, at least in areas where tuberculosis is prevalent, probably has a greater role than previously appreciated (117). The dynamic nature of mycobacteria is highlighted by work showing that exogenously infected *M. marinum* in zebrafish, enters pre-existing granulomas by specific mycobacteria-mediated mechanisms that direct infected macrophages into granulomas (118).

Active *M. tuberculosis* lesions generally contain detectable populations of acid-fast, easily culturable bacilli, but bacilli from tubercles of post-chemotherapy, sputum-negative patients often fail to be cultured (119). Extending culture incubation time from weeks to many months enables fully drug sensitive *M. tuberculosis* from closed lesions of drug-treated patient to be

cultured, proving that bacilli from latent tubercles are still viable (120). The difficulty in eradicating *M. tuberculosis* from a latent infection with drugs has also spurred ideas that alternate forms of bacilli may exist, such as protoplast, L-forms (forms without a cell wall), or spores, that may go undetected *in vivo* and are difficult to culture (121,122). Conditions within closed lesions such as a lowered oxygen tension, long chain fatty acids, lactic acid, and other bacteriostatic agents are hypothesized to reduce bacterial metabolism and render the tubercle bacilli resistant to drugs.

1.7.1. Mechanism of latency.

Formation of granulomas coincides with nutrient deprivation, oxygen restriction and an onslaught of host defenses. Long term survival of *M. tuberculosis* within a granuloma presumably consists of different strategies for persisting in harsh environments and evasion of the host immune response. Many genes that are important for pathogenesis have been identified in expression and mutant screens (117).

M. tuberculosis is likely to have significant alterations in gene expression from its encounter with the host immune response, from its transitions of acute to chronic infection, and from log phase growth to stationary phase growth. *M. tuberculosis* contains 13 sigma factors, including a general house keeping sigma factor SigA and several alternative sigma factors with key roles in pathogenesis at later stages of infection (123). Alternative sigma factors bind to the core RNA polymerase and direct it to the promoters that are not normally recognized. Transcription of several sigma factors have been identified to increase during limiting conditions: *sigB*, *rpoS*-like sigma factor, and *sigE* increase during entry into stationary phase, under conditions of low aeration, heat and hydrogen peroxide (124); *sigE* and additionally *sigF*, are expressed during growth of *M. tuberculosis* in a macrophage (125). Disruption of the *M. tuberculosis sigH* gene and a gene encoding a putative transcriptional regulator, *whiB3*, result in attenuation of virulence in mice, while not having an impact on the bacterial load (126). Mice infected with mutants for each of these genes (*sigH* or *WhiB3*) show an altered inflammatory process in the lungs as compared to mice infected with wild-type *M. tuberculosis*. These studies indicate different regulons may be controlled depending on environmental conditions, and altered expression may be essential for TB persistence.

To circumvent nutritional shortages, *M. tuberculosis* has developed alternative means for generating energy. The pathogen increases breakdown and utilization of fatty acids, which are abundant in caseous environment of the granuloma, as a source of carbon and energy during infection in the lungs (127). Among the pathways required for utilization of fatty acids is the glyoxylate cycle, present in many bacteria but absent in vertebrates. Using bacteria carrying knockout mutations, McKinney has shown that *M. tuberculosis* late-stage persistence in mice was facilitated by isocitrate lyase (ICL), an enzyme that is essential for the metabolism of fatty acids in glyoxylate cycle (128). Disruption of the *icl* gene had no effect on growth in the acute phase of infection in mice, indicating that during late stages of infection, *M. tuberculosis* may reside in an environment with limited carbohydrates and might convert lipids into carbohydrates.

Adaptation to oxygen limitation by *M. tuberculosis* induces enhanced resistance to isoniazid and rifampin, thermotolerance, decreased protein synthesis and thickened cell wall (129). In response to oxygen restriction, microarray analysis identified the induction of two-component system DevR-DevS (130). Inactivation of DevR abolishes the rapid induction of hypoxia induced gene expression, and the *M. tuberculosis* mutant has decreased survival compared to wild type Mtb after 40 days under hypoxic conditions. A microarray study has found that DevR controls the expression of a 48-gene "dormancy regulon," which is induced under hypoxic conditions and by nitric oxide (131). Regulons differentially expressed under hypoxic conditions are likely mechanisms for *M. tuberculosis* adaptation and survival in microaerobic granuloma.

A shift from aerobic to hypoxic conditions also induces the alpha crystallin protein in *M. tuberculosis*, a 16-kD chaperone protein. Decline in α-crystalline protein parallels the loss of *M. tuberculosis* tolerance to anaerobic shock. The protein has also been identified to play a protective role in the survival of bacilli and its growth in macrophages (132).

Morphologic changes are associated with *M. tuberculosis* long-term survival as well. Persistence and virulence of *M. tuberculosis* has been associated with "cording" a formation of rope like tangles of laterally associated bacilli (133). Using a transposon mutagenesis approach, cord formation was found to depend on PcaA, an enzyme responsible for the cyclopropanation of β-mycolates, which are long chain alkyl β-hydroxy fatty acids that are a major constituent of the mycobacterial cell wall. A *pcaA* mutant's enhanced bacterial replication in mice during the acute

phase of infection, followed by a later defect in persistence, may indicate that cell wall components can modulate host immunity in a stage-specific manner (134).

The discoveries of some of the *M. tuberculosis* survival mechanisms indicate that the pathogen is capable of altering its transcription to adapt changing environment. Persistence is due to its ability to conserve energy by shutting down unused biochemical pathways and utilizing pathways consistent with environmental resource availability. The state of dormancy reflects organims's ability to regulate its cellular metabolic activity.

1.8. ROS paradox

Due to lack of literature available on ROS and mycobacterium interaction a paradox is created in drawing a conclusive line on the issue. Mycobacterium bacilli persist in an oxidizing environment but ROS interaction with bacilli is still unclear. Although ROS produced by macrophages is well accounted but ROS produced by bacteria is completely neglected. An earlier report revealed that production of significantly less amount of ROS in D9 macrophage cell line decreases survival of *M. tuberculosis* in comparison to other related cell lines even though the pathogen is relatively resistant to killing by ROS *in vitro* (135). Mice deficient of p47 (136) or gp91 (137, 138) subunit of NADPH oxidase are relatively resistant to *M. tuberculosis* infection. *M. tuberculosis* possesses functionally active antioxidant enzymes like sodA and sodC (70, 79, 139). High extracellular release of SodA in the medium by *M. tuberculosis* prompted to conclude that these enzymes are used as defense mechanism to sustain the oxidative pressure within the host (139). Interestingly, SodC mutant of *M. tuberculosis* is highly susceptible to superoxide and hydrogen peroxide under *in vitro* conditions and also showed decreased survival in murine macrophages (70, 79). An attenuated sodA strain of *Mycobacterium tuberculosis* generated via antisense technology had severely impaired its survival in mice (52). Although the functional role of the anti-oxidant enzymes SOD and KatG has been shown to play important role in the survival and pathogenicity of mycobacteria within host systems, poor survival of their mutants under in vitro condition raises some fundamental issues about their role in axenic cultures (140). Mostly in prokaryotes superoxide is generated by ETC, NADH oxidase and fumarate reductase. In past few years many research groups had identified NADH oxidase

activity in different *Mycobacterial* sp (141). It is found that KatG and Lipoamide dehydrgenase has NADH oxidase activity. KatG catalyzes NADH to NAD+ conversion to produce superoxide and hydrogen peroxide produced as by product at pH 7.0 (142). Very recently, it has been reported that an oxidizing environment actually promotes the intracellular growth of *M. abscessus* and a reducing environment inhibits *M. abscessus* growth (143).

1.9. Strategies for dealing with persistence of TB

In spite of better understanding of the physiology of *M. tuberculosis*, our knowledge about the state of the bacillus during the latent period is far from being complete. Moreover, a true representative model of latent tuberculosis in the laboratory setting is not available. Establishment of such a system would certainly accelerate the efforts to understand the physiology of mycobacteria during the latent period and eventually it will help in the identification of new drug targets that can act on the persistent mycobacteria. Recent advances in modern biology, in combination with bioinformatic tools, proteomics and microarray technology would further facilitate the search of new drug targets against tuberculosis. These exciting techniques are providing new avenues for understanding the biology of mycobacteria. The result of better understanding of the physiology of mycobacteria is manifested by the fact that the list of possible drug targets for tuberculosis is increasing day by day, the utility of these targets, however, cannot be predetermined. The list of potential drug targets encoded in the genome of *M. tuberculosis* include genes involved in persistence or latency, cell wall synthesis, virulence, signal transduction, genes encoding transcription factors and enzymes of other intermediary metabolic pathways (130). All these targets should be explored to identify new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

Besides the choice of drug targets, there are many different approaches one should consider and use in TB drug development. One is the way drug screens are designed. Current TB drugs were mostly discovered based on their activity against growing bacilli *in vitro*. However, activity against nongrowing persister bacilli is correlated with good sterilizing activity that is responsible for shortening therapy *in vivo*, as shown by PZA and RIF. Thus, novel drug screens

that mimic *in vivo* conditions in lesions (i.e. acidic pH and hypoxia) and act against old stationary-phase nongrowing bacilli could be important for identifying drugs that kill persisters and thereby shortening TB treatment. Along this line of combination to screen is the recent interest in the use of systems biology approach for drug discovery. Instead of the conventional reductionist approach of finding a single drug that hits a single target, the systems biology approach proposes using multiple compounds that hit multiple targets in different pathways to achieve the desired outcome. A systems biology approach can be used for identifying novel drug combinations against latent TB.

1.10. Thesis objectives

This thesis was inspired by a major objective in tuberculosis research that is switching from active to dormant and resuscitation of mycobacterium. Most important question was existing ROS paradox with reference to mycobacteria. As mycobacterium is an intracellular pathogen it has to face an environment excess of ROS & RNS, acidic pH and nutrient starvation. Mostly focus of mycobacterial research was focused on ROS generation by host cells but its typical behavior in axenic cultures was neglected. We first investigated the reason of slow growth in *Mycobacterium sp.* in axenic cultures. We modulated concentration of ROS using different antioxidants, SOD inhibitor and ROS generators to understand the relevance of ROS generation in process of bacterial growth. The first part of the study focused on generation of different ROS in actively growing mycobacterium. We found a significant amount of ROS production, so we identified different type of ROS such as superoxide, hydrogen peroxide and hydroxyl radical in vivo. As all kind of ROS is inter-convertible our emphasis was mainly on source of superoxide generation (Chapter 2).

Once the results of ROS generation were confirmed, its source and physiological role was identified (Chapter 3). We extended understanding of bacteriostasis due to scavenging of ROS as dormancy. Here our main emphasis was to confirm the lack of ROS created bacteriostasis was dormancy phenotype of mycobacterium. We elucidated potential role of ROS in switching from active to dormant stage by reversing the process in presence of extraneous addition of ROS. We attempt to priorities specific ROS (whether superoxide, Hydrogen peroxide or hydroxyl radical)

involved in process. How this transition is taking place at molecular level and which are the proteins involved in process? This major question was addressed by global expression of proteins in different conditions like active, dormant and resuscitated bacilli.

As NADH oxidase turned out as major contributor of superoxide generation in actively growing mycobacterial cells. NADH oxidase expression in different conditions such as actively growing, hypoxia and antioxidant induced dormancy as well as ROS resuscitated dormant bacilli was monitored. Further, Cloning, purification and partial characterization of NADH oxidase was done improve our understanding about enzyme (chapter 4).

These studies may help us to understand the fundamental biochemistry and biology of achieving latency and resuscitation in *M. smegmatis*. Furthermore this study reveals the significance in preventive and therapeutic use of antioxidants.

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CHAPTER 2

Generation of ROS in actively growing *Mycobacterium smegmatis*

2.1 Introduction

Reactive oxygen Species (ROS) comprise superoxide radicals (O^{-}_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) formally originating from one, two, or three-electron transfers to dioxygen (O_2). The toxicity of ROS strongly depends on the presence of a Fenton catalyst such as iron ions or peroxidase, giving rise to extremely reactive $^{\bullet}OH$ radicals in the presence of H_2O_2 and O^{-}_2. Superoxide is produced when a single electron is transferred from a donor atom to molecular oxygen. The $E^{'}_0$ for univalent oxygen reduction is low ($E^{'}_0$ = -0.16 V), requiring that donor should be a strong univalent reductant to push the reaction forward. Most of biological molecules cannot macth this standard, for example, instability of NAD^+ prohibits spontaneous transfer of electron from NADH to molecular oxygen (1). This suggests flavin and quinone moieties as potential superoxide generators. Hydrogen peroxide is mostly generated in cell by dismutation of superoxide by Superoxide dismutase (SOD). It is well documented in living system primarily as a molecule for host defense and oxidative biosynthesis. The most stable radical among ROS, H_2O_2 is considered as potential molecule in redox signaling. On contrary, i*n vivo* production of highly reactive hydroxyl ion is a rare biological phenomenon reported in very few prokaryotes (2).

ROS is described in recent years as key physiological regulator of many cellular functions like transcriptional regulation, direct oxidative modification, protein turnover, protein interaction and enzyme modification (3). Although its role is well established in higher eukaryotes, fragmented evidences suggest the presence of a simpler system in unicellular eukaryotes as well as prokaryotes (4). Production of superoxide and hydrogen peroxide is well documented in *E.coli* and *E.faecalis* (2, 5). In fact, SoxRS and OxyR regulon in *E.coli* is thoroughly studied ROS- responsive transcription factor in bacterial system (6, 7). Fe-S cluster associated with aconitase, dihydroxyacid dehydrogenase, fumarases and 6-phosphonogluconate dehydrogenase in *E.coli* are evidenced as target of superoxide attack to alter their physiological role (8, 9).

In this chapter, we quantified significant amount of ROS, such as superoxide, hydrogen peroxide and hydroxyl ion, generation. Also, we attempted to determine the precise site of

superoxide production. In earlier reports, potential anti-mycobacterial activity was reported by different antioxidants (10, 11). But the mode of action of these

2.2. Materials and Methods

2.2.1. Chemicals, strains and Media

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. *M. smegmatis* strain ATCC 607 was used throughout the present study which was grown in a defined medium containing 0.5 gm KH_2PO_4, 0.2 gm sodium citrate, 60mg $MgSO_4$, 0.5 gm asparagine and 2 ml glycerol in 100ml of distilled water at pH 6.6. The stock culture was maintained at -70^0 C and sub cultured once in liquid medium before inoculation to an experimental culture.

2.2.2. Cultivation of oxygen depletion induced dormant bacilli

For the cultivation of anaerobic dormant bacilli Wayne's 0.5 HSR tube model was followed wherein 20x125 mm tubes 25.5 ml of the medium was inoculated with 1% v/v of 1 O.D580 culture (27). The culture tubes were incubated at 37^oC for 8days after making air tight with rubber septa after inserting an 8mm magnetic spin bar and gently stirred at 100rpm on a magnetic stirrer. Viable cells were counted by method described earlier (10).

2.2.3. Susceptibility testing

Susceptibility testing for *M.smegmatis* was done using the Colony Forming Unit per milliliter (CFU / ml). Initial stock solution (10mM) and subsequent dilutions of antioxidants, SOD mimic, SOD inhibitors and superoxide generators was prepared in dimethyl sulfoxide (DMSO).

2.2.4 Assay for superoxide production

2.2.4.1. NBT/ TritonX100/ Hydroxylamine based assay NBT (Nitro blue tetrazolium) stock solution (10 mM) was prepared in potassium phosphate buffer (20 mM, pH 6.0) when required, or stored at –20°C. For the assay, NBT was added to aerobically growing cells at a concentration of 500 μM, and incubated for the indicated time. Reaction with superoxide causes the pale yellow NBT to form its bright blue-colored formazan which was further dissolved in Triton X100 and Hydroxylamine. The dissolved mixture can be measured by monitoring its absorbance at 580 nm.

2.2.4.2 XTT based assay XTT (2,3-bis- [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) stock solution (10 mM) was prepared in potassium phosphate buffer (20 mM, pH 6.0) when required, or stored at –20°C. For the assay, XTT was added to aerobically growing cells at a concentration of 100 μM, and incubated for the indicated time. Reaction with superoxide causes the pale yellow XTT to form its bright orange-colored formazan, the accumulation of which can be measured by monitoring its absorbance at 470 nm.

2.2.4.3. DHE Microscopy The generation of superoxide was confirmed through detection of dihydroethidium (DHE) fluorescence from *M.smegmatis* cells under a microscope by following a method described earlier (12). Briefly, *M.smegmatis* late log phase culture of 1.0 OD_{620} was washed in PBS and resuspended again in PBS containing 25μM of DHE. These cells were incubated for two hours at 37ºC on an orbital shaker at 150 RPM. Cell were washed in PBS and visualized by fluorescent microscopy (LeitzWetzlar, Germany) with I2 excitation filter (530nm) and Emission filter (600nm) at 40x optical zoom.

2.2.4.4 DHE HPLC Initially, cells were washed twice with PBS/DTPA by centrifugation (10000 RPM for 5 min at 4 ºC). PBS containing DTPA (100 μM) and DHE (10 μM) is added and cell suspensions were kept for 60 min at 37 ºC. Excess DHE was removed by washing twice with PBS/DTPA and centrifuged at 10000 RPM for 5 min at 4ºC. The cell pellet was resuspended in 500μl acetonitrile, sonicated (10 s, 2 cycles at 30 hz), and the lysates were centrifuged at 12,000 RPM, 10 min, 4ºC. Thereafter, the supernatant was transferred to an eppendrof tube and acetonitrile dried under vacuum for 2 to 3 h (Speed-Vac Plus SC-110A, Thermo Savant). For HPLC analysis, samples were resuspended in 200 μl PBS/DTPA, and a volume of 100 μl was used for injection.

Chromatographic separation was performed with C18 reverse phase column (Aligent 250 x 4.6 mm) a gradient of solutions A (pure acetonitrile) and B (water/10% acetonitrile/0.1% trifluoracetic acid) was used as a mobile phase at a flow rate of 0.4 ml/min. Runs were started with 0% solution A, increased linearly to 40% solution A during the initial 10 min, kept at this proportion for another 10 min, changed to 100% solution A for additional 5 min, and to 0% solution A for the final 10 min. DHE was monitored by ultraviolet absorption at 245 nm. EOH

and ethidium were monitored by fluorescence detection with excitation 480 nm and emission 580 nm (13).

2.2.5 Assay for Hydrogen peroxide production

2.2.5.1. DCFH-DA assay 2', 7'-dichlorofluorescin - diacetate was prepared freshly in potassium phosphate buffer (20mM, pH 6.0) when required. This solution was added to log phase culture cells of OD ~ 1.00 at 580 nm at 20μM final concentration of DCFH-DA. Immediately this solution was measured for increase in fluorescence (excitation 485nm and emission 520nm) due to oxidation of DCFH to DCF by using a fluorescence spectrophotometer (LS 55, Perkin-Elmer, Foster City, CA).

The percentage increase in fluorescence per well was calculated by formula $[(Ft - Ft_0)/ Ft_0 * 100]$, where Ft = fluorescence at time t and Ft_0 = Fluorescence at time 0 min. This method of analysis has advantage over analyzing just net change n fluorescence in that, only did the calculation directly reflect percentage changes of florescence over time from the cells in same well, they also effectively control for variability among the wells. This method also cancelled out the background fluorescence in each well, and therefore, a no cell control is not needed.

2.2.5.2. Amplex Red/ Horse Radish Peroxidase Assay Production of hydrogen peroxide was estimated by using amplex red (10-acetyl-3,7-dihydroxyphenoxazine) / HRP mix following an earlier method (14). Briefly, 5ml of 0.1 O.D. culture measured at 600nmwas centrifuged at 8000 RPM for 5mins at 4ºC temperature. Then, the pellet was resuspended in 5ml PBS containing 10μ/ml of amplex red and 0.2 units of HRP and incubated for 60 min. The Cells were visualized under a fluorescence microscope and the emission intensity was measure by fluorescence spectroscopy at excitation λ= 560 and emission λ =600 using 10mm bandwidth each for excitation and emission respectively.

2.2.6 Assay for Hydroxyl radical production

Hydroxyl radical production was estimated as described earlier (15) with some modification by incubating *M.smegmatis* in 20 mL of buffer containing 20 mm 2-deoxy-D-Ribose (Sigma). The formation of the breakdown product malondialdehyde was determined by mixing 750 μL of centrifuged incubation medium with 250 μL of 2-thiobarbituric acid (Serva; 10 g L21 in 50 mm NaOH) and 250 μL of trichloroacetic acid (28 g L21). After heating in boiling water for exactly

10 min, cooling in tap water, and clarifying by centrifugation, the reaction product was measured Spectrscopically (λ=560nm) against reagent blanks.

2.2.7 Membrane preparation from *M. smegmatis*

Initially, spheroplast of *M. smegmatis* was prepared by following as earlier described method (16). Briefly, the method includes addition of spheroplast solution having final composition as follows cycloserin (0.006% w/v), lysozyme (0.002% w/v), LiCl(1% w/v) and EDTA (0.2% w/v) in the exponentially growing log phase culture and incubated for 12 hrs at 37°C within a incubator shaker rotating at a speed of 150 RPM for spheroplast formation. Spheroplasts obtained from the above treatment were washed and resuspended in 20 mM K-phosphate buffer saline pH 6.6.The suspension was then sonicated for 5mins in water bath sonicator at 35 kHz (Bandelin electronics, Germany). Due to mechanical sheer, fragile spheroplasts were broken down in smaller membrane fragments. These membrane fragments were kept on ice before use. For each experiment, freshly prepared membrane fragments were used.

2.3. Results

2.3.1. Growth of *M.smegmatis* at different shaking conditions

To identify the effect of different aeration conditions on growth and survival of M*ycobacterium* cells, *Mycobacterium* cultures were kept at three different shaking speeds 130, 200 and 250 RPM. This experiment was done in two different media Dubos (enriched medium) and *M. phlei* (minimal medium). It was found that there is marked decrease in cell count as RPM increased in presence of both type of medium. At 130 RPM in Dubos medium CFU/ml was 24.2 x 10^6 which decreased to 21.8 x10^6 at 200 RPM which further decrease to 0.8 x 10^6 at 250 RPM. Similarly, in *M. phlei* medium at 130 RPM CFU/ml was 10.4 x 10^6 which decreased to 5.4 x10^6 at 200 RPM which further decrease to 0.2 x 10^6 at 250 RPM. There was consistent decrease in cellular survival as RPM was increased. As it is well known that aeration increase generation of ROS in the cells our first assumption was this reduction in cell growth is because of increase in ROS.

Now the major question was if we modulate ROS level in what will be effect on growth and survival of mycobacterial cells.

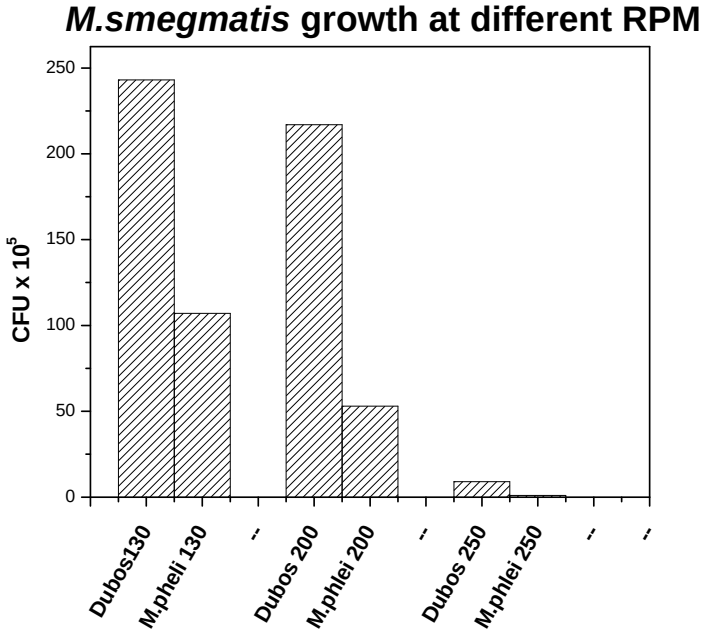
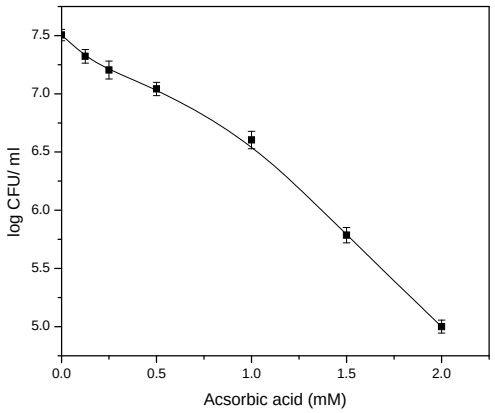
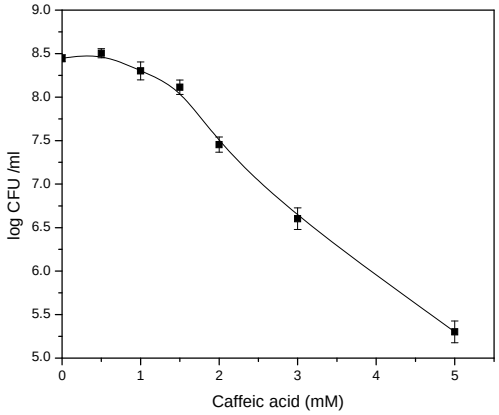


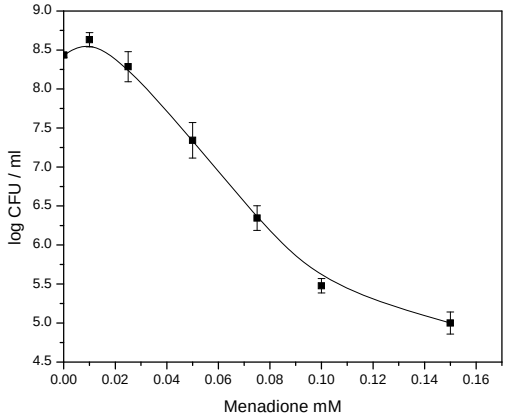
Fig 2. 1 Growth of *M. smegmatis* at different shaking conditions 0.1% of ~1.0 OD620 *M. smegmatis* culture was separately inoculated in Doubos and M. pheli medium. The cultures were kept at 37ºC at 130, 200 and 250 RPM under identical conditions. After 3 days, CFU counts were taken as described in "materials and methods". The result is an average with SD obtained from three identical experiments

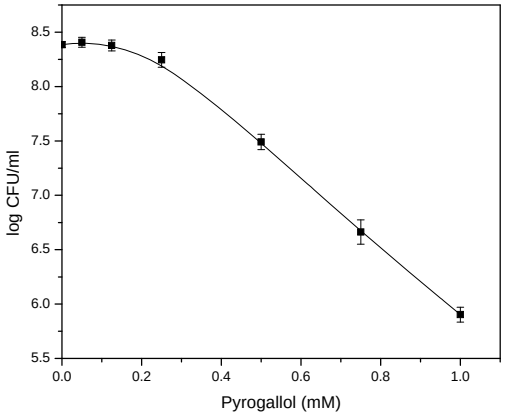
2.3.2. Effect of ROS modulation on growth of *M. smegmatis*

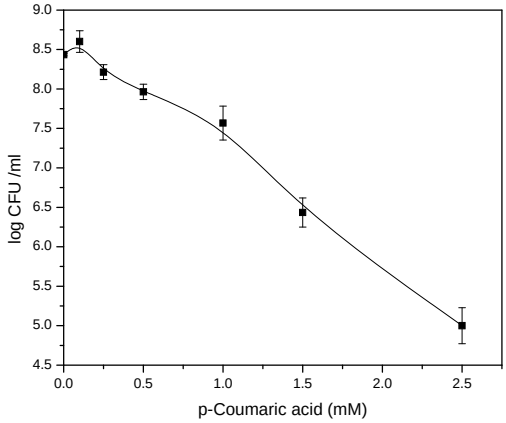
In order to identify the presence of an ROS in mycobacterium, we challenged it with different types of ROS modulators in whole cell culture and monitored the inhibitory effect on growth of the bacilli (Table 1). These modulators were superoxide generators like menadione and pyrogallol, antioxidants like ascorbic acid, p-coumaric acid, caffeic acid and hydroquinone, SOD mimic like TEMPOL and SOD inhibitor like Trine. The ratio of IC_{90}/IC_{50} values obtained from menadione and pyrogallol was≈1.5. Both menadione and pyrogallol are well known superoxide generators which could be assumed to be their mode of action in killing the bacilli (17). IC_{90}/IC_{50} ratio of ≈ 2 in case of Trine, a well-known SOD inhibitor, also indicated a similar bactericidal pattern. For caffeic acid, TEMPOL (SOD mimic) and hydroquinone these values were 2.25, 2.57 and 2.69, respectively (18). For ascorbic acid and p-coumaric acid, the ratio of IC_{90} and IC_{50} values were found to be 6.56 and 3.82, respectively. Ascorbic acid and p-coumaric acid are well known antioxidants also reported to be antibacterial in nature (19). Except TEMPOL, all other anti-oxidants reacts with both superoxide and hydroxyl radicals. The characteristic difference between the dose response patterns suggests their difference in affinities toward oxygen free radicals (20). The CFU data suggests both superoxide generators and SOD inhibitor were bactericidal and free radical scavengers were bacteriostatic in nature. Evidently, similar effect of superoxide generators and SOD inhibitor on *M. smegmatis* was observed possibly due to exposure of higher level of superoxide which created a toxic environment for bacterial survival. On the contrary, scavenger action is supposed to decrease ROS level below the effective range for generating cell growth possibly due to the lack of signal for sustaining multiplication of the bacterium.

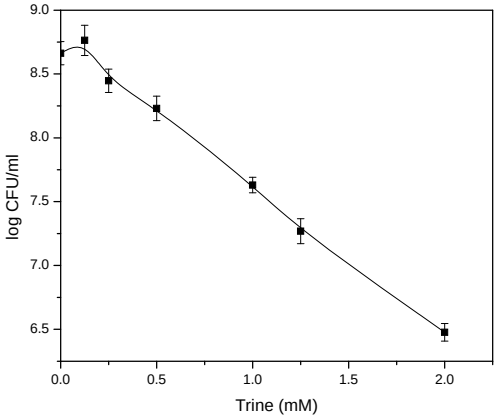

log CFU/ ml
Acsorbic acid (mM)


log CFU /ml
Caffeic acid (mM)


log CFU / ml
Menadione mM


log CFU/ml
Pyrogallol (mM)


log CFU /ml
p-Coumaric acid (mM)


log CFU/ml
Trine (mM)

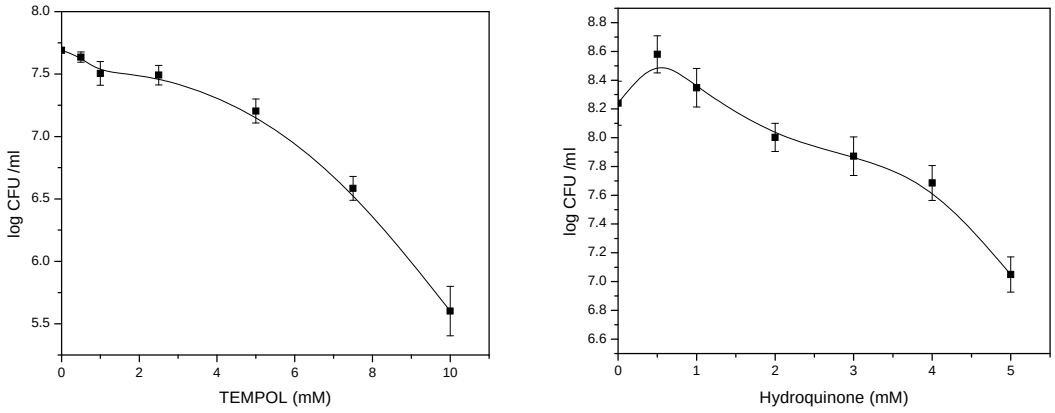


Fig 2. 2 Dose response curve used for IC_{50} and IC_{90} calculation for Table 1. Different ROS modulators were added in the culture medium dose dependent manner at the time inoculation. After two days cultures were spread on dubos agar plates for CFU count.

Table 2. 1 IC_{50} and IC_{90} value of different ROS modulators determined against *M. smegmatis*.

S.No.	ROS modulator	IC_{50}(mM)	IC_{90} (mM)	IC_{90}/ IC_{50}
1	Ascorbic Acid	0.25±0.02	1.64±0.07	6.56
2	Caffeic acid	1.2 ± 0.11	2.71 ± 0.18	2.25
3	Hydroquinone	1.5 ± 0.1	4.04 ± 0.3	2.69
4	Menadione	0.048 ± 0.005	0.07 ± 0.007	1.45
5	p-coumaric acid	0.35 ± 0.03	1.34 ± 0.17	3.82
6	Pyrogallol	0.58 ± 0.03	0.82 ± 0.04	1.51
9	TEMPOL	3.5 ± 0.26	9.01 ± 0.79	2.57

10	Trine	0.87 ± 0.17	1.77 ± 0.29	2.03

2.3.3. Detection of superoxide in aerobically growing *M. smegmatis* cells

Growth of the bacilli in liquid medium occurs best under relatively mild shaking conditions which is indicative of optimum requirement of dissolved O_2 in the medium. In order to investigate the underlying reason behind the required low level of O_2 in the medium , cultures were kept at 130, 200 and 250 RPM with same head space ratio in each flask and incubated at 37^{o}C (Fig. 2. 1). After 72hrs of incubation, when the cultures were plated for CFU, it was found that the viable cell count decreased to 1.0x10^{7}at 200 RPM and 0.8 x 10^{5}at 250 RPM respectively from 2.42 x10^{7} obtained at 130 RPM. A higher degree of aeration became detrimental to viability possibly due to increased oxidative stress generated by increasing rate of aerobic respiration (21). Organisms thus posses a well regulated system for the production of ROS in the form of superoxide which can be sequentially converted to H_2O_2 and $OH^{\cdot}$ (22).

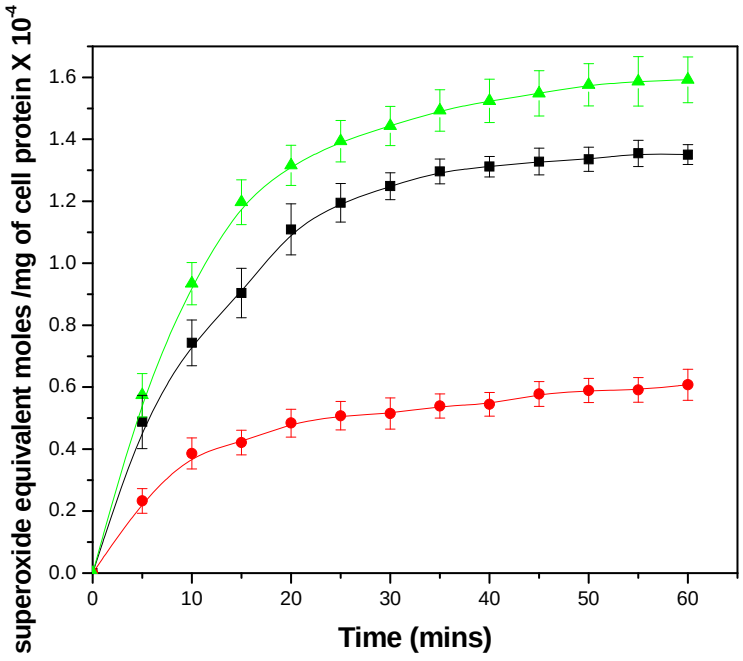


Fig. 2. 3. Kinetics of superoxide production.1.0 OD_{620} *M. smegmatis* culture resuspended in 50mM K-phosphate buffer (pH 7.2) containing 500µM NBT. Then, the OD at 580 nm was followed for an hour in presence of vehicle (■) control, (▲) DEDTC (50µM), (●) DPI (20µg/ml)

Table 2. 2 Effect of antioxidants and SOD inhibitor on NBT reduction by *M.smegmatis*

S.No.	Substrate and inhibitors	Membrane fragment percent activity if control is zero	Whole cell Percent activity if control is Zero
1	DEDTC	+140±12	+142±13
2	Caffeic acid	-78 ± 6	-75 ± 6
3	P-Coumaric acid	-76 ± 4	-61 ± 6
4	TEMPOL	-71 ± 3	-73 ± 3
5	TIRON	-74 ±6	-71 ± 5

In order to detect superoxide production in *M. smegmatis,* bacterial cells were exposed to Nitro Blue Tetrazolium (NBT) dye. The kinetics of NBT reduction showed an initially higher rate up to the first 30 minutes, then gradually attaining saturation (Fig. 2. 3). The initial rate of superoxide production was equivalent to 0.085 mole/minute/mg of whole cell protein under the mentioned condition. In order to specify the superoxide dependent NBT reduction, DEDTC an inhibitor of SOD, and DPI were added (4). Increased reduction of NBT is possibly due to increased availability of superoxide in presence of DEDTC whereas the reverse phenomenon occurred in presence of DPI by inhibiting a potential source of superoxide production. Superoxide production in aerobic culture was further confirmed by fluorescence images using superoxide specific dye, dihydroethidium (DHE) (Fig. 2.4.). Increased DHE specific fluorescence clearly indicated production of superoxide within the aerobically growing mycobacterial cells. It was recently reported that the reaction product of superoxide with

dihydroethidium yields a fluorescent product oxyethidium. Using HPLC with fluorescence detector, we successfully to separated the reaction product after DHE incubation with *M. smegmatis* cells as oxyethidium (13).

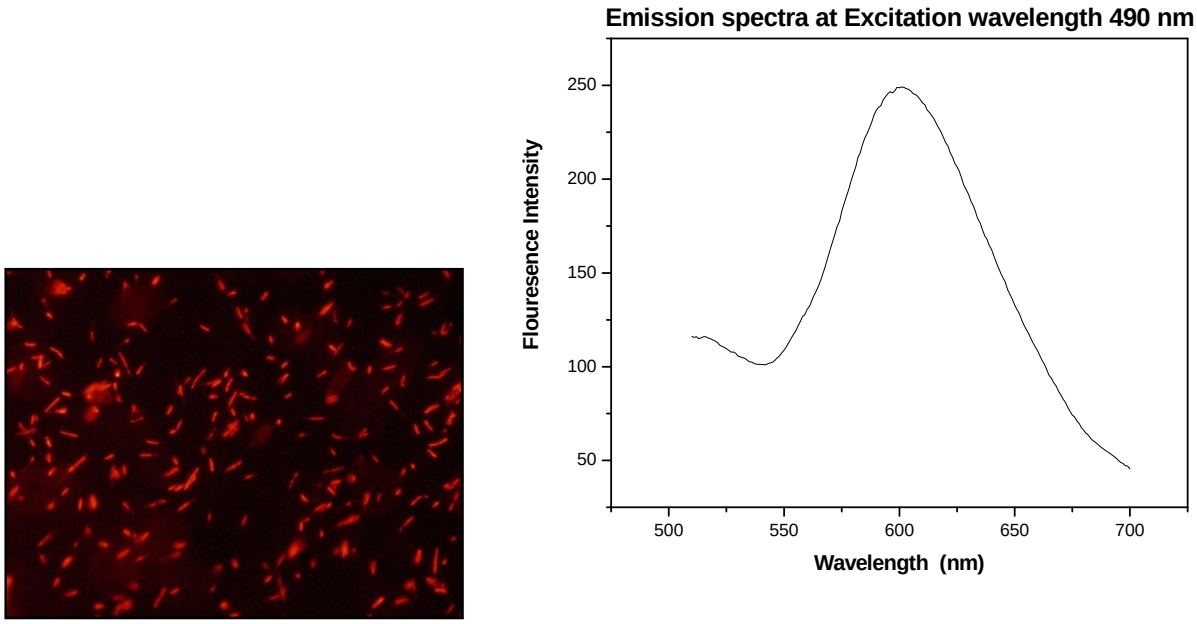
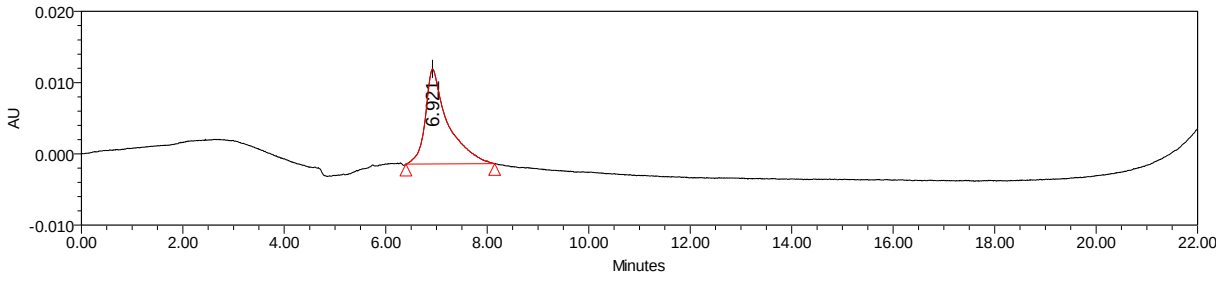
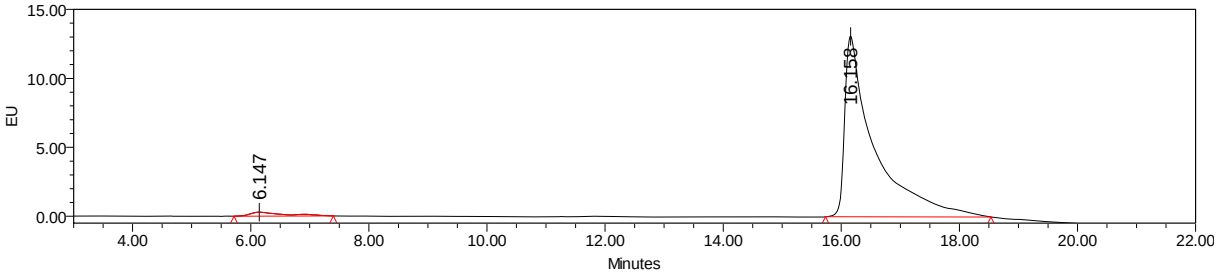


Fig. 2. 4 Demonstration of superoxide production by aerobically growing culture. Late log phase culture of 1.0 OD_{620} was washed in PBS and resuspended again in PBS containing 25µM of DHE and incubated for two hours at 37°C on an orbital shaker at 150 RPM. Photograph was taken at 40x optical zoom with excitation filter 530 nm and emission filter 600nm.



	Name	Retention Time	Area	% Area	Height	Int Type	Amount	Units	Peak Type	Peak Codes
1		6.921	406969	100.00	13346	bb			Unknown	



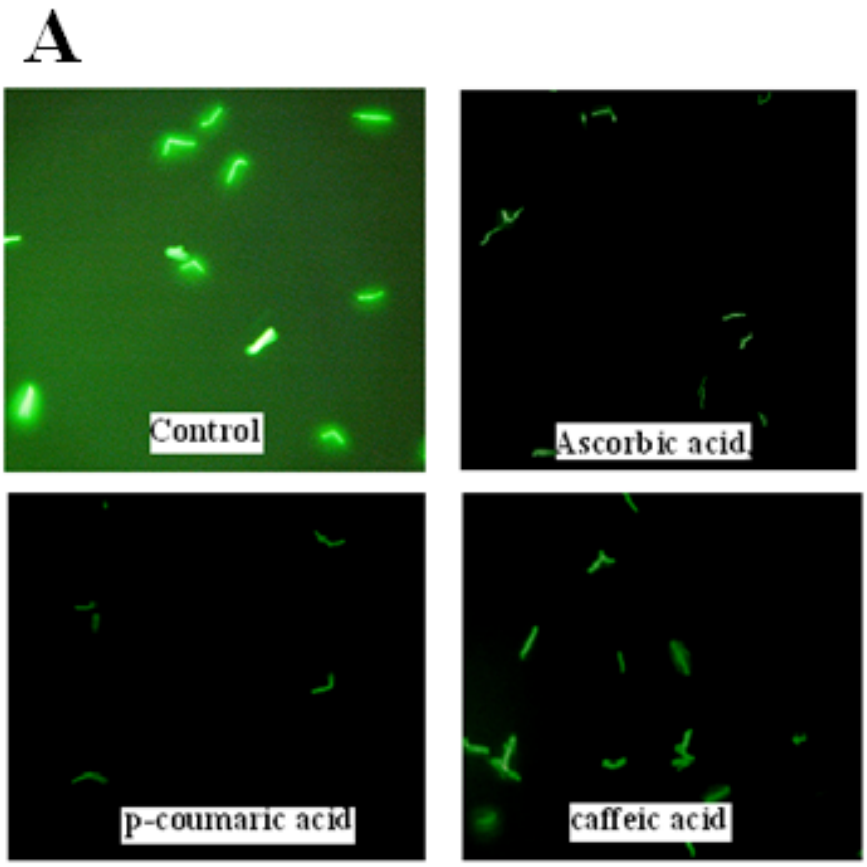
	Name	Retention Time	Area	% Area	Height
1	Dihydroethidium	6.147	119393	2.55	2924
2	Oxyethidium	16.158	4568417	97.45	131271

Fig. 2. 5 HPLC analysis of DHE derived product Reverse phase HPLC was performed with a gradient of acetonitrile (i) 10μM DHE absorbance at 355 nm, it was separated at 6 mins (ii) *M. smegmatis* cells were incubated with 10μM DHE for 60 min in PBS then after acetonitrile extraction resuspended in PBS and injected in HPLC. At excitation λ 480 and emission λ 580 elution of oxyehidium was found at 16 min.

2.3.4 Detection of H_2O_2 in aerobically growing *M. smegmatis* cells

It is imperative that part of superoxide be converted to H_2O_2 by SOD, which can be detected by a specific dye. For this, we have used both DCFH-DA as well as amplex red (14). Initially, H_2O_2 production within the bacilli was monitored by using DCFH-DA by a fluorescent microscope (Fig. 2A). The decrease in fluorescence intensity within bacilli was clearly visible in cells previously treated with ROS scavengers like p-coumaric acid, ascorbic acid and caffeic acid when applied at their respective IC_{50} values. A quantitative estimation of the effect of these free radical scavengers have shown that ascorbic acid and p-coumaric acid decreased DCF fluorescence intensity to almost 65% compared to control whereas caffeic acid reduced it up to 56% (Fig. 2.6.). The differential effect of these scavengers on DCF fluorescence was found to follow similar grouping which again suggested that ascorbic acid and p-coumaric acid have a higher affinity for superoxide radical than others. Amplex red was also used to confirm the intracellular production of H_2O_2 within the bacilli (Fig 2.6). Emission of fluorescence signal

from amplex red treated bacilli clearly indicated presence of H_2O_2 within the bacterium. Further, the H_2O_2 concentration was measured by using amplex red/ HRP assay as 2.6μM per 1x 10^5 cells. In an earlier report using similar method, it was demonstrated that *E. coli* wild type cell produce around 0.04 μM of H_2O_2 which is far less in comparison to 1.8 μM by Ahp^-/ $katG^-$ *E. coli* mutants (23). Thus, the results clearly established that the production of H_2O_2 takes place within actively growing mycobacterial cells under aerobic condition. This also possibly indicated that the intracellular environment of *M. smegmatis* is more towards oxidative in nature.



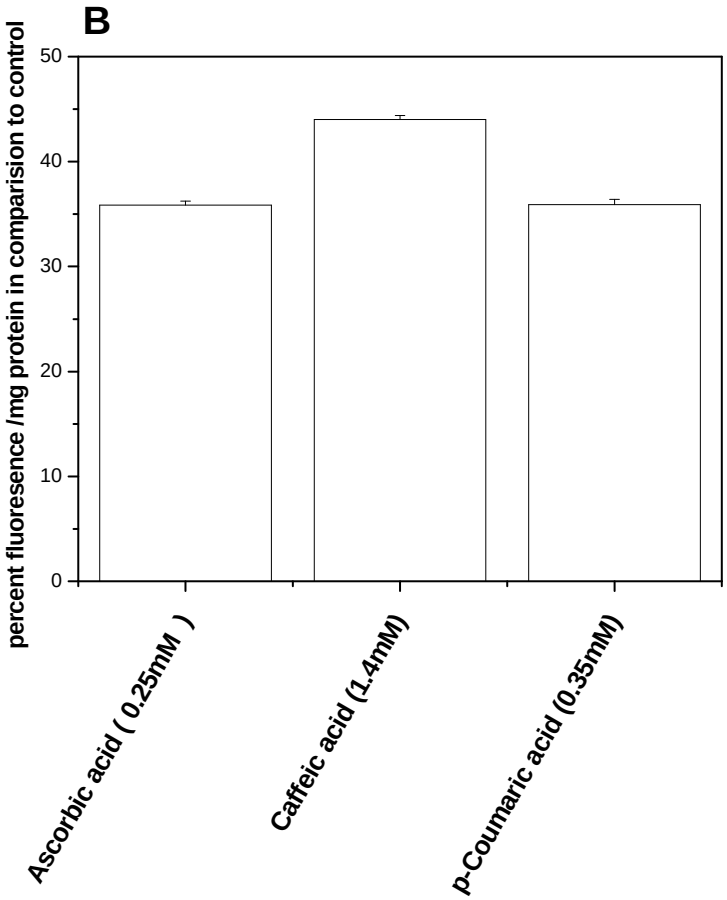


Fig. 2. 6 Hydrogen peroxide generation measured by DCFH-DA Log phase ~1OD_{620} culture of *M.smegmatis*was incubated in PBS containing DCFH-DA (20µM) for 30mins in presence of ascorbic acid, p-coumaric acid, caffeic acid and vehicle control at their respective IC_{50} values. The effect of DCFH-DA fluorescence monitored by (A) Fluorescence microscopy as well as from (B) Fluorescence intensity measurement.

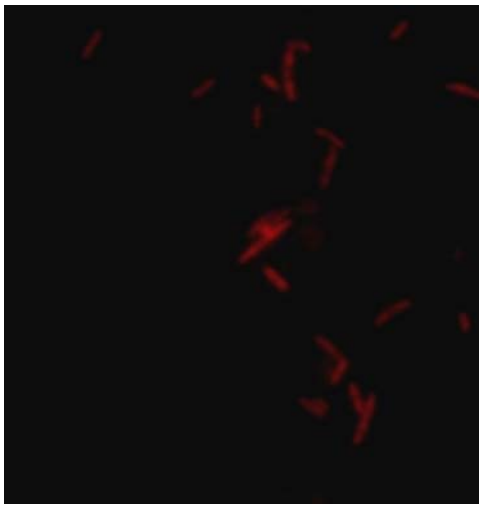


Fig. 2. 7. Fluorescence microscopic image of *M. smegmatis* *M. smegmatis* cells in PBS were incubated with 10µM amplex red and 0.2 units HRP for 60 min. After washing with PBS cells were fixed in paraformaldehyde and mounted in 70% glycerol on a glass slide. Then images were captured by a fluorescence microscope

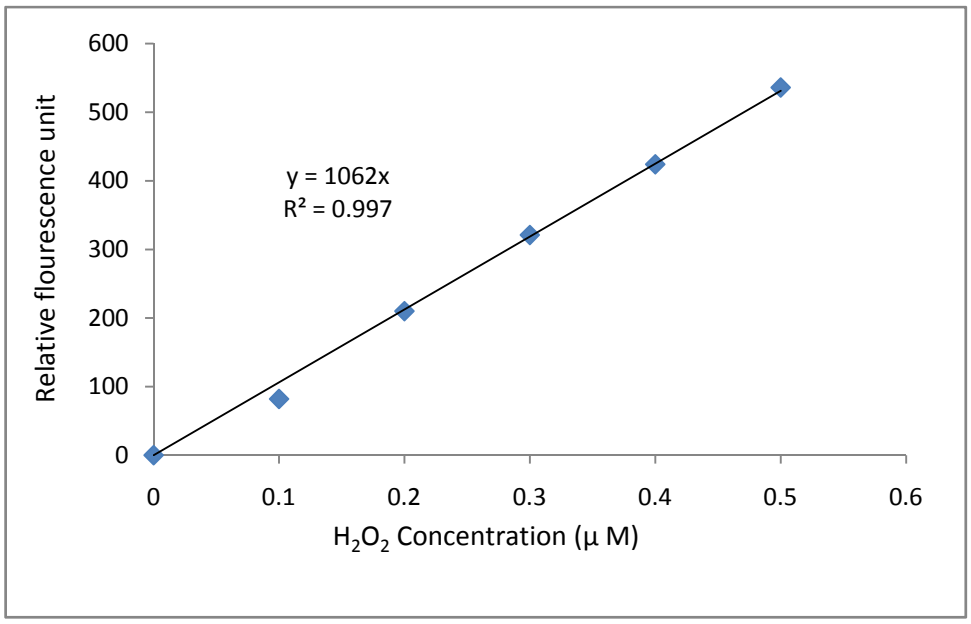


Fig. 2. 8. Standard plot of hydrogen peroxide solution used for quantification of cellular hydrogen peroxide. Hydrogen peroxide solution of concentration range 0.1µM to 0.5µM were incubated with

10μM amplex red and 0.2 units HRP for 60 min. Then fluorescence was measured at excitation λ= 560 and emission λ =600.Results obtained are representative of three identical experiments.

2.3.5. Hydroxyl radical generation by aerobically growing culture of *M.smegmatis*

In leukocytes, superoxide radical gets converted into H_2O_2 by SOD which in presence Fe^{2+} is further converted into ·OH radical. ·OH radicals are very short lived, which makes it difficult to measure in living systems. There is no direct method available for its detection in living systems. After modification and careful standardization of deoxy-D-ribose degradation method we found it to be suitable for estimation of ·OH radical production in *M.smegmatis*. 2-deoxy-D-ribose can be cleaved by hydroxyl radical to form malondialdehyde which further reacts with thiobarbutaric acid to give an adduct which can be measured spectrophotometerically at 560nm. Here, we found that oxidation of 2-deoxy-D-Ribose remained constant up to 8 hours in aerobic culture of *M.smegmatis*.

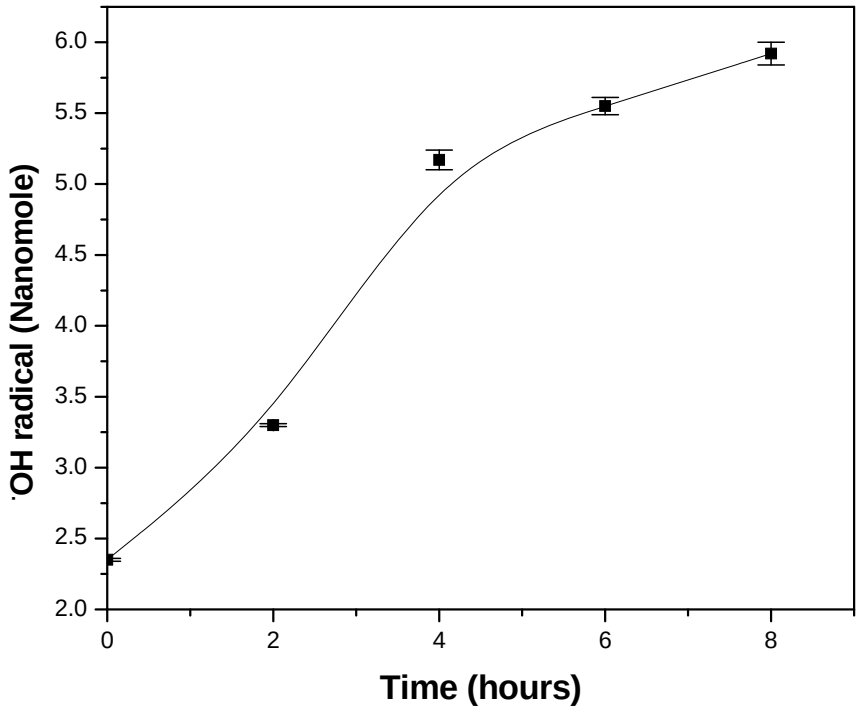


Fig. 2. 9 Hydroxyl radical generation by aerobically growing culture of *M. smegmatis* *M. smegmatis* cells were incubated with 20mM of 2-deoxy-D-Ribose in PBS at 37ºC at 130 RPM. Oxidation of 2-

deoxy-D-Ribose by aerobically growing culture of *M.smegmatis* shows generation of Hydroxy radical by organism. Result is an average of three identical experiments.

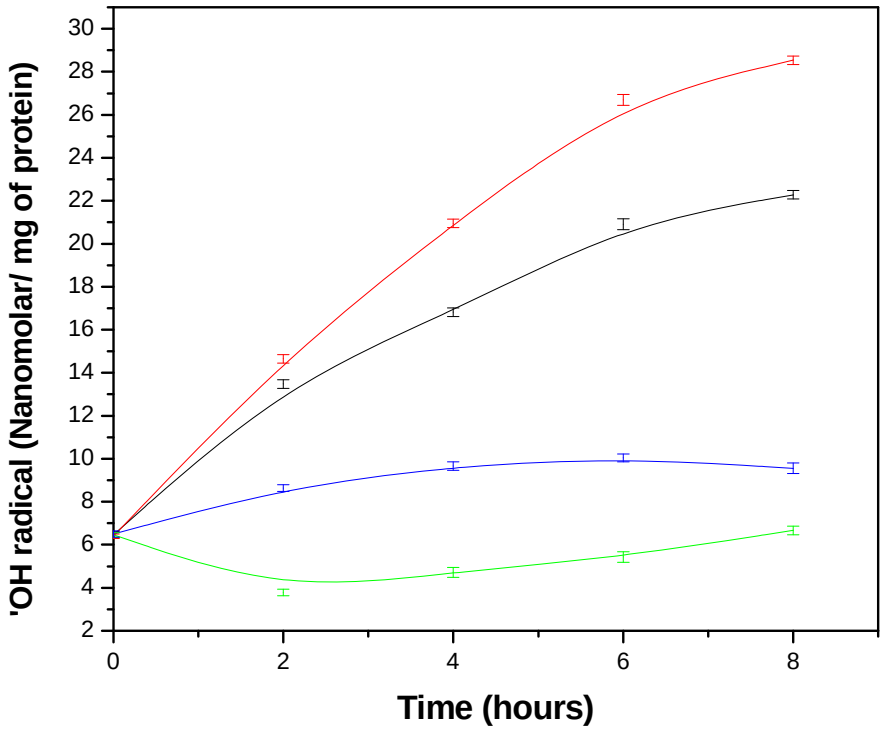


Fig 2.10 Effect of iron salts on oxidation of 2-deoxy-ribose by aerobically growing culture of *M. smegmatis* *M. smegmatis* cells were incubated with 20mM of 2-deoxy-D-Ribose in PBS at 37ºC at 130 RPM in presence of (■) control, (■) $FeSO_4$ (0.1mM), (■) 2%DMSO, (■)$FeCl_3$ (0.1mM). Oxidation of 2-deoxy-D-Ribose by aerobically growing culture of *M. smegmatis* shows generation of Hydroxy radical by organism. Result is an average of three identical experiments.

Oxidation of 2-deoxy-D-ribose was inhibited significantly by Hydroxyl radical scavengers like thiourea, sodium benzoate, sodium formate and Dimethyl thiourea when applied in the culture. Although no significant decrease was observed in case of standard antioxidants like Ascorbic acid, p-Coumaric acid, Hydroquinone and Caffeic acid, specific scavengers of $^{\bullet}OH$ radicals severely affected the oxidation of 2-deoxy-ribose under otherwise identical condition This experiment very clearly indicated the generation of $^{\bullet}OH$ radicals within the bacilli.

Hydroxyl radical generation by aerobically growing culture of *M.smegmatis* in presence of Ferrous and Ferric ion

In leukocytes, superoxide radical gets converted into H_2O_2 by SOD which in presence Fe^{2+} is further converted into ·OH radical. ·OH radicals are very short lived which makes it difficult to measure in living systems. There is no direct method available for its detection in living systems. After modification and careful standardization of deoxy-D-ribose degradation method we found it to be suitable for estimation of ·OH radical production in *M. smegmatis*. Here, we found that oxidation of 2-deoxy-D-Ribose increased significantly in presence of ferric ion but decrease in presence of ferric ion. DMSO (Dimethyl sulfoxide) is well known electron spin trap showed dose dependent decrease in Hydroxyl ion production.

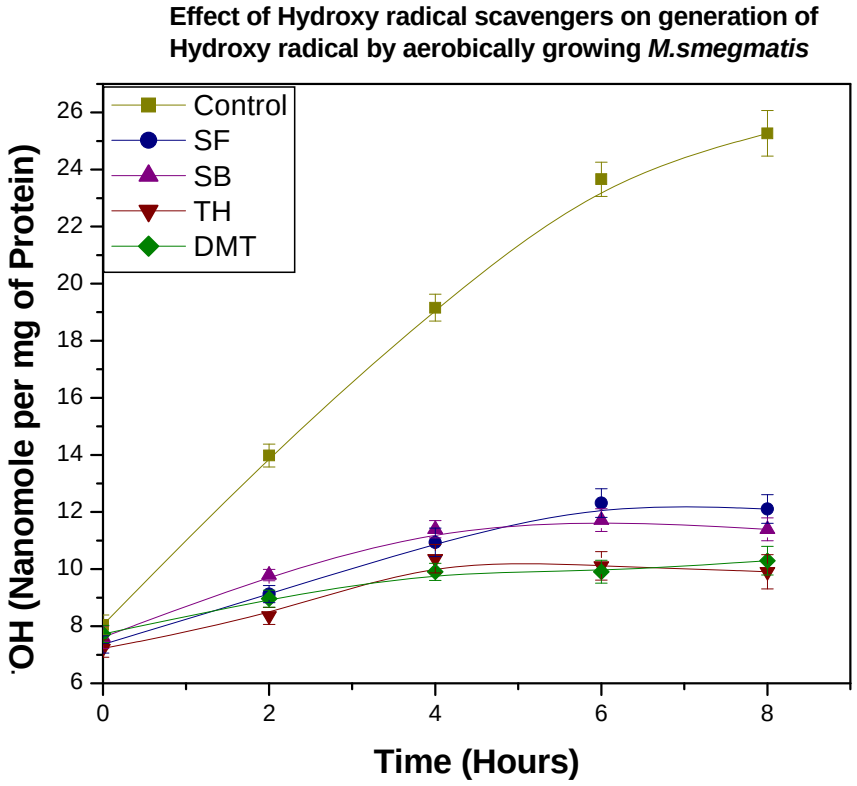


Fig 2.11 Effect of hydroxyl radical scavenger on hydroxyl radical generation by aerobically growing culture of *M.smegmatis* *M.smegmatis* cells were incubated with 20mM of 2-deoxy-D-Ribose in PBS at 37ºC at 130 RPM. Reduced Oxidation of 2-deoxy-D-Ribose by aerobically growing culture of *M.smegmatis* when treated with different different hydroxyl radical scavengers Thiourea (TH) (10mM), Dimethyl Thiourea (DMT) (10mM), Sodium Benzoate (SB) (10mM) and Sodium formate (SF) (10mM). Result is anaverage of three identical experiments.

2.3.5. Identification of NADH oxidase as major source of superoxide production in *M. smegmatis*

Fumarate reductase and terminal quinol oxidase associated with ETC was earlier shown as major source of superoxide production in *E.coli* and *E.faecalis* respectively (23, 24). Although fumarate reductase is an essential enzyme in hypoxia induced dormant mycobacterium, its functional role as superoxide generator is not yet established (25). Also, functional NADH oxidase gene, a bacterial homologue of NADPH oxidase in mammalian cells, is found to be present in *M.smegmatis* (26).

In order to identify the possible source/s of superoxide, we tested the effect of different substrates and inhibitors on the reduction of NBT using both whole cell and membrane fragment of *M. smegmatis* (Table 2.2 & 2.3). In whole cell experiments, NBT reduction was increased to 138% with respect to the control when NADH was added as a substrate to the incubation mix. This NADH dependent increase in NBT reduction was completely inhibited by DPI indicating involvement of the enzyme in superoxide generation.$FADH_2$, NADPH or NAD^+ had no effect on NBT reduction indicating NADH specificity of the superoxide producing enzyme. Moreover, effect of both rotenone and antimycinA on NADH dependent increase in superoxide production was negligible compared to DPI. Further, NADH dependent reduction of NBT in membrane fraction of *M. smegmatis* confirms that NADH oxidase is mainly responsible for the production of superoxide radical within the bacilli. Increase in NBT reduction by 20% in presence of fumarate which was completely inhibited by 2-mercaptopyridine, an inhibitor of fumarate reductase indicated that the enzyme, albeit at a minor level, acts as another potential site of superoxide production in *M. smegmatis* under the mentioned condition. ETC inhibitors like rotenone (complex I) and antimycinA (complex III) showed only ~25% decrease in NBT reduction, even in presence of NADH, indicated that the ETC is a minor contributor to the total pool of superoxide. Moreover, rotenone and antimycinA effect was not additive in nature which limits the involvement of the ETC complex as a single unit in superoxide generation. Also, addition of DPI in XTT-menadione assay completely inhibits the superoxide production by bacterial cells (27).

Table 2.3. Effect of different ROS modulators on superoxide production by *M. smegmatis.*

S.No.	Substrate and inhibitors	Membrane fragment percent activity if control is zero	Whole cell Percent activity if control is Zero
1	NADH (50µM)	+138 ± 16	+ 80 ± 8
2	Fumarate (20mM)	+21 ±2	+22 ± 4
3	2-Mercaptopyridine (250µM)	-23± 2	-24±3
4	Fumarate + 2-mercaptopyridine	-2±0.01	0 ± 0.02
5	Succinate (20mM)	0 ± 0.02	0 ± 0.01
6	Rotenone (200µM)	-24 ± 3	-23 ± 4
7	Antimycin A (20µM)	-26 ± 4	-23 ± 2
8	DPI (10µg/ml)	-45 ± 5	-44 ± 4
9	Fumarate + NADH	+153 ± 7	+91 ± 5
10	NADH + DPI	-23 ± 2	-41 ± 3
11	AntimycinA + Rotenone	-35 ± 6	-32 ± 4
12	NADH + Rotenone	+121 ± 8	+62 ± 7

2.4. Discussion

The effect of differential shaking, superoxide generators, scavengers and SOD inhibitors on *M. smegmatis* indicated that the bacillus possess a controlled intracellular oxidative redox environment directly linked to its growth (Fig 2.1 and Table 2.1).The detection of superoxide and H_2O_2 within this bacillus clearly established that well-developed ROS machinery actively involved in maintaining basic cellular functions (Fig 2.1, 2.2). Withdrawal of intracellular superoxide by using antioxidants severely affected growth of *M. smegmatis* (Table 2.1). Further, membrane bound NADH dependent superoxide production, its inhibition by DPI as well as presence of genes in available genome database clearly demonstrated that the NADH oxidase is the major contributor of superoxide in *M. smegmatis* (Table 2.3). Under aerobic condition, apart from NADH oxidase, fumarate reductase and/or ETC also contributes by almost 20-25% of the total pool of superoxide. Present study provided evidence in favor of *M. smegmatis* producing a significant amount of superoxide, scarcity of which under anaerobic condition could lead to the development of dormancy in bacilli. Noteworthy, decline in redox balance (NAD^+/ NADH) was observed during anaerobic dormancy (28). Evidence of strong up-regulation of NADH dehydrogenase operon (*nuo*A-M) during hypoxia and nutrient starvation can be explained in this context as a requirement to maintain NAD+/NADH balance needed during dormancy (29).

Being the most stable and membrane permeable member among the different ROS, H_2O_2 was reported to play major role in cellular signal transduction processes in many cellular systems (30). Antioxidant enzymes like Cu-SOD, KatG and AhpC in mycobacteria reflect their possible role in maintaining intracellular level of H_2O_2 at 0.65µM (Fig 2.5) (31). Interestingly, our unpublished observation as well as whole genome sequence also suggests the presence of a functional Fe-SOD in *M.smegmatis* (Uniprot data bank) which was first reported in Mtb (32, 33). Effect of Trine, a specific inhibitor of SOD, severely affected the viability of the bacilli. NBT reduction as well as detection of H_2O_2 collectively established the fact that SOD is probably playing a vital role in detoxifying excess superoxide generated within *M. smegmatis* cells under aerobic conditions (Fig 2.1- 2.2 and Table 2.1). The antioxidant enzyme gene cluster was transiently up-regulated when tubercle bacteria stopped growing in mouse lung which can be considered as an adaptation to dormancy that enables the bacillus to transit from acute to chronic infection (34).

In this context, it also should be mentioned that Vitamin D, a potent antioxidant, was earlier used for the treatment of tuberculosis and its deficiency has recently been identified as a major cause of prevalence of the disease in South African population (35). It has been generally observed that patients suffering from tuberculosis have high oxidative stress and lower antioxidant level in the blood serum (36). Earlier reports indicated that antioxidants have both *in vitro* as well as *in vivo* anti-mycobacterial activity (10, 11). The target and actual role of these antioxidants could now be explained in the context of current finding.

Thus, this study suggests that production of superoxide occurs mainly by NADH oxidase and its conversion to H_2O_2 by the endogenous SOD of the bacilli. This H_2O_2 is further converted to hydroxyl radical by cellular Fenton's reaction.

2.5. Reference

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CHAPTER 3

Physiological role of ROS in dormancy and resuscitation of *Mycobacterium smegmatis*

3.1. Introduction

A hallmark of the *Mycobacterium tuberculosis* life cycle is the pathogen's ability to switch between replicative and non-replicative states in response to host immunity. Immunity-induced cessation of bacterial growth leads to a chronic, asymptomatic infection that is maintained by persisting tubercle bacilli. When host immunity falters, tubercle bacilli can resume growth and cause disease. Dormant bacilli present a formidable challenge to tuberculosis control, because they are much less susceptible to antibacterial drugs than growing bacilli (1). The lifelong survival of persistent tubercle bacilli within granulomatous lesions represents a major obstacle to the eradication of tuberculosis (2).Thus, a molecular understanding of events occurring during the transition from actively growing to dormant stage is important for identifying critical targets for new tuberculosis control strategies.

So far, there are different stresses like hypoxia, nutrient starvation, Iron deficiency, NO, CO, ethanol and H_2O_2exposure were used to induce dormancy in mycobacterial bacilli under in vitro conditions (6, 7, 8, 9). However, our understanding about the molecular mechanism of developing dormancy in mycobacterial bacilli still remained at its infancy. As a result of exposure of the bacilli to these chemical and physical stresses different molecular and morphological changes appear in cells. The morphological changes such as reduction in size and change in shape as well as reduced susceptibility towards drugs is widely documented (3). The *DosRS* regulon, a well studied dormancy marker, is induced under a variety of conditions. It is found to be induced also during standing culture condition and centrifugation (both of which generate local hypoxia) (4, 5). Most important is its*in vivo* induction during early infection of macrophages, dendritic cells, mice and guinea pigs (10, 11, 12, 13).

It is reported that Mtb cells within lipid loaded macrophages accumulate lipid droplets containing triacyl-glycerol, lose acid-fast staining and become phenotypically resistant to the two frontline anti-mycobacterial drugs, rifampicin and isoniazid, as well as induce gene transcripts involved in dormancy and lipid metabolism within the pathogen (14, 15, 16, 17, 18).Very recently, antioxidants like vitamins A, C and D3 have also been shown to induce dormancy of Mtb inside macrophages (19, 20). We have earlier shown that superoxide, hydrogen peroxide as well as hydroxyl radical is generated in actively growing *M. smegmatis* cells (chapter 2). Any kind of interference in the production and availability of intracellular ROS is found to cause

dormancy in *M. smegmatis* bacilli. In other words, ROS also acts as signaling molecule in these bacilli as was documented in multi-cellular eukaryotes as well as in many unicellular eukaryotes and prokaryotes (21, 22, 23). Superoxide signaling is evidenced in multi-cellular development of *dictostellium*, *OxyR* regulation in *E.coli*, apoptosis and necrosis, kidney induced hypertension and many other cellular functions (21, 22, 24, 25).Due to its stability, most of the in vivo signaling studies were carried out on H_2O_2 (26, 27). A biologically rare highly reactive hydroxyl radical has signaling role in different diseases (21).A growing pool of evidences suggests that ROS operate in signaling through chemical reactions with specific atoms of target proteins that lead to covalent modification of proteins (22).It is important to note that, earlier studies provide global transcript or proteome analysis data of actively growing and different model of dormant state (29, 30, 31). If we assume that in presence of an inducer, the transition of actively growing bacilli to a dormant one takes place in a stepwise manner, proper analysis of the intermediate stages also should be carried out. So far, there is no such data available for this transition state of active to dormant bacilli and vice versa.

In this chapter, we attempt to identify global changes in sub-cellular expression of *M. smegmatis* proteins after interaction with hydroxyl radicals. Also a comparison of active and dormant state bacilli with respect to HOPT treated resuscitated dormant cells. Here in this chapter, not only global change in resuscitation but also in transition state is underlined.

3.2. Material and Methods

3.2.1. Chemicals, strains and Media

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. *M. smegmatis* strain ATCC 607 was used throughout the present study which was grown in a defined medium containing 0.5 gm KH_2PO_4, 0.2 gm sodium citrate, 60mg $MgSO_4$, 0.5 gm asparagine and 2 ml glycerol in 100ml of distilled water at pH 6.6. The stock culture was maintained at -70^0 C and sub cultured once in liquid medium before inoculation to an experimental culture.

3.2.2. Cultivation of hypoxia induced dormant bacilli

For the cultivation of anaerobic dormant bacilli Wayne's 0.5 HSR tube model was followed wherein 20x125 mm tubes 25.5 ml of the medium was inoculated with 1% v/v of 1 O.D580 culture (32). The culture tubes were incubated at 37^oC for 8days after making air tight with rubber septa after inserting an 8mm magnetic spin bar and gently stirred at 100rpm on a magnetic stirrer. Viable cells were counted by method described earlier (in chapter 2).

3.2.3. Susceptibility testing

Susceptibility testing for *M .smegmatis* was done using the Colony Forming Unit per milliliter (CFU / ml). Initial stock solution (10mM) and subsequent dilutions of 1-Hydroxypyridine -2-thione (HOPT) was prepared in dimethyl sulfoxide (DMSO). At NRP-II stage (after 6 days when culture OD_{600} ~ 0.6) of *M. smegmatis* HOPT was added and accordingly plated on dubos-agar plates.

3.2.4. Scanning electron microscopy of *M. smegmais* cells

Scanning electron microscopy of *M. smegmais* cells was carried out by following an earlier established procedure. Briefly, mid-exponential growth phase cultures were diluted with Dubos medium to a cell density of 0.1 (620 nm) and treated with antioxidant. Both treated and untreated samples were fixed with 2.5% gluteraldehyde overnight at 4^0C, washed four times for 15 minutes with sodium cocodylate buffer at room temperature and then post-fixed with Osmium tetraoxide for 6 hrs at 4^0C. The samples were washed with sodium cocodylate buffer and dehydrated with graded ethanol series of 30%, 50%, 70% 90% ethanol for 30 minutes each and 95% and 100%

ethanol for 1 hour each. Samples were then air dried and microscopy was performed with a Zeiss Supra 55VP (Oberkochen, Germany) microscope. Secondary electron images were taken at low electron energies around 2 keV.

3.2.5. RNA extraction and Reverse Transcriptase-PCR

RNA extraction was done by following a method established in our laboratory (33). Briefly, spheroplast solution consisting of lysozyme (0.002% w/v), D-cycloserine (0.0006% w/v), glycine (1.4% w/v), EDTA (0.2% w/v) and lithium chloride (0.1% w/v) prepared in distilled water was aseptically added to log phase bacterial culture. Different antioxidants (2mM) were simultaneously added in the above mentioned culture. After 12 hrs of incubation, both control and antioxidant treated bacilli were centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 ml of Trizol reagent (Sigma) and then sonicated for one minute at 50 kHz in a water-bath sonicator. After 10 minutes of incubation at 37°C, 200 µl of chloroform:isoamyl alcohol (24:1) was added to the Trizol suspension and mixed vigorously for 15 seconds. Cell debris was removed by centrifugation at 10000 rpm for 10 minutes at 4°C and the upper aqueous phase was collected in a separate tube. 500 µl of isopropanol was added to the aqueous phase, and RNA was precipitated by centrifugation at 15000 rpm for 10 minutes at 4°C. The RNA pellet was washed twice with 70% ethanol and air dried at 37°C for five minutes and then finally resuspended in 50 µl DEPC water. Qualitative as well as quantitative analysis was performed with agarose-gel electrophoresis and a NanoDrop spectrophotometer. For RT-PCR, 1.0 µg of total RNA was reverse transcribed using random primers provided with the 'single stranded cDNA synthesis kit' (Sigma) by following the manufacture's protocol. The synthesized cDNA was used for RT-PCR of DosS, DoSR and HspX genes using their specific primers (34). 40-µl PCR reaction mixtures contained 1.0-2.5 mM $MgCl_2$, 0.2 mMdNTPs, 0.5 µM each of the forward and reverse primers, 0.1 U of Taq DNA polymerase and 2.5 µl cDNA template. The amplification product was visualized by ethidium bromide staining after electrophoresis on a 1.8% agarose gel in 0.5% TAE buffer.

3.2.6. Protein isolation and quantification ELISA

The expression of Dos regulon proteins was analyzed by following an established method (35). Briefly, the protein sample was initially prepared by harvesting the late log phase bacterial

culture of 1 OD by centrifugation at 5000 rpm at 4°C followed by washing with PBS. Pellet obtained was re-solubilized in buffer containing 8M Urea, 2M Thiourea, 0.02% DTT and 0.04% CHAPS. This mixture was then sonicated at 75% amplitude with 30 sec on and 45 sec off cycle for 5 minutes. Obtained cell lysate was centrifuged at 15000 RPM for 45 minutes and to 1 part of supernatant 8 part of acetone and 1 part of 10% TCA was added for protein precipitation. The precipitate was washed 4-5 times by ice cold acetone. 100 µg of each protein sample was dissolved in ammonium bicarbonate buffer pH 7.8. Then, 100µl of each sample along with bicarbonate buffer was coated on 96 well micro titter ELISA plate (Grenier microon), covered with Aluminum foil and incubated at 4ºC for overnight. The plate was then washed thrice with PBS-T (0.05% v/v Tween 20 was added in PBS). The plate was soaked on tissue paper towel after each wash. Free binding sites of the bacterial protein were blocked with 100 µl of 2% BSA in PBS-T for 6 hours at 4ºC. The plate was washed thrice with PBS-T as described earlier. Each well was coated with 100 µl primary antibody (gifted by Dr. Jaya Tyagi, AIIMS, New Delhi, India) (34) in 1:1000 in PBS-T with 0.25% BSA at 37 º C for 2 hours. After washing with PBS-T as described earlier, 100 µl of alkaline phosphate labeled goat anti-rabbit antibody (1:5000 dilution) in PBS-T with 0.25% BSA was added in each well and the plate was incubated at 37 ºC for 2 hrs. After washing with PBS-T followed by PBS, 100 µl of freshly prepared p-nitrophenlyl phosphate (1mg/ml) in each well and incubated in dark at room temperature for 45 minutes. Reaction was terminated by adding 3(N) NAOH and absorbance was measured at 405nm.

3.2.7. Preparation of *Mycobacterium smegmatis* whole cell proteome

Approximately, 250ml 0.5 $O.D_{600}$ of the bacterial cells were harvested by centrifugation at 9000 RPM for 30min. The pellet was resuspended in Extraction buffer (Urea 8M, Thiourea 2M, Dithiothretol 40mg/ml and CHAPS 2%). After resolubilization, sonication was done at 75 Hz with 30 second pulse with a break of 45 second. This cycle was repeated for eight times. Proteins were precipitated by TCA and Acetone extraction in ratio of 1:8 to the protein sample. This consortium was kept at -20 ºC for two hours and then centrifugation was done at 14000 RPM for 1hr. The precipitate was further solubilized in extraction buffer and used for further studies.

3.2.8. Trypsin digestion of *Mycobacterium smegmatis* whole proteome

Approximately, 10µg complex protein mixture was taken and washed twice with 50 µL of 0.1% Rapigest™ (1 vial diluted in 1000µL 50mM Ammonium Bicarbonate (ABC)) using a MW 5000 (or MW 3000) cut off spin column. Protein solution was concentrated to 10-20 µL using a MW

5000 (or MW 3000) cut off spin column. The solution was then heated to 80°C for 15 minutes and then 3 µL of 100mM DTT (made up in 50mM ABC) was added before heating at 60°C for 15 minutes. Then, 3 µL of 200mM Iodoacetamide (made up in 50mM ABC) was added and the solution was left in the dark at room temperature for 30 minutes. 1µL of 1 µg/µl (2ul of 0.5µg/µl) trypsin (made up in 50mM AmBic) was added and the solution was left overnight at 37°C. 1 µL of concentrated HCl was added and the solution incubated at 37°C for 20 minutes before mix and centrifuge at 30000 RPM for 30mins. The supernatant was collected and transferred to a clean eppendorf tube and transferred the digest to nano LC.

3.2.9. LC/MS^E Analysis

For LC/ MS^E an earlier method was used with modifications (36). Briefly, prior to LC/MS^E analysis, each digested protein sample was spiked with a predigested alcohol dehydrogenase from *sacchromyces cervesie* internal standard (Waters, Milford, MA) at a level of 50 fmol per 10 µL injection. For sample analysis, 10 µL aliquots of proteome tryptic digests were analyzed in duplicate (2 technical replicates per sample) by LC/MS^E using a nano ACQUITY ultra pressure liquid chromatograph (UPLC) and Premier Q-Tof mass spectrometer equipped with a nanolock spray ion source (Waters). Samples were injected online onto a Waters Symmetry C18 trapping cartridge (300 µm i.d. × 1 cm length) at a flow rate of 10 µL/min. Next, peptides were separated by in-line gradient elution onto a 75 µm i.d. × 25 cm column packed with BEH C18 Stationary phase (Waters), 1.7 µm particle size, at a flow rate of 300 nL/min using a linear gradient from 2 to 40% B over 120 min (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile). The Q-Tof was operated in the LC/MS^E mode of acquisition, where alternating 2 s scans of low (4 V) or high (10−32 V) collision energies are used to generate either intact peptide ions (low energy) or peptide product ions (high energy). Glu-fibrino peptide at a concentration of 200 fmol/µL (*m*/*z* 785.8426) was infused via the nanolock spray ion source at a flow rate of 600 nL/min and sampled every 30 s as the external mass calibrant. Samples were injected as sets based on treatment from earliest to latest time points. A protein standard (tryptic digest of bovine serum albumin) analyzed prior to the first sample injection and again following the last sample injection showed no significant loss in instrument sensitivity or performance during the course of the analysis.

3.2.10 Data Processing and Database Searching

Each raw data file was processed using Protein Lynx Global Server V 2.2.5 software (Waters) to generate charge state reduced and isotope free precursor mass lists as well as associated product ion mass lists for subsequent protein identification and quantification. Each processed file was then searched against the Uniprot protein database (http://www.uniprot.org/) using the IDENTITY^{E} database search algorithm within PLGS 2.2.5. Prior to searching, the internal standard alcohol dehydrogenase from *Sacchromyces cerevisiae* sequence was added to the database and redundant entries (identical sequences reported more than once) were removed using an *ad hoc* C++ program. Except for the false positive rate, default search parameters were used including the "automatic" setting for mass accuracy (10 ppm for precursor ions and 15 ppm for product ions), a minimum of 1 peptide match per protein, a minimum of 3 consecutive product ion matches per protein, and a minimum of 7 total product ion matches per protein. The maximum false positive rate (FPR) against the randomized forward database was set to 2%, and the absolute protein quantification functionality was enabled using the alcohol dehydrogenase from *Sacchromyces cervesiae* . Only 1 missed tryptic cleavage site was allowed during the search. A fixed carbamidomethyl-Cys modification was used, in addition to the following variable modifications: deamidation of Asn and Gln; oxidation of Met; and dehydration of Ser and Thr (in-source modification).

3.3. Results

3.3.1. Morphological changes in bacteriostatic cells

The role of ROS in the development of dormancy and resuscitation was investigated by characterizing the cells treated with antioxidants. Apart from non-growing nature of the bacilli, earlier studies have clearly demonstrated that the overall size of the bacillus will reduce to a significant extent (3). The electron microscopic observation of antioxidant treated cells was found to be shorter in length than the untreated cells (Fig 3.1). Normally, mycobacterial cells are 4-6μm of length but antioxidant treated cells were found to be ~2μm long as observed earlier which clearly indicated that all these cells reside in the state of dormancy (3). The exposure of the bacilli for longer duration to 48hrs in presence of 10mM of ascorbic acid, these cells was converted into coccoid shape with average length of 500 nm. These antioxidant treated cells were further tested for susceptibility against anti-tubercular drug to ensure the dormancy phenotype. Antioxidant treated cells were found resistant to active cell specific drugs but susceptible to dormancy specific drugs (data not shown).

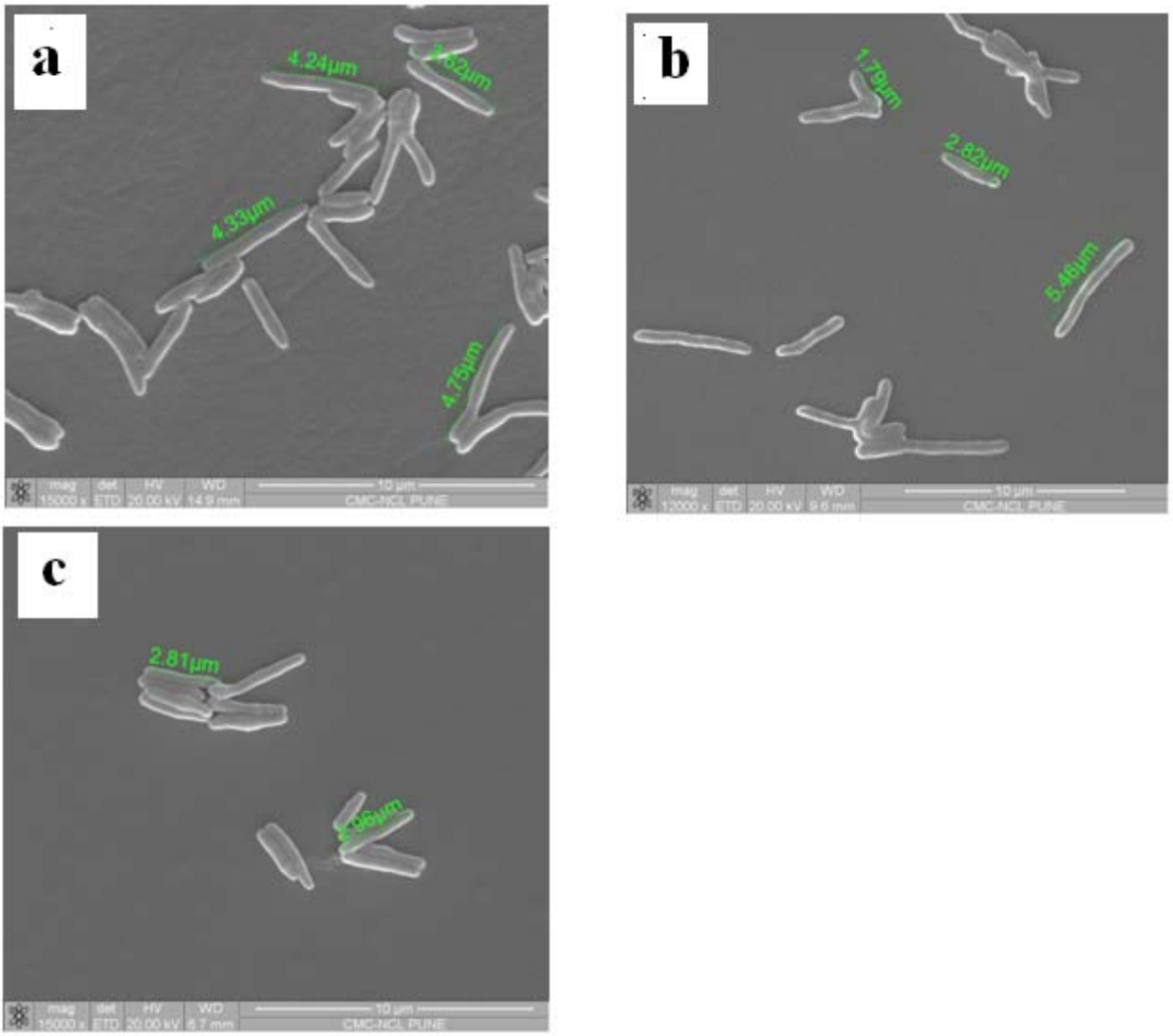
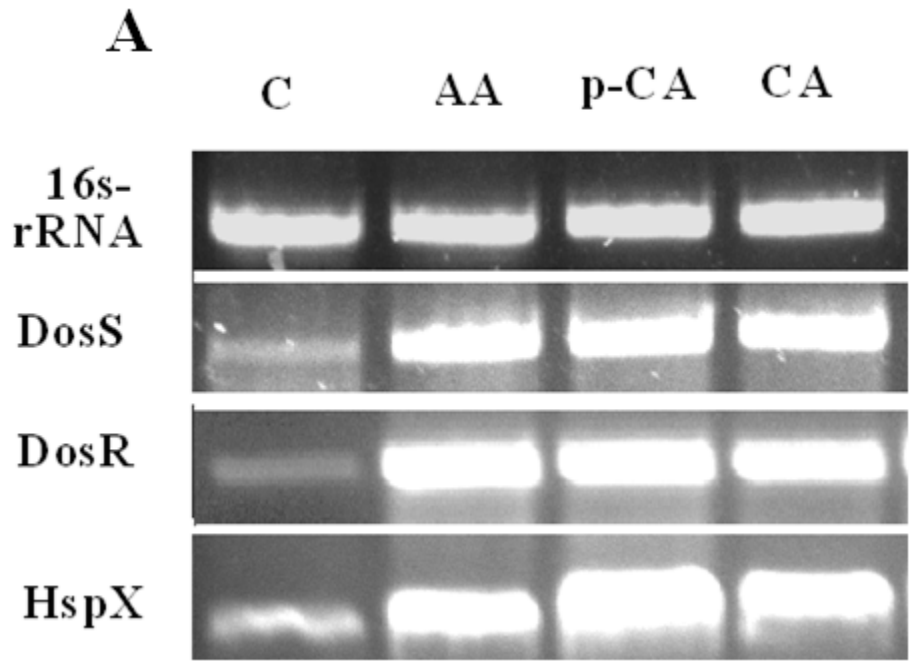


Fig 3.1. Scanning Electron Microscopic images of *Mycobacterium smegmatis* treated with antioxidant ~0.1 $O.D_{600nm.}$cultures from (a) actively growing, (b) Wayne's dormant (c) actively growing *M.smegmatis* treated with Ascorbic acid (10mM)were taken for SEM studies. These cells were first treated with para-glutaraldehyde before taking the SEM pictures. Rest of the experimental details is described in "Materials and Methods". The microscopic pictures are representative of three identical experiments.

3.3.2. Up-regulation of DosR, DosS and HspX genes in presence of antioxidants

The expression of molecular markers like DosS, DosR and HspX proteins are known to be up-regulated during shifting of the bacillus from actively growing to dormant stage (34). In order to

further characterize the bacteriostatic effect of antioxidants, selective expression of the above mentioned genes from total RNA pool of the bacilli were used after treatment with Ascorbic acid, p-Coumaric acid and Caffeic acid (Fig. 3.2 A). RT-PCR results clearly indicated that the level of *dos*S, *dos*R and *hsp*X transcript in antioxidant treated samples increased significantly in comparison to the untreated culture. The normalization of RNA was carried out by using total RNA with equivalent amount of 16S rRNA. Further, the quantitative estimation of the expression of DosR, DosS and HspX protein homologues in antioxidant treated *M. smegmatis* culture was assessed by using ELISA technique (Fig. 3.2B). After treatment with antioxidants, the level of protein expression for DosS increased by 3.8x, 4x and 3.9x respectively in comparison with the control cells. Similarly, DosR expression was also increased by 5.5x, 4.3x and 6x in presence of ascorbic acid, caffeic acid and p-coumaric acid respectively. The expression of co-transcribed gene HspX was also up-regulated almost to a similar extent in presence of antioxidants. HspX is reported to play an important role in stabilizing proteins during the persistent stage (37, 38). The HspX gene was also found to be highly up-regulated within macrophages, hypoxia and presence of NO donors. Interestingly, HspX transcripts are more prevalent at late stages of *M. tuberculosis* infection in mice (39, 40, 41). In presence of all three antioxidants, HspX protein was strongly up-regulated by ~6x.



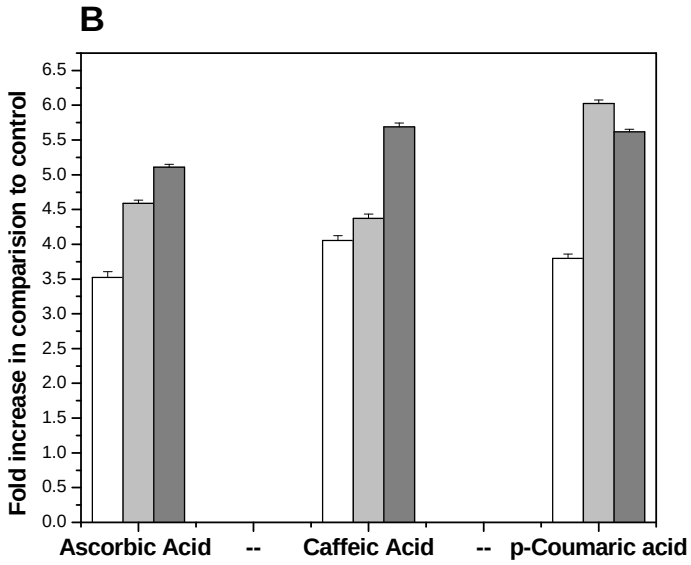


Fig. 3.2.Effect of ROS modulators on expression of *DosRS* regulon in *M.smegmatis*.Log phase ~0.1 OD_{620} culture of *M. smegmatis*was incubated with 1.6 mM Ascorbic acid (AA), 1.3 mM p-Coumaric acid (p-CA), 2.7 mMCaffeicacid(CA)**(A)** RT- PCR analysis of gene expression **(B)** ELISA analysis of protein was carried out in *M. smegmatis* lysate for expression of DosS (white), DosR(grey) and HspX (Black). Rest of the experimental details is described in "Materials and Methods". The results are shown as average of three identical experiments with S.D.

3.3.3. Resuscitation of antioxidant induced dormancy by superoxide

As it was clear from the expression of *DOS-regulon* that antioxidant induced bacteriostasis was actually a result of dormancy state achieved in the bacilli. Our major question was if antioxidants can push the bacterial cells into dormancy, this can also be reverse by extraneous addition of ROS. So, we added different superoxide generators like pyrogallol and menadione, hydroxyl ion generator HOPT, to antioxidant induced dormant cells. We found reversal of the bacilli to active replication of dormant state in presence of ROS generators (Fig 3.3). Interestingly, the reversal was faster in presence of HOPT than menadione and pyrogallol. This affirms that hydroxyl ion is

possibly closely responsible for resuscitation of dormant bacilli than the other ROS species. Further studies are being carried out to confirm the role of hydroxyl ion for resuscitation.

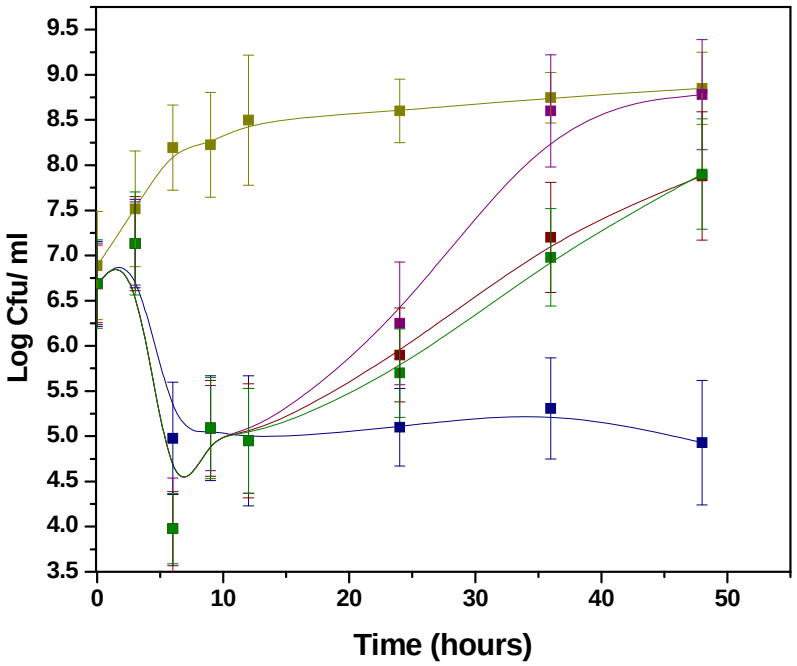
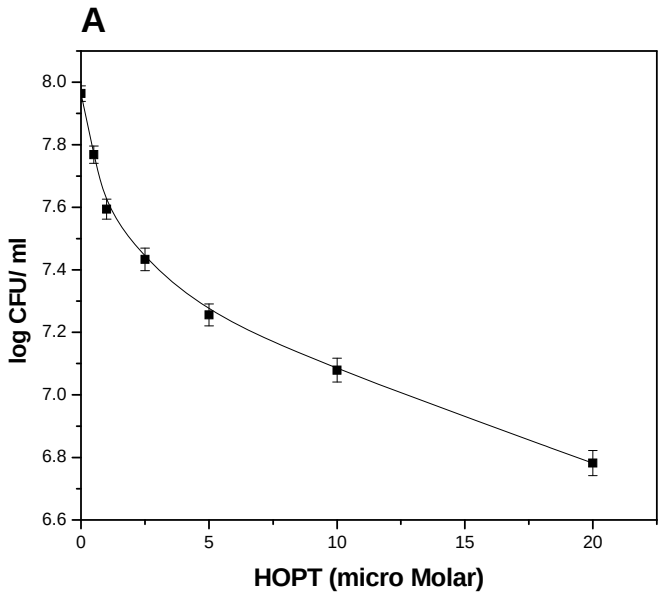


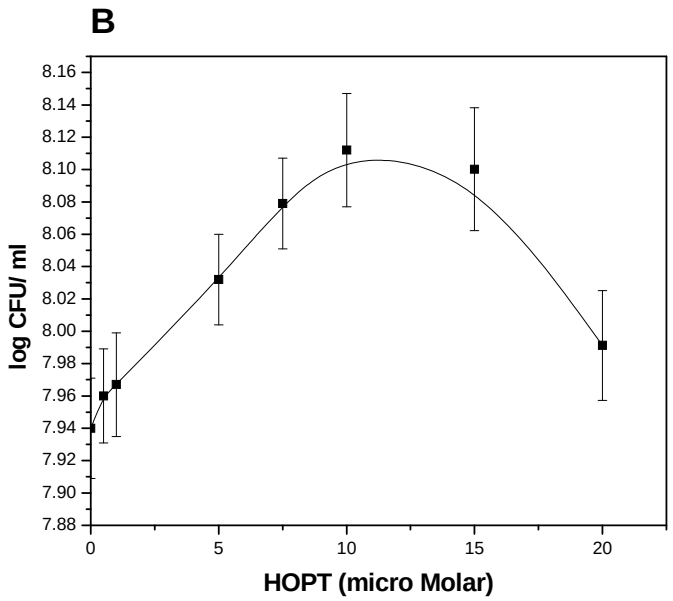
Fig. 3.3.**Resuscitation of dormant *M. smegmatis* bacilli by superoxide and hydroxyl ion generators** Log culture ~ 0.15 OD_{620} treated with ascorbic acid (2mM) (■) and subsequently a parallel set of cells are added with HOPT (10µM) (■), menadione (0.22µM) (■) and pyrogallol (0.65µM) (■) after 12hrs of incubation and the CFU was determined to follow the effect of treatment on cultivability of *M. smegmatis* bacilli. The vehicle control (■)was also kept at 0hr. Rest of the details of the experiment was described in "Materials and Methods". The results are average values with S.D of four different experiments.

3.3.4. Effect of Hydroxyl ion generator (1-Hydroxypyridine -2- thione) on hypoxia induced dormant bacilli

The Wayne's hypoxia culture is also a well established dormancy model of *M. smegmatis* which could be obtained devoid of O_2in the medium (2).Hydroxyl ion generator, HOPT could be used to these cells to study the resuscitation in dormant mycobacterial bacilli. At NRP-2 stage of Wayne culture, HOPT was added into the medium with the help of syringe. Although in aerobic culture HOPT had inhibitory effect but in anaerobic culture HOPT act as growth stimulant (Fig

3.4). This can be understood by significant amount increase in cell growth of dormant bacilli. It is also observed that HOPT and UV alone could not stimulate growth in dormant bacilli. In fact, there was no observable difference identified with normal cells. This clearly indicated that growth stimulation was because of hydroxyl ions generated by HOPT. After HOPT treatment and UV exposure growth stimulation was observed for 24 hrs.





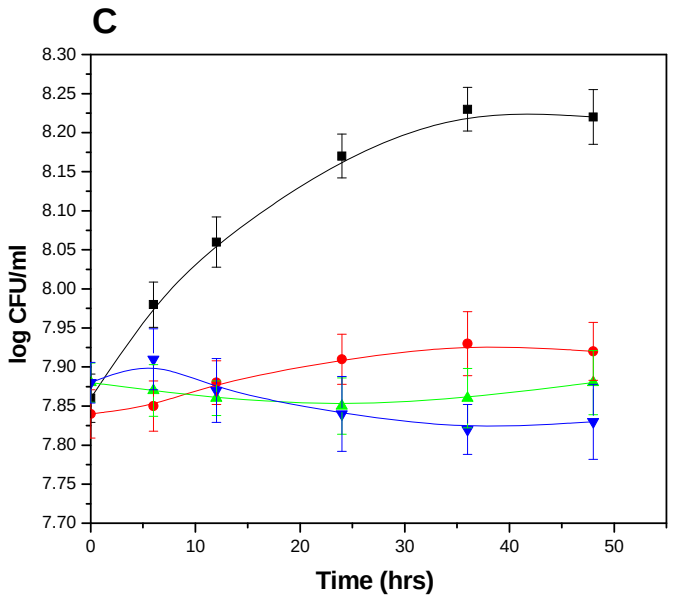


Fig 3.4.Effect of HOPT on dormant *Mycobacterium smegmatis* bacilli A) Effect of HOPT on aerobic culture, B) Dose response effect of HOPT on dormant bacilli and C) requirement of UV exposure along with HOPT treatment on growth of dormant bacilli was monitored by measuring the CFU at different time intervals. In Wayne's dormancy model on 6th day (culture OD_{600} ~ 0.6) (■) 10µM HOPT was added and kept in Uv for 10mins (■)10 µM HOPT was added (■) 10 µl DMSO was added (■) 10µl DMSO was added and kept in Uv for 10 mins. With respective addition culture was plated on dubos agrose at mentioned time. The rest of the details of the experiment were provided in "Materials and Methods". The plot was made using average values ± S.D. obtained from three identical experiments.

3.3.5. Global protein expression of active, dormant and resuscitated bacilli.

For the analysis of global expression of proteins in *M. smegmatis* under different conditions, we used a recently developed method LC- MS^{E} (Waters, USA) in which whole protein mixture was analyzed in single injection. We identified 210 proteins in comparison to 73 secreted proteins identified in an earlier report (36). Number of proteins identified is less in comparision to ~ 1000 proteins analyzed using conventional method using gel electrophoresis coupled with mass spectrometry. In the conventional approach protein mixtures are first separated by two-dimensional (2D) gel electrophoresis. Next, visualized protein spots of interest are individually excised from the gel, digested with trypsin and identified by mass spectrometric analyses. In this

approach, relative quantification of individual proteins between samples is based on the staining intensity of protein spots on the 2D gel. Newer gel-based techniques, such as differential in-gel electrophoresis (DIGE), that use differential fluorescent dye tagging to allow visualization of multiple samples on a single gel, have enhanced the accuracy of relative protein quantification (42). Although these gel-based approaches are widely used, the entire process from gel electrophoresis, staining, protein spot selection to protein identification can be labor intensive and difficult to reproduce quantitatively. In addition, a given stained spot is often composed of several proteins, so a change in spot intensity cannot be unequivocally attributed to a single protein.

For global protein expression change we used a recent method, a new variant of label-free quantification known as LC/MS^E was introduced for quadrupole time-of-flight (Q-Tof) mass spectrometers. For this method, alternating scans of low collision energy and elevated collision energy during LC/MS analysis were used to obtain both protein quantification and protein identification data in a single experiment (43, 44, 45). The low-energy scan mode was used to obtain accurate precursor ion mass and intensity data for quantification, while the elevated collision energy mode generates multiplex peptide fragmentation of all peptide precursors with associated accurate mass product ion information for database searching and subsequent protein identification. In addition to relative quantification between samples based on electrospray intensity, absolute quantification (moles protein) for each identified protein is possible using the LC/MS^E mode of acquisition (45). This was predicated on the observation that average signal intensity measured by LC/MS^E of the three most intense tryptic peptides for any given protein is constant at a given concentration, regardless of protein size. In addition to tracking the relative changes in protein abundance in response to treatment, the ability to derive absolute quantities enables the stoichiometric comparison of proteins within and across samples. Moreover, LC/MS^E provides substantial advantages for protein identification over conventional LC/MS/MS approaches. Unlike data-dependent LC/MS/MS, where the most abundant precursors in an MS scan are sequentially subjected to MS/MS fragmentation, MS^E utilizes parallel, multiplex fragmentation where all peptide precursors are simultaneously fragmented throughout the chromatographic separation process regardless of intensity. This allowed data-independent

identification of lower abundance peptides and provided increased proteome coverage and dynamic range of protein identification compared to data-dependent LC/MS/MS.

3.3.5.1. Protein identification, validation and quantification.

After treatment, proteins were extracted and temporal changes in the proteome were characterized both qualitatively and quantitatively using LC/MS^E. In this study total 209 proteins were identified in study using LC /MS^Etechnique.

The ion detection, clustering, and normalization were performed using Protein Lynx Global Server. Intensity measurements were typically adjusted on those components, *i.e.* deisotoped and charge state-reduced accurate mass retention time pairs, that replicate throughout the complete experiment for analysis at the accurate mass/retention cluster level. Components were typically clustered together with a <10 ppm mass precision and a <0.25-min time tolerance. Alignment of elevated energy ions with low energy precursor peptide ions was conducted with an approximate precision of ±0.05 min. For analysis on the protein identification and quantification level the observed intensity measurements were normalized on the intensity measurement of the identified peptides of the digested internal standard. The observed intensity measurements were normalized for injection volume and protein load variability before conducting quantitative comparisons between conditions by applying scaling. A binary comparison of the peptide precursor intensity measurements of two injections of all three of the investigated conditions was discussed. A 45° diagonal line was obtained (Fig. 3.5.1, 3.5.2, 3.5.3) with almost no variation throughout the detected range. This demonstrated that the expected distribution occurred in the instance with no obvious change between the investigated injections or conditions.

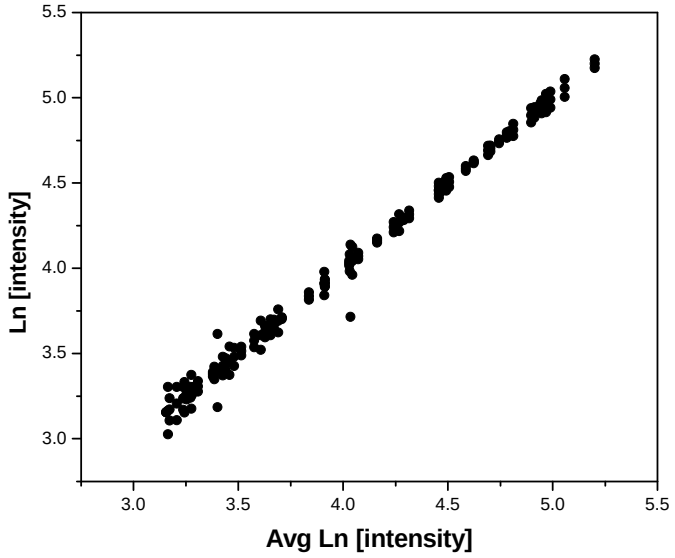


Fig. 3.5.1 Normalization of proteins extracted from aerobic cultures shown by scatter plot comparison of $\log_{10}$ Intensity obtained from three technical replicates

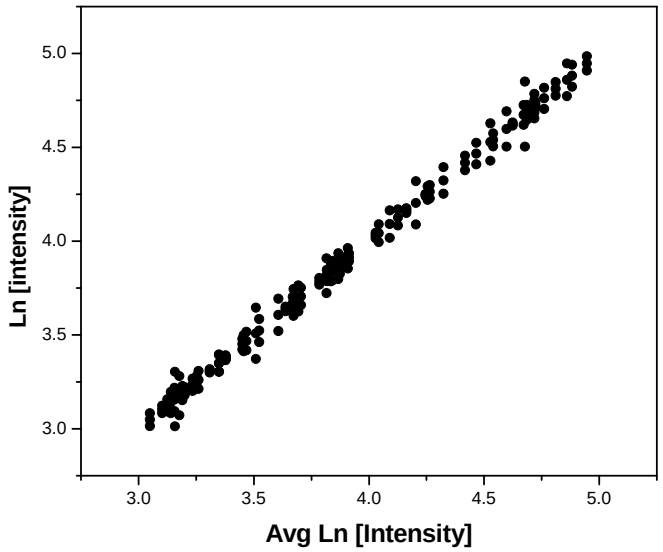


Fig. 3.5.2 Normalization of proteins extracted from Wayne model cultures shown by scatter plot comparison of $\log_{10}$ Intensity obtained from three technical replicates.

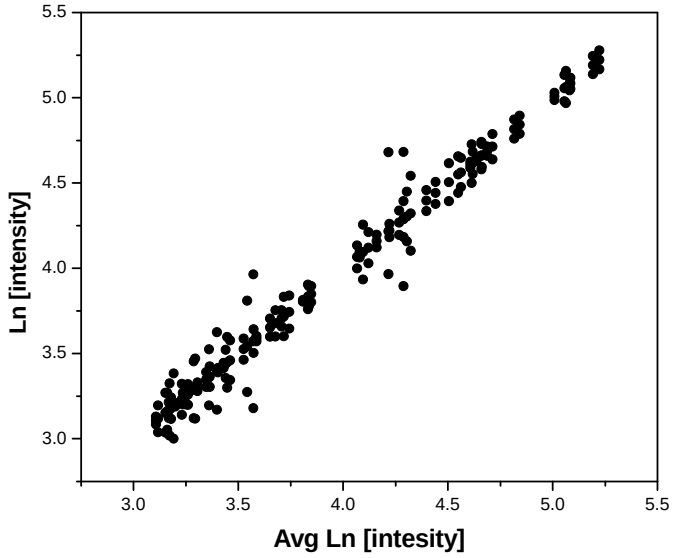


Fig. 3.5.3 Normalization of proteins extracted from HOPT treated Wayne model cultures shown by scatter plot comparison of $\log_{10}$ Intensity obtained from three technical replicates

Table. 3.1. List of identified proteins using LC /MS^E technique.

S.No.	Accession No.	Proteins	mW (Da)	pI (pH)	PLGS Score	Coverage (%)
1	A0R535	Secreted protein MSMEG 6049	40884	4.30	16220.32	48.71
2	A0R1V9	Alkyl hydroperoxide reductase subunit C	21612	4.25	14431.75	74.36
3	A0QSS3	10 kDachaperoningroS PE 1 SV 1	10756	4.31	10027.25	73.00
4	A0QYW6	Universal stress protein family protein	15197	4.75	8787.06	63.95
5	A0R0B3	Meromycolate extension acyl carrier	10730	3.69	6617.02	34.34
6	A0QS98	Elongation factor Tu	43708	5.01	6026.07	42.93
7	A0QQU5	60 kDachaperoningroL	56452	4.61	4473.01	51.76
8	A0QWW2	Glyceraldehyde 3 phosphate dehydrogenase	35925	4.95	4001.02	40.29

9	A0R092	Methyltransferase	54695	5.47	3372.21	22.97
10	A0QWS8	Integration host factor	11628	10.43	2896.69	39.05
11	A0QS63	50S ribosomal rplL	13450	4.34	2772.48	27.69
12	A0QT19	Endoribonuclease L PSP superfamily protein	16207	4.70	2449.88	38.78
13	A0R7F9	30S ribosomal protein S6 rpsF	10178	6.12	2194.03	50.00
14	A0R202	ATP synthase subunit alpha atpA	58852	4.65	2002.13	42.34
15	A0QUV7	Electron transfer flavoprotein alpha subunit	31538	4.58	1968.73	36.42
16	A0QNF6	Peptidylprolylcis trans isomerase	18714	5.33	1777.77	45.14
17	A0QXZ5	Thiol peroxidase	16821	4.18	1770.83	57.93
18	A0R1Y7	Probable acetyl CoA acetyltransferase OS	40041	4.93	1746.11	43.56
19	A0R072	2 oxoglutarate dehydrogenase E2 component dihydrolipoamidesuccinyl transferase	60992	4.45	1685.85	23.59
20	A0QUV6	Electron transfer flavoprotein beta subunit	27770	4.55	1522.25	43.35
21	A0R199	Trigger factor tig	51624	4.11	1400.65	27.08
22	A0R3B8	Enolaseeno	44733	4.29	1310.69	38.88
23	A0QYG2	Forkhead associated protein	14999	4.34	1299.37	63.64
24	A0QPX3	Sulfonate binding protein	34482	4.41	1298.29	45.03
25	A0R198	ATP dependent Clp protease proteolytic subunit 2	21841	4.50	1233.92	36.95
26	A0QQJ4	F420 dependent glucose 6 phosphate dehydrogenase	37252	5.03	1200.07	30.27
27	A0QSD0	30S ribosomal protein S10 rpsJ	11426	9.79	1187.70	24.75
28	A0QX24	ATPase MoxR family moxR PE 4	38027	5.55	1117.44	30.46
29	A0QYG3	Glycine cleavage system H protein gcvH	13831	3.59	1099.74	29.01
30	A0R729	Glycerol kinase 3 glpK	55064	4.55	1083.63	23.76
31	A0QSS4	60 kDa chaperonin 2 groL2	56118	4.72	1061.51	26.30

32	A0R200	ATP synthase subunit beta atpD	51584	4.65	1049.47	38.53
33	A0R0W7	Sulfate binding protein	36660	4.60	1026.82	21.78
34	A0R4H6	Glutathione peroxidase	17192	4.34	992.79	28.57
35	A0R425	Glyoxalase family protein	16253	4.38	992.77	37.75
36	A0QQQ1	Superoxide dismutase Cu Zn sodC	23199	4.68	962.49	11.44
37	A0R753	Integral membrane protein	35527	4.33	887.39	31.82
38	A0QV37	30S ribosomal protein S16 rpsP	16758	9.72	882.28	41.67
39	A0R0I3	Isochorismatase hydrolase	27431	4.90	867.11	30.36
40	A0QWT3	S adenosylmethionine synthase metK	42564	4.78	847.66	34.84
41	A0QNF2	Periplasmic binding protein	33987	4.01	830.90	26.98
42	A0QVX3	Glutamate binding protein	29018	4.35	826.62	43.22
43	A0R7J0	Thioredoxintrx	11615	4.34	817.16	29.09
44	A0QSP9	30S ribosomal protein S9 rpsI	16755	10.43	802.39	33.33
45	A0R5R1	UPF0133 protein	10329	3.96	800.66	22.55
46	A0R4C9	Putative thiosulfate sulfurtransferase	30966	4.79	774.91	36.46
47	A0QSL8	DNA directed RNA polymerase subunit alpha	37896	4.43	771.77	46.86
48	A0QVB8	30S ribosomal protein S2 rpsB	30088	5.77	771.18	23.83
49	A0QWW3	Phosphoglycerate kinase pgk	42068	4.48	756.53	41.42
50	A0R3M4	Succinyl CoA ligase ADP forming subunit beta	40907	4.51	751.31	28.17
51	A0R533	Cation ABC transporter periplasmiccation binding protein putative	33247	4.11	749.70	23.05
52	A0R623	Antigen 85 A	35678	6.18	732.06	18.51
53	A0R2Y1	Beta ketoadipyl CoA thiolase	42564	4.78	723.77	33.09
54	A0QSG3	30S ribosomal protein S8 rpsH	14461	10.35	709.88	40.15
55	Q50441	Alkyl hydroperoxidereductaseAhpD	18824	4.72	703.85	22.60
56	A0QXB0	ABC type amino acid transport system secreted component	30687	4.25	675.69	28.47

57	A0QSG6	30S ribosomal protein S5 rpsE	21897	10.61	640.56	27.10
58	A0R519	Putative uncharacterized protein	52279	4.67	609.19	20.27
59	A0QQ65	Extracellular solute binding protein family protein 5	60522	4.34	600.18	26.50
60	A0R3M3	Succinyl CoA ligase ADP forming subunit alpha	30929	5.08	598.39	23.67
61	A0R5H1	Transcriptional regulator CrpFnr family protein	24760	10.07	597.46	40.63
62	A0R079	Glutamine synthetase 1 glnA	53558	4.80	573.59	26.36
63	A0QSG1	50S ribosomal protein L5 rplE	21109	10.09	571.60	17.11
64	A0QWY0	Transketolasetkt	75108	4.68	532.72	25.86
65	A0R5E1	Probable cold shock protein A cspA	7342	4.71	526.34	14.93
66	O85501	Nucleoside diphosphate kinase ndk	14906	5.21	517.02	24.46
67	A0QVB9	Elongation factor tsf	29387	4.92	500.28	44.73
68	A0QUY3	3 isopropylmalate dehydrogenase leuB	35836	4.98	494.50	12.65
69	A0QYD3	Alanine and proline rich secreted protein apa	35545	4.31	490.67	13.88
70	A0R156	Putative uncharacterized	19902	3.86	486.25	15.20
71	A0QR91	Monooxygenase	56142	7.88	482.72	30.26
72	A0R1X3	ThiS family protein	9819	4.99	477.34	36.56
73	A0R3B5	Immunogenic protein MPT63	33340	3.91	473.65	13.29
74	A0QQW8	Dihydrolipoyl dehydrogenase lpdA	49438	5.36	472.39	17.24
75	A0QQX8	Methoxymycolic acid synthase	33047	4.77	468.78	22.07
76	A0R2X1	Transcription elongation factor greA	17994	4.36	461.95	45.12
77	A0R2U8	Fumaratehydratase class II	49731	4.96	457.78	29.49
78	A0QXC0	Branched chain amino acid ABC transporter substrate binding protein	40476	4.15	454.27	41.60
79	A0R609	Catalase peroxidase katG1	81064	4.54	445.95	35.72
80	A0QP01	O methyltransferaseMdmC	23121	4.67	440.51	42.20

81	A0QS46	50S ribosomal protein L1 rplA	25004	10.04	431.23	29.36
82	A0QS45	50S ribosomal protein L11 rplK	14993	9.97	422.00	9.86
83	A0QV12	Putative serine threonine protein kinase	25554	5.41	420.07	18.60
84	A0R461	Putative uncharacterized protein	36659	5.36	419.79	34.24
85	Q59560	Protein recA	37277	5.10	417.66	26.36
86	A0QTE1	Acetyl propionyl coenzyme A carboxylase alpha chain	63098	5.09	395.64	18.90
87	A0R4G7	Glyoxalase family protein	12181	4.05	389.90	9.48
88	A0QNP2	Extracellular solute binding protein family protein 3	35960	4.29	381.87	21.64
89	A0QWY3	Quinone oxidoreductase	35520	4.77	377.08	25.73
90	A0QQC8	Chaperone protein dnaK	66606	4.52	360.09	22.03
91	A0QYB5	Sugar binding transcriptional regulator LacI family protein	36327	4.30	358.85	21.78
92	A0R197	ATP dependent Clp protease proteolytic subunit	24051	5.08	358.72	9.63
93	A0R1E4	Short chain dehydrogenase	27898	5.72	324.87	30.60
94	A0QVE0	Ribosome recycling factor frr	20811	5.13	320.23	21.62
95	Q9AFI5	Single stranded DNA binding protein	17390	4.95	319.84	30.30
96	A0QUA1	Acyl CoA synthase	55324	4.92	307.85	15.78
97	A0QNX2	Serine esterase cutinase family protein	24045	4.21	303.77	23.71
98	A0QQC1	Monooxygenase	33443	4.59	303.16	26.13
99	Q3I5Q7	HBHA like protein	24547	8.93	300.22	11.64
100	A0R069	Probable cytosol aminopeptidasepepA	53692	6.46	298.81	22.74
101	A0QYY6	30S ribosomal protein S1	53283	4.58	280.55	27.97
102	A0R1Z9	ATP synthase epsilon chain atpC	13256	4.07	272.47	19.83
103	A0QYE7	6 phosphogluconate dehydrogenase decarboxylating	51780	4.88	270.39	13.93
104	A0QUY2	D 3 phosphoglycerate	54325	4.72	265.76	14.39

		dehydrogenase				
105	A0QSX4	Nitroreductase family protein	23755	5.60	262.88	18.78
106	A0QU51	Antigen 85 C	35167	4.79	261.76	8.62
107	A0R7F6	50S ribosomal protein L9 rplI	15913	9.73	256.98	30.46
108	A0QSZ1	O acetylhomoserinesulfhydrylase	46795	4.97	235.95	16.03
109	A0QSG5	50S ribosomal protein L18 rplR	13675	11.49	227.30	19.69
110	A0QWU8	LprG protein	24093	4.26	226.09	14.41
111	A0R2U7	Fructose 1 6 bisphosphatase class II	35843	4.72	220.88	16.96
112	A0R2V4	Putative uncharacterized protein	36198	5.03	218.80	13.07
113	A0QYH7	Mannose binding lectin	21909	4.34	216.44	40.38
114	P42829	Enoyl acyl carrier protein reductase NADH	28508	5.12	207.68	11.52
115	A0R7G8	MmcI protein	28357	4.77	200.64	35.02
116	A0R220	Threonine synthase thrC	37419	5.61	194.69	17.78
117	A0QQP1	Phosphomethylpyrimidine kinase thiD	28714	5.35	193.80	41.01
118	A0QYD5	Putative uncharacterized protein	33951	4.67	187.72	21.04
119	A0QY33	Putative uncharacterized protein	13407	4.12	187.69	19.05
120	A0QVQ5	Polyribonucleotide nucleotidyltransferase	80976	4.57	185.73	15.99
121	A0QPE7	Oxidoreductase short chain dehydrogenase reductase family protein	46470	5.45	184.35	24.00
122	A0QNZ3	3 hydroxyacyl CoA dehydrogenase	27009	5.02	183.39	23.86
123	A0QUA0	Putative acyl CoA	42626	5.69	177.54	38.32
124	A0QWX6	6 phosphogluconolactonasepgl	25571	4.44	174.64	28.69
125	A0QWG8	Pyridoxal biosynthesis lyasepdxSpdxS	32082	4.93	172.38	13.53
126	A0R2V7	Serine hydroxymethyltransferaseglyA	51872	5.28	169.37	17.14
127	A0QSL7	30S ribosomal protein S4 rpsD	23361	10.39	168.68	17.41
128	A0QQ46	Secretion protein Snm4	48230	6.51	164.87	12.00
129	A0R3N8	NADP succinate semialdehyde	50752	4.55	161.23	18.88

		dehydrogenase				
130	A0R5R3	KanY protein	15258	9.72	157.72	35.17
131	A0QZ46	Proteasome subunit alpha prcA	26898	4.82	157.33	4.88
132	A0R0R8	Putative conserved transmembrane protein	71631	5.00	155.72	11.91
133	A0R0B4	3 oxoacyl acyl carrier protein synthase 1	43758	4.91	152.35	33.41
134	A0R766	Putative uncharacterized protein	13897	4.99	150.90	14.84
135	A0QXY1	Acyl CoA synthase	65761	5.28	149.99	14.54
136	A0R534	Cobalamin synthesis protein P47K	40977	4.28	149.96	10.05
137	A0R429	Phosphoserine aminotransferase serC	39300	4.55	149.29	7.57
138	A0QZ56	LppK protein	19232	4.15	147.95	5.88
139	A0QND6	DNA polymerase III beta subunit dnaN	41299	4.52	147.79	6.55
140	A0QSB1	Pyridine nucleotide disulphide oxidoreductase	41896	4.64	144.47	7.85
141	A0QQ47	Subtilase family protein	46403	5.04	142.28	16.99
142	A0QWR9	Thiopurine S methyltransferaseTpmt superfamily protein	23724	4.05	139.21	7.11
143	A0R012	Cell division protein ftsZ	39109	4.28	136.20	24.16
144	A0R5B0	Anti sigma factor antagonist	12021	4.12	135.72	21.01
145	A0R652	Superoxide dismutase MnsodA	22921	5.39	135.56	21.26
146	A0QSD6	50S ribosomal protein L22 rplV	16313	11.38	134.90	14.38
147	A0QSU4	Uncharacterized oxidoreductase	38982	4.93	133.69	19.20
148	A0QVZ3	Iron dependent repressor IdeR	25400	5.04	133.68	32.61
149	A0QXX7	Catalase peroxidase katG2	81947	4.81	133.23	14.71
150	A0QQL0	Thiazole synthase thiG	25628	4.41	130.79	9.52
151	A0R1B3	Aminopeptidase N pepN	94040	4.47	127.44	17.56
152	A0QQS3	Transcriptional regulator TetR family protein	37032	5.39	125.75	12.76
153	A0QSZ6	TryptophanyltRNAsynthetasetrpS	36787	5.98	125.39	9.76

154	A0QSU3	Inosine 5 monophosphate dehydrogenase	53298	5.51	123.89	22.61
155	A0QSH0	Signal peptide peptidase SppA 67K type	62676	5.09	121.96	14.98
156	A0R5N7	Aspartate semialdehyde dehydrogenase	36453	4.93	120.86	32.66
157	A0QW62	Putative uncharacterized protein	46821	4.87	119.87	8.11
158	A0R6D6	Acyl CoA dehydrogenase domain protein	42462	4.72	117.89	18.18
159	A0R1B5	Uncharacterized protein MSMEG 4692	15889	4.44	115.30	26.75
160	A0R417	Citrate synthase gltA	48491	5.45	115.05	17.51
161	A0QY23	Fructose bisphosphatealdolase class I	31905	4.62	101.94	12.46
162	A0R1H9	Putative uncharacterized protein	5366	12.18	101.49	26.42
163	A0R574	Negative regulator of genetic competence ClpCmecB	93496	5.30	99.33	21.58
164	A0R2C0	ABC transporter ATP binding protein SugC	43629	4.84	97.52	10.34
165	A0QYF5	Malate synthase G glcB	78228	4.52	96.11	8.32
167	A0QQ39	Putative uncharacterized protein	53604	5.16	95.48	6.95
168	A0QQI7	O succinylhomoserinesulfhydrylase	43239	4.66	90.49	9.38
169	A0QYZ2	DNA polymerase I	99836	4.76	88.12	11.89
170	A0R536	Solute binding lipoprotein	31724	4.88	87.52	17.67
171	A0QR00	2 3 bisphosphoglycerate dependent phosphoglyceratemutase	27283	5.07	86.46	9.72
172	A0QUK9	Bile acid 7 alpha dehydratase	16513	5.08	86.17	16.89
173	A0QYM9	Putative uncharacterized protein	3409	4.30	85.77	51.52
174	A0QTR2	ATP binding protein	8442	4.55	84.45	8.86
175	A0R2B7	NAD dependent malic enzyme MSMEG 5055	39093	4.65	83.20	18.95
176	A0R157	Saccharopine dehydrogenase	43606	5.76	79.60	12.26
177	A0QZA1	Universal stress protein	31432	5.47	79.40	4.08

178	A0R5Y0	Delta3 5 delta2 4 dienoyl CoA isomerase	28532	4.66	77.58	17.71
179	A0QND7	6 phosphogluconate dehydrogenase decarboxylating	31516	4.69	77.55	13.80
180	A0R293	Alkanalmonooxygenase alpha chain	37273	5.13	76.22	25.79
181	A0QZ40	Sec independent protein translocase protein tatA E homolog	8926	9.48	67.39	17.28
182	A4T197	UPF0234 protein Mflv 5248 Mflv	18097	5.50	83.39	11.04
182	A0R4I0	Phosphoribosylaminoimidazolesuccinocarboxamide synthase	33080	4.59	78.67	25.08
183	A1T8X3	Indole 3 glycerol phosphate synthase trpC	28305	4.81	87.65	30.51
184	A0QUX8	Ketol acid reductoisomeraseilvC	36398	4.67	81.41	17.21
185	A0R072	2 oxoglutarate dehydrogenase E2 component dihydrolipoamidesuccinyl transferase	60992	4.45	896.93	22.56
186	A0QNF6	Peptidylprolylcis trans isomerase	18714	5.33	589.31	24.00
187	A0QNF2	Periplasmic binding protein	33987	4.01	261.61	20.95
188	A0R5B0	Anti sigma factor antagonist	12021	4.12	55.41	12.61
189	A0QQJ4	F420 dependent glucose 6 phosphate dehydrogenase	37252	5.03	572.24	32.64
190	A0QQW8	Dihydrolipoyl dehydrogenase lpdA	49438	5.36	133.26	34.05
191	A0R4B3	Fatty acid desaturase	38429	4.89	94.88	19.46

192	A0QW08	Deoxyuridine 5 triphosphate nucleotidohydrolase	15925	5.09	94.75	16.88
193	A0QWY0	Transketolase OS tkt	75108	4.68	70.37	7.90
194	A0R0B4	3 oxoacyl acyl carrier protein synthase	43758	4.91	399.87	27.64
195	A0R0B5	3 oxoacyl acyl carrier protein synthase 2	43707	5.14	397.37	23.26
196	A0R3I5	Molybdopterin biosynthesis protein	17933	4.07	50.57	8.43
197	A0R758	Dihydroxyacetone kinase	59320	4.49	49.92	6.13
198	A0R2V3	Universal stress protein family protein	30050	5.53	49.90	18.21
199	A0R5L6	Thiopurine S methyltransferaseTpmt superfamily protein	24485	4.81	49.22	7.96
200	A0QT19	Endoribonuclease L PSP superfamily protein	16207	4.70	48.08	9.52
201	A0QV28	Nitrogen regulatory protein P II	12108	5.74	46.79	9.82
202	A0QWT7	Esterase	30949	4.54	44.83	11.19
203	A0R2B7	NAD dependent malic enzyme	39093	4.65	54.42	12.89
204	A0R6V8	CAIB BAIF family protein	38681	4.87	53.77	15.53
205	A0QPU4	Porin	22448	4.35	55.92	8.84
206	A0QT32	Uracil phosphoribosyltransferase 1	21935	4.53	62.70	5.80
207	A0R758	Dihydroxyacetone kinase	59320	4.49	49.92	6.13
208	A0R5R1	UPF0133 protein	10329	3.96	86.28	31.37
209	A0QYB3	Periplasmic sugar binding proteins	36058	4.25	88.58	19.20

Table. 3.2. Proteins up-regulated in aerobic aerobic condition

S. No.	**Proteins**	**Protein ID**	**Aerobic / Dormant fold change**	**HOPT / Dormant fold change**	**HOPT / Aerobic fold change**
1	Alkyl hydroperoxidereductase subunit C	**ahpC**/ MSMEG 4891	4	4	1
2	ATP synthase subunit alpha	**atpA**/ MSMEG 4938	35	40	1
3	Beta ketoadipyl CoA thiolase	MSMEG 5273	4	6	1.5
4	Branched chain amino acid ABC transporter substrate binding protein	MSMEG 3247	3	3	1
5	Chaperone protein	**dnaK** / MSMEG 0709	2	5	1.5
6	D 3 phosphoglycerate dehydrogenase	**serA** / MSMEG 2378	3	5	1.7
7	Electron transfer flavoprotein alpha	**etfA** / MSMEG	3	4.5	1.5

	subunit	2352			
8	Electron transfer flavoprotein beta subunit		3	4	1.4
9	Extracellular solute binding protein family protein 3	MSMEG 0114	2	4	2
10	Extracellular solute binding protein family protein 5	MSMEG 0643	2	2	1
11	Glyceraldehyde 3 phosphate dehydrogenase	**gapA** / MSMEG 3084	3	5	1.6
12	Glutamate binding protein	MSMEG 2727	4	2	0.5
13	Meromycolate extension acyl carrier protein	**acpM/** MSMEG_4326	5	3	0.6
14	Polyribonucleotide nucleotidyltransferase	**pnp** / MSMEG 2656	3	3	1
15	Sulfonate binding protein	MSMEG 0550	5	3	0.6
16	Iron dependent repressor	IdeR	Unique to Aerobic	Unique to Aerobic	Unique to Aerobic

Table. 3.3. Proteins up-regulated in HOPT treated dormant cells

S. No.	**Proteins**	**Protein ID**	**Aerobic / Dormant fold change**	**HOPT / Dormant fold change**	**HOPT / Aerobic fold change**
1	3 oxoacyl acyl carrier protein synthase 1	MSMEG 4327	2	5	2.5
2	3 oxoacyl acyl carrier protein synthase 2	MSMEG 1204	Unique to HOPT	Unique to HOPT	Unique to HOPT
3	Alcohol dehydrogenase	MSMEG 2079	ND in aerobic	4	ND in aerobic
4	Alcohol dehydrogenase iron containing	MSMEG 6242	0.25	1	4
5	ATP synthase subunit beta	**atpD** / MSMEG 4936	28	58	2
6	ATP synthase epsilon chain	**atpC** / MSMEG 4935	0.5	2	4
7	Branched chain amino acid aminotransferase	**ilvE** / MSMEG 4276	Unique to HOPT	Unique to HOPT	Unique to HOPT
8	Cell division protein	**ftsZ** / MSMEG 4222	ND in Dormant	ND in Dormant	3

9	Glycerol kinase 3	**glpK** / MSMEG 6759	1	4	4
10	Nitrogen regulatory protein P II	MSMEG 2426	ND in aerobic	1	ND in aerobic
11	Oxidoreductase short chain dehydrogenase reductase family	MSMEG 0372	0.3	4	13
12	Quinone oxidoreductase	MSMEG 3106	1	3	3
13	Serine hydroxymethyltransferase	**glyA**	1	3	3
14	Superoxide dismutase Cu Zn	**sodC** / MSMEG 0835	1.5	3	2
15	Superoxide dismutase Mn	**sodA/** MSMEG 6427	0.5	1.5	3
16	Universal stress protein family protein OS Mycobacterium smegmatis strain ATCC 700084 mc 2 155 G	MSMEG 5245	Unique to HOPT	Unique to HOPT	Unique to HOPT
17	Universal stress protein MSMEG 3950 OS Mycobacterium	MSMEG 3950	Unique to HOPT	Unique to HOPT	Unique to HOPT

	smegmatis strain ATCC 700084 mc 2 155 GN MS				

Table. 3.4. Proteins up-regulated in Wayne hypoxia (dormant) cells

S. No.	**Proteins**	**Protein ID**	**Aerobic / Dormant fold change**	**HOPT / Dormant fold change**	**HOPT / Aerobic fold change**
1	Adenosylhomocysteinase / S-adenosyl-L-homocysteine hydrolase	**ahcY**/ MSMEG 1843	0.1	0.5	5
2	Elongation factor Tu	**tuf**/ MSMEG 1401	0.2	0.2	1
3	GMP synthase glutamine hydrolyzing	**guaA/** MSMEG 1610	Unique to dormant	Unique to dormant	Unique to dormant
4	Helix turn helix motif	MSMEG 5136	Unique to dormant	Unique to dormant	Unique to dormant
5	Inositol 3 phosphate synthase	**ino1** / MSMEG 6904	0.3	3	10

6	Isocitrate dehydrogenase NADP	MSMEG 1654	0.1	0.5	5
7	MarR family protein regulatory protein	MSMEG 3415	Unique to dormant	Unique to dormant	Unique to dormant
8	Pyruvate kinase	**pyk**	0.1	0.5	5
9	Succinyl CoA ligase ADP forming subunit alpha	**sucD/** MSMEG 5524	0.3	1.5	0.5
10	Succinyl CoA ligase ADP forming subunit beta	**sucC/** MSMEG 5525	0.3	1.5	0.5

3.3.5.2. Global expression change in dormant, HOPT treated dormant and active bacilli

About 210 proteins with pI ranging from 3-12, were identified using MS^E technique in *M. smegmatis* grown in aerobic, Wayne model and HOPT treated Wayne model cultures. Analysis of three independent experiments using PLGS V 2.2.5 analysis software revealed 42 proteins with significant folds of change in expression of proteins.

Metabolic enzymes

Mycobacteria are a group of obligate aerobes that require oxygen for growth, but paradoxically have the extraordinary ability to survive and metabolize under hypoxia suggesting a high degree of metabolic plasticity. Mycobacterium shift to nitrogen metabolism during dormancy is a hallmark event (46, 47, 48). Nitrogen regulatory protein Ms2426 was detected in dormant cells which also persisted in HOPT treated dormant cell approximately to the same extent. The Glutamate binding protein Ms 2727 was down regulated in dormancy by 4 folds and up-regulated by 2 folds in HOPT treated dormant cells. This indicated the shifting of dormant bacilli towards active form due to the addition of HOPT. Similarly, Pyruvate kinase, an essential

enzyme in carbohydrate metabolism, was up regulated by 10 fold in dormant cells and 5 fold in HOPT treated dormant cells. This enzyme of glycolytic pathway, catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP. This up-regulation of Pyruvate kinase indicated that increased importance of glycolysis or gluconeogenesis pathway for carbohydrate metabolism during dormancy (49, 50). Glycerol kinase-3, a key enzyme in the regulation of glycerol uptake and metabolism remained comparable in dormant and aerobic bacilli but surprisingly it was found to be up-regulated 3 folds in HOPT treated dormant *M. Smegmatis* cells. This can be an essential enzyme during resuscitation of dormant bacilli. Fatty acids, which are the main source of carbon and energy for *M. tuberculosis* during infection, are primarily catabolized via successive rounds of β-oxidation (2, 43). The production of glycerol-3-phosphate via glycerol kinase is essential for membrane and fatty acid metabolism involving fatty-acyl glycerol phosphates and inter-conversions between CDP-diacyl-glycerol and phosphatidyl-glycerol phosphates (43, 44). These results were consistent and expected given the importance of membrane metabolism in the role of biomass production (51). 3-oxoacyl acyl carrier protein synthase (Ms4327) of beta-ketoacyl-ACP synthases family was 2 fold down-regulated in dormant state and up-regulated by 2.5 folds in comparison to aerobic cells. A similar enzyme 3-oxoacyl acyl carrier protein synthase (Ms1204) was uniquely present in HOPT treated dormant cells also indicated increased necessity for lipid biosynthesis during resuscitation of dormant cells. Acetyl-coA derived from the catabolism of fatty acids or sugars is assimilated via the TCA cycle, which provides biosynthetic precursors and reducing equivalents for energy generation and biosynthetic reactions (44). We observed a strong up-regulation of isocitrate dehydrogenase, a TCA cycle enzyme, by 10 folds in dormant and 5 folds in HOPT treated dormant cells. Succinyl CoA ligase, Ms5524 and Ms5525, both representing another enzyme in TCA cycle were up-regulated by 3 folds during dormancy as well as1.5 folds during HOPT treated cells in comparison with the aerobic cells. This justified the different variants of TCA cycle in *Mycobacterium sp*. as well as importance of glycolytic and TCA cycle pathway during dormant condition of the bacilli.

ATP synthesis

ATP synthase, an essential enzyme in the energy metabolism, is a validated drug target for the treatment of tuberculosis, and ATP synthase inhibitors, such as diarylquinoline, are promising candidate drugs for the treatment of infections caused by *M. tuberculosis* (52). ATP synthase is a multi-subunit complex consisting of a membrane-embedded F_0 part and a cytosolic F_1 moiety (52). The enzyme can utilize the proton-motive force (PMF) across the bacterial cytoplasmatic membrane for the synthesis of ATP At low PMF, for example in environments with limited oxygen concentrations, this reaction can be reversed in several bacteria, which use the energy released from hydrolysis of ATP to maintain a PMF (53). A strong down- regulation of ATP synthase is characteristic of dormant bacilli in different models (54). We found 30 fold and 45 fold up-regulation of *atpA* and 28 folds and 58 folds up-regulation *atpD* respectively in aerobic and HOPT treted dormant cells in comparison with the dormant cells. This suggested the increased requirement of ATP during resuscitation and growth of the bacilli and potential involvement *atpD* in the process. Surprisingly, the other subunit *atpC* was up-regulated by 2 folds in dormant and by 4 folds HOPT treated dormant cells in comparison to actively replicating bacilli.

Cell division proteins

An optimal level of *M. Smegmatis* FtsZ was required to sustain cell division and that the cell division initiation mechanism was also similar in other mycobacterial species (55). Fstz is a structural homologue of tubulin function as cell division initiator in a GTP- dependent process. Its controlled interaction with other cell division proteins in spatial and temporal manner is considered as key event to bacterial cell division (55). This protein was not detected in dormant cells but up-regulated by 3 folds in HOPT treated dormant bacilli in comparison to aerobic cells. This significant induction in expression of FstZ in HOPT treated dormant cells indicated its possible role in resuscitation.

Cell Wall proteins

Mycobacteria have an unusual cell wall in which mycolic acids play a critical role in structure and function (56). This structure confers the bacteria to resist any chemical injury, low permeability to antibiotics, damage from dehydration and an ability to survive within the phagolysosomes of the macrophages (57). During the entry into dormancy, the overall size of mycobacteria is significantly reduced due to altered cell wall composition (3). β-ketoacyl-ACP thiolase involved in the synthesis of mycolic acids was down-regulated in dormant bacilli by 4 folds and 6 folds in comparison to aerobic and HOPT treated dormant cells. It was also noticed that expression of enzyme in HOPT treated dormant cell were 1.5 folds more than aerobic cells. A previous study of the effect of zinc deprivation on *Mycobacterium bovis* BCG pointed out the potential importance of an alcohol dehydrogenase for maintaining the hydrophobic character of the cell envelope. In this study, we observed that two different alcohol dehydrogenase Ms2079 (iron containing) and Ms6242 (Zinc containing) were detected in the mass spectrometric analysis. Iron containing alcohol dehydrogenase was absent in aerobic cells and 4 folds more in HOPT treated dormant cells than dormant cells. Although zinc containing alcohol dehydrogenase was comparable in dormant and HOPT treated dormant cells it was up-regulated by 4 folds in comparison to aerobic cells. Together, this result indicated an increased hydrophobic character of the cell wall of aerobic bacilli. Inositol 3 phosphate synthase, catalyses the conversion of glucose-6-phosphate to 1D-myo-inositol 3-phosphate was up-regulated by more than 3 folds in dormant and 10 folds in HOPT treated dormant cells in comparison to aerobic cells. Inositol phosphate capping of the non-reducing termini of lipoarabinomannan is an essential step in step in achieving dormancy type cell wall strains of *Mycobacterium* (58).

Methyltrasferase (MarR family regulatory proteins)

Expression of methyl transferase is tightly regulated by MarR family regulatory proteins (59). Earlier studies clearly indicated that very low expression occurred under *in vitro* and non-stressed conditions but strongly up-regulated under in vivo and stressed condition (60). This up-regulation varied from 100 to 150 folds under different stress conditions. We observed that this protein was uniquely detected in dormant (Wayne model) and not in either aerobic or HOPT treated dormant cultures. Genes regulated by MarR family members are repressed in the absence of the inducer and de-repressed in the presence of inducer (60). Many MarR repressors are also autoregulatory, repressing their own expression in the absence of inducer (59). This explained

the observed down-regulation of MarR regulatory proteins in HOPT treated dormant cells. It also suggested that the indication of resuscitation was observed in HOPT treated dormant cells.

Chaperons and Universal stress Proteins

As reported earlier, we found that up-regulation of Elongation Factor Tu occurred by 5 folds in dormant as well as HOPT treated dormant cells (61, 62). A stress protein and chaeperone, *dnaK* was down-regulated by 2 fold in dormant condition but up-regulated by 1.5 fold in HOPT treated dormant cells. Universal stress protein (USP) Ms3811 was also down regulated in hypoxia and in HOPT treated hypoxic cells which suggested that the functional role of these proteins become essential during resuscitation and growth of the bacilli. Function of these genes is putative but their homologues are known for DNA damage repair (63). These proteins might also be involved in DNA damage repair due to ROS, in hypoxia their down-regulation could possibly be justified as less amount of ROS is produced in cells. In case of HOPT treated cells again these genes were up-regulated for DNA damage repair as actively growing bacilli produce significantly increased amount of intracellular ROS. USP Ms5245 and Ms3950 were uniquely found in HOPT treated cells probably because of their important function in resuscitation of dormant bacilli.

Antioxidant enzymes

Antioxidant enzyme such as *SodA* (iron dependent superoxide dismutase), *SodC* (membrane bound copper dependent superoxide dismutase) and *AhpC* (Alkyhydroxy peroxidase) were earlier reported to down-regulate in dormant stage (63, 64, 65). Although all three mentioned enzymes were found to be essential at the time of infection when oxidative burst occurs by host macrophages and subsequently down-regulate after fifteen days post-infection (66). *AhpC* together with *AhpD* constitutes an NADH-dependent peroxidase active against hydrogen and alkyl peroxides as well as serving as a peroxynitrite reductase, thus protecting the bacterium against reactive nitrogen intermediates and oxidative stress generated by the host immune system (65). Here, in case of HOPT treated Wayne cells, we found comparable expression of the proteins in aerobic stage which was 4 fold higher than untreated Wayne hypoxic cells. Antisense RNA of *SodA* was found to significantly decrease the survival of tubercular bacilli under aerobic condition (63). Its significance could be understood from the failure of making *SodA* mutants in

past one decade. As reported earlier, *SodA* was induced in dormancy by 2 fold as well as 3 fold in HOPT treated Wayne culture (64, 66). *SodC*, which was considered as insignificant in infection as its mutants survived in guinea pigs, was down-regulated by 0.75 fold during hypoxia induced dormancy and up-regulated by 3 folds in those cells when treated with HOPT. Thioredoxin reductase, a putative enzyme that was earlier found to inhibit oxidative stress dependent killing of *M. smegmatis* was not detected in both hypoxic and HOPT treated Wayne hypoxic cultures. In tuberculosis this enzyme was reported to be down-regulated in anaerobic and mouse infection model (66).

3.4 Discussion

In the last chapter, major findings related to generation of different ROS species such as superoxide, hydrogen peroxide and hydroxyl radical along with bacteriostasis achieved due to the exposure to antioxidants inspired us to validate the physiological role of ROS in bacterial survival and growth. It also raised two important questions 1) whether antioxidants induced bacteriostasis is basically a state of dormancy? 2) Which ROS is playing crucial role in bacterial cell replication?

To validate antioxidant induced bacteriostasis as actually dormancy we studied cell morphology and *DosRS regulon* after treatment with antioxidants (Fig 1 & 2). As indicated by IC50 and IC90 ratio (in chapter 2) it was implicit that ROS generator and ROS scavengers have different mode of action. ROS generators had bactericidal effect where as ROS scavengers had bacteriostatic role. Bacteriostatic role of ROS scavengers was consistent irrespective of specificity of scavengers like superoxide scavengers, SOD mimic (TEMPOL) and hydroxyl radical scavengers had similar effect. This may be because of inter-convertibility of ROS (discussed in chapter 1). Electron microscopy reveled differences between normal cells and antioxidant treated cells grown in same media. We found that antioxidant treated cells were intact and retained rod shape but shorter than normal cells. These observations were in line with earlier reported morphology of nitrogen limited dormant cells kept for 5 months (2).

Earlier, DosS was proposed as redox sensor but skeptic consideration exists mainly because lack of redox potential measurement and its stability towards autoxidation and not likely exist in met (Fe^{3+}) *in vivo*, a condition necessary for redox sensing function (63, 64) . Recent advance in field suggests that during hypoxic conditions, growth regulon was attenuated by treatment with compounds that occluded electron flow into the menaquinone pool or decreased the size of the menaquinone pool itself. Increased regulon expression during hypoxia was observed when exogenous menaquinone was added, demonstrating that the menaquinone pool is a limiting factor in regulon induction. Taken together, these data demonstrated that a reduced menaquinone pool directly or indirectly triggers induction of the *DosR regulon* via *DosS* (65).Now we can add that up-regulation of *DosRS regulon* in presence of antioxidants suggest that this induction depends on the redox state of cells but we do not rule out the role of oxygen in

silencing the signal. This also strengthens our claim that potential redox machinery exists in *Mycobacterium sp*.

After confirming antioxidant induced bacteriostasis as dormancy major question was whether we can reverse this by extraneous addition of ROS? So we added different ROS generator menadione, pyrogallol and HOPT to the antioxidant induced dormant cells. We found the reactivation of dormant cell by increase in 2 log CFU count. Noteworthy, that these ROS generators were bactericidal in aerobic stage. After studying the kinetics of reactivation it was found that resuscitation faster in HOPT treated cells. This suggested that hydroxyl radical is more potential ROS in comparison to others for resuscitation of dormant cells. This observation was not strong enough to conclude hydroxyl radical as only molecule because of inter-convertibility of ROS. From above mentioned observation and generation of rare hydroxyl radical *in vivo* (chapter 2) prodded us to study hydroxyl radical induced resuscitation.

Despite being highly reactive molecule hydroxyl radical was reported to possess signaling role in bacterial quorum sensing, Ca^{2+}signaling and human tumor cells (66, 67, 68). Like many bacteria, mycobacteria synthesize siderophores (known as mycobactins) to capture iron. Two forms of mycobactins are produced, which differ in the length of an alkyl substitution and, hence, in polarity and solubility (69).The essential nature of siderophores for iron acquisition was demonstrated by the generation of an *M. tuberculosis* mutant strain (*mbtB*), 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 (70). In fact, iron acquisition was considered as an important step in attaining successful infection (71).In our study we found in vivo fenton's reaction (chapter 2) which was coupled with hydroxyl radical generation explains the requirement of high level of iron in mycobacterial cells. This also suggests the significance of low level of iron in tuberculosis patients (72) as well as role of hydroxyl ions in growth of the bacilli.

As hydroxyl radical is highly reactive species, as a signaling molecule its primary targets are amino acids which are the building blocks of proteins. So our next objective was to study global change in protein expression during resuscitation. For lucidity of our study we used well defined Wayne model to study dormancy and HOPT for resuscitation along with actively

growing aerobic cells (Fig 4-6). This also provided us with advantage to study a transition state between actively replicating and dormant stage. As mentioned earlier, there is no report available about transition state of resuscitation this study was unique in itself.

Fig 3.6 Metabolic pathways of Mtb important during infection. (Adapted from FEMS microbiology review, 2012) Growing evidence suggests that pathogenic mycobacteria rely on lipids in vivo. Degradation of fatty acids by b-oxidation leads to acetyl-CoA (C2) and for uneven chain length or methyl-branched fatty acids additionally to propionyl-CoA (C3). The pathogen can directly metabolize C2 units via the tricarboxcylic acid (TCA) cycle, while excessive accumulation of toxic propionyl-CoA is prevented by two metabolic routes: (1) the methylcitrate cycle and (2) the methylmalonyl pathway. The products of both pathways can enter the TCA cycle, either directly (succinate) or after conversion to succinyl-CoA (methylmalonyl-CoA). Moreover, methylmalonyl- CoA is a building block of methyl-branched lipids. Mycobacterial isocitratelyase (Icl) plays a key role in the methylcitrate cycle and the glyoxylate shunt. The intermediate glyoxylate can be used terminally to generate pyruvate (via malate),

from which glycolytic substrates can be replenished by gluconeogenesis. C3 bodies of the glycolysis/gluconeogenesis and acetyl-CoA are required for the biosynthesis of triacylglycerol, a lipid relevant during dormancy.

As reported earlier, we found similar shift in metabolism from the available set of proteins when compared between active and dormant state (31, 32, 33, 41, 49, 50).The resuscitation of dormant bacilli means reestablishment of metabolic and replicative activity. Resuscitation is a poorly studied phenomenon not only in *Mycobacterium sp.* but also in any other species. The most important metabolic changes during resuscitation are not so far documented. The RPF induced resuscitation in *Micrococcus luteus* and germination of spore in *Bacillus sp.* starts with cell wall hydrolysis (73, 74). Here, we highlight the metabolic pathways relevant during dormancy and resuscitation (Fig. 3.6). Our study clearly indicated changes in *M. smegmatis* during HOPT induced resuscitation different cell membrane proteins such as β-ketoacyl-ACP thiolase, Inositol 3 phosphate synthase iron containg alcohol dehydrogenase and Zinc containing alcohol dehydrogenase were up- regulated. The change in cell membrane dynamics in terms of its content, polarity and hydrophobicity is possibly important parameters for resuscitation. Furthermore, FstZ, an important cell division protein which is involved in septa formation was also induced by HOPT indicated its involvement in resuscitation process along with cell replication.

The rapid decrease in ATP level during dormancy leads to cessation of ribosomal as well as other general protein synthesis. A strong induction of ATP synthase during resuscitation suggested that ATP synthesis is the primary event in resuscitation which restores the synthesis of proteins. The up-regulated proteins involved in lipid metabolism during dormancy confirmed that the previous evidence about the involvement of lipids are critical for virulence of Mtb (75). Isocitratelyase (Icl) was identified as gate enzyme of the glyoxylate shunt, a short-cut of the tricarboxylic acid (TCA) cycle, bypassing the steps of carbon loss by CO_2 formation. The asymmetric cleavage of isocitrate by Icl produces glyoxylate, which is converted into malate and succinate. The TCA cycle intermediate malate can be used to generate pyruvate and further to replenish the pool of glycolytic intermediates by gluconeogenesis (Fig. 3.6). This affirmed our finding, up- regulation of Succinyl CoA ligase and pyruvate kinase during dormancy. In dormant cells

we found a shift in carbohydrate metabolism where TCA cycle re-routes towards glyoxalate cycle as described in earlier reports (50). But in HOPT resuscitated cells it was found in between which confirms few cells in dormant and few in resuscitated state. The response to stress also followed similar pattern. As described in results, a transition phase is properly defined in HOPT treated resuscitated cells. Catabolism of cholesterol, odd-chain fatty acids, methyl branched fatty acids, and amino acids funnels into propionyl-CoA, a C3 intermediate, which is toxic in excess (76). However, propionyl-CoA toxicity can be avoided by condensing the C3body with oxaloacetate to form succinate and pyruvate bythe 2-methylcitrate cycle Propionyl-CoA metabolization can also be performed by the methylmalonyl-CoA pathway ending up in methylmalonyl-CoA, which can either be converted into succinyl-CoA by a vitamin B12-dependent mutase or directly incorporated into methyl-branched fatty acids (76) (Fig. 3.6). The expression of unique 3-oxoacyl carrier protein in resuscitated cells suggests the requirement of fatty acid metabolism in resuscitation. The restoration of stress proteins and antioxidants enzymes profile in HOPT induced resuscitated cells as actively dividing cells confirms the complete activation of dormant cells into active state. The expression of unique universal stress proteins in resuscitated cells needs further characterization to understand their role in reactivation.

As described earlier, siderophore molecules are considered as good targets because pathogen survival and virulence is directly related to iron availability (77).*IdeR* (Iron dependent regulator)is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages (78). In our study, *IdeR* proteins were expressed only in actively dividing cells but completely absent in dormant state. This can be explained by requirement of hydroxyl radical in actively replicating cells and iron acquisition is an important step to maintain the concentration of hydroxyl radical by *in vivo* Fenton's reaction. It is important to note that *IdeR* gene was not up-regulated in HOPT treated cells where hydroxyl radical was extraneously provided. This support our idea that iron acquisition by mycobacterium is primarily to generate hydroxyl radical.

Although the role of hydroxyl radical was established but the specific interaction with proteins is not determined. Moreover, identification of redox proteins and the molecular interaction with hydroxyl radical still remained a mystery. This report mainly helps in

understanding the metabolic shift during dormancy and resuscitation. Further, it supports the identification of potential drug targets against tuberculosis.

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CHAPTER 4

Cloning and expression analysis of *Mycobacterium smegmatis* NADH oxidase

4.1. Introduction

Superoxide can be toxic or signaling entity under physiological conditions despite its limited production, short half-life, and limited reactivity, because it can readily dismute into hydrogen peroxide which in the presence of appropriate metal catalysts produce hydroxyl radical (1). Basically, other ROS such as hydrogen peroxide or hydroxyl radical are derived products of superoxide. So far, the physiological role of superoxide in the different cellular systems was executed through its production from respiratory reactions and NADPH oxidase in higher eukaryotes (2). But the major contribution of intracellular superoxide generally comes from fumarate reductase or NADH oxidase in bacteria (3, 4). From an elaborated study in chapter 2 we identified NADH oxidase as major source of superoxide production in *Mycobacterium smegmatis* bacilli. Considering its important role in the development of latency in mycobacterium, further study was carried out on NADH oxidase in this bacillus.

In several bacterial cells, the NADH formed under aerobic conditions by various dehydrogenases is utilized by NADH oxidase to produce superoxide, is considered as responsible for the maintenance of the intracellular redox balance (5). There are two types of NADH oxidases; one catalyzes the four-electron reduction of O_2 with formation of H_2O, and the other catalyzes the two-electron reduction of O_2 to H_2O_2 (5). The latter is found in many microorganisms including thermophilic eubacteria. So far, NADH oxidase had been isolated from anaerobic bacteria such as *Streptococcus* (6, 7, 8, 9), *Thermotoga* (10), *Clostridium* (11, 12), *Eubacterium* (13), and *Lactobacillus* (14, 15, 16) as well as from archaeon such as *Sulfolobus* (17), *Thermus* (18, 19), *Archaeoglobus* (20). In these anaerobic organisms, NADH oxidase plays an important role of oxygen scavenger under oxidative stress (21). In aerobic microorganisms, NADH oxidase activity helps in the electron transfer from NADH to O_2 through cytochrome by membrane-bound enzymes (5). In fact, NADH oxidase of *Corynebacterium* was isolated from membrane (22, 23). Probably because of the difficulty in purifying this membrane protein, characterization of NADH oxidase from aerobic bacteria still remained hardly been reported. The structural studies were carried out using mainly the most abundant enzyme from *Thermus sp.* which elaborates as, the homodimeric enzyme consists of a central 4-stranded anti-parallel β-sheet covered by helices, a more flexible domain formed by two helices, and a C-terminal excursion connecting the subunits (24). The active sites are located

in a deep cleft between the subunits. The binding site of the flavin cofactor lacks the common nucleotide binding fold and is different from the FMN binding site found in flavodoxins. So far, there is a lack of overall knowledge about NADH oxidase function and its role in mycobacterial cells except reports of NADH oxidase activity in cellular fractions of *M. tuberculosis* (25). Cell wall fraction and membrane fraction of *M. smegmatis* exhibit NADH oxidase activity of 0.41 and 1.49 μM /15min /mg of protein. Few other enzymes like KatG, catalase- peroxidase in *Mycobacterium tuberculosis* was earlier reported to possess NADH oxidase activity (26).

Here in this chapter, we attempted to find a functional role of NADH oxidase with the help of available genome database such as UNIPROT and KEGG along with transcript analysis. Also we cloned, expressed and partially characterized one of the NADH oxidases, MSMEG_6603 protein, which may have a prospective role in resuscitation of dormant bacilli.

4.2 Materials and Methods

4.2.1. Chemicals, strains and Media

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. *M. smegmatis* strain ATCC 607 was used throughout the present study which was grown in a defined medium containing 0.5 gm KH_2PO_4, 0.2 gm sodium citrate, 60mg $MgSO_4$, 0.5 gm asparagine and 2 ml glycerol in 100ml of distilled water at pH 6.6. The stock culture was maintained at -70^0 C and sub cultured once in liquid medium before inoculation to an experimental culture.

4.2.2. Cultivation of aerobic M. smegmatis and E. coli

For aerobic cultivation, The bacterial cultures were grown in 20 ml defined medium in 100 ml flask under aerobic conditions in a shaker incubator (Thermo Electron Corporation Model 481) maintained at 150 rpm and 37^oC till logarithmic phase ($O.D._{620}$ ~ 1.0) was reached.

While *E. coli* cells were grown in different volume in sterilized LB (Luria-Bertani) broth medium. The cells were kept in different volume of flask under aerobic condition in rotatory shaker rotating with speed of 200rpm at 37 ^oC till logarithmic phase ($O.D._{600}$ ~ 0.6) was reached.

4.2.3. Genomic DNA isolation from M. smegmatis

M. smegmatis was used for isolation of genomic DNA (g-DNA) using standard protocol. Briefly, 2ml of cells (O.D~1.0) was pellet down at 10,000rpm at room temperature for 5min. After discarding the medium, pellet cells were resuspended in 550µl of Solution A (2mg/ml of Lysozyme in Tris EDTA buffer, 10mM, pH 8.0) and vigorously mixed the cells by pippeting 5-6 times and kept at water bath maintained at 37^oC for 1 hr. for incubation. After 1hr., 70µl of solution B was added (10mg/ml proteinase K in 10% of SDS solution made in DW) and mixed it properly and heat at 60^oC for 10mins. After 10mins of heating, 80µl of 5M NaCL was added in cells and mixed by shaking it and 100µl of CTAB+NaCL (10% CTAB dissolved in 5M NaCL solution) was and mixed it vigorously and heated again at 60^oC for 10mins. After incubation 500µl chloroform and isoamyle mixure (24:1) was added and mixed gently

4-5times and then centrifuged at 10,000rpm for 10mins at room temperature. Upper clear phase was taken without disturbing or touching middle layer and pipette out into another eppendrof tube. 500µl of isopropanol was added and again mixed it very gently up and down for 4-5 times which will cause the precipitation of genomic DNA which was visualized as woolen tread like structure and sample was kept for 1hr at -20^{o}C.

After incubation, sample was centrifuged at 12,000rpm for 15mins at room temperature which will leads to precipitation of g-DNA as pellet which was washed with 70%ethanol and air dried for 5mins. After proper drying, g-DNA was resuspended in 50µl TE buffer (10mM, pH 8.0) and stored at 4^{o}C.

4.2.4. RNA extraction and Reverse Transcriptase-PCR

RNA extraction was done by following a method established in our laboratory (27). Briefly, spheroplast solution consisting of lysozyme (0.002% w/v), D-cycloserine (0.0006% w/v), glycine (1.4% w/v), EDTA (0.2% w/v) and lithium chloride (0.1% w/v) prepared in distilled water was aseptically added to log phase bacterial culture. Different antioxidants (2mM) were simultaneously added in the above mentioned culture. After 12 hrs of incubation, both control and antioxidant treated bacilli were centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 ml of Trizol reagent (Sigma) and then sonicated for one minute at 50 kHz in a water-bath sonicator. After 10 minutes of incubation at 37°C, 200 µl of chloroform:isoamyl alcohol (24:1) was added to the Trizol suspension and mixed vigorously for 15 seconds. Cell debris was removed by centrifugation at 10000 rpm for 10 minutes at 4°C and the upper aqueous phase was collected in a separate tube. 500 µl of isopropanol was added to the aqueous phase, and RNA was precipitated by centrifugation at 15000 rpm for 10 minutes at 4°C. The RNA pellet was washed twice with 70% ethanol and air dried at 37°C for five minutes and then finally resuspended in 50 µl DEPC water. Qualitative as well as quantitative analysis was performed with agarose-gel electrophoresis and a NanoDrop spectrophotometer. For RT-PCR, 1.0 µg of total RNA was reverse transcribed using random primers provided with the 'single stranded cDNA synthesis kit' (Sigma) by following the manufacture's protocol. The synthesized cDNA was used for RT-PCR of NADH oxidaseI, NADH oxidase II, NADH oxidase III and NADH oxidase IV genes using their specific primers. 40-µl PCR reaction mixtures contained 1.0-2.5

mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of the forward and reverse primers **(**Table 4.1**)**, 0.1 U of Taq DNA polymerase and 2.5 µl cDNA template. The amplification product was visualized by ethidium bromide staining after electrophoresis on a 1.8% agarose gel in 0.5% TAE buffer.

Table. 4.1 Primers of NADH oxidase genes used for RT-PCR

1) NADH oxidase I (MSMEG_1645)

Forward Primer 5' ATG AAC ACT GAG CCC AAT 3'

Reverse primer 5' GCA TCC AGA TCT GCC AGC CGT 3'

2) NADH oxidase II (MSMEG_2889)

Forward Primer 5' GTT GCA CTT CGG ACA TAT GTA CCT GC 3'

Reverse primer 5' CCA TGC ACT TGT TGT TGT GGT TGC AC 3'

3) NADH oxidase III (MSMEG_2969)

Forward Primer 5' CGT GAGA AGT AGT GCC GCC GAC AT 3'

Reverse primer 5' CCG GTA CTC GAA GTC GTT GAC CTT 3'

4) NADH oxidase IV (MSMEG_6603)

Forward Primer 5' GTC GCG TAC AAG CCC TAT CCG 3'

Reverse primer 5' GGT CTT GCC ATC GAC CTT GG 3'

4.2.5. Primer designing for NADH oxidase clone preparation

Primers were designed using the gene sequence data available in KEGG pathway for M. smegmatis NADH oxidase IV using ITDNA primer designing software. For full length amplification NADH oxidase IV which was to be cloned in pET160 Topo vector, CACC was added before initiation codon in forward primer while Stop codon was removed from the reverse primer.

Forward primer ATG CAC CCG TTC CGT CAG GC
Reverse primer CG CTT CCC CGA GTG CTT CCC

The designed primers were checked for annealing temperature, GC content, self ligation, hairpin formation etc with the ITDNA software and found optimum parameter for amplification.

Volume	Reagents	Final concentration
5µl	10X Buffer for LA DNA pol.	1X
2.5µl	Deoxy ribonucleotide mix	200µM
1µl	Forward Primer	0.5µM
1µl	Reverse primer	0.5µM
1µl	LA DNA pol.	0.1unit/µl
2.5µl	DNA template	500pg/µl
37µl	PCR grade water	
Total= 50µl	PCR reaction mixture	

Steps		Temperature	Time
1.	Initial Denaturation	95^{o}C	2min
2.	Denaturation	95^{o}C	30sec

3.	Annealing	60°C	30sec
4.	Extension	72°C	2.5min
5.	Repeat step 2-4 for 30 cycles		
6.	Final Extension	68	10min
	Hold	4°C	Overnight

4.3.6. A. Preparation of Ligation mixture

The amplified PCR product was gel eluted after cutting the gene specific band from agarose gel using Gel-Elute PCR Kit (Sigma) following manuals instructions. The PCR product was quantified using nano-drop spectrophotometer. The ligation mixture consist of cloning vector, salt and PCR product and DW as follows

Mixture	**Volume**
Fresh PCR product	1µl
Salt solution	1µl
Sterile WATER	3µl
TOPO vector	1µl
Total	6µl

After mixing all the components, ligation mixture was kept at room temperature for 30mins. in water bath.

4.2.7. Transformation into *E. coli* with plasmid carrying NADH oxidase gene

6ul of ligation mixture was transformed into *E. coli* competent cells (TOP10, provided in kit) using heat shock method. Initially the cells were kept with ligation mixture on ice for 5 min.

After incubation, cells were given heat shock treatment for 30-45 secs. at 42^{o}C in water bath and immediately incubated in ice for 5mins.

After incubations, 200µl of fresh SOC medium (provided in kit) was added to effendrof containing transformed cells and kept for 1hrs at rotatory shaker rotating with speed of 150rpm at 37^{o}C. After 1 hrs of growth on rotatory shaker, cells were spread on preformed 1.5% agar plate with ampicillin (100µg/ml) in aseptic conditions. After spreading, plate was kept in incubator maintained at 37^{o}C for overnight.

4.2.8. The isolation of plasmid carrying NADH oxidase clone

For plasmid isolation from *E. coli* cells, individual colony grown on agar containing antibiotic plate, was separately inoculated in 10ml LB medium containing 100µg/ml of ampicilin and was grown up to $O.D_{600}$~ 0.6. The cells were then centrifuged at 10,000rpm for 5min at room temperature. The cell pellet was processed for plasmid isolation using plasmid extraction kit (Sigma) by following instruction provided in manual. The plasmid was then analyzed on 1% agarose gel and quantified using nano-drop spectrophotometer.

4.2.9. The lysis of *E. coli* clone cells of NADH oxidase

E. coli cells were lysed using sonicator kept on ice box. Grown cells were pellet down by centrifugation at 10,000rpm for 5min at room temperature. After discarding the medium, the lysis buffer consist of lysozyme, EDTA, Chaps, was added to the pellet cells and was mixed properly by pippeting down 5-6 times. The cells in lysis buffer were sonincated using 80kz for 30sec. for 3 cycles on ice box. The lysed cells were centrifuged at 15,000rpm for 30mins at 4^{o}C, to separate soluble portion in supernatant fraction and membrane or un-dissolved portion as pellet fraction. Both fractions were subjected for protein estimation, enzyme assay or SDS PAGE.

4.2.10. Analysis of the cloned and expressed protein by SDS-PAGE

Different proteins having difference in over all molecular weight in whole supernatant fraction as well as pellet fractions of protein sample were separated by carrying out SDS-PAGE (sodium dodecyl sulfate-Poly acryl amide gel electrophoresis) (28). Briefly, protein samples were first mixed with 1X loading buffer containing 5% β meceptoethanol (Sigma). The samples were then

heated for 10min at 80^{o}C. 30µl of samples was then loaded onto 12.5% acryl amide bis-acryle amide Tris buffer pre cast polyacrylamide gel and electrophoresis was carried out using mini-cell system (Amersham, USA). After electrophoresis, gel was subjected to Comassie Blue staining for overnight. Protein bands were seen on the gel after de-staining with DW:ethanol:acetic acid in 45:45:10 ratio.

4.2.11 NADH oxidase enzyme assay

The activity of NADH oxidase was measured spectrophotometrically by following a standard method (29). The standard solution for the assay contained NADH-buffer (50 mM Tris–base, 0.1 mM $FADH_2$, and 0.066 mM NADH) and enzyme NADH oxidase. The activity of NADH oxidase was assayed by measuring the decrease of absorbance at 340 nm corresponding to the NADH oxidation at 25°C. One unit of activity was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of NAD+ from NADH in 1 min. For activity calculation, 6.22 x 10^{-3} was taken as molar extinction co-efficient.

4.2.11. Protein estimation

Protein quantification was done by following Bradford method using BSA as standard protein (30). The standard protein graph was plotted using serial dilutions of BSA and the plot obtained was used for determining protein concentration in given samples.

4.2.12. Tryptic In-Gel Digestion Procedure

The gel spot was cut and transferred into a 1.5 ml microcentrifuge tube, 30 µl of H_2O was added and left at RT for 15 minutes. The supernatant was removed and 200 µl of 50% acetonitrile was added and left for another 15 mins. The supernatant was removed and 300 µl of 100% acetonitrile was added and kept for 15mins. The supernatant was removed and 200 µl of 0.1 M NH_4HCO_3 added and left at RT for 5 minutes. Then, 300 µl of 100% acetonitrile added and mixed for 15 minutes. The supernatant was completely dried with a Speed Vac (20 minutes). Then, 100 µl of 10mM DTT in 0.1 M NH_4HCO_3 was added in the dry tube and incubated at 56 ºC for 45 minutes. The supernatant was removed and 50-100 µl of 55mM IAA (iodoacetamide) in 0.1 M $NH4HCO_3$ was added and left in the dark for 30min at 4 ºC. Then, the steps were repeated 2-6X and then added 20 µl of 0.1% RapiGest^{TM} SF solution in 50mM $NH4HCO_3$ and

incubated at 37 ºC for 10mins. Excess of solution was removed and completely dried the gel pieces with a Speed Vac (20mins). 20ng/ul of trypsin was added to the gel slice until the gel was re-swollen and incubated for 45min at 4 ºC. Excess of solution was removed and 20 ul of 50mM NH_4HCO_3 added and incubated at 37 ºC for 16 hrs. Three times extraction with 50 µl 0.1% TFA/60%ACN was done. The extract was centrifuged at 15000g for 10-15min the supernatant was collected and completely dried the solution with a Speed Vac (1 hr). The digest was reconstituted in 5-6µl 3% ACN and transferred the digest to nano-LC bottles by using gel-loading tips or through 10ul syringe.

4.2.13. LC/MS^E Analysis and Data Processing and Database Searching

As described earlier in Chapter 3 of this thesis.

4.3. Results and Discussion

4.3.1 Transcript analysis of different NADH oxidase present in *M. smegmatis*

After database search such as KEGG and UNIPROT, we found four putative NADH oxidases are present in *M. smegmatis*. To confirm the functionality of different NADH oxidase transcript analysis was done using specific primers (mentioned in materials and methods). Bioinformatics database available about NADH oxidase I (MSMEG_1645), NADH oxidase II (MSMEG_2889) and NADH oxidase III (MSMEG_2969) predicts oxidoreductase and histidine biosynthesis domain (KEGG : Kyoto Encyclopedia of Genes and Genomes http://www.genome.jp/dbget-bin/www_bfind_sub?maxhit=1000&dbkey=msm&mode=bfind&keywords=nadh+oxidoreductase) in these proteins. Also, NADH oxidase IV (MSMEG_6603) considered as consisting SnoaL-like polyketide cyclase domain involved in nogalamycin biosynthesis (KEGG). SnoaL has a different mechanism to that of the classical aldolase for catalysing intramolecular aldol condensation (KEGG). Here, we attempt to identify role of NADH oxidases in the development of dormancy and resuscitation in *M. smegmatis.* The RT-PCR analysis of NADH oxidase I clearly indicated expression of the transcript in all the condition except in HOPT treated Wayne model culture (Fig 4.1). Its expression was comparable in active, antioxidant (such as ascorbic acid and p-coumaric acid) induced dormant and Wayne dormancy model suggested its requirement in both the states. NADH II expression was significantly observed in antioxidant induced dormant states and was absent in both HOPT treated Wayne dormant and Wayne dormant cells. It clearly showed that NADH II has a possible role in maintaining REDOX balance during aerobic growth. NADH oxidase III expression was found to be significant in Wayne hypoxia and antioxidant induced dormancy model which probably plays important role during hypoxic conditions. NADH oxidase IV was expressed in active, antioxidant induced dormant state and HOPT treated Wayne hypoxic cultures. Here, it is important to note that NADH oxidase IV expression was not there in Wayne hypoxia dormant cultures was induced to a significant level after addition of HOPT. This supported the idea to speculate the possible role of NADH oxidase IV in resuscitation of Wayne hypoxia induced dormancy.

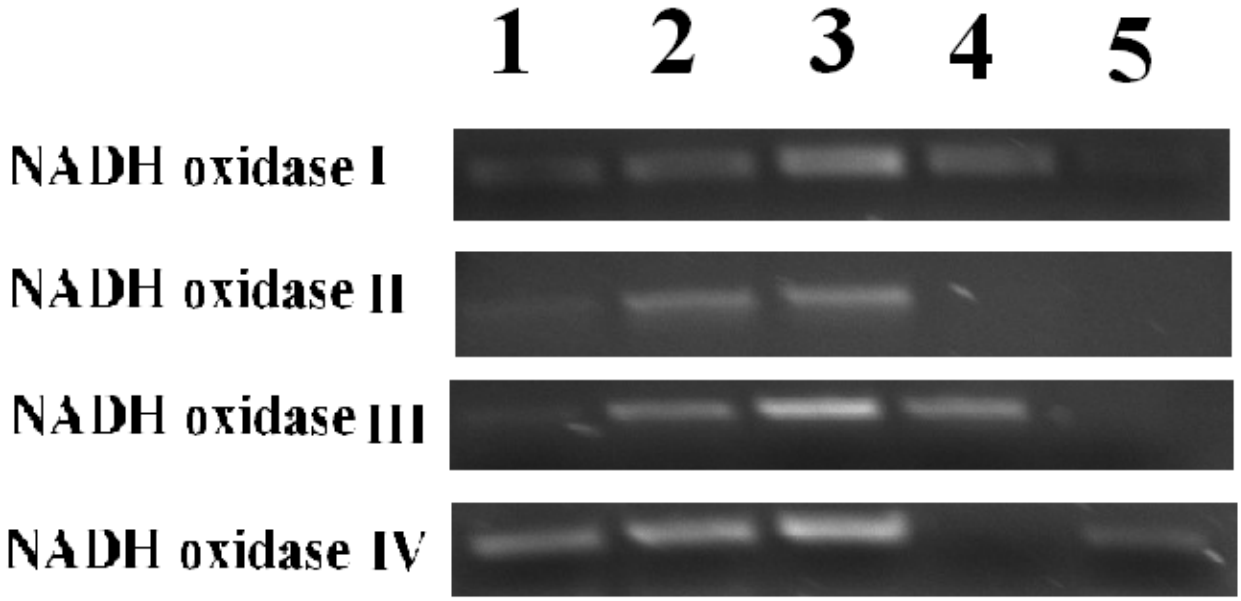

Fig 4.1. Transcript analysis of all four putative NADH oxidase under different condition. RNA was isolated from (1) log phase ~0.1 OD_{620} culture of *M. smegmatis* (2) with 1.6 mM Ascorbic acid (AA) (3) 1.3 mM p-Coumaric acid (p-CA) (4) Wayne hypoxia model cultures (5) HOPT treated Wayne hypoxia model cutures. 1µg/ 20µl cDNA reaction mixture concentration of RNA was used for cDNA synthesis. Further these cDNA was normalized by 16s rRNA amplification. Then these cDNAs were subjected to PCR with specific primers (mentioned in Materials and methods)

4.3.2. Cloning, expression and enzyme activity of NADH oxidase IV (MSMEG_6603)

4.3.2.1. Full length amplification of NADH oxidase (MSMEG_6603)

Full length gene of larger subunit of NADH oxidase was amplified by gene specific primers using proof reading DNA polymerase. The amplified product having size of 396 base pair was confirmed by running on 1% agarose gel with 1kb ladder (Fig 4.2). The gel picture indicated that the length of the PCR amplification product is 396 bp. The PCR product was then confirmed by sequencing which indicated the PCR product is 100% identical with NADH oxidase (MSMEG_6603) of *M. smegmatis* and also of specific gene size. The eluted PCR product of NADH oxidase was quantified with nano-drop spectrophotometer.

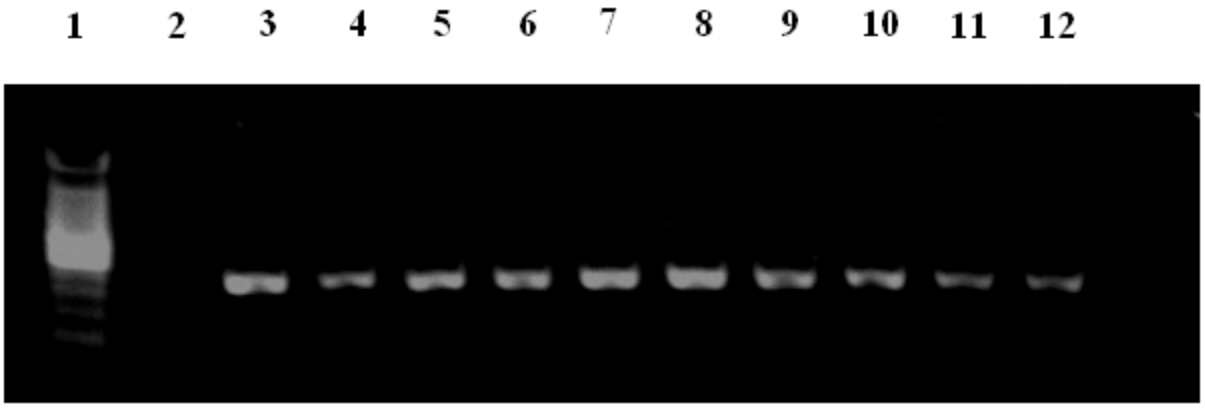

Fig. 4.2. Gel image of full length PCR amplification of NADH oxidase. A gradient PCR with *M. smegmatis* genomic DNA at different annealing temperatures Lane 1- Marker Lane 2- Negative control (without DNA template) Lane 3- 12 PCR amplification with annealing temperature ranges from 58 ºC to 68 ºC.

4.3.2.2 Expression of NADH oxidase protein in *E.coli* cells

The recombinant plasmid was then transformed into *E. coli* expression system (BL21star, provide in kit). The transformed cells were grown in LB + Ampicillin medium for upto $O.D_{600}$ ~0.8 and then IPTG (isopropyl-thio-galactose) was added to induce the recombinant clone. After 3hrs of induction, cells were harvested and lysed by sonication for 1hr at 25MHz by keeping the culture in water bath sonicator. The supernatant and pellet obtained after centrifugation at 15000RPM for 1hr of lysed cells were analyzed by SDS PAGE (Fig 4.3). The SDS PAGE data clearly indicated induction of NADH oxidase in pellet fraction of IPTG induced cells as compared to non-induced cells. The induced protein corresponds to ~30kD molecular weight when compared with marker, which corresponded with the expected size of the NADH oxidase IV **(**MSMEG_6603) along with 4kD tag on C-terminal.

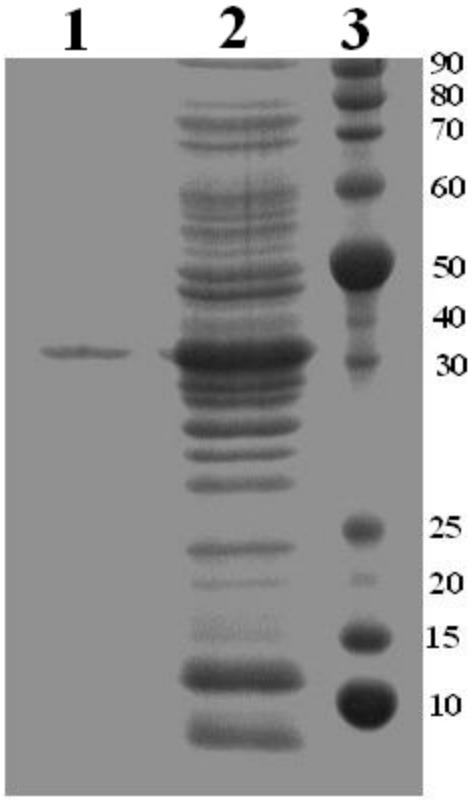

Fig.4.3. SDS-PAGE analysis during different steps of purification of NADH oxidase. Lane 1 and Lane 2 were loaded respectively with Ni-NTA column purified HIS- tagged NADH oxidase and crude cell membrane extract. Molecular weight marker was loaded in Lane 3. Purified protein corresponds with protein molecular weight marker at ~ 30 kD.

The induced pellet had protein concentration of 24.4 mg/ ml and NADH oxidase activity was 0.1 µmol/min/mg of protein. Whereas, purified protein was 1.3 mg/ ml and NADH oxidase activity was 2.2 µmol/min/mg of protein.

4.3.3. MS identification and analysis of cloned and over-expressed protein

Almost without exception, protein identification is based on the analysis of peptides generated by proteolyic digestion. The most widely used enzyme is trypsin, which hydrolyzes the protein on the C-terminal side of lysine and arginine, unless the subsequent amino acid in the sequence is a proline. This is advantageous as every peptide other than the protein C terminus has at least two sites for efficient protonation, the N-terminal amino group and the C-terminal basic residue, so peptides are readily ionized and detected as positive ions. LC/MS^E is a recently introduced, novel mode of data-independent acquisition where alternating MS scans of lower and higher collision

energy are used to simultaneously capture peptide precursor intensity data and peptide fragmentation data, allowing both protein quantification and identification in the same analysis. LC/MSE spectra provide excellent product ion coverage across each peptide with relatively high mass measurement accuracy (<12 ppm rms mass errors). Total eight peptides were sequenced with clearly detectable intensities (Table 4.1). Overall coverage of identified protein was more than 70% which is considered as excellent coverage (31).

Table. 4.2. Details of peptides identified by MS^E

Precursor MH+ (Da)	Peptide MH+ (Da)	MH+ Error (Da)	MH+ Error (ppm)	Start	End	Sequence	Intensity
2885.43	2885.42	0.009	3.209	12	38	(R)DTDAMTALLADDVVFTSPVAYKPYPGK(A)	1244151
1431.68	1431.66	0.013	9.717	12	25	(R)DTDAMTALLADDVV(F)	2768
1882.95	1882.95	0.001	0.455	22	38	(A)DDVVFTSPVAYKPYPGK(A)	1882
1553.84	1553.83	0.009	6.279	25	38	(V)VFTSPVAYKPYPGK(A)	1772
1454.76	1454.76	0.001	-0.57	26	38	(V)FTSPVAYKPYPGK(A)	4220
1307.69	1307.69	-0.001	-1.445	27	38	(F)TSPVAYKPYPGK(A)	1147
1206.65	1206.65	0.005	4.287	28	38	(T)SPVAYKPYPGK(A)	2005
1332.6	1332.59	0.001	0.770	12	24	(R)DTDAMTALLADDV(V)	2059

As the expressed protein indicated a molecular weight of 30 kD, in gel trypsin digestion was carried out followed by nano LC- ESI- MS/ MS. From raw data file the ion detection, clustering, and normalization were performed using ProteinLynx GlobalServer. Each processed file was then searched against the Uniprot protein database (http://www.uniprot.org/) using the IDENTITY^{E} database search algorithm within PLGS 2.2.5. The identified protein was NADH oxidase IV from *M. smegmatis* (Table 4.2) Around 70% of coverage was obtained which prove the robustness of identification.

Table. 4.3 ESI – MS/ MS analysis of the cloned protein

Uniprot Accession No.	A0R6M6
Description	NADH flavin oxidoreductase (nadh oxidase) *Mycobacterium smegmatis* strain ATCC 700084 mc ^{2} 155
Molecular Weight (Da)	14392
pI (pH)	4.6267
PLGS Score	691.9645
Peptides	8
Theoretical Peptides	10
Coverage (%)	70.61
Precursor RMS Mass Error (ppm)	4.5516
Products RMS Mass Error (ppm)	12.8627
Products RMS RT Error (min)	0.01392

4.3.4 Partial characterization of the purified NADH oxidase

4.3.4.1. Effect of pH and temperature on Enzyme activity

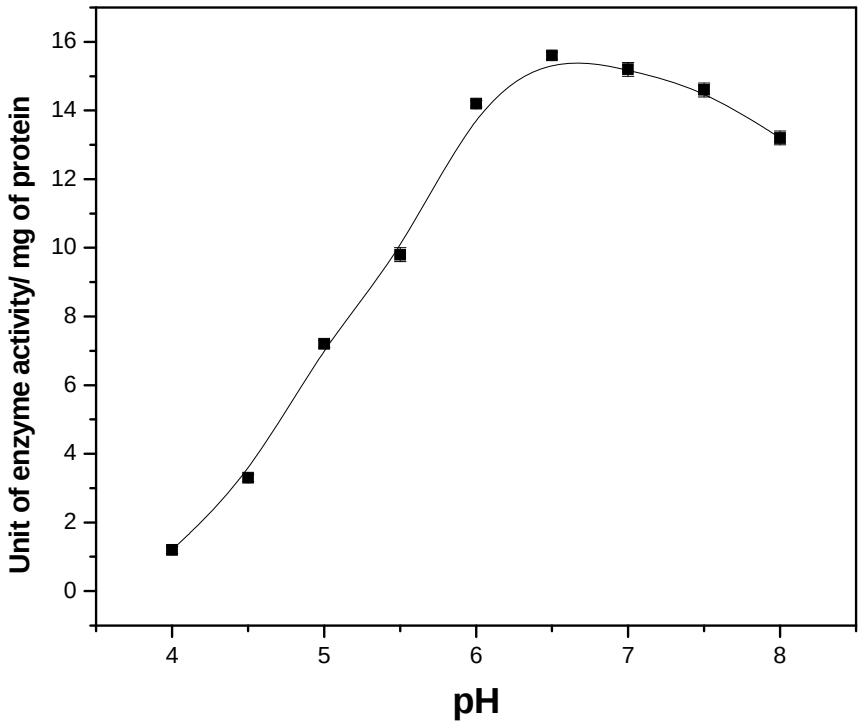

Fig. 4. 4. Effect of pH on NADH oxidase activity of purified enzyme. The activity was assayed in different pH phosphate buffers. The reaction was carried out at 37 °C and 10 minutes incubation time in final assay volume of 300µl containing 50mM sodium phosphate buffer, 100µM of $FADH_2$, 20µg/ml of enzyme and 30µM of NADH. Results were mean +/- SD of three identical experiments.

NADH oxidase activity was optimal at pH 6.5 (Fig. 5.6). It maintained more than 90% of the optimal activity from pH 6 to 7.5 and more than 50% activity in the range of 5-8. This indicated the enzyme has ability to adapt to a wide range of pH and functioning in environment at different pH. The optimal temperature of the enzyme was found to be 37^0C and more than 90% of it was retained in the range of 25-60^0C (Fig. 5.7).

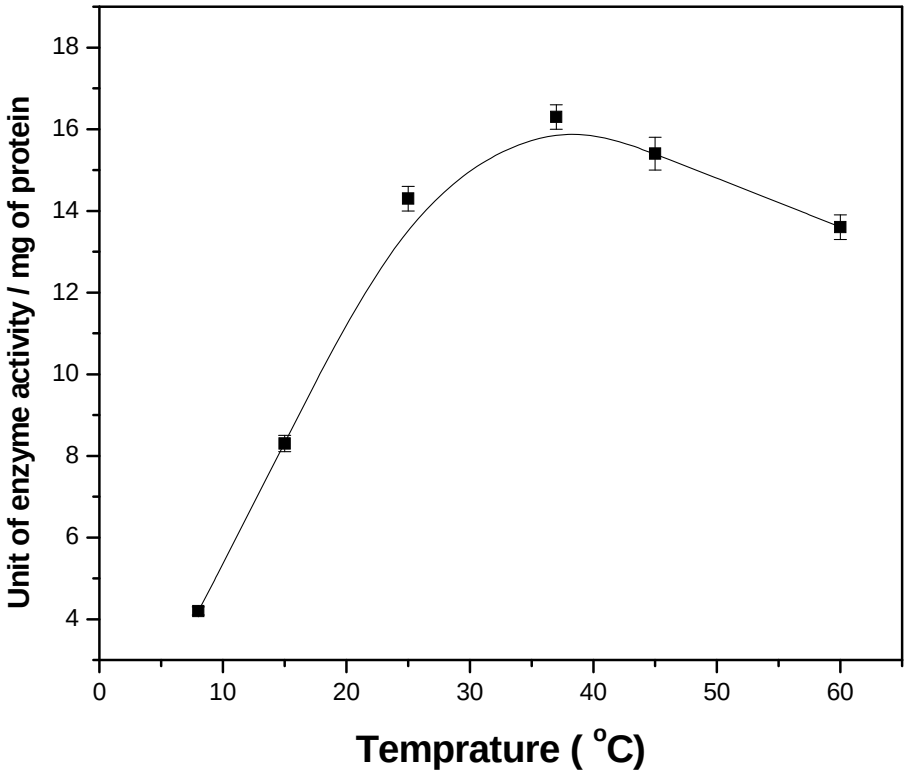

Fig. 4. 5 Effect of temperature on NADH oxidase activity of purified enzyme. The reaction was carried out at pH 6.5 and 10 minutes incubation time in final assay volume of 300µl containing 50mM sodium phosphate buffer, 100µM of $FADH_2$, 20µg/ml of enzyme and 30µM of NADH. Results were mean +/- SD of three identical experiments.

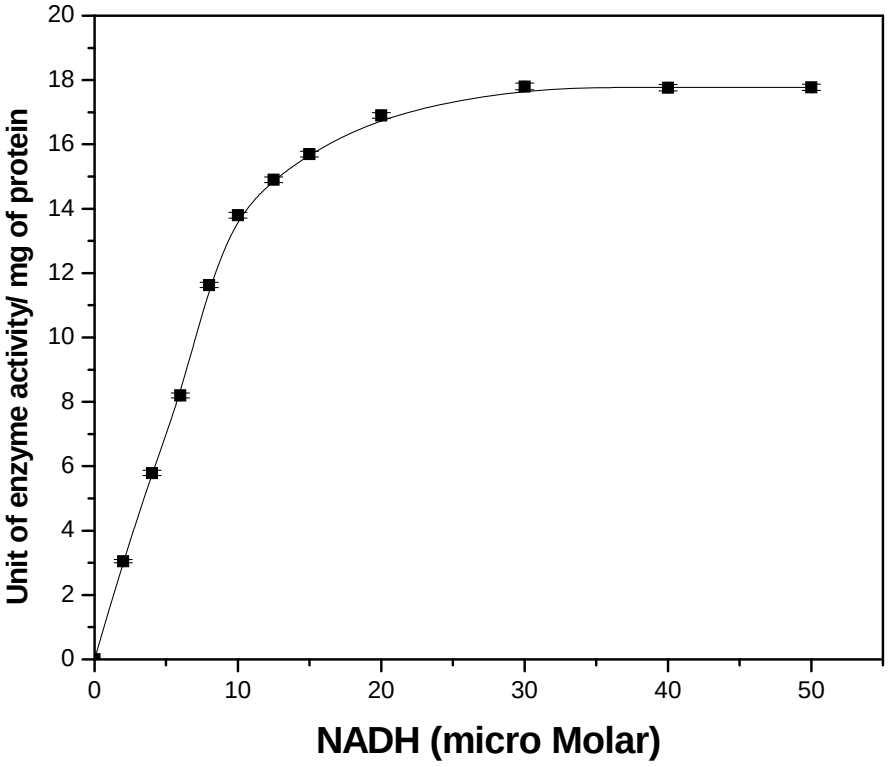

Fig 4. 6 The NADH oxidase activity of purified enzyme at different NADH concentrations. The reaction was carried out at pH 6.5, 37 ºC and 10 minutes incubation time in final assay volume of 300µl containing 50mM sodium phosphate buffer, 100µM of $FADH_2$, 20µg/ml of enzyme and different concentration of NADH. Results are mean +/- SD of three identical experiments.

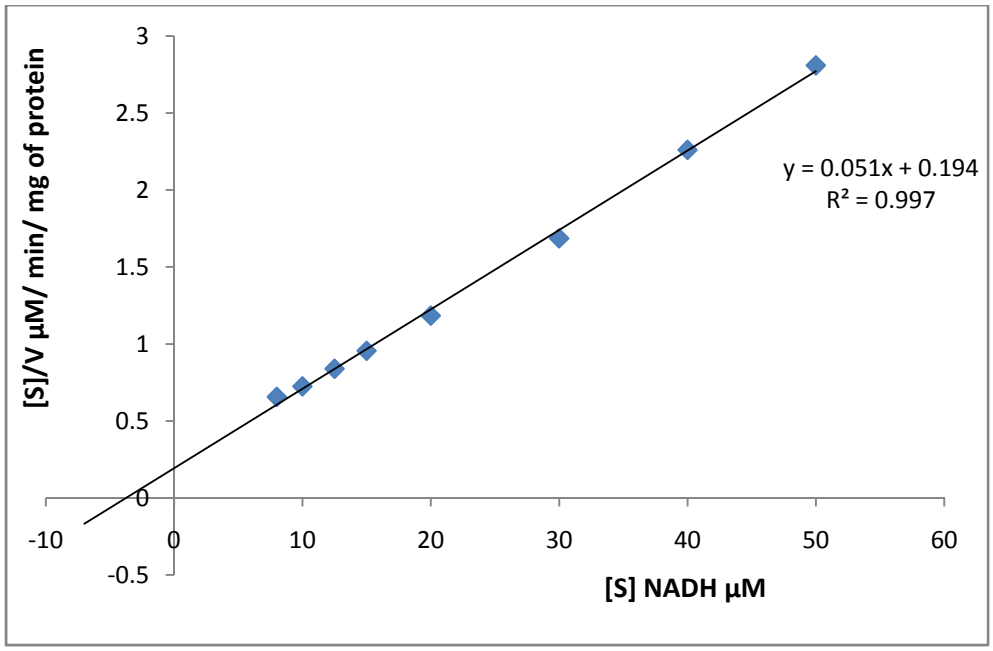

Fig. 4.7 Hanes–Woolf plot to determine the K_m and V_{max} of NADH for NADH oxidase. Experiment was carried out at 20µg/ ml of enzyme concentration in the final assay volume of 300 µl. The Enzyme reaction was carried out at pH 6.5, 37 ºC and 10 minutes incubation time. Other details of experiment are described in materials and methods. Results were mean of three identical experiments.

Table 4. 4 Effect of different DPI concentration on NADH oxidase activity

DPI concentration (µM)	NADH oxidase activity (µM / min/ mg of protein)
0	17.8
0.1	12.1
0.5	1.3
1	0

4.3.4.2. Determination of kinetics parameters of *Mycobacterium smegmatis* NADH oxidase and its inhibition by DPI

NADH oxidase activity was assayed against different concentrations of NADH in the reaction to determine the specificity and affinity of enzyme for substrate. A very high activity was seen at very low concentration of nitrate. Maximum activity of enzyme could be achieved at a concentration of 20 μM (Fig. 4.12). K_m for NADH was calculated to be 3.8 μM as determined by Hanes–Woolf plot (fig 4.13). This low K_m value indicated the high affinity of enzyme towards NADH. V_{max} for the reaction was calculated 19.6 μM/ min /mg of protein. The K_m value of enzyme was comparable with earlier reported NADH oxidase from different organisms such as, aerobic *Brevibacterium sp.* 22, obligate anaerobic bacteria *T. hypogeal* and *C. aminovalericum* 7.2 and 19.5 respectively

DPI is a general flavoprotein inhibitor, commonly used to inhibit NADH and NADPH oxidases (32). DPI is reported to sensitize cells to Fas-mediated apoptosis (33), providing a link between plasma membrane redox activities and resistance to apoptosis. In our study we found that there was complete inhibition of NADH oxidase activity by 1μM of DPI which is contrary to the effect of DPI on human NADPH oxidase. Human NADPH oxidase is insensitive to DPI up to 1 μM concentration this may be due to difference in structure and homology (34).

Altogether, this work suggested that these NADH oxidases in *M. smegmatis* are functionally important in the survival of the bacilli. Noteworthy, different NADH oxidases have different possible role in active, antioxidant and hypoxia induced dormant and HOPT induced resuscitated bacilli. NADH oxidase appears to be an important enzyme in the process of switching from one state to another state. This preliminary study was an attempt to partially characterize one of the NADH oxidases that has possible role in resuscitation but for clearer picture further studies are necessary. As all four NADH oxidase has own significant role, extension of this work should also acknowledge rest three enzymes.

4.4 Reference

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