Abstract

Mycobacterium tuberculosis (*Mtb*) has evolved as an important clinical pathogen due to its ability to gain multidrug resistance, to enter into latency to persist there and to get reactivated from the latent infection in aged, immunocompromised persons to cause the disease. *Mtb*, which can pesist within the granulomatous parenchymal lesions in a nonreplicating persistent (NRP) stage called dormancy (clinically called latency), experience hypoxic stress. In order to understand the response of the bacilli to the hypoxic environment, several *in vivo* and *in vitro* model systems have been designed and used. Amongst the *in vitro* model systems, the one which closely resembles the hypoxic stress induced within the host wherein the bacilli experience a gradual depletion of oxygen, was found to be the Wayne's *in vitro* hypoxia model. Therefore, it is considered to be the best model system to study the response of *M. tuberculosis* to the hypoxic stress *in vitro* as here as well the bacilli experience a gradual depletion of oxygen to progress from the actively growing mid-log phase to NRP-I and NRP-II stages. Even though, several works have been carried out at the gene expression and proteomic levels to know the response of the bacilli using Wayne's *in vitro* hypoxia model, surprisingly there is hardly any information available on the morphological and cellular level changes, which occur in response to hypoxia, and their correlation to molecular changes. Therefore, in the present study, using Wayne's *in vitro* hypoxia model, this lacuna of information was addressed by studying morphological and cellular changes occurring to NRPI and NRP-II *M. tuberculosis* cells at the ultrastructural level, correlating the changes with respective gene expression level changes, and finding out their physiological significance.

The **Chapter 1**, which forms the **Introduction** to the thesis, gives an extensive literature survey on all the different aspects of the research performed on mycobacterial cells under hypoxia, which are linked to the present study, and under other stress conditions such as nutrient depletion, pH and so on.

The **Chapter 2** presents in detail all the materials and methods used to perform the experiments. A large number of biochemical, biophysical, and molecular level methods, such as scanning and transmission electron microscopy, atomic force microscopy, Fourier transform infrared spectroscopy, dynamic light scattering, flow cytometry, fluorescence microscopy, real time RT-PCR analysis, and others were used to perform the experiments.

The data **Chapter 3** presents ultrastructural analysis of the *M. tuberculosis* cells exposed to hypoxic stress condition using Wayne's *in vitro* hypoxia model. Among the different ultra-structural changes observed, this study focused mainly on the unique thickening of the outer layer (OL), during the progression of the bacilli from mid-log phase to NRP-I and then to NRPII stages. On the contrary, the NRP-I and NRP-II cells of the saprophytic mycobacterial species, *Mycobacterium smegmatis*, did not show thickening of the OL. Dynamic light scattering

experiments using Zetasizer showed the NRP-I and NRP-II *M. tuberculosis* cells to be longer in size. Experiments using Zeta potential analyzer revealed high level of negative charge on the surface of the NRP-I and NRP-II *M. tuberculosis* bacilli. Ultrastructural studies, using scanning and transmission electron microscopy and atomic force microscopy revealed that the surface of the NRP-II *M. tuberculosis* bacilli was extensively rough and uneven, unlike the smooth and even surface of the mid-log phase cells. Fourier transform infrared spectroscopy of the groups present in the polysaccharides extracted from thickened outer layer were found to be highly anionic in nature. Polysaccharide-specific calcofluor white staining showed the thickened outer layer of *M. tuberculosis* to be rich in polysaccharides. Transcriptome analysis of the respective genes involved in the synthesis of capsular polysaccharides showed significant upregulation in comparison to the same genes in the MLP cells, thus supporting the observed hypoxic adaptation of the thickening of the OL in NRP-II *M. tuberculosis* cells.

The data **Chapter 4** presents studies on the physiological significance of the thickening of the OL in terms of the permeability to the anti-TB drug, rifampicin. Since it was necessary to choose a non-bioactive variant of rifampicin in order to avoid the growth inhibitory antibiotic action on the cells, rifampicin conjugated to 5-carboxyfluorescein (5-FAMrifampicin), which showed only 2.5% bioactivity, was used for the permeability assay, instead of 14C-rifampicin. The thickened OL of the NRP-I and NRP-II cells were found to significantly restrict the entry of 5-FAM-rifampicin into the cells. Mild bead beating of the NRP-II cells, to remove the thickened OL without affecting the outer membrane integrity, as confirmed using transmission electron microscopy, restored the permeability of 5-FAM-rifampicin ascomparable to that into mid-log phase cells. The entry of 5-FAM-rifampicin into the cells was monitored using flow cytometry analysis. *M. tuberculosis* cells, at 48 hrs. post-release from= the NRP-II stage, also showed restoration of the permeability of 5-FAM-rifampicin as comparable to that into mid-log phase cells. These observations suggested that the recalcitrance of dormant bacilli to anti-TB drugs might be due to the presence of thickened OL generated by the bacilli as a strategy to evade bactericidal effects.

The data chapters in the thesis are concluded with discussion of the findings presented in the respective chapter.

The thesis finally lists the highlights of the present study, followed up with an extensive bibliography.