Abstract

Rifampicin is a non-competitive inhibitor of bacterial RNA polymerase (RNAP). The knowledge about the mechanism of action of this drug has emanated from the genetic and the biochemical studies carried out on *Escherichia coli* RNAP. The complete picture about the steric mechanism was obtained from structural studies on *Thermus aquaticus* core RNAP in complex with rifampicin. Resistance to rifampicin has been attributed to mutations in its binding pocket lying within the β-subunit of RNAP. The phenomenon of molecular tolerance to this drug came to light with the discovery of differential inhibition of transcription from σ70- and σ32- dependent promoters by rifampicin. After the discovery of the differential inhibition of transcription from σ70- and σ32- dependent promoters by rifampicin, similar instances of proteins were reported. In all such cases, their association with RNAP reduced its susceptibility to rifampicin. In an independent line of study, a new protein, RbpA, was discovered in *Streptomyces coelicolor*. This protein has the capability of interacting with RNAP, causes rifampicin tolerance to RNAP activity *in vitro* and leads to basal levels of rifampicin resistance *in vivo.* Moreover, this protein has sequence homologs exclusively in the actinomycete family, with nearest neighbors in mycobacteria. Interestingly, when *rbpA* null mutants in *S. coelicolor* were transformed with *M. tuberculosis rbpA* gene, the resistance to rifampicin grew from 0.75μg/ml to 2μg/ml *in vivo*, which pointed towards an analogous role of *rbpAMTB*. Saturation mutagenesis studies carried out on *M. tuberculosis* have placed *rbpAMTB* (Rv2050) on the list of genes imperative for optimal growth. The similarities and the speculations over mycobacterial RbpA made a compelling case in its favour for deciphering the role it played in the mycobacterial paradigm especially in the backdrop of rifampicin tolerance. This work focuses on the discovery of MsRbpA in *M. smegmatis* as an RNAP-interacting protein, characterization of MsRbpA with respect to its role in conferring phenotypic tolerance to rifampicin, molecular mechanism of the release of rifampicin from RNAP-rifampicin complex, location of MsRbpA on RNAP and studies on the probable role of MsRbpA in a rifampicin-resistant strain.

The thesis is organized as follows:

Chapter 1 deals with the literature survey on rifampicin with an all-inclusive perspective. It provides a brief history on the evolution of antibiotics as principal inhibitors against essential macromolecular assemblies, like RNAP and ribosomes, which control major cellular processes. Subsequently, we discuss the inhibitors of the transcription process, with a major emphasis on the mechanisms of inhibition of transcription activity by rifampicin. We also present an in-depth bibliomic analysis of the response to rifampicin across the microbial spectrum, which scratches the physiological and molecular landscape in a comprehensive manner. Finally, we present the justification for undertaking this work, and the proximate aims of this study.

Chapter 2 informs about the identification of MSMEG_3858 as an RNAP-interacting protein. It details the cloning, expression and purification of MsRbpA. The focus then shifts towards the interact omics of MsRbpA and RNAP. Studies on the *in vivo* expression of MsRbpA form the concluding part of this chapter. Investigation into the outcome of interaction between MsRbpA and RNAP forms the central theme of

Chapter 3. In this regard, first of all, we report the *in vitro* reconstitution of a heterologous mycobacterial core RNAP and its competence in carrying out gel-based *in vitro* promoter-specific transcription assays. Consequently, we apply this transcription apparatus in judging the role of MsRbpA in preventing the rifampicin-mediated inhibition of transcription activity, both in single- and multiple-round gel-based transcription assays. As a corroboration of this work, it is shown as to how the induction of MsRbpA *in vivo* causes an increase in the rifampicin-tolerance levels of *M. smegmatis*. Finally, we probe the existence

of any possible interaction between MsRbpA and rifampicin as a prelude to ensuing investigations into the mechanism of phenotypic tolerance to rifampicin.
In **Chapter 4** we decipher the molecular mechanism of MsRbpA-mediated release of rifampicin from RNAP-rifampicin complex. A two-pronged strategy comprising of fluorescence-based and mass spectrometry-based approaches helps us to elucidate the aforesaid mechanism in greater detail. As a result, the location of interaction of MsRbpA on *M. smegmatis* RNAP is found to be at the junction of β and β' subunit.

Chapter 5 attempts to explain the existence of RbpA homologs in microorganisms with two copies of β-subunit, one of which is Rifampicin susceptible (RifS) and the other being Rifampicin resistant (RifR). Using *M. smegmatis* as a model organism, we induce rifampicin resistance in *M. smegmatis* and create 3 variants with graded rifampicin resistance. After confirming the non-revertant nature of these strains, we assay the role of MsRbpA in rescuing the activity of RifR RNAPs at their respective IC50 values. The results show a redundancy of MsRbpA in rescuing RifR RNAPs. Alongside, we compare the colony morphology of MsRbpA overexpressing strain and the RifR strains at subinhibitory concentrations of rifampicin. The comparison shows a similarity in colony morphology typical to stress-induced conditions. Combining these results, we attempt to visualize the role of MsRbpA in species carrying two copies of the β-subunit.

Chapter 6 studies the coevolution of RbpA and RNAP in the actinomycete phylum. The studies presented involve bioinformatics and statistics to decipher the cause of the exclusive existence of this protein in actinobacteria. The results show that RbpA appears to have co-evolved with actinobacterial RNAP as a defense system. This could be one of the ways of survival in the soil environment where metabolites like rifampicin are secreted by the producing organisms during struggle for existence.

Chapter 7 summarizes the work presented in this thesis.