Description of Research Work

Research work objectives have been carefully articulated after detailed discussion with the host laboratory (Prof. Kirtimaan Syal's laboratory from BITS Pilani).

Introduction

The frequent failure of antibiotic treatment is one of the world's most pressing public health problems. Such frequent failure is predominantly attributed to the emergence of drug resistance. The bacteria have developed resistance towards most if not all clinically used antibiotics. All growing bacteria produce a small fraction of cells that escape the lethal action of antibiotics by entering a physiological state in which the antibiotics do not attack them. This phenomenon is called bacterial persistence. Bacterial persistence is distinct from resistance because, unlike resistant mutant cells, it is not genetic. Also, the persister cells do not grow in the presence of the antibiotic but upon restoration of normal conditions switch back to antibiotic sensitive growing state. Tuberculosis requires a long treatment regime because of its capacity to persist inside host. In tuberculosis, persistence is predominantly governed by different second messengers including ppGpp and cAMP. Earlier, Petros Karakousis' group from John Hopkins University showed that without second messenger (p)ppGpp, M. tuberculosis cannot cause infection in animal models. The stringent response mediated by (p)ppGpp has been proven to be essential for virulence of *M. tuberculosis* and its inhibition lead to compromised long term survival and biofilm formation. In the proposed study, we aim to study novel enzyme $RelZ_{Msm}$ and its homolog Rv1366Mtb which can make ppGpp and pGpp but how it decides on making one over the other is unclear. Is it the substrate concentration or a regulatory factor that triggers pGpp synthesis? Interestingly, the RelZMsm enzyme also has a RNase H activity but the function and biological significance of RNase H coupled with (p)ppGpp synthetase is not clear. Are (p)ppGpp synthesis and RNaseH activities mutually exclusive? We will be studying these activities in its homolog Rv1366 from M. tuberculosis. In which growth phase Rv1366 is most active will be determined in the proposed study and the network of Rv1366 interactome will be deciphered with an aim to reveal the defense network of mycobacteria involved in stress response and long-term survival inside the host.

Objectives

My objective is to investigate the biosynthesis, regulation, and functional roles of second messengers, particularly ppGpp, in mycobacterial persistence.

We propose the following three broad objectives in this project.

1. Deciphering the role of RelZ and its homolog Rv1366 in antimicrobial tolerance in mycobacteria.
2. Biochemical and biophysical characterization of RelZ and Rv1366.

3. To dissect the interactome of RelZ and Rv1366 in mycobacteria and determine its significance in adaptability.

Brief description of pilot data

Earlier, we observed an increase in the expression of RelZ (second messenger ppGpp synthetase) in the stationary phase and its mutants are defective in surviving antibiotics including rifampicin. Persister assays suggested that RelZ mutant strain capacity to produce persister cells is compromised. Molecular mechanism remains unclear.

Methodology

1. **Deciphering the role of RelZ and its homolog Rv1366 in antimicrobial tolerance in *M. tuberculosis***

Earlier, it has been shown that second messenger synthetase RelZ, and not Rel, from Msm (an ortholog of Rv1366 in Mtb) is responsible for conferring antimicrobial tolerance (Petchipachhan et al. J Bacteriol, 2019). How RelZ is responsible for antimicrobial tolerance and persistor phenotype will be investigated in this proposal. We have prepared a list of antimicrobials of various classes including transcription inhibitors, cell wall inhibitors, DNA replication inhibitors and translation inhibitors to which RelZ mutant will be tested. We will further test the highlighted antimicrobials against knock out of Rv1366 and investigate their susceptibility. We will test different mutants to each of the antimicrobials with an attempt to understand the role of each of the enzyme activities.

2. **Biochemical and biophysical characterization of RelZ and Rv1366.**

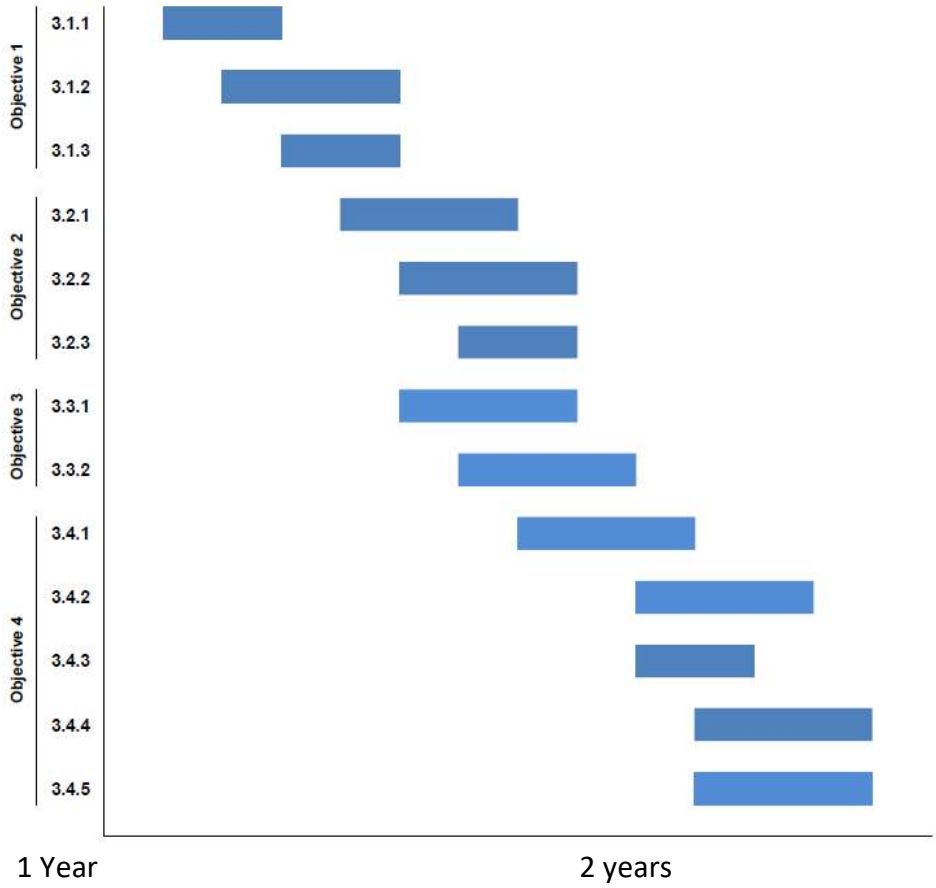
This objective will be undertaken to understand the biochemical and biophysical properties of Rv1366, in order to gain a better understanding of the protein and its behaviour. Cloning, expression, and purification of RelZ and Rv1366. The gene will be PCR amplified and cloned in pET vector that will also provide a hexa-histidine tag to ease purification. The cloning will be carried out following established methods in PIs laboratory. Clones will be confirmed by DNA sequencing. Overexpression of the target gene will be achieved in *E. coli* BL21(DE3) cells by inducing them with IPTG.

3. **To dissect the interactome of RelZ and Rv1366 in mycobacteria.** This objective will allow us to address how RelZ and Rv1366 interacts with other proteins and allow the bacteria to adapt during stress conditions. It will be outsourced to mass spectrometry facility at IISc Bangalore. Bioinformatic analysis will be carried out at BITS Pilani.

Anticipated outcomes

The objectives of the proposal will potentially reveal the defense network of mycobacteria involved in persistence, antimicrobial tolerance and long-term survival. The biological significance of a bifunctional enzyme which possess second messenger (p)ppGpp synthetase along with RNase H activity will further our understanding about the role of (p)ppGpp in DNA replication. RelZ can synthesize pGpp however its role remains unclear. Our study will help in the identification of therapeutic targets responsible for long term survival, persistence, and the emergence of multiple drug resistance in mycobacteria. Such therapeutic targets can also compromise stress response and long-term survival, and shorten the long treatment regime.

Timeline



3.1. Deciphering the role of RelZ and its homolog Rv1366 in antimicrobial tolerance in M. tuberculosis

3.1.1. Generation of Rv1366 knockout in Mtb.

3.1.2. Preparation of complemented strain

3.1.3. Complementation of loss of Rv1366 with RelZ of Msm.

3.1.4. Evaluation of antibiotics tolerance in knockout and complemented strains.
3.1.5. In silico clustering of the defects to understand the implication of RelZ and Rv1366 deletion.

3.2. Biochemical and biophysical characterization of RelZ and Rv1366.
3.2.1. Cloning, expression, and purification of RelZ and Rv1366.
3.2.2. In vitro activity assays with RelZ and Rv1366.
3.2.3. Identification of conserved residues and mutants preparation.
3.2.4. Biophysical analysis of wildtype and mutated proteins.
3.2.5. Effect of Rv1366 overexpression on the physiology of *E. coli*, *Msm*, and *Mtb*.

3.3. To dissect the interactome of RelZ and Rv1366 in mycobacteria. [divided into two objectives (3&4) in the timeline]
3.3.1. Cloning of RelZ and Rv1366 with GST tag for pull down experiments.
3.3.2. Pull down of RelZ and Rv1366-interacting proteins and MALDI-based identification of the proteins.
3.3.3. Delineating the differences in the interactome under different growth conditions.
3.3.4. Identification of the interacting region and its mutational analysis to disrupt interaction.
3.3.5. Activity analysis of RelZ and Rv1366 in the presence of interacting proteins.