Synopsis

Adaptation to any undesirable change in the environment dictates the survivability of many microorganisms. Such changes generate a quick and suitable response, which guides the physiology of bacteria. Stringent response is one of the mechanisms that can be called a survival strategy under nutritional starvation in bacteria and was first observed in *E. coli* upon amino acid starvation, when bacteria demonstrated an immediate downshift in the rRNA and tRNA levels (Stent and Brenner 1961). Mutations that rendered bacteria insensitive to amino acid levels were mapped to an 'RC gene locus', later termed *relA* because of the ***relAxed*** behavior of the bacteria (Alfoldi *et al.* 1962). Later on, Cashel and Gallant, showed that two "***magic spots***" (MSI and MSII) were specifically observed in starved cells when a labeled nucleotide extract of these cells was separated by thin layer chromatography (Cashel and Gallant 1969). These molecules were found to be polyphosphate derivatives of guanosine, ppGpp and pppGpp (Cashel and Kalbacher 1970; Sy and Lipmann 1973), and were shown to be involved in regulating the gene expression in the bacterial cell, demonstrating a global response, thus fine-tuning the physiology of the bacterium. Two proteins in *E. coli*, RelA and SpoT, carry out the synthesis and hydrolysis of these molecules, respectively, and maintain their levels in the cell (Cashel *et al.* 1996; Chatterji and Ojha 2001). On the other hand, Gram-positive organisms have only one protein Rel carrying out the functions of both RelA and SpoT (Mechold *et al.* 1996; Martinez-Costa *et al.* 1998; Avarbock *et al.* 1999).

Although Rel or RelA/SpoT has been studied from several systems in detail pertaining to the physiological adaptation, less information is available on the regulation of the protein activity under different conditions. Our studies show that the Rel_{Msm} is composed of several domains (HD, RSD, TGS and ACT) with distinct function. HD and RSD domains, present in the N-terminal half of the protein, harbor catalytic sites for the hydrolysis and the synthesis of (p)ppGpp, respectively. TGS and ACT domains, on the other hand, are present at the C-terminal half of the protein and have regulatory function. It, therefore, appears that a communication exists between these domains, to regulate protein activity. It was shown earlier, while studying Rel from *S. equisimilis*, that there exists an interaction between the C-terminal and the N-terminal of the protein which determines the kind of activity (synthesis/hydrolysis),

the protein should demonstrate (Mechold *et al.* 2002). Later, the N-terminal half crystal structure of the same protein suggested an inter-domain "cross-talk" between the HD and the RSD domain that controls the synthesis/hydrolysis switch depending on cellular conditions (Hogg *et al.* 2004).

In the present work, studies have been carried out to understand a Gram-positive Rel in greater detail and to find out how the opposing activities of Rel are regulated so that a futile cycle of synthesis and hydrolysis of (p)ppGpp, at the expense of ATP, can be avoided. The work has been divided into several chapters describing studies on various aspects of the protein.

Chapter 1 outlines the history of the stringent response and summarizes the information available about the stringent response in various systems including plants. Several roles that (p)ppGpp plays in different bacteria have been examined. A special mention on the crystal structure of Rel_{Seq} has been made with respect to the regulation of activity. Also, the information available regarding the effects of (p)ppGpp on RNA polymerase has been documented. Role of ppGpp in plants has been discussed in great detail with special emphasis on abiotic stresses.

Since different functional domains have been identified in Rel_{Msm}, the protein has been divided into two halves and they have been discussed separately in the form of two chapters.

Chapter 2 describes the N-terminal half of the Rel protein of *M. smegmatis* in greater detail. Out of the several domains identified, the role of the two domains present in the N-terminal half of the protein has been studied. The N-terminal half shows both synthesis and hydrolysis activities. Importantly, we find that the protein is active even in the absence of accessory factors such as ribosome and uncharged tRNA, unlike RelA of *E. coli*. Moreover, deletion of the C-terminal half of the protein leads to a much higher synthetic activity, clearly indicating that the C-terminus is involved in regulating the activity of the protein. Both TGS and ACT domains (the two domains found in the C-terminal half of the protein) have been found to play a regulatory role. The results also indicate that all the deleted constructs are active both *in vitro* and *in vivo*.

Chapter 3 discusses the C-terminal half of the protein and its role in the multimerization observed in Rel_{Msm}. We show that multimerization of Rel protein is due to the inter-molecular disulfide cross-linking. Furthermore, we find that the

monomer is the active species *in vivo*. One of the fascinating points about the C-terminal half is that it is largely unstructured. Additionally, the C-terminal half cannot complement the N-terminal part of the protein when provided in *trans*, demonstrating further, the requirement of an intact protein for bringing about regulation of Rel activity. This requirement in *cis* suggests the presence of an intra-molecular communication between the N- and the C-termini, as a mediator of protein regulation. Further, presence of uncharged tRNA increases pppGpp synthesis and down-regulates its hydrolysis in the wildtype protein. However, the uncharged tRNA-mediated regulation is absent in the deleted construct with only the N-terminus half, indicating that uncharged tRNA binds to the C-terminal half of the protein. Several cysteine mutants have been constructed to understand their role in the regulation of Rel activity. The results suggest that one cysteine, present at the C-terminus, is required for intra-molecular cross-talk and the uncharged tRNA-mediated regulation.

A detailed characterization of the communication between the two halves of the protein has been attempted in ***Chapter 4***. Surface plasmon resonance experiments carried out on the different cysteine mutants discussed in Chapter 3, for uncharged tRNA binding indicate that all the mutants bind to uncharged tRNA with near-equal affinities as the wildtype protein. This study suggests that the non-responsiveness for tRNA seen in one of the cysteine mutants is due to the loss of inter-domain interaction, while the binding of protein to accessory factors is unaffected. Fluorescence resonance energy transfer has been carried out to observe domain movement in the presence of accessory factors. Distances between the different domains scattered in this ~90 kDa protein, measured by FRET technique, are suggestive of an inter-domain cross-talk, specifically between C338 and C692, thereby regulating the activity of this enzyme. We show, for the first time, that the product of this protein, (p)ppGpp can bind to the C-terminal half making it unstructured, and can, therefore, regulate the protein activity.

Chapter 5 is an effort to characterize the promoter of *rel* from *M. tuberculosis*. This study was undertaken in order to develop an expression system in mycobacteria. The +1 transcription and the translation start sites have been identified. The –10 hexamer for the RNA polymerase binding has also been mapped using site-directed mutagenesis and is found to be TATCCT. This promoter is also unusually close to the +1 transcription start site. The promoter is specific for mycobacteria and does not

function in *E. coli*. Additionally, the promoter is found to be constitutive in *M. smegmatis*; however, the possibility of it being regulated in *M. tuberculosis* cannot be ruled out.

Appendix section discusses, in short, the phylogenetic analysis of the mycobacterial Rel sequences. Diagrams of the plasmids used in this study have been provided. Mass spectra recorded for the *in vitro* synthesized and purified pppGpp and the trypsin digest of the full-length Rel protein have also been given.