Summary

Riboflavin biosynthesis pathway is essential to microorganisms but it is absent in human, and thus the enzymes of this pathway are potential targets for designing novel antimicrobials. Most of the microorganisms can synthesize riboflavin and some of them can produce it more than their own requirement. *Bacillus subtilis* (*B. subtilis*) is a gram positive, non-pathogenic bacterium which has been extensively used for production of many industrially relevant compounds. Riboflavin is one of those bulk commodity which is biotechnologically produced by *B. subtilis*. Understanding the riboflavin biosynthetic and regulatory pathway in *B. subtilis* will be helpful in improving the organism as a super producer of riboflavin and will also improve our understanding towards the development of novel therapeutics. In the current study, the riboflavin biosynthetic and /or regulatory genes *ribT* and *ribC* from *B. subtilis* has been targeted for structural and functional characterisation.

In *B. subtilis ribT* gene (*bribT*) is positioned at the distal terminus of its *rib* operon. With the aim to understand the structure and function of this gene we initiated our study with the cloning, expression and purification of *bribT*. The *bribT gene* is cloned in pET21b vector carrying C-terminal 6X histidine tag and purified using Ni-NTA chromatography followed by size exclusion chromatography. The bribT exists as monomer in size exclusion chromatography. The Far-UV circular dichroism study shows that bribT is well folded and consists of mixed α-helix and β-sheet. In order to characterise bribT structurally, we initiated the crystallisation studies which could be successful after several attempts in the presence of one of its substrate Acetyl-CoA (AcCoA). In the absence of any close structural homolog, the crystal structure of bribT is solved by Se-SAD phasing. The crystal structure analysis reveals that bribT belongs to a member of GCN5-related N-acetyltransferase superfamily of enzymes preserving all the hallmarks of the superfamily. For the identification of possible substrate for bribT, many class of substrates including antibiotics, polyamines, and amino acids has been screened using DTNB assay with no success. Based on the previous report (Kim *et al.*, 2013) we try to check the auto-acetylation capability of bribT. We have made alanine mutants of Lys-24 of bribT as bribT has been shown to be acetylated on this residue. The acetylation signal observed in the assay carried out using [^{14}C]-labelled AcCoA for K24A was comparable to that of bribT. The result indicates that bribT is not capable of auto acetylation of K24 suggesting acetylation of K24 might be nonenzymatic or is mediated by some other proteins or could be achieved in the presence of some specific conditions or appropriate substrate. Moreover, N-acetyltransferases are reported to catalyse acetylation reaction via direct transfer or ping pong

mechanism. The structural analysis of bribT shows the presence of Glu-67 and Ser-107 at the position where they could act as possible catalytic base and catalytic acid respectively for direct transfer mode of catalysis in bribT. Additionally, Cys-112 is also observed close to binding site of CoA and is speculated as possible candidate for ping pong mechanism of catalysis. Thus, we have made mutants for residues which could be putatively involved in catalysis via direct mechanism (E67A and S107A) or ping pong mechanism (C112A and C112S). The decrease in acetylation signal is observed in case of cysteine mutants. This indicates the involvement of Cys-112 residue in the binding of AcCoA/CoA or in the catalysis. To further confirm the role of Cys-112, the acetylation assay of bribT performed in presence of thiol blocking agents (MMTS and H_2O_2) which also shows loss of acetylation signal. However, the mutation of potential residues (Glu-67, Ser-107) which is thought to be involved in direct transfer mechanism doesn't show any significant change in acetylation signal. Although, these results strongly suggests that bribT may follow ping-pong mechanism for its catalysis, location of Glu-67 and Ser-107 at the appropriate position suitable for direct transfer mechanism restricts us to conclude the same. Thus, from this part of study we have characterised bribT as another member of GCN5 related superfamily of enzyme and have shown the role of a non-conserved cysteine residue (Cys-112) in the binding of AcCoA/CoA molecule.

The other part of the thesis involves the structural and biochemical characterization of ribC protein. The *ribC* gene encodes for the bifunctional RFK/FADS enzyme which is involved in the conversion of riboflavin to important cofactors FMN and FAD. The *ribC* gene has been shown to be essential for *B. subtilis* (Tanaka *et al.*, 2013). The involvement of *ribC* gene in the regulation of riboflavin biosynthesis of *B. subtilis* has already been reported (Mack *et al.*, 1998). However, the enzyme remained to be structurally characterized. Moreover, the specific requirement of reduced substrate reported for this enzyme (Kearney *et al.*, 1979) is not clear till date. In the present study, we have cloned *ribC* gene from *B. subtilis* (*bribC*) in pET28c vector, expressed in BL21 (DE3) and purified using Ni-NTA chromatography followed by size exclusion chromatography. The N-terminal FADS domain and C-terminal RFK domain has also been cloned, expressed and purified independently. The size exclusion chromatographic studies reveals that all the proteins exists as monomer in solution. The circular dichroism studies reveals that all the purified proteins are well folded and bribC showed melting (Tm) at 51°C. The biochemical characterization has been done for bribC and the kinetic parameters has also been reported in this study. The catalytic activity studies on bribC shows that the enzyme is specific for ATP. The RFK activity is observed in the broad range of pH from 5.0 to 10.0

with maximum activity at pH 8.0 whereas the FADS activity is observed in the alkaline range of pH 7.0-10.0, with maximum activity at pH 9.0. Maximum RFK activity is observed at 50°C whereas maximum FADS activity is observed at 30°C. The enzyme activity of bribC with different metal ions shows that Mg^{2+} is optimum for both the activities. Interestingly, contrary to previous finding, our study confirms that for FADS activity bribC essentially requires reduced form of substrate whereas RFK activity could be observed even in the absence of any reducing agent. The independently expressed C-terminal RFK domain is active whereas independently expressed N-terminal FADS domain doesn't show any activity indicating the importance of full length enzyme for the FADS activity. The serine and alanine mutants of Cys-152 and Cys-242 are made to check whether substrate specificity is due to reducing environment around the enzyme or the enzyme requires reduced form of substrate. It is observed that for FADS activity, there is (10%-15%) reduction in activity in case of C152S and C242S mutants when compared to wild type bribC whereas for C152A mutant there is a drop of 30% in activity compared to wild type bribC. This indicates that reducing environment around the protein might not be required as there is not much loss in activity in case of cysteine to serine mutants. Interestingly, similar observation was also made in case of an archaeal FADS which has been found to be active only under reducing conditions. Multiple sequence alignment of bribC with archaea *M. janschii* reveals the presence of cysteine at similar position in both the organisms. In *M. janschii*, the mutation of cysteine to serine has no effect in the enzyme activity, but total loss of activity is observed upon alkylating the cysteine residue. We have also solved the crystal structure of bribC complexed with riboflavin, ATP in RFK domain and phosphate to the N-terminal FADS domain. Analysis of structure showed the conservation of most of the residues involved in catalysis. Structural comparison also reveals the presence of some tyrosine residues which lines the FMN/FAD binding pocket of FADS domain and are exclusive to *B. subtilis*. Thus, from this part of study we conclude that stringency for the reduced substrate is more for the FADS activity of the enzyme. Also, we believe that tyrosine residue alone or cysteine along with the tyrosine might be playing some role in conferring the substrate specificity to bribC.

Future directions

In the present study, the structural characterisation has been done for *bribT* gene which is shown to have N-acetyltransferase fold. Moreover, the cysteine residue has been shown to participate in the binding of AcCoA. Although, the structural characterization of bribT and the involvement of cysteine residue in the binding of AcCoA/CoA represents a significant advancement towards the understanding of riboflavin biosynthesis pathway in *B. subtilis* many questions still remain unanswered. The most important one is the identification of the correct substrate for bribT which will further help in answering many questions. For example, while the N-acetyltransferase activity is not required for riboflavin biosynthesis, the existence of *bribT* gene in *rib* operon and its biological significance remains to be explored. Additionally, identification of correct substrate will also help in understanding the catalytic mechanism followed by bribT which is not yet clear.

The structural characterisation of bribC represents the third structure for the bifunctional RFK/FADS in bacteria. Although the structural characterization of bifunctional RFK/FADS provided the information regarding its fold and binding sites for the substrates the mechanistic details are still unclear. Especially, while the mechanistic details for RFK domain of bifunctional enzyme is well characterized, such details are lacking for the FADS domain of bifunctional enzyme. The mechanistic details regarding the conversion of FMN to FAD by FADS domain and the requirement of full length enzyme by the FADS domain to perform its function remains to be explored. In fact, in spite of several attempts made by our group and other groups, no crystal structure bound with FMN or FAD to the FADS domain in bifunctional enzyme is available till date. Moreover, to catalyse the same reaction the prokaryotic and eukaryotic FADS strikingly differs in their size. FADS domain of bifunctional enzyme in bacteria is encoded by around 180 amino acid residues which is way too small when compared to its counterpart in yeast and human where it is encoded by 300 and 600 amino acids respectively. Thus, further studies are required to completely understand the FADS domain of bifunctional enzyme.