Chapter 5: Summary
Summary

The surface exposed lipid, Pthiocerol dimycocerosate (PDIM), which is an unusual nonetheless characteristic component of the cell wall of all pathogenic *M. tuberculosis* strains, has been iteratively implied as an important virulence determinant for the tubercle bacilli. Therefore, the PDIM biosynthesis cluster is of special significance to the growth and pathogenesis of *M. tuberculosis*, a point well asserted by the numerous knock-out studies involving the gene cluster members (Camacho et. al., 1999; Cox et. al., 1999). Considering the ever-increasing threat imposed by *M. tuberculosis*, the causative agent of tuberculosis- a threat further compounded by an upsurge in multidrug resistant strains- the study of the route leading up to PDIM synthesis in *M. tuberculosis* is of immediate interest. The phenolphthiocerol/phthiocerol polyketide synthase (ppsA-E), the mycocerocic acid synthase (mas), two acylCoA synthases or acoas (fadD26 and fadD28), and a few other genes have been found to be involved in PDIM synthesis and transport in *M. tuberculosis* (Fernandes and kolattukudy, 1997; Camacho et. al., 2001). The present study is an effort to extend the scope of a model postulated by Cox and co-workers (1999) that illustrates the functioning of the above-said virulence cluster, by staging a case for the involvement of a type II thioesterase called TesA, a hitherto unnoticed member of the virulence cluster. By confirming an interaction between the C terminus of PpsE and TesA protein *in vitro* by using GST “pull-down” assay, and *in vivo* by using a bacterial two-hybrid system, the inclusion of tesA in the workings of this virulence cluster is proposed. This inclusion of tesA in the revised Cox model now concomitantly strengthens the hypothesis of a decarboxylative step at the end of phthiocerol biosynthesis by the PpsA-E PKS. In keeping with these findings, the roles assigned previously for the two acoa enzymes, FadD26 and FadD28 have also been reviewed.

The present study represents the first example of the use of a two-hybrid system for studying the interactions between PKS proteins. The use of this technique may also be helpful in studying the hitherto less understood PKS-PKS interactions that govern the synthesis of medicinally important polyketides. Build-up of knowledge of such interactions may be of particular relevance for designing inhibitors against one or many members of this important virulence cluster. Finally, detailed *in vivo* knock-out studies of tesA and ppsE genes in *M. tuberculosis* now gain more importance in the context of firmly establishing the role of these genes in the pathogenesis of *M. tuberculosis*. 
The work done can be summarized as follows:

- With the amino acid sequence analysis of *M. tuberculosis* TesA within and across the genus, it was concluded that it is a type II thioesterase that might play a role in the processing of the end product of Pps PKS of the PDIM operon.

- To look for the significance of TesA in PDIM operon, protein-protein interaction studies were carried out between the three proteins of this operon namely, TesA, PpsE and FadD26.

- The genes coding for TesA, FadD26 and C-terminus of PpsE proteins were PCR amplified from *M. tuberculosis* BAC library and were successfully cloned in B2H vectors (pBT and pTRG) as well as expression vectors, pET28a(+) and pGEX4T2.

- Using Bacteriomatch™ B2H system an in vivo protein-protein interaction assay was carried out. For the assay Bacteriomatch reporter strain was cotransformed with all the possible combinations of recombinant pBT and pTRG plasmids carrying genes under study.

- The MIC values for carbenicillin for these cotransformants resulted into only one pair of proteins under study namely, TesA and PpsE exhibiting values of 1750 μg/ml that was closer the MIC value of positive control pair which is 3000 μg/ml.

- Subsequent plate and liquid β-galactosidase assay confirmed that cotransformants carrying TesA and PpsE proteins together gave blue color on plates containing X-gal (as is the case of positive control pair proteins) as well as higher β-galactosidase activity (370 Miller units) compared to all negative controls (ranging from 0-45 Miller units) in the presence of ONPG. Quantitative estimation revealed that β-galactosidase activity in case of test pair TesA and PpsE is far less than that of positive control (950 Miller units). Therefore, in vivo B2H suggested that TesA interacts with C-terminus of PpsE, although, the interaction is weaker than the positive control.

- Similarly, from the in vivo B2H assay, it was concluded that FadD26 does not interact with C-terminus of PpsE protein.

- For the purpose of in vitro pull down assay, all the three proteins were expressed in large amounts. The PpsE protein and FadD26 protein were
expressed as N-terminal 6X-His tagged protein and PpsE protein was then purified using Ni-NTA chromatography to 90% homogeneity.

- TesA protein was expressed as a TesA-GST fusion protein and purified to 90% homogeneity using Glutathione sepharose (GS) affinity chromatography.
- GST pull down assay carried out with equal amount of purified proteins TesA-GST and PpsE resulted into elution of both the protein together from the GS resin thus confirming that these two proteins are interacting with each other in \textit{in vitro} as well.
- Based on these results, the inclusion of tesA into the workings of the PDIM biosynthetic gene cluster can be proposed and accordingly a revision of the Cox model (1999) is suggested.
- With tesA as a member, the proposed functions of acoas enzymes as per Cox model are revised:
  - That FadD26, which has recently been reported to be transcriptionally as well as translationally coupled with PpsA, might be acting as a surrogate acyltransferase domain for PpsA protein rather than facilitating the removal of PK moiety from the pps cluster. Thus, FadD26 might be acting like a default loading domain. This explains how fatty acids of specific lengths are taken up by pps cluster.
Application of the Codon-Shuffling Method in Designing De Novo Antibacterials.