INTRODUCTION
Molecular characterization of functionally important proteins of microbes using recombinant DNA technologies and advances in protein structure and function facilitated better understanding on the microbial antigens and pathogenesis. Pathogenic microbes secrete number of proteins which are immunologically and biochemically important. The interactions of these proteins secreted by the pathogen with the host lead to diversified spectrum of clinical conditions.

This interaction of host and pathogen results in releasing various proteins from the pathogen, some of them are considered as potent virulent factors, due to their protease, ribonuclease, DNase, ATPase, elastase and cytotoxic activities; some of these proteins facilitate colonization of the microbe inside the host. Colonization of the microbe triggers a series of immunological responses, which are of both cell mediated and humoral in nature. However, intracellular microbes growing inside the phagocytes induce cell mediated type response, more specifically delayed type of hypersensitivity reactions. In this response cytokines secreted by T-cells are important, which activate macrophages to achieve more effective killing of intracellular pathogens.

Recent technological advances in the studies of genomics and proteomics have provided tools for studying and understanding the biology of both pathogenic and non-pathogenic microbes. In the light of the available genome sequence information of pathogenic organisms it is now possible to get similar information on closely related non-pathogenic microbes and such information will be useful to analyze and identify the
factors related to pathogenicity. Understanding of these biochemically important proteins on related non-pathogenic organisms at molecular level will help in elucidating the functional role of these proteins and their role in the pathogenesis. This information is anticipated to be useful in designing and developing therapeutic reagents or drugs.

**Recombinant DNA technology, application to Molecular characterization of antigens.**

The ability to amplify individual DNA sequences, and to purify, break and rejoin DNA molecules at virtually any desired site, has generally accelerated the progress of research in almost every field of life sciences and has already led to major medical and commercial applications. It is now possible to identify genes coding for biologically active proteins, to analyse them in detail with respect to its structure and function; and to transfer them from one organism to another, so as to obtain highly efficient synthesis of their products. It is also possible to produce gene coding for modified or entirely new proteins, in order to obtain novel products that have reduced side effects and/or enhanced biological activity. The first commercially produced biological product by recombinant DNA technology was insulin. However, now there are several medicinal products derived from genetic engineering such as Hepatitis B Vaccine, interferons, tumor necrosis factor, various cytokines etc., which have extensive applications in the prevention or treatment of very diverse conditions like hepatitis, cancer, diabetes, or myocardial infarction. The first recombinant vaccine developed was against Hepatitis which is caused by Hepatitis B virus (HBV). The generation of recombinant vaccine for Hepatitis was the culmination of the studies carried out on the surface antigens of HBV.
These Hepatitis B surface antigens (HBsAg) occurs in the serum of hepatitis patients in the form of 22 nm aggregates [Moriarty, A.M et al (1981)]. When this gene was brought under the control of the late SV-40 promoter, it was expressed, albeit weakly (2.5μg per 2 x 10^7 cells), and the product aggregated in the form of 22 nm particles. However, Michel, M. L et al (1984) expressed more efficiently under SV-40 early promoter. This particular construction included a 55 amino acid long precursor of HBsAg and led to the formation of HBsAg 22 nm particles which elicited a strong immune response in mice. The generation of hepatitis B vaccine has been summarized by Tiollias, P et al (1985).

The fastest method to clone a gene is amplification of the desired DNA fragment by the polymerase chain reaction (PCR). PCR has been successfully used to clone antigens and proteins, however, strong limitation of PCR technology lies in the requirement of sequence information, at least from the N-terminal sequence of the protein, in order to be able to design primers for the PCR amplification reaction [Mcpherson. M. J. et al (1993)].

A rational approach to clone and identify the genes encoding various antigens from various organisms is by generating genomic or cDNA expression libraries. To identify genes / cDNA from the genomic or cDNA library using different probes which are derived from the native protein. These probes may be antibody probes or oligonucleotide probes[Micerendrof, R.C. et al (1987) and Young et al (1984)]. Several genes from different pathogenic and non-pathogenic organisms have been successfully
cloned and expressed by these approaches for example, cloning and isolation of genes encoding 71 KDa protein of *M. tuberculosis* which had sequence homology with the *M. leprae* and *M. bovis* and MPB70 of *M. bovis* BCG were isolated from the genomic library constructed in λgt 11 using antibodies and oligonucleotide approach respectively [Garsia. R. J et al (1989) and Terasaka, K et al (1989).

Another method of isolating the specified gene is by phage display system where the sequence and its product are in tandem from the same clone and are seen on the surface of the phage. The gene III (gIII) coat protein located at the tip of the filamentous phage of the bacterial F pilus and has been used to display peptide epitopes proteins and immunoglobulin fragments on the surface of phage. Phages expressing fusion proteins of interest were isolated from phage display libraries by selection with a variety of solid phase immobilized ligands [Kang et al (1991), Barbas, C. F et al (1991) and Cwirla, S.E et al (1990)].

*M. tuberculosis*

*M. tuberculosis* is the principal causative agent of tuberculosis. Although, the genome sequence of this pathogen has been deciphered completely, understanding on its virulent factors and the pathogenecity requires detailed study. Pathogens like mycobacteria survive inside the macrophages by down regulating its own enzymes like super oxide dismutases, catalases, peroxidases and other enzymes, and probably utilizing these enzymes of the host.[Bloom, B.R and C. J. L. Murray (1992), Bretscher, P.A (1992). Cooper, A.M and J.L. Flynn (1995), Huebner, R.E. and K.G. Castro (1995) and
Weiss, R (1992) and Zhang et al (1992)]. Although diseases caused by pathogenic species of Mycobacterial genus are very well established, their factors contributing to pathogenicity are not identified and characterized.

Studies on the virulence factors associated with tuberculosis (TB) are made difficult by two factors, the generation time of *M. tuberculosis* is about 18 to 24 h and the second factor is that there are no effective methods for constructing disruptions in a specific chromosomal gene. Recently, introduction of foreign genes into the *M. tuberculosis* has been achieved, and a transposon that integrates randomly enough to be used for transposon mutagenesis has also been found. [Hatfull, G. F (1993), Hatfull. G. F and G. Sarkis (1993) and Jacobs, W.R. Jr et al (1993)].

Non-pathogenic mycobacteria were isolated from the inanimate environment about a decade after Koch's (1882) discovery of the tubercle bacillus. These included *M. smegmatis* and *M. phlei*. Other species were subsequently isolated from environmental sources and very occasionally, from lesions in man but in view of the overwhelming importance of *M. tuberculosis*, little attention was paid to *M. phlei* and *M. smegmatis*. Immune responsiveness to mycobacteria is reported to be affected by exposure to mycobacterial species [Stanford, J. L and G.A.W. Rook (1983)]. In this context researcher's are now concentrating on closely related organisms like *M. smegmatis* and *M. phlei* to understand the basic biology of mycobacteria, mycobacterial proteins and lipids to implicate their role in the pathogenicity.
Antigens of Mycobacteria

The mycobacterial antigens may be broadly classified as (1) cytoplasmic (soluble) or cell wall lipid-bound (insoluble), (2) according to their chemical structure (carbohydrate or protein) or (3) by their distribution within the genus. Antigens have been extensively used to classify, identify and type the mycobacteria. Up to 15 lines of precipitation are demonstrable when ultrasonicates of mycobacteria are tested against homologous antisera by immunoelectrophoresis [Daniel, T. M and B.W. Janicki (1978)] or double diffusion in agar gel [Stanford, J.L and J. M. Grange (1974)] while up to 90 antigens are demonstrable by the more sensitive technique of crossed immunoelectrophoresis [Closs et al (1980)]. Soluble antigens are divisible into 4 major groups [Stanford, J.L and J.M. Grange (1974)]: those common to all mycobacteria (group i); those occurring in slowly growing species (group ii); those occurring in rapidly growing species (group iii) and those unique to each individual species (group iv). This antigenic distribution suggests that the mycobacteria must have evolved from a common ancestral form and that there was an early division of the genus into the slow and rapid growers. Some of the (group iii) antigens are also found in the genus Nocardia. Many of the common (group i) antigens are also found in the nocardiae and some are present in related genera such as Corynebacterium and Listeria. This intergeneric sharing of antigens is probably responsible for the lack of specificity of serological tests for tuberculosis (TB). Immunelectrophoresis analysis of culture filtrates of M. tuberculosis [Daniel, T.M and B.W. Janicki (1978)] revealed 11 arcs of precipitation which were numbered for reference purposes. Antigens 1, 2 and 3 are, respectively, arabinomannans,
arabinogalactans and glucans that are distributed throughout the genus; antigens 6,7 and 8 are widely distributed proteins. A more elaborate reference system based on 30 numbered antigens BCG demonstrable by two-dimensional polyacrylamide gel electrophoresis was proposed by Closs et al (1980). Several of these antigens have been further characterized [Harboe, M et al (1986)] and some have been shown to be enzymes [Harboe, M and H. G. Wiker (1986)]. Until recently, very little was known of the nature and properties of the mycobacterial protein antigens because of their complex nature.

Immunological studies on mycobacteria have largely employed antigens derived from disrupted bacterial cells, but those actively secreted by living cells may be more relevant to protective immunity or immunopathology [Rook, G.A.W et al (1986)]. Secreted antigens may be identified by cultivating mycobacteria for a few days in \(^{35}\text{S}\) methionine, separating antigens in the culture supernatants by high resolution electrophoresis and visualizing them by autoradiography [Abou-Zeid, C et al.,(1986)]. These actively secreted antigens form only a small proportion of those released from old cultures by autolysis. It has been shown that BCG strains are separable into 2 major groups by the secretion of large amounts of a protein of mol.wt 46,000 daltons and that strains of \textit{M. tuberculosis}, except for the South-Indian variant secrete a dimeric protein of mol. wt 23,000 [Abou-Zeid ,C et al (1988)]. Mycobacteria that form smooth suspensions may be identified or typed by agglutination tests. The agglutination of the \textit{M. avium}, \textit{M. intracellularare} group has been studied extensively by Schaefer and his colleagues [Wolinsky, E and W. B. Schaefer (1973)]. Serotypes are identifiable in several other species but not, unfortunately, in \textit{M. tuberculostis} which has rough surface
structure and readily autoagglutinates. The responsible antigens for autoagglutinations have been identified as the sugar moieties on mycosides.

Mycobacteria contain a wide range of lipids, several of which are unique to this genus. Many of the lipids are long-chain fatty acids (general formula $\text{CH}_3-(\text{CH}_2)_n\text{-COOH}$) which are often modified by the presence of unsaturated bonds, cyclopropane rings or methyl side chains. They include the mycolic, mycostearic, phthienoic and tuberculostearic acids. The mycolic acids are long -chain $\alpha$-alkyl, $\beta$-hydroxyl fatty acids, i.e. they have an alkyl chain attached to the $\text{CH}_2$ group adjacent to the terminal carboxylic ($\text{--COOH}$) group. Mycolic acids contain between 60 and 90 carbon atoms. Mycolic and other fatty acids are often covalently linked to sugars, particularly arabinose and trehalose. Thus, mycolic acids forming the main cell-wall palisade are linked to the arabinose residues of arabinoglactomannan-B (LAM-B) is one of the dominant antigens of *M. leprae* and, despite the fact that closely related, serologically cross-reacting analogues are found in other species, it has been used in serodiagnostic tests for leprosy, [Brennan P.J. (1986)]. The lipoarabinomannans also contain inositol and phosphorous which are usually associated with the cell membrane; it has been suggested that these lipids traverse the cell wall linking it to the cytoplasmic membrane. It is likely that they include the 'lipodial' antigens in earlier descriptions and the antigenic phosphatidyl inositol mannosides used in serodiagnostic tests for tuberculosis. The lipid in the lipoarabinomannans is tuberculostearic acid is unique to the genus *Mycobacterium*. Thus, its detection by mass spectroscopy is a sensitive means of detecting mycobacteria in clinical specimens such as cerebrospinal fluid.
The sulpholipids are strongly acidic compounds consisting of mycostearic acid covalently linked to trehalose sulfate. Several different sulpholipids are synthesized by *M. tuberculosis*, the most abundant being sulpholipid I (2,3,6,6'-tetra-acyl α-α'-D-trehalose-2-sulfate) [Goren, M. B (1970)]. It was thought that sulpholipid was a major determinant of virulence of *M. tuberculosis*; indeed, the 'cytochemical neutral red test for virulence strains' is based on the binding of this dye to sulpholipid. [Dubos, R. J and G. Middlebrook (1948)]. It now seems likely that sulpholipids determine virulence because some fully virulent strains of *M. tuberculosis* contain very small amounts of them [Goren, M. B et al (1982)]. They may, nevertheless, contribute to virulence by inhibiting phagosome-lysosome fusion within the macrophages, neutralizing lysosomal enzymes and forming toxic complexes with cord factor.

**Phospholipids of Mycobacteria**

Phospholipids are the most common polar lipids and these molecules are amphipathic being composed of polar head groups and long alkyl chains having usually less than 20 carbon atoms. The studies of Sabin (1932) have revealed that crude phosphatide fractions of mycobacteria when injected intraperitoneally into rabbits elicited typical tuberculosis lesions. Owing to their serological and antigenic activities, phospholipids of mycobacteria are well studied. The mycobacterial phospholipids include diphosphatidyl glycerol i.e cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and phosphatidylinositolmannosides (PIMS). In tubercle
bacilli, they comprise 3-7% of the total dry weight of the cells and 25 percent of the total lipids [Witcoff (1951), Reddy and Venkitasubramanian (1975)]. Virulent strains of mycobacteria contain a higher percentage of phospholipids as compared to the avirulent strains [Long (1958)].

The essential constituent of the Wasserman antigen in the diagnosis of syphilis isolated from beef heart [Pangborn, M.C (1942)] and was characterized as glycerol rich phospholipid "cardiolipin". Its structure is revealed as diphosphatidyl glycerol [Faure, M and M. J. Morelec-Coulon (1956) and Gray, G. M and M. G. MacFarlane (1958)]. Both in pathogenic as well as in saprophytic species of mycobacteria, cardiolipin is often the most abundant (30-50%) of total phospholipids. Cardiolipin, localized mainly in membranes has been shown to have a dynamic role [Prasad, R et al (1975)] and thus account for its high turnover rate observed earlier [Akamatsu, Y et al (1967)]. The unique functional role of cardiolipin due to its structure is very well documented in prokaryote's. CL is known to activate dnaA protein responsible for replication of the DNA and also activates gyrase in the cell [Ohta, I et al (1985), Sekimizu, K and A. Kornberg (1988), Sekimizu, K et al (1988) and Yung, B. Y. M and A. Kornberg (1988)].

Phosphatidyl ethanolamine (PE) is found in virulent, avirulent and saprophytic strains of mycobacteria by Subrahmanyam et al (1962). Later studies have revealed the presence of PE in M. bovis BCG and M. phlei [Akamatsu, Y and S. Nojima (1965)] and in atypical mycobacteria [Motomiya et al (1968) and Sasaki (1970)]. PE comprises 20-36% of the total lipids depending on the age of the culture [Chandramouli and
Venkitasubramanian (1974) had a higher turnover in some mycobacteria [Dhariwal (1978)] and its biosynthesis in *M. smegmatis* ATCC 607 via CDP-ethanolamine pathway is also known [Nandedkar (1975)]. Other phospholipids present in minor amounts are phosphatidyl glycerol (PG), phophatidyl serine (PS), lysophosphatidyl ethanolamine (LPE) [Subrahmanyam (1964)].

The phosphatidyl inositol mannosides (PIMS) are widespread in mycobacteria. Their limited occurrence to certain actinomycetes and related organisms are well reviewed by Lechevalier (1977). The most common PIMS are mono-and diacyl mannosides but pentamannosides and hexamannosides have also been characterized [Nojima, S (1959), Brennan, P.J and C. E. Ballou (1967)]. The phosphatidyl myoinositolides of *M. tuberculosis* have been established by Vilkas and Lederer (1960) as 1,2-diacyl-Sn-glycerol, 3-phosphoryl alpha-1-myoinositol ring, they have been classified as phosphatidyl inositol monomannoside (PIM₁), phosphatidyl inositol diamannoside (PIM₂) [Nojima, S (1959)].

The mycobacterial phospholipids are located in the protoplasmic membrane [Akamatsu, Y et al (1966)] as well as in the cell wall [Motomiya et al (1968)]. Phosphatidyl myoinositol oligomannosides comprise the main cell wall phospholipids while cardiolipin predominates in the protoplasmic membrane [Akamatsu, Y et al 1966; Penumarti and Khuller. G.K (1983) and Rita Dewan (1983)]. Palmitic, hexadecenoic, octadecenoic and tuberculostearic acid are the major fatty acid constituents of the mycobacterial phospholipids [Subrahmanyam (1965) and Pangborn. M. C and Mckinney
Mycobacterial phospholipids play an essential role in enzymatic transalkylation reactions [Akamatsu, Y and Law (1970)]. Antibodies to phospholipids have been demonstrated in patients suffering from tuberculosis as well as infected animals [Takahashi (1962), Subrahmanyam et al (1964)].

Cardiolipin Synthetase

There is a great variation in the composition of phospholipids found in the cytoplasmic membrane of prokaryotic cells and in the inner mitochondrial membrane of eukaryotic cells. The membranes of *E. coli* have simple lipid composition, containing three important classes of phospholipids: PE, (75-85%), PG (10-20%), and CL (5-15%). [Rock, C.O and J. E. Cronan, (1985)]. However, the CL content in mycobacterial genus is between (30-50%) [Mathur, A.K et al (1975)]

The enzymes for phospholipid synthesis are located on the inner plasma membrane of *E. coli* and other prokaryotic organisms, including the genus mycobacteria, except the phosphatidylserine synthase, which is mostly found associated with the ribosomes. CDP-diacylglycerol is a branch point intermediate and has two points, one point for this compound is reaction with serine, catalysed by phosphatidyl serine synthase, to form phosphatidyl serine. In the final reaction of this sequence, phosphatidyl serine is decarboxylated by phosphatidyl serine decarboxylase to yield phophatidyl ethanolamine. In an alternative sequence the CDP-diacylglycerol is converted to phosphatidyl glycerol phosphate. Then the phosphatidyl glicerophosphate is dephosphorylated by phosphatidylglycerol phosphate phosphatase, yielding phosphatidyl...
glycerol. Subsequently, cardiolipin can be made by the reaction of two phosphatidyl glycerol molecules in a reaction catalyzed by cardiolipin synthase.

**Biosynthesis of phospholipids in prokaryote's**

\[
\begin{align*}
\text{Phosphatidic Acid} & \quad \xrightarrow{CTP} \quad \text{CDP-Diacylglycerol} \\
\text{L-Serine} & \quad \xrightarrow{(\text{phosphatidyl serine synthase})} \quad \text{Phosphatidyl serine + CMP} \\
\text{Sn-Glycerol-3-P} & \quad \xrightarrow{(\text{phosphatidylglycerol phosphate synthase})} \quad \text{Phosphatidyglycerophosphate + CMP} \\
\text{Phosphatidyl ethanolamine + CO}_2 & \quad \xrightarrow{(\text{Phosphatidyl serine decarboxylase})} \quad \text{Phosphatidyglycerol + phosphatidyglycerol} \\
\text{CARDIOLIPIN (biphasphatidyl glycerol) + GLYCEROL} & \quad \xrightarrow{(\text{Cardiolipin synthetase})}
\end{align*}
\]

CL, one of the phospholipid of *E.coli*, is unique in its structure among membrane lipids and has been postulated to play specific role(s) in membrane functions. It is synthesized from two molecules of PG by cardiolipin synthetase [Hirschberg, C.B and E.P. Kennedy (1972) and Tunaitis, E and J.E. Cronan, Jr. (1973)] which differs from its eukaryotic counterpart that utilizes CDP-diacylglycerol and PG as substrates. The gene *cls* is responsible for CL formation, and several lines of evidence indicate, though not
definitely, that it is the structural gene [Ohta, A et al (1985)]. The bacterial reaction of CL formation was proposed by Rampini and Coworkers to explain their observation the bacterial cells that can convert phosphatidylglycerol to CL under energy deprivation conditions, which presumably prevent CDP-diacylglycerol formation. Rampini, C et al. (1970). De Siervo and Salton. (1971) provided direct evidence for this reaction by demonstrating that membranes isolated from Micrococcus lysodeikticus convert two molecules of phosphatidyl glycerol to one molecule of CL. Subsequent studies demonstrated that a similar enzyme activity was present in E. coli, Staphylococcus aureus, Mycobacterium smegmatis, Lactobacillus plantarum, Bacillus megaterium, Bacillus firmus OF4 and Pseudomonas putida. Careful examination of growth characteristics in Shibuya's laboratory revealed three important differences between wild type and cls mutants: (i) cls mutants have somewhat longer doubling times, (ii) cls mutants have lower final cell densities and (iii) cls mutants are approximately $10^4$-fold less viable when incubated in the stationary phase for 5 days [Nishijima, S et al (1988) and Hiraoka, S et al (1993)]. Doubling time difference becomes even more pronounced when the pH of the growth medium is increased, suggesting that CL may be required for cell growth under alkaline conditions [Hwang, Y. W et al (1984)]. The role of CL has been well established in prokaryotic plasma membranes. Kornberg and co-workers reported that anionic phospholipids CL and PG play an important role in DNA-replication in E. coli. CL in E. coli regulates ATP binding to dna A protein is essential for its action in initiating the replication at ori C site. ADP bound to that site renders dnaA protein inactive for replication. CL a diacidic membrane phospholipid, displaces the bound nucleotide, and in the presence of components that reconstitute replication,
fully reactivates the inert ADP form of dna A protein. This activation of dnaA protein by PG is is one-tenth as active as CL, whereas, the neutral phospholipids like phosphatidylethanolamine, the principle E coli phospholipid, is totally inactive. Fluphenazine, a tranquilizer drug blocks CL activation of dna A protein. Inoue K and co-workers have showed that phospholipid compositions affect transcriptional expression of certain regulatory genes in E.coli and mutation of this gene makes the cell sensitive to novobiocin [Yung, M.B.Y and A. Kornberg (1988), Sekimizu, K et al (1988), Sekimizu, K and Kornberg, A (1988), Inoue, K et al (1997) and Milija, J et al (1999)].

Understanding on the molecular structure of the cls enzyme is anticipated to give an insight into the function of CL in both pathogenic and non-pathogenic mycobacteria. Mycobacteria has high amount of phospholipids in their cell membranes. Among the phospholipids of mycobacteria, cardiolipin is present in large amounts in their plasma membranes. The high content of cardiolipin (CL) appears to have an important role on the stability of the pathogenic and non-pathogenic mycobacteria [Mathur, A.K. et al (1975)]. The important role played by CL in the growth, regulation of various enzymatic reactions and stability in prokaryotic organisms necessitated to study the role of cls in M. phlei. This study is anticipated to give an insight into the function of cls in non-pathogenic mycobacteria, which may provide scope for comparison of M. tuberculosis with non-pathogenic mycobacteria and the observations may be useful for applications.
Calmodulin like protein

The distribution of calmodulin (CaM), the major intracellular Ca$^{2+}$-binding protein is well documented in eukaryote's. The general functions of CaM include (i) modulation of intracellular levels of Ca$^{2+}$ (ii) regulation of cAMP metabolism and (iii) phosphorylation / dephosphorylation of specific intracellular proteins [Cheung, W.Y., (1980) and Ortega Perez et al., (1983)]. In fungi, CaM associated activities have been noted to be important for morphogenesis, sexual development as well as for germination, growth and enzyme secretion. [Muthukumar, G et al (1985), Paranjpe, V and A, Datta (1990), Lydan, M and D. H. O'Day, (1988) and St.Leger, R.J., D.W. Roberts and R. C. Staples, (1989)].

Calmodulin is a cytoplasmic protein of 148 amino acids modulates most of the regulatory functions of Ca$^{2+}$ ions. The chain has four domains (numbered I through IV), each binding one Ca$^{2+}$ion. Each binding site is a loop containing aspartate and glutamate side chains that form ionic bonds with the Ca$^{2+}$. The oxygen atoms on the side chains of threonine, serine, tyrosine and asparagine also participate in Ca$^{2+}$ binding. On binding Ca$^{2+}$, calmodulin undergoes a major conformational change that allows it to bind to other proteins, modifying their enzymic activity. The Ca$^{2+}$ calmodulin complex, binds to activate cAMP phosphodiesterase. Binding of Ca$^{2+}$ to calmodulin is cooperative, a small change in the level of cytosolic Ca$^{2+}$ causes large change in the level of "active" calmodulin [Cheung, W. Y. (1982) and Babu, Y. S et al (1985)].
Several authors have reported presence of CALPs in prokaryotes. The analogy drawn between CaM and these prokaryotic proteins, however, has relied on physical properties characteristic of CaM, such as amino acid sequence, motifs of Ca$^{2+}$ binding, and binding to anti calmodulin antibodies. Prokaryotic organisms like *Bacillus subtilis*, *Bacillus cereus*, *E.coli*, *Myxococcus xanthus* and *Streptomyces erythreus*, are reported to have calmodulin like protein. These molecules stimulated bovine brain phosphodiesterase and NAD kinase in a Ca$^{2+}$ dose dependent manner [Inouye, S et al (1983), Iwasa, Y., et al(1981), Fry, L. L et al (1991), Leadlay, F.P., (1984), Swan, D.G., et al (1987). Swan, D.G., et al (1989) and Shyu, Y and Foegeding, P.M (1989)].

The presence of CALP in five species of the genus *Mycobacterium*, namely *Mycobacterium smegmatis*, *M. phlei*, *M. bovis* BCG, *M. tuberculosis* H$_37$Ra and *M. tuberculosis* H$_37$Rv [Falah, A.M.S et al (1988) and Sarma, P.V.G.K et al (1998)] was examined. Positive co-relations were observed between the levels of CALP and total phospholipids and growth in glucose [Ratnakar, P and P.S. Murthy (1992 and 1993)]. It has been shown that trifluoperazine (TFP), the calmodulin antagonist, inhibits the growth of strains of pathogenic tubercle bacilli *M. tuberculosis* H$_37$Rv susceptible and resistant to isoniazid and streptomycin. It was recently found that TFP inhibits the growth of *M.avium* as well. TFP was shown to inhibit the incorporation of $^{14}$C-acetate into lipids, $^{32}$Pj into phospholipids [Hemalata Reddy, P et al (1991) and Sastry, S. B et al (1991)].

Presence of large amounts of CL in the plasma membrane of *mycobacteria* and possible regulation by CALP, necessitated studies on the molecular structure of the
CALP present in the cytoplasm of *Mycobacterium phlei*. This study is anticipated to give an insight into the function of CALP in non-pathogenic *mycobacteria* and further may help in understanding the differences in the biology of pathogenic and non-pathogenic *mycobacteria* thus, may help in understanding the mechanism of pathogenesis of *mycobacteria*.

**Catalase**

Catalase is a heme containing enzyme widely distributed in nature. This enzyme particularly plays an important role in the survival of strict and facultative aerobes. Catalase has a double function, (1) decomposition of H$_2$O$_2$ to give H$_2$O and O$_2$ (catalase activity; equation 1) (2) oxidation of H donors for example, methanol, ethanol, formic acid, phenols, with the consumption of 1 mole of peroxide (peroxidase activity; equation 2).  

\[ \text{1) } 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

\[ \text{2) } \text{ROOH} + \text{AH}_2 \rightarrow \text{H}_2\text{O} + \text{ROH} + \text{A}. \]

The physiological function of catalase is still obscure, although various theories have been put forward. Catalase located in the cell organelles plays the role of a specific peroxidase. The enzyme pattern of the peroxisomes is noteworthy for the simultaneous presence of H$_2$O$_2$ - producing (e.g., D-amino acid oxidase, uricase) and consuming enzymes (catalase). In erythrocytes catalase-like glutathione peroxidase exerts a protective function for haemoglobin and other SH-proteins (enzymes, stroma), the importance of which can vary with the species and the experimental conditions.
For *M. tuberculosis* and *M. bovis*, 22 enzyme activities were detected in the culture filtrates and/or on the surfaces, of which eight were absent from the culture fluids of non-pathogens: alanine dehydrogenase, glutamine synthetase, nicotinamidase, isonicotinamidase, superoxide dismutase, catalase, peroxidase, and alcohol dehydrogenase. [Table-I]. These activities, which correspond to secreted enzymes, formed a significant part (up to 92%) of the total enzyme activities of the bacteria and were absent from the culture fluids and the cell surfaces of the non-pathogenic species *M. smegmatis* and *M. phlei*. The extracellular location of superoxide dismutase and glutamine synthetase seemed to be restricted to the obligate pathogens examined. The difference in the enzyme profiles was not attributable to the growth rates of the two groups of bacteria. The presence of the eight enzyme activities in the outermost compartments of obligate pathogens and their absence in those of non-pathogens provide further evidence that these enzymes may be involved in the pathogenicity of mycobacteria [Raynaud, L et al (1998)] [Table-I].

**TABLE-I**

Localization of the ten enzyme activities present in the culture of *M. tuberculosis* and absent from that of *M. smegmatis* and in different mycobacterial species.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>M. smegmatis</th>
<th>M. phlei</th>
<th>M. fortuitum</th>
<th>M. kansasii</th>
<th>M. tuberculosis</th>
<th>M. bovis</th>
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<tr>
<td></td>
<td>H37Rv</td>
<td>H37Ra</td>
<td>Canetti</td>
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<td>Alanine aminopeptidase</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>E</td>
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<td>E</td>
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<td>Cystine aminopeptidase</td>
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<td>S</td>
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<td>Alanine dehydrogenase</td>
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<td>I</td>
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<td>E</td>
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<tr>
<td>Glutamin synthetase</td>
<td>I</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>E</td>
<td>E</td>
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<tr>
<td>Nicotinamidase</td>
<td>I</td>
<td>I</td>
<td>S</td>
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<tr>
<td>Isonicotinamidase</td>
<td>I</td>
<td>I</td>
<td>E</td>
<td>E</td>
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<td>E</td>
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<tr>
<td>Superoxide dismutase</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>E</td>
<td>E</td>
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</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>Catalase</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>E</td>
<td>E</td>
</tr>
</tbody>
</table>

E: extracellular; I: intracellular; S: surface location.

19
The therapeutic drugs for tuberculosis include a set of antibiotics that are effective primarily against mycobacteria: isoniazid, ethionamide, ethambutol and pyrazinamide. Isoniazid and ethionamide act by inhibiting synthesis of mycolic acids, a major component of the mycobacterial cell wall. Ethambutol may interfere with carbohydrate metabolism. Resistance to isoniazid, ethionamide, and rifampin occurs at a relatively high frequency when these drugs are used singly. Isoniazid-rifampin has been the most effective two-drug combination and the one most commonly used [Abigail A. Salyers and Dixie D. Whitt (1994)].

The drug isonicotinic acid hydrazide (Isoniazid, INH) contributes to the core of the antitubercular regime. It has been demonstrated that the catalase -peroxidase of M. tuberculosis, encoded by the Kat G gene mediates susceptibility to INH [Zhang, Y et al (1992)]. It is currently postulated that INH is a prodrug which is active after transformation by the peroxidase into radicals that can either react with vital targets in mycobacterial cells, or ultimately produce isonicotinic acid (INH), explaining INH resistance of Kat G defective mutants. The only defined target of INH is the biosynthesis of mycolic acids [Quemard, A., et al (1991)], which are specific and major compounds of all mycobacterial cell walls. A cell free system of mycolic acid synthesis is inhibited by the drug, and an enoyl-[acyl-carrier protein] reductase which may participate in mycolic acid synthesis was identified and characterized. The enzyme was found to bind to INH only after oxidation of the drug in the presence of Kat G [Quemard, A., et al 1995 and 1996]. It has recently been shown that INH reacts with enoyl-[acyl-carrier protein] reductase, and is covalently bound to the nicotinamide ring of NADH in the active site of
the enzyme [Rozwarski, D.A., et al (1998)]. The Kat G gene alone can restore INH susceptibility to resistant mutants of *M. smegmatis* and *M. tuberculosis*, it has been shown that its expression in *E.coli* can render certain strains of *Escherichia coli* sensitive to the drug. This phenotype is intriguing, as *E. coli* also produces a catalase-peroxidase, which is commonly referred to as hydroperoxidases I (HP I), yet this bacterium does not normally display INH susceptibility. Furthermore, the Kat G gene has been deleted from some of the highly drug resistant clinical isolates of *M. tuberculosis* [Morris, L et al (1995) and Li, Z et al (1998)].

Loss of catalase activity has been consistently observed in *Mycobacterium tuberculosis* upon acquisition of resistance to isoniazid (INH) [Zhang, Y et al (1992)]. It has been demonstrated in *M. smegmatis* oxidative activation of isoniazid and its conversion into a bacteriocidal agent is initiated or mediated by Mn (III) generated by catalase-peroxidase *in vivo* [Magliozzo, S.R and J.A. Marcinkevieniene (1997)]. However, the precise role of catalase in the mechanism of action of the drug remains obscure. It is of interest that atypical and saprophytic mycobacteria may generally be considered clinically resistant to INH because they require higher minimal inhibitory concentrations than does *M. tuberculosis* [Youatt, J (1969)]. As a result, the antimicrobial therapy of mycobacterial infections poses a serious problem. Most of the saprophytic and atypical mycobacteria show only a partial loss of catalase activity upon acquiring resistance to INH [Youatt, J (1969) and Winder, F(1964)]. *M. tuberculosis* possesses a single heat-labile catalase also containing peroxidase activity, as demonstrated by polyacrylamide gel electrophoresis and association of both activities during purification.
[Devi, B.G., et al (1975) and Diaz, G.A and L. G. Wayne (1974)]. Atypical and saprophytic mycobacteria possess two catalases, one of which is heat labile and lost upon acquisition of resistance to INH [Hawkins, J.E and W. Steenken (1993)]. Thus, studying the catalase gene in *Mycobacterium phlei* will help in understanding the differences in molecular nature of this gene in pathogenic and non-pathogenic mycobacteria and possible mechanism of drug in mycobacteria.

**Aspergillus allergens / epitopes**

The diseases caused by fungi are generally the result of immunological responses which are primarily of type I and type III hypersensitivity reactions. These reactions occur due to various proteins secreted by the pathogens and which have wide range of functions such as proteases, ribonucleases, elastases etc. [Jaton-Ogay, K et al (1992), Moser, M et al (1994), Reichard, U et al (1995), Moser, M et al (1993), Borga, A (1990), and Piechura, J. E et al (1983)].

**Diagnostic antigens of Aspergillus**

The different diseases related to an important opportunistic fungus *A. fumigatus* which can be broadly classified according to their clinical presentations into systemic mycoses, saprophytic colonization and allergic diseases. These are as follows: Systemic invasive aspergillosis, Saprophytic bronchopulmonary colonization, aspergilloma, allergic rhinitis and allergic sinusitis, hypersensitivity pneumonitis, allergic asthma and allergic bronchopulmonary aspergillosis [Bodey, G.P., et al (1989), Bardana, E.J., (1980) and Bardana, E. J., (1980)]. Also, there are a number of reports indicating co-existence.
of aspergillosis and tuberculosis, and tubercular cavities being occupied by Aspergillus species in the aspergilloma patients [Singh, P et al (1989)].


**Cloning Strategies**

A wide variety of cloning techniques have been successfully used to isolate complementary DNAs (cDNA) encoding allergens and during the past few years. An
impressive number of primary structures of allergens have been elucidated. Starting from cDNA, the fastest method to clone a gene is amplification of the desired DNA fragment by the polymerase chain reaction (PCR). PCR has been successfully used to clone allergens and allergen isoforms [Moser, M et al (1992), Scheiner, O and Kraft, D (1995), Valenta, R and Kraft, D (1995), King, T.P et al (1995) and Crameri, R, et al (1996)]. However, a strong limitation of the PCR technology lies in the requirement of sequence information, at least from the N-terminal sequence determination is not expected to be easily achievable for gene products of rare mRNA species expressed at a low level. The most frequently used strategy to clone allergens consists of the construction of cDNA expression libraries which can be screened with IgE-containing sera from allergic patients or with antibodies raised against purified antigens [McPherson, M. J et al (1993), Larsen, J.N et al (1992), Breitender, H et al (1993), Micrendroff, R.C., et al (1993) and Helfman, D.M., et al (1983)]. Another approach has been adopted to clone allergen *A. fumigatus* cDNAs is by phage display technique [Kang, A.S et al (1991), Barbas, C. F., et al (1991), Wirla, S.E., et al (1990) and Bass, S et al (1990)]. The amino acid sequences deduced from the nucleotide sequences of inserts generated by a phage displaying IgE binding proteins from *A. fumigatus* can be subdivided into two classes: those with homology to described proteins and others without any homology to sequences deposited in the gene banks.

**Cloning Strategies of Asp f1 of Aspergillus fumigatus**

An insert of 690bp codes for Asp f1, one of the major allergens of *A. fumigatus*. The allergen first described as an 18-kD allergen has been cloned, sequenced and
produced as a recombinant protein in *E.coli*. Asp fl is identical to restrictocin cloned from *Aspergillus restrictus* > 99% identical to the Asp fl sequences derived from different clinical isolates of *A. fumigatus* [Moser, M et al (1992), Aruda, K.L., et al (1992) and Aruda, K.L et al (1990)]. All these proteins are related to ribotoxins of the mitogillin family [Lamy, B and Davies, J (1991), Moser, M et al (1993), Lamy, B et al (1991) and Fando, J et al (1985)] produced by members of the genus *Aspergillus*. Ribotoxins inhibit translation by cleaving a conserved region of the 28S rRNA and are potent inhibitors of eukaryotic protein synthesis [Moser, M., et al (1992)]. The gene Asp fl has been cloned, sequenced and expressed in *E.coli* by different groups. The rAsp fl was found to be enzymatically active resulting in the cleavage of 28S rRNA within a universally conserved region. rAsp fl/a was cytotoxic to EBV immortalized or PHA stimulated human PBMC and RAW macrophage cell lines [Moser, M et al., (1992)]. Furthermore, rAsp fl/a was recognized by murine monoclonal antibodies (MoAb) made against an 18 kDa ribotoxin. IgE of individuals allergic to *A. fumigatus* bound to rAsp fl/a as shown by ELISA, dot blots and western blots elicited positive immediate type I skin reactions in individuals allergic to *A. fumigatus* but not in healthy control individuals [Moser, M et al (1993)].

T cell reactivity to *Aspergillus fumigatus* (Af) antigens, T cell clones (TCC) specific to the Asp fl antigen, an 18kDa protein of Af, were established from the peripheral blood of three ABPA patients. The majority of TCC isolated from ABPA patients, and specific to the Asp fl antigen, an 18 kDa protein of Af, were established from the peripheral blood of three ABPA patients, and specific for the Asp fl allergen of 25
Af, are IL-4 producing CD4+ cells of the Th2 phenotype. Further, analysis in this study revealed that the majority of TCC reacted to mainly two epitopes of Asp fI while remaining TCC reacted to three additional "minor" epitopes [Chauhan, B et al 1996]. The IgE binding epitopes or the T-cell stimulatory peptides of this molecule have not been studied. Kurup et al 1996 have synthesized linear decapeptides spanning the whole molecule of Asp fI and analyzed their IgE binding properties. They have also synthesized peptides based on their possible T-cell stimulatory properties and studied demonstrated distinct IgE antibody binding response against sera from ABPA patients and proliferative response against peripheral blood mononuclear cells from the patients. From the results, they have obtained it can be concluded that the carboxy-terminal region of Asp fI representing amino acid residues 115-149 involved in both humoral and cell mediated immunoresponses in ABPA, also the major IgE binding epitopes of Asp fI are at the C-terminal end of the protein [Kurup et al (1998)]. Specific antibodies raised in the host to these immunodominant proteins have diagnostic significance and may also play a role in pathogenesis. Identification of functional domains of epitopic sequences would offer a better understanding on the mechanism of pathogenesis of aspergillosis and also for the development of diagnostic technologies based on synthetic epitopes.

In the light of possible application value of the epitopes of Asp fI, expressions of such epitopes by cDNA methods may offer better method of preparations of diagnostic epitopes.

In the current work, recombinant DNA methods are exploited for molecular characterization of few immunologically and biologically functional proteins of
Mycobacterium phlei and an important epitope of a major allergen of Aspergillus fumigatus

OBJECTIVES

- Isolation, purification and characterization of cardiolipin synthetase and calmodulin like protein from Mycobacterium phlei.
- Cloning, expression and characterization of the Calmodulin like protein, cardiolipin synthetase and catalase encoding genes from Mycobacterium phlei.
- Cloning, expression and characterization of an immunodominant epitope of Asp f1 of A. fumigatus.

Chapter I deals with the isolation, purification and characterization of calmodulin like protein (CALP) in M. phlei and also cloning, sequencing, expression and characterization of CALP encoding gene.

Chapter II deals with the isolation, purification and characterization of cardiolipin synthetase (cls) in M. phlei and also cloning, expression and characterization of cls encoding gene.

Chapter III deals with the cloning, expression and characterization of Kat G gene of M. phlei.

Chapter IV deals with the cloning, expression and characterization of an immunodominant epitope of Asp f1.