After establishing the function of hemoglobin (Hb) in transportation of oxygen, scientists have so closely prejudiced with role of hemoglobins , that they have been surprised to discover hemoglobin in organisms that have no apparent need for oxygen transport, as in case of single celled organisms. Presence of hemoproteins in microorganisms (yeast, fungi, bacteria) and higher plants (Wittenberg *et al.*, 2002) is not new (Keilin discovered hemoglobin in yeasts and other fungi in the early 1950s), but their exploration has been slow to gain a momentum, in part because of the apparent lack of function. The discovery of new members of the Hb superfamily (trHbs) in diverse and ancient life forms continues, to revitalize the investigations of Hb functions ahead of the classical function (O_2 transport and storage).

Incredibly wide distribution of globin genes among prokaryotes has stimulated comprehensive studies aimed, in understanding the functional implications of unicellular hemoglobins on the physiology of its host and also recognizing the evolutionary relationships that link these proteins to their homologs in higher organisms. Among hemoglobin family, flavohemoglobins, (one of the members) in particular, have received a fair degree of attention because of their exclusive occurrence in a large number of different microbial species (bacteria and lower eukaryotes). FlavoHbs represent an exclusive paradigm of multidomain protein where two domains having different functional properties, interact together and perform entirely new function(s) by carrying out diverse redox reactions. These chimeric proteins (flavoHb) are the oxygen-binding proteins made up of a typical globin domain (having a b-type heme), which is fused with a reductase domain. The N-terminal globin domain of flavohemoglobin has a characteristic 3-over-3 globin fold and its C-terminal domain has the ferredoxin reductase-like FAD- and NAD(P)H-binding motif where FAD and NADH, cofactors can bind and actively participate in electron transfer reactions. (Vasudevan *et al.*, 1991; Zhu and Riggs, 1992). Physiological functions of flavoHbs have been pondering over the last decade and several functions have been anticipated. However experimental evidences support its key role in protection from the deleterious effects of NO by detoxifying it to nitrate via NO-dioxygenation reaction (Gardner *et al.*, 1998; Membrillo *et al.*, 1999; Frey *et al.*, 2002; Gilberthorpe and Poole, 2008). FlavoHbs have also been found to modulate the oxidative stress response in several cases by conferring

sensitivity or resistance against the stress (Zhou *et al.*, 2009). Diverse function of flavoHbs in cellular metabolism is also supported by the fact that more than one flavoHb are present in some bacteria, yeast and fungi (Illima *et. al.*, 2000; Frey *et. al.*, 2002; Zhou *et al.*, 2009) that may carry out different cellular functions. The advantageous role of flavohemoglobins in protection from nitrosative stress and oxidative stress under aerobic and anaerobic condition has been demonstrated for several microorganism (Hausladen *et al.*, 2001; Gardner., 2002; Frey and kallio 2003). Additionally, the significance of flavoHbs for parasitic life has already been established in case of *Cryptococcus* (Dejesus-Berrios *et al.*, 2003; Idnurm *et al* 2004), *Erwinia* (Favey *et al.*, 1995) and *Salmonella* (Crawford and Goldberg *et al.*, 1998a; Stevanin *et al.*, 2002). Roles of flavoHb in providing protection from nitrosative stress are of great interest for clinical microbiology. Pathogenic bacterium *S. typhimurium* acquires the benefit of this resistance machinery upon infection of the potential host. One such highly successful human pathogen, *M. tuberculosis,* seems not to be encoding flavohemoglobin like gene. This bacterium is an obligate aerobe that requires oxygen for its growth, but paradoxically has got remarkable ability to survive under hypoxic condition (Schnappinger *et al* 2003).

The adaptability of tubercle bacillus within the intracellular environment, where high level of toxic reactive species and scarcity of oxygen limit the survival of a pathogen, depends primarily on its specialized multi-tier defence system (Schnappinger *et al* 2003) and metabolic flexibility to balance the oxidative/reductive reactions for the energy generation. As described earlier flavohemoglobins constitute an integral part of virulence and stress response in several pathogenic microbes due to their ability to maintain the cell redox homeostasis at the aerobic/anaerobic interface by scavenging nitric oxide (NO) with high efficiency (Frey *et al* 2002) and in *M. tuberculosis* similar function is carried out by a single-domain truncated hemoglobin, HbN, that displays a potent nitric oxide dioxygenase (NOD) activity and detoxifies NO 15-fold faster than myoglobin (Pathania *et al* 2002b), similar to flavoHbs. Therefore, it has been imagined that *M. tuberculosis* may not need a flavoHb-like protein. In 1999 a report came on flavohemoglobin of *M. tuberculosis*, indicating the induction of flavohemoglobin like protein in nitrosative stress but later on, careful investigation of this gene, turned out to be a reductase lacking a globin domain, thus opening new debate on the presence of flavohemoglobin in

mycobacteria. Recently, we acknowledged that the Rv0385 gene of *M. tuberculosis* (Gupta *et al* 2011) may encode a novel flavoHb like protein (MtbFHb). Its similarity with conventional flavoHbs, however, appeared less than 25% as compared to 40-47% that exists among various bacterial, yeast and fungal flavoHbs (Frey *et al* 2002). Mining of microbial genome data indicated that the occurrence of this unusual flavoHb may be restricted to only few microbes, mainly belonging to actinomycetes. Interestingly, homologs of MtbFHb were detected in majority of mycobacteria including both virulent and avirulent species. Bioinformatic analysis of mycobacterial genome indicated its co-existence along with a conventional flavoHb in some fast growing mycobacterial species like *M. smegmatis, M. gilvum, M. vanvallani, and mycobacterial species mcs,kms, jls.* All these species are avirulent (The correlation between number of flavohemoglobins present in mycobacteria and virulence is not known at present).The diversity in the number and type of flavoHb-encoding genes in the genome of mycobacteria might reflect the difference in requirement of these proteins in individual species. Thus, it may be possible that this protein may be playing different function (s) in cellular metabolism of its host. Since there is no report on flavohemoglobins of mycobacteria and primary sequence analysis of flavoHb identified in *M. tuberculosis* indicates crucial differences from conventional flavohemoglobins. Therefore, we undertook studies on this new class of flavohemoglobin with an aim to understand its functional relevance in the biology and pathogenecity of Mtb by comparing it with flavoHb of non pathogenic species *M. smegmatis.*

Amino acid sequence alignment and comparison of mycobacterial flavoHbs indicated that a novel class of flavoHb, exhibiting unconventional heme and reductase domains, is present in mycobacteria (type II flavohemoglobin) apart from conventional flavoHb (type I) which is present only in limited number of mycobacterial species. Occurrence of two distinct classes of flavoHbs in mycobacteria was interesting as well as intriguing. Sequence comparison of mycobacterial type I and type II flavoHbs with other known bacterial flavoHbs indicated that, the structural features required for adopting a three over three globin fold and signature sequences of typical microbial globins, e. g., B10-Tyr, CD1-Phe, E7-Gln, F8-His etc., (Bonamaro and Boffi 2008) are present in type II flavoHbs of mycobacteria, but there are several decisive differences within the functionally

conserved regions of their heme and reductase domains. The most notable differences within the globin domain of type II flavoHb is the lack of conserved hydrogen bonding and disruption of tetra-interactions between HisF8-GluH23-TyrG5 and contact between G5Tyr and H12Tyr within the proximal site due to mutation at Glu23 and H12Tyr residues. These observations indicated that the peroxidase like catalytic site, present in conventional flavoHbs (Mukai *et al* 2001), is absent in this class of flavoHbs. The reductase domain of type II flavoHbs of mycobacteria is also modified within the cofactor binding sites, e.g., the RXYS motif of the FAD binding and GXGXXP motif of the NADH binding sites (Dym *et al* 2001; kimura *et al* 2001). A RKY/ RW sequence motif, known as high affinity lipid binding motif (Hunte 2005), appeared to be conserved within the proximal site of heme in type II flavoHbs. Interestingly, mycobacterial type II flavoHbs exhibited very high (>70%) overall sequence conservation among them but displayed less than 25 % homology with conventional (type I) flavoHbs. These unusual structural features indicated that type II flavoHbs of mycobacteria may be structurally and functionally different from conventional flavoHbs. To gain an insight into evolutionary co-relation between type I and type II flavoHbs of mycobacteria, phylogenetic analysis of two classes of flavoHbs was done. BLAST search within the microbial genome and protein data bank, using *E. coli* HMP and MtbFHb, retrieved FMN reductase of *E. coli* and Cytochrome b5 reductase of *S. cerevisiae* as orthologs of MtbFHb (type II flavoHbs), whereas Benzoate 1,2, dioxygenase appeared one of the closest orthologs of type I flavoHbs of mycobacteria. Therefore, a phylogenetic tree was developed by focusing on type I, type II flavoHbs of mycobacteria and their first orthologs present in different groups Phylogenetically, type II flavoHbs appeared related to electron-transfer proteins like FMN-reductase of *E. coli* and cytochrome b5 reductase of *S. cervicea* whereas, type I flavoHbs of mycobacteria and other conventional flavoHbs exhibited phylogenetic closeness with dioxygenases. Overall structure of phylogenetic tree and duplication nodes suggest an ancestral duplication and diversion of type I and type II mycobacterial flavoHbs. In order to gain an insight about expression of flavohemoglobin in native host, we did RT PCR under native and NO exposed condition.

The RT-PCR analysis of *M. tuberculosis* and *M .smegmatis fhb* (type II) showed that it expresses during the early stationary phase of mycobacterial growth

and it was not induced on exposure to nitrosative stress . On the other hand type I *fhb* present in *M. smegmatis* was induced under nitrosative stress condition. To characterize the mycobacterial flavohemoglobins in detail, one flavohemoglobin (type II) from *M. tuberculosis* and two (type I and type II) from *M. smegmatis* was cloned and overexpressed in hetrologus host *E. coli.* type II flavohemoglobin from *M. tuberculosis* and *M. smegmatis* appeared to be associated with membrane, whereas type I flavoHb from *M. smegmatis* was in soluble form. Type II flavohemoglobins was purified to near homogeneity by using chaotropic agent sarcosyl, while Type I flavohemoglobin was purified using Ni-NTA affinity cromatography. Gel filtration analysis of all three flavoHb revealed the monomeric nature of the protein. The absolute spectral analysis of purified FHbs (type II) from *M. tuberculosis and M. smegmatis* showed characteristic Soret and α, β peaks similar to oxyhemoglobin , which reacts with CO. On addition of sodium dithionite to type II flavohemoglobins, the and β peaks become more prominent, this behaviour of absorption spectra suggests the cytocrome like nature of pigment. To get further insight, Raman resonance spectral analysis of *M. tuberculosis* flavohemoglobin was done in low and high frequency region. The Raman spectra observed, indicate the characteristics hexacordinated low spin form of heme. This result was consistent with the absorption spectral data which also suggests the hexacordinated nature of the heme in *M. tuberculosis* FHb. Further, the Fe-His stretching mode (vFe-His) of the ferrous protein is identified at 220 cm-1 in *M. tuberculosis* FHb which is lower than that of HMP (Lu *et al* 2007,Mukai *et al* 2001). Hexacordinated nature of heme and lower reductive potential (required for electron transfer) may influence its native function. To unravel the nature of the 6^{th} ligand, a truncated version of MtbFHb, with only the heme domain ($MtbFHb_{HD}$), was over expressed.

In contrast to the full length protein (FHb), MtbFHb heme domain appeared mainly in the cytoplasmic fraction and to characterize the heme domain, protein was purified by Ni-NTA affinity chromatography. The spectral analysis (Absorption and Raman) of globin domain signify that the ferrous species has a penta coordinate high spin (5CHS) configuration, while the ferric species has a mixed hexa coordinated high spin and low spin configuration. These spectral features of globin domain are comparable to typical globins (conventional hemoglobin) (Egawa and Yeh 2005), indicating that the structure of heme domain is regulated by the reductase domain in

MtbFHb and that the binding of the 6th ligand in the active site is stabilized by interdomain interactions. In addition, the inverse correlation diagram indicate the presence of a single conformation (closed) of globin and FHb instead of the dual conformations (open and closed) found in typical flavoHbs (Mukai *et. al.*, 2001; Lu *et. al.*, 2007). These results suggest a restricted conformational freedom of the protein. Since MtbFHb have an unusual hexacoordinated nature of heme, which has been reported for the lipid-bound *Vitreoscilla* hemoglobin (Rinaldi *et al.*, 2006), we further determined whether the purified protein(MtbFHb) is associated with any lipid moiety.TLC analysis of *M. tuberculosis* FHb lipid extract (obtained from chemical hydrolysis) shows, lipid components associated with the protein. We could not remove theses lipids from *M. tuberculosis* FHb by Hydroxylapatite column and it was only obtained after extraction by an organic solvent. To characterize the type of lipid, Gas Chromatography (GC) profile of isolated lipid was generated. Gas Chromatography (GC) profile of fatty acid methyl ester analysis confirmed the presence of fatty acids in the lipid component extracted from MtbFHb identified as androst-4-ene-3, 17-dione, spiro(5,7,8-trimethoxynapthaline) and methyl ester of palmitic acid. The fatty acids apparently bind to the heme active site of the protein, may be accounting for the unusual 6CLS configuration as revealed by the spectroscopic studies. This scenario is unswering with the observation that *M. tuberculosis* FHb heme domain lost its membrane-association property, in the absence of the reductase domain, and it exhibited a typical water-bound configuration (in the ferric form) or a penta-coordinated high spin (5CHS) configuration in the ferrous form, instead of the unusual 6CLS configuration. Taken together the ensemble of experimental observations, provide an unusual picture on the mycobacterial flavohemoglobin (Type II). To understand the implications of these unusual features of Mtb FHb on its physiological properties or its function, we further focused our attention on functional analysis of *M. tuberculosis* flavohemoglobin(type II) and compared it with non pathogenic mycobacteria , *M. smegmatis,* which encodes both type I (conventional) and type II flavohemoglobin.

To elucidate whether expression of flavohemoglobins had any metabolic effect on its host, all three FHbs (One from *M. tuberculosis* and two from *M. smegmatis*) were cloned and over expressed in *E. coli* and *M. smegmatis.* Interestingly, upon over-expression of type II FHbs from *M. tuberculosis* and

M. smegmatis , marked enhancement in the growth of *E. coli and M. smegmatis* was seen under both aerobic and microaerophillic condition. Similar results were seen in *M. smegmatis* (type I) FHb. The observed beneficial effects of FHbs over expression on the metabolism might be due to increased respiratory activity, *i.e.* higher oxygen uptake, higher specific activities of terminal oxidases and, therefore, an overall improved cellular energetic charge. The over expression of type II FHbs form *M. tuberculosis and M. smegmatis* failed to protect the *E. coli* and *M. smegmatis* cells against reactive nitrogen species (nitrosative stress) in both aerobic and micro aerophillic conditions . Contrary to this type I Fhb from *M. smegmatis,* protected the *E. coli* and *M. smegmatis* cells from acidified nitrite. Type II FHbs of mycobacteria displayed lower NO dioxygenase activity, it was >20 fold lower than that of *E. coli* HMP , which makes a NO detoxification function for mycobacterial flavohemoglobin (type II) less plausible. While NO dioxygenase activity of type I FHb from *M. smegmatis* was comparable to *M. tuberculosis* HbN. It has been observed that, not all flavohemoglobins function as nitric oxide dioxygenases (NODs) (Ullman *et al.*, 2004). They may also be involved in modulating the oxidative stress. Therefore, we decided to investigate the role of mycobacterial flavohemoglobins in oxidative stress condition.

Studies on mycobacterial FHbs under oxidative stress condition (cells exposed to hydrogen peroxide) revealed that (Type II) FHbs have a profound protective effect on the growth and survival of *E. coli* and *M.smegmatis* which was unusual because no flavoHb till date has been reported to be protective solely against oxidative stress. On the contrary, upon exposure to hydrogen peroxide, over expression of the type I FHb in *E. coli* and *M. smegmatis* conferred more sensitivity towards oxidative stress .This result was expected as, similar effect have been found in majority of cases, where flavohemoglobins have been over expressed.

From above observations, it becomes apparent that type I flavohemoglobin of *M. smegmatis* have similar functionalities (under both, nitrosative and oxidative stress) when compared to classical flavohemoglobin, however type II flavohemoglobins of *M. tuberculosis* and *M. smegmatis,* which represent the new class among flavohemoglobin family, perform antagonistic function under oxidative stress conditions. Therefore, next we sought the mechanism, how over expression of type II flavohemoglobin is involved in protection from oxidative stress. To elucidate

the mechanism, we checked, the level of lipid hydro peroxide in the cell under oxidative stress condition along with control and the possibility whether any peroxidase like activity is present in type II *M. tuberculosis* FHbs. Results obtained, indicated that there was no apparent difference in peroxidase activity of cells present in Mtb FHb expressing cells as compared to the control.This finding was consistent with our *in silico* protein sequence analysis of mycobacterial flavohemoglobin (type II) which states the loss of catalytic triad which resemble to peroxidase like catalytic site, hence responsible for peroxidase activity. However the level of lipid hydro peroxide in flavohemoglobin over expressing cells were nearly 1.5 times lower than the control cells , when exposed to H_2O_2, suggesting that the lower level of lipid hydroperoxide in Mtb FHb expressing cells may be the plausible cause of antioxidant effect.

Further, we analysed our next hypothesis, stating that under oxidative stress condition enhanced level of membrane lipid peroxidation, generate accumulation of D-lactate as the byproduct that may interact with Fe^{3+}, and result in generation of OH^- causing enhancement in the level of oxidative stress. MtbFHb may relieve this over stress probably by metabolizing the D-lactate (conversion of D- lactate into pyruvate) and diverting it towards energy generation as part of the respiratory chain. The D-lactate metabolizing activity of MtbFHb may occur due to specific binding of D-lactate and its oxidation by FAD (FAD dependent D-LDH), for which we have identified motif similar to D-LDH. Therefore we determine the lactate metabolizing activity of *M. tuberculosis* FHb by HPLC. Results indicate that MtbFHb metabolizes D-lactate only when the heme domain remains in the ferric form that may possibly acts as an additional electron acceptor for the utilization of lactate and participate in electron transport chain to balance the stress level and promote lactate/Fe^{3+} mediated Fe^{3+}/Fe^{2+} redox cycling. Since mycobacteria are naturally deficient in D-lactate dehydrogenasese, it may be possible that mycobacterial FHbs (type II) acquired a FAD motif similar to D-lactate dehydrogenase and function as D-lactate dehydrogenase under specific conditions, to protect the cells from oxidative stress and accomplished its energy requirements in nutrient deficient environment. Repeated attempt were made to knock down the rv0385 gene in *M. tuberculosis,* so that we can authenticate these results in flavohemoglobin negative background of *M. tuberculosis.* However we were not successful to get any positive clones.

In due course, to understand how genes are regulated the way they are, the preliminary regulation analysis and comparisons of regulatory regions of the flavohemoglobin of *M. tuberculosis* and *M. smegmatis* was explored to gain new insight into the properties and functionalities of these novel flavohemoglobin. The analysis of genetic organization of the mycobacteria showed that the flanking genetic loci are quite conserved in case of FHb. However, they make a distinct group when compared to the conventional flavohemoglobin in both cases of type I and type II FHBs. Gene organization helps in speculating the probable function of the gene based on its positional counterparts; therefore, it is quite possible that mycobacterial FHb may be playing distinct role(s) in different mycobacterial species. Further, SigF and SigE binding site was found in the upstream sequence of *fhb* gene in *M. tuberculosis* , which stressed, upon the importance of the FHb protein under the conditions where SigF is expressed. However despite the presence of consensus we did not find any binding of sigma factors to FHb promoter. Here, it is note worthy that we cannot rule out the binding possibility, as sigma factors may bind with DNA in presence of core RNA polymerase.

On the whole, conclusion based on the structure-function studies suggest that genes encoding novel flavohemoglobin might be playing an important but distinctly different roles (as compared to conventional flavohemoglobin) in the physiology of *M. tuberculosis*, which together would form a multi-tier system to help in the survival of organism . MTb FHb appears to be evolved for host specific lifestyle based on its presence in only a limited number of organisms, and the selection of this two domain Hb to carry out a function more efficiently, under stress condition. On the other hand, FHb would also protect the mycobacterium from the oxidative stress, indirectly by consuming the D lactate, accumulation of which generates more oxidative stress.

The rehabilitated interest in TB research has led to a considerable explode of activities in exploring the bacilli at cellular and molecular level. Tuberculosis teaches that a balance can waver for years between host immunity and a pathogen's resistance. A grand challenging work remains if humans are to outsmart to this ancient and clever rival. A better understanding of the *in vivo* activity of the flavohemoglobin requires more functional experimentation. The mycobacterial flavohemoglobin (type II) has several unique features which suggest a novel function and to understand the mechanism and the role of each of these features it's important

to ensue a search. The evaluation of the protective mechanism of FHb in knock down strain of Rv0385 would add a wealth of information to the existing reactions carried out by the Mtb FHb. Pathogenic microorganisms, such as *M. tuberculosis* might profit from the flavohemoglobin assisted resistance mechanism upon infection of a potential host. Bearing in mind the steadily increasing number of antibiotic-resistant pathogens, the flavoHbs might represent a novel drug target for therapy.