CHARACTERIZATION OF MYCOBACTERIAL ESTERASES/LIPASES USING COMBINED BIOCHEMICAL AND COMPUTATIONAL ENZYMOLOGY

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2013

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Ankit Shukla

28 December 2012

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Summary

Esterases/Lipases are evolutionarily related enzymes mostly belonging to hydrolases superfamily sharing a common α/β-hydrolase protein fold. Recent studies have suggested that they play a pivotal role in disease manifestation due to disruption of lipid metabolizing enzymes and their pathways, yet only very few have been functionally annotated.

This study focuses on Mycobacterial lipolytic/non-lipolytic enzymes comprising of 31 putative lipases and/or esterases belonging to α/β-hydrolase fold family. *In silico* molecular modeling, sequence analysis and ligand docking experiments provide insights into molecular structure of these classes of enzymes. We performed a unique structure based virtual ligand screening to predict natural substrates of 4 putative Mycobacterial esterases/lipases namely *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase).

The information obtained from molecular docking and molecular dynamics (MD) simulations was preceded with an *in vitro* study, where we performed enzymatic assays with whole cell lysates of *Mycobacterium bovis* BCG over-expressing lipases and/or esterases using synthetic substrates. We provide evidence at structural and biochemical level that the short-chain esterase activity of *BCG_1460c* (lipH probable lipase) is inhibited by tetrahydrolipstatin (THL) an FDA approved drug. Overall, the *in silico* and *in vitro* analysis show high correlation and provide an effective approach to characterize and distinguish mycobacterial lipolytic/non-lipolytic enzymes.

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Lists of Abbreviations

THL: Tetrahydrolipstatin

pNP: para-nitrophenol

PDB: Protein Data Bank

PMSF: phenylmethanesulfonylfluoride

E600: diethyl-p-nitrophenylphosphate

EC : Enzyme classifier

TAGs: Triacylglycerol

MTB: *Mycobacterium tuberculosis*

LB: Lipid bodies

ILI: Intracytoplasmic lipid

OD: Optical Density

SBDD: Structure Based Drug Design

VHTS: Virtual High Throughput Screening

FBDD: Fragment Based Drug Design

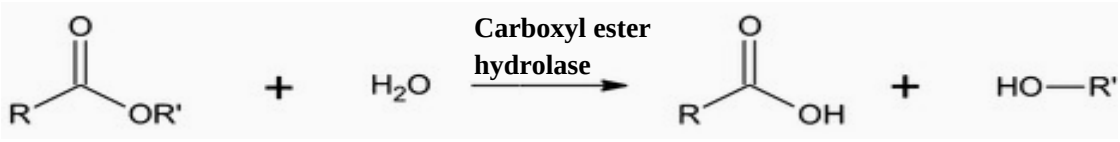
3DQSAR: Three Dimensional Quantitative Structure Activity Relationship

1. Introduction

1.1. Esterases/Lipases

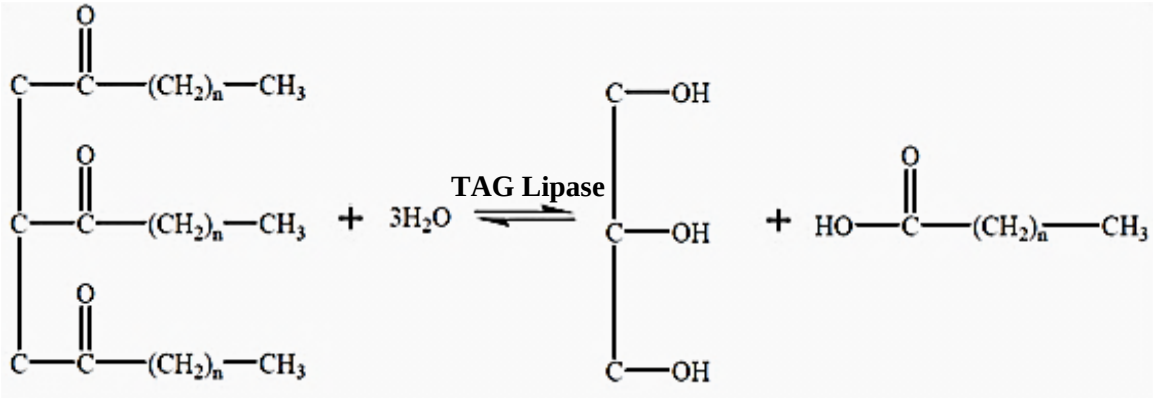
The biological relevance and coexisting variability of lipids has led to the development of wide range of lipid metabolizing enzymes. Esterases (EC 3.1) are widely distributed amongst bacteria, fungi, plants and animals defined by their ability to catalyse the formation and cleavage of ester bonds. They are classified based on the nature of the ester bonds (carboxyl ester, thio ester, phosphomonoester, etc.) they catalyse. Among them, carboxyl ester hydrolases (EC 3.1.1) are enzymes that catalyses ester bond hydrolysis of carboxylic esters (Figure 1.1a). Lipases/TAG lipases (EC 3.1.1.3) are lipolytic enzymes which constitute a special sub-class of carboxyl ester hydrolases (EC 3.1.1) (Ali, Verger et al. 2012) capable of releasing long-chain fatty acids from natural water-insoluble esters such as lipids (Figure 1.1b).

(a)



Carboxylic ester **Water** **Carboxylic acid** **Alcohol**

(b)



Triacylglycerol **Water** **Glycerol** **Fatty Acid**

Figure 1.1: (a) Hydrolysis of a carboxylic ester catalysed by carboxyl esterase enzyme. **(b**) Hydrolysis of a triacylglycerol substrate catalysed by TAG lipase enzyme (Source: Thomson, Delaquis et al. 1999)

1.1.1 Esterases/Lipases in Infectious Diseases

Several studies on lipid metabolism have been undertaken and the outcome of these studies has opened up new ways and avenues to analyse and characterize a host of diseases as well as providing newer insights and approaches into the mechanisms involved when cells start functioning abnormally and hence enable establishing of an important link between lipid metabolism and the disease. The concept and hypothesis was further extended to infectious diseases which are known to be accompanied by an altered host lipid metabolism via a unique sequence i.e. the disruption of lipid metabolizing enzymes (esterases/lipases) and their pathways and there also exists a close and significant inter-relationship between lipid metabolism and host responses to infection.

It is indeed important to explain the mechanism and the factors by which the lipid metabolism is regulated. The frontline and exclusive studies have shown that the lipid metabolism is regulated by lipid metabolizing enzymes (esterases/lipases) which are considered to be one of the known virulence factors in many bacteria such as *Pseudomonas cepacia, Staphylococcus aureus* (Lonon, Woods et al. 1988 and Rollof, Braconier et al. 1988) and fungal species like *Candida albicans, Fusarium gramearium.* Further insight reveals that the lipid metabolizing enzymes of *Propionibacterium acnes* and *Staphylococcus epidermis* are probably involved in incidence of commonly prevalent human skin infections where they help triggering colonization and subsequent persistence of bacteria on the human skin.

EFFECTS OF INFECTION ON LIPID METABOLISM OF HOST	
Presence of invading microorganism	**Secondary effects due to infection**
1. Direct Effects	• Decreased dietary intake of fats.
• Utilization of host lipids required by replicating microorganisms.	
• Disruption of host cell metabolism by intracellular microorganisms.	• Minimal interference with intestinal absorption of fats.
• Localized destruction of fat cells at sites of an infectious process.	• Altered lipid metabolism within host cells.
2. Indirect Effects	• Altered rates of hormone-mediated lipolysis within fat depots to supply increased metabolic demands.
• Alterations in host lipid metabolism caused by bacterial exotoxins, endotoxins or enzymes.	
• Activation of lipase and other lysosomal enzymes within host phagocytes.	• Altered rates of lipid synthesis within the liver
• Release of mediator substances from host cells, that is, endogenous pyrogen, interferon.	• Altered rates of fat utilization by peripheral tissues.

Table 1.1: Effects of infectious pathogens on host lipid metabolism
(Source: (Beisel and Fiser 1970))

According to some hypothesis it has been suggested that these enzymes may also be responsible in contributing to the invasiveness and proliferation by inducing the destruction of the host tissues thereby supplying hydrolysed material as nutrient to the microorganisms. Lipid precursors needed for the replication of the invading pathogens (bacteria, viruses and protozoans) are derived and supplied from the source metabolic pools within the host by lipid

metabolizing enzymes thereby altering the host lipid metabolism. In the subsequent process of progression and infection may allow for the re-distribution of nutrients to cells which are considered extremely important in ensuring host defence or tissue repair.

It has now been well established and recognized that certain pathogens have the inherent capabilities to co-opt the lipid metabolism and some illustrative examples can be listed as:

a.) The ability of *Mycobacterium tuberculosis* to catabolize cholesterol as an energy source which might as a result facilitate and regulate its ability to survive within the macrophages.

b.) Utilization of host cholesterol by *Toxoplasma gondii* for its persistence, growth and proliferation.

c.) *Heliobacter pylori* being unable to synthesize cholesterol and hence requires exogenous cholesterol from the host.

d.) Ebola virus (EBOV) in lysosomal compartments binds to cholesterol transporter protein Niemann-Pick C1 (NPC1).

Fatty metamorphosis of cells particularly from liver, kidney and heart is a common histologic finding during a host of bacterial infectious diseases. An increase in esterified fatty acids has been observed in viral hepatitis whereas free fatty acids and triacylglycerol (TAGs) have been reported to be elevated in gram-negative bacillus infections in humans. Since the action of hormone-sensitive lipase in adipose tissue is a major event contributing to free fatty acids to blood, such infection-related hormonal responses may have a pivotal role to play in altering/affecting the rates of lipolysis or fatty acid utilization.

1.1.2 Biology of Mycobacteria

It has now been reported that the genus *Mycobacterium* is known to comprise of more than 100 species (Tortoli 2006). The cultivable (grown in lab) members of *Mycobacterium* are clinically grouped either as the *Mtb* complex or the non-tuberculous mycobacteria. The *M. leprae*, which is responsible for causing leprosy is an obligate parasite and therefore not cultivable *in vitro* (van Beers, de Wit et al. 1996). On the other hand, diseases caused by members of *Mtb* complex include *M. tuberculosis*, *M. bovis*, *M. microti, M. africanum* and *M. Canettii* subspecies they are known to possess and demonstrate very similar clinical features. Pulmonary diseases caused by *M. tuberculosis* and *M. bovis* are clinically, radiologically and pathologically indistinguishable. However, *M. bovis* appears to have a diminished propensity and potentiality to reactivate and spread from person to person (O'Reilly and Daborn 1995) through human chain. Calmette and Guérin (BCG) attenuated a strain of *M. bovis* to generate BCG which is used as a vaccine by continuous passaging through culture media. Mycobacteria are aerobic and non-motile rod (bacillus) shaped that are identified to be weakly Gram-positive and acid-fast by Ziehl-Neelsen staining. The bacilli belong to the actinobacterium family and all *Mycobacterium* species are known to share a characteristic cell wall architecture which is relatively much thicker than other bacteria and are known to be hydrophobic, rich in mycolic acids. In the laboratory, *Mtb* can be grown, *in vitro*, on the agar-based Middlebrook medium or the egg based Lowenstein-Jensen medium (Parrish, Dick et al. 1998). Considering that it is a relatively slow growing bacteria, it takes a time period of around 4-6 weeks to

have visual bacterial colonies formed on these solid media (Parrish, Dick et al. 1998).

1.1.3 Mycobacterial Esterases/Lipases

In Mycobacterium such as *Mycobacterium tuberculosis* lipids play a vital role wherein it is stated that a large fraction of the genome encodes putative enzymes said to be involved in lipid metabolism (Cole, Brosch et al. 1998). Indeed, *Mycobacterium tuberculosis* genome contains 250 genes encoding putative enzymes involved in the synthesis or degradation of lipids compared to 50 genes in *Escherichia coli*, which is known to have a similar genome size. This feature, combined with the extremely large quantum of lipids representing 30–40% of the dry weight of *M. tuberculosis* tends to suggest that lipids and lipid metabolizing enzymes play an important role in the mycobacterial life cycle and perhaps also in virulence. In the study conducted by (Deb, Daniel et al. 2006) group reported the expression status of all the twenty-four putative lipase/esterase genes of lipase gene family of *M. tuberculosis* H37Rv in *Escherichia coli* BL21.

In silico analysis has identified the presence of around 31 putative genes encoding lipid metabolizing enzymes (enzymes involved in lipids degradation) including 24 lipid/ester hydrolases belonging to the so called “Lip family” (LipC to LipZ). These have been annotated as putative esterases or lipases based on the presence of the consensus sequence GXSXG which is considered to be the characteristic feature of the α/β hydrolase-fold family members (Ollis, Cheah et al. 1992). The functional classification of *Mycobacterium tuberculosis* genes has been depicted in fig.1.2

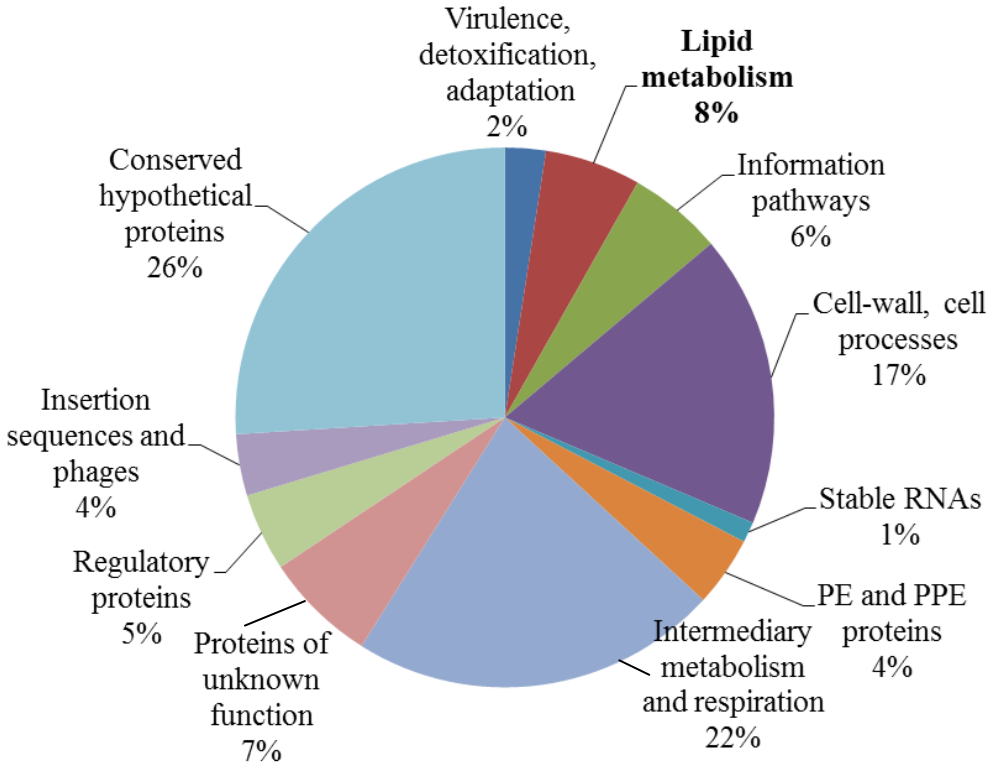


Figure1.2: Functional classifcation of *Mycobacterium tuberculosis* genome
(Source: data from (Camus, Pryor et al. 2002))

1.1.4 Role of Esterases/Lipases in Mycobacterial Infection Cycle

There are a large number of mycobacterial species such as *M. tuberculosis* (Garton, Christensen et al. 2002), (Schue, Maurin et al. 2010), (Peyron, Vaubourgeix et al. 2008), (Daniel, Deb et al. 2004), (Deb, Daniel et al. 2006), (McKinney, Bentrup et al. 2000), *Mycobacterium bovis* BCG (Low, Rao et al. 2009 and Low, Shui et al. 2010), *Mycobacterium leprae* (Mattos, D'Avila et al. 2010) and *Mycobacterium smegmatis* (Garton, Christensen et al. 2002 and Dhouib, Ducret et al. 2011) which predominantly demonstrate the accumulation of lipids derived from host cells. In addition, the consumption pathways involving lipid metabolizing enzymes (esterases/lipases) have also

been identified and expressed. In particular, the tubercule bacilli enter the body by inhalation of aerosol route and reach the lungs where they are phagocytosed by the frontline pulmonary alveolar macrophages. Subsequently, host response ensues which consists of recruitment of lymphocytes, macrophages and dendritic cells leading to the formation of a highly organised structure termed as 'granuloma' a major histopathological hallmark of tuberculosis (Singh, et al. 2010). In these granuloma macrophages containing bacilli accumulates intra-cytoplasmic lipid inclusion bodies (LB) which are predominantly composed of neutral lipids surrounded by a phospholipid layer that reveals and assigns the macrophage their foamy appearance within the foamy macrophage, phagocytised bacteria preferentially metabolize lipids rather than carbohydrates (Wheeler and Ratledge 1988), a view point that is supported by an evidence showing up-regulation of several mycobacteria genes involved in lipid metabolism (McKinney, Honer zu Bentrup et al. 2000). At this stage of progression, the intra phagosomal bacteria acquire and accumulate intra cytoplasmic lipid inclusion (ILIs) in their cytoplasm (Figure 1.4(B) and 1.4(C)) and persist in a non-replicating state ultimately and eventually leading to dormancy i.e. latent infection. It has been demonstrated in an *in vitro* model of human granulomas (Peyron, Vaubourgeix et al. 2008) that these lipid bodies (LB and ILIs) serve as sources of carbon and energy for dormant bacilli. The infection cycle of *Mycobacterium tuberculosis* has been shown in fig.1.3

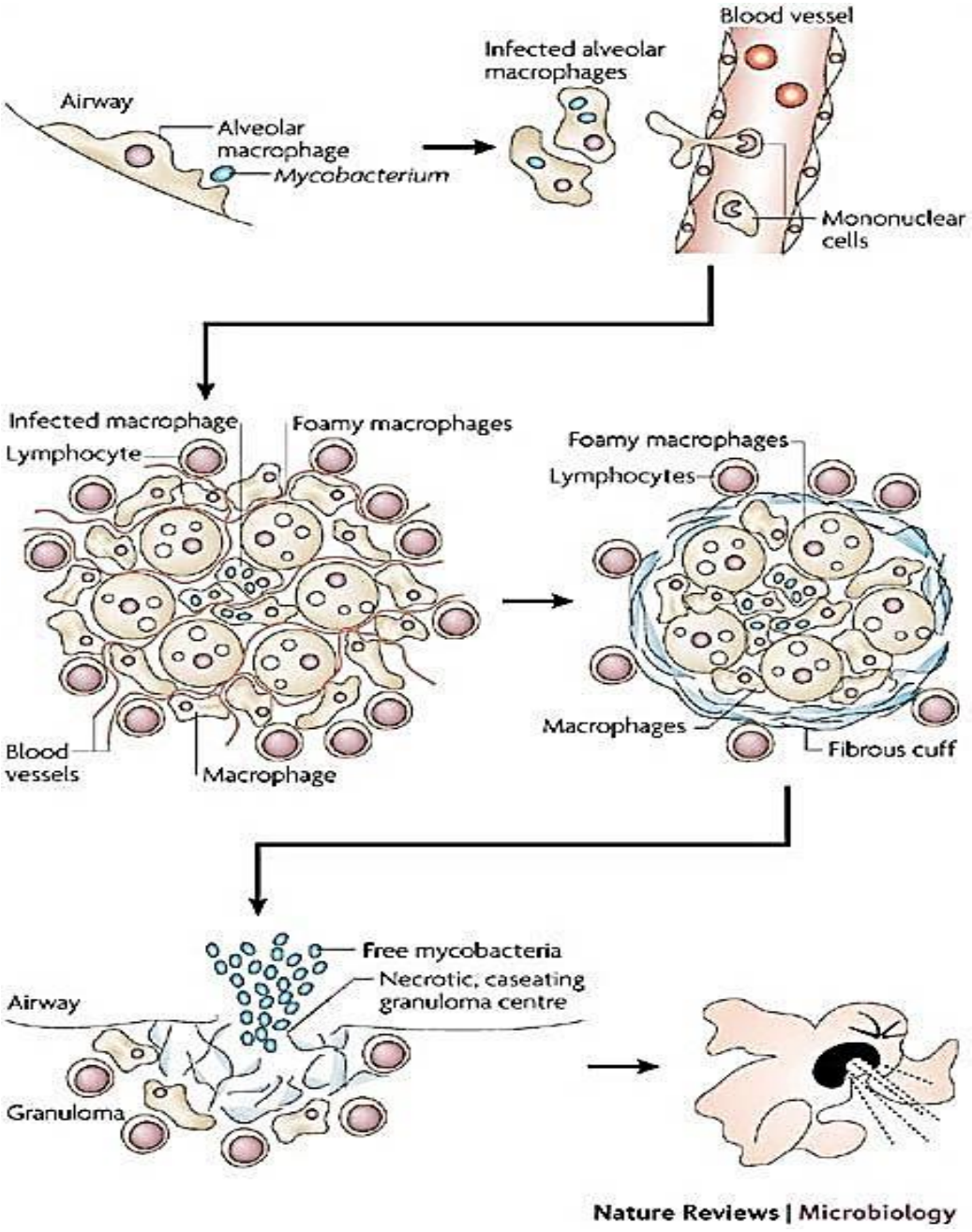


Figure 1.3: *Mycobacterium tuberculosis* infection cycle

(Russell 2007)

within granulomas and as a consequence aiding in reactivation that can ultimately lead to an active tuberculosis infection (Parrish, Dick et al. 1998). The typical foamy macrophages having lipid bodies, LB surrounded by *Mtb*

bacilli and intracytoplasmic lipid within *M. tuberculosis* bacilli have been shown in fig 1.4

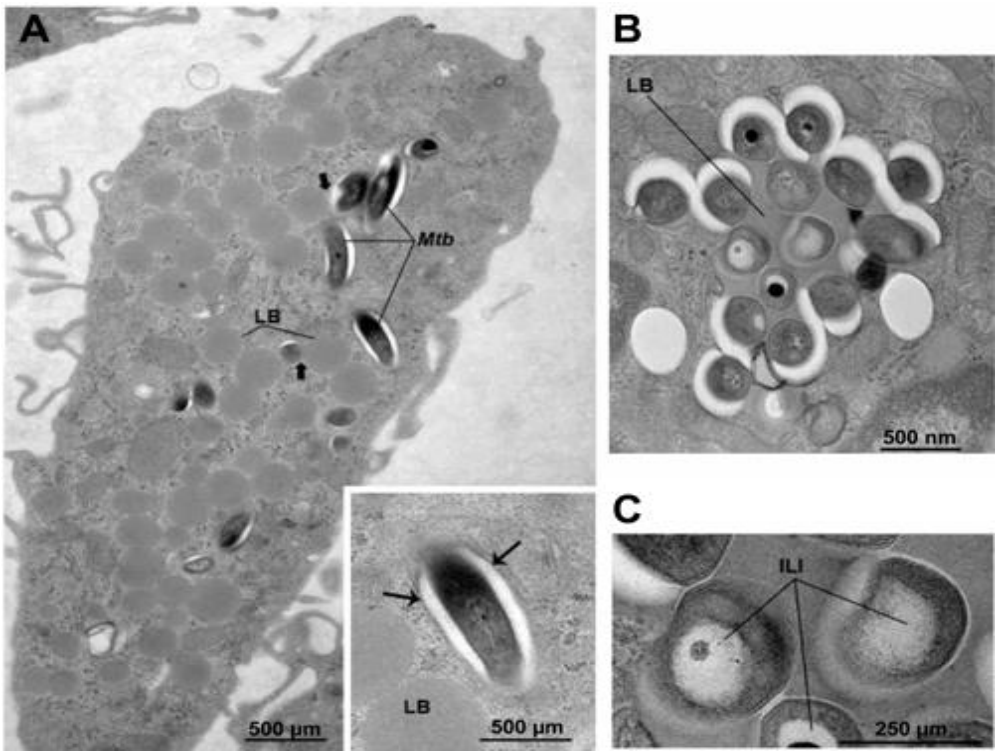


Figure 1.4: (A) Typical foamy macrophage having lipid bodies (LBs) and *M. tuberculosis* containing phagosomes: arrows depict phagosomal membrane around bacterium. **(B)** LB surrounded by several *M. tuberculosis* bacilli intracytoplasmic lipid (ILIs) **(C)** Enlarged view of (B) showing large intracytoplasmic lipid within *M. tuberculosis* bacilli [Adapted from (Peyron, Vaubourgeix et al. 2008)]

In addition to attention drawn on the foamy macrophages, ILI accumulation has also been reported in *M. tuberculosis* infected adipocytes as well as in *Mycobacterium leprae* infected macrophages and Schwann cells (Mattos, Lara et al. 2011). Further biochemical analysis and associated experimentation has revealed that *M. tuberculosis* lipid inclusion bodies mainly comprise of triacylglycerol (TAGs). These TAGs are derived from free fatty acids that may

be imported from host or result from denovo synthesis (Daniel, Maamar et al. 2011). The pattern indicated that Triacylglycerol (TAGs) accumulate during mycobacterial growth and the amount of intracellular TAGs peak in the late exponential growth phase (Kremer, de Chastellier et al. 2005) and non-replicating phase (Daniel, Deb et al. 2004).

Further it has also been shown that the expression of *M. tuberculosis* specific lipase gene family is significantly elevated during dormancy (Deb, Daniel et al. 2006) and that in the re-activated bacilli, a reduction in triacylglycerol (TAG) levels coincides with an increase triacylglycerol (TAG) lipase activity (Low, Rao et al. 2009). Thus lipid metabolizing enzymes (esterases/lipases) appear to play an important central role and associate with important physiological functions and also contribute to the extraordinary capacity of survival of *M. tuberculosis* within the infected host. These enzymes are peculiar molecules that provide a metabolic turnover of lipids and can be defined as essential biocatalysts for the hydrolysis of esters containing long chainfattyacids.

1.2. Esterases/Lipases in physiopathology and disease progression

Pathogenic bacteria have been known to follow a number of mechanisms and pathways to cause and allow subsequent persistence of diseases in human hosts. The molecular strategies used by the bacteria to interact with the host can be unique and characteristic to specific pathogens, and follow conserved pattern across several different species. Hydrolytic enzymes like esterases/lipases contribute to invasiveness and proliferation by causing

destruction of the host tissue thereby supplying hydrolysed material to the organisms as nutrients. These esterases/lipases are one of the known and critical virulence factors in a host of bacterial species such as *Pseudomonas cepacia, Staphylococcus aureus* (Lonon, Woods et al. 1988, Rollof, Braconier et al. 1988) and also in fungal species like *Alternaria brassicicola, Candida albicans* and *Fusarium graminearum* (Berto, Commenil et al. 1999). *M. tuberculosis* is a bacterial pathogen that can persist in for decades in an infected patient in dormant state without causing symptoms or disease with clinically evident features. In fact, prior to entering into dormancy, it has been hypothesized that the bacteria accumulate lipids originating from the host cell membrane degradation as precursors and re-synthesize complex lipid molecules. Fluorescence studies (Garton, Christensen et al. 2002) have shown that large amounts of intracellular lipids forming inclusion bodies can be detected in the cytoplasm (Anuchin, Mulyukin et al. 2009) supporting the view that bacteria tends to accumulate lipids. The presence of lipid inclusions confers and indirectly correlates the existence of lipid metabolizing esterases/lipases. During the re-activation phase of the bacteria, these stored lipids are hydrolysed and the infection process acquires further impetus to demonstrate its detectable occurrence (Cotes, Bakala et al. 2008). What is interesting to mention here is that a critical link between storage-lipid accumulation and development of phenotypic drug resistance in *M. tuberculosis* has also been established and the findings of several studies on non-mycobacterial pathogens suggested the involvement of lipid metabolizing enzymes in pathogenicity.

In pathogenic bacteria, for example it has been shown that *Mycobacterium tuberculosis* Rv3097c (lipY) is able to hydrolyse long-chain triacylglycerol (TAG). The role of esterase Rv3487c (lipF) has been implicated in pathogenesis (Zhang, Wang et al. 2005). In addition, Rv0220 (lipC) has been reported to be an immunogenic cell-surface esterase actively involved in modulation of the host immune response (Shen, Singh et al. 2012). This entity Rv0220 (lipC) is also known to be capable of stimulating pro-inflammatory cytokines and chemokines in macrophages as well as to pulmonary epithelium cells (Shen, Singh et al. 2012). It is also important to mention that the lipase Rv0183 has been identified as a monoglyceride lipase involved in degradation of host cell lipids and may strongly induce immune responses of the host (Xu, Jia et al. 2010). Based on these facts, it can therefore be categorically stated that lipid metabolizing enzymes (esterases/lipases) are involved throughout the life-cycle of the pathogen and they assume important physiological role during dormancy and reactivation i.e. during the course of entire infection process. The released fatty acids by these enzymes are then taken up by intracellular mycobacteria and stored in the form of triacylglycerol (TAGs) to be subsequently used as sources of carbon during the persistence stage. Conversely, intracellular triacylglycerol hydrolases maybe required for assimilation of intra-cytoplasmic lipid inclusions to exit dormancy.

1.3 Esterases/Lipases Enzyme Classification System

1.3.1 Hydrolases (EC 3.)

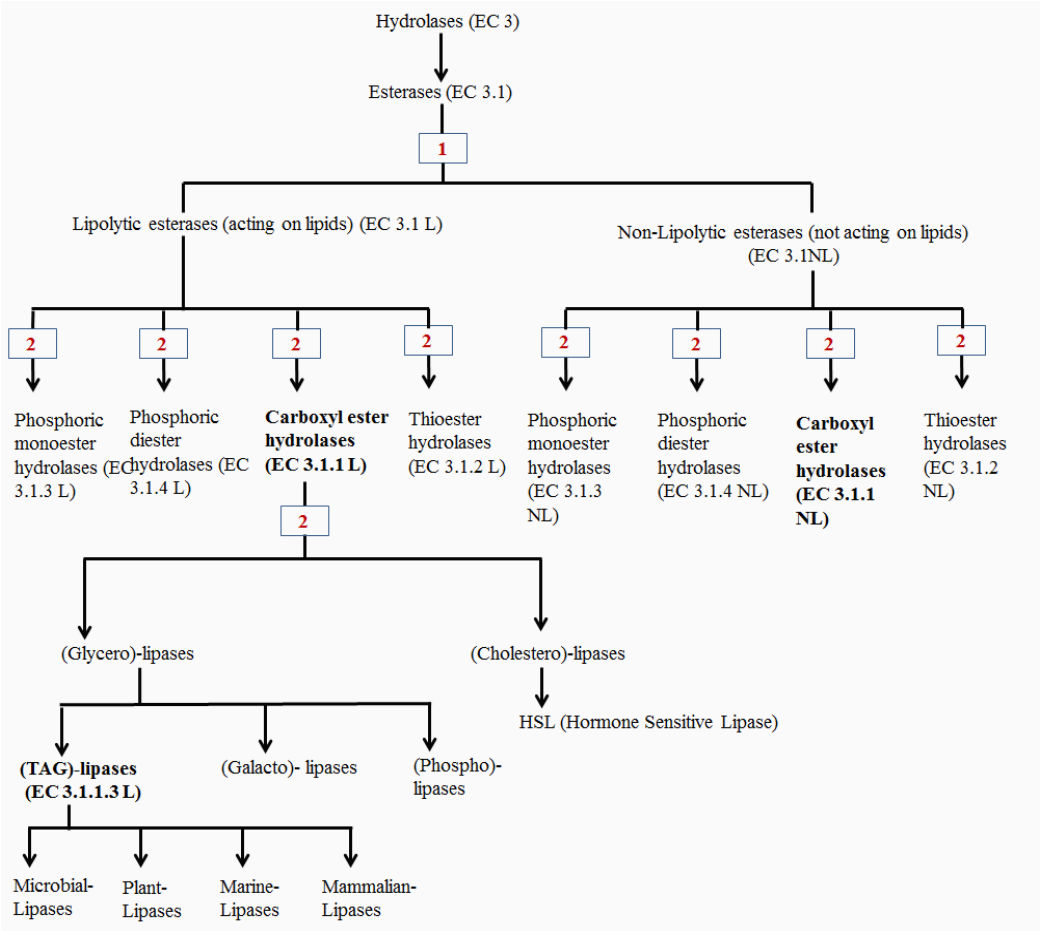
Are group of enzymes which catalyse the hydrolysis of a chemical bond. In general an enzyme capable of catalysing the following reaction is a hydrolase:

$$X–Y + H_2O \rightarrow X–OH + Y–H$$

In the enzyme classification (EC) number system, they have been classified as EC 3. Hydrolases can be further classified into several subclasses based upon the bonds they act upon, for example, ester hydrolases, peptidases, amidases etc.

1.3.2 Carboxyl ester hydrolases (EC 3.1.1)

This group of enzymes act on ester substrates mainly derived from the condensation reaction of a carboxylic acid and an alcohol. Members of this group have been classified chronologically based on their known substrate specificity (fig.1.5) into two major classes: carboxyl esterases (EC 3.1.1.1) and triacylglycerol (TAG) lipases (EC 3.1.1.3).



(from previous page) Figure 1.5: Esterases' classification based on *1* : Physico-chemical; *2* : chemical criteria (L means lipolytic those enzymes capable of acting on lipid while NL: non-lipolytic those enzymes which do not act on lipids) (EC is the enzyme classifier) [Source: adapted from (Ali, Verger et al. 2012)]

1.3.3 Carboxyl esterases (EC 3.1.1.1)

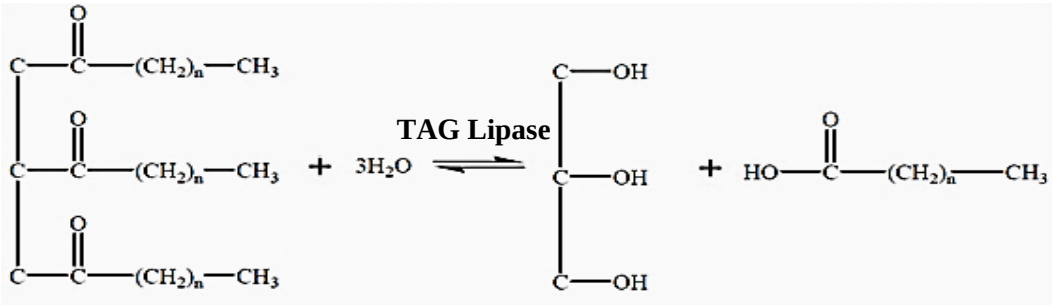
This group of enzymes shows diverse substrate specificity and catalyse the hydrolysis of ester bond of acyl chain esters forming a carboxylic acid and an alcohol.

$$\underset{\textbf{Carboxylic ester}}{RCOOR} + \underset{\textbf{Water}}{H_2O} \rightarrow \underset{\textbf{Carboxylic Acid}}{R{-}COOH} + \underset{\textbf{Alcohol}}{R{-}OH}$$

Most members of this group are hydrolases especially those involved in the hydrolytic cleavage of carboxylic ester bonds are found to share a common alpha/beta (α/β) hydrolase folding pattern. Enzymatic assays using chromogenic substrates such as acyl esters of p-nitrophenol (pNP) allow for the spectroscopic and calorimetric determination of esterase activity.

1.3.4 Triacylglycerol (TAG) Lipases (EC 3.1.1.3)

They constitute a special class of carboxyl esterases (Ali, Verger et al. 2012) capable of releasing long-chain fatty acids from natural water-insoluble esters (lipids) as depicted below:



Triacylglycerol **Water** **Glycerol** **Fatty Acid**

In bacterial species such as Mycobacteria, Pseudomonas, Burkholderia TAG lipases have been shown to completely hydrolyse triacylglycerol substrates although ester bonds are more preferable (Jaeger et al., 1994) and they possess both lipolytic as well as esterolytic activity. Bacterial TAG lipases have also been found to share a common alpha/beta (α/β) hydrolase folding pattern. A large number of enzymatic assay methods using fluorescent substrates allow for the fluorimetric and spectroscopic detection of lipase activity.

1.4 Alpha/Beta (α/β) Hydrolase fold family

The alpha/beta (α/β) hydrolase fold is considered to be a common characteristic to a number of hydrolase enzymes of largely different phylogenetic origin and catalytic function (Ollis, Cheah et al. 1992). Each enzyme has a conserved alpha/beta (α/β) hydrolase core (fig.7b) consisting of alpha/beta sheet having 8 strands connected by helices. They all have a similar arrangement of a catalytic triad composed of nucleophilic serine charge relay network aspartate and proton carrier histidine (shown in fig.1. 6) which are the best-conserved structural features in the fold. The canonical *α/β* hydrolase fold is an eight-stranded and mostly parallel *α/β* structure (figure 1.6), (1.7a&b) (Ollis, Cheah et al. 1992).

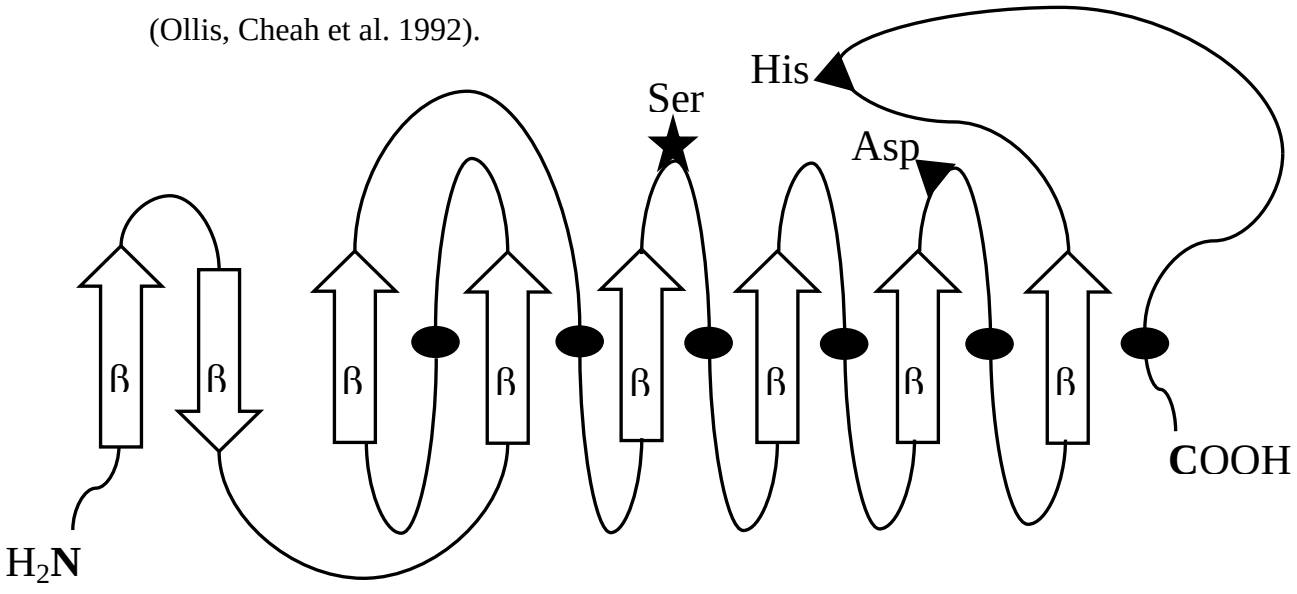


Figure 1.6: Topology diagram of α/β hydrolase fold enzymes α Helices and β strands are represented by black spheres and arrows, respectively while catalytic triad members are highlighted by black star and triangles.

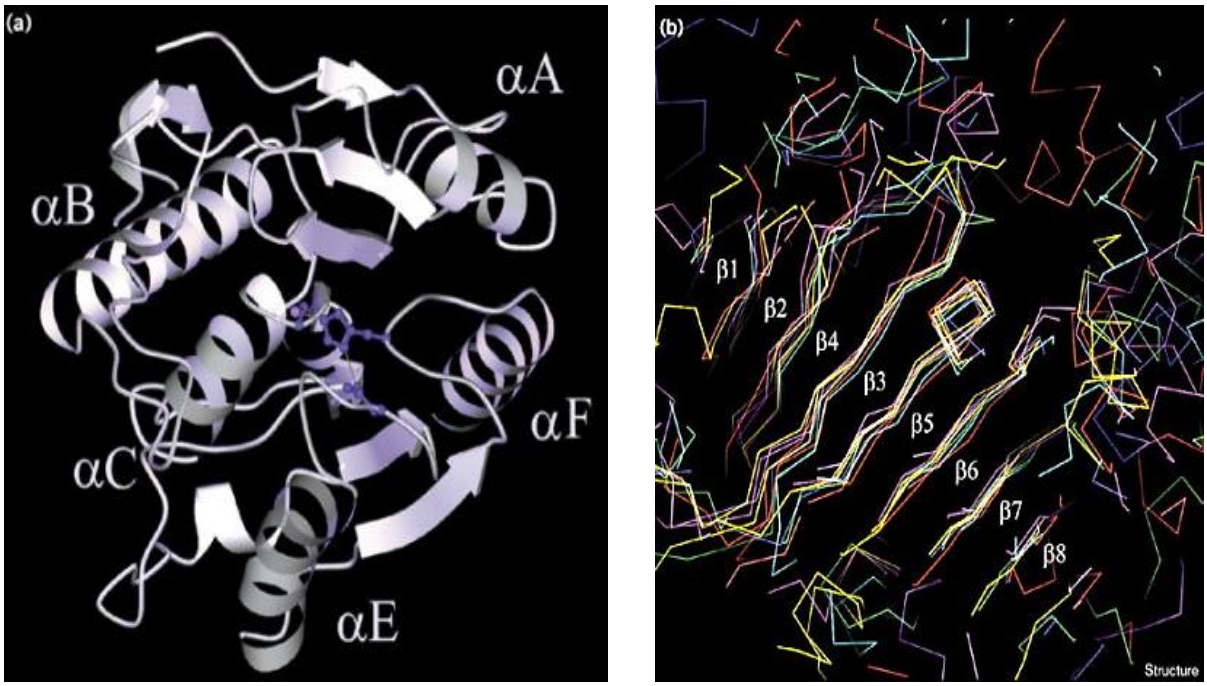


Figure 1.7: (a) 3D structure of *Pseudomonas fluorescens* carboxyl esterase (PfCES) belonging to α/β hydrolase fold family revealing mostly-parallel *β* sheets. **(b)** Superposition of the conserved *α/β* hydrolase core of 9 representative *α/β* hydrolase fold enzymes (Source: Heikinheimo et. al, 1999)

The enzymes adopting alpha/beta hydrolase fold share no significant sequence similarity suggestive of a divergent evolution from a common ancestor. The members of alpha/beta (α/β) hydrolase fold family include: hydrolases, esterases, lipases, proteases, peroxidases, dehalogenases.

1.4.1 Alpha/Beta (α/β) Hydrolase fold family in Mycobacteria

Most members of the alpha/beta (α/β) hydrolase fold family are esterase/lipase enzymes that catalyse ester hydrolysis reactions (Schrag et al., 1997, Nardini et al., 1999). In *Mycobacterium tuberculosis*, out of the 250 genes encoding putative enzymes involved in lipid metabolism, 94 gene products would have the characteristic alpha/beta (α/β) hydrolase fold (Hotelier, Renault et al. 2004), ESTHER database http://bioweb.ensam.inra.fr/esther) of which 47 are

annotated as esterases (ester hydrolases) and also including 24 lipid/ester hydrolase belonging to lip family (lipC to lipZ) (Cole, Brosch et al. 1998). The total *M. tuberculosis* functionally annotated genes and α/β hydrolase fold distribution in *M. tuberculosis* are shown in fig.1.8 (a&b).

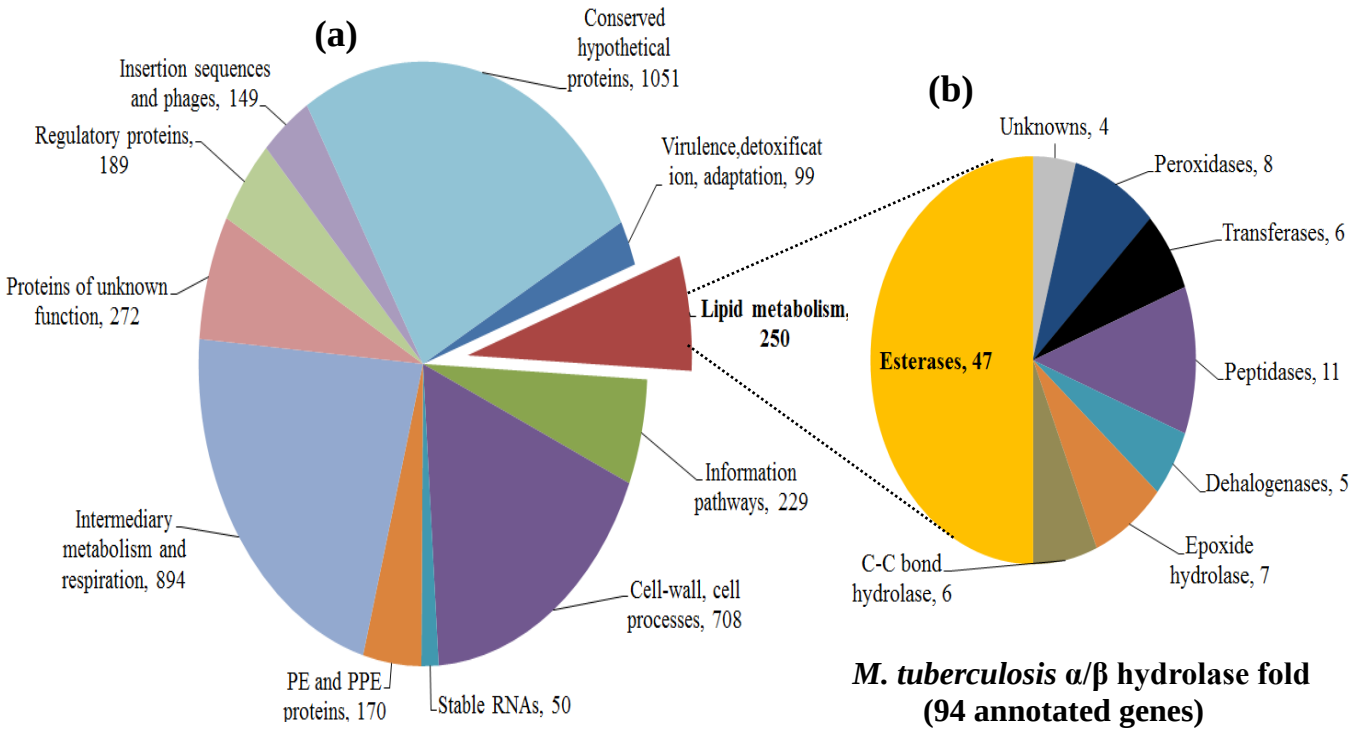


Figure 1.8: (a) Total *M. tuberculosis* functionally annotated genes.
(b) 94 α/β hydrolase fold distribution in enzymes involved in mycobacterial lipid metabolism having 250 lipid encoding genes [http://bioweb.ensam.inra.fr/esther].

Overall alpha/beta (α/β) hydrolase fold family members in Mycobacteria are mainly esterases/lipases suggesting an important structural link of this family of enzymes in mycobacterial lipid metabolism.

1.4.2 Mycobacterial Lipase gene family

This sub-family of alpha/beta (α/β) hydrolase fold family comprises of 24 genes annotated as putative esterases/lipases (lipC to LipZ) genes (Cole, Brosch et al. 1998) and all pose a catalytic triad with active site as nucleophilic serine showing a characteristic G-X-S-X-G sequence motif and are typically involved in various biological processes. Their probable function has been represented and listed in:

Table 1.2 Probable functions and identities of Mycobacterial lipase gene family enzymes

Protein	Gene	Mol wt.	Activity type	Active site	Biological function	References
lipC	BCG 0257 (Rv0220)	44.3	Probable esterase (77% αβ-hydrolase)	GCSAG	Low TG lipase activity, induced under hypoxic resuscitation Carboxyl esterase type B, Upregulated in starvation Located in cell wall and capsule, elicit strong immune response, expresses only during active tuberculosis, hydrolyze short chain esters	(Deb, Daniel et al. 2006) (Singh, Singh et al. 2010)
lipD	BCG 1962 (Rv1923)	47.2	Probable esterase/β-lactamase (69% β-lactamase)		A hydrolase lipase similar to esterases and beta-lactamases Role in defence	

lipE	BCG 3837 (Rv3775)	45.3	Caboxyl esterase (79% β-lactamase)		Lipolytic enzyme involved in cellular metabolism Defense mechanism, Induced under hypoxic resuscitation	(Deb, Daniel et al. 2006)
lipF	BCG 3551c (Rv3487c)	29.4	Caboxyl esterase (75% αβ-hydrolase)	GDSAG	Member of Hormone sensitive lipase family. Non-lipolytic hydrolase, hydrolyze short chain esters Induced at low pH, related to virulence No TG lipase activity, intermediary metabolism and respiration Important for bacilli persistence Membrane protein, hydrolyzes short chain esters /phosphatidylcholine	(Zhang, Wang et al. 2005) (Richter and Saviola 2009) (Camacho, Ensergueix et al. 1999) (Deb, Daniel et al. 2006)
lipG	BCG 0695c (Rv0646c)	32.9	Probable hydrolase (80% αβ-hydrolase)	GASMG	Lipolytic enzyme, involved in cellular metabolism, highly similar to various hydrolases, especially lipases from Acinetobacter calcoaceti	(Deb, Daniel et al. 2006)

lipH	BCG 1460c (Rv1399c)	33.9	Possible lipase (70% αβ-hydrolase)	GWSLG	Member of Hormone sensitive lipase family. lipid transport and metabolism Non-lipolytic esterase	(Deb, Daniel et al. 2006) (Canaan, Maurin et al. 2004)
lipI	BCG 1461c (Rv1400c)	34.0	Probable lipase (69% carboxylesterase family)	GDSAG	A probable lipase invloved intermediary metabolism and respiration, Member of Hormone sensitive lipase family. Intermediary metabolism and respiration	
lipJ	BCG 1939c (Rv1900c)	49.7	Putative lignin peroxidase (75% αβ-hydrolase)		Alkaloid biosynthesis II	
lipK	mbtJ, BCG 2399 (Rv2385)	32.9	Probable acetyl hydrolase (71% αβ-hydrolase)		Intermediary metabolism, Low TG lipase activity	(Deb, Daniel et al. 2006)
lipL	BCG 1560 (Rv1497)	45.8	Probable esterase (70% β-lactamase)		Transpeptidase, Beta-lactamase class C Intermediary metabolism and respiration, Low TG lipase activity	(Deb, Daniel et al. 2006)

lipM	BCG 2299 (Rv2284)	46.7	Probable esterase (78% αβ-hydrolase)	GGSAG	Involved in intermediary metabolism and respiration, predicted transmembrane protein Lipid transport and metabolism	(Gu, Chen et al. 2003)
lipN	BCG 2991c (Rv2970c)	40.1	Lipase like enzyme (77% αβ-hydrolase)	GDSAG	Member of Hormone sensitive lipase family. Lipid transport and metabolism	
lipO	BCG 1487c (Rv1426c)	46.1	Probable esterase (79% αβ-hydrolase)	GGSAG	Lipid transport and metabolism	
lipP	BCG 2483 (Rv2463)	42.8	Probable esterase (76% β-lactamase)		Involved in defense mechanism	
lipQ	BCG 2503c (Rv2485c)	45.2	Carboxyle sterase (74% αβ-hydrolase)	GGSAG	Intermediary metabolism and respiration	
lipR	BCG 3109 (Rv3084)	32.6	Probable acetyl-hydrolase/ esterase (68% αβ-hydrolase)	GDSAG	Member of Hormone sensitive lipase family. Based on sequence analysis belongs to 'GDXG' family of lipolytic enzymes, Domain search reveals it contains a partial Thioesterase	(Fisher, Plikaytis et al. 2002)

lipS	mesTa, BCG3201c (Rv3176c)	35.2	Probable epoxide hydrolase (99% amidase)		Virulence, detoxification and adaptation	
lipT	BCG2064c (Rv2045c)	56.1	Probable carboxylesterase (71% carboxylesterase)	GESAG	Converts unknown esters to correspondinf free acid and alcohol, a probable carboxyesterase, Contains Carboxylesterases type-B serine active site. Member of Hormone sensitive lipase family. Lipid transport and metabolism, Upregulated in starvation Induced under hypoxic resuscitation	(Betts, Lukey et al. 2002) (Deb, Daniel et al. 2006)
lipU	BCG1134 (Rv1076)	31.7	Probable lipase (76% αβ-hydrolase)	GDSAG	Member of Hormone sensitive lipase family. αβ-hydrolase, Upregulated in starvation	(Betts, Lukey et al. 2002)

lipV	BCG 3229 (Rv3203)	23.6	Probable lipase (71% αβ-hydrolase)	GHSFG	Presumed to be a lipolytic enzyme, Contains serine active site signature of lipases. Contains TAG lipase signature as well. Intermediary metabolism and respiration	
lipW	BCG 0254c (Rv0217c)	32.2	Esterase (75% αβ-hydrolase)	GASAG	Lipolytic enzyme involved in cellular metabolism, Possible esterase, showing similarity with others esterases Alkaloid biosynthesis II	
lipX	PE11, BCG 1232c (Rv1169c)	10.8	PE family protein, Esterase/lipase (73% αβ-hydrolase)		Hydrolase or acyltransferase, Upregulated in starvation	(Betts, Lukey et al. 2002)
lipY	PE30, BCG 3122c (Rv3097c)	45.0	PE-PGRS family, Membrane associated TG lipase (99% PE-PGRS family)	GDSAG	Member of Hormone sensitive lipase family. TG lipase activity, mutant has less TG degradation, Lipid transport and metabolism, induced under hypoxic resuscitation	(Mishra, de Chastellier et al. 2008)

lipZ	BCG 1869 (Rv1834)	31.6	Probable hydrolase (76% αβ-hydrolase	Unknown function	(Deb, Daniel et al. 2006)

1.4.2.1 Hormone-sensitive lipase sub-family (HSL)

The hormone sensitive lipases are also known as triacylglycerol (TAG) lipases wherein their main function is to hydrolyse first fatty acid of triacylglycerol thereby yielding a diacylglycerol and a free fatty acid. They are, in turn, highly regulated enzymes catalysing the hydrolysis of lipids in adipocytes. *In silico* sequence analysis of mycobacterial lipase gene family members show significant sequence homology with hormone-sensitive lipase family having a characteristic HGG motif and a conserved active-site motif GDSAG. There are also 12 mycobacterial lipolytic enzymes which belong to the hormone-sensitive lipase family of which 8 are derived from lip gene family namely lipF (Rv3487c), lipH (Rv1399c), lipI (Rv1400), lipN (Rv2970), lipR (Rv3084), lipT (Rv2045c), lipU (Rv1076), lipY (Rv3097c) and are of high functional importance.

1.5 Issues and Problems with functional characterization of Mycobacterial putative Esterases/Lipases

A literature survey of the studies conducted in the past revealed that they were aimed at the functional characterization of mycobacterial esterases/lipases and reflects that the following issues and problems need to be addressed:

- Expression of mycobacterial esterase/lipase enzymes in their non-natural (non-mycobacterial) expression systems such as *E. coli*.(Deb, Daniel et al. 2006)
- Active conformation of enzymes lost after the purification step and consequently re-folding in *E. coli* (Canaan, Maurin et al. 2004).
- Mycobacterial esterase/lipase enzymes such as Rv1399c (lipH) and Rv1400c (lipI) are insoluble and reported to form inclusion bodies (Canaan, Maurin et al. 2004).
- Poor solubility of mycobacterial esterase/lipase makes it difficult to obtain an X-ray crystal structure. By far, only one 3D structure has been solved of mycobacterial esterase (lipW) (PDB ID: 3QH4) belonging to the lipase gene family.

1.6 Tetrahydrolipstatin (Orlistat)

Commonly called Orlistat and marketed by Roche pharmaceutical company under the name of Xenical is a semisynthetic hydrogenated derivative of naturally occurring lipase inhibitor lipstatin produced from *Streptomyces toxytricini (Hochuli, Kupfer et al. 1987).* It has been well characterized as an irreversible inhibitor of serine esterases (Hadvary, Lengsfeld et al. 1988) covalently modifying its biological target. It was identified originally as a specific inhibitor of pancreatic lipases and later developed as an anti-obesity drug. The inhibitor has a reactive β- lactone ring (Fig.1.9) leading to an ester

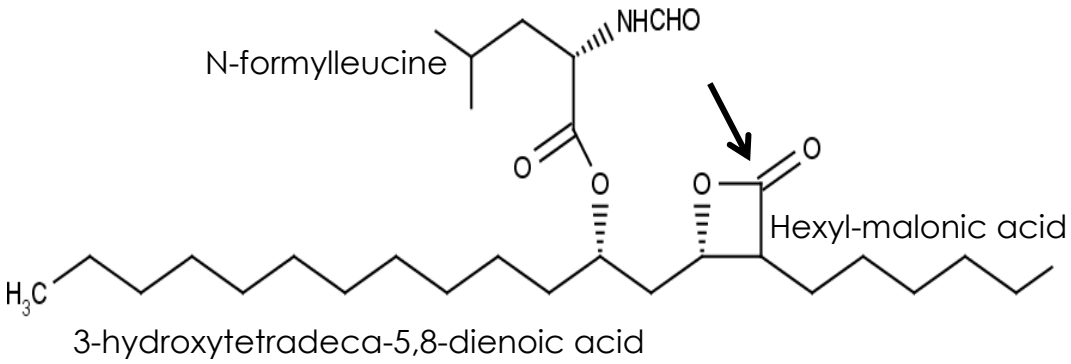


Figure 1.9: Chemical Structure of Tetrahydrolipstatin (Orlistat): arrow depicting the β lactone ring in the chemical structure

Serine hydroxyl group of the catalytic triad of esterase/ lipase (Hadvary et al., 1988).

1.6.1 An FDA approved anti-obesity drug

Orlistat was introduced in the pharmaceutical market by Roche under the name of 'Xenical' launched in the year 1998, was approved by the Food and Drug Administration (FAD), USA in 1999; it was represented as a 'magic medicine' for control of obesity for the sole reason that it inhibits potently and specifically breakdown of dietary triglycerides into absorbable fatty acids and monoglycerides (Cudrey, van Tilbeurgh et al. 1993) thereby resulting into 30% fat absorption and hence consequential weight loss.

1.6.2 An anti-cancer agent

Recent studies have demonstrated that tetrahydrolipstatin (Orlistat) possess antitumor properties to prostate cancer cells due to its ability to induce inhibition of thioesterase domain of human fatty acid synthase (FAS) lipogenic activity which is found to be significantly up-regulated in many tumors and is an indicator of poor prognosis (Menendez, Vellon et al. 2005)

(Menendez et al., 2005). For example, 50% of breast cancers exhibit high expression levels of FAS. THL eliminates tumour cells by inducing apoptosis.

1.6.3 An anti-mycobacterial agent

In mycobacteria it has been shown that tetrahydrolipstatin (Orlistat) interferes with the cell wall formation of mycobacteria by decreasing mycolic acid synthesis (Kremer, de Chastellier et al. 2005), leading to a defective mycobacterial cell wall. Tetrahydrolipstatin is shown to inhibit Rv3802c an essential cell wall lipase enzyme which is probably involved in the mycolic acid biosynthetic pathway. Therefore tetrahydrolipstatin (THL) eliminates both mycobacterial and tumor cells by interfering with the lipid metabolism.

1.7 Aims and Objectives of the present study:

Carboxyl ester hydrolases (EC 3.1.1) comprising of evolutionarily related enzymes mostly belonging to hydrolases superfamily sharing a common α/β-hydrolase protein fold and even though recent studies have revealed the findings suggestive of their pivotal role in disease manifestation by way of disruption of lipid metabolizing enzymes esterases/lipases and their pathways, yet only very few have been functionally annotated. This study therefore focuses attention on 4 putative mycobacterial lipases/esterase namely *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) with the following objectives:

1. To explore computational enzymology as an effective tool for *In silico* characterization of mycobacterial putative esterases/lipases and identifying potential inhibitors.

2. To investigate structural features of mycobacterial putative esterases/lipases and distinguishing lipolytic and non-lipolytic enzymes at the structural level itself.

3. *In vitro* biochemical characterization of Mycobacterial esterases/lipases and experimental validation of *in silico* predictions.

2. Materials and Methods

2.1 Molecular modelling

2.1.1 Sequence analysis:

The sequences of *BCG_1460c* (lipH probable lipase) (Uniprot Id: A1KII8), *BCG_2991c* (lipN probable lipase/esterase) (Uniprot Id: A1KMW4), *BCG_2950* (tesA probable thioesterase) (Uniprot Id: A1KMS3) and *BCG_3229* (lipV possible lipase) (Uniprot Id: A1KNK2) and their representative homologs were collected from the Uniprot database and multiple sequence alignments were performed using ClustalW (Larkin, Blackshields et al. 2007). This was found on the website [http://www.ebi.ac.uk/Tools/msa/clustalw2/] to identify the catalytic triad position, motifs and amino acid conservation.

2.1.2 Comparative modelling:

Structural homologs were identified using protein BLAST (Basic Local Alignment Search Tool) [http://blast.ncbi.nlm.nih.gov/] against Protein Data Bank (PDB) database and were selected as templates based on the highest sequence identity presented. Swiss PDB viewer was used to thread the protein sequence on its structural homologs using MUSCLE package of Swiss PDB viewer (Guex and Peitsch 1997) and thereafter initial structural alignments were generated. Most favourable rotamers were added to the structure using the rotamer library embedded in Swiss PDB viewer and a modelling request was submitted to Swiss model server [http://swissmodel.expasy.org/]. There were top ten models generated for each protein and validated using 4 different

validation soft wares on NIH MBI Laboratory for Structural Genomics and Proteomics [http://nihserver.mbi.ucla.edu/SAVES/]. The evaluation of native protein fold on validated 3D structure models was performed using ProSA program (Wiederstein and Sippl 2007) and were further refined by fragment-guided molecular dynamic simulations FG-MD program (Zhang, Liang et al. 2011) located at [http://zhanglab.ccmb.med.umich.edu/FG-MD/]. The refined model was energy minimized with GROMACS 4.0.7 package [http://www.gromacs.org/]. The resulting models were found to be energetically stable. The schematic representation of the steps involved in comparative modelling has been shown in figure 2.1

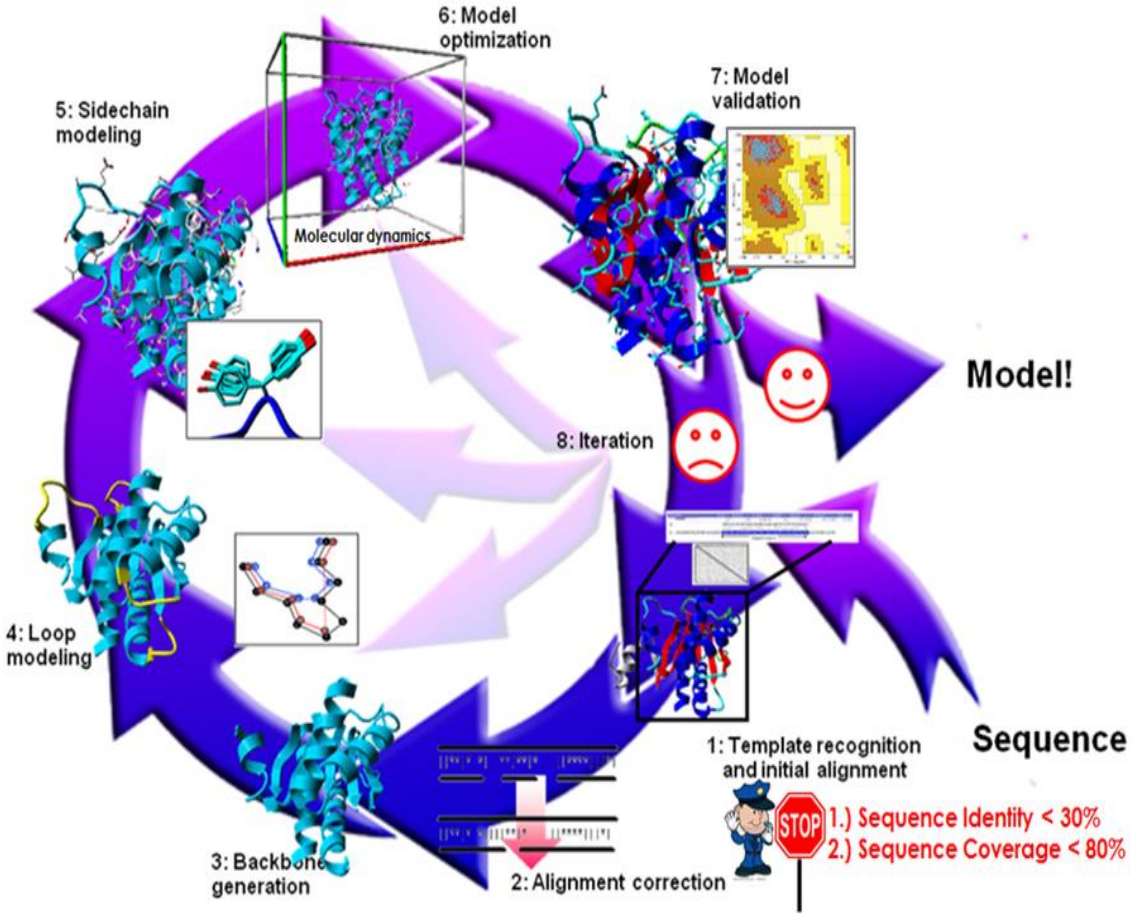


Figure 2.1: Schematic diagram of steps involved in Molecular modelling

(Source: adapted from http://swift.cmbi.ru.nl/teach/EMHOMX/EMBMOD_1.html)

2.2 Molecular Dynamics Simulations:

Protein stability of the energy minimized models in a solvent system was assessed using molecular dynamics simulations and periodic boundary conditions were applied to the structures in three dimension. By adding the sodium ions and replacing the water molecules that are 3.5 Å from the protein surface, the net charge of the system was neutralized. Simulations were run for 100 pico seconds with the solvent being equilibrated by a harmonic force constant 100 KJ nm^{-2} and the solute atoms were restrained. A production run of 10 nano second was used to check for the stability of protein models which is a plot of root mean square deviation of the backbone structure versus time. All simulations were performed at 300K temperature with velocity rescaling thermostat using Parrinello-Rahman barostat. The pressure was maintained at 1 atmosphere and the long-range electrostatic interaction with cut-off 12 Å was calculated using Ewald (PME) summation method while the hydrogen bonds in the atoms were constrained with the linear constraint solver (LINCS) algorithm (Hess, Bekker et al. 1997) and (Hess 2007).

2.3 Virtual Ligand Screening:

Computational docking of ligands in protein 3D structure model was performed using Autodock Vina (Trott and Olson 2010). All essential hydrogen atoms, solvation parameters and the united atom charges were added using the help of AutoDock tools (Morris, Goodsell et al. 1998). Affinity grid maps were constructed via and with the aid of Autogrid program. The Vander Waals and electrostatic calculations were done using distance dependent

dielectric functions and parameters set functions of Autodock Vina. Computational docking simulations have been performed using the Lamarckian genetic algorithm and local search method (Solis and Wets, 1981). The initial orientations, position, torsion angles of ligands were set randomly and during docking all rotatable torsions were released. For every docking experiment, 500 independent docking runs were performed involving 30000 maximum energy evaluations and the population size was set to 200. The ligand 2D-structures were drawn in ChemDraw and the 3D structures of ligands were constructed with BUILDER module of molecular modelling program Insight-II assigning standard geometric parameters. The atomic charges were assigned to ligand using Amber potential. These ligands were then subjected to force field until the root mean square energy gradient became less than 0.005 Kcal/mol. All hydrogen atoms were added to the 3D structure models and Amber all-atom charges were assigned to the whole protein.

The active site was then analysed using the CASTp program (Dundas, Ouyang et al. 2006) to recognize and characterize accessible pockets in the protein 3D model based on two parameters: the solvent accessible surface and the molecular surface. The active site was defined within a radius of 25 Å which covered 94% of the total residues in the protein ensuring that the size of the active site is sufficiently large enough to accommodate the whole ligand binding pocket and also allow for rotation and translation of ligands. The schematics of computational docking are shown in fig.2.2

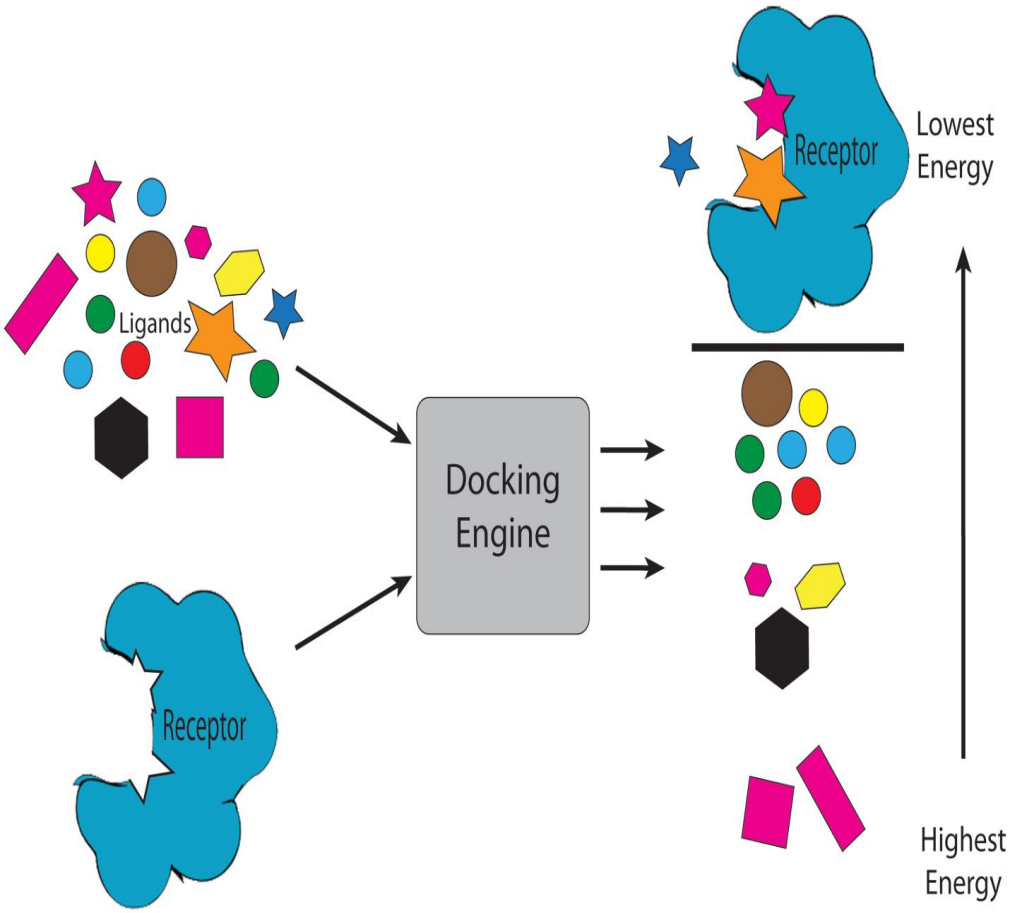


Figure 2.2: Schematic diagram of Computational Docking

(Source: (Jacob, Andersen et al. 2012))

2.4 Bacterial strains and cultures

2.4.1 Bacterial Strains

M. bovis BCG Pasteur strain (ATCC 35734) and *M. smegmatis* (mc2155) were used in the experiments.

2.4.2 Bacterial Culture Media

Cultures of mycobacteria were grown under aerobic conditions. Inoculum was prepared from colony of growing cells and pre-culture was grown in 7H11 media. Aerobic *M. bovis* BCG were grown in roller bottles with initial OD (optical density) measured at 600nm (OD_{600}) of 0.5. The roller culture bottles were rotated at 1rpm for three days.

Middlebrook 7H11: The mycobacterial cultures were grown using this culture media. Middlebrook 7H11 (Biomed Diagnostics, BD Difco Mycobacteria 7H11 Agar, Catalogue No. 283810) supplemented with 10% oleic acid-dextrose-albumin-catalase enrichment and 0.5% of glycerol (Biomed Diagnostics, BD Difco Middlebrook OADC Enrichment catalogue no.212351) at 37 °C. Whenever required, antibiotics were added to the *M. bovis* BCG culture media with the following concentrations: kanamycin (Sigma-Aldrich, USA) at 25 µg/ml and hygromycin (Roche,Germany) at 80 µg/ml .

Middlebrook 7H9 broth: This liquid media was used for growing mycobacterial cultures. The Middlebrook 7H9 (Biomed Diagnostics, BD Difco Mycobacteria 7H9 Broth, Catalogue No. 2713100) supplemented with 0.2% glycerol, 0.05% Tween-80 and 10% (v/v) Albumin-Dextrose-Saline (ADS: 950ml dH20, 8.1g NaCl, 50g Bovine Serum Albumin Fraction V, 20g

D-dextrose). The whole medium was filter sterilized with o.22µM filter and stored at 4°C until further use.

2.4.3 Glycerol stock of bacteria

M.bovis BCG and *M. smegmatis*: Glycerol stocks of cultures were prepared by re-suspending in Middlebrook 7H9 broth containing 25% glycerol (v/v) and stored as 0.5ml aliquots at -80ºC.

2.5 Cloning Procedures

2.5.1 Genomic DNA Isolation (Mycobacteria)

The mycobacterial cells were grown to an OD of 0.8 (measured at 600nm), cells were then harvested by centrifuging at 3000g for 10mins at 4°C, washed with PBS-T (0.05% of Tween-80). The pellet obtained was re-suspended in 600µL buffer having 3% SDS, 1mM Tris-HCL with 100Mm sodium chloride. Cells were transferred to tubes having silica beads (0.1mm in diameter) and then displaced using bead beater at 50rpm for 5mins at 4°C. Mixture of phenol/isoamyl alcohol/chloroform in the ratio (49:2:49 v/v/v) was added and then final volume made up to 2 X. Upon centrifuging at 16,000g for 5mins at 4°C the aqueous phase was collected then washed using 500µl of 70% ethyl alcohol. Supernatant was then recovered pellet was dried at room temperature for 10mins. Precipitated DNA pellets were then re-suspended in 100µl of TE buffer and then storing it at 20°C until further use.

2.5.1 Preparation of Over-expression Plasmids

The putative esterases and/or lipases genes namely, *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) were amplified by using PCR with genomic DNA isolated from *M. tuberculosis* H37Rv with primer details as given below:

Primers used for gene cloning and amplification		
BCG_1460c (lipH)	Forward	GCAGATCTATGACCAAGAGTCTGCCAGGT
	Reverse	GCAAGCTTTTATGCGTGCAACGCCCTCTT
BCG_2991c (lipN)	Forward	GCAGATCTATGACCAAGAGTCTGCCAGGT
	Reverse	GCAAGCTTTCAAACCCGGCTAAGGTGCGC
BCG_2950 (tesA)	Forward	GCAGATCTATGCTGGCCCGTCACGGACCA
	Reverse	GCAAGCTTCTAAGCTCGATCATGCCATTG
BCG_3229 (lipV)	Forward	GCAGATCTTTGCCCGAAATCCCCATCGCC
	Reverse	GCAAGCTTCTAGCGCGGACCCAGTCGACT

Note: Restriction sites are underlined: forward BglII; reverse HindIII

The BglII and HindIII sites of the E.coli-Mycobacterium shuttle vector pMV262 were inserted with PCR fragments under a constitutive HSP60 promoter.

2.6 *Mycobacterium bovis* BCG competent cell preparation, transformation (electroporation) and selection of transformants

Inoculum from growing BCG cells was grown in 7H9 broth media (no antibiotics) the culture was grown to ~0.8 optical density (OD_{600}). The culture was centrifuged at 3700rpm and cells were re-suspended in 1ml of 0.05% tween80. 200 µl aliquot was stored at -80ºC. *M.bovis* BCG cells grown up to an OD_{600}. Cuvettes (Biorad) with an electrode gap of 2mm were used for electroporation and 1µl of plasmids was added to each cuvette and mixed gently. Using Biorad gene pulser Xcell and pulsed at 2500V, 800 ohms, 25µF transformants carrying the plasmids were selected on 7H11 plates containing suitable antibiotic (hygromycin) the plates were left at 37 ºC to allow for colonies to appear and thereafter an incubation period was allowed for four weeks to have them grown until a visual turbidity was reached.

2.7 Preparation of Whole cell lysate (WCL)

The mycobacterial cultures were grown on Middlebrook 7H11 (Biomed Diagnostics, BD Difco Mycobacteria 7H11 Agar, Catalogue No. 283810) supplemented with 10% oleic acid-dextrose-albumin-catalase enrichment and 0.5% of glycerol) (Biomed Diagnostics, BD Difco Middlebrook OADC Enrichment catalogue no.212351) for four weeks. Bacterial colonies were harvested and washed thrice with phosphate buffered saline with tween80 (PBST) and cells were vortexed. Bacterial cells were lysed using probe sonicator with the following parameters: 60% amplitude, 5 mins, 10 seconds

pulse on 5 seconds off and the lysed cells were centrifuged at 14000rpm; protein was estimated using the Lowry's protein estimation method.

2.8 Fluorescent click chemistry

Samples were prepared for click reaction by washing the *M. bovis* BCG cells with phosphate buffered saline with tween80 (PBST) thrice and once with phosphate buffered saline (PBS) and centrifuged at 3500g for 15 minutes at 4 ºC thereafter lysed in phosphate buffered saline (PBS) at 50% amplitude. 100µg of protein was used for the reaction in which drug was incubated with protein. The amounts of reagents used for each reaction have been described in table 2.1. After incubation; samples were prepared for gel electrophoresis run.

Sample	Protein conc.	PBS	Lysate	THL Probe	DMSO	Incubation time
Stock conc.	µg/µl	1x	µg/µl	1 mM	100%	
Final conc./rxn.			100 µg	5 µM	Final 2.8%	No shaking for 1 hour/RT
No probe	3.6	73 µl	27 µl	–	0.5 µl	1 hour
With probe	3.6	73 µl	27 µl	0.5 µl	–	1 hour

Table 2.1 Click reaction

2.9 Gel Electrophoresis

Samples were prepared for the gel run by adding 600 µl of acetone and left at -20 ºC for 24 hours cells were centrifuged at 14,000 rpm then washed with 200 µl of methanol. Subsequently, cell lysis was done using sonicator with the following parameters: 60% amplitude, 5 mins, 10 sec pulse on/ 5 sec off after cell lysis 50 µl of SDS dye was added and boiled at 95 ºC for 5 mins. Equal amounts of 10 micro gram protein was separated on 12% SDS-polyacrylamide gel, ran at 120 V for 110 mins followed by in-gel fluorescence scanning with Typhoon 9410 Variable Mode Imager scanner (GE Amersham, UK) . The cyc3/cyc5 scan protocol adopted was as follows: $3*10^8$ cells for each staining

and washed once with phosphate buffered saline with 0.05%tween80 (PBST) and centrifuged at 3000g for 5mins at 4 ºC. Subsequently washed with phosphate buffered saline (PBS) after which 10ng of LD540 dye was added and incubated for 10 mins at room temperature. 500µl of 4% formaldehyde was added and tubes were centrifuged at 3000g for 5mins at 4 ºC. Cells were again washed with phosphate buffered saline (PBS) and permealized with 500 µl of 0.25% Triton-X 100 in PBS for 15 mins .

2.10 Enzymatic Assays

The enzymatic assays were performed with whole cell lysates of BCG cells overexpressing proteins *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) using p-nitrophenyl (pNP) esters (Sigma Aldrich) with carbon chain lengths of C_2, C_4 and $C_{16.}$ The pNP release was measured at 400nm by using a 96 well plate spectrophotometer. The enzymatic reaction was performed with 225µl of NaDC tris buffer (Tris 15.14mg, Nacl-0.8766g, Sodium deoxycholate -1mM (20.7mg) measured and dissolved in 25ml of milliq water and the pH of the whole medium was adjusted to pH 8.0). This was added to each reaction along with 2.5µg/µl of protein concentration and 1mM of pnP-C_2/C_4/C_{16} substrate incubated for 30mins at 37 ºC. The final volume of each well was kept at 250 µl and the absorption was measured at 400nm using a 96 well plate spectrophotometer and readings were recorded at 0, 1, 2.5, 5 and 10 minutes time points. All reactions were performed in triplicates. The schematics of pNP assay are depicted in fig.2.3

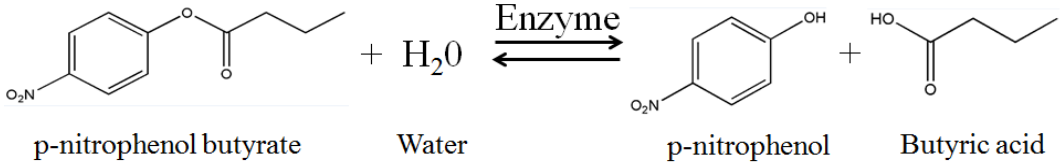


Figure 2.3: Scheme of pNP Assay

Table 2.2 Concentrations of reagents used

Enzymatic assay of *M. bovis* overexpressing clones using *p*-nitrophenylphosphate $C_2/C_4/C_{16}$ as substrate			
	Stock Conc.	Working conc.	Volume
Buffer	-	-	225µl
Overexpression protein whole cell lysate	-	2.5µg	1µl
pNP substrate	10 mM	1 mM	25 µl

2.11 Inhibitor assays

Fresh stock of frozen cells at -80 ºC was used for every experiment. The vials were thawed in ice for 30 minutes and fresh substrate p-nitrophenol butyrate (pnP-C_4) used in the inhibition experiments was prepared to 1mM concentration (dissolving 2µl of pnP-C_4 in 9.7ml of dimethyl sulfoxide), buffer NaDC tris buffer (Tris 15.14mg , Nacl-0.8766g, Sodium deoxycholate -1mM (20.7mg) measured and dissolved in 25ml of milli-Q water and the pH of the whole medium was adjusted to pH 8.0). 225 µl of NaDC tris buffer, 2.5µg of protein and different concentrations of inhibitors such as THL

(tetrahydrolipstatin) E600 (diethyl-p-nitrophenylphosphate) was used from .01µM to 100µM (volume kept at 1µl) were added to each well of 96 well plate. DMSO (dimethyl sulfoxide) concentration was maintained at 0.004% in each well, incubation time was 30 mins at 37 ºC and the absorption was measured at 400nm using a 96 well plate spectrophotometer. Measurement readings were recorded at 0, 1, 2.5, 5 and 10 minutes time points. All reactions were performed in triplicates. The template indicating the component of each well of the 96 well plate has been shown in figure 2.4

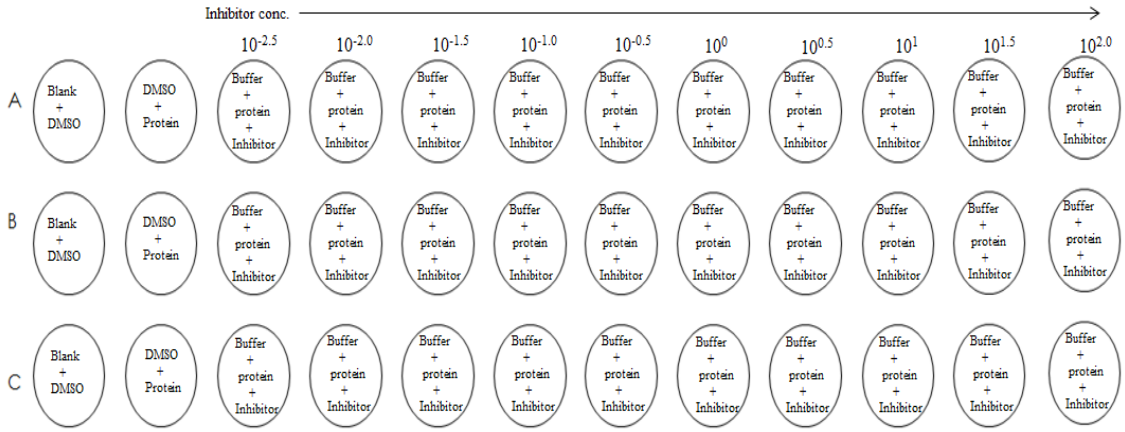


Figure 2.4: Template design of a 96 well plate used for Inhibitor assays

3. Results

3.1 Molecular modelling of protein 3D structures

In order to understand the insight as well as structural details and characterize four putative esterases/lipases belonging to (α/β) hydrolase family viz. *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase), a computational prediction of their ligand specificity for catalytic triad was performed. 3D model structures were generated using molecular modelling protocol, at first we validated the molecular modelling protocol by predicting *in silico* the 3D structure of protein named thioesterase domain of fatty acid synthase (Human FAS-TE) (Protein Databank ID: 2PX6) whose X-ray 3D structure is well known this acted as our positive control. Molecular overlay of the modelled 3D structure with known 3D structure of FAS-TE (figure 3.1 (a)) revealed that *in silico* modelled FAS-TE structure could mimic the known FAS-TE structure reasonably well with a carbon alpha backbone (Cα) root mean square deviation (rmsd) of 0.192Å (figure 3.1b) and an acceptable statistics in Ramachandaran plot (Suplatov, Besenmatter et al. 2012). The 3D model structure of *BCG_1460c* (lipH probable lipase) was generated based on structural homologues of 3 thermophilic esterases with significant sequence identity in the range of 40 to 44% namely *Alicyclobacillus acidocaldarius* (carboxyl esterase Est2) (Protein data bank ID: 1U4N) with 44% sequence identity, *Archaeoglobus fulgidus* (carboxyl esterase AFEST) (Protein data bank ID: 1JJI) with 41% sequence identity and bacterial acetyl esterase (HerE) (Protein data bank ID: 1LZL) had 40%

sequence identity. It was also revealed that *BCG_2950* (tesA probable thioesterase) has 2 bacterial thioesterases structural homologues with sequence identity in the range of 31-33% namely *Streptomyces coelicolor* (Thioesterase) (Protein data bank ID: 3QMV) having 33% sequence identity and thioesterase from *Amycolactopsis mediterranei* (Protein Data Bank ID: 3FLB) had 31% sequence identity with protein sequence of *BCG_2950* (tesA probable thioesterase). For *BCG_2991c* (lipN probable lipase/esterase) the BLAST searches found that it has structural homologs in 2 carboxyl esterases with sequence identity ranging from 39% to 41% : Hormone sensitive lipase-like Carboxylesterase from *Sulfolobus Tokodaii* (Protein data bank ID: 3AIO) (41%) , carboxyl esterase (Est2) *Alicyclobacillus acidocaldarius* (Protein data bank ID: 2HM7) (39%) while *BCG_3229* (lipV possible lipase) shows structural homologs in the sequence identity range of 27% to 31% with two esterases: thioesterase domain from Curacin biosynthetic pathway (Protein data bank ID: 2HM7) (31%) and Rv0045c from *Mycobacterium tuberculosis* (Protein Data Bank ID: 3P2M).

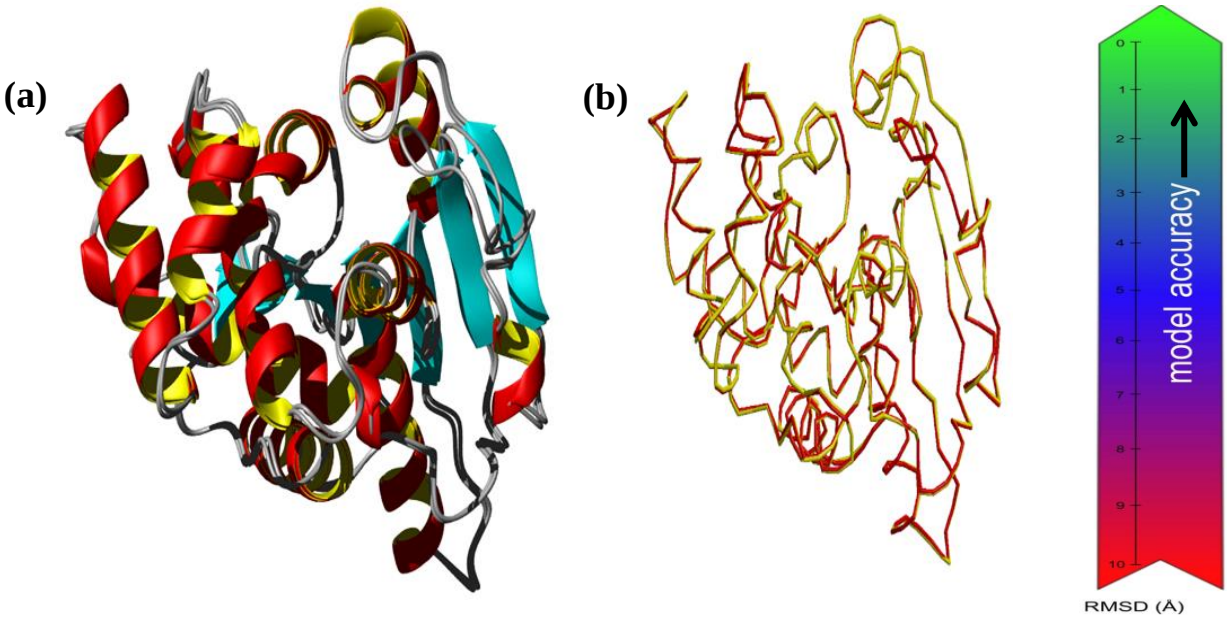


Figure3.1: (a) 3D structure Overlay of FAS-TE modelled structure over FAS-TE known X-ray 3D structure in protein data bank (2PX6) **(b)** Cα – backbone overlay r.m.s.d **0.192Å** (**red:** *in silico* modelled 3D structure**, yellow:** X-ray known 3D structure Protein Databank ID: 2PX6)

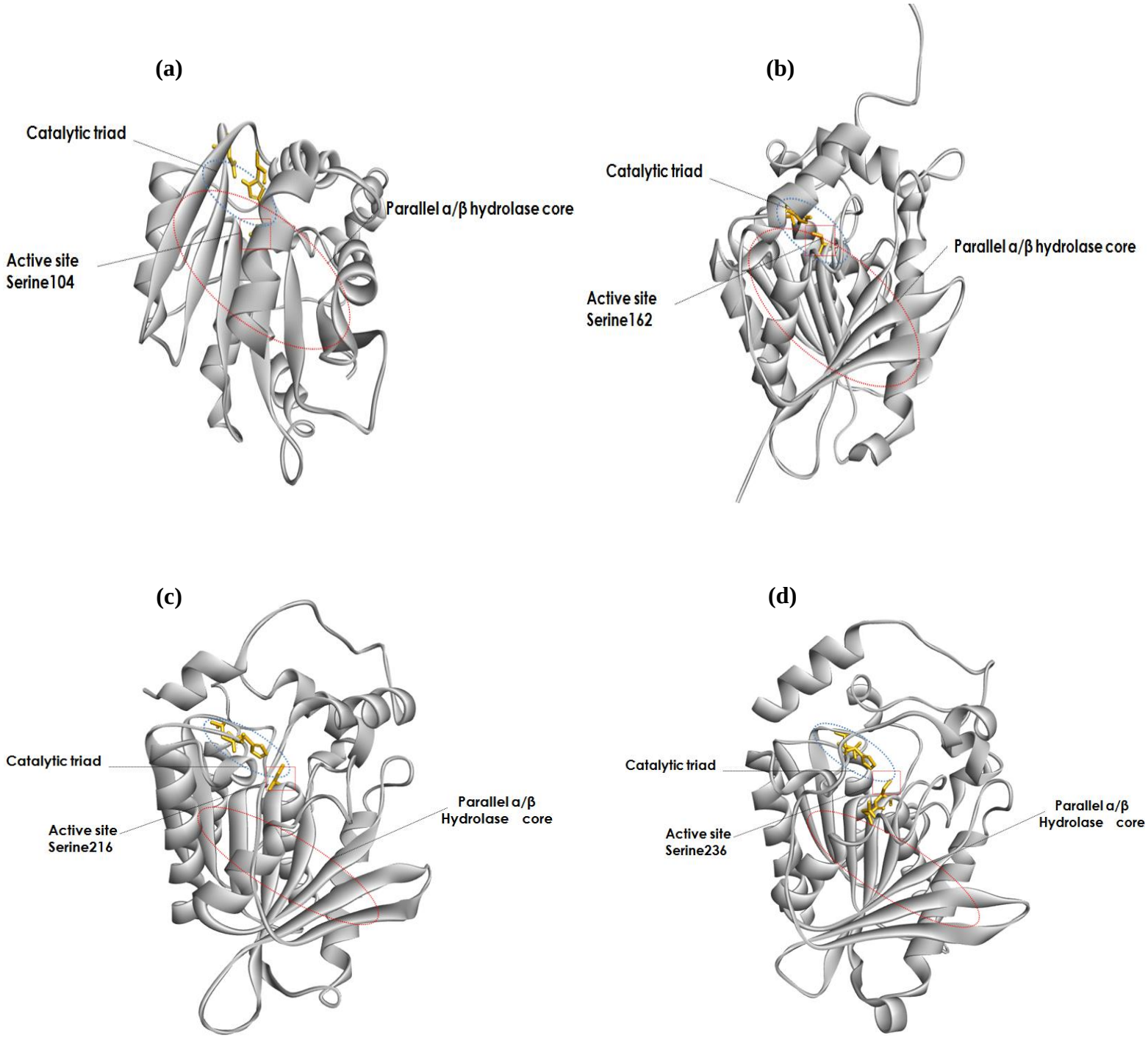


Figure3.2 3D structure models: **(a)** *BCG_2950* (tesA probable thioesterase) **(b)** *BCG_1460c* (lipH probable lipase) **(c)** *BCG_2991c* (lipN probable lipase/esterase) **(d)** *BCG_3229* (lipV possible lipase)

The 3D structure models (figure 3.2) indicated that all 4 putative esterases/lipases namely *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) are globular proteins similar to other alpha/beta (α/β) hydrolase enzymes and consist of 11 α- helices and a central β-sheet core containing 6 parallel β- strands. The GXSXG motif characteristic of esterases/lipases was found to be conserved in all 4 enzymes viz. *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) whereas the invariant motif HGG of hormone sensitive lipase family was conserved in *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase). The catalytic triad was known to be composed of nucleophilic serine which is the active site, a charge relay network aspartate and proton carrier histidine. The structure validation report from four different validation soft wares on NIH MBI laboratory [http://nihserver.mbi.ucla.edu/SAVES/] for structural genomics and proteomics are shown in fig.3.3 whereas angle distribution of amino acids in 3D structure models is shown in fig.3.4

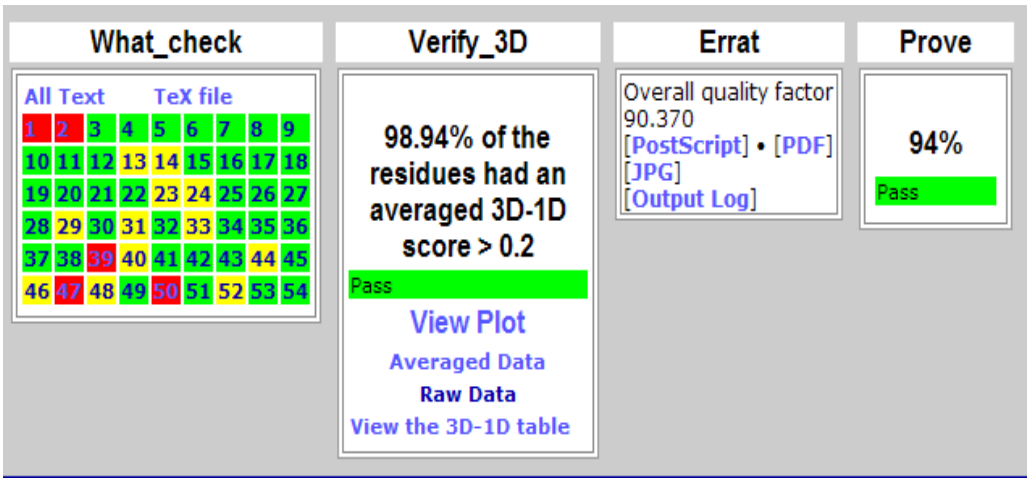
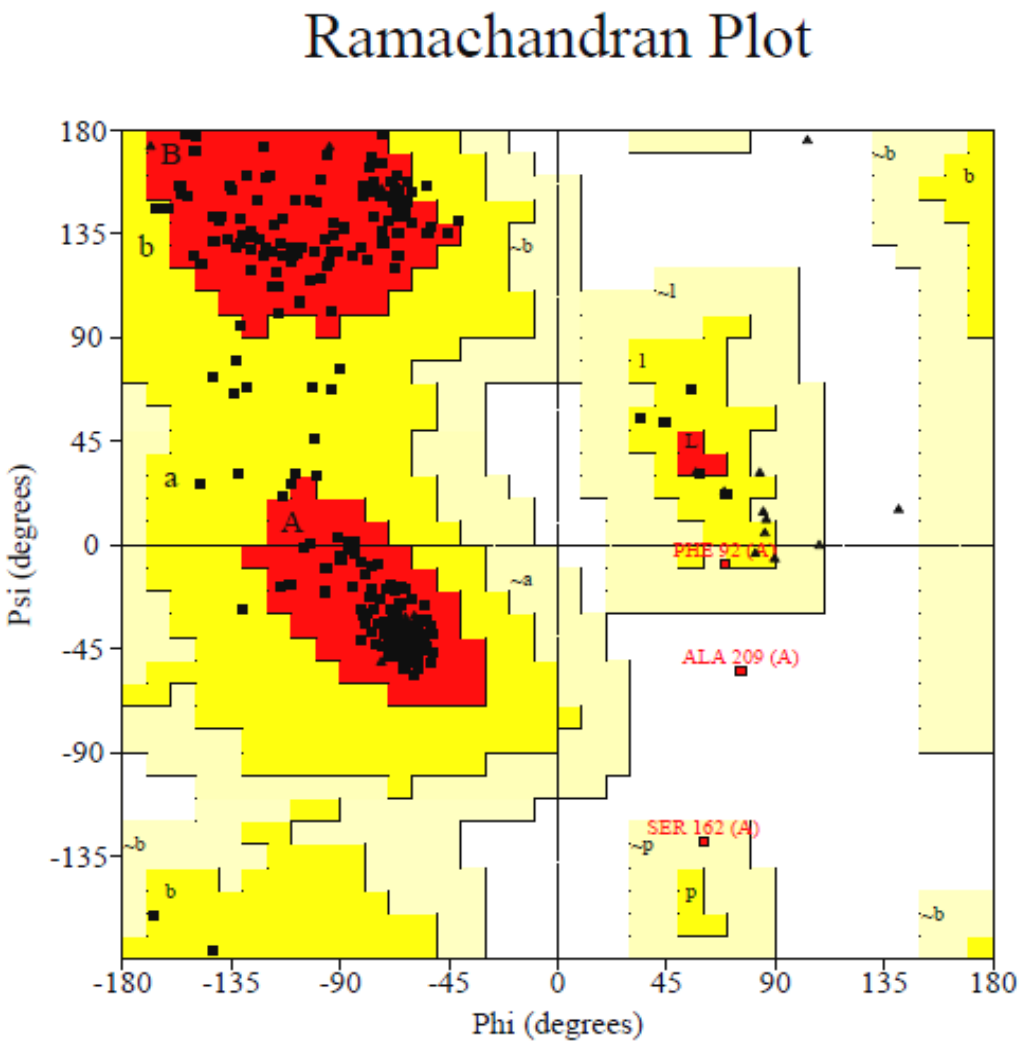


Figure 3.3: Structure validation report from 4 different validation soft wares on NIH MBI Laboratory for Structural Genomics and Proteomics [http://nihserver.mbi.ucla.edu/SAVES/]



Plot statistics

Residues in most favoured regions [A,B,L]	90.7%
Residues in additional allowed regions [a,b,l,p]	8.6%
Residues in generously allowed regions [~a,~b,~l,~p]	0.7%
Residues in disallowed regions	0.0%

	100.0%

Figure 3.4: Ramachandran plot statistics of backbone dihedral angle distribution of amino acids in the 3D structure model having no disallowed regions.

Fig. 3.5 illustrates protein structure analysis from PROSA (protein structural analysis) web server as depicted below whereas fig 3.6 illustrates molecular dynamic simulation on validated 3D structure models.

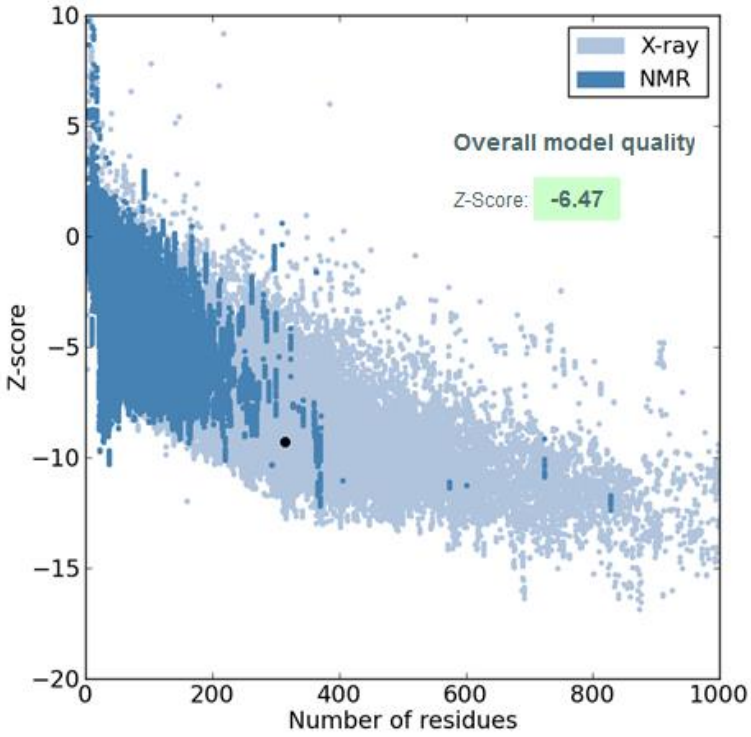


Figure 3.5: Protein structure analysis from PROSA (protein structural analysis) web server shows 3D structure models have Z-score -6.47 in the acceptable range for near native conformation of X-ray crystal structures

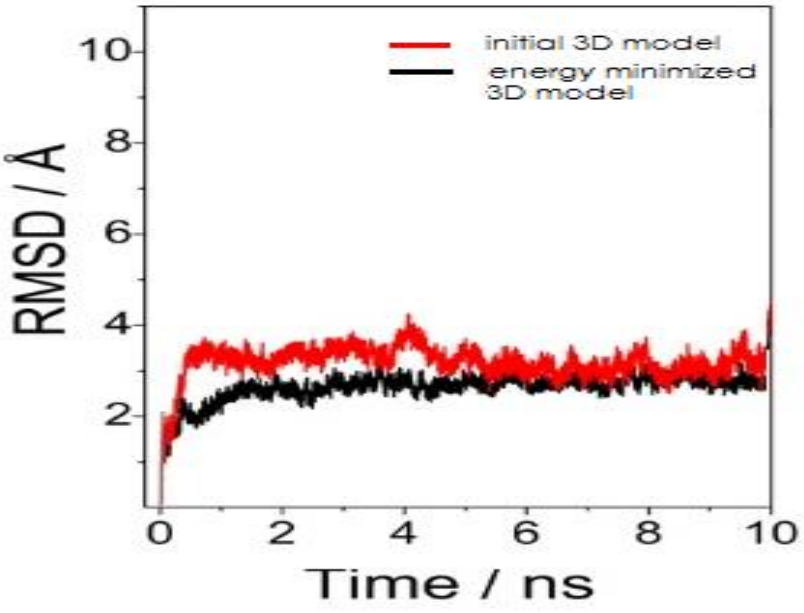


Figure 3.6: Molecular dynamic simulations of modelled 3D structures show that the energy minimized 3D model structures are energetically stable

3.2 Molecular Structure based Ligand Screening

In order to identify natural substrates/potential inhibitors and to characterize the substrate specificity of *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase), a structure based virtual ligand screening was performed. At first a *in silico* library of potential ligands was constructed based on literature review of substrate preferences of well characterized carboxyl esterases in mycobacterial species. This resulted into a *in silico* ligand library of natural substrates such vinyl esters of varying acyl chain lengths from short chain vinyl acetate (C_2) to long chain palmitate (C_{16}) along with triacylglycerol substrates (TAGs) varying from short chain TAGs such as tripropionate (C_3) to long chain TAGs trioleate (C_{18}) with potential inhibitors of serine hydrolases such as THL, PMSF and E600.

The *in silico* potential ligand library was thereafter screened using molecular docking engine Autodock (Trott and Olson 2010) into the active site of 3D structures of BCG*_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) and results were subjected to further analysis and interpretation.

3.2.1 Evaluation of Ligand Screening Results

The ligand screening results were evaluated based on two scoring functions:

a.) Ligand binding affinity/energy towards its target enzyme.

b.) Distance to active site (serine in all cases) which describes a near attack conformation (NAC) (Hur, Bruice et al. 2003) of the ligand in the binding site.

The enzyme-ligand docked structures were ranked by frequency binding in near attack conformation obtained from 500 independent docking runs (p-value <0.05). The ligand specificity to its target enzyme was confirmed based on the highest binding affinity and the least distance to active site (serine). A threshold for an acceptable near attack conformation of ligand distance to active site should be less than 3.5Å (Suplatov, Besenmatter et al. 2012) while the ligand binding affinity should fall within the range of -4 Kcal/mol to -8 Kcal/mol (fig 3.7 a,b,c,d).

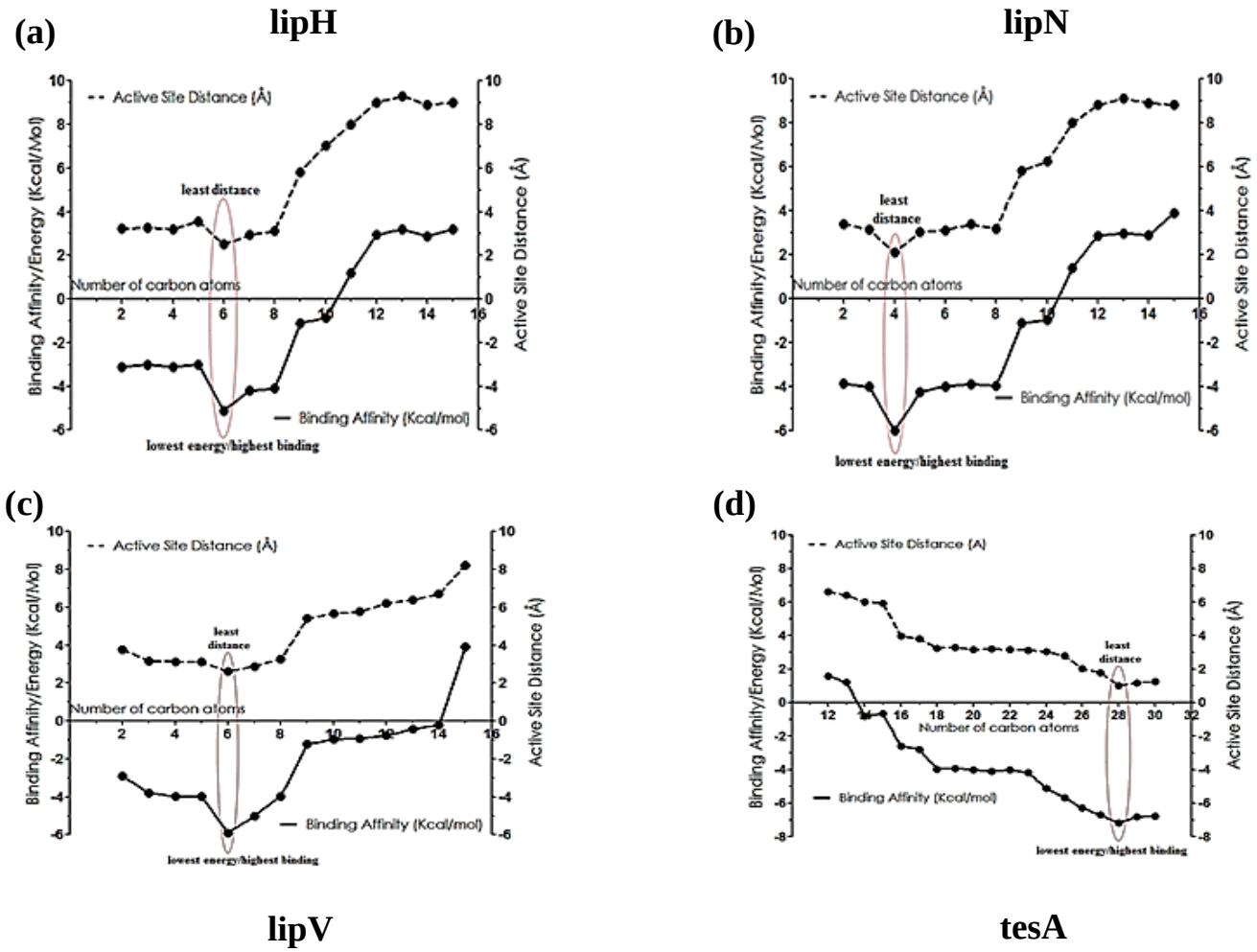


Figure 3.7: Short (C_2) to long (>C_{12}) acyl chain ester ligands screening results, highlighted circle in red show most favourable ligand (highest ligand specificity) based on its lowest energy and least distance to active site (Serine). Graphs showing the ligand specificity pattern for (a) lipH (b) lipN (c) lipV (d) tesA

Table 3.1 Summary of potential substrates screening results

	Vinyl esters		TAGs	
Protein name	**Best**	**Up to**	**Best**	**Up to**
lipH *BCG1460c*	C6 (-5.11 Kcal/mol)	C4-C12	C4 (-4.16 Kcal/mol)	C3-C4
lipN *BCG2991c*	C4 (-5.9 Kcal/mol)	C4-C12	C4 (-5.3 Kcal/mol)	C3-C4
lipV *BCG3229*	C6 (-6.0 Kcal/mol)	C4-C14	C4 (-4.16 Kcal/mol)	C3-C6
tesA *BCG_2950*	C28(-5.9 Kcal/mol)	C14-C30	C24 (-5.11 Kcal/mol)	C12-C28

Based on the two criteria evaluation of structure based virtual screening, results revealed that *BCG_1460c* (lipH probable lipase) favours docked near attack conformations with short acyl chain esters from vinyl butyrate (C_4) up to vinyl laurate (C_{12}) and short chain TAGs such as tripropionate having identical fatty acyl chains (3:0/3:0/3:0) and tri butyrate (4:0/4:0/4:0) (table 3.1) with highest ligand specificity for vinyl caproate (C_6) and a binding affinity of -5.11 Kcal/mol and 2.5Å distance to active site (serine) (figure 3.7(a)). For *BCG_2991c* (lipN probable lipase/esterase), it had a similar binding affinity towards short acyl chain vinyl esters and TAGs ranging from vinyl butyrate (C_4) up to vinyl lauraterate (C_{12}) and tripropionate having identical fatty acyl chains (3:0/3:0/3:0) to tri butyrate (4:0/4:0/4:0) (table 3.1) with highest ligand specificity for vinyl butyrate (C_4) and a binding affinity of -5.9 Kcal/mol with 2.62 Å distance to active site (serine) (figure 3.7(b)). On the other hand *BCG_3229* (lipV possible lipase) also depicted ligand preference towards short chain vinyl esters such as vinyl butyrate (C_4) up to (C_{14}) and TAGs ranging from tripropionate having identical fatty acyl chains (3:0/3:0/3:0) to tricaproate (6:0/6:0/6:0) (table 3.1) with highest ligand specificity for vinyl caproate (C_6) having a binding affinity of -6.0 Kcal/mol and 1.92Å distance to active site (serine) (figure 3.7(c)). However, interestingly *BCG_2950* (tesA probable thioesterase) showed preference for very long chain lengths ($>C_{16}$) with the highest ligand specificity for phthiocerol (C_{28}) having a binding affinity of -7.2 Kcal/mol and 1.02Å distance to active site (serine) (figure 3.7(d)).

Table 3.2 Summary of potential inhibitors ligand screening results

Protein	Ligand	Ligand function	Distance to active site	Binding affinity/energy (Kcal/mol)
lipH	**THL**	Lipase inhibitor	**1.82 Å**	**-7.10**
lipH	E600	Lipase inhibitor	2.02 Å	-6.70
lipH	PMSF	Protease Inhibitor	2.12 Å	-5.60
lipN	**THL**	Lipase inhibitor	**2.08 Å**	**-6.5**
lipN	E600	Lipase inhibitor	2.23 Å	-5.2
lipN	PMSF	Protease Inhibitor	2.32 Å	-4.9
tesA	**THL**	Lipase inhibitor	**1. 91Å**	**-7.2**
tesA	E600	Lipase inhibitor	2.37 Å	-5.6
tesA	PMSF	Protease Inhibitor	2.38 Å	-5.4
lipV	**THL**	Lipase inhibitor	**1. 84Å**	**-7.6**
lipV	E600	Lipase inhibitor	2.06 Å	-5.2
lipV	PMSF	Protease Inhibitor	2.62 Å	-4.4

The screening for potential inhibitors resulted in the prediction of THL (tetrahydrolipstatin) as the most potent inhibitor of *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA

probable thioesterase) and *BCG_3229* (lipV possible lipase) as compared to E600 and PMSF as shown above in table 3.2.

3.3 Over expressing mycobacterial putative esterases/lipases.

BCG_1460c (lipH), *BCG_2991c* (lipN), *BCG_2950* (tesA) and *BCG_3229* (lipV) were found to be over expressed and successfully transformed. The fluorescent gel image (fig.3.8) clearly demonstrates an over-expression in protein levels of *BCG_1460c* (lipH probable lipase) and *BCG_2950* (tesA probable thioesterase) having molecular weight of 34.0 kDa and 29.0 kDa respectively were seen as thick bands compared to wild type *M. bovis* BCG (non-over expressed).

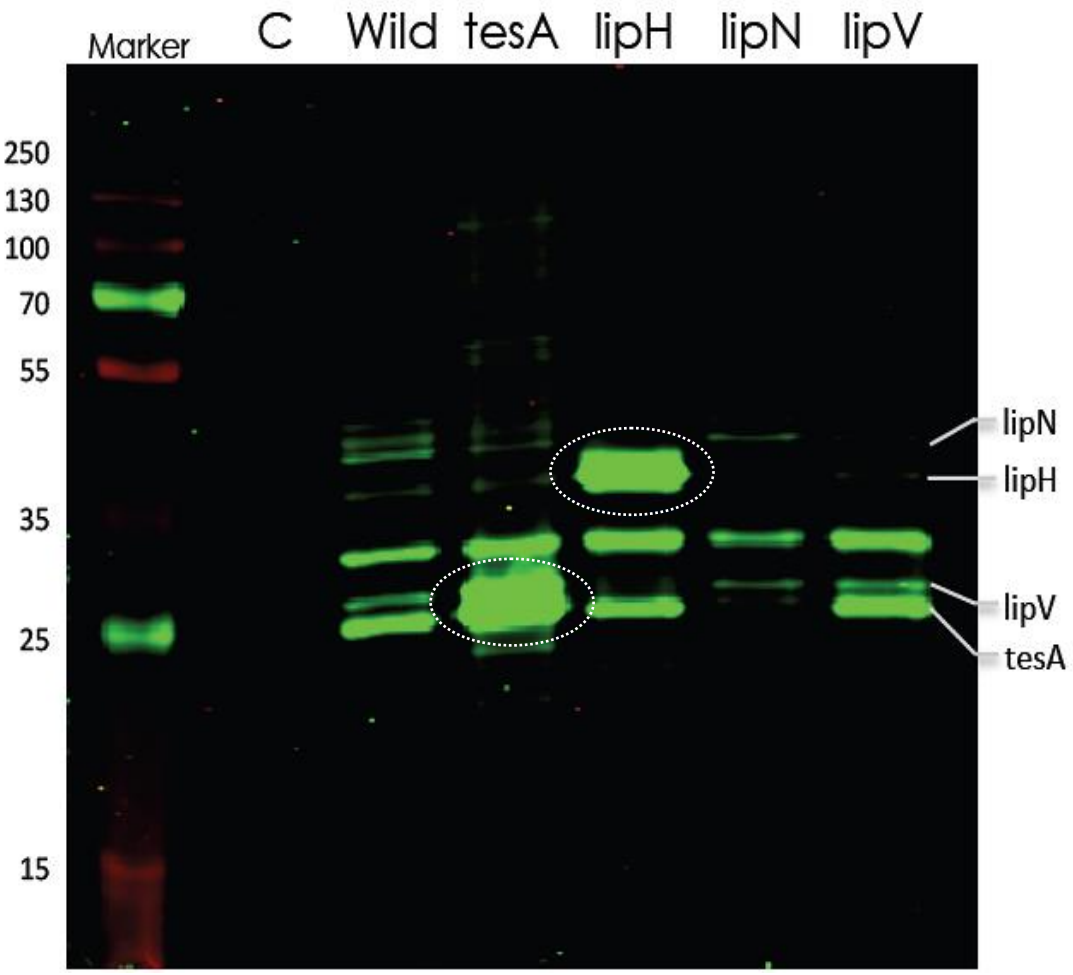


Figure 3.8: SDS-PAGE fluorescent gel image showing change in protein levels of *M. bovis* BCG over expressing lipH and tesA

3.4 Biochemical Characterization

3.4.1 Enzymatic Assays

To characterize the activities of *BCG_1460c* (lipH) and *BCG_2950* (tesA) enzymes, the whole cell lysates of over expressed *BCG_1460c* (lipH) and *BCG_2950 (*tesA) were assayed with synthetic vinyl ester substrate para-nitrophenol-butyrate (C_4). A ~2.5 times fold change was observed in the absorption (measured at 400nm) for *BCG_1460c* (lipH) as compared to wild type *M. bovis* BCG (non-over expressed) (fig.3.9)

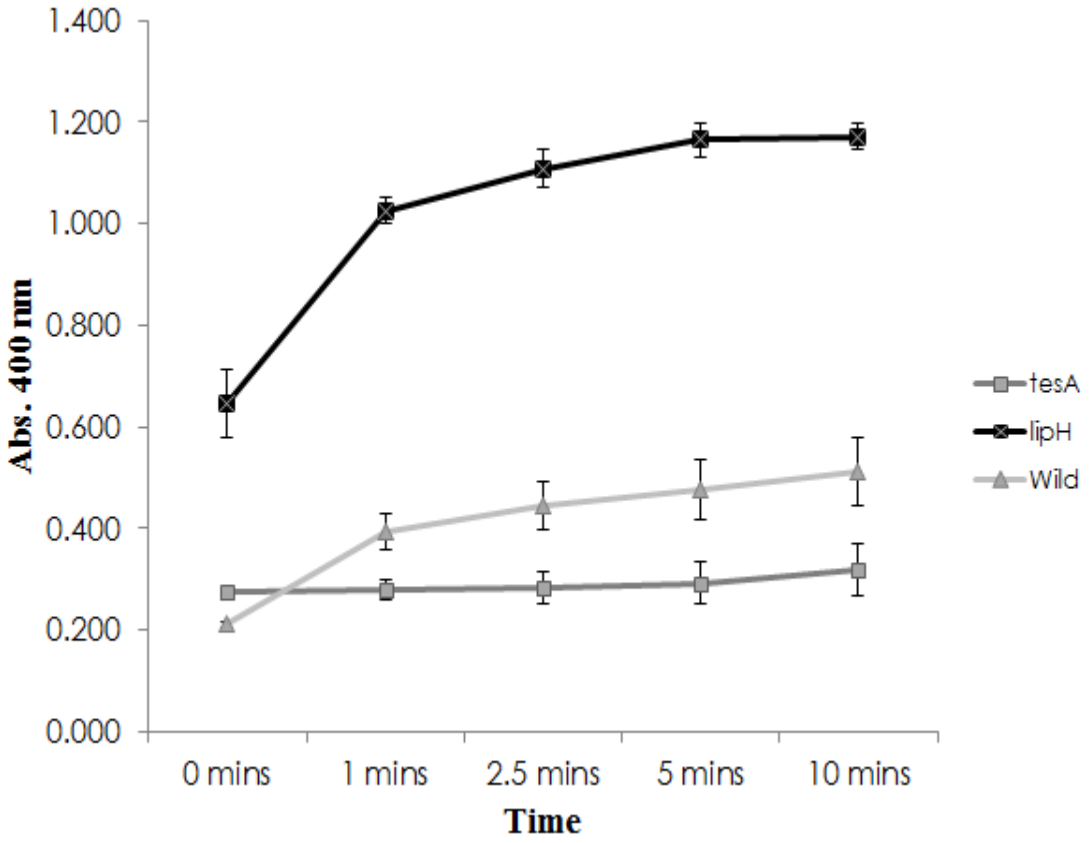


Figure 3.9: Enzymatic assay with whole cell lysate of overexpressed *BCG_1460c* (lipH) and BCG_*2950* (tesA) using substrate para-nitro phenol-butyrate (C_4) (n=2 biological replicates)**

Within 2.5 mins of the enzyme reaction almost 80% of substrate para-nitrophenol-butyrate (C_4) was converted into products i.e. butyric acid and para-nitrophenol the latter showing maximum absorption at 400 nm. However, overexpressed BCG_*2950* (tesA) indicated no change in absorption and was lesser compared to the wild type (non-overexpressed) (fig3.9). In order to ensure adequate accuracy, the enzymatic assay was performed with two biological and two technical replicates.

3.4.2 *BCG_1460c* (lipH probable lipase) shows short-chain esterase activity

To further investigate the substrate specificity of *BCG_1460c* (lipH), enzyme assay was performed using synthetic substrates of vinyl ester substrates including short–chain esters such as para-nitrophenol-acetate (C_2) and para-nitrophenol-butyrate (C_4) and long-chain ester para-nitrophenol-palmitate (C_{16}) with whole cell lysate of over expressed *BCG_1460c* (lipH).

The assay results revealed that *BCG_1460c* (lipH probable lipase) shows a short-chain esterase activity towards para-nitrophenol-acetate (C_2) and para-nitrophenol-butyrate (C_4) (fig3.10) and there was no activity observed towards long chain-vinly esters (C_{16}) i.e. para-nitrophenol-palmitate (C_{16}) as shown in fig3.10.

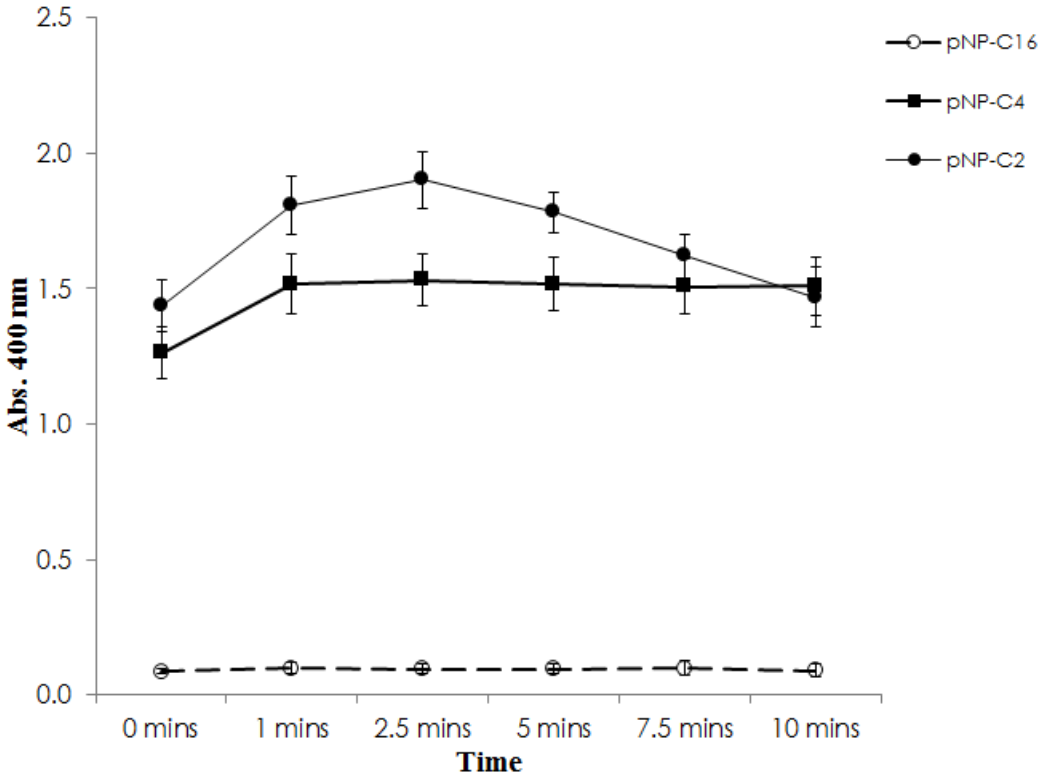


Figure 3.10: Enzymatic assay of over expressed *BCG_1460c* (lipH) with varying acyl chain length substrates para-nitrophenol-acetate (C_2), butyrate (C_4), palmitate (C_{16}) (n=2 biological replicates)**

3.5 THL (Tetrahydrolipstatin) strongly inhibits short-chain esterase activity of *BCG_1460c* (lipH)

Inhibition experiments were performed using well known lipase inhibitor tetrahydrolipstatin (THL) and E600 (diethylparanitrophenyl phosphate) the latter being a specific inhibitor of catalytic site serine were assayed with whole cell lysate of over expressed *BCG_1460c* (lipH) with the choice of substrate being p-nitrophenol butyrate (pNP-C_4) since all our previous enzymatic reactions of over expressed *BCG_1460c* (lipH) were most stable with pNP-C_4. Tetrahydrolipstatin (THL) was found to strongly inhibit the short-chain

esterase activity of *BCG_1460c* (lipH) as indicated by its IC_{50} value of 0.12±0.035µM (fig3.11) and E600 (diethylparanitrophenyl phosphate) inhibitor having an IC_{50} value of 0.399±0.172µM (fig3.12). All the inhibition assays were performed with 2 biological and 2 technical replicates. Of note, IC_{50} values are expected to be even lower for purified *BCG_1460c* (lipH) enzyme since we used whole cell lysates for our inhibition assays.

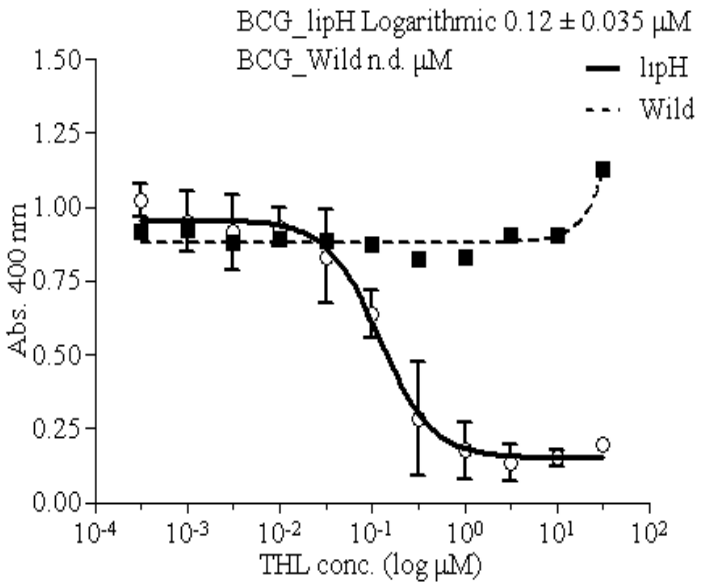


Figure 3.11: THL (Tetrahydrolipstatin) inhibitor assay (IC_{50}) towards BCG overexpressing *BCG_1460c* (lipH) from log phase culture (* n=3)**

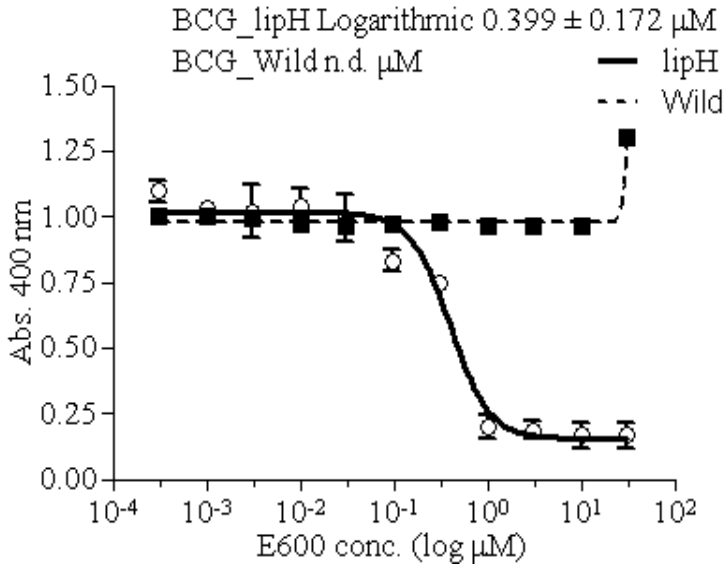
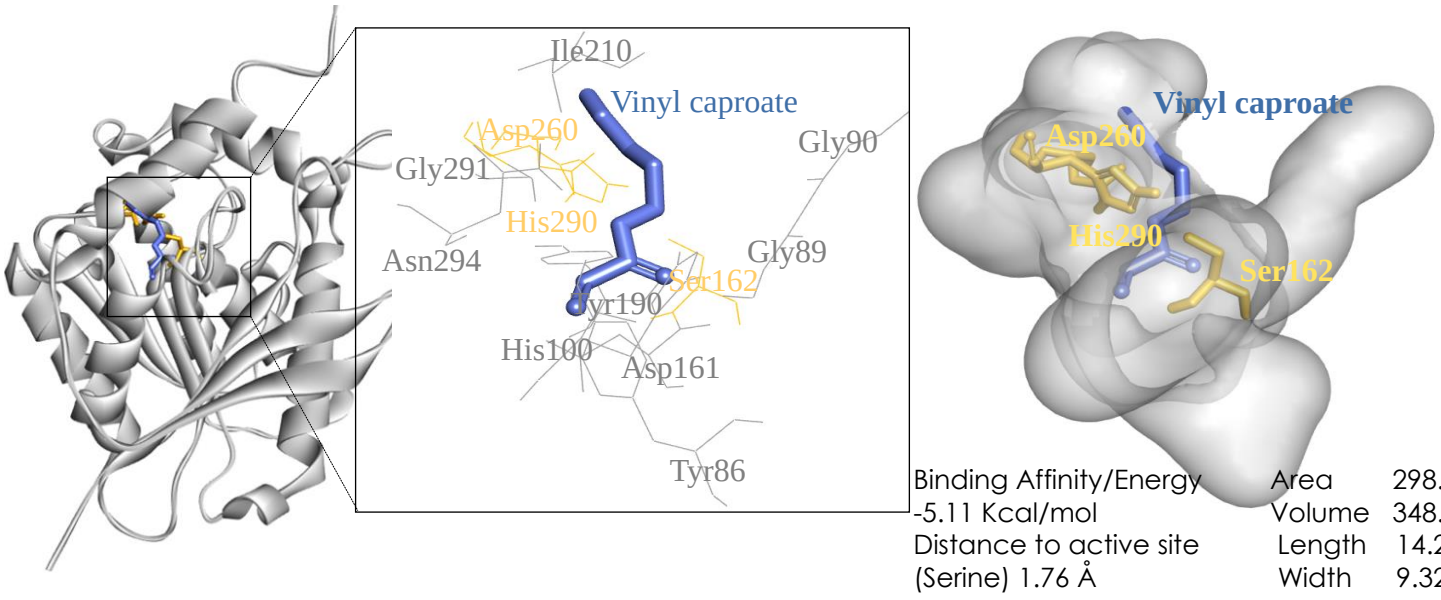


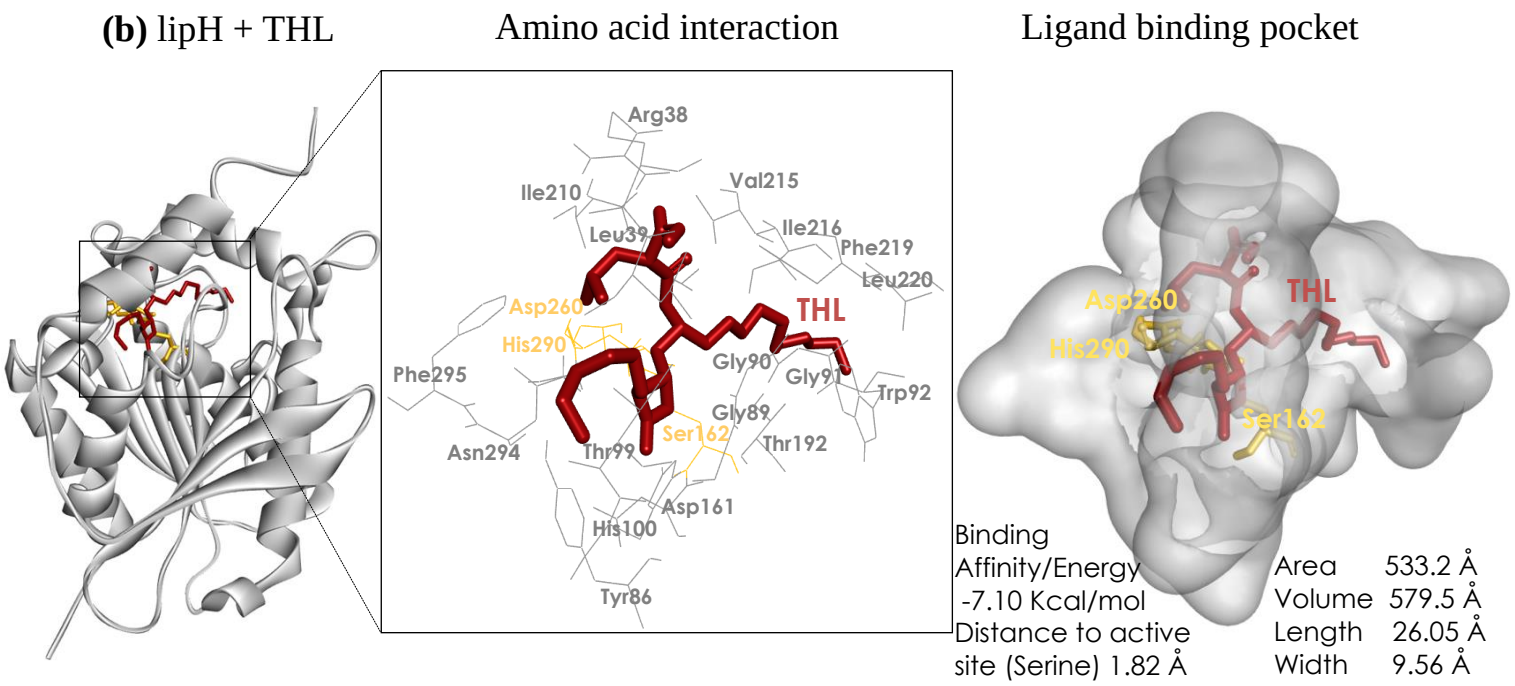
Figure 3.12: E600 (diethylparanitrophenyl phosphate) inhibitor assay (IC_{50}) towards BCG overexpressing *BCG_1460c* (lipH) from log phase culture (* n=3)**

3.6 Predicted binding mode model of THL inhibition in *BCG_1460c* (lipH) 3D structure

To understand at the structural level THL inhibition of short-chain esterase activity of *BCG_1460c* ligand docking experiments were performed. 3D structure model of lipH is shown in fig.3.13 (a) with its potential docked substrate (Vinyl caproate) and inhibitor (THL) predicted from *in silico* ligand screening results. Interactions of substrate (Vinyl caproate) within binding pocket have been shown in fig.3.13 (a) while interactions of THL (tetrahydrolipstatin) within the binding pocket shown in fig. 3.13(b) revealed a near attack conformation of β-lactone ring from the active site serine further the molecular overlay of catalytic triad (serine, histidine, aspartate) from substrate bound over inhibitor (THL) bound shown in fig. 3.13(c) revealed

(a) lipH + substrate Amino acid interaction Ligand binding pocket





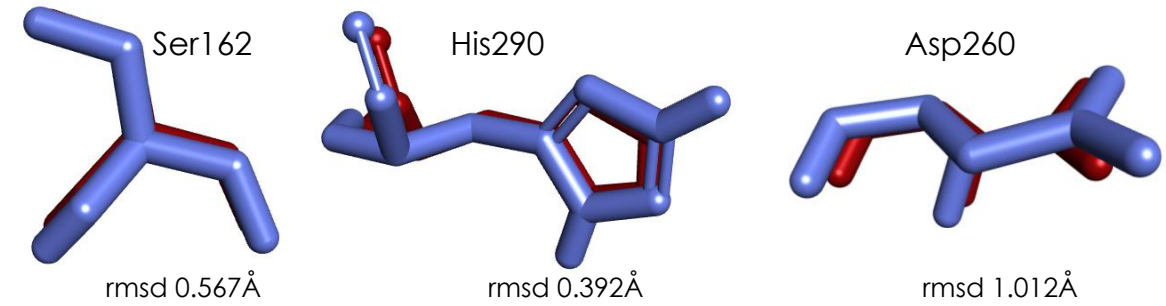


Figure 3.13 (a & b). Three dimensional model of lipH structure depicting the core α/β-hydrolase fold with catalytic triad (yellow). The 3D model is docked with lipH probable substrate, vinyl caproate (blue) and probable inhibitor, THL (red). The insert (middle) depicts the catalytic triad and other amino acids interacting with docked ligand. And the binding energy of ligand binding pocket is represented on far right to show that area, volume and width of the pocket decide the specificity of ligand conformation and the folding pattern. **(c)** Overlay of catalytic triad docked with substrate (blue) and with THL (red). The change in C—C atom r.m.s.d value of lipH catalytic site triad with substrate and THL is mentioned at the bottom of the figure.

that there is only a very small root mean square deviation (difference) of the catalytic triad restudies with active site serine having root mean square deviation of 0.567 Å, histidine 0.392 Å and aspartate 1.012 Å (fig. 3.13(c)). Therefore, it was predicted that THL inhibitor binds in the substrate pocket of *BCG_1460c* (lipH) where the hexanoyl (C_6) arm of THL binding to the substrate pocket may well be mimicking the short-chain substrate (vinyl caproate C_6) binding in the ligand binding pocket of *BCG_1460c* (lipH) this hypothesis has been discussed in details in the discussion section which would now follow.

4. Discussion

Mycobacterium tuberculosis incorporates an unusually high number of genes in its lipid metabolism (Cole et al., 1998) and it is known that there are 250 genes encoding putative lipid synthesis/degrading enzymes as compared to only 50 genes in *E.coli* having a similar genome size. Recent studies have suggested that success of *Mycobacterium tuberculosis* pathogen relies on its exceptional capacity to latently infect and re-infect its host which is mainly attributed to its lipid metabolism. The mycobacteria prior to entering into non-replicating state have been shown to accumulate intracellular lipids (TAGs) (Garton, Christensen et al. 2002). The studies of Coles group, in 2008 (Cotes, Bakala N'goma J et al. 2008) have reported that during the mycobacterial re-infection cycle i.e. exit from non-replicating state, the bacteria hydrolyses its intracellular lipid deposits (TAGs) using hydrolytic enzymes like esterases/lipases. Sequence analysis has revealed 31 of such putative lipolytic

enzymes out of which 24 have been classified under lip family as putative esterases/lipases but very few have been functionally annotated and the 3D structure of only one esterase lipW (PDB ID: 3QH4) has been reported to have been established so far. Our results from this study conducted in *Mycobacteirum bovis* BCG provides insights into molecular structure of 4 putative mycobacterial esterases/lipases namely *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) and predicting natural substrates and a potent inhibitor THL (tetrahydrolipstatin) by structure based virtual ligand screening apart from biochemical characterization of short-chain esterase activity *BCG_1460c* (lipH probable lipase) inhibited by THL (tetrahydrolipstatin). However, detailed results would now be discussed in following sections

4.1 *In silico* Studies:

4.1.1 Role of Virtual Screening in Antibacterial Drug Discovery

The golden era of antibacterial chemotherapy began in the 1950s and by the advent of 1970s almost all major classes of antibacterial agents currently in use (Simmons, Chopra et al. 2010) were discovered this was a major scientific and medical achievement that had enormous benefits for treatment of deadly infectious diseases (caused by bacteria) affecting the human race. However, the emergence of widespread global occurrence of bacteria resistant to the antibiotics and synthetic drugs threatens to reverse our ability to treat infectious diseases (Chopra 2013). Furthermore the new drugs in pharmaceutical pipelines are mostly derivatives of older classes of

antibacterial currently in use and therefore making them more prone to the existing mechanisms of bacterial resistance.

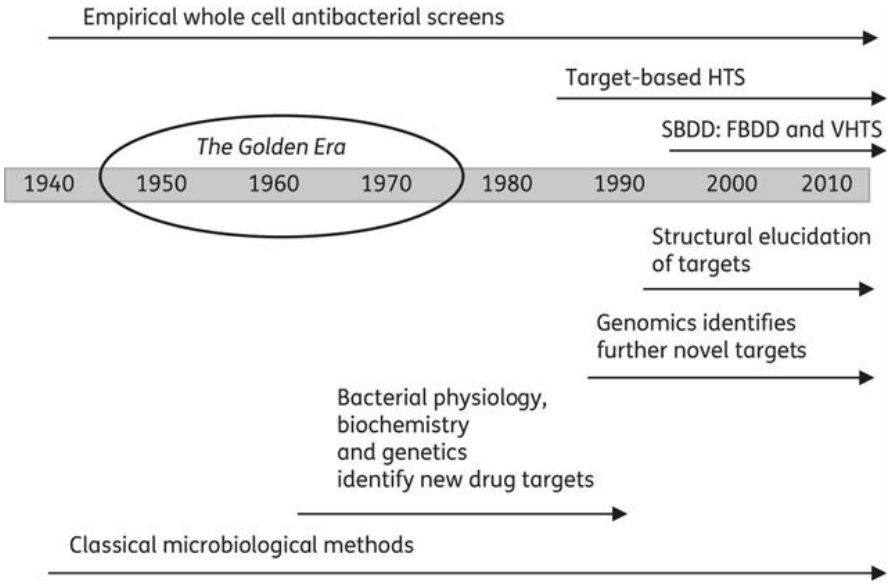


Figure 4.1 Showing principal antibacterial drug discovery strategies (above the date line) in the period from 1940 to the present day and supporting technologies (below the date line). The golden era of antibacterial drug discovery(~1945–76) is also indicated (Adapted from Chopra 2013)

As shown in figure 4.1 the most critical step in the process of antibacterial drug discovery is the viable lead identification step. The early predictions on the lead quality set the stage for the subsequent efforts to enhance therapeutic efficacy through selectivity, pharmacokinetics, potency and toxicity (Polgar 2007). In a retrospective view of the antibacterial drug discovery process, the essential step of lead identification was mainly driven by *in vivo* methodologies, but the limitations of *in vivo* models were later found to be major factors in evaluating attrition rates which therefore suggested researchers to introduce rational approaches such as structure based virtual screening and *in vitro* methodologies at the frontline of drug discovery campaigns. Virtual screening (VHTS) combines both rational approaches such

as structure-based screening with high throughput approaches (HTS). The typical workflow of a high throughput virtual screening has been depicted in fig. 4.2

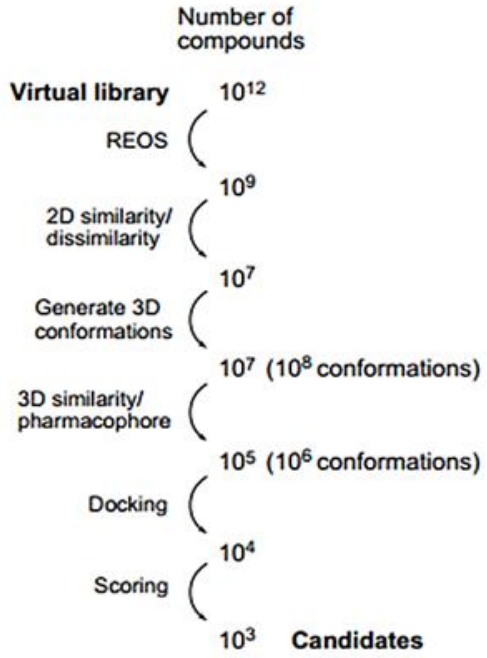


Fig. 4.2 State of the art in Virtual Screening

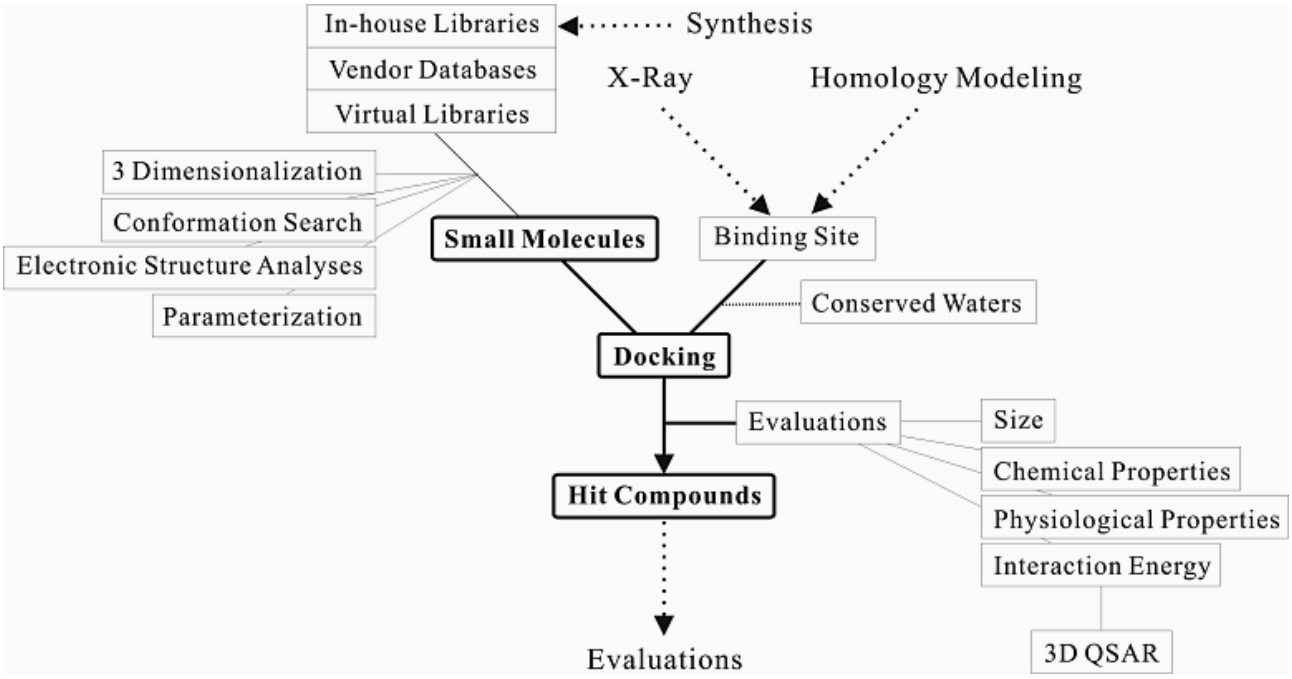


Figure 4.3 Detailed workflow of a high throughput virtual screening (VHTS)
(Source: http://www.molfunction.com/virtual.htm **)**

4.1.2 Concepts, Feasibility and Drawbacks of Virtual Screening

Virtual screening is a strategy aimed at bringing together a more focused approach to high throughput screening by applying rational computational analysis to a subset of compounds considered to be appropriate for a given receptor. It is quite clear that this strategy implies that there is some starting point information which is available pertaining to either the nature of the binding site of receptor and/or ligand type that would be expected to favourably bind within the active site of the receptor. However, it should be realized that virtual screening itself combines a variety of computational screens, which encompasses simplistic to sophisticated algorithms which explore different types of information describing receptor. Similarly, it can be used to produce either a highly focused compound subset (for instance if only the close structural analogs of a lead compound are of interest) or a highly open ended subset (for instance a constraint on size, described by molecular weight, may be applied), although this largely depends on the objectives and interests of the HTS project.

4.1.3 Molecular 3D Structure Modelling and Virtual Ligand Screening

In our *in silico* study, we have demonstrated 3D structure models of *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase), with an accuracy of >95% having near native conformation of energetically stable and validated 3D structure models.

Partially modelled 3D structure of Rv1399c (*BCG_1460c* lipH) in *Mycobacterium tuberculosis* has been reported in the study of Canaan group in 2004 (Canaan, Maurin et al. 2004) and Rv2928 (*BCG_2950* tesA) from Chavadi group in 2011 (Chavadi, Edupuganti et al. 2011) whereas in the present study we have predicted complete domain modelling of BCG*_1460c* (lipH probable lipase), *BCG_2950* (tesA probable thioesterase), *BCG_2991c* (lipN probable lipase/esterase), and *BCG_3229* (lipV possible lipase).

We have uniquely extended the concept of molecular modelling to a structure based ligand screening to predict and understand the ligand specificities particularly those pertaining to *BCG_1460c* (lipH probable lipase), *BCG_2950* (tesA probable thioesterase), *BCG_2991c* (lipN probable lipase/esterase), and *BCG_3229* (lipV possible lipase). Our results suggest that *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase) and *BCG_3229* (lipV possible) show an inclined tendency of ligand affinity towards short-chain vinyl esters such as vinyl butyrate (C_4) and vinly caproate (C_6) whereas for triacylglycerol (TAGs) substrates were found to be tripropionate having identical fatty acyl chains (3:0/3:0/3:0) to tricaproate having identical fatty acyl chains (6:0/6:0/6:0). Our results were performed with 500 independent docking runs involving 300000 maximum energy evaluations to achieve desired statistical significance ($p\text{-value}<0.05$).

Further our molecular modelling and ligand screening (docking) protocols were validated to serve as effective computational tools towards *in silico* characterization of mycobacterial putative esterases/lipases.

4.1.4 Predicted binding mode model of THL inhibition in *BCG_1460c* (lipH) 3D structure

Our results predict the binding mode and ligand interactions of THL (tetrahydrolipstatin) within the ligand binding pocket of *BCG_1460c* (lipH) (fig.3.13). 3D structure and molecular overlay of catalytic site in fig.3.13(c) shows a very small root mean square deviation (difference) of substrate bound and inhibitor bound catalytic triad residues with active site serine having an root mean square deviation of 0.567 Å, histidine 0.392 Å and aspartate 1.012 Å. This observation tends to suggest that THL binds within the substrate pocket of *BCG_1460c* (lipH). Further it was also found that β-lactone ring of THL known to interact covalently with the active site serine of esterases/lipases adopts near attack conformation within the substrate binding pocket of *BCG_1460c* (lipH) and is located at a distance of 1.82 Å from active site serine (hydroxyl group-OH). This again is a favourable indicator of a possible nucleophilic attack by active serine on the carbonyl carbon of β-lactone ring. Further comparisons between ligand interactions of short-chain substrate vinyl caproate (C_6) and inhibitor THL tempted us to predict that hexanoyl (C_6) arm of THL might well be mimicking short-chain vinyl caproate (C_6) substrate specificity of *BCG_1460c* (lipH) within its substrate pocket.

4.2 *In vitro* Studies:

4.2.1 *BCG_1460c* (lipH probable lipase) is a short-chain carboxyl esterase

Our in vitro biochemical assays suggest a short-chain esterase activity of *BCG_1460c* (lipH probable lipase) (fig 3.13) which correlates well with the

findings of a study conducted by Canaan et al., 2004 in *Mycobacterium tuberculosis* on Rv1399C(lipH putative lipase) where they have cloned, expressed and folded the enzyme in *E.coli*. This is not the natural environment (non-mycobacterial) and the enzyme was found to be insoluble, expressed as inclusion bodies and the active state was lost after the purification step and was consequently re-folded in *E.coli* using an alternative *in vitro* protocol. In this study we performed enzymatic assays with whole cell lysates of *Mycobacterium bovis* BCG over-expressing *BCG_1460c* (lipH probable lipase) using synthetic substrates and results suggest a short-chain esterase activity of *BCG_1460c* (lipH probable lipase) with para-nitrophenyl actetae (C_2) and para-nitrophenyl actetae (C_4) (fig.4.1). We also transformed *Mycobacterium smegmatis* MC2 with *BCG_1460c* (lipH probable lipase) over-expression clone and our biochemical assays again showed a short-chain esterase activity of *BCG_1460c* (lipH probable lipase) in *Mycobacterium smegmatis* MC2 (fig.4.2)

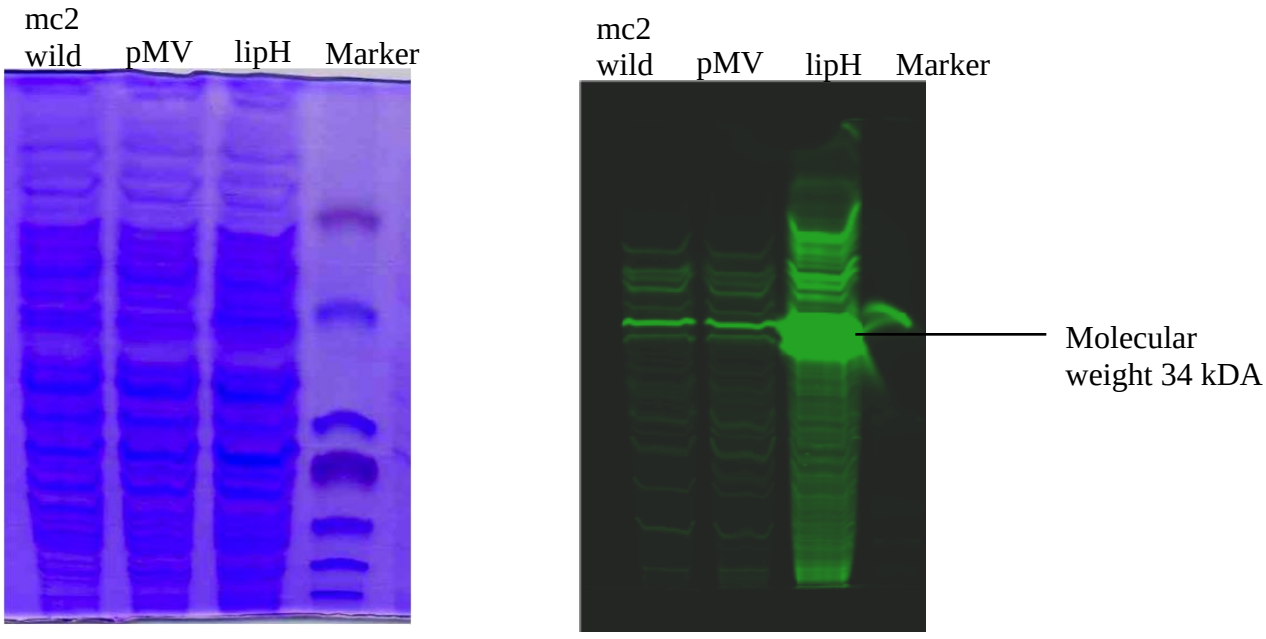


Figure 4.4: SDS-PAGE fluorescent gel image showing change in protein levels of *M. smegmatis* MC2 over expressing lipH (BCG_1460c)

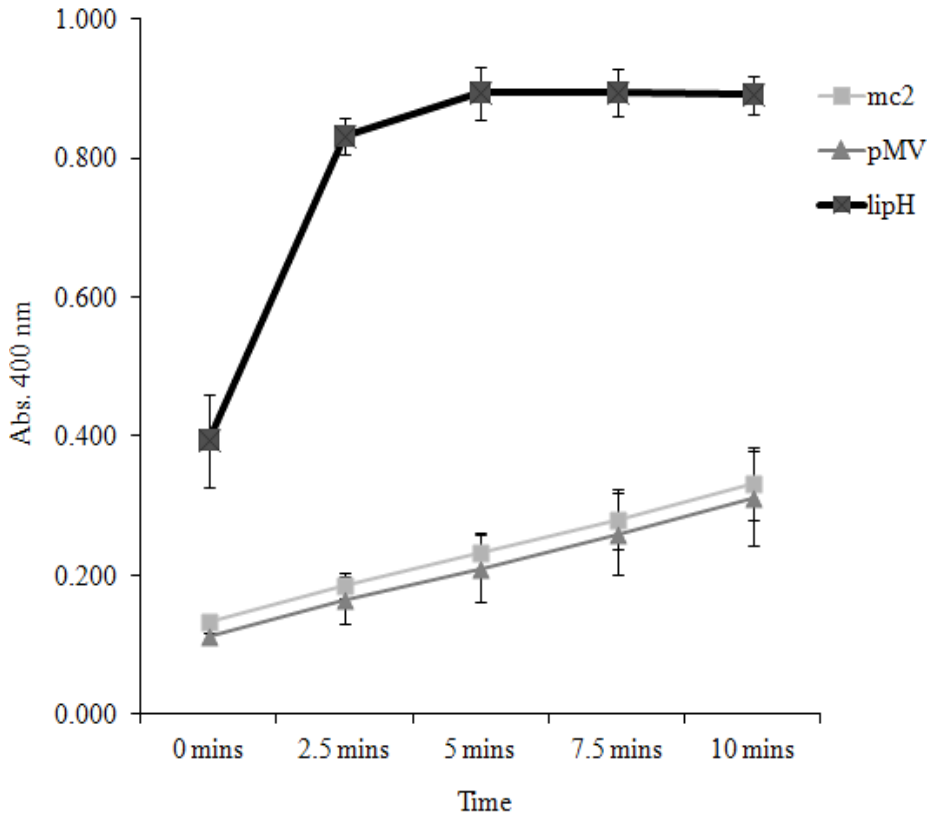


Figure 4.5: Enzymatic assay with whole cell lysate of *M. smegmatis* MC2 over expressing lipH (BCG_1460c) using substrate Pnp-C4 para-nitrophenol-butyrate (C_4) (n=2 biological replicates)**

4.2.2 THL (Tetrahydrolipstatin) strongly inhibits short-chain carboxyl esterase activity of *BCG_1460c* (lipH probable lipase)

Further we found that this short-chain esterase activity of *BCG_1460c* (lipH probable lipase) was inhibited by THL (tetrahydrolipstatin) as indicated by IC_{50} value of 0.12±0.035µM (fig.3.11) while E600 (diethylparanitrophenyl phosphate) inhibitor having an IC_{50} value of 0.399±0.172µM (fig.3.11). These values are expected to be even lower for purified enzyme since we performed

our enzymatic assays using whole cell lysates. The 3D protein structure model supports our biochemical data wherein at the structural level our data suggests a near attack conformation of β-lactone ring from the active site serine and a favourable nucleophilic attack on the carbonyl carbon of β-lactone ring therefore opening the β-lactone ring cycle.

4.2.3 Possible functions of *BCG_1460c* (lipH probable lipase)

The mycobacterial lip family comprises of 24 putative esterases/lipases of which hormone sensitive lipase (HSL) family is a sub-family. *BCG_1460c* (lipH probable lipase) is a member of hormone sensitive lipase (HSL) family in this family Rv3097c (lipY) has been reported previously in studies conducted by Deb and Mishra groups (Deb. et al., 2006 and Mishra et al., 2008) that Rv3097c (lipY) hydrolyses long-chain TAGs releasing long-chain fatty acids which are used in the glyoxylate cycle while other HSL member enzymes such as *BCG_1460c* (lipH probable lipase) and it is contemplated that these maybe involved in short-chain substrate pathways such as regulation, detoxification, signalling or support to the membrane and may provide mycobacteria an alternate source of carbon. A non-redundant database search from BLAST results shown in (table1) reveals that *BCG_1460c* (lipH probable lipase) gene although a non-essential gene, is found to be significantly well conserved right from non-pathogenic soil mycobacteria to human pathogenic *Mycobacterium tuberculosis* as shown in table and suggesting that this gene maybe be conditionally important for the bacteria.

Table 4.1 BCG_1460c (lipH) homologs in bacterial species

lipH homologs (BLAST non-redundant database search)			
Organism	**Gene name**	**Coverage**	**Identity**
Mycobacterium tuberculosis SUMu001	lipase lipH	100%	100%
Mycobacterium bovis BCG str. Pasteur 1173P2, Mycobacterium bovis BCG str. Tokyo 172, Mycobacterium bovis BCG str. Mexico, Mycobacterium bovis BCG str. Moreau	lipase lipH	100%	100%
Mtb. H37Rv, Mycobacterium bovis AF2122/97 ,Mycobacterium H37Ra, Mycobacterium tuberculosis F11, Mycobacterium tuberculosis KZN 1435, Mycobacterium tuberculosis CPHL_A, Mtb K85 ,Mtb. T85, Mtb. 210, Mtb KZN 4207, Mtb. KZN R506	lipase lipH	100%	100%
Mycobacterium marinum	lipase lipH	99%	79%
Mycobacterium ulcerans Agy99	lipH gene product	99%	79%
uncultured organism	hypothetical protein	98%	79%

Mycobacterium parascrofulaceum	lipase lipH	99%	79%
Mycobacterium tuberculosis '98-R604 INH-RIF-EM'	putative lipase	76%	99%
Mycobacterium colombiense	putative lipase LIPH	98%	76%
Mycobacterium rhodesiae NBB3	esterase/lipase	97%	65%
Mycobacterium xenopi RIVM700367	putative lipase	97%	63%
Mycobacterium rhodesiae JS60	Triacylglycerol lipase	93%	67%
Mycobacterium canettii CIPT 140010059	putative lipase LIPH	97%	63%
Mycobacterium bovis AF2122/97, Mycobacterium bovis BCG str. Tokyo172,Mycobacterium africanum	lipase/esterase , lipase lipH	97%	63%
Mycobacterium thermoresistibile ATCC 19527	esterase	97%	64%
Mycobacterium gilvum Spyr1	esterase/lipase	96%	63%
Mycobacterium smegmatis str. MC2 155	esterase	97%	62%
Mycobacterium avium subsp. avium ATCC 25291	α/β hydrolase domain-containing protein	97%	63%
Mycobacteriu Abs.	lipase LipH	97%	60%

Mycobacterium intracellulare MOTT-64	lipase LipI	97%	61%
Mycobacterium marinum M	lipase LipI	97%	58%
Mycobacterium ulcerans Agy99	lipase LipI	97%	58%
Mycobacterium sp. MCS	α/β hydrolase	97%	62%
Segniliparus rugosus ATCC BAA-974]	α/β hydrolase	93%	57%

The data pertaining to cloning of *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) were derived from the doctoral thesis work of Mr. Ravindran in Prof. Wenk's lab. The molecular modelling and biochemical assay data (figures) for *BCG_1460c* (lipH probable lipase) were also included in the manuscript under preparation for research publication.

5. Conclusions and Future directions:

The present study was undertaken with pointed objectives as enumerated right in the beginning. Both *In silico* as well as *In vitro* experimental approach were adopted to demonstrate the convergence and correlation of the results. The outcome provided for the evidence at structural and biochemical level that the short-chain esterase activity of *BCG_1460c* (lipH) was inhibited by tetrahydrolipstatin (THL). As pointed out *in silico* and *in vitro* analysis correlate and the data so obtained provided an effective approach to characterize and distinguish lipolytic/non-lipolytic enzymes. To summarize the conclusive findings of the present study following points need to be emphasized:

- In the present study, it has emerged that the 3D structure models of 4 mycobcaterial putative esterases/lipases namely *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase) were well validated achieving the degree of accuracy to the tune of >95%. In addition complete domain modelling of all structures and their respective ligand specificities was also undertaken. It is to be pointed out that this was a unique feature particularly in view of the fact that only a partial modelling of lipH (Rv1399c) and tesA (Rv2928c) was so far done in the studies reported by (Canaan et. al.2004) and (Chavadi et. al. 2006) respectively. Further for the first time this study validated models for *BCG_3229* (lipV possible lipase) and *BCG_2991c* (lipN probable lipase/esterase) 3D structures.

- In order to experimentally characterize these and also validate the *in silico* ligand specificity predictions an *in vitro* biochemical characterization was carried out with whole cell lysates of BCG overexpressing *BCG_1460c* (lipH probable lipase), *BCG_2991c* (lipN probable lipase/esterase), *BCG_2950* (tesA probable thioesterase) and *BCG_3229* (lipV possible lipase). Out of these four, one *BCG_1460c* (lipH probable lipase) was shown to have a short chain esterase activity and further inhibitor studies indicated that this short chain esterase activity was strongly inhibited by a well-known FDA approved drug called THL (Tetrahydrolipstatin).

- *In silico* molecular modelling predictions put forth were observed to be in line with biochemical experimental data making it an effective and validated characterization approach to mycobacterial esterases/lipases.

- Future work in continuation to the present work would preferably be to understand the exact functional role of *BCG_1460c* (lipH probable lipase) and the pathways in which this enzyme acts in mycobacteria so as to understand its exact functional role. At this point of time we can only hypothesize its probable function in mycobacteria that it might be acting on short-chain substrate pathways such as regulation, detoxification, signalling or support to the membrane and may provide mycobacteria an alternate source of carbon. However, it would be worthwhile to design and undertake a systematic study to unfold the truth of the current hypothesis.

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