Construction of *lipU* anti-sense knock out mutant of *Mycobacterium fortuitum* and its *in-silico* analysis to determine its potential role in pathogenesis

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CERTIFICATE

This is to certify that Ms. Divya has carried out the M.Tech Biotechnology dissertation project work on "**Construction of *lipU* anti-sense knock out mutant of *Mycobacterium fortuitum* and its *in-silico* analysis to determine its potential role in pathogenesis"** from Jaypee University of Information Technology, Solan, Himachal Pradesh under my supervision from August 2017 to May 2018. This work has not been submitted partially or wholly to any other university or institute for the award of this or any other degree or diploma.

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Date :

DECLARATION

I hereby declare that the work presented in this project dissertation entitled, **"Construction of anti-sense knock out mutant of *Mycobacterium fortuitum* and its *in-silico* analysis to determine its potential role in pathogenesis"** submitted as partial fulfillment of M.Tech in Biotechnology was carried out at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Himachal Pradesh. The work presented in this project dissertation is original and will remain intellectual property of Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Himachal Pradesh.

Divya (133801)

ACKNOWLEDGEMENT

No venture can be completed without the blessings of the Almighty. I would like to thank all the people who helped us in any form in the completion of my project "Construction of anti-sense knock out mutant of *Mycobacterium fortuitum* and its *in-silico* analysis to determine its potential role in pathogenesis.

I owe my profound gratitude to my project supervisor Dr. Rahul Shrivastava, who took keen interest and guided me all along in my project work till this moment in the project by providing all the necessary information for developing the project. I am really thankful to him for his constant support, careful supervision and critical suggestions.

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ABSTRACT

Mycobacterium fortuitum is a non-tuberculous mycobacterium (NTM) causing various clinical symptoms such as cutaneous infections, respiratory infections, joint infections and disseminated infections in immuno-compromised patients. Since the mechanism of pathogenesis of *M. fortuitum* is not known, be that as it may, it has been reported that it taints macrophages in an indistinguishable way from that of *Mycobacterium tuberculosis*. Out of all the genes identified for the virulence of *M. tuberculosis* infection, *lipU* also called as cystathionine beta synthase, plays a major role coding for lipid hydrolyses necessary for the survival and virulence of *M. tuberculosis* and other mycobacteria. This lipase is very much similar to that of esterases of mycobacterium which implicates modification of the mycobacterial cell wall as an adaptive response to acid damage, also degrades host lipids during infection, making fatty acids available as building blocks for lipid biosynthesis. This gene helps in compensating with un-favorable acidic conditions in macrophages, where mycobacterium resides. With an aim to study the pathogenesis of *M. fortuitum*, multiple primer sets were designed from conserved regions of *lipF* of *M. tuberculosis* to amplify full length gene sequence of its homologue in *M. fortuitum* on the basis of the homology between the two genomic sequences (*M. fortuitum* and *M. tuberculosis*). Full length fragments were confirmed by sequencing. Longest sequence showing highest similarity with *M. tuberculosis* was submitted to GenBank database (Accession number - MH197269) as *lipU* of *M. fortuitum* ATCC 6841 homologue found using *in-silico* analysis. The study also followed *in-silico* anaylsis for sequence based homological studies and protein based homological studies using bio-informatics tools. To further confirm the role of *lipU* in virulence of *M. fortuitum*, an anti sense mutant was constructed by cloning the gene in *pMV261* vector followed by electroporation into the wild type *M. fortuitum*. This construct can be further used to establish the role of this gene in pathogenesis of *M. fortuitum* which may act as a potential drug target against the infection caused by the bacteria.

Rationale

- Understanding the potential role of *lipU* gene in the virulence of *M. fortuitum* by *in-silico* analysis.

Objectives

- To construct *lipU* anti-sense knock out mutant of *Mycobacterium fortuitum.*
- To perform sequence based *in-silico* homological studies.

Chapter 1
Introduction

Pulmonary diseases caused by mycobacterium are increasing day by day throughout the world and hence there is an immediate need for the thorough study of the mechanisms used by these bacterial pathogens when they conquer human body cells and cause disease. In the contemporary era, the importance of the resistance acquired by these bacteria for multiple antibiotics has gained the spotlight. Yet there is no as such accurate data available because of the less number of reportable conditions in maximum countries. Ample number of mechanisms has been employed by bacteria to cause certain infectious diseases in human hosts. Though, variation occurring on the basis of geographic conditions signifies the widespread presence of these bacteria in the environment. Bacterial pathogens manifest a huge variety and range of biological entities which bind to host cell targets to facilitate diversified host responses. Different mechanisms used on the molecular level by bacteria to interact with the host can be distinctive for a particular pathogen or perpetuate beyond various divergent species. A cue to battle with these bacterial diseases is the identification and characterization of all these unconventional strategies. Some definite virulence facets are obligatory for complete pathogenicity of the bacteria irrespective of the host cell.

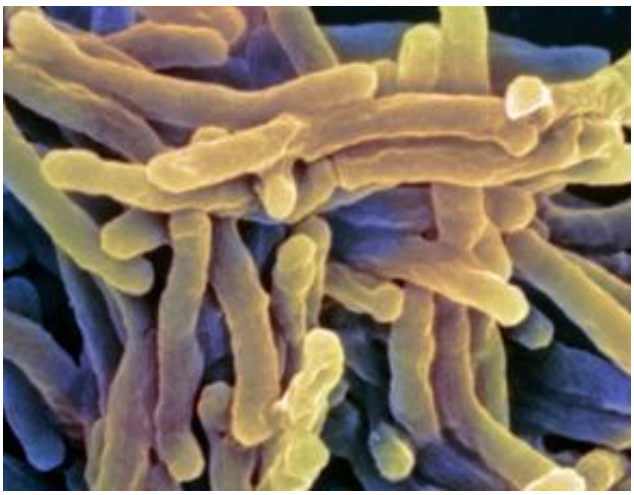
Nontuberculous mycobacteria (NTM), also known as environmental mycobacteria, atypical mycobacteria and mycobacteria other than tuberculosis (MOTT), are mycobacteria which do not cause tuberculosis or leprosy (also known as Hansen's disease). NTM do cause pulmonary diseases that resemble tuberculosis. Mycobacteriosis is any of these illnesses, usually meant to exclude tuberculosis. They occur in many animals, including humans.

Mycobacterium fortuitum is a fast-growing species that can cause infections. The term "fast growing" is a reference to a growth rate of 3 or 4 days, when compared to other Mycobacteria that may take weeks to grow out on laboratory media. Pulmonary infections of *M. fortuitum* are uncommon, but *Mycobacterium fortuitum* can cause local skin disease, osteomyelitis (inflammation of the bone), joint infections and infections of the eye after trauma. *Mycobacterium fortuitum* has a worldwide distribution and can be found in natural and processed water, sewage, and dirt.

M. fortuitum infection can be a nosocomial (hospital acquired) disease. Surgical sites may become infected after the wound is exposed directly or indirectly to contaminated tap water. Other possible sources of *M. fortuitum* infection include implanted devices such as catheters, injection site abscesses, and contaminated endoscopes. Some researches on Rapidly Growing Mycobacteria (RGM)

provides the following aspects of RGM: (i) its sources, predisposing factors, clinical manifestations, and concomitant fungal infections; (ii) the risks of misdiagnoses in the management of RGM infections in dermatological settings; (iii) the diagnoses and outcomes of treatment responses in common and uncommon infections in immuno-compromised and immuno-competent patients; (iv) conventional versus current molecular methods for the detection of RGM; (v) the basic principles of a promising MALDI-TOF MS, sampling protocol for cutaneous or subcutaneous lesions and its potential for the precise differentiation of *M. fortuitum*, *M. chelonae*, and *M. abscessus*; and (vi) improvements in RGM infection management as described in the 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines, including interpretation criteria of molecular methods and antimicrobial drug panels and their break points [minimum inhibitory concentrations (MICs)], which have been highlighted for the initiation of antimicrobial therapy.

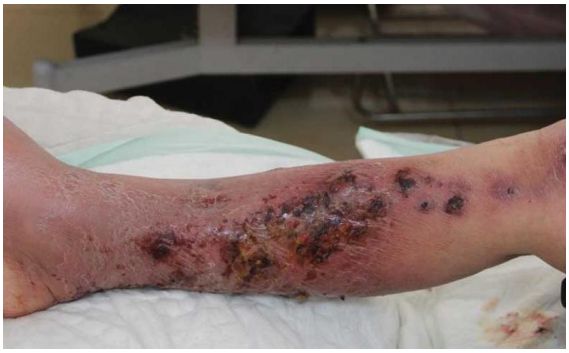
(Fig. 1.1)



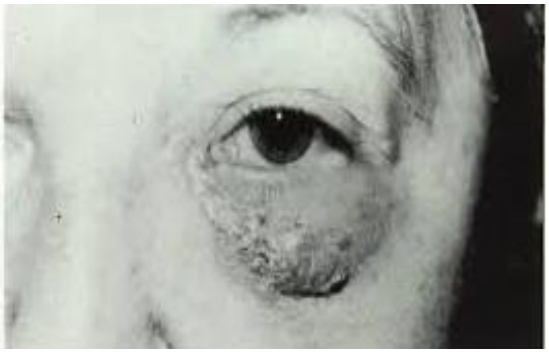
(Fig. 1.2)

(Figure : *Microscopic view of rod shaped Mycobacterium fortuitum)*[30,31]

Infection in healthy humans caused by *Mycobacterium fortuitum* is rare, but exposure to large and repeated amounts of the organism can activate the immune system and cause disease. Infections most likely occur in immuno-compromised patients. Diseases and infections caused vary from each other and involve almost every organ and tissue system. The most common infections involve NTM lung disease, leprosy, local cutaneous disease, lymphadenitis, surgical site infections and injection site inflammation. Nearly all surgical site infections and injection site inflammation occur when the open wound gets contaminated under infectious or commonly used tap water.



(Fig. 1.3)



(Fig. 1.4)

(Figure: *Lesions due to infection from M. fortuitum*)[32,33]

Lipid metabolism plays an important role in the sustenance of *M. tuberculosis* in the macrophages and out of the 250 genes involved in the lipid metabolism of bacteria encodes a polypeptide with similarity to lipid esterases. The association of lipase production with pathogenicity has been suggested by several works concerning non-mycobacterial pathogens. Lipase implicates modification of the mycobacterial cell wall as an adaptive response to acid damage, also degrades host lipids during infection, making fatty acids available as building blocks for lipid biosynthesis. *lipF* gene helps in compensating with all the un-favorable acidic conditions in macrophages, where bacterium resides. Hence, virulence of tuberculosis is chiefly dependent on the expression of gene. Since, the gene is important for the pathogenesis and persistence of *M. tuberculosis* in the macrophages, this project has mainly focused on the role of *lipU* (*lipF* in *M. tuberculosis*) in the pathogenesis of *Mycobacterium fortuitum* whose significance has been checked first by the construction of *lipU* anti-sense knock-out mutant in *M. fortuitum* and further by its *in-silico* analysis using bio-informatics tools.

The strategy being used for this project was criterion on the homology similarity of the genomic sequence of *M. tuberculosis* and *M. fortuitum.* Multiple primer sets were designed from conserved regions of *lipF* of *M. tuberculosis* to amplify full length gene sequence of its homologue in *M. fortuitum.* Full length fragments were confirmed by sequencing. Longest sequence showing highest similarity with *M. tuberculosis* was submitted to GenBank database (Accession number - MH197269) as *lipU* of *M. fortuitum* ATCC 6841 homologue on the basis of similarity found using *in-silico* analysis. Thus, the present study was based on the construction of *lipU* anti-sense mutant of *M. fortuitum* followed by its sequence based homological studies using *in-silico* analysis.

Chapter 2
Review of Literature

2.1 *Mycobacterium*:

Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. Over 190 species are recognized in this genus. This genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) in humans. The Greek prefix *myco-* means "fungus," alluding to the way mycobacteria have been observed to grow in a mold-like fashion on the surface of cultures. It is acid fast and cannot be stained by the gram stain procedure.

Mycobacteria are a family of small, rod-shaped bacilli that can be classified into 3 main groups for the purpose of diagnosis and treatment:

- *Mycobacterium tuberculosis* complex which can cause tuberculosis: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti*.
- *M. leprae* and *M. lepromatosis* which cause Hansen's disease, also called leprosy.
- Nontuberculous mycobacteria (NTM) are all the other mycobacteria which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease. Although over 150 different species of NTM have been described, pulmonary infections are most commonly due to *Mycobacterium avium* complex (MAC), *Mycobacterium kansasii*, and *Mycobacterium abscessus*

2.2 *Mycobacterium tuberculosis* complex:

Microscopic organisms of the *Mycobacterium tuberculosis complex* show remarkable abilities to subvert and to oppose the bactericidal reactions of their tainted host. These limits have driven this bacillus to colonize 33% of the total populace and to slaughter about three million individuals every year. Amid the contamination by means of the airborne course, *M. tuberculosis* bacilli are breathed in into the alveoli of the lung where they achieve macrophages. This cooperation of *M. tuberculosis* with the mononuclear phagocytes is a key stride in the pathogenic procedure.

In vitro, *M. tuberculosis* has been appeared to tie either straight forwardly, or after opsonization, to a wide range of phagocyte receptors that permit the disguise of the microbes. In any case, the relative significance in vivo of these conceivable courses of section stays to be set up. It is likewise vague what ligands are included, in vivo, in the adherence. Without a doubt, numerous mycobacterial envelope

atoms and in addition opsonins can intervene the in vitro official of *M. tuberculosis* to the phagocyte receptors, however their accessibility at the disease site (for the host ligand) or their nearness at the bacterial surface (for the mycobacterial particles) is still under scrutiny. Once phagocytosed, *M. tuberculosis* duplicates inside a particular compartment that does not ferment, conceivably in light of the avoidance of the proton ATPase from the layer of this organelle. This phagosome does not continue through the standard development pathway, and is by all accounts obstructed at a halfway phase of development. From these reviews, it appears to be certain that *M. tuberculosis* has advanced harmfulness systems that enable it to regulate the development of the phagosome and to oppose the lethal particle delivered by the macrophages.[1] Be that as it may, the mycobacterial parts in charge of these bizarre elements stay obscure. These various reviews concerning this association between *M. tuberculosis* and the host underscore our poor comprehension of the mycobacterial elements, and their part in the diverse strides of the irresistible procedure.

A hereditary way to deal with recognize these mycobacterial elements is disconnect mutants influenced in one or a few stages of the irresistible procedure, and to distinguish the aggravates no longer created by these strains. Noso-comial flare-ups and pseudo-episodes brought on by the nontuberculous mycobacteria (NTM) have been perceived for over 20 years and keep on being an issue. Comparative sequence analysis of the *M. tuberculosis* genome has uncovered that it contains 250 proteins required in lipid digestion contrasted with just 50 in *Escherichia coli*. Among these proteins, a group of 21 carboxyl ester hydrolases, called *Lip* (A to W, aside from K and S), have been commented on as putative esterases or lipases, in light of the nearness of the agreement succession GXSXG normal for individuals from the α/β hydrolase overlap family. Inside this family, the current gem structure of the *M. tuberculosis* antigen 85C (Ag85C), a mycolyltransferase required for survival of mycobacteria, alongside that of the noncatalytic *M. tuberculosis* MPT51 protein (FbpC1), which is included in mycobacteria pathogenicity, have uncovered that they have the same α/β hydrolase crease. In this way, a nitty gritty biochemical portrayal of all individuals from the *Lip* family ought to be performed past the computational investigation. For a long time, it was by and large expected that lipases are ineffectively dynamic against solvent esters and turn out to be notably dynamic when the dissolvability furthest reaches of the substrate is surpassed, a wonder called interfacial actuation. Conversely, esterases don't share this conduct and show their maximal movement on esters in arrangement. Sadly, biochemical investigations of a few lipases have demonstrated that the interfacial enactment wonder can't be viewed as a general (and adequate) administer to separate between a lipase and esterase.

An unmistakable qualification amongst lipases and esterases was built up as of late from the examination of the K estimations of these two classes of carboxyl ester hydrolases utilizing halfway dissolvable triacylglycerols and vinyl esters as substrates. Where a lipase can likewise be viewed as an esterase, the K values speak to a solid foundation to separate between these two classes of catalysts portrayal, the *Lip* expression could be befuddled to allude to a NLH, and the first promotion number ought to be utilized to maintain a strategic distance from disarray.[7]

2.3 *Non-tuberculous Mycobacterium :*

Non-tuberculous mycobacteria (NTM), also known as environmental mycobacteria, atypical mycobacteria and mycobacteria other than tuberculosis (MOTT), are mycobacteria which do not cause tuberculosis or leprosy (also known as Hansen's disease). NTM do cause pulmonary diseases that resemble tuberculosis. Mycobacteriosis is any of these illnesses, usually meant to exclude tuberculosis. They occur in many animals, including humans. NTM are widely distributed in the environment, particularly in wet soil, marshland, streams, rivers and estuaries. Different species of NTM prefer different types of environment. Human disease is believed to be acquired from environmental exposures, and unlike tuberculosis and leprosy, there has been no evidence of animal-to-human or human-to-human transmission of NTM, hence the alternative label "environmental bacteria".

NTM diseases have been seen in most industrialized countries, where incidence rates vary from 1.0 to 1.8 cases per 100,000 persons. Most NTM disease cases involve the species known as *Mycobacterium avium complex* or MAC for short, *M. abscessus*, *M. fortuitum* and *M. kansasii*. *M. abscessus* is being seen with increasing frequency and is particularly difficult to treat.

Rapidly growing NTMs are implicated in catheter infections, post-LASIK, skin and soft tissue (especially post-cosmetic surgery) and pulmonary infections.

2.4 Lipase family of *M. tuberculosis* and their plausible capacity :[3]

Gene Product	Theoretical Molecular Mass	Predicted subcellular localization	Activity Type	Probable Role/ Function	Maximum Identity
LIPC (Rv0220)	44.3	Cytoplasmic	Probable esterase	Carboxylesterase type B	77% with alpha/beta hydrolase
LIPD (Rv1923)	47.2	Periplasmic	Similar to esterases, beta-lactamases	Defense mechanisms	69% with β-lactamase
LIPE (Rv3775)	45.3	Periplasmic	Carboxylic-ester hydrolase	Defense mechanisms	79% with β-lactamase
LIPF (Rv3487c)	29.4	Cytoplasmic	Unknown but similar to esterases and lipases	intermediary metabolism and respiration	75% with alpha/beta hydrolase
LIPG (Rv0646c)	32.9	Cytoplasmic	Homology to hydrolases	Lipid transport and metabolism	80% with alpha/beta hydrolase
LIPH (Rv1399c)	33.9	Cytoplasmic	Possible lipase	Lipid transport and metabolism	70% with alpha/beta hydrolase fold
LIPI (Rv1400c)	34.0	Cytoplasmic	Possible lipase	Intermediary metabolism and respiration	69% carboxylesterase family protein
LIPJ (Rv1900c)	49.7	Cytoplasmic	Similarity to esterases	Alkaloid biosynthesis II	75% with alpha/beta hydrolase fold
LipK, MBTJ(Rv2385)	32.9	Cytoplasmic	Probable esterase/acetyl hydrolase	intermediary metabolism	71% with alpha/beta hydrolase fold
LIPL (Rv1497)	45.8	Cytoplasmic	Probable esterase	Intermediary metabolism and respiration	70% with β-lactamase
LIPM (Rv2284)	46.7	Cytoplasmic	Probable esterase	Lipid transport and metabolism	78% with alpha/beta hydrolase fold
LIPN (Rv2970c)	40.1	Cytoplasmic	Lipase-like enzyme	Lipid transport and metabolism	77% with alpha/beta hydrolase fold
LIPO (Rv1426c)	46.1	Cytoplasmic	Possible esterase, also similar human arylacetamide deacetylase	Lipid transport and metabolism	79% with alpha/beta hydrolase fold
LIPP (Rv2463)	42.8	Periplasmic	Similar to esterases from Pseudomonas spp.	Defense mechanisms	76% β-lactamase
LIPQ (Rv2485c)	45.2	Cytoplasmic	Carboxylesterase	Intermediary metabolism and respiration	74% with alpha/beta hydrolase fold
LIPR (Rv3084)	32.6	Cytoplasmic	Similar to acetyl-hydrolase	intermediary metabolism and respiration	68% with alpha/beta hydrolase fold
LipS (MesT, Rv3176c)	35.2	Cytoplasmic	Similarity to esterases/ lipases/epoxide hydrolase	Virulence, detoxification, adaptation	99% with amidase
LIPT (Rv2045c)	56.1	Cytoplasmic	Probable carboxylesterase	Lipid transport and metabolism	71% Carboxyesterase
LIPU (Rv1076)	31.7	Cytoplasmic	Unknown but very similar to esterases and lipases	Alpha/beta hydrolase	76% with alpha/beta hydrolase fold
LIPV (Rv3203)	23.6	Cytoplasmic	Unknown but shows some similarity to lipases	intermediary metabolism and respiration	71% with alpha/beta hydrolase fold
LIPW (Rv0217c)	32.2	Cytoplasmic	Esterase	Alkaloid biosynthesis II	75% with alpha/beta hydrolase fold
LipX, PE Family Protein (Rv1169c)	10.8	Cytoplasmic	Esterase/lipase	Hydrolases or acyltransferases	73
LIPY (Rv3097c)	45.0	Inner-membarane	Triacylglycerol lipase, PE-PGRS family	Lipid transport and metabolism	99% with PE-PGRS family
LipZ (Rv1834)	31.6	Cytoplasmic	Probable hydrolase	Intermediary metabolism	76% with hydrolase

Gene Product	Identity of the gene of *M. tuberculosis* with				
	M. smegmatis	*M.* marinum	*M.* leprae	*M. abscessus*	*M. bovis*
LIPC (Rv0220)	71% with α, β hydrolase fold family protein	79%	—	—	100%
LIPD (Rv1923)	69% with β-lactamase	80%	64% esterase	68% with putative lipase/ β-lactamase	99% with putative lipase LipD
LIPE (Rv3775)	72% with β-lactamase	81%	79% with probable hydrolase	74%	100%
LIPF (Rv3487c)	—	72%	67% with probable esterase	—	100%
LIPG (Rv0646c)	73% with hydrolase, α, β hydrolase fold	79%	75% with probable hydrolase	68%	100%
LIPH (Rv1399c)	70% with esterase	78%	——	—	100%
LIPI (Rv1400c)	71% with esterase	77%	67% with probable lipase	67% with LipH	100%
LIPJ (Rv1900c)	—	75% with lignin peroxidise	71% with probable esterase	—	99% with putative lipoprotein
LipK, MBTJ(Rv2385)	—	74% with acetyl hydrolase	—	65% with putative lipase	100% with putative acetyl hydrolase
LIPL (Rv1497)	68% with β-lactamase	81%	69% with probable esterase	68% with putative lipase	99% with putative esterase
LIPO (Rv1426c)	72% with peptidase	79%	—	—	100% with putative esterase
LIPP (Rv2463)	74% with putative carboxylesterase	81%	69% with probable esterase	71%	100%
LIPQ (Rv2485c)	66% with peptidase	76%	—	—	100% with putative carboxlesterase
LIPR (Rv3084)	—	—	—	—	99% with probable acetyl hydrolase
LipS (MesT, Rv3176c)	—	—	—	—	99% with amidase
LIPT (Rv2045c)	71% with para-nitrobenzyl esterase	79% with carboxylesterase	70%	71% with putative carboxylesterase	100% with putative carboxylesterase
LIPU (Rv1076)	69% with esterase	81%	76% with putative esterase	—	100% with putative lipase & 73% with putative LipF
LIPV (Rv3203)	—	79%	—	—	100%
LIPW (Rv0217c)	73% with α, β hydrolase fold	67% with esterase/ lipase	—	70%	99%
LipX, PE Family Protein (Rv1169c)	—	—	—	—	100% with PE family protein
LIPY (Rv3097c)	—	80% with PE-PGRS family protein	—	—	99% with PE-PGRS family protein
LipZ (Rv1834)	—	76% with hydrolase	—	—	100% with putative hydrolase

2.5 Role of lipase in the virulence of *M. tuberculosis* :

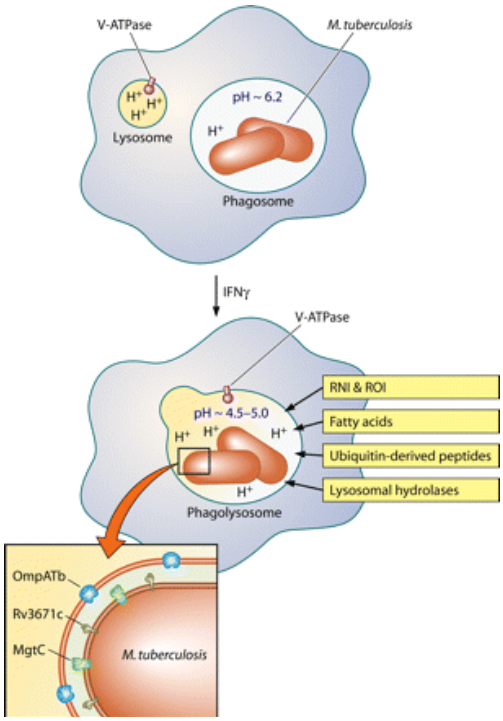
Fatty acids are stored in the form of triacylglycerol which has been widely recognized as carbon source for dormant *M. tuberculosis*. Lipases/esterase, triacylglycerol acylhydrolases, participates in the metabolism of acyl-glycerols in the active stage or stress conditions. *M. tuberculosis* lipases are involved in the virulence and pathogenesis. Stresses (i.e. acidic, oxidative, nutritive) can regulate the expression of mycobacterial lipases. Among 24 putative esterases/lipases of *M. tb*, *LipD* (Rv1923), *LipL* (Rv1497), *LipF* (Rv3487c), *LipN* (Rv2970c) and *LipV* (Rv3203) play a major role in the pathogenesis of *M. tuberculosis* belong to HSL family based on the presence of consensus pentapeptide motif 'GXSXG'. Ser140, as well as Asp244 and His269 of Rv1076 located in pentapeptide have been confirmed as a crucial active site residue by site-directed mutagenesis in this respect. Contrary to *LipD* and *LipY*

which shows a highly hydrolyzed activity to long-chain ester, *lipU* prefers shortchain ester. Some other lipases (*LipC, LipF, LipI, LipL, LipN*) of this family also hydrolyzes short-chain ester. Lipases vary from esterases because of their capacity to hydrolyze substrates with long-chain acylglycerols, though esterases can just hydrolyze substrates with short-chain acylglycerols. Consequently, *lipU* may be esterase rather than lipase. The 3D models shows that the catalytic active sites Ser144 Asp243 and His268 of *lipU* are spatially adjacent, which might underlie the preference over short-chain esters. The optimal temperature for enzyme activity of *lipU* is 40°C and the stability of catalytic activity declines over this temperature. This enzyme displays maximal hydrolytic activity at pH 8.0. And it is stable in over a broad pH range from 6.0–9.0. *lipU* might play a role in mycobacteria survival within host. Detergents interact with hydrophobic and hydrophilic areas of molecule to affect the tertiary structure of proteins. Hence, it also exhibits higher tolerance to non-ionic than ionic detergents. Sodium acetate might be an optimal carbon source for the denitrifying phosphorus bacteria. In other mycobacteria, lipases are also capable of degrading short-chain p-NP esters. Hence, *lipU* is majorly considered as esterase**.**

2.6 Acidic nature of *M. tuberculosis*:

In vitro investigations of microscopic organisms at low pH are educational on the grounds that they can show whether the microorganisms are probably going to be acid safe or delicate amid disease. These reviews can likewise distinguish bacterial elements that give security against low pH and may do as such also in the host condition. In this manner, to start understanding whether *M. tuberculosis* opposes acid in vivo, it is valuable to first audit survival of *M. tuberculosis* in acid in vitro. Critically, in any case, the translation of in vitro studies is confounded by the perceptions that survival of numerous microscopic organisms in acid is reliant on the way of life conditions, for example, bacterial thickness and piece of the test medium. These factors likewise drastically impact the survival and development of mycobacteria at low pH. When all is said in done, the quickly developing, saprophytic mycobacteria become over a more extensive pH run than the pathogenic, moderate developing mycobacteria. This may mirror that the situations in which saprophytic mycobacteria dwell, for example, soil and water, are frequently acidic. Strikingly, mycobacterial species were discovered extraordinarily enhanced in greatly acidic volcanic shake at pH 1. With people being its lone regular habitat and inward breath its most normal course of passage into the body, *M. tuberculosis* does not have to keep up such a high resilience for acidic conditions. *E. coli* additionally shows more prominent acid resistance at

high densities, and a cell-cell contact-based instrument seems, by all accounts, to be included. Defensive variables emitted by *E. coli* may likewise assume a part in its imperviousness to acidic. The in vitro perceptions of *M. tuberculosis* in acidic condition welcomes the hypothesis that the bacterium may be profoundly vulnerable to the low pH of the phagolysosome, especially on the off chance that one considers the bacterial thickness (that is, number of microorganisms per unit liquid volume) in a phagosome "low." However, one may likewise consider the bacterial thickness in a phagosome to be to a great degree high. More critical, executing of *M. tuberculosis* at pH 4.5 is significantly affected by the creation of the medium in a way that can be viewed as artifactual . Due to their inclination to bunch, mycobacteria are usually developed in cleansers to take into account scattered development and readiness of moderately uniform bacterial suspensions for trial contemplates. Free unsaturated fats are lethal to *M. tuberculosis*, especially at low pH. It was accounted for that an assortment of strains of *M. tuberculosis* are impervious to executing at a pH of 4.5 in phosphatecitrate cradle. The bacilli are likewise ready to keep up a close nonpartisan intra-bacterial pH when set in phosphate-citrate support at pH 4.5, demonstrating that they can counter the passage of protons. In this way, in basic support *M. tuberculosis* opposes phagolysosomal centralizations of corrosive.[6]



(Figure 2.1 *M. tuberculosis inside the macrophage. In resting macrophages, M. tuberculosis impedes phagosome development and lives in a somewhat acidic compartment. Enactment with IFN-γ brings about phagosome development and phagosome-lysosome combination.*

This opens the microscopic organisms to have gotten push including protons from the vacuolar ATPase, RNI and ROI, free unsaturated fats, ubiquitin-inferred peptides, and lysosomal hydrolases. M. tuberculosis opposes fermentation with the assistance of the Rv3671c-encoded layer bound serine protease, the putative magnesium transporter MgtC, and the pore-shaping M. tuberculosis external layer protein (OmpATb). The correct systems by which these proteins give acid resistance stay to be distinguished)[6]

The already said observations laid ground for the demonstrating that when lysosomes consolidate with M. tuberculosis containing phagosomes in immunologically authorized macrophages, the phagolysosomal pH tumbles to 4.5 to 5.0. Different audits have certified these discernments. In one of the reviews where fluorescent mannosylated dots were utilized, it was accounted for that the pH of the macrophage phagolysosome falls quickly, inside 15 to 60 min, to a pH somewhat underneath 5.0. Along these lines, the pH of the macrophage phagolysosome seems to shift with the immunological condition of actuation of the macrophage and the way of the phagocytic molecule however by and large achieves a pH related with suspension of development of numerous facultative intracellular bacterial pathogens in soup culture. Absolutely, overabundance protons can harm DNA, proteins, and lipids and upset biochemical responses. Regardless of whether phagosomal acid is really a noteworthy bactericidal effector mechanism of macrophages is hard to set up.[5]

2.7 *Mycobacterium fortuitum*:

Mycobacteria other than *Mycobacterium tuberculosis complex* and *Mycobacterium leprae* are by and large alluded to as Non Tuberculous Mycobacteria (NTM). Human sickness coming about because of NTM contamination is ordered into four particular clinical disorders: aspiratory infection, lymphadenitis, cutaneous malady, and dispersed illness. Among these, unending aspiratory malady is the most widely recognized limited clinical condition. Thinks about have exhibited that sickness inferable from NTM is on the ascent. NTM are in charge of an expanding extent of mycobacterial illness in many created and creating nations.[8]

Non Tuberculous Mycobacteria are omnipresent creatures and are much of the time disconnected from natural sources, including surface water, tap water, and soil. In like manner, the segregation of NTM species from a respiratory example is lacking confirmation for the nearness of NTM lung sickness. A few patients are contaminated with NTM without confirmation of aspiratory illness. Such disease may demonstrate colonization or transient contamination. *Mycobacterium fortuitum* is one of the quickly developing mycobacteria (RGM), which are recognized from other NTM by their

capacity to frame settlements in under one week and there in vitro imperviousness to anti-mycobacterials. *M. fortuitum* is a generally detached creature from respiratory examples in clinical research facilities in numerous nations. To date, in any case, the clinical importance of this living being has not been all around examined.[9]

M. fortuitum contamination can be a nosocomial (healing center obtained) illness. Surgical locales may end up noticeably tainted after the injury is uncovered straightforwardly or by implication to sullied tap water. Other conceivable wellsprings of *M. fortuitum* disease incorporate embedded gadgets, for example, catheters, infusion site abscesses, and debased endoscopes. Late production on Rapidly developing Mycobacteria (RGM) is accessible gives the accompanying parts of RGM: (i) its sources, inclining variables, clinical signs, and associative contagious contaminations; (ii) the dangers of misdiagnoses in the administration of RGM diseases in dermatological settings; (iii) the conclusions and results of treatment reactions in like manner and remarkable contaminations in immuno-compromised and immuno-competent patients; (iv) traditional versus flow sub-atomic strategies for the location of RGM; (v) the fundamental standards of a promising MALDI-TOF MS, inspecting convention for cutaneous or subcutaneous sores and its potential for the exact separation of *M. fortuitum*, *M. chelonae*, and *M. abscessus*; and (vi) changes in RGM disease administration as depicted in the current 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines, including interpretation criteria of molecular methods and antimicrobial drug panels and their break points [minimum inhibitory concentrations (MICs)], which have been highlighted for the initiation of antimicrobial therapy.[10]

M. fortuitum and *M. chelonae* have been one of the reason for abscesses and post surgical injury diseases.[11] They are spreaded by airborne, clean or polluted faucet water. In one review the post agent wounds had been at first mended agreeably after surgery however it was seen after a time of 12-28 days they ended up noticeably erythematous and began releasing discharge in little amount which later handed overflowing and serous over character. These injuries did not have impact of anti-toxins on them.

Twisted contaminations after operation set aside some opportunity to show up, it by and large happened as talked about before when the operation scar separates and a non-recuperating shallow ulcer creates with releasing sinus.[12] Sethi et al announced seven patients with *M. fortuitum* diseases post laproscopic tubectomies.[13] There was development of mellow inconvenience, in-duration, with/without neighborhood torment, swelling and sero-sanguineous release which happened from a moment opening over the

worked scar for past 2-3 weeks, after the onset of the disease. Suction examples demonstrated no living being on gram stain were sterile for oxygen consuming and anaerobic creatures. Subsequently, all these sterile examples must be recolored by ZN strategy for corrosive quick bacilli (AFB) and subjected to culture on LJ media.[14] Surgical site contaminations because of *M. fortuitum* are all around reported, and are extraordinarily connected with cardio thoracic surgery.[15] Deferred wound mending, chronicity of disease with delayed course of costly anti-toxins makes it a genuine nosocomial contamination. The wellspring of disease is mostly sullying of twisted because of any methods. On the off chance that there is appropriate observation of natural culture from faucet water, bowl and operation theater (OT) it may not diminish development of NTM. Some healing center gained diseases or say nosocomial contaminations with this living being incorporate diseases of embedded gadgets extraordinarily catheters and infusion site abscesses. There ought to be appropriate and strict cleansing of all OT hardware and legitimate hand washing, purification and offices must be under taken to avert wound contaminations. To begin with line anti-tubercular drugs like ethambutol and rifampicin have a cidal impact against the living being however as a rule blend of antibacterial operators like amikacin, fluorinated quinolones, doxycycline, imipenem and clarithromycin are utilized.[16] In India, there is requirement for high level of doubt for recognizable proof of *M. fortuitum* as few instances of *M. fortuitum* contaminations are being under announced because of low doubt of its nearness among clinicians and microbiologists.[17] Particularly in instances of constant post agent wounds. The quantity of diseases brought on by *M. fortuitum* is on the ascent.[16] A few clinical elements like osteomyelitis, catheter contaminations, postsurgical diseases, peritonitis and aspiratory diseases have additionally been accounted for as signs of its disease. The shortcoming of one's resistant framework can likewise impact the hazard calculate of a man getting an illness because of *M. fortuitum* presentation. The primary target gatherings of mycobacterioses (malady because of atypical mycobacteria) are the elderly, and the HIV-contaminated with traded off insusceptibility. Along these lines, individuals with stifled insusceptible framework have a more prominent possibility of building up a contamination. Intrigue has been kept up in the astute "atypical" mycobacteria in connection to their capacity to bring about illness in the immuno-compromised people, and progressively in their conceivable part in adjusting invulnerable reactions like the major pathogenic species.[18]

2.8 Clinical significance of *M. fortuitum* :

M. fortuitum contamination can bring about different clinical disorders. It is an exceptional reason for NTM lung infection. Neighborhood cutaneous sickness, osteomyelitis, joint contaminations, and visual ailment (eg, keratitis, corneal ulcers) may happen after injury *M. fortuitum* disease is an uncommon reason for separated lymphadenitis. Dispersed malady, more often than not with scattered skin sores and delicate tissue sores, happens solely in the setting of serious immuno-suppression, particularly AIDS. Endocarditis has been recorded.

Surgical-site diseases because of *M. fortuitum* contamination are very much reported, particularly in relationship with cardiothoracic surgery. The source is as often as possible tainting of the injury, specifically or by implication, with colonized faucet water. Other noso-comial diseases with this living being incorporate contaminations of embedded gadgets (e.g. catheters, etc.) and infusion site abscesses. Pseudo-flare-ups have been related with debased endoscopes. Late episodes have additionally been depicted in immuno-competent has after utilization of debased whirlpool footbaths in nail salons.[19]

There are three critical perceptions that are made in regards to pathogenesis of NTM diseases:

1. Disseminated NTM contaminations happened in patients who were tainted by HIV after the estimation of CD4 T-lymphocyte esteem diminishes beneath 50/l, proposing that particular T-cell items or exercises are required for mycobacterial resistance.[20,21]

2. In second gathering where patients were HIV-uninfected, transformations in interferon (IFN)- γ and interleukin (IL)- 12 blend and reaction pathway. (IFN-γ receptor 1 [IFNγR1], IFN-γ receptor 2 [IFNγR2], IL-12 receptor β1 subunit [IL12Rβ1], the IL-12 subunit p40 [IL12p40], the flag transducer and activator of interpretation 1 [STAT1], and the atomic variable ƙβ fundamental modulator [NEMO]) have been related with hereditary disorders of scattered NTM.

3. There is likewise a relationship between bronchiectasis, nodular aspiratory NTM contaminations and a specific body habitus, transcendently in postmenopausal ladies (e.g., pectus excavatum, scoliosis, mitral valve prolapse)[18]

Non tuberculous mycobacteria are known to bring about illnesses in immuno skilled people too. The instruments by which they taint and remain in macprophages are not that outstanding. So in one review THP-1 macrophages were contaminated with *M. fortuitum , M. celatum, M. abscessus* and *M. tuberculosis*. Comes about demonstrated that moderate

developing mycobacteria got access to the THP-1 macrophages in contrast with other quickly developing mycobacteria. It can be along these lines presumed that quickly developing microorganisms were effectively phagocytosed. Reasonability (CFUs/ml assurance) test was performed to check whether intracellular mycobacteria were alive. Quickly developing microbes indicated 2-log of reduction in CFUs of *M. abscessus* display inside THP-1 macrophages . Number of *M. fortuitum* stayed same between various conditions however it expanded by 2-log from 6h to 48h post disease. Quickly developing mycobacteria likewise harmed monolayer trustworthiness of THP-1 macrophage. Uprightness of THP-1 macrophage monolayer diminished when *M. abscessus* and *M. fortuitum* were available. It was likewise watched that moderate producers did not adjusted morphology of THP-1. Along these lines obviously macrophage monolayer was harmed at 48h post contamination.

Quickly developing mycobacteria instigated the generation of ROS by THP-1 macrophages at 6 and 24 h post contamination. Conversely, no ROS could be recognized with the moderate developing mycobacteria, *M. celatum* and *M. tuberculosis*, even after 24 hour of contamination. It is plausible that the large amounts of ROS prompted by quickly developing mycobacteria could be related with the broad cell harm.

Cytokines assume vital part in regulating resistant reaction and furthermore in assurance obviously of disease. After time of 24 hours quickly developing microbes indicated abnormal state of cytokine. The quickly developing mycobacteria and *M. celatum* incited the generation of elevated amounts of IL-8 at 6 and 24 h post contamination, while no perceptible levels of this cytokine were found within the sight of *M. tuberculosis*.

Macrophage obliteration can likewise be an outcome of a strong generation of receptive oxygen species (ROS) in THP-1 cells tainted with *M. abscessus* and *M. fortuitum*, as has been accounted for beforehand with various host cell/pathogen frameworks.These comes about affirm the theory that quickly developing mycobacteria initiate an extremely intense safe reaction when contrasted with run of the mill pathogenic mycobacteria, (for example, *M. tuberculosis*. Hence, development rate may be connected, sometimes, to the intracellular survival of mycobacteria and the safe reaction that they incite in THP-1 macrophages. Development rate, be that as it may, is not by any means the only determinant of the result of the cooperation of mycobacteria macrophages; different elements, for example, envelope cell lipids and the specific destructiveness elements of every

mycobacterium ought to be additionally considered. There is a recommendation that the capacity to square responsive oxygen species generation by moderate developing mycobacteria is an invulnerable avoidance methodology that putatively advances their survival and cytokine creation in the host, even in NTM species. At long last, the information gives understanding into the novel components that *M. celatum* utilizations to hold on inside its host cell, which ought to further be described keeping in mind the end goal to pick up learning about the pathogenic NTM species that cause sickness in immuno able patients. *M. fortuitum* lives significantly in soil and water and can flourish even in most antagonistic conditions. Human contaminations because of this microorganisms have been found in each segment of the world this is on account of it is discovered wherever and duplicates when supplement substance and temperature are at right level. High centralization of nourishment is a bit much for improvement of *M. fortuitum* rather high temperatures upgrade nearness of these microscopic organisms. The way that it is observed in soil turns out to be valuable since it helps in the breakdown of natural matter in soil, in this way recharging the dirt with supplements.[23,24]

2.9 Virulence of *M. fortuitum* :

"Virulence" is gotten from the Latin words "infection" ("harm") and "lentus"("fullness") signifying "loaded with toxin", and the expression "infection" might be identified with the Sanskrit word "visham," signifying "harm". “The term virulence is utilized to describe the relative limit of a microorganism to bring about infection. It is the normal for harmfulness that recognizes pathogens, from non-pathogens. It is the capacity of a microorganism to bring about ailment because of the statement of specific microbial qualities. Such qualities, or virulent elements, have been characterized traditionally as segments of a pathogen that hinder destructiveness when erased, yet not feasibility. Microbial characteristics, for example, the container of *S. pneumoniae,* the poisons of *C. diphtheriae* and *Vibrio cholerae,* and the M protein of gathering *A. Streptococcus*, are reliable with this definition.

As it was realized that *M. fortuitum* is pathogenic in this way there are numerous destructiveness calculates yet to be found. It goes under family "Mycobacterium" which additionally incorporates *Mycobacterium tuberculosis*, the subsequent specialist of tuberculosis. Studing the pathogenicity of its assorted is undoubtly testing. Destructiveness being the fundamental component of pathogenicity can be measured at the season of disease of macrophages and creatures, utilizing different examines and different procedures can be upgraded to produce mutants as in *M. tuberculosis*. Thus, diverse

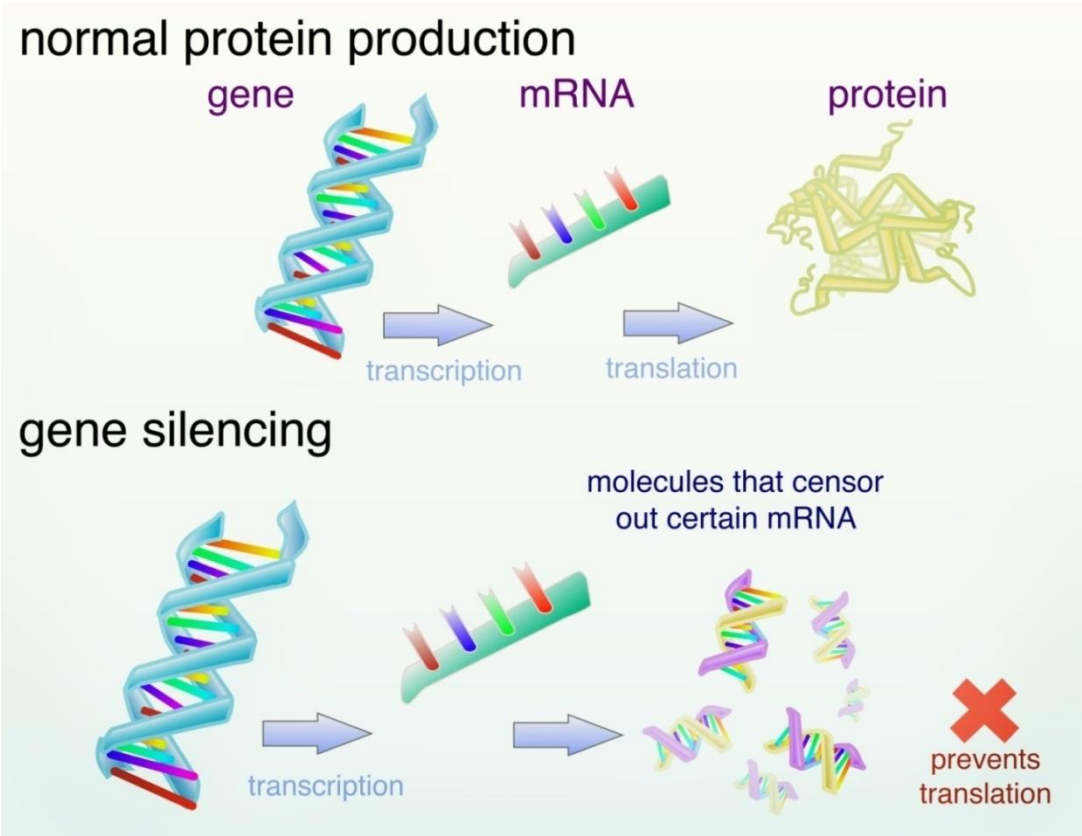
methodologies can be joined in same route as of plenty to recognize virulence determinant of *M. fortuitum*.

A superior instrument TnphoA is utilized to produce influenced mutants in secretory or film crossing proteins. Replication of TnphoA contains transposon of Tn5 which encodes kanamycin and promoter less basic Gene of E.coli alkane phosphates which needs flag peptide arrangements.[25] Antacid phosphatase is dynamic just when sent out on the cell surface. Action of antacid phosphate is not distinguished until and unless the coordination of TnphoA has happened in casing into the qualities coding for secretory proteins. It is a superior columnist framework for the determination of clones having joining in secretory qualities. TnphoA is a framework accommodated insertional mutagenesis of secretory proteins and contemplating of these proteins as harmfulness determinant(s). Since by and large all bacterial proteins involved as destructiveness variables are extracellular, surface related, or periplasmic, consequently utilization of TnphoA ought to give a solid enhancement to inclusion changes that influence pathogenic properties of microscopic organisms. Utilizing the transposon in *lipU*[25] ought to bring about the recognizable proof of layer or secretory proteins which assumes a part in destructiveness. Distinguishing proof of qualities of *M. fortuitum* brings about the ID of their homologues in *M. tuberculosis* as appeared with other atypical mycobacterial strains like *M. marinum* and *M. smegmatis*. Cloning and sequencing of the disturbed quality taken after by complementation examination of the mutant indicated inclusion of the upset quality in destructiveness and constancy of *M. fortuitum*, bearing abnormal state of homology with administrative grouping Rv3291c of *M . tuberculosis*. Contemplates on destructiveness in mycobacteria other than the *M. tuberculosis* complex have been completed before and homologues of *M. ulcerans* toxin and *M. avium* subsp. avium mig (macrophage initiated quality) in *M. tuberculosis* have been effectively distinguished.[26]

Focal points in distinguishing virulent components incorporate the arrangement of focuses for new potential therapeutics and the recognizable proof of qualities that can be changed to permit the advancement of new immunizations. At a more basic level, the distinguishing proof of bacterial destructiveness figures and additionally the host elements required at various phases of contamination and illness is basic for the explanation of mycobacterial pathogenesis. Improvement of a reproducible and solid model for the investigation of industrious disease ought to demonstrate important data towards understanding the science of mycobacterial idleness.

2.10 Gene Silencing and Knock Out :

Gene silencing: Gene silencing is by and large used to portray direction of gene expression. A specific cell averts articulation of certain quality this is characterized as Gene Silencing. For research planned quality quieting can happen either amid interpretation or interpretation. It is thought to be like quality knockout however it has some distinction for sure. Amid quality knockout quality is totally expelled from genome and have no expression yet in gene silencing, expression is for the most part decreased. Hence gene silencing is considered as quality knockdown.



(Figure 2.2 : *Mechanism of gene silencing)*[34]

Gene knockout: A gene knockout is a hereditarily built creature that conveys at least one genes in its chromosomes that have been made out of commission (have been "knock out" of the organism). This is accomplished for research purposes. Otherwise called knockout living beings or just knockouts, they are utilized as a part of finding out about a gene that has been sequenced, however which has an obscure or not completely known capacity. Scientists draw derivations from the contrast between the knockout life form and ordinary people. The term likewise alludes to the way toward making such a living being, as in "knocking out" a gene. Knockout is expert through a mix of systems, starting in the test tube with a plasmid, a

bacterial artificial chromosome or other DNA construct, and continuing to cell culture. Singular cells are hereditarily changed with the build and for knockouts in multi-cell life forms - at last intertwined with an undifferentiated cell from an early incipient organism. The construct is designed to recombine with the objective gene, which is proficient by joining arrangements from the gene itself into the develop. Recombination then happens in the locale of that arrangement inside the gene, bringing about the inclusion of a foreign gene to disrupt the gene. With its sequence intruded on, the altered gene by and large will give rise to a nonfunctional protein, in the event that it is interpreted or translated by any means. A restrictive knockout permits quality cancellation in a tissue particular way. Since recombination is an uncommon occasion on account of most cells and most constructs, the sequence decided for insertion is usually a reporter. This empowers simple determination of cells or people in which knockout was effective.

Gene Knockout-Directed Gene Disruption Directed gene inactivation involves the inclusion of an antibiotic resistance cassette in center of the gene of interest, trailed by allelic substitution of the chromosomal gene by the transformed one, bringing on disturbance of the gene capacity in the host cell. Gene knockout is normally a two-stage handle including incorporation of plasmid containing desired disrupted gene by a solitary hybrid occasion (Campbell-sort combination) into the genome at the area of homology. In the second hybrid occasion, the plasmid spine is extracted by methods for recombination, bringing about the desired gene disruption. Another strategy for directed gene inactivation, named specific transduction, utilizing a bacteriophage delivery system has been portrayed and is the present technique for decision for the directed mutation.[27] This system is exceptionally productive in light of the fact that basically the majority of the beneficiary cells can be transduced and the selection is powerful.

2.11 Anti-sense Technology:

In anti-sense technology, artificially – created corresponding atoms search out and tie to messenger RNA (mRNA), hindering the last stride of protein generation. mRNA is the nucleic acid molecule that conveys hereditary data from the DNA to the next cell apparatus required in the protein generation. By Binding to mRNA, the antisense drugs hinder and restrain the generation of particular illness related proteins. "Sense" alludes to the first arrangement of the DNA or RNA particle. "Anti-sense" alludes to the reciprocal grouping or complementary sequence of the DNA or RNA molecules.

The fundamental thought is that if an oligo-nucleotide (a short) RNA or DNA atom integral to a mRNA created by a quality) can be brought into a cell, it will particularly tie to its objective mRNA through the stunning specificity of correlative based blending a similar mechanism which ensures the constancy of DNA replication and of RNA translation from the gene. This coupling shapes a RNA dimmer in the cytoplasm and stops protein synthesis. This happens in light of the fact that the mRNA no longer has entry to the ribosome and cytoplasm by ribonucleotide. Along these lines, the presentation of short chain of DNA reciprocal to mRNA will prompt a particular decrease, or blockage, of protein blend by a specific gave. In actuality, the gene will be turned off.[28] A Sense strand is a 5' to 3' mRNA atom or DNA particle. The complementary strands or mirror strand to the sense is called an antisense. Antisense technology is the procedure in which the antisense strand hydrogen bonds with the focused on sense strand. At the point when an antisense strand ties to a mRNA sense strand, a cell will perceive the twofold helix as outside to the cell and continue to debase the faculty mRNA particle in this way keeping the generation of undesired protein. Despite the fact that DNA is now a twofold stranded protein, antisense innovation can be connected to it constructing a triplex development. A DNA antisense molecule must be around seventeen packs to function, and roughly thirteen bases for a RNA particle RNA antisense strands can be either catalytic, or non catalytic. The catalytic antisense strands, likewise called ribozymes, which will cleave the RNA molecule at specific sequences. A Non catalytic RNA antisense strand squares assist RNA preparing, i.e. changing the mRNA strand or translation. The correct mechanism of an antisense strand has not been resolved. The present theories incorporate. "Blocking RNA grafting, accelerating degradation of the RNA particle, and keeping introns from being spliced out of the mRNA, obstructing the exportation of mRNA into the cytoplasm, hindering translation, and the triplex arrangement in DNA.[29]

Anti-sense technology includes utilization of complementary RNA, which ties to the mRNA of a particular gene along these lines preventing its translation. Antisense technology is especially helpful for silencing of fundamental genes as their knockout mutants can't survive, and in frameworks where gene inactivation is troublesome. Regulatable acetamide/acetamidase framework is for the most part utilized for restrictively controlling the generation of antisense RNA in mycobacteria. Uses of the antisense strategy were in *M bovis* to bring down the levels of *AhpC* and in *M. tuberculosis* H37Rv to decrease the measures of *SodA*. As of late, the antisense approach was utilized to diminish the level of *sigA*, and Rv3303c in *M. tuberculosis*.

2.12 *In-Silico* Analysis:

In silico does not mean anything in Latin. The proper Latin phrase would likely be *in simulacra* to describe experiments done on the likeness (simulacrum) or model of a phenomenon. The expression *in silico* was first used in public in 1989 to characterize biological experiments carried out entirely on a computer.

In silico biology refers to computational models of biology. *In silico* is an expression used to mean performed on a computer or via computer simulation. It is used in systems biology. Due to the vast amounts of data that is now generated by molecular and cell experimental biologists, computational biology is increasingly necessary to manage it. *In silico* biology draws from the vast amounts of biological information available, and applies sophisticated algorithms or simulations to advance scientific understanding. The results of these simulations can then be tested experimentally or serve as a guide for future physical experimentation.

In bioinformatics *in silico* biology can be used to measure or even preserve biodiversity. Biodiversity of an ecosystem might be defined as the total genomic complement of a particular environment, from all of the species present, whether it is a biofilm in an abandoned mine, a drop of sea water, a scoop of soil, or the entire biosphere of the planet Earth. Databases are used to collect the species names, descriptions, distributions, genetic information, status and size of populations, habitat needs, and how each organism interacts with other species. Specialized software programs are used to find, visualize, and analyze the information, and most importantly, communicate it to other people. Computer simulations model such things as population dynamics, or calculate the cumulative genetic health of a breeding pool (in agriculture) or endangered population (in conservation). One very exciting potential of this field is that entire DNA sequences, or genomes of endangered species can be preserved, allowing the results of nature's genetic experiment to be remembered *in silico*, and possibly reused in the future, even if that species is eventually lost.

Bio-informatics tools

1. BLAST :

BLAST for **B**asic **L**ocal **A**lignment **S**earch **T**ool is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to

compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

Different types of BLASTs are available according to the query sequences. For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence. The BLAST algorithm and program were designed by Stephen Altschul, Warren Gish, Webb Miller, Eugene Myers, and David J. Lipman at the National Institutes of Health and was published in the *Journal of Molecular Biology* in 1990 and cited over 50,000 times.[1]

Mechanism : Using a heuristic method, BLAST finds similar sequences, by locating short matches between the two sequences. This process of finding similar sequences is called seeding. It is after this first match that BLAST begins to make local alignments. While attempting to find similarity in sequences, sets of common letters, known as words, are very important. For example, suppose that the sequence contains the following stretch of letters, GLKFA. If a BLAST was being conducted under normal conditions, the word size would be 3 letters. In this case, using the given stretch of letters, the searched words would be GLK, LKF, KFA. The heuristic algorithm of BLAST locates all common three-letter words between the sequence of interest and the hit sequence or sequences from the database. This result will then be used to build an alignment. After making words for the sequence of interest, the rest of the words are also assembled. These words must satisfy a requirement of having a score of at least the threshold *T*, when compared by using a scoring matrix. One commonly used scoring matrix for BLAST searches is BLOSUM62, although the optimal scoring matrix depends on sequence similarity. Once both words and neighborhood words are assembled and compiled, they are compared to the sequences in the database in order to find matches. The threshold score *T* determines whether or not a particular word will be included in the alignment. Once seeding has been conducted, the alignment which is only 3 residues long, is extended in both directions by the algorithm used by BLAST. Each extension impacts the score of the alignment by either increasing or decreasing it. If this score is higher than a pre-determined *T*, the alignment will be included in the results given by BLAST. However, if this score is lower than this pre-determined *T*, the alignment will cease to extend, preventing the areas of poor alignment from being included in the BLAST results. Note that increasing the *T* score limits the amount of space available to search, decreasing the number of neighborhood words, while at the same time speeding up the process of BLAST.

The BLAST program can either be downloaded and run as a command-line utility "blastall" or accessed for free over the web. The BLAST web server, hosted by the NCBI, allows anyone with a web browser to perform similarity searches against constantly updated databases of proteins and DNA that include most of the newly sequenced organisms.

The BLAST program is based on an open-source format, giving everyone access to it and enabling them to have the ability to change the program code. This has led to the creation of several BLAST "spin-offs".

There are now a handful of different BLAST programs available, which can be used depending on what one is attempting to do and what they are working with. These different programs vary in query sequence input, the database being searched, and what is being compared. These programs and their details are listed below:

BLAST is actually a family of programs (all included in the blastall executable).

Major programs used in this project are:

1. Nucleotide-nucleotide BLAST (blastn)

This program, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies.

2. Protein-protein BLAST (blastp)

This program, given a protein query, returns the most similar protein sequences from the protein database that the user specifies.

2. Multiple Sequence Alignment:

In bioinformatics, a **sequence alignment** is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns. Sequence alignments are also used for non-biological sequences, such as calculating the edit distance cost between strings in a natural language or in financial data.
Multiple sequence alignment is an extension of pair-wise alignment to incorporate more than two sequences at a time. Multiple alignment methods try to align all of the sequences in a

given query set. Multiple alignments are often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionarily related. Such conserved sequence motifs can be used in conjunction with structural and mechanistic information to locate the catalytic active sites of enzymes. Alignments are also used to aid in establishing evolutionary relationships by constructing phylogenetic trees. Multiple sequence alignments are computationally difficult to produce and most formulations of the problem lead to NP-complete combinatorial optimization problems. Nevertheless, the utility of these alignments in bioinformatics has led to the development of a variety of methods suitable for aligning three or more sequences.

Chapter 3
Materials
And
Methods

lipF gene with full length of 927 base pair having an open reading frame (orf) of 729 base pair is present in the genome of *Mycobacterium tuberculosis*. The confirmation of this gene in *M. fortuitum* is done by PCR. Multiple sets of primers were designed from flanking region of *lipU* constant domain regions (CDS) and then PCR was done using the *M. fortuitum* genome. The PCR products were sequenced and checked for the homology with the *lipU* of *M. tuberculosis*. After doing the homology search, the longest sequence having the maximum homology was determinded which is yet to be submitted to NCBI. *pMV261* vector was used to construct the antisense mutants of *M. fortuitum lipU*. The orientation has been confirmed using *BamHI* site in *pMV261* vector. Presence of full length gene and its orientation was confirmed by restriction digestion and colony PCR using the primers designed . *In-silico* analysis of the whole data has been done using bio-informatics tools such as BLAST, MSA, etc.

3.1 Primer designing for *lipU* sequence of *M. fortuitum* :

Primers were designed using following method manually:

1. Full genomic sequence of *lipU* gene of *M. tuberculosis* was taken and full length primers of *M. tuberculosis* were designed using software primer 3.
2. For the forward primer, nucleotides of the sequence for the region that binds to the ORF was taken and full length primer was designed.
3. Similarly, for reverse primer designing, reverse complement of the bases were taken to get the PCR amplification including the stop codon using software primer 3.

3.2 Genomic DNA isolation of *M. fortuitum* :

Chemical requirements: TES buffer, lysozyme, buffer phenol, chloroform: isoamylalcohol, isopropanol (chilled), ethanol, autoclaved distilled water, culture of *M. fortuitum*

Equipments used: Centrifuge, centrifuge tubes, micro-centrifuge tubes, incubator, discard box, gel doc, pipettes, and tips.

Procedure:

1. The culture was taken and pelleted in 50 ml centrifuge tube at 7000 rpm for 15 minutes.
2. The supernatant was discarded and 2 mL TES buffer was added to pellet.
3. Then it was incubated at 80°C for about 1 hour. After that 2mg/ml of lysozyme was added to it i.e. 80µL and incubated at 37°C for 1 hour.

4. 1.5 % SDS and 100 µg/mL proteinase K was added and incubated at 50°C for 1 hour.
5. Buffer phenol was added in each tube and they were centrifuged at 12000g for 15 minutes.
6. Then chloroform: isoamylalcohol was added in 24:1 ratio in each tube and centrifuged them at 12000g for 15 minutes.
7. The aqueous layer was transferred to fresh tubes. Again chloroform: isoamylalcohol was added and centrifuged at 12000g for 10 minutes.
8. The aqueous layer was transferred to fresh tubes and equal volume of chilled iso-propanol was added.
9. It was incubated at 4°C for overnight and then centrifuged at 12000g for 15 minutes. Supernatant was removed and 1mL 70% ethanol was added and centrifuged at 12000g for 15 minutes.
10. Again, supernatant was discarded and ethanol was evaporated. 100 µL of autoclaved distilled water was added.
11. It was then placed in ice for about 2 hours for proper suspension of DNA.
12. Finally, electrophoresis was done to check the DNA in 0.8% agarose gel.

3.3 PCR amplification of genomic DNA of *M. fortuitum* by full length *lipU* primer of *M. tuberculosis*:

Chemical requirements: Isolated genomic DNA of *M. fortuitum*, forward primer, reverse primer, green master mix, nuclease free water.

Equipments used: Thermocylcer, mini-spin, PCR - vials, discard box, gel doc, pipettes, and tips.

Procedure :

1. PCR reaction was carried out with the following mixture:

- gDNA	- 2.0 µL
- Forward Primer	- 0.5 µL
- Reverse Primer	- 0.5 µL
- Master Mix	- 5.0 µL
- Nuclease free water	- 2.0 µL
Total	- 10 µL

2. PCR conditions were kept as follows:
    - Initial Denaturation - 95°C
    - Denaturation - 95°C
    - Annealing - 58°C / 60°C / 62°C
    - Extension - 72°C
    - Final Extension - 72°C

    Total number of cycles – 35
3. Electrophoresis of the PCR product was done on 1.5% agarose gel.

3.4 Primer designing for ORF region of *lipU* sequence of *M. fortuitum* :

For PCR based plasmid cloning, primers were designed using following method manually:

1. For the forward primer, primers were designed using software Primer 3 and *BamHI* restriction site (GGATCC) was added to the 5' end of this primer.
2. Similarly, for reverse primer designing, again primers were designed using software Primer 3. Finally *BamHI* restriction enzyme was added to the same but in a reverse complement order.

3.5 PCR amplification (gradient) of genomic DNA of *M. fortuitum* using designed *lipU* primers :

Chemical requirements: Isolated genomic DNA of *M. fortuitum*, forward primer, reverse primer, green master mix, nuclease free water.

Equipments used: Thermocylcer, mini-spin, PCR - vials, discard box, gel doc, pipettes, and tips.

Procedure :

1. PCR reaction was carried out with the following mixture:
    - gDNA - 2.0 µL
    - Forward Primer - 0.5 µL
    - Reverse Primer - 0.5 µL
    - Master Mix - 5.0 µL
    - Nuclease free water - 2.0 µL

    Total - 10 µL

2. PCR conditions were kept as follows:
    - Initial Denaturation - 95°C
    - Denaturation - 95°C
    - Annealing - 58°C / 60°C / 62°C
    - Extension - 72°C
    - Final Extension - 72°C

    Total number of cycles – 35
3. Electrophoresis of the PCR product was done on 1.5% agarose gel.

3.6 TA Cloning of the amplified PCR product of *lipU* of *M. fortuitum* :

Chemical requirements: Amplified PCR product of *M. fortuitum*, DNA Ligase, pGEM-T easy vector, buffer, nuclease free water.

Equipments used: Thermo-cylcer, mini-spin, vials, discard box, gel doc, pipettes, and tips.

Procedure :

1. Cloning reaction of the PCR product was carried as follows :

PCR Product	- 2 µL
DNA Ligase	- 1 µL
pGEMT easy	- 1 µL
Buffer	- 5 µL
Nuclease free water	- 1 µL
Total	- 10 µL

2. The above reaction was kept at 16°C for overnight.

3.7 Transformation of ligated product into competent DH5α *E.coli* cells:

3.7.1 Preparation of competent cells :

Chemical requirements: Transformation buffer-1 (RbCl, $MnCl_2.4H_2O$, Potassium acetate, $CaCl_2.2H_2O$, 15% glycerol), transformation buffer-2 (MoPs, RbCl, $CaCl_2.2H_2O$, 15% glycerol), LB.

Equipments used: Centrifuge, PCR-vials, micro-centrifuge tubes, vortex, incubator, discard box, gel doc, pipettes and tips.

Procedure:

1. Single colony was picked and inoculated in 10 mL LB. It was incubated at 37°C for overnight.
2. 1 ml of overnight culture was added to 100 mL pre-warmed LB medium and incubated with constant shaking at 37°C.
3. The culture was cooled in ice for 5 minute and transferred to sterile round bottom centrifuged tube.
4. The cells were collected by centrifuged at low speed 4000g for 5 minute at 4°C. Supernatant was carefully discarded. The cells were always kept in ice.
5. The cells were gently re-suspended in transformation buffer 1 and kept on ice for an additional 90 minute.
6. Cells were collected by centrifuging for 5 minute at 4000 rpm at 4°C. The supernatant was discarded.
7. The cells were then carefully re-suspended in 4 ml ice cold transformation buffer 2.
8. Aliquots of 100-200 µL were prepared in sterile centrifuge tubes and stored at -80°C.

3.7.2 Transformation of TA cloned gene into *E.coli* :

Chemical requirements: Ligated product, competent cells, LB.

Equipments used: PCR - vials, micro-centrifuge tubes incubator, water bath, petri plates.

Procedure:

1. Competent cells were thawed in ice. 15µL of ligated product was added in 200 µL of competent cells.
2. It was mixed by swirling and incubated in ice for 30 minutes.
3. After that, it was incubated in water bath set at 42°C for 90 minutes. Then, it was chilled on ice for about 5-15 minute.
4. Then, 800µL of LB was added to the vials, and incubated for 45 minute a 37°C.
5. After 45 minutes of incubation, the cells were spread on selection plate with appropriate antibiotics.

3.8 Screening and selection of transformed mutants on Ampicillin resistant plates along with the preparation of master plate:

Chemical requirements: LB Agar plates , ampicillin, IPTG, X-gal, transformed mutant cells, distilled water.

Equipments used: Petri-dishes, weighing balance, autoclave, micro-centrifuge tubes, centrifuge, pipette, incubator, tips.

Procedure :

1. Culture plates of LB Agar were prepared along with the standard concentration of ampicillin. IPTG and X-gal were spread on the same culture plates.
2. 100µL of transformed cells were spread on LB agar plates.
3. Plates were kept in incubator for overnight at 37°C.
4. White colonies observed the very next day were transferred to master plate containing same concentration of ampicillin.
5. Plates were stored at 4°C.

3.9 Colony PCR of *lipU* transformants :

This step was performed for the confirmation of transformed mutants on the plate.

Chemical requirements: Isolated colony of transformant from the master plate, forward primer, reverse primer, green master mix, nuclease free water.

Equipments used: Thermocylcer, mini-spin, vials, discard box, gel doc, pipettes, and tips.

Procedure :

1. PCR reaction was carried out with the following mixture:
    - gDNA - 1 isolated colony
    - Forward Primer - 0.5 µL
    - Reverse Primer - 0.5 µL
    - Master Mix - 5.0 µL
    - Nuclease free water - 4.0 µL

    Total - 10 µL

2. PCR conditions were kept as follows:
    - Initial Denaturation - 95°C
    - Denaturation - 95°C
    - Annealing - 58°C
    - Extension - 72°C
    - Final Extension - 72°C

    Total number of cycles – 35
3. Electrophoresis of the PCR product was done on 1.5% agarose gel.

3.10 Plasmid isolation from the *lipU* transformants from master plate:

3.10.1 Inoculation of seed culture followed by primary culture:

Chemical requirements: LB nutrient media, distilled water, ampicillin, *lipU* transformed colony from master plate.

Equipments used: Flask, test-tubes, autoclave, incubator, petri-plates, pipette, tips.

Procedure:

1. Seed culture was inoculated in 10mL LB nutrient media with one isolated colony of *lipU* transformant taken from the master plate.
2. Test tubes were kept at 37°C for overnight growth.
3. Next day, 1mL of the growth culture was inoculated in 100mL LB nutrient media in two flasks separately.
4. After overnight growth, plasmid was isolated manually and from kit from the cultures.

3.10.2.1 Plasmid isolation from *lipU* transformants primary culture (Manually):

Chemical requirements: Primary culture, GTE (Glucose tris EDTA), ALS (Alkaline lysis buffer), SDS-NaOH solution, acetate solution, isopropanol, 96% ethanol, 70% ethanol, autoclaved distilled water.

Equipments used: Centrifuge, micro-centrifuge tubes, discard box, pipette, tips.

Procedure :

1. The culture was centrifuged at 7000 rpm for 10 minutes.
2. Then pellet was dried properly.
3. 300µL of GTE (Glucose tris EDTA), (ALS I) was added and vortexed.
4. 600µL of SDS-NaOH (ALS II) solution was added and mixed gently.
5. It was then incubated at 37°C for 5 minutes.
6. 450µL of acetate solution (ALS III) was added.
7. It was gently mixed for 5-6 times.
8. It was then incubated in ice for 30 minutes and centrifuged at 7000 rpm for 25 minutes at 4°C.
9. Supernatant was transferred to the fresh eppendorf or tarson tube and 270µL of isopropanol was added (or in 1:1 ratio).
10. Samples were incubated at -20°C for overnight.

11. The next day, samples were centrifuged at 12000 rpm for 15 minutes at 4°C.
12. Supernatant was discarded and 1mL of 96% ethanol was added to each sample.
13. Centrifugation was done at 10,000 rpm for 12 minutes at 4°C and again supernatant was discarded.
14. 1 mL of 70% ethanol was added to each sample and centrifuged at 10,000 rpm for 5 minutes at 4°C.
15. Supernatant was removed and 1mL of 70% ethanol was added to each eppendorf again.
16. Centrifugation is done at 10,000 rpm for 3 minutes at 4°C and the pellet is dried properly afterwards to evaporate ethanol.
17. 50µL of distilled autoclaved water was added to each sample and kept in ice for 1-2 hours for proper suspension of plasmid.
18. Plasmid from all micro-centrifuge tubes are pooled together and stored at 20°C.
19. Electrophoresis was done in 0.8% agarose gel.

Similarly, plasmid pMV261 was also isolated (manually).

3.10.2.2 Plasmid isolation from *lipU* transformants (Using kit):

Chemical requirements: Primary culture, plasmid isolation kit, autoclaved distilled water.

Equipments used: Centrifuge, vortex, centrifuge tubes, micro-centrifuge tubes, discard box, pipette, tips.

Procedure:

1. Primary culture was centrifuged at 7000 rpm for 10 minutes at 4°C and supernatant was discarded afterwards.
2. Pellet was dried properly and dissolved in 500µL of suspension buffer.
3. Culture was vortexed and 500µL of lysis buffer was added. The solution was mixed smoothly.
4. 650µL of neutralization buffer was added to the solution and mixed properly.
5. Centrifugation was done at 7000 rpm for 10 minutes at 4°C.
6. Supernatant was transferred into the gel column provided in the kit and centrifuged at 10,000 rpm for 2 minutes at 4°C.
7. 500µL of wash buffer was added to it and again it was centrifuged at 10,000 rpm for two minutes.
8. The above step was performed twice.

9. Column was again spun at 10,000 rpm for 2 minutes.
10. 40µL of autoclaved distilled water was added to it and it was incubated at room temperature for 10 minutes.
11. Centrifugation was done at 10,000 rpm for 2 minutes.
12. Again 30µL of autoclaved distilled water was added to it and it was incubated at room temperature for 10 minutes for the proper suspension of plasmid in the autoclaved distilled water.
13. Centrifugation was done again at 10,000 rpm for 2 minutes.
14. Finally, the micro-centrifuge tubes were stored at -20°C.
15. Electrophoresis was done on 0.8% agarose gel.

3.11 Digestion of ligated plasmid (*lipU* and *pMV261*) with *BamHI* and *EcoRV* :

Chemical requirements: *lipU* plasmid, cut smart buffer, enzymes (*BamHI* and *EcoRV*), nuclease free water.

Equipments used: Vials, mini-spin, ice-box, incubator, discard box, pipette, tips.

Procedure :

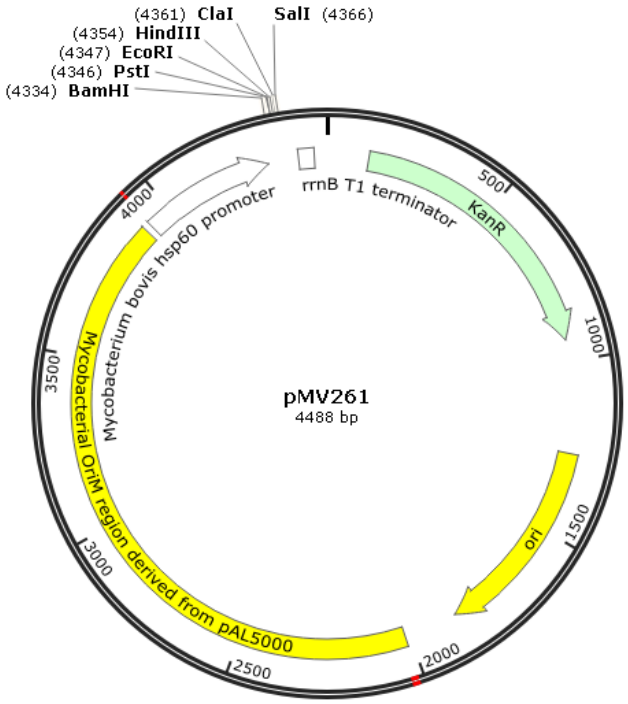
1. The following reaction was prepared in a vial separately for *lipU* and *pMV261* with both the enzymes:

- Nuclease free water	- 3.5µL
- Cut smart buffer (10X)	- 1.0µL
- Plasmid	- 5.0µL
- Enzyme	- 0.5µL
Total	- 10.0µL

2. The vials were kept at 37°C for 3 hours reaction.
3. Electrophoresis was done at 0.8% agarose gel.
4. After observing successful results, bulk/ mass reaction was prepared with different parameters:

- Nuclease free water	- 6.0µL
- Cut smart buffer (10X)	- 6.0µL
- Plasmid	- 45µL
- Enzyme	- 3.0µL
Total	- 60µL

5. Again, the vials were kept at 37°C for 3 hours digestion reaction and stored at -20°C afterwards.



(Figure 3.1 *Vector map of pMV261)*[35]

3.12 *In-silico* analysis using various bio-informatics tools :

3.12.1 Sequence based homological studies:

A. Nucleotide based homological studies :

1. Query sequence obtained from sequencing was taken.
2. *lipU* sequence of *M. fortuitum* obtained from ncbi (Ac. no - WP_065071208) was taken.
3. *lipU* sequence of *M. tuberculosis* obtained from ncbi (Ac. No.- EFP14259.1) was taken.
4. *lipU* sequence of *M. smegmatis* obtained from ncbi (Ac. No. - ELQ87153) was taken.
5. All the nucleotide sequences were produced to local alignment using bio-informatics tool BLAST (BlastN).
6. Similarly, all the nucleotide sequences were produced to multiple sequence alignment using bio-informatics tool T-Coffee and visualized using Jalview.

B. Protein based homological studies :

1. Query sequence of protein obtained from translating nucleotide sequence was taken.
2. *lipU* protein sequence of *M. fortuitum* obtained from ncbi (Ac. no - WP_065071208) was taken.
3. *lipU* protein sequence of *M. tuberculosis* obtained from ncbi (Ac. No.- EFP14259.1) was taken.
4. *lipU* protein sequence of *M. smegmatis* obtained from ncbi (Ac. No. - ELQ87153) was taken.
5. All the protein sequences were produced to local alignment using bio-informatics tool BLAST (BlastP an d BlastX).
6. Similarly, all the protein sequences were produced to multiple sequence alignment using bio-informatics tool T-Coffee and visualized using Jalview.

Chapter 4
Results

4.1 Primers designed for the *lipF* full length sequence :

Forward Primer : 5’ TTACCASGTGGCCTCT 3

Reverse Primer : 3’ ATGCATGCCGATGGC 5

4.2 *lipU* sequence of *M. fortuitum* obtained after Sanger sequencing (Accession number : MH197269):

Nucleotide sequence :

TTACCASGTGGCCTCTCGAATGTACTCACCGATCTGGCGCAACGAGCGCTTCGCT
TCGGCCACCATCGGCGACGCGAGCTGGAATACGTGCATCTGACCGGGCCAGATC
CTGAGCTCGACGGGAACGCCGGCCGCAGCCAGCATGTGCGCGGCTTTCCGCGCA
TCGCTGAGCAGAGCCTCCGAGCCCGATGCGTGGATCAACGTGCGAGGCAGACCG
GGCTCGATGTGGTCGAGAGGTTCGTAGACGTCCTCCCCCTTGCGGGCGGCCGCCC
GCTCGATGAGCTCGACCAGAGCATCGAAGGCCTTGGGCGGGAACATCGCGTCGG
TGTGGATGTTGGGGTGGTTGGCCCGTGATTCGTTGTCGATCTCGAACAACGGCGA
CATCGTCACCAGCGCGGCAGGCATCTCTCCCTCGTCGACCAGGCGCTCGGCCAGC
GCCAGCGACAGGTAGCCGCCCGCCGAGTCGCCGGCCAACACGATCTGGTCCGGC
WGGTARCCGGTCAGCCGCAGCCAGCGGTAGGCGTCGTAACAGTCGTCGATCGCC
GTGCCCACCGAGTGCTTGGGCACCATCCGGTAATCGACGACCAGGACCGGGCTG
TCGGCATATCCCGACAGCGCCGTGACGATCCCGGAGTGCGTGTTGGCCCCGCAG
GMCAKGAACGCACCGCCGTGCAAGTAGAGGATGWCGCTGCGTTTCYCATCGRY
GGGCAGCACGCCATCGGCATGCAT

Protein sequence :

MHADGVLPXDXKRSXILYLHGGAFMXXXANTHSGIVTALSGYADSPVLVVDYRMV
PKHSVGTAIDDCYDAYRWLRLTXYXPDQIVLAGDSAGGYLSLALAERLVDEGEMPA
ALVTMSPLFEIDNESRANHPNIHTDAMFPPKAFDALVELIERAAARKGEDVYEPLDHI
EPGLPRTLIHASGSEALLSDARKAAHMLAAAGVPVELRIWPGQMHVFQLASPMVAE
AKRSLRQIGEYIREATW

4.3 Primers designed for the *lipU* sequence of *M. fortuitum* :

- Forward Primer : 5’ **GGATCC**ATGCATGCCGATGGCGTG 3

- Reverse Primer : 3’ **GGATCC**TTACCACGTGGCCTCTCG 5

4.4 Gradient PCR for the amplification of *lipU* sequence of *M. fortuitum* using manully designed primers :

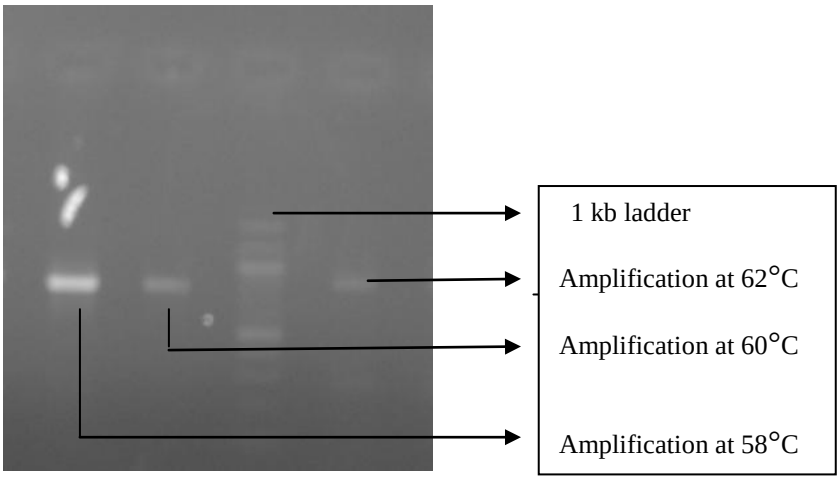


Fig. 4.1 *Electrophoresis of gradient PCR amplicons of genomic DNA isolated from M. fortuitum*

lipU showed the best amplification at 58°C, hence it was always amplified at 58°C afterwards.

4.5.1 Screening of transformants on LB agar plates (ampicillin resistant):

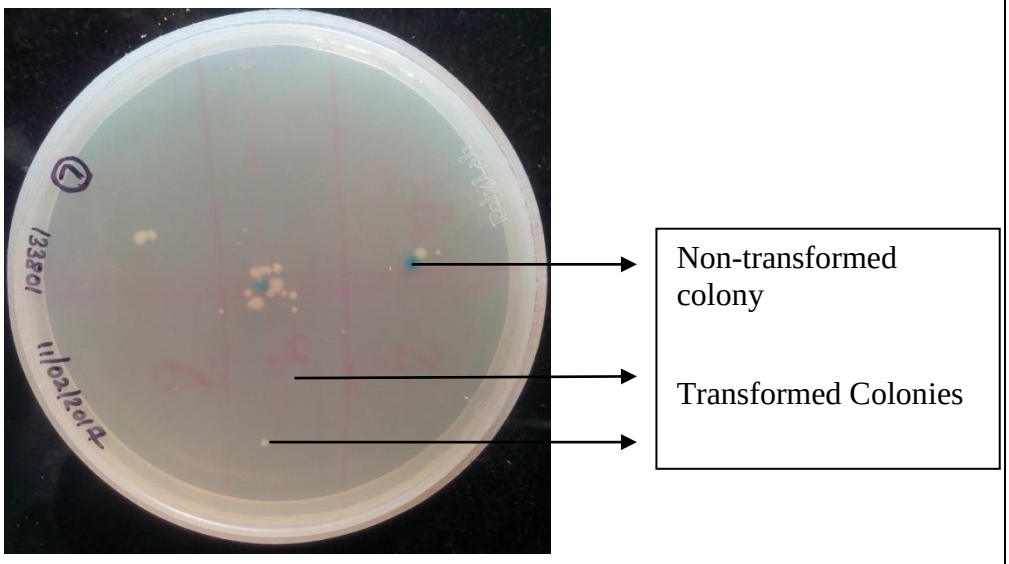


Fig. 4.2 *Blue-white screening of transformants on LB agar ampicillin plate containing X-gal and IPTG*

4.5.2 Master plate preparation from screened colonies :

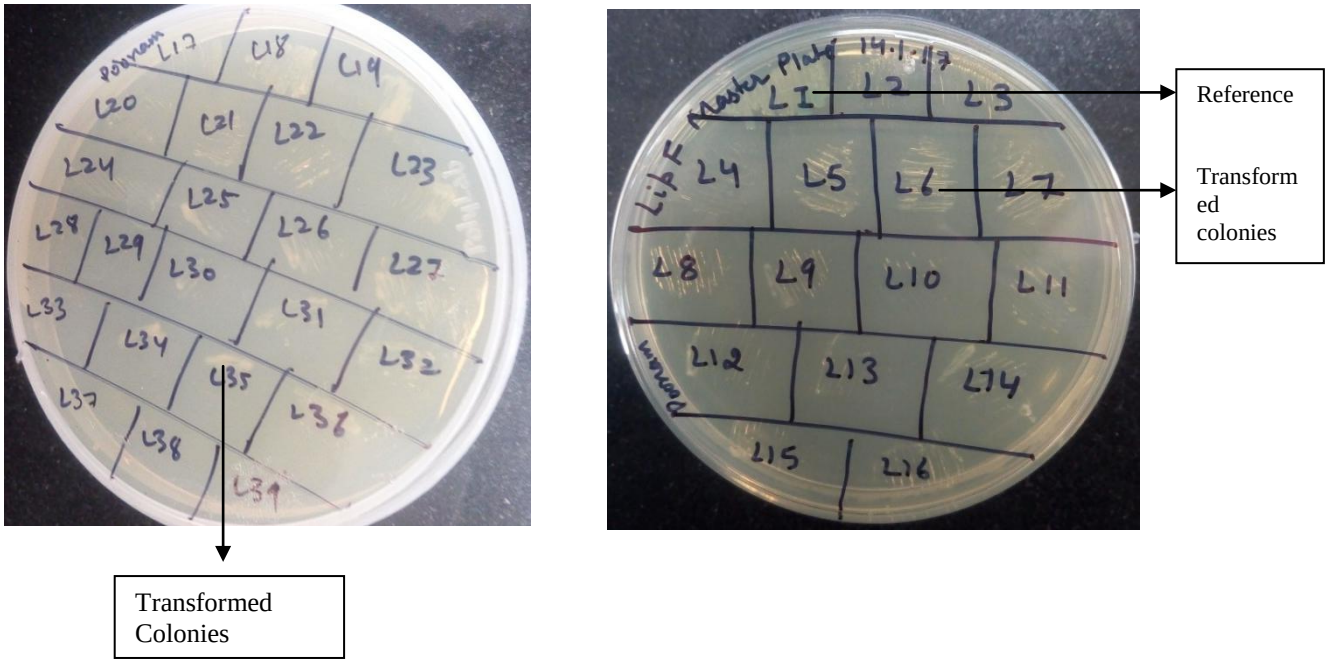


Fig. 4.3 *Master plates of transformant strains on LB agar ampicillin plate containing X-gal and IPTG*

4.6 Colony PCR of *lipU* transformed cells :

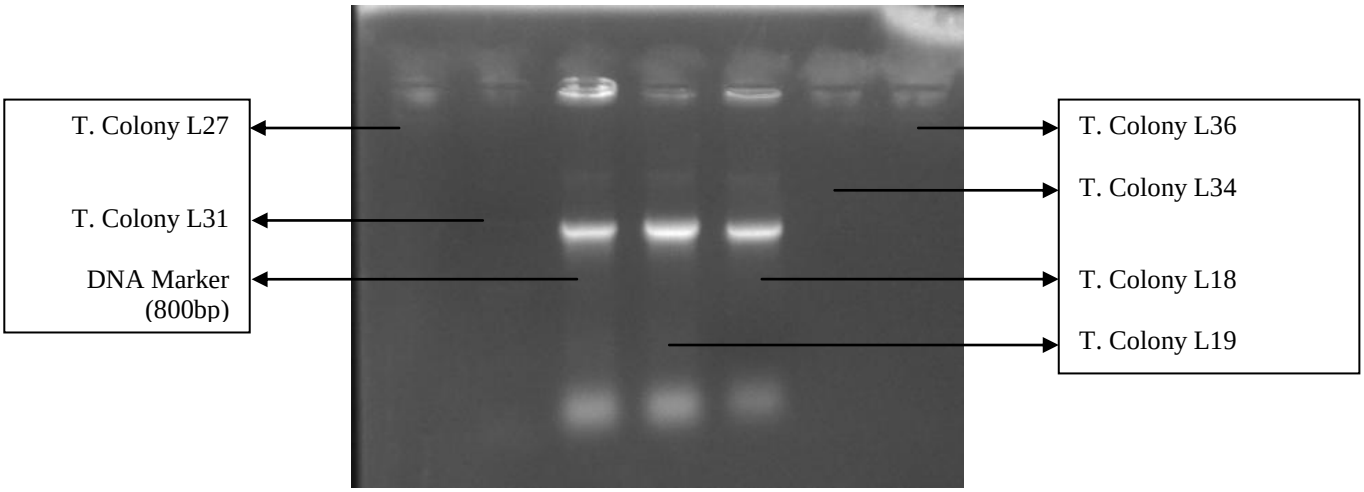


Fig. 4.4 *Electrophoresis of colony PCR amplicons of lipU transformed cells*

4.7 Plasmid isolation from transformants (*lipU and pMV261*) :

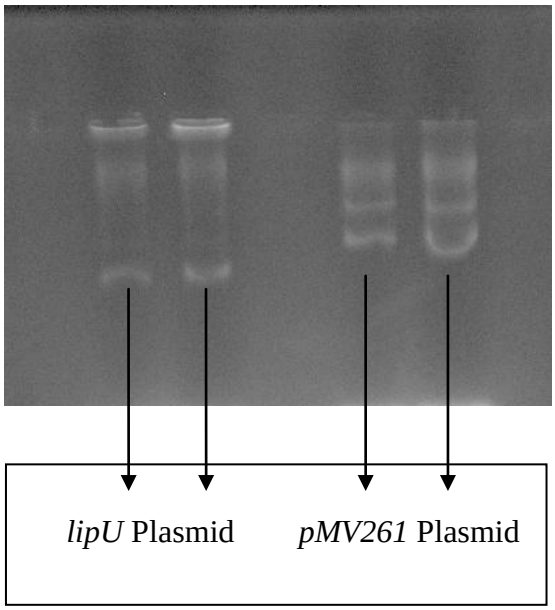


Fig. 4.5 *Electrophoresis of isolated plasmid from lipU transformants and pMV261vector*

4.8 Digestion of *lipU* plasmid with *BamHI* and *EcoRV*:

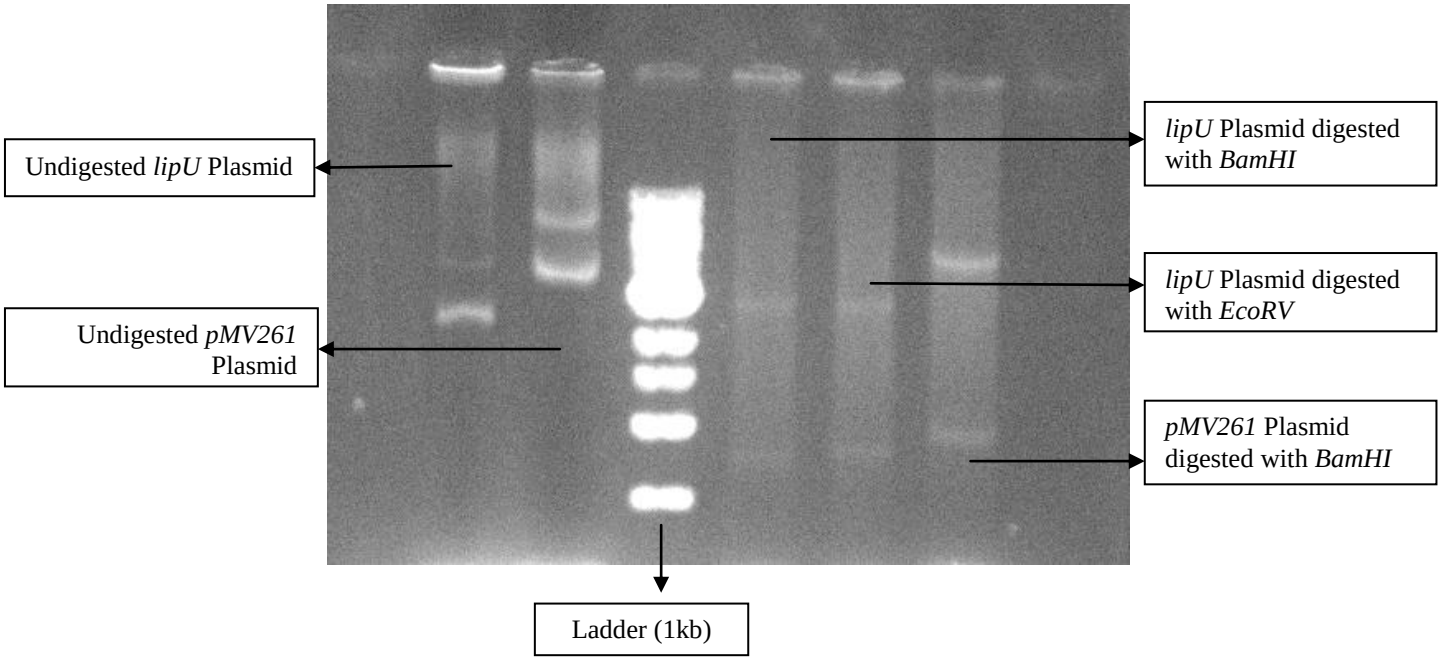


Fig. 4.6 *Electrophoresis of digested plasmid of lipU transformant with BamHI and EcoRV and pMV261 with BamHI*

4.9 : BlastN of query sequence :

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycobacterium sp. VKM Ac-1817D, complete genome	1295	1295	99%	0.0	98%	CP009914.1
Mycobacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841 genome	1282	1282	99%	0.0	98%	CP014258.1
Mycobacterium fortuitum strain CT6, complete genome	1245	1245	99%	0.0	97%	CP011269.1
Mycobacterium goodii strain X7B, complete genome	534	534	98%	1e-149	80%	CP012150.1
Mycobacterium phlei strain CCUG 21000, complete genome	405	405	97%	1e-110	77%	CP014475.1

Alignments

Mycobacterium sp. VKM Ac-1817D, complete genome
Sequence ID: **CP009914.1** Length: 6324222 Number of Matches: 1
Range 1: 4816397 to 4817124

Score	Expect	Identities	Gaps	Strand	Frame
1295 bits(701)	0.0()	716/728(98%)	0/728(0%)	Plus/Plus	

Fig. 4.7 *Nucleotide BLAST results of query sequence with other Mycobacterium species*

4.10 : Multiple Sequence Alignment results of nucleotide sequences (Jalview):

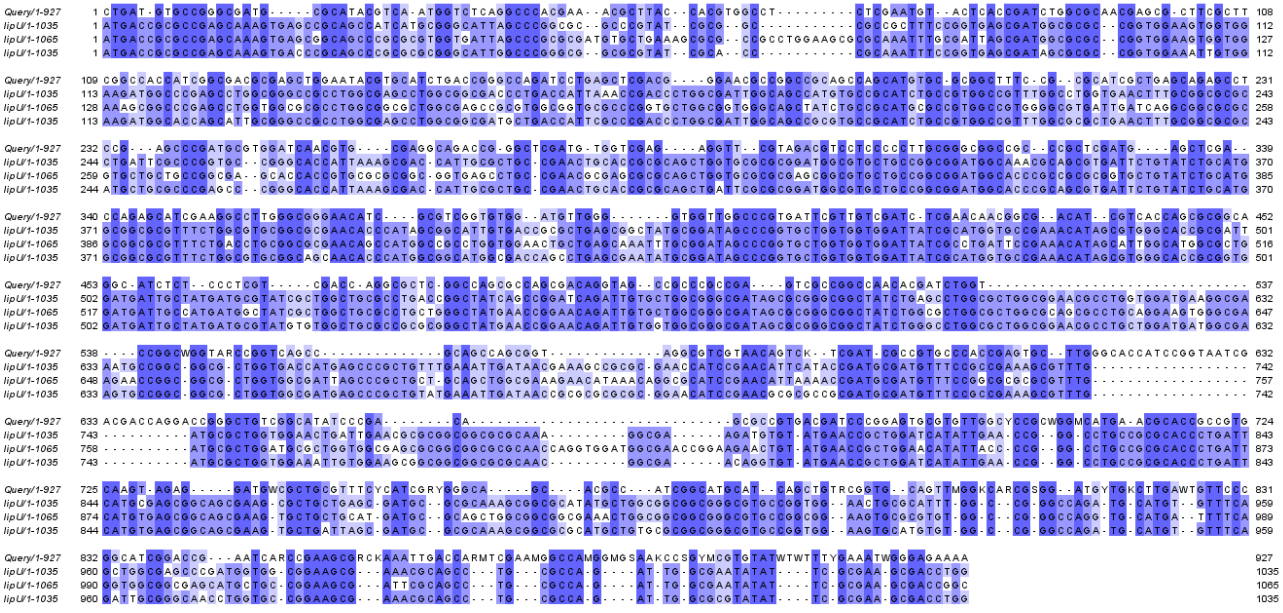


Fig. 4.8 *Nucleotide Multiple Sequence Alignment (MSA) results of query sequence with other Mycobacterium species viewed using Jalview*

Query	*M. fortuitum*	*M. tuberculosis*	*M. smegmatis*
% identity	50.46	51.01	52.76
Score	24170	24690	25240

4.11 : BlastP results of query sequence :

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lipase LipU [Mycobacterium sp. VKM Ac-1817D]	469	469	100%	1e-165	95%	AIY49705.2
MULTISPECIES: alpha/beta hydrolase [Mycobacterium]	468	468	100%	4e-165	95%	WP_003884377.1
alpha/beta hydrolase [Mycobacterium fortuitum]	468	468	100%	5e-165	95%	WP_065071208.1
esterase [Mycobacterium fortuitum]	466	466	100%	2e-164	95%	ALI28509.1
alpha/beta hydrolase [Mycobacterium fortuitum]	467	467	100%	2e-164	95%	WP_064913759.1
alpha/beta hydrolase [Mycobacterium fortuitum]	465	465	100%	7e-164	95%	WP_054604254.1
alpha/beta hydrolase [Mycobacterium fortuitum]	465	465	100%	8e-164	95%	WP_064897559.1
alpha/beta hydrolase [Mycobacterium fortuitum]	465	465	100%	1e-163	95%	WP_064847947.1
alpha/beta hydrolase [Mycobacterium fortuitum]	465	465	100%	1e-163	95%	WP_065022296.1
alpha/beta hydrolase [Mycobacterium fortuitum]	464	464	100%	3e-163	95%	WP_064926667.1
esterase [Mycobacterium conceptionense]	461	461	100%	7e-163	94%	CQD17480.1
MULTISPECIES: alpha/beta hydrolase [Mycobacterium fortuitum complex]	462	462	100%	2e-162	94%	WP_036395087.1
alpha/beta hydrolase [Mycobacterium conceptionense]	462	462	100%	2e-162	94%	WP_064894831.1

Fig. 4.9 *Protein BLAST results of query sequence with other Mycobacterium species*

4.12 : BlastX results of query sequence :

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lipase LipU [Mycobacterium sp. VKM Ac-1817D]	491	491	89%	2e-173	89%	AIY49705.2
alpha/beta hydrolase [Mycobacterium fortuitum]	491	491	89%	1e-172	89%	WP_065071208.1
MULTISPECIES: alpha/beta hydrolase [Mycobacterium]	491	491	89%	1e-172	89%	WP_003884377.1
esterase [Mycobacterium fortuitum]	489	489	89%	2e-172	88%	ALI28509.1
alpha/beta hydrolase [Mycobacterium fortuitum]	489	489	89%	4e-172	88%	WP_064913759.1
alpha/beta hydrolase [Mycobacterium fortuitum]	488	488	89%	1e-171	88%	WP_054604254.1
alpha/beta hydrolase [Mycobacterium fortuitum]	488	488	89%	2e-171	88%	WP_064897559.1
alpha/beta hydrolase [Mycobacterium fortuitum]	488	488	89%	2e-171	88%	WP_064847947.1
alpha/beta hydrolase [Mycobacterium fortuitum]	487	487	89%	3e-171	88%	WP_065022296.1
alpha/beta hydrolase [Mycobacterium fortuitum]	486	486	89%	5e-171	88%	WP_064926667.1
esterase [Mycobacterium conceptionense]	484	484	89%	2e-170	87%	CQD17480.1
MULTISPECIES: alpha/beta hydrolase [Mycobacterium fortuitum complex]	484	484	89%	5e-170	87%	WP_036395087.1
alpha/beta hydrolase [Mycobacterium conceptionense]	484	484	89%	5e-170	87%	WP_064894831.1

Fig. 4.10 *Translated nucleotide to protein results of query sequence with other Mycobacterium species*

4.13 : MSA results of protein sequences (Jalview) :

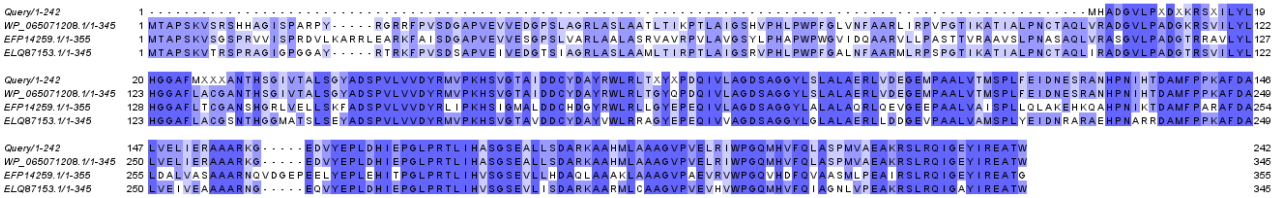


Fig. 4.11 *Protein Multiple Sequence Alignment (MSA) results of query sequence with other Mycobacterium species viewed using Jalview*

Query	***M. fortuitum***	***M. tuberculosis***	***M. smegmatis***
% identity	95.45	64.78	72.27
Score	11900.0	8180	9970

Chapter 5
Discussion and Conclusion

Discussion:

There is a continuing need to study intensively the strategies used by bacterial pathogens during invasion and infection. This has been highlighted in recent times by the enhancement in resistance to multiple antibiotics of certain bacteria. Pathogenic bacteria utilize a number of mechanisms to cause disease in human hosts. Bacterial pathogens express a wide range of molecules that bind host cell targets to facilitate a variety of different host responses. The molecular strategies used by bacteria to interact with the host can be unique to specific pathogens or conserved across several different species. A key to fighting bacterial disease is the identification and characterization of all these different strategies. Certain virulence factors are necessary for full pathogenicity regardless of the host. The lipolytic enzymes are one of the known virulence factor in many bacteria such as *Pseudomonas cepacia*, *Staphylococcus aureus*, and in fungal species like *Alternaria brassicicola*, *Candida albicans*, and *Fusarium graminearum*. Many different bacterial species produce lipases, which hydrolyze esters of glycerol with preferably long-chain fatty acids. Hydrolytic enzymes like lipase may contribute to the invasivity and proliferation by causing the destruction of the host tissues, thereby supplying hydrolyzed material to the organism as nutrients.

Lipase family contributes to the major virulence factors of *Mycobacterium tuberculosis* which was taken as the basic criteria for this study. Initially *lipF* gene was taken to study the virulence of *Mycobacterium fortuitum* using TA cloning method followed by transformation to construct *lipF* anti-sense homologue of *M. fortuitum* . After the completion of the construction of anti-sense knock out mutant followed by its *in-silico* analysis, it was found that *lipF* gene of *M. tuberculosis* showed the highest homology with the *lipU* gene of *M. fortuitum.*

Conclusion:

In this project study, the importance of *lipU* in the pathogenesis of *M. fortuitum* has been tried to identified using construction of anti-sense knock out mutant followed by *in-silico* analysis. As disruption of the *lipF* gene of *M. tuberculosis* reduces survival of the bacteria under persistent conditions, it must play an important role in survival and proliferation of *M. fortuitum* under different acidic conditions.

Thus, *lipU* gene of *M. fortuitum* can be a potential drug target for the treatment of the infections caused by the bacteria, in both antibiotic formulation as well as for vaccine production.

Appendix

6.1 Bacteriological media

All the media were prepared in Milli RO grade water and autoclaved at 15 pounds per square inch for 15 min. unless otherwise indicated.

6.2 LB Broth (Luria Bertani Broth)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000 ml with Milli RO water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

6.3 Nutrient Broth

Peptone	5 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5 g

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000 ml with Milli RO water.

6.4 Nutrient Agar Tween80 (NAT)

Nutrient Broth	13 g
Tween 80	2 ml

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000 ml with Milli RO water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

6.5 Reagents for Acid Fast Staining

1. Carbol fuchsin (Primary stain)

Basic fuchsin	3g

Phenol	5%
Ethanol (96%)	10 ml

Mixed 10 ml of Basic fuchsin to 90 ml of phenol and the solution was filtered through Whatman filter paper.

2. Acid alcohol (Decolorizer)

HCL (conc.)	3 ml
Ethanol (96%)	97 ml

Reagent	**Stock solution**	**Final Conc.** (in *E. coli*)	**Final Conc.** (in Mycobacterium)
Ampicillin	5 mg/ml in H_2O	50 µg/ml	-
Kanamycin	5 mg/ml in H_2O	50 µg/ml	25 µg/ml
Cycloheximide	5 mg/ml in H_2O	100 µg/ml	100 µg/ml
Streptomycin	5 mg/ml in H_2O	50 µg/ml	25 µg/ml
X-gal	40 mg/ml in DMF	40 µg/ml	40 µg/ml

3. Malachite green solution (Counter stain)

Malachite green	0.25 g in Milli RO water

6.7 Antibiotics and Substrates

All antibiotic solutions were filter sterilized by a 0.22 µm filter (Millipore) and stock solutions were stored at -20°C for long-term use.

6.8 Reagents and Buffers

All the reagents and buffers for DNA and protein work were prepare in Milli Q grade water and sterilized by autoclaving for 15 minutes at 15-psi pressure unless otherwise indicated.

6.8.1 Commonly used Buffers

i. Tris HCL buffer

Tris-HCL buffer of desired strength was prepared by dissolving appropriate amount of Tris in distilled water and adjusting the pH with concentrated HCl. For bacteriological work 10 mM Tris-HCl (pH 8.0) was used.

ii. Ethylene diamine tetra acetic acid (EDTA)

0.5 M solution of disodium salt of EDTA was prepared in Milli Q, pH adjusted to 8.0 with NaOH pellets and stored at 4°C.

iii. Normal Saline

NaCl	8.50 g
Milli RO water	1000 ml (final volume)

iv. Tween Normal Saline

0.02% Tween -80 was added to normal saline.

6.8.2 Reagents for Genomic DNA isolation from Mycobacterium

i. TE Buffer

Tris-HCl (pH 8.0)	10 mM
EDTA	1 mM

ii. Tris EDTA Saline (TES) Buffer

TRIS-HCL (pH 8.0)	10 mM
EDTA	1mM
NaCl	150 mM

iii. Lysozyme

Lysozyme	50 mg/ml in Milli Q

iv. Proteinase K

Proteinase k	20 mg/ml in Milli Q

v. Buffer Phenol

Molten phenol containing 0.1% 8-hydoxyquinoline was equilibrated with 1MTris-HCl (pH 8.0) and twice with 0.1 Tris-HCl (pH 8.0) till the pH > 7.8 and then it is stored submerged in 10 mM Tris-HCl (pH 8.0) in dark bottle at 4°C away from direct light.

vi. Chloroform: Isoamyl alcohol

Solution contains 24 parts chloroform and 1 part Isoamyl alcohol. The solution is stored in dark bottle at 4°C.

6.8.3 Buffer for Plasmid Isolation from *E. coli*

i. Glucose Tris EDTA Buffer (GTE)

TRIS-HCL (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM
Glucose	50 mM

ii. NaOH-SDS Mix

NaOH	0.2 N
SDS	1.0%

iii. Acetate Mix

Solution contains 3 volumes of 3 M sodium acetate and 4 volume of 7.5 M ammonium acetate.

6.8.4 Buffers for Electrophoresis

i. TAE Buffer (50 X)

Tris Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Final Volume	1000 ml

ii. TBE Buffer (50 X)

Tris Base	54 g
Boric acid	27.5 g
0.5 M EDTA	20 ml
Final Volume	1000 ml

iii. Tris-glycine

Tris Base	3.0 g
Glycine	14.4 g
SDS	2.0 g
Final Volume	1000 ml

6.8.5 Buffer for Transformation

i. Transformation Buffer 1 (TFB I)

MOPS Buffer (pH 6.5)	100 mM
RbCl	10 mM

ii. Transformation Buffer II (TFB II)

MOPS Buffer (pH 6.5)	100 mM
RbCl	10 mM
$CaCl_2$	50 mM

6.8.6 Buffer for Gel Loading

i. 6X dye for agarose gel electrophoresis :

Bromophenol Blue	0.25%
Sucrose	40%”

The volume was then made up to 10 ml with RNase free water. For loading onto the gel the buffer was added to a final concentration of 1X to the sample, the mixture incubated at 65°C for 3-5 minute and chilled on ice. Before loading 1µl of EtBr (1mg/ml) was added to the sample.

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Publications

1. **Identification and construction of *lipU* anti-sense knock out mutant of *Mycobacterium fortuitum* and its potential role in pathogenesis**

   Divya, Anandita Govil, Jitendraa Vashistt, Rahul Shrivastava*

   Poster presentation at 58th Annual Conference of Association of Microbiologists of India, organized by BBAU, Lucknow from November 16 - 19, 2017

Identification and construction of *lipU* anti-sense knock out mutant of *Mycobacterium fortuitum* and its potential role in pathogenesis

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Introduction

Mycobacterium fortuitum is a non-tuberculous mycobacterium (NTM) causing various clinical symptoms such as cutaneous infections, respiratory infections, joint infections and disseminated infections in immuno-compromised patients. Since the mechanism of pathogenesis of *M. fortuitum* is not known, be that as it may, it has been reported that it taints macrophages in an indistinguishable way from that of *Mycobacterium tuberculosis*. Out of all the genes identified for the virulence of *M. tuberculosis* infection, *lipU* also called as cystathionine beta synthase, plays a major role coding for lipid hydrolyses necessary for the survival and virulence of *M. tuberculosis* and other mycobacteria. This lipase is very much similar to that of esterases of mycobacterium which implicates modification of the mycobacterial cell wall as an adaptive response to acid damage, also degrades host lipids during infection, making fatty acids available as building blocks for lipid biosynthesis. This gene helps in compensating with un-favorable acidic conditions in macrophages, where mycobacterium resides. With an aim to study the role of *lipU* in pathogenesis of *M. fortuitum*, multiple primer sets were designed from conserved regions of *M. tuberculosis lipU* to amplify full length gene sequence of its homologue in *M. fortuitum* on the basis of the homology between the two genomic sequences (*M. fortuitum* and *M. tuberculosis*). Full length fragments were confirmed by sequencing. Longest sequence showing highest similarity with *M. tuberculosis lipU* was submitted to GenBank database as *M. fortuitum* ATCC 6841 homologue of *lipU*. To further confirm the role of *lipU* in virulence of *M. fortuitum*, an anti sense *lipU* mutant was constructed by cloning the gene in *pMV261* vector followed by electroporation into the wild type *M. fortuitum*. This construct can be further used to establish the role of *lipU* gene in pathogenesis of *M. fortuitum* which may act as a potential drug target against the infection caused by *M. fortuitum*.

Objective

- To get an insight into the pathogenesis of *M. fortuitum*.
- Identification of the role of gene involved in the virulence of *M. fortuitum* using anti-sense technology.

Method

Amplification of *lipU* sequence of *M. tuberculosis* in the genomic DNA of *M. fortuitum*

↓

Cloning of the gene was done in pGEM-T vector followed by sequencing

↓

Cloned product transformed into *E.coli* was screened on ampicillin resistant plates followed by the plasmid isolation from *E.coli DH5α*

↓

Plasmid was isolated from *lipU* transformed cells and from *pMV261* separately

↓

Both the plasmids were digested with *BamHI* and ligated later on

↓

Screening was done on NA plates (Kanamycin resistant) and plasmid was isolated

↓

Restriction profiling of the isolated plasmid to confirm sense/anti sense construct

Conclusion

This project study shows the importance of lipase esterase also called as cystathionine beta synthase esterase in the pathogenesis of *M. fortuitum*. *lipU* is an important gene required for pathogenesis and persistence of *Mycobacterium tuberculosis*. Disruption of the *lipU* gene reduces survival of *M. tuberculosis* under persistent conditions. It also plays an important role in survival and proliferation under different acidic conditions, thus can be a potential drug target for the diseases caused by *M. fortuitum*, in both antibiotic formulation as well as for vaccine production.

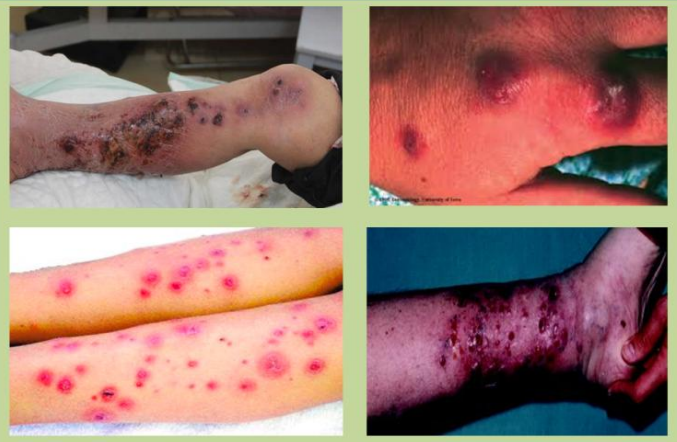


Fig 1. Infections caused by *M. fortuitum*[1,2,3]

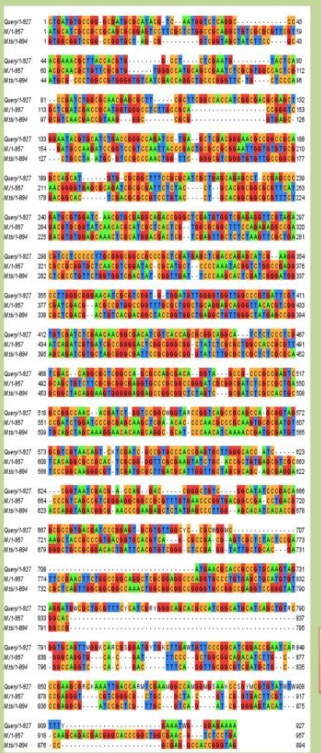
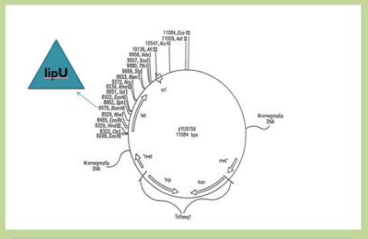


Fig 2. lipU query sequence showing 98% homology with *Mycobacterium tuberculosis* and 63% homology with *M. smegmatis*



pMV261 Shuttle Vector

Fig 5. Ligated product of *pmv261* and *lipU* transformed in *E.coli* cells

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