OVEREXPRESSION OF ISOCITRATE LYASE IN *MYCOBACTERIUM FORTUITUM*: *IN VITRO* STUDIES

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By

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DECLARATION

I hereby declare that the work reported in the M-Tech thesis entitled **"Overexpression of Isocitrate Lyase in *Mycobacterium fortuitum*: *In vitro* studies"** submitted at **Jaypee University of Information Technology, Waknaghat India,** is an authentic record of my work carried out under the supervision of **Dr. Rahul Shrivastava.** I have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that the work reported in the M-Tech. thesis entitled **"Overexpression of Isocitrate Lyase in *Mycobacterium fortuitum*: *In vitro* studies",** submitted by **Arpita Prasad** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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ABSTRACT

Tuberculosis has been a health menace for the human race since time immemorial. *Mycobacterium tuberculosis* is the etiological agent of tuberculosis, whose preferred host cell is macrophage. In order to attenuate the survival of *M. tuberculosis*, the macrophage creates many stressful conditions like generation of ROI and RNI, antimicrobial peptides, hypoxia, nutrient deprivation, oxidative stress etc. But *M. tuberculosis*, being a prudent and an efficient organism has evolved many strategies to protect itself from the myriad of killing and trapping mechanisms of the macrophage. Isocitrate Lyase is an enzyme of glyoxylate cycle involved in host fatty acid degradation. Its role has been registered in persistence of *M. tuberculosis* inside the host cell, helping it to overcome the adversities like nutrient deprivation, hypoxic condition and maintaining antibiotic tolerance. Considering these vital functions, many *ICL* inhibitors had been designed but proved to be inefficient due to their toxicity and non specificity. Surplus studies on *icl* knockouts have already been done. However, the consequences of overexpression of this gene have not been elucidated yet. Therefore, by referring to the previous work on gene overexpression and its result on the survival of the organism, the present work is designed to have an idea of overexpression of *icl* on the bacterial growth under various stress conditions existing inside the host granuloma: acidic, nutrient deprivation, oxidative, detergent, heat, hypoxia. Over expression was done by using *icl* homologue of *M. fortuitum* (served as model organism here) in *pMV261* vector. Surprisingly, the results showed diminished growth of *M. fortuitum* under hypoxic and acidic stress. It may be suggestive of the fact that the over expression may prove inhibitory to *icl* either by feedback inhibition mechanism or by regulation of genes responsible for survival in stress conditions. A lesson emerging from the gene knockout studies is that loss of function mutations is alone not enough to deduce the function of gene. In accordance with the other studies, this result signifies that the overexpression can also be disruptive to the organism. Hence, it should also be considered being studied to find out important drug target.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BCG	Bacillus Calmette Guerin
BP	Base Pairs
CFU	Colony Forming Units
CR	Complement Receptors
DOTS	Directly Observed Treatment Shortcourse
DC	Dendritic Cell
Esat	Early Secretory Antigenic target
HIV	Human Immunodeficiency Virus
ICL	Isocitrate lyase
IFNγ	Interferon gamma (γ)
IL	Interleukine
IUAT	International Union Against Tuberculosis
KD	Kilo Dalton
LAM	Lipoarabinomannans
LJ	Lowenstein- Jansen
MB7H9	Middlebrook 7H9
MDR	Multi Drug Resistance
MMP	Matrix Metalloproteinase
MTB	*Mycobacterium tuberculosis*
NRP	Non Replicating Phase
NTM	Non Tuberculous Mycobacteria
PAMP	Pathogen Associated Molecular Pattern
PMN	Polymorph nuclear
PRR	Pattern Recognition Receptor
RNI	Reactive Nitrogen intermediate
ROI	Reactive Oxygen Intermediate
TB	Tuberculosis

TCA	Tricarboxylic Acid Cycle
TLR	Toll Like Receptors
TNF	Tumour Necrosis Factor
WHO	World Health Organisation
WT	Wild Type
Pca	Proximal Cyclopropanation of Alpha-Mycolates

CHAPTER 1

INTRODUCTION

INTRODUCTION

Tuberculosis (TB) has existed for hundreds of year and remains a noteworthy medical issue worldwide. It causes sick wellbeing for around 10 million people each year and is one of the top ten reasons for morbidity around the world. For as way back as 5 years, it has been the main reason behind deaths, positioning above HIV/AIDS. The causative agent of tuberculosis is *Mycobacterium tuberculosis.* Amid development in fluid media Mycobacterium form mold like pell*Icl*e and subsequently named as 'Mycobacteria' which means fungus like bacteria. Two main pathogens of genus are *M. tuberculosis* and *M. leprae*. Third group consists of isolates from varied sources called as 'atypical mycobacteria [1]. *Mycobacterium fortuitum* is a NTM, which is rapidly growing and causes opportunistic , nosocomial infections and is frequently associated with local dermatological infections, osteomyelitis (inflammation of the bone), joint infections and ophthalmic infections after trauma [2].

Tuberculosis is communicated from one person to the other via airborne droplets of size 1-5 cm enclosing *Mycobacterium tuberculosis.* After inhalation the droplet lands in alveoli, where it is phagocytosed by the alveolar macrophages. Depending on the efficiency of the immune system to evade the bacteria, the infection is categorised into active and latent. The failure of the immune system to restrain the bacteria leads to the development of active TB, which harbours actively dividing mycobacteria that gets disseminated to exhibit the symptoms. Whereas the latent tuberculosis, also called as persistent infection doesn't manifest any symptoms and therefore act as a potential reservoir of the infection. Persistent form of tuberculosis is a foremost obstacle in managing the disease as the current course of therapy of antimycobacterials is mostly ineffective in eradicating persistent infection [3]. In latent tuberculosis, the body do not manifest any symptoms since the infection is contained in an immune structure called as granuloma, which is a cluster of immune cells(T cells, macrophages) recruited at the site of infection on antigen presentation by the infected macrophage [4]. The granuloma subjects the bacilli residing inside it, to different stressful situations like generation of reactive oxygen and nitrogen species, nutrient deprivation, generation of antimicrobial radicals, prevalence of hypoxic and oxidative conditions etc to combat the mycobacterial infection [5].This also form the fundamental of the *in vitro* models of latent TB infections currently

present. Many *in vitro* models have been put forward making use of the stress conditions prevailing inside granuloma (3). But *M. tuberculosis*, being wise bacteria has evolved many ways to escape the immune response and the hostile environment of the host granuloma and multiply within it. This includes the presence of virulent factors like, *Icl, lipf, esat6, pcaA, lam* etc, which are responsible for its pathogenicity. The defence mechanisms like, preventing phagosome- lysosomal fusion makes it capable of fooling the host.

During infections, *Mycobacterium tuberculosis* needs to acquire nutrients and oppose host defence. In the nutrient deprived condition inside the granuloma host derived lipids not only become the major source of nutrition but they are also required for pathogenesis of the *M. tuberculosis.* *Icl* (gene length 1287bp) encoding for isocitrate lyase is an enzyme fundamental for metabolism of fatty acids. *Icl* is the first enzyme of glyoxylate shunt (an alternative pathway of TCA) catalyses the conversion of isocitrate to glyoxylate. The glyoxylate cycle is imperative for carbon anaplerosis in the Kreb's cycle, bypasses a two step decarboxylation step. These functions enables the bacteria to refill the TCA cycle intermediate pool as well avoid the loss of two carbon dioxide molecules respectively, later which can be integrated into cellular structures. It has been reported previously Isocitrate lyase aid the persistence of *M. tuberculosis* in mice [6]. *Icl* not only takes part in lipid metabolism of the *M. tuberculosis* but also assists this bacteria in combating other stresses like hypoxia and antibiotic tolerance [7]. It was reported that the bacterial persistence as well as virulence was impaired on disruption of the *Icl* gene in immune-competent mice but it didn't affect the growth of bacteria all over the acute phase of infection [8]. Considering its role in persistence of bacteria and survival during the course of infection, gene *Icl* became an eligible target for chemotherapy, which led to the synthesis of many inhibitors till date, like itaconate , 3- nitropropionate , and 3 bromopyruvate. However, these inhibitors are not appropriate to be used as drug due to their toxicity and their ability to inhibit key metabolism enzymes *in vivo* [9].
This explains the fact that there lies some research gap still to be discovered between *icl* functions and its inhibition.

A myriad of molecular mechanisms occur inside an organism which makes sure that genes are expressed at the desired level and at the appropriate conditions. If there is an upsurge in expression of a wild type gene, then this can also prove distorting to a cell or life forms, but phenotypes caused by over expression abound

[10]. In a study done on *Salmonella enterica*, it was found that mutation in the promoter site of the gene *recA*, which serves as a binding site of *LexA* (the repressor), caused it to overproduce the protein which in turn decreased the fitness of orally inoculated *recAo6869* cells dramatically [11]. The above studies throws light on the possibility that the over expression of a gene involved in pathogenesis may also prove to be disruptive to the organism. Numerous knockout experiments of *icl* gene in *M. tuberculosis* have already been performed in past to see its potential as a drug target. But no major conclusive results were obtained. Therefore, keeping these perspectives in mind, the present study is designed to see the outcome of over expression of the gene coding for isocitrate lyase (*icl*) on the survival of the *M. fortuitum* (the model organism) under various stress conditions existing inside the host granuloma.

To study the effect of over expression of *icl*, *Mycobacterium tuberculosis* is required, but it cannot be used for the defined purpose due to constraints in accessibility of the Bio safety Level 3 (due to elevated risk of infection) and also due to long doubling time of the bacteria. *M. fortuitum* has been established as surrogate model organism for studies and screening strategies related to *M. tuberculosis* persistent infection (11). Therefore, it is being preferably exploited in the present study as a surrogate organism because of the following reasons:

- It has been established and validated as a model to study persistence and can be worked upon in BSL2 (against BSL3 required for *M. tuberculosis* [12].
- Fast growing organisms (as compared to other mycobacterial species).
- It is a mildly pathogenic Non Tuberculous Mycobacteria.

Objectives of the study:

- To study the effect of overexpression of *icl* gene on the growth of *M. fortuitum* under following stresses existing inside granuloma:
  - Nutrient deprivation
  - Heat stress
  - Oxidative stress
  - Detergent stress
  - Acidic stress
  - Hypoxic stress

CHAPTER 2
REVIEW OF LITERATURE

2.1 Tuberculosis

Tuberculosis is one of the primitive infectious diseases that has tormented human populace since the spreading out of the modern human out of Africa [13]. Despite the discovery and advancement of the effective chemotherapy more than 50 years ago, tuberculosis up to this time happens to be one of the leading grounds of deaths worldwide. Records and incidence of tuberculosis can be traced back to ancient times when people were completely unaware of this disease and referred it as phthisis, consumption and “the white plague”. The period of 17th, 18th and 19th century, saw the dawn of industrialisation and urbanisation which consequently gave rise to crowded living condition, pollution, malnutrition. These factors fortified the growth and spread of bacteria due to which the disease reached to epidemic extent in the commencement of 19th century. This prompted increment in death-rates because of tuberculosis in Stockholm to 1000 deaths for every 100 000 people/year [14] . The reason for the infection was obscure up till the 24th of March 1882 whilst the German scientist Robert Koch proclaimed that he had isolated the causative agent of the disease, the bacterium *M. tuberculosis*, a deed for which he later on got the Nobel Prize.

This disclosure prompted revived endeavours to discover medicines against the infection, however it had been not till 1943 when one more Nobel Prize laureate, Selman A. Waksman, discovered streptomycin which for the first instant, opened the door for the treatment of tuberculosis [15].

Over the resulting 14 years, most antimycobacterial mixes directly being used were found, which likewise incorporates para-amino salicylic corrosive (PAS), that was first portrayed by the Swedish doctor and physicist Jörgen Lehmann . In any case, as a result of enhanced living conditions and to some degree separating the patients by sending them to specific nursing homes known as sanitoria, a decrease in death rates by T.B was seen some time before the presentation of anti-toxins. Today, tuberculosis still remains an outsized risk against general wellbeing on a worldwide scale and in spite of being an antiquated illness, new difficulties as anti-microbial protection and co-contamination by HIV gives a thought that this irresistible. Over the subsequent 14 years, most antimycobacterial compounds presently being used were found, which likewise incorporates para-amino salicylic acid (PAS), that was first delineated by the Swedish physician and chemist Jörgen Lehmann. In any case, because of enhanced living conditions and to some degree secluding the patients by transporting them to

specialized nursing homes known as sanitoria, a decrease in death rates by T.B was observed way earlier than the advent of antibiotics. At present, in spite of an appreciable antibiotics regime being used in curing this illness, TB still happens to be an outsized menace for people on the global level. Although, it's a primitive disease but challenges antibiotic resistance and co-infection by HIV gives a clue that this infectious disease is distant from complete eradication.

2.2 The bacterium: *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, the etiological agent of T.B., is an intracellular bacterium whose chosen host is the human macrophage cell. The group constitutes bacilli which are straight or slight curved rods, the size varying from 2-3µm × 0.3-0.5µm, occurring separately, in pairs or small clumps. It's a slow-growing bacterium with a protracted doubling time of 24 hours and manifestation of colony takes 4-6 weeks. Categorised as a gram-positive, rod shaped, *M. tuberculosis* doesn't forms spore and possess an atypical cell wall with an **e**xtra outer layer of unusual lipid of long fatty acid chains, mycolic acid. The existence of this additional layer of mycolic acid in mycobacteria confers many unique characteristics and this layer also forms the basis of identification of mycobacteria in the laboratory by Ziehl-Neelsen staining, also called as acid fast staining and hence the name Acid Fast Bacteria (AFB). The staining was first developed by FranzZiehl and Friedrich Neelsen [16]. Apart from being acid fast, the mycobacteria are mainly non motile, intracellular pathogens, obligate aerobes and eugonic (luxuriant grower). Optimal growth temperature and pH is 37ºC and 6.4 - 7.0 respectively.

Both solid as well as liquid media are used for cultivation - solid media containing egg (Lowenstein-Jensen [LJ]), Petragnini, and Dorset), blood (Tarshis), serum (Loeffler) or potato (Pawlowsky). The IUAT (International union Against Tuberculosis) recommends LJ media (without starch) as the most extensively used solid medium for the growth of *M. tuberculosis*. Dubos', Middle brook's, Prokauer and Beck's, Sula's and Sauton's media are some liquid media which are used. Liquid media are chiefly used for sensitivity testing, chemical analyses and preparation of antigens and vaccines. In liquid media growth originates from bottom and progress upwards along sides and then to form a surface pell*Icl*e. A filamentous growth is observed. The colony morphology of tubercle bacilli on solid media is dry, rough and

raised irregular wrinkled surface On further incubation they turn creamy white to yellow to buff colored (1).

People infected with active disease develop tuberculin sensitivity within 6-8 weeks of exposure [17]. Thus, Mantoux test based on tuberculin sensitivity remains the major diagnostic tool. Other diagnostic techniques include acid fast staining of sputum, followed by smear microscopy, culture based techniques and chest X-ray are some of the conventional diagnostic tests performed for the identification. Some new and rapid detection methods introduces are: Nucleic acid amplification, Mycobacteriophage based methods [18]. Nevertheless with time, more receptive and precise tests such as interferon-gamma (IFN-γ) release assay (IGRA) have been developed which detect T cell response against *M. tuberculosis*-specific antigens, ESAT-6 and CFP-1 [19].

WHO has recommended DOTS (Directly Observed Treatment Short course) as the standardised and most effective method to cure TB. DOTS include standardized, short-course treatment of fixed-dose drug combinations (FDCs) under strict observation. Four drugs are included in DOTS, these are: Rifampicin, Isoniazid, Ethanebutol and Pyrazinamide, which target different elements of the bacilli. Bacillus Calmette–Guérin (BCG) vaccine is principally used against tuberculosis. Mainly administered in infants and neonates, has been used since 80 years as the most extensively used medicines [20].

2.3. Global epidemiology of tuberculosis

Tuberculosis strikes each part of the globe and stays one among the most elevated ten reasons for demise around the world. In 2016, according to the report, Asia ended up being the landmass with biggest magnitude of new TB cases with 45 of most recent cases, trailed by Africa, with twenty fifth of most recent cases. In 2016, 10.4 million folks were indisposed with TB, and 1.7 million were deceased because of the malady (which also includes 4 million amid folks hit with HIV). Greater than ninety fifth of TB demises happen in low-and middle salary nations. Seven nations which represent 64% of the aggregate TB cases all-encompassing, are as followed chronologically: India (driving the check) trailed by Indonesia, China, Philippines, Pakistan, Nigeria, and South Africa) .In 2016, Associate in Nursing calculable one million children turned out to be sick with TB and 250 000 kids faced death out of TB (counting kids with HIV associatedwTB).

TB may be a leading killer of HIV-positive people. In 2016, four-hundredth of HIV passings was because of TB Multidrug-resistant TB (MDR-TB) has become a leading public health hazard and a health security menace. WHO has made an approximation of 600 000 recent cases remaining unaffected by dosage of rifampicin – the mostly recommended first-line drug, of that 490 000 had MDR-TB. All around, TB frequency is falling at concerning two every year This must pick up the pace to a 4 – 5% yearly decrease to achieve the 2020 objective of the programme End TB Strategy [21].

2.4. *Mycobacterium fortuitum:*

The atypical bacteria are also referred to as Non Tuberculous mycobacteria which cause pulmonary disease which bear a resemblance to tuberculosis, lymphadenitis, and skin disease. Nearly 150 diverse species of NTM have been classified. *Mycobacterium fortuitum* is one such NTM. This organism is an omnipresent (found in natural water, sewage and dirt), rapidly growing bacteria since it takes just 3-4 days to grow which is way more faster than other mycobacteria [22].
M. fortuitum doesn't cause any severe infection but are definitely associated with mild, nosocomial infections in humans. Also, it is the causal organism behind the formation of local abscesses and disseminated infections in human. Alternative sources of *M. fortuitum* infection embody implanted devices such as catheters, injection site abscesses, and contaminated endoscopes .The target groups of *M. fortuitum* are following: elderly people, people diseased with HIV and people who have undergone surgery. Although, the infections caused by this bacterium is communicated from one person to the other. *M. fortuitum* could be a normally isolated organism from respiratory samples in clinical laboratories in several countries. Till date, though, the clinical implication of this organism has not been well studied [23]. Although, it has been established and validated as a model to study persistence, which makes it a preferable organism to simulate the conditions thriving inside the host (12).

2.5. Pathogenesis of *M. tuberculosis*:

Apart from pulmonary tuberculosis, there also exists extrapulmonary tuberculosis like pleural, meningeal, pericardial, skeletal, gastrointestinal, genitourinary and miliary TB [24]. When the infected person (active TB) sneezes or

coughs, small aerosols containing the active bacteria are dispersed and get transferred from one person to another. Post inhalation by an individual, the aerosol makes way and lands into the distal lung, where it is recognised by the host macrophages and dendritic cells. After inhalation, there are several possible fates of the mycobacteria inside the host which depends on many parameters for instance mycobacterial pathogenicity, host immune status, genetic determinants:

- complete elimination by host immune system
- establishment of latent infection, which outbreaks on immune suppression
- active tuberculosis on failure of the immune system in eliminating the invader.

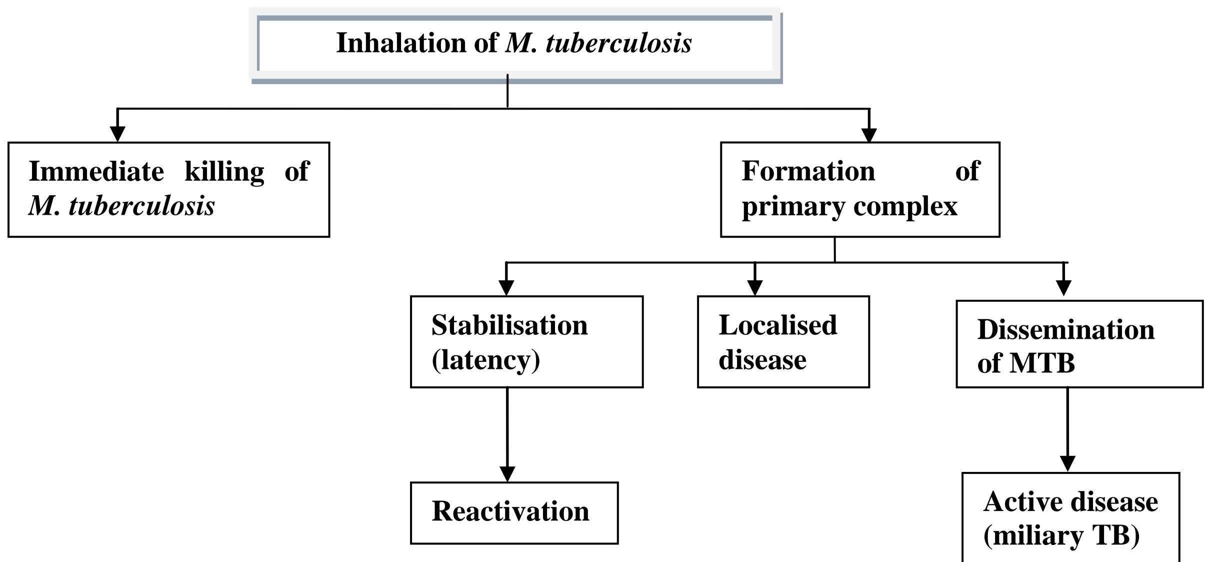

Figure 2.1: Possible fates of *M. tuberculosis* after inhalation [25].

Although the chances of infection becoming active tuberculosis (with clinical manifestations) is very less since in 90% of the cases the bacteria gets eliminated or acquire a latent form. Merely, 10% probability of relapse is observed in the case of latent TB. Immune impairment, such as HIV co-infection, increases the risk considerably. The landing of bacteria in lungs is followed by a cascade of events, starting with the phagocytosis of the bacteria which in turn is initiated by their recognition of Pathogen Associated Molecular Patterns (PAMP) in mycobacteria by Pattern Recognition Receptors (PRRs) and complement receptors present on surface of host cells.

- **Toll Like receptors2 (TLR2):** Recognises lipoproteins and lipomannan.
- **TLR 9:** Recognises mycobacterial DNA and produces cytokines by macrophages and dentritic cells.
- **C type lectin receptor:** This includes DC-SIGN and Mannose receptors which recognise mycobacterial surface mannose, N-acetylglucosamine.
- **Cytosolic PRR:** It includes NOD2, NOD, NLRs which recognises mycobacterial peptidoglycan subunit N-glycosyl muranyl dipeptide [26].

After internalisation, the *M. tuberculosis* resists the host antibacterial mechanisms and initiates intracellular replication. In addition, post recognition, the second event is the production and secretion of pro-inflammatory cytokines, sensing which an army of circulating monocytes, neutrophils, dendritic cells are recruited to the site of infection. As dendritic cells have a paramount role in the poll of APCs proving their significance in causing immune response during initial phase of infection. Being a migratory cell, dendritic cells also play a crucial role in dissemination of *M. tuberculosis*.

On arrival at the infected location, immune cells of both the innate and adaptive immunity forms the typical **granuloma**; these are heterogeneous cellular topology made up of infected lipid rich macrophages at the centre surrounded by polynucleate (giant cells). The infected macrophage present at the centre is further surrounded by a layer of fibrous tissue with both B and T lymphocytes in the periphery. Other immune cells like neutrophils and DCs are also present in the granuloma. It is responsible for containment of the bacteria for decades forming a latent and persistent infection. Granuloma is said to be the survival niche of *M. tuberculosis.* It contains the latent mycobacterium infection even for decades. The host develops active infection only when the body becomes immunocompromised, like in the case of aging, HIV (Human Immunodeficiency Virus) infection, diabetes etc

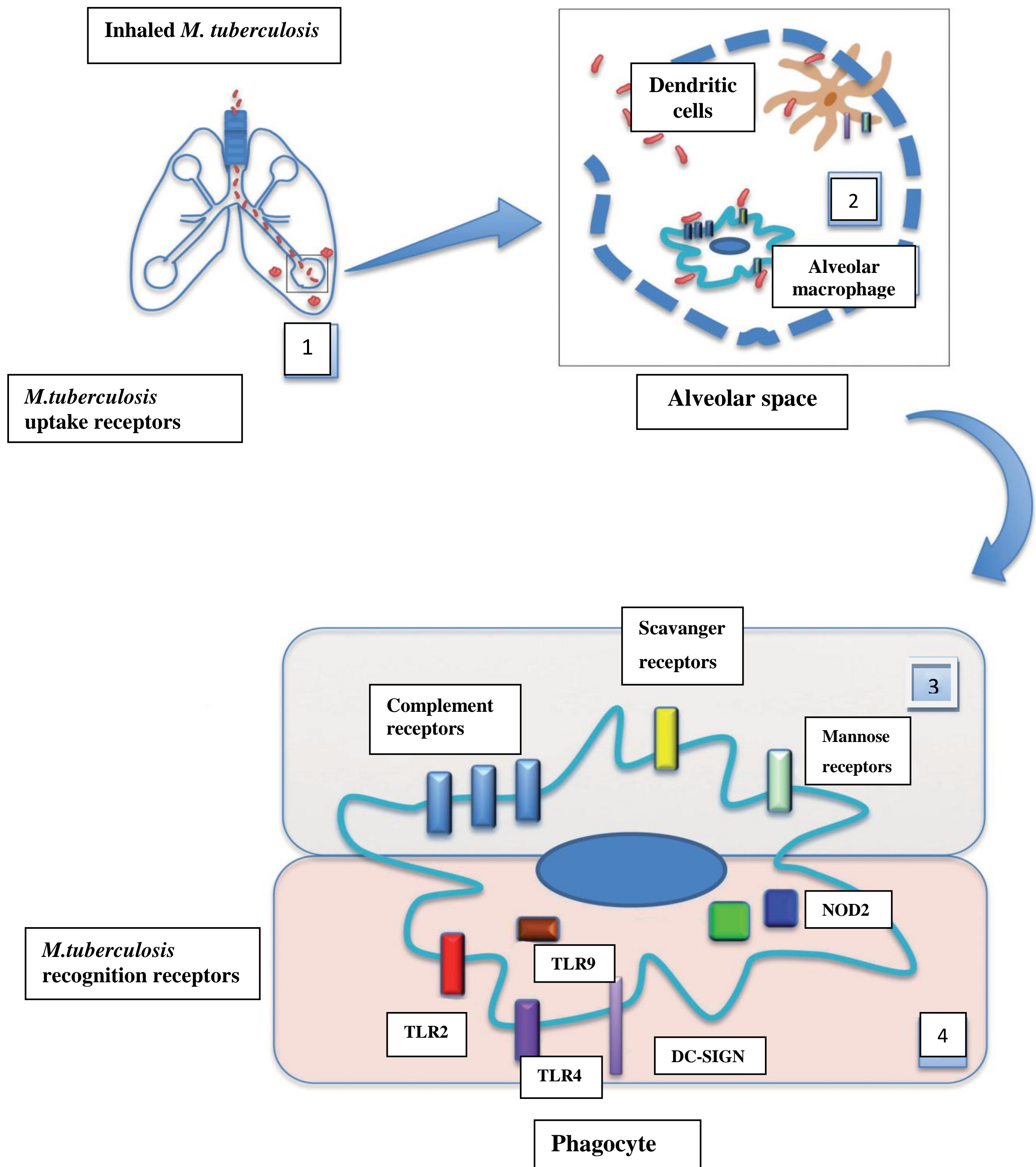

Figure 2.2: Early steps of phagocyte infection.: 1) Inhalation through respiratory pathways. 2) Phagocytosis by APCs. 3) CRs uptake opsonised *M. tb* whereas Mannose and Scavenger receptors for nonopsonised bacteria. 4) Recognition receptors express itself on surface of phagosome and APCs as well [27].

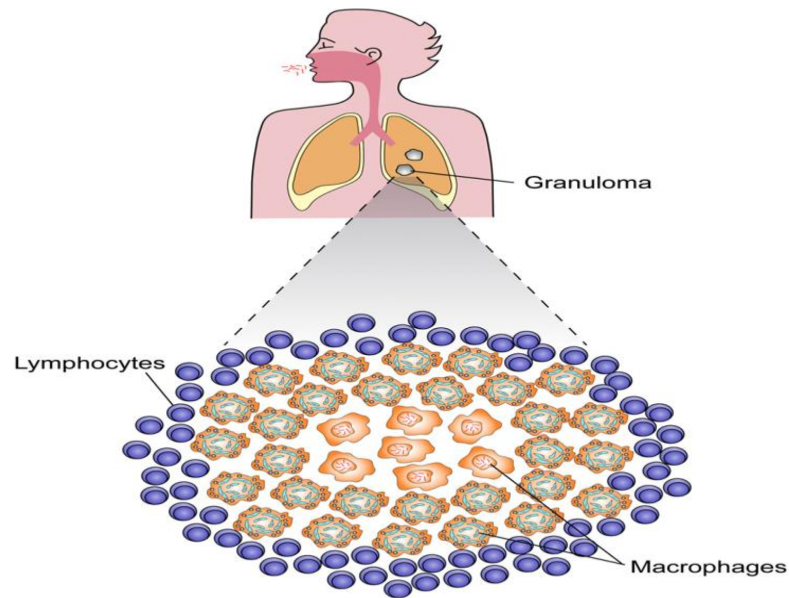

Figure 2.3: Granuloma formation: *M. tuberculosis* is inhaled via aerosols into the lung, where they are internalized within alveolar macrophages. Inside nonactivated macrophages (those within the centre of the granuloma), mycobacteria (indicated in red) resist destruction, surrounded by T lymphocytes and macrophages (4).

2.6. Mechanisms for killing of *M. tuberculosis*

Macrophages have adopted a plethora of defence strategy to withstand infection with *M. tuberculosis*. These are:

a) **Production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI):** The macrophages on activation produce reactive nitrogen intermediates (RNI) and reactive oxygen intermediates as well (ROI). The production of RNI is mediated by the activity of nitric oxide synthase (NOS2). NOX2 (consisting of 5 catalytic subunits) aids in production of ROI. Once these intermediates cross the phagosomal membranes, they implement their antimicrobial functions by oxidative damage of membrane lipids, DNA, thiol and tyrosine, focusing on iron sulphur bunch of enzymes involved in key metabolism. It has been validated in studies that the mutations of NOX2 or NOS2 genes causes more severe form of tuberculosis [28].

b) **Generation of toxic antimicrobial radicals:** Nramp1 gene codes for natural-resistance-associated macrophage protein (Nramp). It is an integral membrane protein which belongs to the group of metal ion transporters. These metal ions, particularly

Fe2_, functions to activate macrophages and also in production of toxic antimicrobial radicals. It takes part in the activation of macrophages as well extermination) of mycobacteria [29].

c) **Apoptosis:** Apoptosis is yet another means of defence adopted by the infected host cell to constrain the growth and proliferation of *M. tuberculosis*. Apoptosis of the infected phagocytic cells helps to prevent the spread as well as attenuate the survival of the intracellular mycobacteria [30]. It is known that TNF prompts apoptosis in reaction to infection by *M. tuberculosis.*

d) **Nutrient deprivation:** Glucose and lipids are the prime nutrients required *by M. tuberculosis* and macrophage has efficiently developed mechanisms to inhibit or restrict the access to these nutrients. To deprive the bacteria of glucose, the macrophage inhibits a vital enzyme fructose-1, 6-bis phosphanate aldolase (FBA which assists mycobacteria in accessing the carbon source from carbohydrates. Thus the inhibition of this essential enzyme impedes the growth and division of *M. tuberculosis* making it susceptible to host cell's immune system [31]. Another defence mechanism undertaken by the bacterium to restrict the growth of bacteria is depriving them of amino acids, which is again done by NOS2 upon activation of macrophage. This do not just influence microbial energy consumption but additionally causes autophagy and consequent clearance of *M. tuberculosis* [31]**.**

e) **Granulocytes assist macrophages in *M. tuberculosis* infection:** Polymorph nuclear leukocytes (PMN) get assembled to the site of infection as soon as the macrophages are apoptized. The PMNs take a couple of seconds to completely destroy the pathogens by secreting three different kinds of granules: after the apoptosis of macrophage. It takes few seconds for the PMNs to kill the pathogen. PMNs release 3 types of granules: primary azurophilic, secondary specific, and tertiary gelatinase granules, which contains antimicrobial molecules like AMP, lactoferrin , myeloperoxidase (MPO), elastase, cathepsins, and proteinase- 3 accelerating disruption matrix like metalloproteinase 9 (MMP-9), collagenase, and gelatinase. PMNs aids in restraining of mycobacteria post their discharge after the necrosis of macrophages [32].

f) **Vitamin D2:** This vitamin is related to modulation of lipid configuration inside the mycobacterial phagosome. It inhibits peroxisome proliferator-activated receptor γ (PPARc), which consequently prevents accumulation of lipid droplets

induced by *M. tuberculosis*. The accumulation of lipid droplets aids in multiplication of *M. tuberculosis* [33].

2.7. Defence mechanisms of *M. tuberculosis* against the host cells

The successful survival inside the host for elongated time period and the capability to infect the individual can be attributed to the fact that this prudent organism easily flees the immune system of the host.

a) Phagocytosis into the macrophage type of receptor used for internalisation: The internalisation of *M. tuberculosis* into the host cells is mediated by a special kind of cell surface receptors of the macrophages (like complement receptors, mannose receptor, in addition to the dendritic cell-specific intercellular adhesion molecule). The viability of the *M. tuberculosis* is also reliant upon the types of receptors used for its internalisation. The type of host cell receptors used for internalisation has a significant effect on the sustainability of *M. tuberculosis* within the host. For example, phagocytosis of the mycobacteria via CR3 receptor prevents macrophage activation. The cholesterol present on the plasma membrane of macrophages also affects the uptake of *M. tuberculosis* by CR3 as well as prevents the fusion of phagosome and lysosome [34].

b) Establishment of a balance: the granuloma

After internalisation of *M. tuberculosis*, depending on the host immunity, there may be a formation of granuloma, a clustered immunological structure which functions to keep the spread of mycobacteria in check. In granuloma, the macrophages infected with the *M. tuberculosis* reside at the centre, encapsulated with different immune cells (which are recruited at site on cytokine signalling) like T lymphocytes, and macrophages. Granuloma acts as a survival niche for the bacteria and helps in persistence of *M. tuberculosis* for an unknown time duration which may be extended for decades or life. [35].

c) Prevention of phagosome-lysosome fusion: Phagosome- lysosome fusion is an important tactic employed by the phagocytes for the elimination of internalised *M. tuberculosis.* Phosphatidylinositol 3-phosphate (PI3P), a host membrane protein, act as a docking site for many essential proteins which take part in maturation of phagosome to lysosome. But being an efficient organism, *M. tuberculosis*, utilises its glycolipids to manage to escape this fusion event via a two step strategy:

- It thwarts PIP3 production on phagosomal membrane. This is accomplished by inhibiting the activity of PIP3 kinase (which helps in generation of PIP3) [36].
- It prevents PIP3 accumulation on phagosome: The mycobacteria do it by releasing phosphatases, SapM within the cytosol of the host cell on the onset of infection. SapM hydrolyses PIP3 [37].

(d) Metals in macrophage responses to *M. tuberculosis*: Microbes like *M. tuberculosis* occupy transition metals by numerous pathway, and adequate acquirement of these metals by pathogens is linked to their pathogenicity and division.

2.8. *M. tuberculosis* virulence factors: Virulence factors are the genes that encode cellular components and secretory proteins which are concerned with the pathogenicity of the *M. tuberculosis.*

2.8.1. Cell Secretion and envelope function: This group comprise of the genes which encode specific proteins which are exposed to their surrounding where the bacilli grows. This group contains secreted proteins and enzymes that are crucial for the synthesis of various surface molecules [38].

a) **Culture filtrate proteins:** While growing in culture media *M. tuberculosis* release culture filtrate proteins (CFPs). Till now almost 200 such proteins are reported in Literatures [39]. The following are some CFPs.

- **HspX:** Its is a key antigen of *M. tuberculosis* which is identified in the sera of TB patients. HspX is a domineering protein required for *M. tuberculosis* dormancy or persistence [40].
- **Esat6/CF-10:** These belong to the Esat (Early Secretory Antigenic Target) family of small secreted proteins produced by actinobacteria. Both the proteins act as marker antigens and are extensively used for serological detection of tuberculosis in Quanti-FERON TB test. A recent study has reported that the mycobacterial secretory protein ESAT-6, acts as mediator of foamy macrophage differentiation process, an important step in pathogenesis [41].
- **19-kD protein:** The antigen has a size of 19-kDa sufficing it for immunorecognition by T cells and in the infected serums as well [42].

b) **Cell surface components:** Mycobacteria contain a distinctive cytomembrane and envelope that assists the bacterium to flee the

immune system of the host cells. Thus, the genes are of great importance and an excellent target for further studies on virulence of *M. tuberculosis*.

- **MmpL7:** MmpL7 was recognized as a significant virulence factor for *M. tuberculosis*. It is necessary for PDIM translocation [43]. MmpL7 is reported to be a part of the same cluster that codes the enzymes involved in the biosynthesis of the pthiocerol (ppsA-E) and mycocerosic acid (mas) residues of PDIM [44].
- **PcaA (proximal cyclopropanation of alpha-mycolates):** The gene encodes a methyltransferase. It takes part in the formation of cyclopropane residues in mycolic acids. At first it got detected in *M. bovis* BCG [45]. It may be as a prospective drug and vaccine target.
- **LAM:** LAM, a complex glycolipid constitutes an extensive part of the *M. tuberculosis* cytomembrane. It was observed that on exposing LAM to murine macrophages, the production of IFNγ severely decreased, due to which the genes whose expression was dependent on the induction by IFNγ, also got blocked. In *in vitro* conditions, LAM was found to perform scavenging of oxygen radical too. The manifold functions executed by this gene is suggestive of the fact that it is one of the important virulent genes which efficiently lower down the immune response of the host thus rescuing bacterium from host cell's immune system [46].

2.8.2. Enzymes engaged in cellular metabolism:

(a) Fatty acid metabolism: The host cell creates the condition of nutrient deprivation for the *M. tuberculosis*. In this case the bacteria switch its metabolism from carbohydrate to the pathway that uses fatty acid as a major source of energy. On annotating the genome of *M. tuberculosis* there are roughly 200 genes found to be involved in fatty acid metabolism [47]

- **Icl:** Inside granuloma, there exists a condition of nutrient deprivation. Fatty acid remains the only possible source to be utilised by the bacteria and *Icl* is involved in fatty acid metabolism. It functions to convert isocitrate to glyoxylate in glyoxylate shunt. Hence the bacteria continue to survive for longer period of time inside host causing latent infection which is not easy to eradicate [40].
- **LipF:** LipF gene codes for protein a lipase/ esterase that may have a role in lipid degradation. Its role is confirmed in pathogenesis and its promoter is upregulated under acidic stress condition [48].

(b) Genes synthesising amino acids and purine:

- **LeuD:** The gene encodes for the enzyme isopropylmalate isomerase that functions in the biosynthesis of leucine. After its inactivation in *M. tuberculosis* H37Rv by using plasmid in a two step process, it was observed that the mutant was unable to grow in primary murine macrophages or killed SCID mice [49]
- **TrpD (Rv2192c, *trpD*):** TrpD is a gene which codes for anthranilate phosphoribosyl transferase that takes part in the pathway involved in biosynthesis of tryptophan. The *M. tuberculosis* gene was disrupted in *M. tuberculosis* with help of a two step process involving plasmid. This procedure led to the creation of a mutant phenotype that couldn't survive in murine macrophage [50].

2.8.3. Metal uptake: Metals forms an essential part of the survival of *M. tuberculosis* because of their involvement in many metabolic pathways. In case of deficit of metal uptake, the bacterial survival will weaken.

- **MgtC:** The *Salmonella* MgtC is a transporter which helps in utilisation of Mg^{2+}. In media and in macrophages having low concentration of Mg^{2+}, this transporter becomes vital for this pathogen to grow, signifying that this environment is restraining for this divalent cation [51].
- **2.8.4. Anaerobic respiration and oxidative stress:** In spite of being an aerobic microbe, *M. tuberculosis* is confronted by anaerobic or microaerophilic surrounding inside the granuloma**.**
- **KatG:** KatG is a catalase: peroxidase whose task is to degrade H2O2 and organic peroxides. It is the mere enzyme with a subunit of the prokaryotic respiratory (anaerobic) nitrate reductase that plays a significant role in respiration in the absence of oxygen, and anaerobic nitrate reductase activity increases when *M. tuberculosis* becomes microaerophilic. It helps in *M. tuberculosis* persistence by degrading the ROIs produced by the host cells in order to kill the bacteria [52].

2.8.5. Transcriptional Regulators:

- **Sigma factors:** Use of RNA holoenzymes with diverse specificity for promoters of the genes to be transcribed is yet another tactic used by *M. tuberculosis* to escape the adverse conditions created by the host. This is accomplished by the arrangement of new holoenzymes which contain diverse sigma factors, which permits

the transcription of the genes required for new circumstances. The genes belonging to these criteria include *SigA, SigE, SigF, and SigH* [50].

2.9. The enzyme: Isocitrate lyase

Isocitrate lyase (Rv0467, *Icl* or aceA), was one of the first *M. tuberculosis* genes shown to be required for persistent infection (2). *Icl* (gene length 1287bp) encodes for isocitrate lyase, an enzyme fundamental for metabolism of fatty acids, present in plants, archaebacteria, fungi and bacteria also. It was identified in *Mycobacterium tuberculosis* in 1999 in retaliation to phagocytosis by human macrophages [53]. *ICL* is the first enzyme of glyoxylate shunt (an alternative pathway of TCA) catalyses the conversion of isocitrate to glyoxylate. In the TCA cycle, D-isocitrate is first converted into 2-oxoglutarate, and subsequently into succinyl-CoA. As a means of defence strategy, TCA cycle becomes suppressed or less active on invasion of the bacteria in order to starve them of nutrients. So, to restore the nutritional acquirement of the organism the genes coding for enzymes of glyoxylate cycle becomes upregulated [54].

ICL being the beginner enzyme is seen as an important enzyme of this process. Glyoxylate has following important roles:

- it bypasses the two decarboxylation steps of the TCA cycle. Hence, the loss of two carbons (decarboxylation steps in TCA) in the form of carbon dioxide is avoided. The carbon sources of *M. tuberculosis* comprise of fatty acids, which are present in abundance inside the host macrophages. The major being acetyl CoA which is the product of many pathways one being β-oxidation of fatty acids [55].
- Second role is that the cycle is predominantly essential for latent *M. tuberculosis* that resides in a dormant state inside macrophages.
- This anaplerotic pathway recharges the intermediate pool of TCA cycle intermediates which take part in many other biosynthesis pathways.

The evidence of fatty acids being crucial for mycobacterium pathogenesis is supported by the fact that the genome of *M. tuberculosis* itself comprises of over 250 genes coding for proteins engaged in fatty acid metabolism. This fact has been backed by plenty of studies conducted which directly proves the role of fatty acids and hence *icl* in the persistence and pathogenesis of bacteria [47].

In studies done formerly, Segal and Bloch observed that it was fatty acid and not carbohydrate that triggered the respiration of *M. tuberculosis* if they were isolated from mice lungs. This formed the basis of hypothesis of reliance *M. tuberculosis* on fatty acid as a sole source of nutrition [56].

After forty four years, McKinney et al. delineated a *M. tuberculosis* mutant, in which a gene (unknown at that time) coding isocitrate lyase had been dysfunctional. On comparing the growth with the wild type, mutant bacterium ceased to survive during the chronic infection phase which employed a mouse model of TB**.**
Persistent mycobacteria barely secure its survival amid the adverse surrounding within the macrophage but also manage to get obscured by the potent anti-TB drugs. The bacteria switch its metabolism to C2 substrates, the end product of fatty acid metabolism, which is a tactful approach of *M. tuberculosis* during chronic infection.

Muñoz-Elías and McKinney exhibited the presence of two forms of MTB *icl* (*Icl*1: prokaryotic-like isoform and *Icl*2: eukaryotic-like isoform). He confirmed that both the forms are collectively required for the survival of MTB. It was being shown by them that the disruption of either of these two isoforms at one time didn't affect the growth and survival of the bacteria. However, absence of both the forms did compromise the survival, leading to the elimination of MTB from the host cell. The *icl*1 is coded by the gene *icl* and *icl*2 is encoded by gene ace A. The *icl* isoforms are encoded by *icl* gene (*Icl*1) and *ace A* gene (*Icl*2), respectively. The *aceA* gene, is however less active in comparison to *icl* gene and does not gets expressed in all forms of mycobacteria [57].

Another important role of isocitrate lyase is found in survival of mycobacteria during hypoxic conditions. As the tuberculous granuloma is hypoxic in nature, this subjects the mycobacteria to the challenge of survival in low oxygen level. Therefore, it induces many genes as a means of defence against the low oxygen. *Icl* and *ald* are the chief genes which are incited in reaction to the conditions that inhibit respiration. This has been proposed to function in redox balancing throughout entry into the Non Replicating Persistence (NRP) state. Each mRNA levels and enzymatic activities of isocitrate lyase, the primary catalyst of the glyoxylate cycle, and amino acid dehydrogenase amplified at the time of entry into no replicating persistence [7].

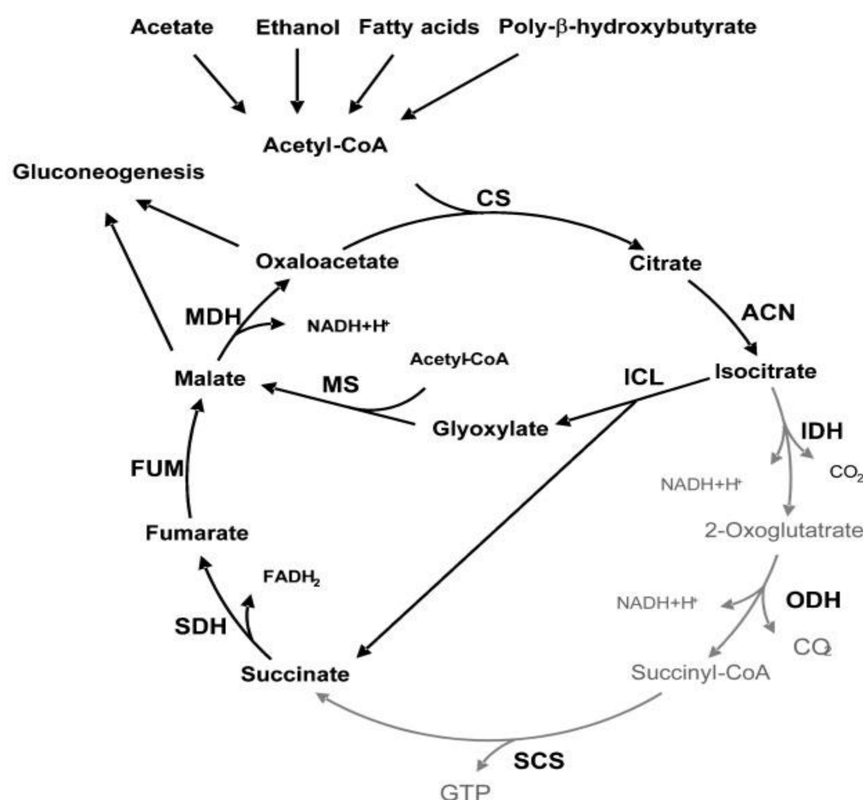

Figure 2.4: Enzymatic reactions of the glyoxylate and TCA cycles. A variety of metabolic processes can` generate acetyl-CoA, from which can be preserved by metabolism via the glyoxylate cycle, which bypasses the CO2- generating steps of the TCA cycle [58].

2.9.1. Structure of *ICL*:

ICL is a tetramer (81 Å ´ 86 Å ´ 92 Å) with 222 symmetry. Every subunit of the enzyme consists of 14 α-helices and 14 β-strands. Eight α-helices (α4–α11) and eight β-strands (β 2– β 5, β 8, β 12– β 14), representing the biggest domain and therefore the core of the structure, form an atypical a/b-barrel. This domain is crucial since it contains many of the active site residues. Residues 184–200 and 235–254 (connecting the third and fourth b-strands to their consecutive helices, respectively) form a small b-domain consisting of a small five stranded b-sheet (β 6, β 7, β 9, β 10, β 11) that lies atop the a/b-barrel [59].

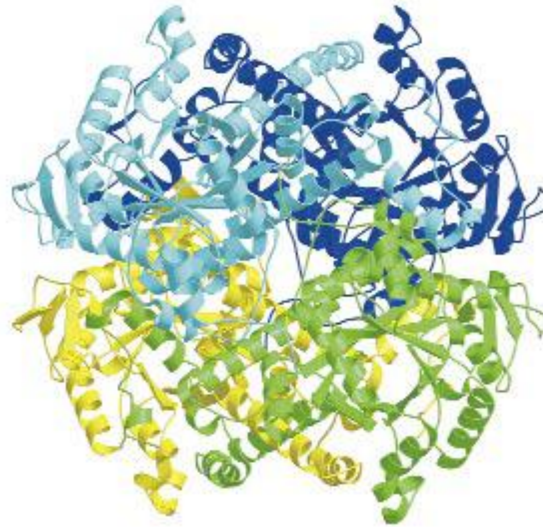

Figure 2.5: Ribbon illustration of the *ICL* homotetramer: with each subunit colored differently. The four subunits of the tetramer are linked by 222 symmetry. The cyan and blue and the yellow and green subunits show widespread interactions (59).

ICL is an eligible inhibitory target for the drugs in order to treat latent TB. Its prospective as a drug target is attributed to its major role in survival of bacteria inside host for long period and secondly *icl* is absent in humans. Existing *ICL* inhibitors like itaconate, 3- nitropropionate and 3-bromopyruvate are imitation of the native substrate (or products). Although these inhibitors didn't prove successful as drug candidates because of their toxicity along with activity of hindering essential metabolic enzymes *in vivo*. The inherent toxicity of these inhibitors may be due to their non specificity of binding to enzymes involved in human metabolic pathways, for instance, enzymes taking isocitrate or succinate as their substrates [60].

2.10. Overexpression

A myriad of molecular mechanisms occur inside an organism which makes sure that genes are expressed at the desired level and at the apt conditions. If there is an upsurge in expression of a wild type gene, then this can also prove distorting to a cell or life forms [10]. In a study done on *Salmonella enterica*, it was found that mutation in the promoter site of the gene recA, caused it to overproduce the protein which in turn decreased the fitness of orally inoculated *recAo6869* cells decreased dramatically [11]. The above studies throws light the fact the overexpression of a

functional gene may also prove to be disruptive to the organism or have adverse effect on the organism. Linking genes to biological pathways is many a times accomplished by intended overexpression, which acts as a constructive tool for individual genes.

The probable usefulness of overexpression phenotypes did not skip ignored. In 1983, a particular paper trying to classify drug objectives defined screens to pick out overexpressed genes that undo the growth inhibitory consequences of tunicamycin, compactin/mevastatin, and ethionine which is in accordance to the preceding findings that overexpression of drug targets pass on resistance to their resultant drugs in bacteria, mouse, hamster and human cells [61].

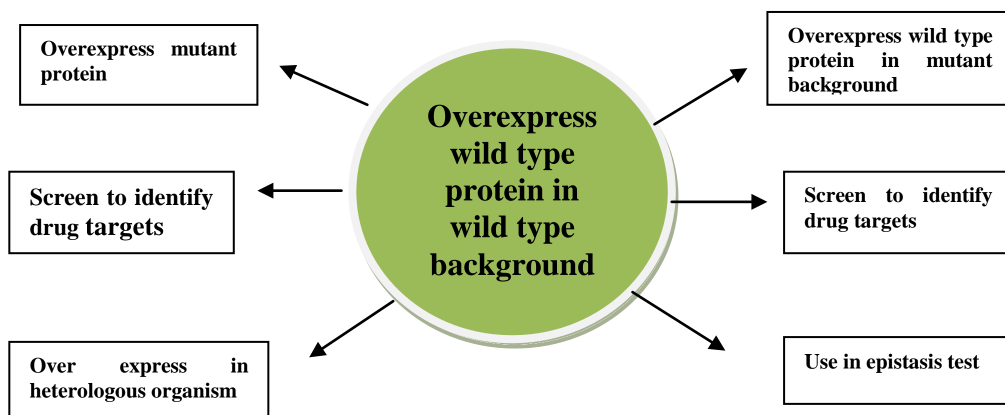

Figure 2.6: Common uses of overexpression (10).

The two central mechanisms that explains the production of mutant phenotypes owing to overexpression:

- **Inhibition-** In this mechanism it's presumed that steady levels of other proteins can be reduced because of hindrance in transcription or translation. Functional inactivation of the proteins by overexpression is observed which is not dependent upon competition based machinery. Additionally, another possible reason for inhibition may be disruption of a protein -protein interaction after the post-translational modification of one subunit.
- **Activation-** Over expression generates a phenotype through activation a step in a pathway. Overexpression can prompt new pathways via neomorphic effects. A repressor with the aid of any wide variety of mechanisms, inclusive of degradation of the repressor, inactivating it through posttranslational modification or by way of direct competition could activate a pathway (10).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials used:

3.1.1 Bacterial strains and plasmids used in the study: *M. fortuitum* ATCC 6841 and *Escherichia coli* DH5α cultures were used for the present study. *pGEM T* easy vectors (Promega) and *pMV261* [62] were used for TA cloning and sense construction of *icl* gene in *M. fortuitum* respectively.

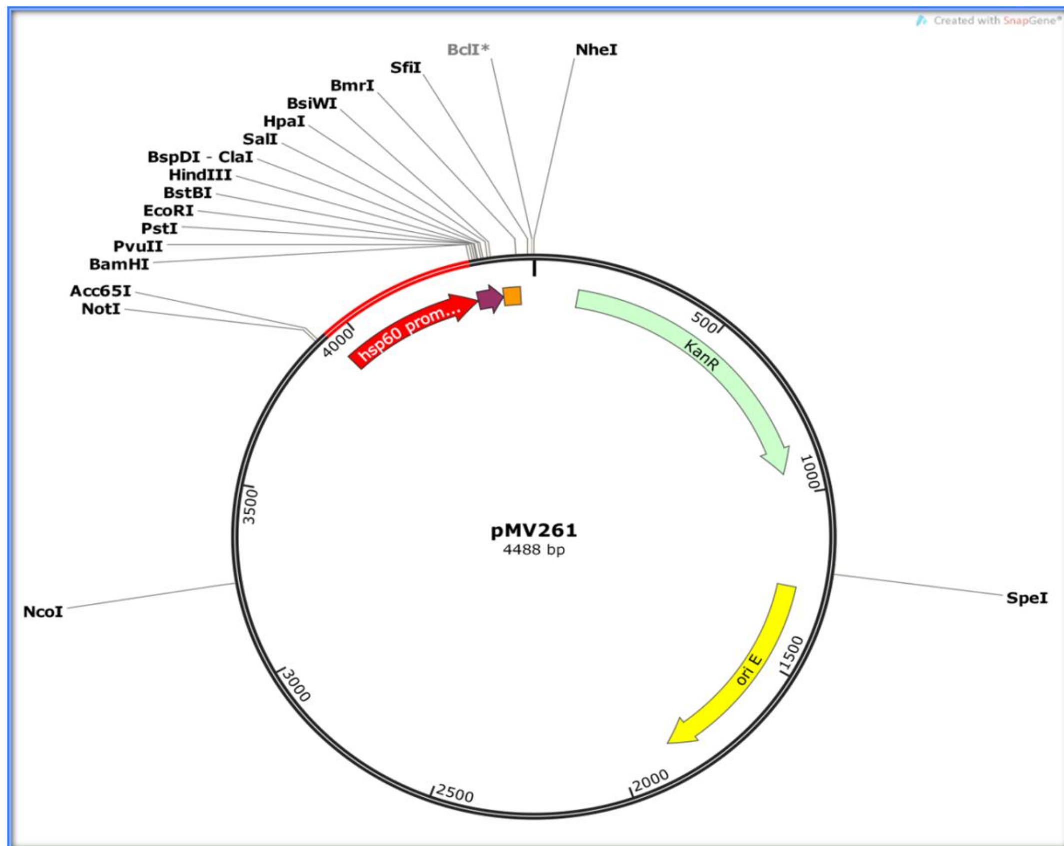

Figure 3.1 : Vector map of *pMV261*

3.2 Methods:

3.2.1 Construction of sense construct of *icl* gene of *M. fortuitum* (Acknowledgment Ms. Shivani Sood)

Isocitrate lyase gene with length of 1287 bp is present in the genome of *Mycobacterium tuberculosis*. It is likewise present in *M. fortuitum* the *icl* sense construct was taken from the work done formerly, as described briefly in this section. ATCC 6841. Multiple sets of primers were designed from flanking region of *Icl* 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 *icl* of *M. tuberculosis*. After doing the homology search, the longest sequence having the maximum homology was submitted to NCBI [GenBANK ID - KM275229) as *icl*

homologue. *pMV261* vector was used to construct the sense mutants of *M. fortuitum icl*. The orientation was confirmed using BamHI site in *pMV261* vector. Presence of full length gene and its orientation was confirmed by restriction digestion and PCR using hsp60 primer (promoter). Obtained sense mutants were electroporated in *M. fortuitum*

3.2.2. Media used for culturing *M. fortuitum*:

a) Middlebrook 7H9 Broth (MB7H9 media): This media is used in the growth of *M. fortuitum* is MB7H9 (Hi-media) with 0.5% glycerol and 0.2% tween 80. Cycloheximide (50µg/mL) (Hi-Media) was added to the growth medium to prevent fungus contamination. The media was also supplemented with Kanamycin (30 µg/mL) in order to allow the growth of only the transformed bacteria.

b) Nutrient agar tween: The media used for maintaining *M. fortuitum* culture and colony forming unit (CFU) determination was nutrient agar (Hi-Media) with 0.05% Tween 80 (NAT).

3.3. *In vitro* studies of *the M. fortuitum icl* sense constructs under various stress conditions

3.3.1. Growth of *Mficl* sense construct under *in vitro* nutrient starvation stress:

For nutrient starvation stress, the cells were grown to mid logarithmic phase and pellet was collected after centrifugation at 5000 rpm for 10 minutes. Pellet was washed with PBS two times and then resuspended in MB7H9 medium. The cells were then inoculated into 50 mL PBS and incubated at 37^0C. Samples were taken after 0, 2, 6, 12, 24, 36 and 48 hour for CFU determination.

3.3.2. Growth of *Mficl* construct under *in vitro* oxidative stress:

Icl sense constructs and wild type *M. fortuitum* cultures were grown in MB7H9 medium till the achievement of 0.4 O.D at 600 nm. Centrifugation of cultures was done at 7000 rpm for 10 minutes which was further followed by two times washing with PBS at same speed. The pellet of cells was then resuspended in MB7H9 medium and used for inoculation in MB7H9 medium containing 10mM H_2O_2. The CFU of the culture in 10mM H_2O_2 was then determined after 0, 2, 6, 12, 24, 36 and 48 hour.

3.3.3. Growth of *Mficl* sense construct under *in vitro* detergent stress: The *icl* sense was subjected to detergent stress by exposing the bacilli to 0.05% SDS in MB7H9 medium. The cells were grown in MB7H9 medium till O.D reaches to 0.4 at 600nm and then cells were collected by centrifugation and then the cells were washed with PBS two times. The cells were then resuspended in 5mL MB7H9 medium and then inoculate in the medium MB7H9 containing 0.05% SDS (Deborah et al., 2006). The aliquots from the culture were taken after 0, 2, 6, 12, 24, 36 and 48 hour and CFU was determined.

3.3.5. Growth of *Mficl* sense construct under *in vitro* acidic stress conditions: The method for the exposure of sense construct to *in vitro* acidic stress conditions was adopted as previously described (3)**.** *Icl* sense constructs and wild type *M. fortuitum* were grown in Middlebrook MB7H9 (Hi-media) media with 0.5% glycerol (Fischer Scientific) and 0.2% tween 80 (Bio Basic Inc) and incubated at 37^0C for 2 days. Seed cultures were passaged in 100ml of MB7H9 with glycerol and tween 80. The cultures were incubated till the optical density of the cultures reached to 0.4 at 600 nm. Cultures were then aliquoted into two flasks (50ml each) and incubated at 37^0C for 2 hours. Cultures were centrifuged at 5000 rpm for 10 minutes followed by washing of pellet with Phosphate buffer saline (PBS) two times. The pellet was suspended in 5 mL of MB7H9 media. This suspension of microbes was then used for inoculation of antisense construct and wild type to MB7H9 medium of different pH including 3.5, 4.5, 5.5 and 6.5. The samples from each medium with different pH were taken at different time interval 0, 2, 6, 12, 24, 36 and 48 hour and CFU of those samples were done by spreading on NAT plates after 10 fold serial dilutions up to 10^{-6}.

3.3.6. Exposure of *Mficl* to hypoxic conditions: To determine the effect of hypoxia on expression on *M. fortuitum*, the sense construct was subjected to hypoxic stress condition. Cells were grown in MB7H9 until the O.D of culture reaches 0.8-1. Methylene blue as oxygen depletion indicator at concentration of 1.5µg/mL was added to the culture and then the cells were dispensed into 15mL glass vials and capped with rubber septa and vacuum Greece to prevent the oxygen intake into the vials. The glass vials were kept at 37^0C for 30 days and CFU at different time intervals was taken to determine the viability of the cells under hypoxic conditions.

CHAPTER 4

RESULTS

RESULTS

Growth curve of *M. fortuitum icl* sense (*Mficl*) construct under different *in vitro* stress conditions

4.1. Nutrient starvation

- The growth curve of wild type (WT) *M. fortuitum* remained constant throughout the experiment.
- On the other hand, the growth of *Mficl* remained constant till 36th hours. However, at 48th hour *Mficl* showed 4 log decrease in growth in comparison to wild type.(Fig: 4.1).

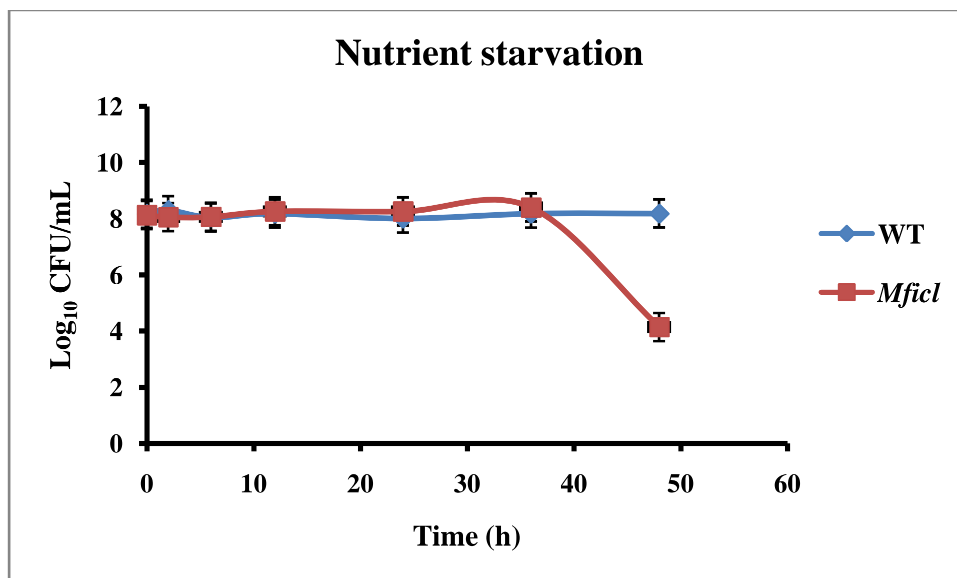

Figure 4.1: CFU of WT and *Mficl* under nutrient starvation stress. The growth of WT was constant but that of *Mficl* showed decline after 36th hour.

4.2. Heat stress

- The growth of WT as well as *Mficl* declined after 24 hours (Fig: 4.2)
- On comparing growth curve of WT and *Mficl*, there was no distinct difference of growth pattern observed.

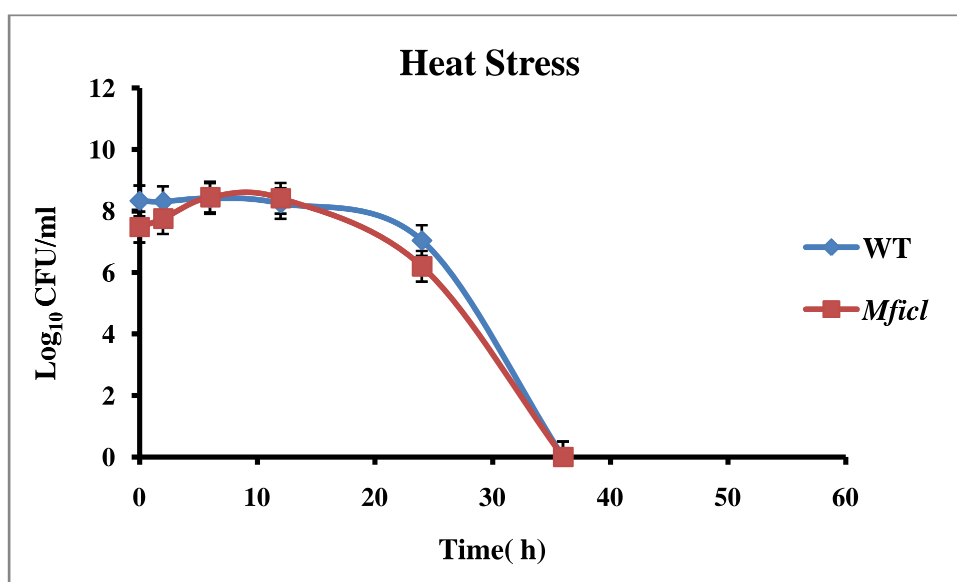

Figure 4.2: CFU of *WT and Mficl* under heat stress: No difference in growth pattern was observed.

4.3. Oxidative stress

- The WT as well as *Mficl* showed increased CFU till 12th hour. But after 12th hour the CFU and hence the growth curve became constant. (Fig: 4.3).
- On comparing the growth curve of WT and *Mficl* showed no difference.

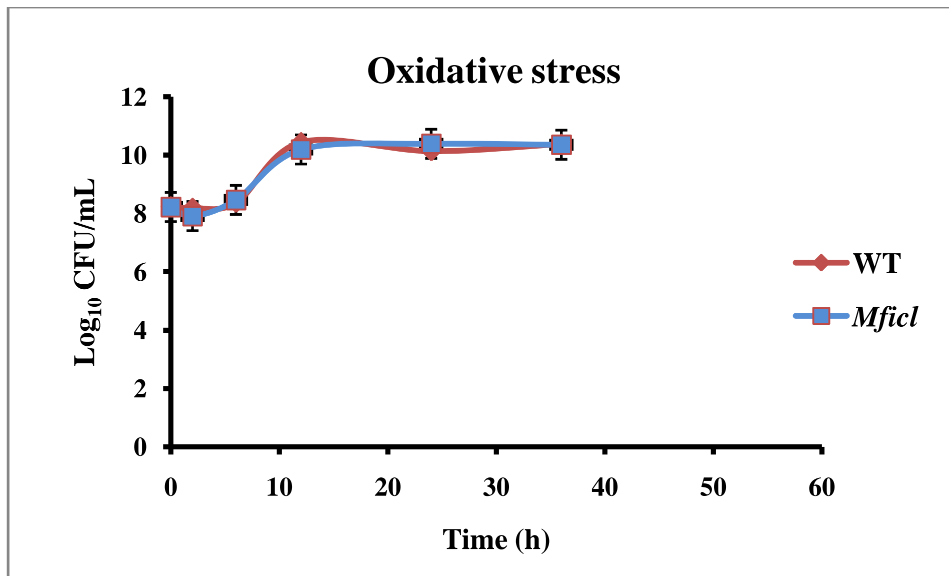

Figure 4.3: CFU of WT and *Mficl* under oxidative stress. The growth curve of both WT and *Mficl* was found to be constant after 12th hour.

4.4. Detergent stress

- Under detergent stress, the CFU of both the WT and *Mficl* was constant.
- On comparison, the growth curve of WT and *Mficl* showed no distinct difference (Fig: 4.4).

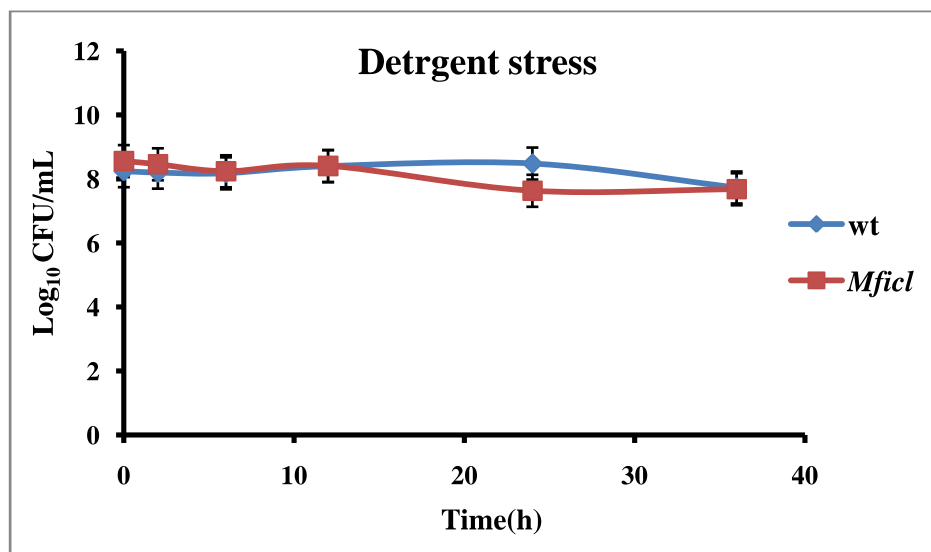

Figure 4.4: CFU of WT and *Mficl* under detergent stress. The growth of both WT as well as *Mficl* was found to be constant with no distinct differences.

4.5. Acidic stress

- On comparing the CFU of the WT *M. fortuitum* and *Mficl* in different pH ranges it was found:
- Under pH 3.5 the growth of WT showed a constant decline after 6th hour whereas the growth of *Mficl* also showed a constant decline after 12th hour (Fig: 4.5)
- Under pH 4.5, the growth of both wild type *M. fortuitum* as well as *Mficl* declined but the declination was not as prominent as it was in pH 3.5. The CFU didn't become 0.The growth pattern of WT and *Mficl* as well didn't show any distinct variation (Fig: 4.6)
- In pH 5.5 and 6.5 the growth of both the WT and *Mficl* was constant. It didn't show any variation throughout the experiment (Fig: 4.7 and 4.8)

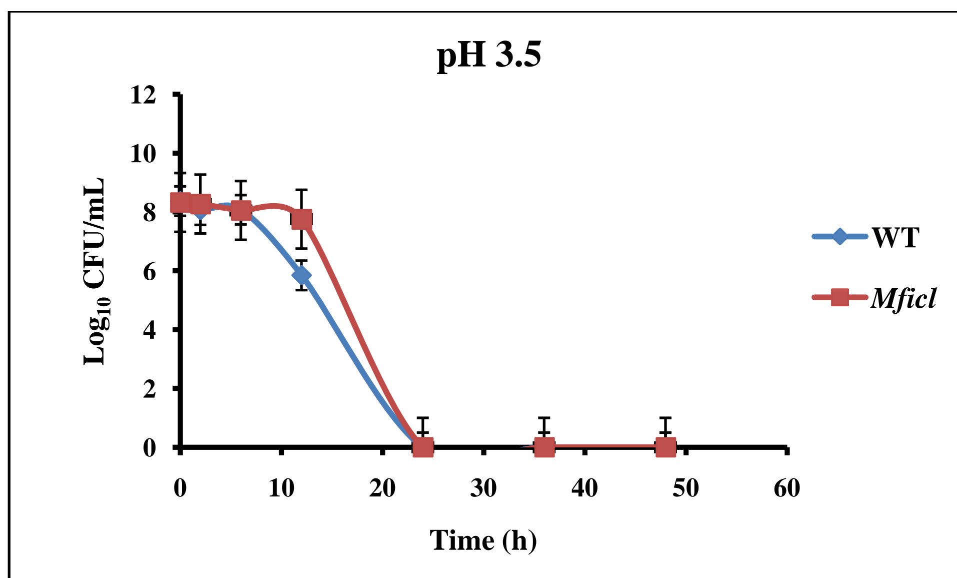

Figure 4.5: CFU of WT and *Mficl* under pH 3.5.

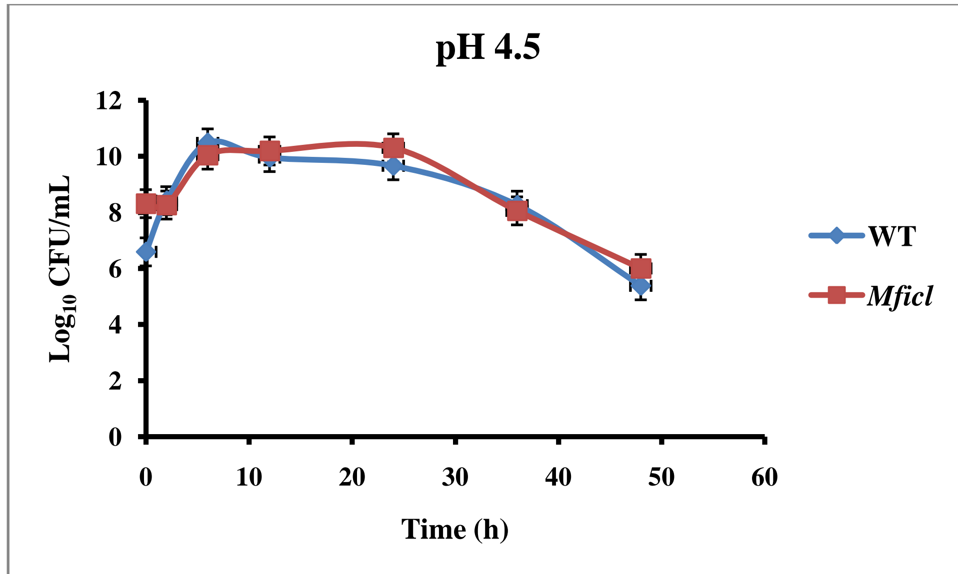

Figure 4.6: CFU of WT and *Mficl* under pH 4.5

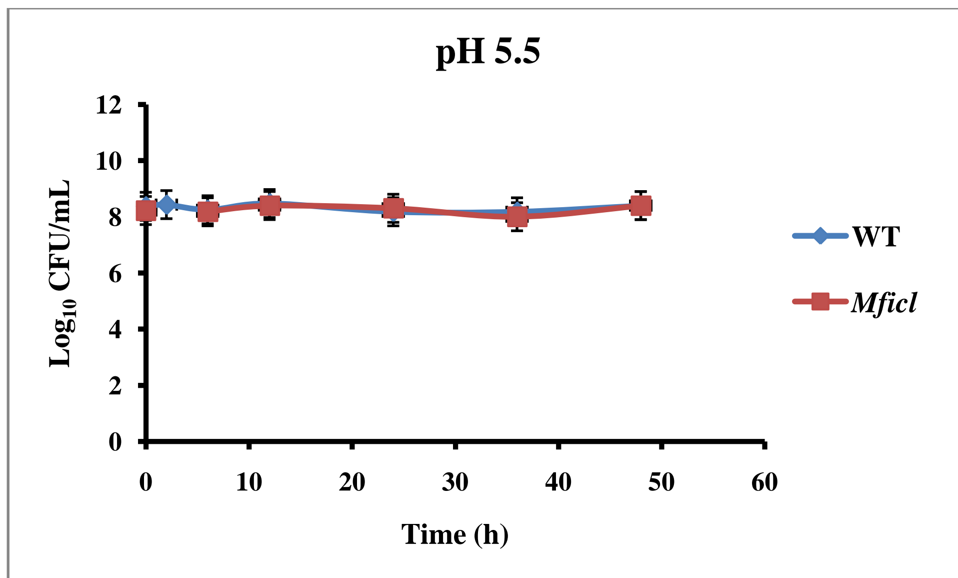

Figure 4.7: CFU of WT and *Mficl* under pH 5.5.

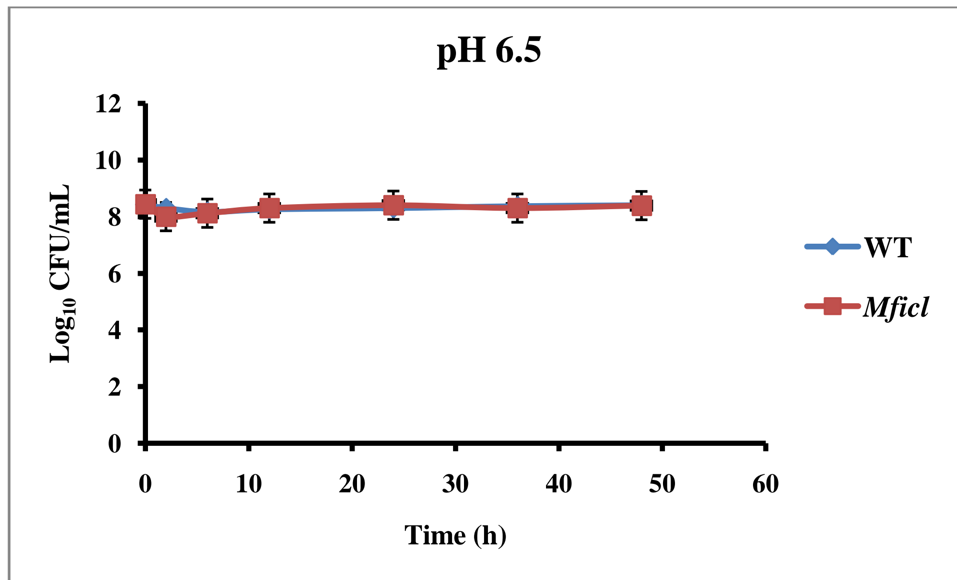

Figure 4.8: The CFU of WT and *Mficl* under pH 6.5.

4.6. Hypoxia

- The growth curve of the wild type *M. fortuitum* was constant throughout the experiment. (Fig: 4.9)
- But the growth curve of *MfIcl* decreased by 2.21 log value after 6th day of CFU count in comparison to WT.

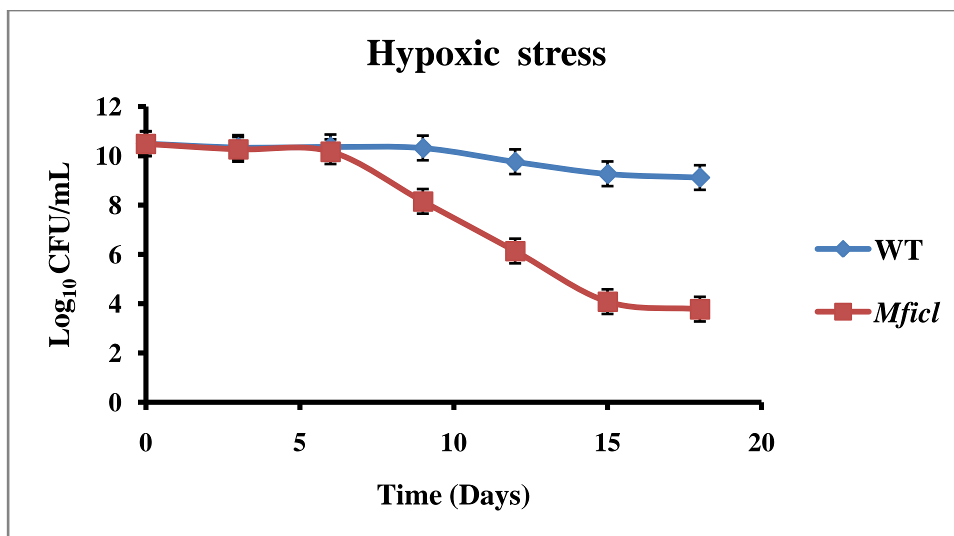

Figure 4.9: CFU of WT and *Mficl* under hypoxic condition.

CHAPTER 5
DISCUSSION AND CONCLUSION

Discussion and Conclusion

The success of *M. tuberculosis* to survive amid the hostile circumstances existing inside the host cell granuloma may be attributed to the fact that it has evolved myriad of defence mechanisms to combat and escape the host immune system. It is capable of persisting inside the host cell even for decades being asymptomatic. Macrophage, the preferred host cell of the bacterium, uses many effector mechanisms to eradicate the mycobacteria by making the environment unfavourable for the them. It produces different stress conditions like nutrient deprivation, acidic, hypoxic, oxidative conditions in order to evade the mycobacteria. In spite of these adverse conditions, *M. tuberculosis,* being a prudent bacteria secures its survival because of various tactics and presence of some unique virulence factors. One of the most important virulence factors of *M. tuberculosis* is Isocitrate Lyase (*ICL*) which discharges many important functions like persistence inside the host, prevention under hypoxic condition, antibiotic tolerance.

Fatty acid is the major source of nutrition and energy for *M. tuberculosis* inside the host cell and *ICL* is the enzyme which is involved in fatty acid metabolism, which makes this gene a crucial one for *M. tuberculosis*. *ICL* is basically the first enzyme of glyoxylate cycle (a bypass of TCA cycle) which catalyses the conversion of isocitrate (an intermediate of TCA cycle) to glyoxylate. Disruption of the *M. tuberculosis icl* gene reduces survival of *M. tuberculosis* under persistent condition, and shows attenuation in virulence in immune-competent mice without affecting bacterial growth during the acute phase of infection (8). The macrophage has developed mechanisms for restricting the utilisation of glucose by inhibiting an enzyme fructose-1,-bisphosphate aldolase (FBA) which assists mycobacteria in accessing the carbon sources from carbohydrates, hence creating the condition of nutrient deprivation for the bacteria. Considering its important roles in *M. tuberculosis*, many inhibitors were synthesized against *icl*, but didn't prove to be much efficient due to their toxicity and non specificity. In many studies it has been reported that the over expression of any functional gene can also be disruptive to the cells (11). Therefore, this project is done to study the effect of over expression of *icl* on *M. tuberculosis* taking *M. fortuitum* as surrogate organism under various stress conditions existing inside the host granuloma.

When compared to the growth pattern of wild type *M. fortuitum, Mficl* showed a decline in growth under two stress conditions: Nutrient starvation and Hypoxic stress. Considering the role of *icl* gene in providing a source of nutrition to the *M. tuberculosis* within the host granuloma, the growth of *icl* sense construct was expected to be normal or unaffected with the passing time but surprisingly, the CFU count declines after 36 hour. Similarly, *icl* performs a vital function to prevent the bacteria under hypoxic conditions inside the granuloma. Therefore, *M. fortuitum* with over expressed *icl* gene was expected to survive under hypoxic stress condition. But again unexpectedly, the CFU of *Mficl* under hypoxic stress also decreased after 4th day while that of wild type remained constant. At the same time, there wasn't any major difference found in the growth of wild type *M. fortuitum* and *Mficl* under the remaining stress conditions (acidic, detergent, oxidative, heat).

So, in the work done, the *Mficl* in *M. fortuitum* when compared to wild type, showed a decreased growth unexpectedly. It may be suggestive of the fact that the over expression may prove inhibitory to *icl* either by feedback inhibition mechanism or by regulation of other genes responsible for survival in stress conditions. In accordance with other studies being done previously, this work on *Icl* gene signifies that the overexpression of an otherwise important gene meant for bacterial survival, can also be disruptive to the organism. Hence, gene knockout studies is alone not enough to deduce the functions, overexpression of genes should also be studied to find out important. However, this work of overexpression of *icl* gene can be further be extended to be studied in *M. tuberculosis* for validation.

CHAPTER 6
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REFERENCES

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PUBLICATIONS

1) ***In-Vitro* studies of the overexpressed gene Isocitrate Lyase of *Mycobacterium fortuitum* under stressed conditions.**

Arpita Prasad, Rahul Pramjeet, Gopal Singh Bisht, Rahul Shrivastava*

Poster Presentation at 58th Annual Conference of Association of Microbiologists of India (AMI), organised by Babasaheb Bhimrao Ambedkar 9ACentral) University Lucknow, Uttar Pradesh from November 16-19, 2017.

Poster

In-Vitro studies of the overexpressed gene Isocitrate Lyase of *Mycobacterium fortuitum* under stressed conditions.

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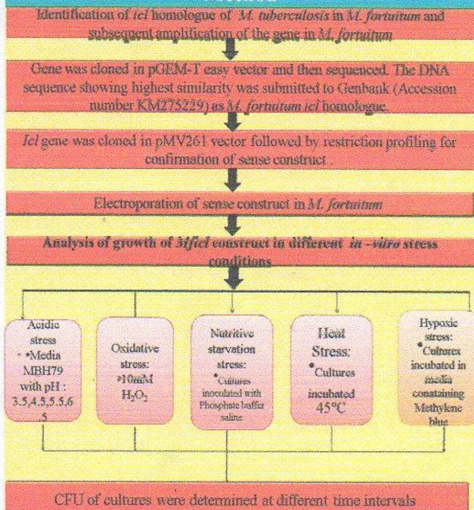
Introduction

Mycobacterium tuberculosis is a global health threat ,infecting about one third of the human population in an annual casuallity of ~2 milliion people worlwide. Infection of a host with *M. tuberculosis* is initiated following the inhalation of droplets (aerosols) containing a small number of bacilli. Once in the lung, bacilli are internalized through phagocytosis by the alveolar macrophages. *M. tuberculosis*, a well-adapted facultative intracellular bacterium, counter-balance the host's immune defense strategies to secure survival or multiplication within this otherwise hostile environment. There are certain genes which are expressed during infection, one of them being Isocitrate lyase(*icl*).,which is Involved in the persistence and virulence of *M.tuberculosis*. Catalyzes the reversible formation of succinate and glyoxylate from isocitrate, a key step of the glyoxylate cycle, which operates as an anaplerotic route for replenishing the tricarboxylic acid cycle during growth on fatty acid substrates. With an aim to explore the role of *icl* for *in vitro* survival, and response to various stress conditions and pathogenesis of *Mycobacterium fortuitum*, an opportunistic bacteria causing infections ranging from nosocomial infections, skin diseases to osteomyelitis, specially in immunocompromised patients, the gene was overexpressed by amplification and subsequent cloning it in vector *pMV261* followed by its electroporation in wild type *M. fortuitum*. Expression of the construct was confirmed by Real-time PCR. *M. fortuitum* mutants were subjected to various *in-vitro* stress conditions existing in macrophages, like, acidic stress, hypoxic condition, nutritive stress, heat stress, oxidative stress

Method



Results

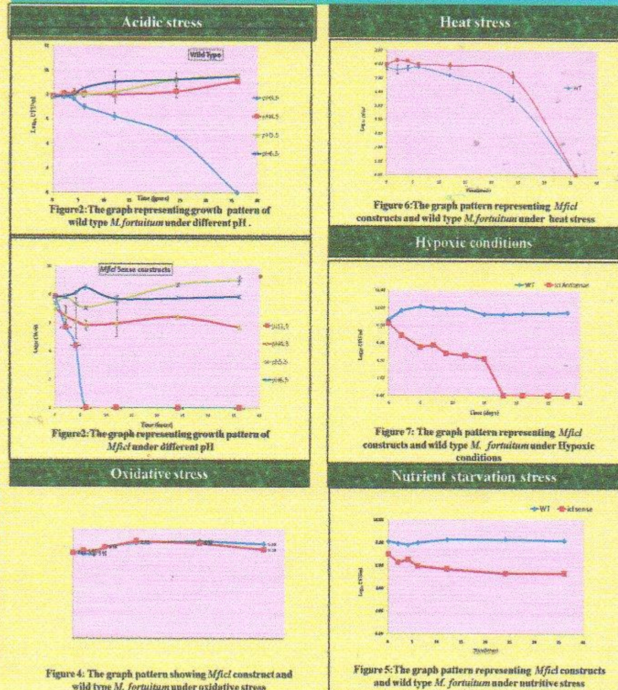


Figure2: The graph representing growth pattern of wild type *M. fortuitum* under different pH .

Figure 6: The graph pattern representing *Mficl* constructs and wild type *M. fortuitum* under heat stress

Figure2: The graph representing growth pattern of *Mficl* under different pH

Figure 7: The graph pattern representing *Mficl* constructs and wild type *M. fortuitum* under Hypoxic conditions

Figure 4: The graph pattern showing *Mficl* construct and wild type *M. fortuitum* under oxidative stress

Figure 5: The graph pattern representing *Mficl* constructs and wild type *M. fortuitum* under nutritive stress

Figure1: Multiple Sequence alignment of *M. fortuitum* gene *icl* with *M. tuberculosis* and *M. smegmatis*

Discussion & Conclusion

•CFU count of *Mficl* construct was found to decrease significantly when compared to the wild type at pH 3.5. and no CFU was observed after 6 hours . This shows that *Mficl* is susceptible to acidic pH and hence can't grow in acidic environment, indicating the probable role of overexpressed *icl* gene in acidic stress.

• Under oxidative stress,the CFU count of *Mficl* as well as the wild type *M. fortuitum* was found to be almost constant and same . Therefore, it may be concluded that *the* overexpression of *icl* gene doesn't have any effect in mycobacterium persistence.

•Under nutritive starvation stress, wild type *M. fortuitum* was able to survive and have constant cell count whereas the *Mficl* showed decrease in cell count.

•When exposed to heat stress, the cell count of wild type *M. fortuitum* showed a constant decrease and became zero after 35 hours whereas the *Mficl* sense construct did not show any significant decrease in cell count suggesting no effect of overexpression of *icl* gene in heat stress.

• Under hypoxic stress, cell count of wild type *M.fortuitum* was found to be constant , but there as a significant decrease in the cell count of the *Mficl* construct.

Since it has been reported in many studies that overexpression of a wild-type gene can also be disruptive to a cell or organism. Therefore, it may be concluded from the results above that overexpression of *icl* in acidic and hypoxic stress does not promotes the persistence of bacteria but creates adverse conditions inside macrophages which leads their death.

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