Mycobacterial lipid transport and metabolism

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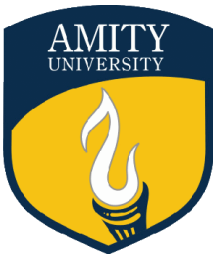
By
RISHAD AHMED
A91700222011

Under the supervision of
Dr. Prabuddha Gupta



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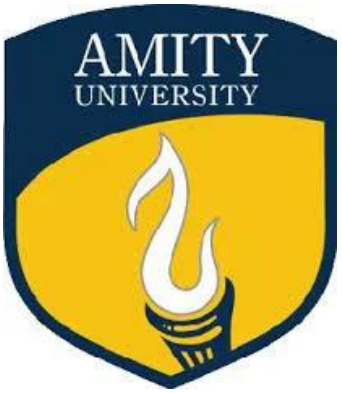

DISSERTATION
ON

Mycobacterial lipid transport and metabolism

SUBMITTED BY: RISHAD AHMED
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COMPLETED UNDER THE SUPERVISION OF
DR. PRABUDDHA GUPTA

DECLARATION

This is to certify that the dissertation entitled "Mycobacterial lipid transport and metabolism" submitted by me to Amity Institute of Biotechnology, Amity University, Kolkata for the award of the degree of Masters of Science in Biotechnology is a bonafide record of research work carried out by me under the guidance of Dr. Prabuddha Gupta. The contents of this dissertation, in full or in parts, have not been submitted to any other institute or university for the award of any degree or diploma.

_____

DATE: **SIGNATURE OF CANDIDATE**

Dr. Prabuddha Gupta
Assistant Professor
Amity Institute of
Biotechnology

AMITY UNIVERSITY
KOLKATA
Major Arterial Road, South-
East, Action area II, Newtown,
Kolkata, 700135

TO WHOM IT MAY CONCERN

This is to certify that this project report is the bonafide work of Mr, Rishad Ahmed (Enrollment No. A91700222011), a final semester student of MSc Biotechnology, Amity Institute of Biotechnology, Amity University, Kolkata, who carried out the project entitled "**Mycobacterial lipid transport and metabolism**" under my supervision from 26thJanuary 2024 to 4thMay 2024.

_____

Dr. Prabuddha Gupta
Assistant Professor,
Amity Institute of Biotechnology,
Amity University, Kolkata.

DATE:
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PLAIGARISM CERTIFICATE

This is to certify that the thesis entitles Mycobacterial lipid transport and metabolism submitted by Rishad Ahmed for the partial fulfilment of the degree of Master's in biotechnology has been checked by Dupli checker software for plagiarism. The thesis has zero percent plagiarism.

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RISHAD AHMED

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Abstract

The principal infectious agents responsible for tuberculosis (TB) and leprosy are Mycobacterium tuberculosis and Mycobacterium leprae, respectively. These diseases primarily afflict populations in tropical regions, particularly in less developed countries where access to healthcare and completion of extensive antibiotic treatment regimens can be challenging. Despite the effectiveness of antibiotics in curing most cases, a significant proportion of patients face obstacles in adhering to the prolonged treatment, contributing to the emergence of drug-resistant strains of M. tuberculosis. Leprosy presents additional complexities due to its intracellular nature and extended incubation period, making early diagnosis and complete eradication daunting tasks.

An in-depth understanding of the core metabolism of these bacterial pathogens is essential for developing targeted interventions. M. tuberculosis and M. leprae exhibit a remarkable ability to persist within specific host cells—pulmonary granulomas and Schwann cells, respectively—where they induce a distinctive 'foamy' phenotype characterized by lipid droplet accumulation. This adaptation allows the bacteria to utilize host-derived lipids, including cholesterol and fatty acids, for energy production and survival.

Within the Mycobacterium organisms, which includes pathogenic species like Mycobacterium leprae and Mycobacterium tuberculosis, adenylating enzymes from the ANL (acyl-CoA synthetase) family are pivotal in orchestrating lipid synthesis processes. Notably, members of the ANL enzyme subfamily, such as FAALs (Fatty Acyl-AMP Ligase) and FACLs (Fatty Acyl-CoA Ligase), are closely intertwined with mycobacterial lipid metabolism, a fundamental aspect contributing to the pathogens' ability to establish and sustain infections. Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis, while M. leprae is responsible for leprosy. Despite extensive research on tuberculosis, our understanding of the detailed mechanisms underlying Mycobacterium leprae's pathogenicity remains limited, hindering the development of specific and reliable therapeutic interventions.

The study explores the pivotal role of FadD proteins in the lipid metabolism of M. tuberculosis and M. leprae, crucial for bacterial persistence and pathogenicity. Through meticulous research, potential drug targets within this metabolic pathway have been identified, particularly focusing on enzymes involved in lipid uptake and utilization, with a specific emphasis on cholesterol transport—a key factor in M. leprae's intracellular survival strategy. This interdisciplinary investigation, integrating genomics, bioinformatics, and molecular biology approaches, provides valuable insights into the intricate mechanisms of lipid metabolism underlying leprosy pathogenesis.

It also represents a significant stride in unravelling the complex nexus between M. leprae and host lipids. By elucidating the genetic foundations of lipid transport and metabolism, researchers not only advance our understanding of leprosy pathogenesis but also lay a solid foundation for the development of precision therapies aimed at disrupting the pathogen's survival strategies and ultimately mitigating the burden of this ancient disease.

By leveraging the wealth of information available on M. tuberculosis and identifying homologous proteins and their functions in M. leprae, we can elucidate potential drug targets, such as the FadD proteins highlighted in this study.

Introduction

Mycobacterial pathogens are the causative agents of two highly contagious diseases, Tuberculosis (TB) and leprosy, with global prevalence. Mycobacterium tuberculosis and Mycobacterium leprae, responsible for TB and leprosy, are believed to share a common ancestral origin. Despite their common lineage, these bacteria infect different organs, resulting in distinct symptoms.

Tuberculosis primarily affects the lungs but can also impact other organs and spread to others through airborne droplets. Many infected individuals do not exhibit symptoms and cannot transmit the disease, known as latent TB, where bacteria reside within granulomas. However, addressing latent tuberculosis is crucial due to its complexity and the increasing mortality rates observed in recent years.

Advancements in molecular biology and genomic sequencing of Mycobacterium tuberculosis have enhanced our understanding of drug resistance mechanisms. This knowledge is vital for improving diagnostic methods and developing new drugs targeting drug-resistant strains. Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) pose significant public health challenges due to inadequate treatment regimens and suboptimal control programs.

Leprosy, caused by Mycobacterium leprae, is a prevalent infectious cause of non-traumatic peripheral neuropathies worldwide. The unique cell wall structure of M. leprae, rich in lipids, contributes to its pathogenesis and resistance to host immune responses and antimicrobial agents. Upon infection, M. leprae induces the accumulation of cholesterol-enriched lipid droplets in Schwann cells, crucial for bacterial survival.

Leprosy remains a significant health concern, especially in developing countries, where challenges in diagnosis and treatment persist due to limited scientific research. Both tuberculosis and leprosy continue to pose serious health threats globally, emphasizing the need for advanced strategies, including the development of novel medications and combating drug resistance, to effectively manage these diseases.

Understanding the intricate processes of mycobacterial lipid transport and metabolism is essential for developing effective treatments against diseases such as tuberculosis and leprosy. Adenylating enzymes from the ANL family, including FAALs (Fatty Acyl-AMP Ligase) and FACLs (Fatty Acyl-CoA Ligase), play crucial roles in synthesizing intricate lipids that contribute to the success of mycobacterial infections. Despite extensive research on tuberculosis, gaps persist in our understanding of the detailed mechanisms underlying Mycobacterium leprae's pathogenicity, emphasizing the urgent need for focused investigations into lipid-related processes.

The mycomembrane structure of mycobacteria, composed of unique lipids and glycolipids, forms a protective barrier against the host immune system and antibiotics. This lipid-rich outer layer, including phenolic glycolipids (PGLs) and mycolic acids, is fundamental to mycobacterial survival within the host environment. Furthermore, the intricate interplay between lipid metabolism, cholesterol uptake, and virulence factor secretion underscores the importance of elucidating these processes for developing targeted interventions against mycobacterial infections.

In this comprehensive project, we undertook an extensive investigation into various aspects of lipid metabolism and transport in M. leprae, with a primary focus on key enzymes such as FadD proteins (FAAL and FACL). Leveraging resources such as Mycobrowser and bioinformatic prediction tools like I-Tasser, we analysed genomic and protein sequences to classify, analyze, and predict the roles of these enzymes. Our investigation delved deep into the intricate pathways involved in cholesterol uptake, lipid metabolism, and transport systems, unraveling key insights into how M. leprae interacts with host lipids during infection. Additionally, we extensively reviewed literature to understand the roles of specific proteins and pathways implicated in M. leprae pathogenesis. By synthesizing information from diverse sources and comparing findings with Mycobacterium tuberculosis, we aimed to construct a comprehensive picture of lipid utilization and metabolic adaptation in this pathogen, advancing our understanding of leprosy pathogenesis and paving the way for innovative therapeutic interventions.

This report focuses on the investigation of FadD proteins, crucial for fatty acid degradation, which are essential for bacterial infections within host cells. These enzymes play a vital role in lipid production, contributing to bacterial virulence and serving as potential targets for the development of new pharmaceuticals. The study involves analysing the structure, confidence score (c-score), and Gene Ontology (GO) annotations of FAAD proteins using I-Tasser software. Additionally, the research examines cholesterol uptake and breakdown mechanisms, including the identification of enzymes or channels involved (such as mce proteins 1, 2, 3, and 4) in both Mycobacterium leprae and Mycobacterium tuberculosis. Furthermore, the study investigates the utilization of lipid droplets by mycobacteria and conducts RNA sequence analysis of M. leprae. Lastly, it explores the ESX-1 secretion system in M. tuberculosis and identifies homologous proteins present in M. leprae.

Methodology

Steps were performed one after another starting from identification of structure and function of FadD proteins (FAAL and FACL) in mycobacterium leprae then classifying them, analysing their structures, functions; Then looking in to cholesterol uptake, lipid metabolism study to their transport system study, analysis and findings.

Mycobrowser (https://mycobrowser.epfl.ch/) was mainly our main databank which we used nearly throughout all the time. From here many FAAL and FACL details, ESX-1 secretion system proteins and more were found in M. leprae. Information starting from the genomic sequence, protein sequence to found function or predicted function were given. Among them the FAAL and FACL were tried to classified and a FadD which could be most involved in the process was searched.

Many research papers, review articles were studied trying to find out which proteins and how they would help in mycobacterium leprae infection in host.

A website named I-Tasser (https://zhanggroup.org/I-TASSER/) was used to predict the structure and function of these proteins along with more information including graphical representations, C-score and GO annotations of each. I-Tasser works by first identifying structural templates from the PDB by multiple threading approach with full-length atomic models constructed by iterative template-based fragment assembly simulations. Function insights of the target are then derived by re-threading the 3D models through protein function database BioLiP.

Then came the cholesterol part and mainly research papers were studied mostly to gather information and also web tools such as BLAST(https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used.

From these we found data on the structure of the mycobacterium cellular membrane, discovery of mce1 and mce4 proteins, lipid droplet formation in the host along with RNA sequence study and how the absence of mce4 in M. Leprae may be replaced by a secretion system, namely ESX secretion system 1.

All information was gathered for mycobacterium tuberculosis first and then the same was done for M. Leprae; since MTB has got a lot of research done and many confirmed information about it is available all over, we basically used it as a control for the findings and possibilities of function types of the proteins in M. Leprae. That was done by finding the homologs of the proteins of MTB in M.leprae and then performing pairwise alignment in between them using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EMBOSS (https://www.ebi.ac.uk/jdispatcher/psa) respectively.

Results

Structure of cellular membrane

Mycobacterium leprae has a distinctive cell wall structure known as the mycomembrane, which is a waxy layer crucial for bacterial protection and immune evasion. Recent research indicates that damage to this mycomembrane exposes underlying structures, increasing the bacterium's affinity for cholesterol. The cell wall consists of inner and outer layers, with the outermost layer containing phenolic glycolipids (PGLs), particularly phthiocerol dimycocerosate (PDIM) and PGL-I that form capsules and a variety of lipids. The innermost layer beyond the plasma membrane is rigid and dense, composed of peptidoglycan (PGN), arabinogalactan (AG), and mycolic acids. Additionally, the outer cell wall includes lipid-linked polysaccharides like lipomannan (LM), lipoarabinomannan (LAM), and phthiocerol-containing lipids such as PDIM and dimycolyltrehalose. The pseudo-lipid bilayer's inner leaflet comprises linked mycolic acids and arabinan chain termini, while the outer leaflet is made up of PGLs, mycolic acids with trehalose mono-mycolate (TMM), and mycocerosoic acids from PDIM.

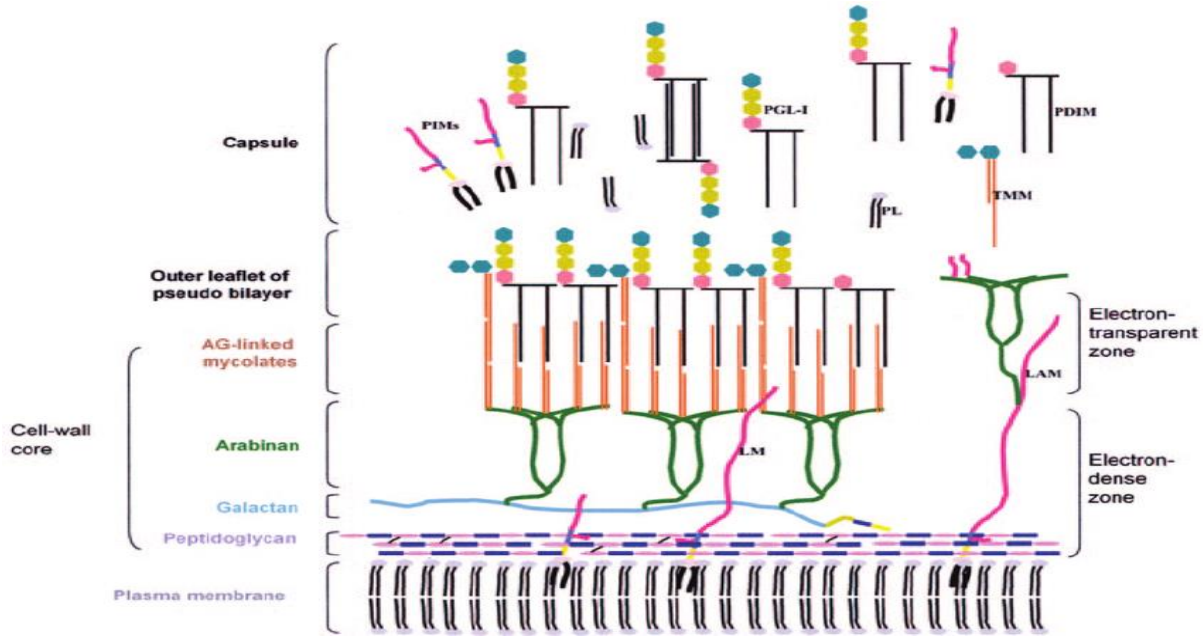

Fig 1: *Schematic model of the cell envelope of M. leprae. The plasma membrane is covered by a cell wall core made of peptidoglycan covalently linked to the galactan by a linker unit of arabinogalactan. Three branched chains of arabinan are in turn linked to the galactan. Mycolic acids are linked to the termini of the arabinan chains to form the inner leaflet of a pseudolipid bilayer. An outer leaflet is formed by the mycolic acids of trehalose monomycolates (TMM) and mycocerosoic acids of phthiocerol dimycocerosates (PDIMs) and PGLs as shown. A capsule presumably composed largely of PGLs and other molecules such as PDIMs, phosphatidylinositol mannosides, and phospholipids surrounds the bacterium. Lipoglycans such as phosphatidylinositol mannosides, lipomannan (LM), and lipoarabinomannan (LAM), known to be anchored in the plasma membrane, are also found in the capsular layer as shown.*

Image taken from- https://www.researchgate.net/figure/Schematic-model-of-the-cell-envelope-of-M-leprae-The-plasma-membrane-is-covered-by-a_fig3_7166491

FadD proteins

Mycobacterium produces a variety of lipophilic molecules, including mycolic acids that can be simple or highly complex. Lipid metabolism, particularly β-oxidation, is crucial for the bacterium's survival alongside virulence molecule production. This metabolic process involves the FadD gene family, which in M. tuberculosis comprises up to 36 genes. These genes encode enzymes responsible for both lipid catabolism and biosynthesis.

The fatty acid adenylating enzymes fall into distinct groups that are FACLs (fatty acyl-CoA ligases) are primarily involved in lipid and cholesterol breakdown and FAALs (long-chain fatty acyl-AMP ligases) produce lipids essential for virulence and other critical functions.

FAALs, unlike FACLs, are non-redundant and better understood. Gokhale et al. suggested renaming FadD enzymes based on their functions; for instance, FadD13 could be renamed FACL13, and FadD22 could be renamed FAAL22. However, the specific functions of each FACL and FAAL enzyme remain to be fully elucidated.

In experiments with M. tuberculosis grown on cholesterol, increased expression of FACL3, FACL17, FACL18, and FACL19 suggests their involvement in cholesterol degradation pathways. Cholesterol serves as an important carbon source for M. tuberculosis. FACL3 is involved in degrading the C and D chains of cholesterol, while FACL17 and FACL19 degrade the C17 side chain.

FAAL enzymes play unique roles in connecting fatty acids to polyketide synthases. For example, FAAL23 is involved in sulfolipid production. The production of PDIM, a virulence lipid, requires FAAL26, FAAL28, and FAAL29.

The fundamental differences in structure and function between FAAL (fatty acyl-AMP ligases) and FACL (fatty acyl-CoA ligases) enzymes are pivotal for understanding lipid metabolism in Mycobacterium and other organisms. Fatty acids, inert metabolically, require activation into coenzyme-A derivatives for integration into metabolic pathways. This activation occurs in two phases, involving the formation of an acyl-AMP intermediate catalysed by fatty acyl-CoA ligases (FACLs).

The detailed structural analysis of FadD13 (FACL13) by Lundgren et al. sheds light on the interaction of FACL13 with cellular membranes, making it a valuable model to comprehend the characteristics of FACL enzymes. FACL13 exhibits a specific affinity for long-chain fatty acids, with ligase activity extending up to C26 carbon chain lengths. Encoded within the mymA operon, FACL13 expression is induced under acidic conditions reminiscent of those found within host macrophages during Mycobacterium tuberculosis (MTB) infection.

Structurally, FACL13 comprises two distinct domains: a larger N-terminal domain and a smaller C-terminal domain connected by a flexible 6-amino acid linker (Andersson et al., 2012). The active site of FACL13 is situated between these domains, featuring a notable hydrophobic pocket that, despite its length, cannot fully accommodate long-chain fatty acids. Interestingly, a positive patch within the N-terminal domain facilitates membrane attachment, essential for catalytic activity.

FACL13 functions as a dimer in solution, with the membrane-attaching surface of the N-terminal domain partially covered during this state, which restricts catalytic activity. This dimeric configuration likely serves to prevent non-specific interactions within the cellular milieu.

In contrast, FAAL enzymes belong to the family of long-chain fatty acyl-AMP ligases, playing crucial roles in connecting fatty acids to polyketide synthases for the synthesis of essential lipids involved in virulence and other cellular functions. The functional and structural characteristics of FAALs are distinct from those of FACLs, emphasizing their unique roles in mycobacterial lipid metabolism and pathogenesis. Understanding these distinctions is vital for elucidating the mechanisms underlying lipid utilization and survival strategies of pathogens like MTB.

Lipid metabolism and lipid droplet accumulation

Mycobacteria rely on a sophisticated lipid metabolism pathway crucial for their survival, pathogenicity, and evasion of host defences. This pathway is essential for sustaining their energy needs and structural components, enabling their persistence within the host. It involves systems for fatty acid synthesis (FAS) and mycolic acid production, which are key for forming the protective outer layer of the bacterial cell. Additionally, phospholipid synthesis, which constructs cell membranes, ensures cell stability through enzyme-mediated fatty acid activation and subsequent molecule additions. Mycolic Acid Biosynthesis involves two metabolic phases followed by chemical modifications that reinforce the bacterial cell wall against external threats and enhance antibiotic resistance. Furthermore, Mycobacteria store triacylglycerols (TAGs) as energy reserves, composed of glycerol and three fatty acids, providing essential fuel during periods of dormancy or stress. TAG breakdown supplies fatty acids to power bacterial activities when energy demands increase.

Therefore, the import of host lipids into the bacterial cell membrane is vital for its survival within the host. Recent studies have highlighted the importance of lipid bodies for Mycobacterium tuberculosis during its dormant stage inside host cells, correlating with the observation of lipid-laden bacteria in the sputum of tuberculosis patients.

Lipid droplet accumulation within macrophages represents a hallmark feature of lepromatous leprosy, where it is hypothesized that bacteria derive essential nutrients from these lipid reservoirs. Pathogenic mycobacteria as Mycobacterium tuberculosis, exhibit a unique ability to sustain themselves within host cells by utilizing host lipids as a primary energy source, highlighting the importance of lipid transport across bacterial cell membranes for survival. Recent investigations have underscored the critical role of lipid bodies in facilitating the dormancy of M. tuberculosis within host cells, consistent with the observation of lipid-laden bacteria in the sputum of tuberculosis patients.

Lipid droplets (LDs) are versatile organelles within eukaryotic cells, originally recognized for their role in lipid storage and metabolism but increasingly appreciated for their multifaceted involvement in cellular physiology, including immune defense against microbial pathogens. While LDs were historically viewed as passive lipid reservoirs, recent research has unveiled

their dynamic engagement in host-pathogen interactions and innate immunity, expanding our understanding of their biological significance.

Pathogenic bacteria have evolved sophisticated strategies to exploit host LDs, utilizing them as a nutrient source for growth and survival within infected cells. However, the host immune system has adapted to recognize and respond to these intracellular invaders by mobilizing LD-associated defense mechanisms. Emerging studies, such as the investigation by Bosch et al. (2020), have illuminated the antimicrobial properties of LD proteins against bacterial pathogens like Escherichia coli, underscoring the active role of lipid droplets in innate immunity.

The exposure of immune cells, such as human monocyte-derived macrophages (HMDMs), to fatty acids like oleic acid (OA) triggers lipid droplet formation, enhancing the cells' ability to combat bacterial infections. This response highlights the dynamic nature of LDs in immune defense, where lipid metabolism intersects with host defense mechanisms to promote antibacterial activity.

Moreover, lipid droplets serve as reservoirs of endogenous lipids critical for pathogen survival, with specific components of the LD proteome responding to bacterial components like lipopolysaccharide (LPS) during infection (Bosch et al., 2020). The identification of immune-associated protein clusters on LDs, including established antimicrobial proteins like viperin and newly discovered factors such as IIGP1, TGTP1, IFI47, and CAMP, underscores the complex interplay between lipid metabolism and immune defense within infected cells.

Further investigations into the trafficking and membrane interactions of LDs across various pathogens have shed light on the mechanisms underlying LD-mediated immune responses. These findings suggest that LDs not only store essential lipids but also act as dynamic platforms for immune signaling and antimicrobial protein deployment, contributing significantly to the cellular defense against infectious agents.

Understanding the immunological potential of lipid droplets offers promising avenues for developing novel anti-infective therapeutics, particularly relevant in the context of antimicrobial resistance. Efforts to harness the innate immune functions of LDs could lead to innovative strategies for combating infectious diseases and addressing global health challenges associated with drug-resistant pathogens.

It can be said that lipid droplets represent a nexus of lipid metabolism and immune defense, playing pivotal roles in host-pathogen interactions and offering new opportunities for therapeutic interventions against microbial infections. Further exploration of LD-associated immune responses holds significant promise for advancing our understanding of infectious disease pathogenesis and developing effective strategies to combat emerging antimicrobial resistance, thereby advancing the field of infectious disease research and therapeutics.

FadD proteins, also known as acyl-CoA synthetases, are essential enzymes that catalyse the activation of fatty acids by converting them into acyl-CoA thioesters. These activated fatty acids serve as fundamental building blocks in the synthesis of complex lipids crucial for the physiology and pathogenicity of Mycobacterium tuberculosis. FadD proteins can be categorized into three distinct families—FadD13, FadD28, and FadD32—based on their structural and functional similarities.

FadD13, a key member of the FadD protein family in M. tuberculosis, plays a critical role in activating phenolic glycolipids (PGLs) and phthiocerol dimycocerosates (PDIMs). Both PGLs and PDIMs are implicated in tuberculosis pathogenesis, with PGLs contributing to immune evasion and modulation of host immune responses, while PDIMs strengthen the mycobacterial cell wall. FadD13 facilitates the integration of fatty acids into these lipids, essential for their synthesis and transport.

FadD28 is another significant protein involved in lipid metabolism within M. tuberculosis. It catalyses the activation of long-chain fatty acids, a crucial step in the biosynthesis of complex lipids such as mycolic acids. Mycolic acids are integral components of the mycobacterial cell envelope, contributing to the unique architecture and impermeability of M. tuberculosis. Activation of long-chain fatty acids by FadD28 is essential for mycolic acid production, vital for the survival and virulence of M. tuberculosis.

FadD32, part of the FadD gene family, is associated with the catabolism of host-derived lipids during M. tuberculosis infection. In the context of infection, M. tuberculosis can utilize host-produced lipids as a carbon source. FadD32 facilitates the activation of host fatty acids, enabling their incorporation into Mtb's metabolism and supporting the bacterium's survival within the host environment.

Mycobacterium leprae, with its significantly reduced genome size compared to M. tuberculosis, heavily relies on host-provided catalytic activities for survival. While M. tuberculosis utilizes fatty acids as a primary carbon source during dormancy, the specific carbon source for M. leprae remains unclear, despite evidence of upregulated genes involved in host lipid metabolism in leprosy.

Cholesterol transport, fatty acid oxidation and CHO island

Cholesterol is a key host lipid that accumulates during leprosy infection. Mattos et al. demonstrated that all enzymes involved in cholesterol biosynthesis increase in lepromatous leprosy cases. In investigating the origin of cholesterol in M. leprae, Mattos et al. found evidence that cholesterol is not only synthesized de novo but is also taken up from plasma lipoproteins. Experimental studies revealed that Mycobacterium leprae disrupts normal cholesterol homeostasis in host cells by promoting increased absorption of native LDL-cholesterol. Furthermore, M. leprae infection enhances the expression of LDL receptors both in vivo and in vitro.

Fatty acids represent one of the most abundant lipids found within human granulomas. Although it is known that Mycobacterium tuberculosis (Mtb) can metabolize fatty acids, genome sequencing has revealed a diverse array of fatty acid β-oxidation genes (FadD genes) within this bacterium. Initially, it was believed that approximately 250 genes were involved in fatty acid β-oxidation, but subsequent research has shown that many of these enzymes participate in lipid biosynthesis and may form part of multimeric protein complexes unrelated to fatty acid degradation.

During infection, Mtb not only scavenges fatty acids from the host but also synthesizes them using the fatty acid synthase (FAS 1) enzyme. Given the high energy cost associated with de

novo fatty acid synthesis, Mtb commonly employs scavenging or direct incorporation of fatty acids into required metabolic pathways.

Fatty acid oxidation plays a crucial role in the survival of M. tuberculosis within the lungs of mice. M. tuberculosis possesses approximately 100 genes dedicated solely to fatty acid degradation, known as fad genes, although their exact functions are yet to be fully elucidated, their putative role is thought to be in β-oxidation of fatty acids. In contrast, M. leprae has approximately one-third the number of fad genes compared to M. tuberculosis.

A functional glyoxylate cycle, which utilizes acetyl-CoA from the β-oxidation of fatty acids, is present in M. leprae, with all the requisite genes for its execution. The initial steps of β-oxidation of fatty acids involve the activation of fatty acids, whereby free fatty acids are converted into fatty acyl-CoA.

Mycobacterium leprae's genome contains the Cho Island operon, comprising genes associated with cholesterol metabolism. One critical enzyme within this operon, known as 3β-hydroxysteroid dehydrogenase (3β-HSD), is responsible for converting cholesterol into cholestenone. However, the precise role of this conversion and the enzyme's specific location are not fully understood. Research indicates that 3β-HSD could potentially be situated on the cytosolic side of the mycomembrane, potentially facilitating the import and conversion of cholesterol within the bacterium.

Infection with Mycobacterium leprae leads to increased expression of LDL (Low Density Lipoprotein) receptors, which promotes the uptake of LDL-cholesterol by infected cells. The Mce1 complex found in Mycobacterium species is responsible for fatty acid transport and consists of an ATP-binding cassette transporter associated with specific assemblies of substrate-binding proteins. In Mycobacterium leprae, the Mce4 complex has become a pseudogene, whereas in Mycobacterium tuberculosis, this complex is implicated in cholesterol uptake. However, it is possible that the LucA protein, known to stabilize the protein subunits of Mce1 and Mce4 transporters and facilitate fatty acid and cholesterol uptake in M. tuberculosis, may also be present in M. leprae. Unlike M. tuberculosis, M. leprae lacks the Mce4 protein involved in cholesterol uptake. However, M. leprae infection still enhances the expression of low-density lipoprotein (LDL) receptors and key enzymes in cholesterol biosynthesis, indicating alternative mechanisms for lipid acquisition.

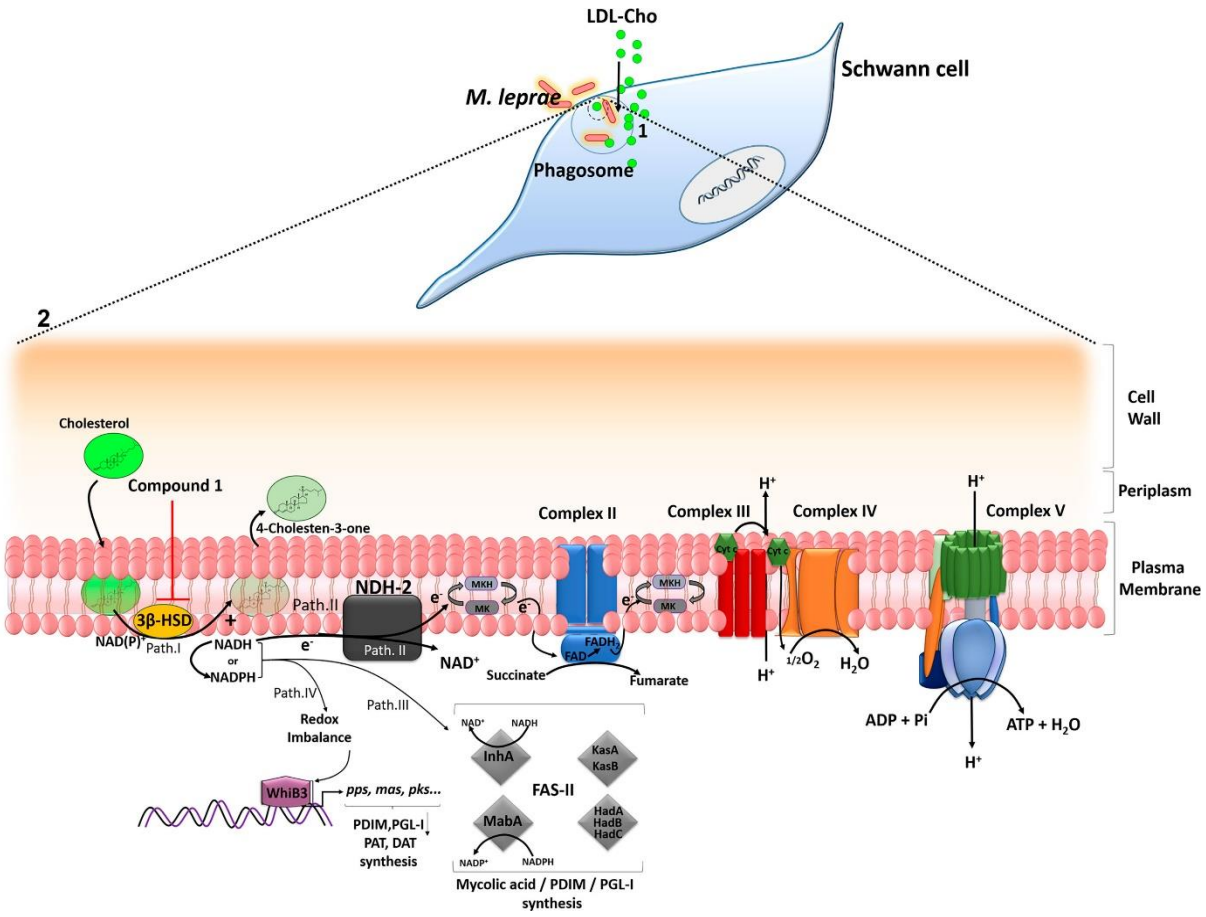

Fig 2: *A model proposing a key role of cholesterol oxidation for M. leprae survival inside SCs. (1) After internalization, M. leprae induces LDL-cholesterol (LDL-Cho) uptake that is recruited to bacterium-containing phagosomes. (2) Magnification of M. leprae cell envelope. Path I - 3β -HSD oxidizes cholesterol to cholestenone generating NADH, which can be converted to NADPH. Path II - NADH derived from 3β-HSD feeds bacterial electron respiratory chain via type-II NADH dehydrogenase (NDH-2), which reduces menaquinone (MK) to menaquinol (MKH), a substrate of the succinate dehydrogenase (complex II). The proton motive force generated during electron transport chain will be used by the ATP synthase (complex V) to generate ATP. Path III – NADH/NADPH can be used by fatty acid synthase II (FASII) for the synthesis of PDIM and PGL-I. The FASII complex trans-2-enoyl-AcpM reductase (InhA) and β-ketoacyl-AcpM reductase (MabA) subunits use NADH and NADPH, respectively. Path IV - WhiB3, a probable transcriptional regulatory protein, senses variations in the redox balance through NADH and NADPH levels and activates the promoter region of polyketide biosynthetic genes inducing the synthesis of mycobacterial lipids such as polyacyltrehaloses (PAT), diacyltrehaloses (DAT), PDIM and PGL-I. Inhibition of 3β -HSD by compound **1** reduces the levels of NADH/NADPH affecting the metabolic pathways described and impacts M. leprae intracellular viability. The arrows indicate some of the destinations of NAD(P)H already described in the literature and propose routes fed by the reducing power generated from 3β–HSD*

Image taken from- https://www.frontiersin.org/articles/10.3389/fcimb.2021.709972/full

Computational analyses revealed that Mycobacterium leprae retains the ability to oxidize cholesterol to cholest-4-en-3-one (cholestenone), a critical step in cholesterol degradation catalysed by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which is the only preserved segment of the cholesterol catabolic pathway observed in Mycobacterium tuberculosis. Experiments using radiolabelled cholesterol confirmed these computational predictions. In M. leprae, 3β-HSD exclusively produces cholestenone, and inhibiting this enzyme reduces intracellular bacterial viability, underscoring the significance of cholesterol metabolism during host cell interactions. This inhibition also impacts the biosynthesis of bacterial cell wall lipids such as PDIM and PGL-I. The reductive power generated by cholesterol oxidation can fuel the respiratory chain, potentially influencing microbial ATP synthesis. Consequently, inhibition of 3β-HSD affects the production of ATP and lipids in M. leprae, highlighting its role as a crucial element in bacterial-host interactions.

Cholesterol Uptake and Conversion Dynamics in Mycobacterium, fatty acid synthesis and insight on ESX-1 system

Studies have aimed to assess the level of cholesterol uptake and processing by M. leprae. Findings from radiolabelling experiments indicate that the bacterium absorbs significant quantities of cholesterol. However, the conversion rate to cholestenone appears relatively low, suggesting that only a portion of the acquired cholesterol is actively utilized, with the remainder possibly stored or eliminated. Understanding the specific cellular location where cholesterol is converted would offer valuable insights into these mechanisms. Notably, electron microscopy studies by Xing et al. (2013) have observed cholesterol accumulation within M. leprae phagosomes, suggesting a potential role of the phagosomal environment in cholesterol metabolism.

Numerous laboratories worldwide have emphasized the importance of cholesterol for the growth and persistence of Mycobacterium tuberculosis in various animal models. The process of cholesterol import, breakdown, and regulation involves approximately 80 genes that encode proteins responsible for these complex functions.

The import of such a large molecule as cholesterol across the unique diderm cell envelope undoubtedly requires a protein complex, identified through mutant studies as Mce4. The Mce1-4 operons encode Mce4 and share homology with ATP-binding cassette (ABC) transporter permeases. Within the Mce4 complex, ten putative proteins are encoded by the mce4 operon, including two integral membrane protein permeases, Rv3501/YrbE4, and Rv3502/YrbE4B, believed to translocate cholesterol.

It can be said that Mce1 protein contributes to the pathogenesis of tuberculosis as introduction of a transposon mutant into mice resulted in mutations in the mce1 operon, leading to observed growth defects in the pathogen during the early stages of infection. Subsequent studies demonstrated that a Mce1 mutant exhibits lower levels of cell wall lipids and membrane phospholipids, along with overexpression of the FAS-I enzyme. These findings suggest depletion of fatty acid pools in the Mce1 mutant, prompting the bacteria to compensate by increasing de novo fatty acid production within their cells.

The process of fatty acid import into M. tuberculosis remains an area of ongoing exploration. Nazarova and colleagues conducted unbiased experiments to investigate the import mechanism, identifying the Mce1 protein complex as a key player in this process. This complex comprises two putative permease subunits, four accessory subunits, and six Mce proteins. Removal of the Mce proteins led to a significant reduction in fatty acid uptake in Mycobacterium tuberculosis, highlighting the importance of the Mce1 complex. In cases where the mce1 gene is absent, fatty acid uptake is compromised.

The role of the Mce1 protein in fatty acid uptake is conserved in M. leprae, which, due to genome reduction, possesses only one Mce gene (Wiker et al., 1999). Studies have shown that M. leprae is capable of importing and metabolizing fatty acids ex vivo, suggesting that the Mce1-mediated fatty acid import mechanism is relevant in related mycobacterial diseases (Franzblau, 1988).

Recent research by Lundgren et al. (2021) demonstrated that FACL13 is bound to the lower membrane, suggesting a potential interaction between Mce1-mediated fatty acid import and subsequent processing by FACL proteins.

It was hypothesized that the role of Mce1 is to recycle mycolic acids, as Mce1 mutants show an accumulation of free mycolic acids in the cell envelope. However, it is unlikely that mycolic acids are imported through Mce1, as a Mce1 mutant did not exhibit growth deficiency when provided exogenous mycolic acids as the sole carbon source in axenic medium. After Mce1 proteins facilitate fatty acid import across the mycolic acid layer, FACL proteins interact with the membrane-bound fatty acids and convert them to their corresponding CoASH derivatives. These collective findings support the hypothesis that Mce1 functions as a fatty acid importer, and M. tuberculosis relies on host-derived fatty acids to establish and sustain infections.

Therefore, it can be concluded that FACL and FAAL enzymes may utilize fatty acids as substrates only when they are imported by Mce1. This highlights the critical role of Mce1 in mediating fatty acid uptake, which is essential for the pathogen's adaptation and virulence during infection.

Additionally, six other putative cell wall proteins within the Mce4 complex, namely Rv3499/Mce4A, Rv3498/Mce4B, Rv3497/Mce4C, Rv3496/Mce4D, Rv3495/Mce4E, Rv3494/Mce4F, facilitate the movement of cholesterol across the pseudo-periplasmic space or mycolic acid layer. The complex also includes two accessory proteins, Rv3493/Mam4A and Rv3492/Mam4B, which according to Nazarova et al., contribute to maintaining the stability and controlling the assembly of the Mce4 protein complex.

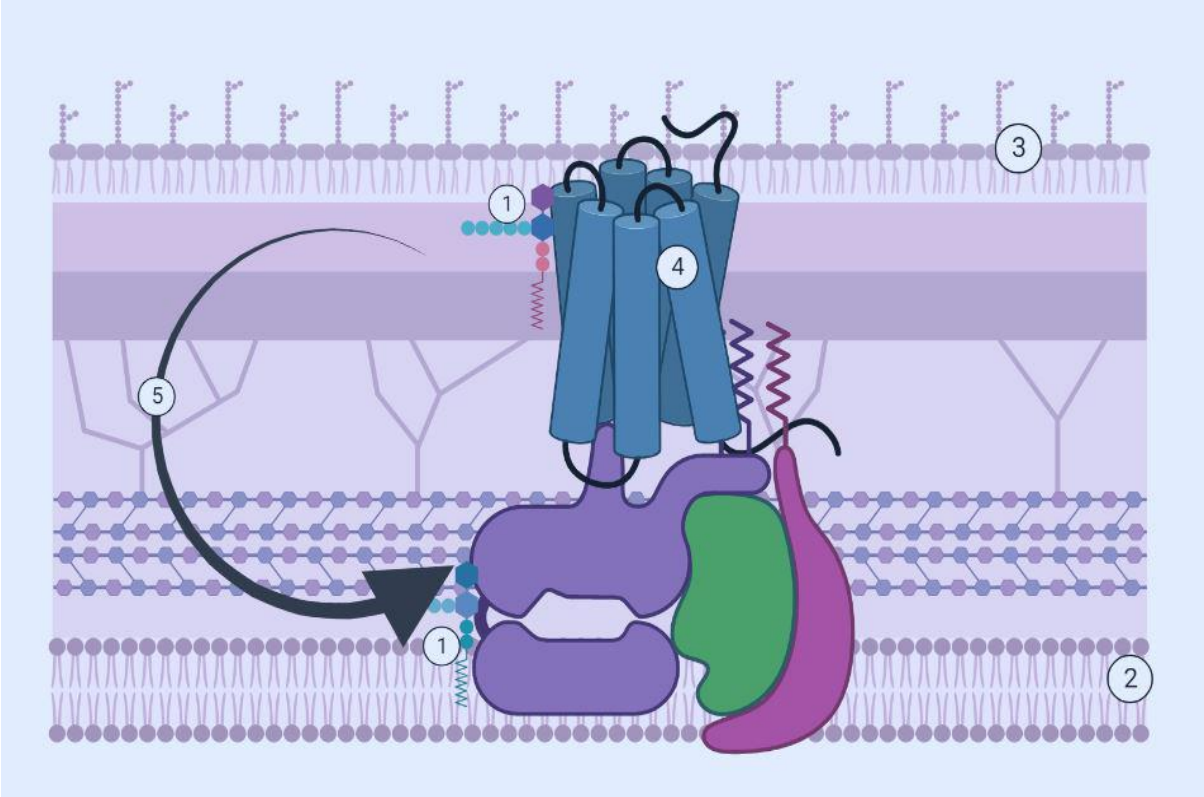

Fig 3: *Mce4 protein complex facilitating the import of cholesterol.*

1. *Cholesterol substrate*
2. *Cell membrane*
3. *Mycolic acid*
4. *Mce4 protein complex*

Cholesterol imported by the Mce4 protein from the external environment towards the inside

Cholesterol degradation in Mycobacterium tuberculosis (Mtb) primarily yields propionyl-CoA as a major metabolite. Propionyl-CoA, a three-carbon compound, is generated by Mtb through three different pathways:

a) β-oxidation of odd-chain fatty acids. b) Degradation of cholesterol. c) Degradation of branched-chain fatty acids.

Mammalian cells do not typically produce even-chain fatty acids, making them an unlikely source of propionyl-CoA during Mtb infection. Hence, propionyl-CoA must be generated by the bacterium itself. Although Mtb can readily import host-derived cholesterol and amino acids, the mechanism by which it obtains branched-chain fatty acids from the host, and thus contributes to propionyl-CoA production, remains unclear. Recent research suggests that cholesterol is the primary source of propionyl-CoA, as inhibition of Mce4-based cholesterol uptake significantly reduces propionyl-CoA levels.

Propionyl-CoA serves several purposes- It can be assimilated by the methyl citrate cycle (MCC) to form intermediary metabolites.
It can be assimilated through the methylmalonyl pathway (MMP) to form intermediary metabolites and can be incorporated into methyl-branched polyketide lipids.

Ultimately, propionyl-CoA generates pyruvate and succinate for central metabolism by condensing with oxaloacetate in the methylmalonyl pathway. In M. tuberculosis, the final reaction of the MCC is catalysed by the enzymes Icl1 and/or Icl2. These enzymes are bifunctional and are essential in the glyoxylate shunt to sustain bacterial growth on even-chain fatty acids. Additionally, propionyl-CoA contributes to central metabolism by converting to methylmalonyl-CoA through the MMP pathway.

The ESX-1 secretion system, controlled by the RD1 locus, serves as a critical virulence factor in mycobacteria by facilitating the secretion of specific proteins involved in host-cell interactions and immune evasion. While M. leprae lacks certain ESX-1 components found in M. tuberculosis, it does contain genes encoding proteins associated with this secretion system. These findings raise intriguing questions about the functional adaptations of ESX-1 in M. leprae and its role in leprosy pathogenesis. Further research is necessary to uncover the specific functions and contributions of the ESX-1 system in M. leprae's interactions with host cells and its overall pathogenic mechanisms. Understanding the nuances of ESX-1 in M. leprae could provide valuable insights into the unique strategies employed by this pathogen to establish and maintain infections, offering potential targets for therapeutic intervention and disease control strategies.

It can be summarised from the data that Mycobacteria like Mycobacterium leprae demonstrate complex lipid transport and metabolism systems crucial for survival and pathogenicity. The unique mycomembrane structure of M. leprae plays a key role in bacterial protection and immune evasion, particularly in cholesterol affinity and metabolism. Lipid metabolism pathways involving fatty acid and mycolic acid synthesis, as well as phospholipid production, are essential for cell wall formation and stability. Cholesterol metabolism, mediated by enzymes like 3β-hydroxysteroid dehydrogenase (3β-HSD), is vital for bacterial viability and cell wall lipid synthesis. Studies on cholesterol uptake dynamics and the role of proteins such as LucA highlight alternative lipid acquisition mechanisms. The investigation of the ESX-1 secretion system in M. leprae underscores its importance in pathogenesis. These findings deepen our understanding of mycobacterial biology and provide potential targets for combating mycobacterial infections.

ESX-1 secretion system

Recent investigations into mycobacteria have uncovered a specialized secretion system that enables the transport of extracellular proteins across their hydrophobic and impermeable cell wall. This unique pathway, proposed by Abdallah et al. and termed type seven secretion system (T7SS), is notable for its distinct composition and widespread presence among Gram-positive bacteria. In M. tuberculosis, five T7SS systems, known as ESX systems, have been identified, characterized by conserved gene content and organization. These systems include

essential genes called ESX conserved components (Ecc) and proteins known as ESX-1 secretion-associated proteins (Esp). Notably, among the secretion systems encoded in the M. tuberculosis H37Rv genome, only type II and type VII systems are associated with virulence.

ESX-1 and ESX-5 have emerged as key players influencing the cell-to-cell migration of pathogenic mycobacteria, contributing significantly to their virulence. ESX-1, a 9.5 kb segment absent in BCG, encodes critical T7SS genes, including ESAT6 (ESXA) and CFP10 (ESXB), which are pivotal virulence factors in the Mycobacterium tuberculosis complex (MTBC). These proteins are secreted via the specialized ESX-1 secretion system and are essential for the full virulence of MTBC species. Importantly, T7SS genes are absent in M. microtti and the BCG vaccine strain, with ESAT6/CFP10 genes localized within a segment known as Region of Difference 1 (RD1).

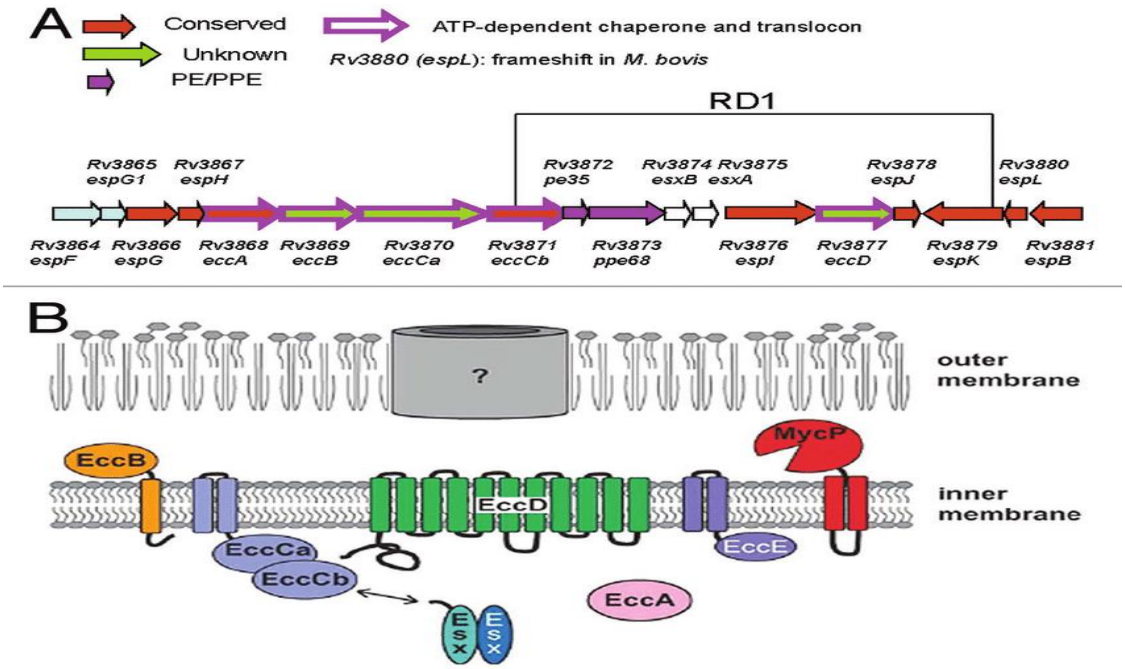

Fig 4**:** *Schematic representation of the ESX-1 secretion system. (A) Schematic organization of the M. tuberculosis genomic region containing the RD1 genes. (B) Model. The abbreviation ecc stands for esx conserved component, whereas esp stands for ESX-1 secretionassociated proteins. The topology of the different proteins in the cytoplasmic membrane is shown and refers to the ESX-1 cluster based on predictions made using the MEMSAT3 algorithm. Note that the channel drawn in the outer membrane of this model refers to a hypothetical pore, whose existence has not been experimentally demonstrated.*

Image taken from- https://www.researchgate.net/figure/Schematic-representation-of-the-ESX-1-secretion-system-A-Schematic-organization-of-the_fig3_232280670

Genetic manipulations involving RD1 segment deletions or insertions in M. tuberculosis and BCG have provided insights into RD1's role in virulence. RD1-deleted M. tuberculosis mutants exhibit attenuated virulence compared to the wild type but are more virulent than BCG, which harbors additional genomic deletions. The conservation of ESX-1 extends to other mycobacteria, such as M. smegmatis, involved in genetic material transfer, and M. leprae, but is absent in M. avium complex species. Complementation studies with full RD1 DNA fragments have confirmed its role in ESAT6/CFP10 secretion and virulence restoration.

M. tuberculosis utilizes the ESX-1 secretion system to deliver virulence proteins during host cell infection. Detailed analyses of ESX-1 proteins have revealed ATPase activity in EccA, transmembrane domains in EccB, EccCa, and EccDd, and C-terminal signal sequence recognition by EccCb for CFP10 secretion. Disruption of this sequence impairs ESAT6/CFP10 secretion. Similar T7SS characteristics have been observed in Mycobacterium marinum and M. smegmatis, which have been localized using confocal and electron microscopy, highlighting the conservation and importance of T7SS across various mycobacterial species.

These discoveries emphasize the significance of the T7SS in mycobacterial pathogenesis and highlight its potential as a target for developing novel therapeutic strategies against tuberculosis and related diseases caused by mycobacteria. Understanding the intricate mechanisms of T7SS-mediated virulence could pave the way for innovative approaches to combat these challenging infections.

Further investigations showed the pivotal role of ESAT6 and CFP10 in mediating the translocation of M. tuberculosis from the phagosome into the host cell cytoplasm during later stages of infection. Notably, studies using human dendritic cells infected with M. tuberculosis revealed that a substantial proportion of these cells contained translocated bacteria between days four to seven post-infection. This phenomenon was conspicuously absent when BCG or M. tuberculosis ESX-1 transposon mutants were used for infection, suggesting a critical role of the ESX-1 system in facilitating bacterial escape from the phagosomal compartment of phagocytic cells. Moreover, the release of ESAT6 proteins into the cytoplasm may enable interaction with the host's class I-processing machinery, which likely contributes to the recruitment and activation of CD8+ T cells observed in the lungs of mice following aerosol infection with M. tuberculosis. These findings strongly support the notion that ESX-1 secreted proteins can effectively access the eukaryotic cytoplasm.

Additionally, recent research by Pathak et al. has demonstrated that ESAT6 disrupts antigen-presenting cell function by interfering with TLR signaling pathways. This disruption leads to reduced IL-12 production by THP1 macrophages and inhibits macrophage apoptosis signals, highlighting the multifaceted impact of ESAT6 on host immune responses. Furthermore, the transcription factor EspR, encoded by Rv3849, plays a critical role in the regulation of the espACD operon. EspR binds to the espACD operon promoter and is secreted by the ESX-1 system in M. tuberculosis, exerting positive regulatory control over espACD transcription. However, upon secretion via ESX-1, EspR leads to downregulation of espACD expression, illustrating the intricate regulatory mechanisms governing ESX-1 activity.

Transcriptomic analyses have revealed that PhoP regulates the expression of the espACD operon, with significantly reduced expression observed in phoP-inactivated strains. This highlights the influence of PhoP-PhoR regulation on ESX-1 activity and underscores the complex interplay of regulatory pathways involved in M. tuberculosis pathogenesis. Moreover, the serine protease MycP1 plays a crucial post-transcriptional role in modulating ESX-1 secretion activity. Deletion of mycP1 enhances the secretion of ESX-1 substrates and attenuates the virulence of M. tuberculosis in chronic infection models, emphasizing MycP1's role in fine-tuning the secretion of ESAT6 and CFP10 to maintain the delicate balance between virulence and immunogenicity necessary for persistent M. tuberculosis infection.

Hence it can be said that the ESX-1 secretion system possesses unique regulatory and functional characteristics, representing one of the most extensively studied virulence factors

in the M. tuberculosis complex. The indispensable role of ESX-1 in M. tuberculosis pathogenesis is underscored by the profound loss of virulence observed upon deletion of RD1 or esxAB, highlighting the critical importance of this secretion system in the intricate interplay between the bacterium and its host during infection.

Measures

The investigation and development of specific inhibitors to elucidate the role of FadDs in lipid metabolism represent a significant endeavor with far-reaching implications. By identifying and characterizing these inhibitors, researchers can deepen their understanding of the intricate pathways and functions involving FadD enzymes, particularly in Mycobacterium tuberculosis (Mtb), a pathogen responsible for tuberculosis.

The discovery of inhibitors capable of targeting different classes of FadDs (such as FACL or FAAL) or selectively inhibiting specific FadD enzymes is essential for advancing this field. These inhibitors serve as valuable tools for dissecting the roles of individual enzymes within the lipid metabolic network. Moreover, they may open doors to the development of innovative therapeutic strategies aimed at disrupting critical nodes in lipid metabolism, thereby potentially impeding the growth and survival of Mtb.

Despite the recent identification of FadDs as adenylate-forming enzymes in Mtb, the quest for effective inhibitors has been relatively nascent. Notable findings include the inhibition of FAAL28 and FACL19 by 5′-O-[N-(dodecanoyl) sulfamoyl] adenosine and the inhibitory effects on FAAL32 by an analogue of dodecylphosphate-AMP.

Furthermore, the development of class-specific inhibitors like 5′-O-[N-lithocholoyl) sulfamoyl] adenosine (LCA-AMS), targeting acyl-CoA synthetases involved in steroid metabolism, exemplifies the potential of this approach (Niu et al., year). LCA-AMS's selective activity against M. smegmatis FadD17 and FadD1 underscores its promise as a tool for studying specific enzyme functions.

Another noteworthy advancement is the identification of a coumarin analog as a potent inhibitor of FadD32, which was discovered through phenotypic screening and whole-genome sequencing. This discovery highlights the value of employing diverse screening strategies to uncover novel inhibitors with therapeutic potential.

The pursuit of FadD inhibitors is not only crucial for fundamental research into lipid metabolism but also holds considerable promise for the development of targeted therapies against tuberculosis and potentially other infectious diseases. Future efforts in this area will likely benefit from interdisciplinary collaborations and innovative drug discovery approaches.

Conclusion

In conclusion, our comprehensive investigation has significantly advanced our understanding of the intricate interplay between Mycobacterium leprae and host lipids, emphasizing the critical roles played by FadD proteins, cholesterol uptake systems, and the ESX-1 secretion system in leprosy pathogenesis. Leveraging a multidisciplinary approach encompassing experimental data, computational analyses, and extensive literature review, we have unveiled novel insights into the metabolic strategies employed by M. leprae to thrive within the host environment and evade immune surveillance.

There is an urgent need to identify novel pharmacological targets for tuberculosis (TB) and leprosy, given the persistent prevalence of these diseases globally. This study underscores the significance of exploring the structural and functional attributes of a group of proteins central to infection pathogenesis, aiming to identify new therapeutic targets. The article emphasizes inhibitors of these proteins, which can impede their normal functions or render them inactive.

The rise of drug-resistant strains of Mycobacterium tuberculosis highlights the critical need for new therapeutic targets, given the limitations of current chemotherapy lasting nine months and prone to drug resistance. Identifying a group of proteins with undefined structure and function presents a promising avenue for developing novel pharmacological interventions to combat drug resistance and improve TB treatment outcomes.

Mycobacterium leprae, the causative agent of leprosy, is a major public health concern alongside tuberculosis. Despite multidrug therapy, the challenges of drug resistance and potential adverse effects necessitate the identification of new therapeutic targets for leprosy. The discovery of novel therapeutic targets represents a significant opportunity to enhance leprosy management and improve the well-being of affected individuals.

This paper primarily focuses on characterizing Mce proteins (also known as channel proteins), which likely facilitate cholesterol uptake and lipid metabolism. The study explores the role of these channel proteins and ESX systems in cholesterol uptake and lipid metabolism, particularly in relation to fatty acid and cholesterol metabolic pathways facilitated by FadD proteins. Additionally, the study examines lipid absorption mechanisms, emphasizing the importance of the Mce1 and Mce4 protein complexes in fatty acid and cholesterol uptake in the context of tuberculosis. Interestingly, individuals affected by leprosy exhibit alternative cholesterol accumulation pathways, despite the absence of Mce4 in their physiological systems.

The identification of selective inhibitors targeting FadD proteins represents a significant advancement in developing innovative therapeutics for tuberculosis and leprosy, while also shedding light on the yet uncharacterized functions of these proteins in lipid metabolism and pathogen infection processes.

Looking ahead, further research efforts should prioritize unraveling the specific functions and regulatory mechanisms governing these lipid-related pathways in M. leprae. By elucidating the complexities of lipid metabolism and transport, we seek to pinpoint promising targets for therapeutic intervention and devise innovative strategies to combat leprosy effectively.

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