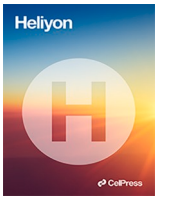
Heliyon 10 (2024) e32003

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Review article

Mycobacterial biofilms: A therapeutic target against bacterial persistence and generation of antibiotic resistance

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ARTICLE INFO

Keywords:
Mycobacterium tuberculosis
Biofilm
Persistence
Drug resistance
Dormancy
Anti-tuberculosis therapy

ABSTRACT

Mycobacterium tuberculosis (*M. tb*) is the causative agent of Tuberculosis, one of the deadliest infectious diseases. According to the WHO Report 2023, in 2022, approximately 10.6 million people got infected with TB, and 1.6 million died. It has multiple antibiotics for treatment, but the major drawback of anti-tuberculosis therapy (ATT) is, its prolonged treatment duration. The major contributors to the lengthy treatment period are mycobacterial persistence and drug tolerance. Persistent *M. tb* is phenotypically drug tolerant and metabolically slow down which makes it difficult to be eliminated during ATT. These persisting bacteria are a huge reservoir of impending disease, waiting to get reactivated upon the onset of an immune compromising state. Directly Observed Treatment Short-course, although effective against replicating bacteria; fails to eliminate the drug-tolerant persisters making TB still the second-highest killer globally. There are different mechanisms for the development of drug-tolerant mycobacterial populations being investigated. Recently, the role of biofilms in the survival and host-evasion mechanism of persisters has come to light. Therefore, it is crucial to understand the mechanism of adaptation, survival and attainment of drug tolerance by persisting *M. tb*-populations, in order to design better immune responses and therapeutics for the effective elimination of these bacteria by reducing the duration of treatment and also circumvent the generation of drug-resistance to achieve the goal of global eradication of TB. This review summarizes the drug-tolerance mechanism and biofilms' role in providing a niche to dormant-*M.tb*. We also discuss methods of targeting biofilms to achieve sterile eradication of the mycobacteria and prevent its reactivation by achieving adequate immune responses.

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https://doi.org/10.1016/j.heliyon.2024.e32003
Received 22 January 2024; Received in revised form 24 May 2024; Accepted 27 May 2024
Available online 29 May 2024

1. Introduction

In recent times, SARS-COV2 has become widely-spread infection of grave concern. However, the absolute percentage of mortality (3.2 %) caused by SARS-COV2 [1] is not more than Tuberculosis (6.6 %) [2]. Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M.tb*), a microbial pathogen, that was first identified by Robert Koch in 1982 [3]. It has co-evolved with humans since prehistoric times [4] and, being a contagious disease, is still considered a global threat to humankind due to exacerbated pathogenicity with a morbidity rate second highest only to AIDS [5]. The only available TB treatment, Directly Observed Treatment Short course (DOTS therapy), is a multidrug therapy which is unfortunately too lengthy (6 months) for both drug-sensitive as well as resistant TB cases (up to 12 months), leading to harmful consequences like hepatotoxicity, hyperuricemia, ototoxicity, and neuropsychiatric manifestations [6]. Because of extensive duration of the treatment, there is an upsurge in non-compliance to therapy, which is one of the foremost reasons, for the emergence of drug-resistant *M. tb* populations. Even after treatment, bacteria may persist in immune-compromised areas of the body [7] (Fig. 1). New forms of **M**ulti **D**rug-**R**esistant (MDR), **E**xtensively **D**rug-**R**esistant (XDR) TB [8] and **T**otal **D**rug-**R**esistant (TDR) TB [9] cases are surfacing every year which possess a serious concern and is an area of emerging research. Treatment of drug-resistant forms of TB is even more challenging as new second-line drugs have a wide variety of side effects, low efficacy and are quite expensive [10]. Therefore, there is substantial need for new drug targets that result in shorter treatment durations with fewer side effects.

Resistance, tolerance and persistence are often used interchangeably in TB pathogenicity. However, although related, these phenomena, are not the same. Tolerance or resistance develops in response to host-factors and anti-microbial agents which hinder the growth of *M. tb* after infection. However, persistent *M. tb* is a heterogeneous bacterial population with different degrees of drug sensitivity. Drug resistant bacteria may form persisters [11].

Resistance to anti-mycobacterial drugs can be attributed to three fundamental characteristics. These include the unique feature of the mycobacterial cell wall, intracellular surviving ability in phagocytic cells and rapid mutation rate in target receptors of bacteria [12]. Moreover, a new concept of the mycobacterial biofilm has emerged as an additional factor for anti-mycobacterial drug resistance, in addition to contributing to mycobacterial persistence in the host [13]. The bacterial cell wall is embedded in a layer of extra polymeric substances (EPS) during biofilm formation [14]. In this EPS, the pathogen protects itself from external stress conditions such as a hostile environment, an external harmful chemical gradient, UV radiation, temperature fluxes, pH change, and limitations in growth supplements. *Mycobacterium* has mycolic acid, making it more hydrophobic than non-mycolic acid-containing bacteria, providing greater hydrophobic properties to the membrane thus aiding in biofilm formation [15]. The *in-vivo* environment, which is highly acidic, anoxic and lacking in nutrient availability, forces the bacteria to restrict their growth, alter their physiology, and enter into a static dormant stage. Biofilms help the dormant and drug tolerant bacteria to survive within the host by escaping host immunity.

The rationale behind the long-term treatment for a patient suffering from Tuberculosis is the presence of non-replicating persister bacteria, and was first established in the year 1958 [16]. The non-replicating bacteria persist in various niches where these bacilli remain non-susceptible during antibiotic therapy and can rejuvenate themselves later [17]. Therefore, understanding the mechanisms behind these phenomena is of major importance in predicting drug efficacy and outcome as well as designing treatment approaches in order to target tolerant bacteria, which are recalcitrant and survive standard anti-TB drug regimens. Therefore, to attain more effective, short-duration TB treatment, we require eradication of both actively replicating bacilli and persister TB ensuring complete omission of dormant and persister populations. Hence understanding how bacterial biofilms support dormant TB would help to develop new strategies to target these non-replicating bacteria. Here in this review, we discuss the phenomenon of persistence, dormancy and drug-resistance highlighting the role of biofilms inducing latency in *M.tb*.

2. Dormant TB and the phenomenon of persistence

In 1944, the first report about phenotypic tolerance of bacteria to drugs was stated by Joseph Bigger, who while treating staphylococcal infection with penicillin, noticed a small population of bacteria surviving the antibiotic dose, regrew in fresh media when re-cultured [18]. He named those cells as “persisters” whose sustainability is relatively high compared to normal replicating cells [19]. In

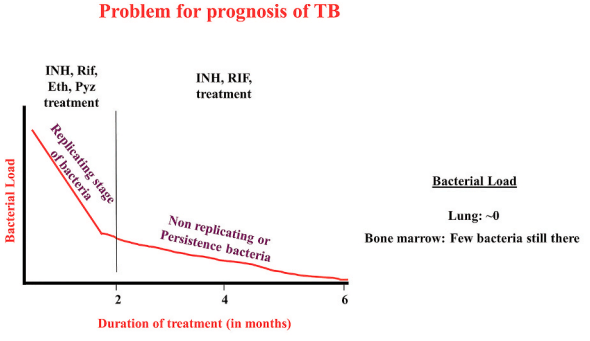

Fig. 1. Problem for prognosis of TB.

1950, Georges Canetti, a Bulgarian-French physician explained the pathology of human TB lesions, carried a varying number of bacilli with some acellular groups which were found in the matrix. This theory gave a new direction towards the persistence phenomenon of *M.tb* [20].

The lung of TB patients has remarkable lesions known as Granuloma. Granuloma is the clinical hallmark of TB. Granuloma creates a microenvironment in which the infection can be controlled; however, it also contributes to the long-time survival of the bacteria by keeping the host immune response at bay and hence becomes a favourable niche in which bacteria can persist for an extended duration. The granuloma harbors active as well as drug-tolerant tubercle bacilli. ATT readily eliminates the actively replicating bacilli. However, some of the bacteria become drug-tolerant by moving themselves into the hypoxic centres of granuloma thereby stimulating the dormant genes of *M.tb* and becoming slow replicating and drug-tolerant [21]. The hypoxic necrotic granulomas are linked to disease severity and provide a niche for drug-resistant *M.tb.* The necrotic lesions of granuloma are known as Caseous granuloma and serve as hypoxic niches [22] These peculiar lesions are a safe place for shielding of non-replicating bacteria. *M.tb* resides in these lesions, avoids itself from immune system detection and antimycobacterial drugs and goes into a state of dormancy. Dormancy is a changeable metabolic closure [19], often termed as "latent TB" or "persistent TB" for non-replicating cells that enable bacteria to circumvent and survive the host defences [23]. Joseph Warwick was the first to introduce the concept of dormancy in *Staphylococcus pyogenes,* but later to be valid for many more pathogens [18]. Latency, a clinical term, is a phase of disease where it does not show any indication of disease. Entry into dormancy is an active process in which specific signals activate master regulatory genes to drive *M. tb* cells toward the non-replicating state (Fig. 2).

3. Mechanism of development of dormant TB and persister population

M. tb are obligate aerobes that shield themselves in mature granulomas which are avascular, with low oxygen levels, and are responsible for inducing the dormancy phenotype in the bacilli. This state of hypoxia, along with high Nitric Oxide (NO) and Carbon monoxide (CO) levels, also induces the expression of the transcription factor **D**ormancy **S**urvival **R**egulan (DosR, *Rv3133c*), thus, facilitating the long-term survival of the bacilli in the host. DosR is a two-component response regulator and is associated with two sensor kinases (DosS and DosT). Remarkably, the DosR is inhibited in presence of oxygen but it is induced in stationary phase of growth or in settled cultures, when the *M. tb* is not actively dividing [24]. DosR or DevR was identified by **D**ifferentially **e**xpressed **v**irulent genes (DevR) induced during hypoxia by *M.tb*. DosS and DosT, the two sensor Kinases of *M. tb* phosphorylate the DosR which ultimately activates the transcription of approximately 50 genes downstream, collectively forms DosR regulon [25]. These DosR-dependent genes have 20 mer degenerate palindromic sites which acts as binding sites for DosR; studies showed that DosR, a tetramer, interacts with the DNA using three amino acid residues per subunit- Lysine 179, Lys182 and Asn183 [24,26]. DosR binding initiates metabolic shifts that allow the bacteria to enter into dormancy [27,28]. Disrupting the DosR in *M.tb* is linked to an increase in virulence in mice but attenuation in *Cavia porcellus* (Guinea pig) [29]. The contrasting virulency in different hosts is probably due to the peculiar lesions formed by the *M. tb* in these hosts [30]. Both kinases are associated with divalent gases such as NO, CO, and O_2, which regulate their activity [31]. These Kinases bind to heme as a prosthetic factor. DosS has a ferrous iron binding groove which shifts to ferric ion-binding site upon reduction and acts as indicator for net redox state. At the same time, DosT binds to oxygen with its heme component and senses the oxygen tension in vicinity [32].

DosR (DevR, *Rv3133c*) is a response regulator of DosR regulon. During aerobic environment PhoP (*Rv0757*) is responsible for basal level expression of DosR and thus, describing DosR as a simple latency trigger is a false idea [33]. When hypoxia is set, the expression of

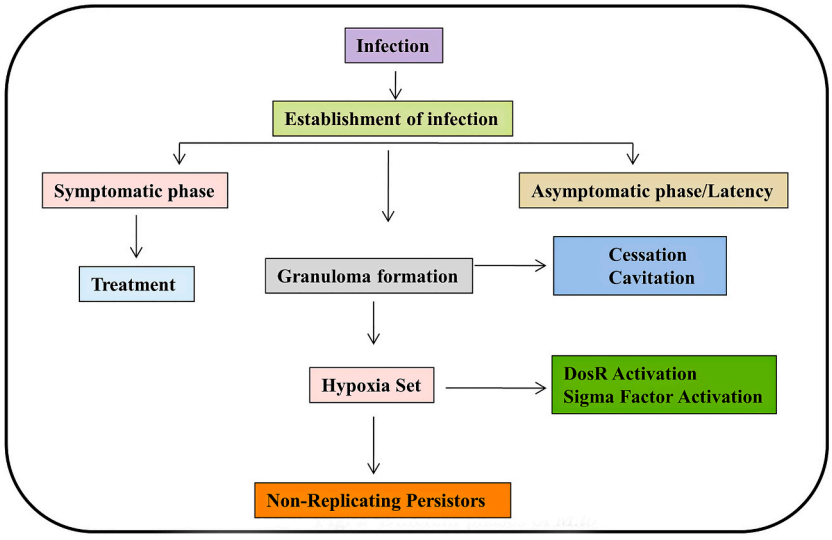

Fig. 2. Different phases of *M.tb* infection.

DosR increases five times, and even a small change in expression can lead to some cellular changes enabling the first set of genes to get transcribed which are related to protein stability and homeostatic regulation, such as *hspx* (*Rv2031*) and *Rv1738*, respectively [34]. Due to low oxygen levels in the late granulomas, which are avascular, inflammatory, and necrotic *M. tb* being aerobic might cause hypoxia during the period of infection [35]. Research has demonstrated that bacteria residing in oxygen-rich environments are vulnerable to chemotherapy, resulting in reduced growth. This phenomenon has also been observed in live animal models [36]. The caseous necrotic lesions are surrounded by a thick multicellular wall, which insulates it from outer surroundings and thus, bacteria become less responsive to the chemotherapies. Oxygen tension could lead to a stage of non-replication, which is an adaptation process causing latent infection [37]. Before hypoxia initiates, the transcription factor PhoP (*Rv0757*) takes responsibility for basal expression of *Rv3133c*. The initiation of the DosR regulon must correlate with complete hypoxia, allowing adaptation mechanisms which are to be transcribed and translated right before depletion of energy sources [38]. After activation of DosR regulan, a second set of 230 genes are activated by persistent hypoxia. These cluster of genes called *enduring hypoxic response* (EHR) take over the entire phenomenon of dormancy. These genes are independent of DosR regulon [26]. SigE and SigH genes play a major role in EHR. They are known as master regulators as they control stress induced transcriptional responses. The Sigma factor interacts with RNA polymerase at the promoter region and may play an important role against heat shock.

4. Paradigms of *M. tb* persistence

Early records of *M. tb* unique adaptability emerged out in two remarkable early studies. First, Corper and Cohn observed that *in vitro* cultures of human and bovine *M.tb* isolates were culturable even after 12 years of incubation in sealed containers [39]. This *in vitro* study demonstrated the characteristic persistent feature of *M. tb* in bacteriostatic conditions. In another *in vitro* study, Opie and Aronson disclosed the presence of virulent *M.tb* bacilli in about 26 % of lung lesions collected from individuals dying of causes which were not related to TB [40]. Though this study revealed asymptomatic infection of *M. tb*, it also raises the question that how bacilli are able to evade the immune system. Through subsequent follow-up studies, it was confirmed that the bacilli unexpectedly reside in some tissues instead of primary lesions [41]. These *in vivo* studies hinted at the clearance of bacilli by a competent immune system at the primary lesions, which failed to clear the bacilli residing at secondary sites, probably in a non-replicating state.

The persistent nature of *M. tb* once again attracted focus during the prephase of era of antibiotics in the mid-20th Century. Even though individuals were TB negative, the bacilli were observed in some lesions even after antibiotic treatment [42–44]. McDermott and colleagues later studied the relapse of TB in mice after chemotherapy which sufficiently reduced the life span of the bacilli to an undetectable levels [45]. No report explained the site of persistence till then. Still, it was expected that some hypoxic lesions could have supported the non-replicating persisters developed in the bacteriostatic environment of the lesions [42,46].Though the presence of non-replicating persisters was confirmed but, the exact mechanism of persistence in latent infection was still unknown. This notion was later demonstrated by two related studies. Sherman and his colleagues 2009 took an unstable plasmid as a reporter where they found that *M. tb* bacilli actively replicates during the chronic phase of infection in an *in vivo* mouse model. This was a phase where neither the host developed any symptoms, nor there was any change in the number of live bacteria [47]. Fortune and colleagues recently showed that in latent and active infections of non-human primates, mutations in *M. tb* populations pile up at the same rate, which is comparable to logarithmically growing *in vitro* culture, showing functional DNA replication and cell division [48]. In short, persistence of *M. tb* in a long-standing infection is likely to ease by a series of mechanisms including the adaptive changes in the bacilli in response to nutrient starvation microenvironments during cell division and active growth. These changes could be seen in surface structure or phenotypic physiology leading to low antibiotic permeability and high antibiotic resistance within the bacteria.

5. Enzymes involved in *M. tb* persistence

McKinney and colleagues focused on seminal studies on the first emergence of the specific role of metabolic enzymes in persistence [49]. The study identified an isocitrate lyase 1, one of two isocitrate lyases (ICLs) in *M. tb*, as an unessential gene for *in vitro* growth, which plays a vital role in the survival of *M. tb* in resting macrophages rather than initiation of infection in mice. Based on the authorized role of ICL in the cells, these were used as a marker for existence of *M. tb* persistence, required for catabolism of even-chain fatty acid substrates into 2-carbon acetyl coenzyme A (CoA) units of TCA cycle and for gluconeogenic intermediates.

Another study of functional ICLs showed a similar essential role for pckA, which encodes the gluconeogenic enzyme phospho-enolpyruvate carboxykinase (PEPCK) [50]. PEPCK has no role for *in vitro* growth; rather, it is essential for growth in the course of acute infection [48]. But clearance of the bacilli from mouse lungs and spleens in chronic phase infection can be carried out through transcriptional silencing of pckA. These findings were quite supportive in playing a role of persistence, can be separated from normal replication.

6. Membrane function in *M. tb* persistence

From a biochemical perspective, maintenance of membrane function plays a vital role in *M. tb* persistence more than metabolic enzymes. This evidence stems, in large part, from studies of hypoxic *M.tb*. Though classified as an obligate aerobe, evidence shows that *M.tb* hides particularly in intra- and extracellular niches, either present where oxygen concentration is low or are functionally hypoxic in NO inducing macrophages [51]. Studies have shown that upon abrupt changes in oxygen concentration, the bacilli are unable to survive, but can persist in a slow or non-replicating, antibiotic-resistant state for years with oxygen tensions [36]. After conducting ^{13}C tracing experiments it was seen that hypoxia induces a change in TCA cycle in *M. tb*, giving succinate as an end product [52,53].

Abundant production of succinate plays an essential role in cell viability because secretion of succinate as an electrogenic substrate maintains membrane potential, ATP synthesis, and anaplerosis at a proportionate rate [54–56]. Various secretion systems such as ESX-I, type VII, and secretory proteins (phthiocerol dimycocerosates PDIM) of the *M.tb* membrane also play an essential role essential role in persistence [57].

7. Biofilm formation

Very often it becomes difficult to understand the persistent nature of bacteria which is because of their nature to survive under stressful conditions by forming a three-dimensional structure made up of an extracellular matrix called Biofilm [58]. The heterogeneous bacterial population residing in Biofilm shows a high level of drug tolerance and is regarded as persisters who stay in the host even for decades and cause latent infection [59,60]. The significance of Biofilm is not only for antibacterial activity and nutrient sources but also for communicating with the bacteria in the niche. This entire complex communication process is called Quorum Sensing (QS) [61–64]. The extracellular signals are diverse in nature. Sometimes they may be nucleotides, amino acid derivatives, small peptides, or proteins. Generally, these are diffusible and are known as Autoinducer1, Autoinducer 2 and peptides. The modulation in the quantity of signal often leads to trigger the cell metabolism. Generally, there are many surface receptors that have histidine kinases effector domains. These domains act as catalysts in the synthesis and hydrolysis of cyclic nucleotides such as Adenyl cyclase (AC), Diguanylate cyclase (DGC) and phosphodiesterase (PDE) [65]. The binding of ligands on receptor leads to generation of amplified second signals like cAMP, cGMP, ppGpp,c-di-AMP or c-di-GMP which leads to generation of signaling cascades. Different bacteria recognize different signals for Quorum sensing (QS); while conventionally Autoinducer 1 which is also known as Acyl-homoserine lactone (acyl-HSL) is employed by Gram negative bacteria and Gram-positive bacteria use peptides. Remarkably, Autoinducer −2(AI-2), furanosyl borate diester, is considered the universal signal molecule for interspecies communication [66]. Interestingly, gram-negative bacteria show a huge response towards QS compared to gram-positive bacteria. Gram-negative bacteria most often utilize a *LuxR-LuxI* system for sensing and responding signals for homoserine lactone levels which is a part of changing environment or cell density. The Lux System is a two-component system that acts as a quorum sensing regulator. *LuxI*–like proteins are signaling molecules while *LuxR* like proteins bind to primary signal and activate the downstream gene transcription. The signal transduction often leads to bacterial movement, biofilm formation, secretory system, and virulence of pathogenic bacteria. Though several gram-positive bacterial species *Bacillus subtilis*, *Staphylococcus aureus*, and some other gram-positive species, follow QS their mechanism of action is still poorly understood. Bioinformatics analysis of the *M.tb* genome confirms the presence of the *LuxR* gene but has not been experimentally investigated [67]. Those compounds that are anti-QS are known as Quorum Quenchers (QQ). These molecules do not have bactericidal activity rather than have bacteriostatic activity. Studies suggested that QQ molecule increases drug susceptibility in both *in vitro* as well as *in vivo* models [68].

Quorum sensing frequently correlates with biofilm formation, as numerous genes play pivotal roles in both phenomena, establishing direct or indirect interrelationships between them. Biofilm formation comprises a series of stages involving initiation, growth, and maturation. The initiation is served by attachment to the substratum of planktonic cells, and their growth, followed by maturation and development is accompanied by the production of an extracellular matrix [69,70]. Within a mature biofilm, a diverse population of cells is established through the aggregation and cooperative behaviour of cells experiencing limited access to nutrients and oxygen,

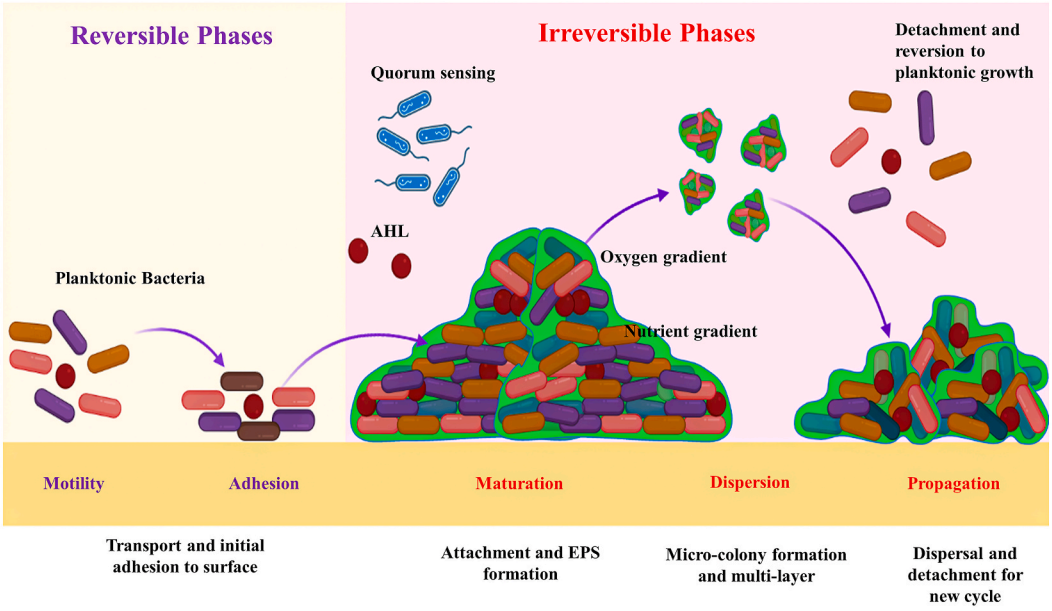

Fig. 3. Lifecycle of microbial Biofilms.

resulting in the formation of a three-dimensional structure (Fig. 3).

The key genes of Biofilm (*sigE*, *sigB*, *WhiB3* [77], *Rel* [78], *DcpA* [79], *DevR* [80], and *GroEL1* [81], *pks10* [82]) & QS regulates the entire process to drive this heterogeneous mass of the bacterial population to become antibiotic-tolerant persisters. Mutant species do not show any defect in the planktonic stage but require some particular genes for biofilm development. Thus, the frequency of drug-resistant persisters in a biofilm is quite relatively high compared to average planktonic growth.

There are three models for biofilm formation found in *M.tb*. These include pellicle biofilms, leukocyte lysate-induced biofilms and thiol reductive stress induced biofilms. Pellicle biofilms are formed at the liquid-air interface of cultures. Lipids, mainly keto-mycolic acids, are the primary components of the EPS constituting the pellicle biofilm. In contrast, polysaccharides are the primary component of EPS of the leukocyte lysate-induced and thiol reductive stress-induced biofilms [71–73]. Mycobacterial mutants that display defective biofilm development, without any defect in planktonic growth, demonstrate that specialized genes are required for biofilm development. Earlier it was shown that anti-biofilm drugs like 2 amino-imidazole restore the isoniazid tolerant *M. tb* to drug susceptibility and enhance the killing of these bacteria [72]. The frequency of drug-tolerant persisters in *M. tb* biofilms are higher than in planktonic cultures, and their occurrence is tightly linked to the development of a mature three-dimensional architecture [71,74,75], similar to that observed in other bacterial species. Downregulation of ribosomal proteins is a hallmarks of *M. tb* and *Mycobacterium smegmatis* Biofilm [76]. However, the precise role of these genes has not deeply investigated.

Biofilm formation is a coordinated process having a standard mode of adaptation, which is exhibited by a variety of non-tuberculous mycobacterial species like *M. smegmatis, M. marinum*, *M. fortuitum, M. chelonae, M. bovis* and some more are known to have the property of forming Biofilm under *in vitro* as well as *in vivo* conditions. Evidence gathered through SEM (Scanning Electron Microscope), TEM (Transmission Electron Microscope) and staining dyes suggests that Biofilm is composed of protein, polysaccharides, and DNA. One of the peculiar functions of Biofilm hypothesized is that it also acts as nutrient source when bacteria encounter starvation conditions [83]. Earlier it was thought that Mycobacterial Biofilms are formed by free mycolic acids (FM) that are released in the mycobacterial culture, which also participates in pellicle maturation [71]. Remarkably, acetylated Glycopeptidolipid (GPLs) derivatives and mycolyl-diacylglycerol (MDAG), Poly-a-L-glutamine (PLG) and cellulose also play an important role in biofilm formation [73,84,85]. While FM synthesis is initiated during the maturation of biofilms through a *GroEL1*-dependent modulation of type II fatty acid synthases [71,81,85,86], the mycolyl-diacylglycerol (MDAG) synthesis is regulated by a nucleoid-associated protein, *Lsr2*. *Lsr2* is a histone-like protein in *M. tb* which is required for the interaction between the enzymes of FASII and includes *GroEL1* and β-keto-acyl ACP synthases (*KasA* and *KasB*), is specifically induced during the later stages of biofilm formation. This fact demonstrates that biofilm development in *M. smegmatis* moves through a distinct stepped process in which association is not involved with planktonic growth [81]. Intracellular iron generated by the Siderophore synthesis also facilitates the biofilm formation of *M. smegmatis* [76]. The other proteins such as *furA* involved in iron uptake, are also upregulated. Other example is the Type VII secretion system ESX-3, required for iron uptake. Interestingly thiol reductive stress (TRS) induced biofilm formation shows increased level of SenX3/RegX3, a two component system that is involved in bacterial growth [73]. However, how the mycobacterial Biofilms form and what induces their expression in native conditions remains an area of major research.

Though Mycobacterial Biofilm gives a recalcitrant environment, it also becomes a space for the conjugal transfer of DNA which helps bacteria to exist and survive in their niche [87]. Up-regulation of several genes related to DNA replication and repair, transport of solute across membrane, existence under carbon and oxidative stress, and surface remodeling are some of the imperative steps that happen during Biofilm maturation in *M. smegmatis* [76]. It has been demonstrated by Ojha et al. that *M. tuberculosis* also forms Biofilm under *in vitro* conditions where the appearance of pellicle Biofilm is quite similar to the above-mentioned mycobacterial species. Three genetic loci namely *pks16*, *helY*, and *pks1* are known to be involved in *M. tb* Biofilm formation [71,88]. Mutants defective of all the above three genes fail to generate mature Biofilms but do not show an effect on planktonic growth. Biofilm formation in *M. tb* is also related to the gaseous environment, especially to the air–media interface, which is supported by the idea that a particular gaseous configuration could generate intercellular or cell-surface interactions in slow-growing mycobacterial heterogeneous cells [71].

Though the growth of *M. tb* in large multicellular structures has been described in long back using histopathological studies of infected lungs, however, till date, there is no clear evidence about the genetic pattern of persistent biofilms [89]. Early pieces of evidence say that biofilms might be a part of *in-vivo* lifestyle of *M. tb* that gets enhanced for increasing their tolerance towards antibiotics. A pathological study designed by Lenaerts et al., 2007 claimed that small colonies of bacteria still existed in the acellular rim of granulomas in infected guinea pigs [90]. After some more research, it was elucidated that a pilin like protein was encoded in *M.tb* that not only expresses itself *in-vivo* but strictly adheres to the eukaryotic extracellular matrix engaging the bacilli for surface attachment [91]. The drug-resistant and persistent cells of *M. tb* are considered as a heterogeneous population of either slow-replicating or non-replicating cells developed as an adaptation process of the bacteria in the hypoxic environment [23,92].

Including hypoxia, some additional physiological and cellular factors, like constrained permeability, restriction of the mycobacterial pellicle, non-hypoxic stress-induced metabolic plasticity, and irregular growth factors also play an essential role in driving mycobacteria towards antibiotic resistance [93–97]. Biofilm surrounded Mycobacteria can tolerate the antibiotics with greater concentration than their planktonic partners [71]. The presence of drug tolerant persisters in the Biofilm formed by *M. tb* suggests that this could potentially harbor bacterial survival even after drug treatment. Therefore, it would be worthy of understanding and studying the mycobacterial physiology in the host while forming biofilms and deciphering signaling between cell to cell while biofilms form and interact for their survival.

8. Mycobacterial biofilms and genetic control of biofilm development

Mycobacterial Biofilms form as monolayer or as multilayers in which each bacterium is attached to the surface and to the

neighboring bacteria by an extracellular matrix consisting of polysaccharides, proteins, and DNA [98]. Biofilm formation in mycobacteria, like any other bacterial biofilm is a multistep process which includes attachment to the carrier surface, reversible and irreversible binding to the surface with the help of adhesion molecules-adhesins, development of microcolonies, and maturation of biofilm architecture [99].

Numerous environmental and genetic signals regulate biofilm development and distribution. The genetic control of biofilm development is mainly through quorum sensing, cyclic diguanosine-5′-monophosphate, and small RNAs [100]. Quorum sensing has been discussed in detail in the above sections.

The second most important crucial biofilm regulator, the c-di-GMP signaling network, deemed to be the most complex secondary signaling system discovered in bacteria regulates the bacterial transcriptional activity, enzymatic activity, and performance of cellular structures [101]. c-di-GMP plays a decisive role in the bacterial decision to grow as planktonic culture or in biofilm [102]. The transcriptional factors in control of c-di-GMP execute the role of biofilm structure development via the synthesis of exo-polysaccharides and adhesion molecule synthesis. Small non-coding RNAs, including riboswitches, are also considered crucial is biofilm formation. Horizontal gene transfer and toxin–antitoxin systems also contribute to biofilm formation [103].

9. Formation of biofilms as a cause of drug resistance and the development of persistence

The metabolic state of all the bacteria which form the biofilms are similar. However, they are different in terms of their access to nutrition and extracellular environment. This leads to different metabolic state of these bacteria within the Biofilm which leads to phenotypic drug tolerance [72]. Drug tolerance has been explained through two hypotheses.

According to the first hypothesis, *M.tb* upon sensing the stressful factors associated with the host environment such as hypoxia, nitric oxide concentration and starvation shift to a non-replicating persistent state which is characterized by slow replication and metabolic quiescence [51]. Another hypothesis states that biofilms formation is the cause of phenotypic drug tolerance [71]. Ojha et al. have demonstrated that *M. tb* residing in the biofilm exhibit drug tolerance and are populated with persister bacteria [71]. Different mechanisms have been proposed by different groups to explain drug tolerance due to biofilms. These factors which decide the fate of biofilms include permeability, metabolic state, activation of resistance genes such as inducible methylases and persister cells [104–106]. Microorganisms capable of forming biofilms display resistance to antibiotics and disinfectants, resulting in their failure to be effectively eradicated. Clinical studies have shown that biofilms have to be physically removed to get rid of the infection [107] The mechanism which bestows biofilms antibiotic resistance or tolerance are lower antibiotic penetration through EPS due to EPS acting as physical barrier [108,109], presence of antibiotic degrading enzymes in the EPS (such as beta-lactamase) [110], and due to the presence of extracellular DNA which increases biofilms resistance to antibiotics [111,112]. Other reasons contributing to antibiotic resistance in biofilms are lack of nutrients, oxidative stress, and efflux pumps [113,114]. The EPS biofilm matrix aids *M.tb* bacterial

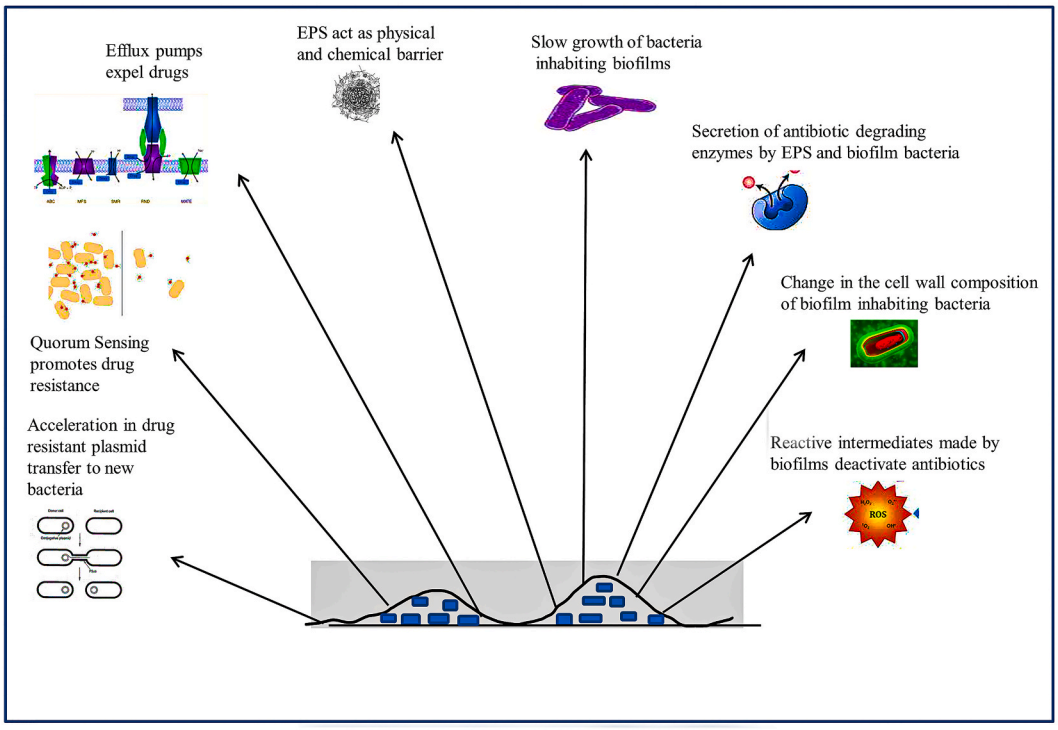

Fig. 4. Mechanisms of drug resistance in biofilms.

communities to survive in close proximity [115] and provides a suitable ground for the exchange of plasmids encoding for resistance genes to conventional antibiotics, thus promoting the spread of bacterial resistance among the bacterial population [14]. It has been demonstrated that horizontal transfer of resistance associated genes between bacterial cells in the biofilm matrix confers antibiotic resistance within the population. It has been reported that Biofilm residing bacteria are 700 times more efficient than the free-living, planktonic bacteria [116]. Therefore, biofilms play a significant role in the development of drug tolerant persister cells. Better understanding of the bacteria forming the biofilms and their signaling pathways will help in the elimination of active as well as persister cells which are responsible for the lengthy anti-TB treatment (Fig. 4).

10. Targeting of drug tolerant *M. tb* in their survival niches for the effective management of TB

Different researchers have been working on eliminating the persister population of *M. tb* along with the actively growing population. An understanding of Biofilm is essential for appropriating managing patients with dormant or drug tolerant *M.tb*. Recent works also revealed that biofilms are developed in animal or patients and that are drug tolerant [13].

Several studies have found mycobacterial biofilms which are resistant to disinfectants and antibiotics, including amikacin and clarithromycin. Even when given at minimal inhibitory concentrations (MIC), bacteria which should have been susceptible to amikacin and clarithromycin, were tolerant to the drugs; when they formed part of biofilms [117,118]. Muñoz-Egea et al. found ciprofloxacin to be the most active antibiotic against bacterial biofilms, compared with clarithromycin or amikacin [119]. Further studies show that, early antibiotic treatment was more effective for biofilm development, when the bacterial cells had not fully conformed to biofilm growth. Several researchers are using compounds or drugs along with conventional anti-tuberculosis therapy to target these dormant bacterial population in the biofilms to eliminate the active *M.tb* along with the drug tolerant dormant population [13]. Biofilm infections can be cured by use of various antibiotics in combination together with drugs known as biofilm disrupters. The combination of antibiotics with the biofilm-dispersing medicines have shown some promising results. The biofilm-dispersing agents do not kill the pathogenic bacteria alone and have to be used with an antibiotic [120]. Other therapeutic strategies include inhibitors of quorum sensing [121], EPS disrupters [122] and agents which target persisters [123]. These approaches could lead to the sterile clearance of TB disease.

11. Conclusion

Mycobacterial persistence is a very serious concern while developing strategies to target *M.tb*. It is the major cause of emergence of drug resistance in the bacterial populations. After increasing cases of multi-drug resistance in the bacterial populations, there is an urgent need to treat biofilm-related infections in view of improving public health. Currently, treatment of Biofilm relies mostly on antibiotics and the highly antibiotic resistant property of biofilms urgently need better and novel antimicrobial agents and Biofilm targeting strategies. We need better strategies for targeting mycobacterial persistence to combat *M. tb* persisters. Moreover, drugs which target multiple types of persisters rather than a single type should be called for. With recent advances in the research on the factors responsible for the development of persisters including biofilm formation, we have different signaling pathways and mechanisms to explore for understanding the mechanisms involved in the development of persisters which do not get eliminated by standard antibiotics. Despite, development of few innovative and effective antibiotic strategies such as dispersion of biofilms using disrupters, and the combination of antibiotics with quorum sensing inhibitors we are still in the infancy of research in targeting biofilms and their associated drug tolerance. Although the above-mentioned strategies are important in the elimination of Biofilm associated pathogenicity, they are still to be studied in clinical research and are still not available commercially. Therefore, we urgently need to investigate methods and strategies targeting both host and bacteria and therapies stimulating the immune response to antigens associated with persistence and decreasing immune-suppressive mechanisms, for the effective development of combination therapies for total treatment of the disease and prevention of reinfection and reactivation.

Funding statement

No funds have been provided for this review article.

Data availability statement

No data was used for the research described in the article.

CRediT authorship contribution statement

Ashirbad Sarangi: Writing – review & editing, Writing – original draft. **Shashi Prakash Singh:** Writing – review & editing, Writing – original draft, Supervision. **Bhabani Shankar Das:** Writing – review & editing, Writing – original draft. **Sristi Rajput:** Writing – review & editing. **Samreen Fatima:** Writing – review & editing, Visualization, Supervision, Investigation, Conceptualization. **Debapriya Bhattacharya:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We acknowledge Kathleen Bushman-Sahay for language editing of our manuscript.

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