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Mycobacterium tuberculosis: Dormancy, Persistence and Survival in the Light of Protein Synthesis

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1. Introduction

The chapter introduces and discusses the metabolic robustness and survival strategies of mycobacteria with focus on glyoxylate shunt and protein synthesis. Primarily, it is an attempt to understand the significance of glyoxylate pathway, which provides an adaptive advantage in metabolically starved situation. The other issue addressed here are the key players behind resistance mechanism in all thriving forms of the bacteria. The protein synthesis process, its challenges and advantages that can effectively be harnessed have been discussed in details. It also gives a comprehensive account of the strength, weaknesses opportunities and threats in targeting mycobacterium both in active and dormant state. Last but not the least we also highlight further perspectives to control the pathogenesis by the bacteria.

Definitions:

Dormancy: the reversible state of bacterial metabolic shutdown (Kell et al., 1998; Mukamolova et al., 1998; Barer & Harwood, 1999).

Persistence: the phenomenon whereby otherwise drug susceptible microorganisms have the capacity to survive indefinitely within mammalian tissues despite continued exposure to the appropriate drug or drugs (Mc et al., 1957).

Latency: *in vivo* situation where bacteria and the host have established a balanced state without causing apparent symptoms in the host, as in latent infection (Orme, 2001a)

1.1 A prelude to mycobacterium: The culprit in stealth

The enigma caused by the mycobacterium has been a challenge to the scientific community by virtue of their adaptive skills and evasion mechanism to combat immunologically educated host. It is a gram positive, acid fast pathogenic bacterium with unique cell wall. It

can survive even within the hostile environment of alveolar macrophages. Statistically speaking as per the WHO fact sheet 2010/2011, Tuberculosis is reported to kill 1.7 million people in 2009 globally. It accounts for about 4700 death per day (Organization, 2010). Further complications in eradicating these 'notorious culprits of mass destruction' is the emergence of drug resistant forms as MDR-TB and XDR-TB i.e. multi drug resistance and extensive drug resistance respectively. Their smart stealth behaviour to get away from host defence together with metabolic fine-tuning in hostile environment makes it world's most successful pathogen in action.

1.2 *Modus operandi* of hide out

It has been quite puzzling to understand the metabolic fluctuations in the changing pathophysiological microenvironment of mycobacteria. Our understanding pertaining to the process for acquisition of essential nutrients for thriving in these environments by intracellular bacterium is still in the stage of infancy. Detailed analyses have revealed a transformative process where environmental hostility brings about a lifestyle change following a reductionist agenda to minimize nutritional needs leading to dormant and/or persistent cells, collectively described as latent tuberculosis (Gomez & McKinney, 2004; Lewis, 2007). A very recent transcriptiome based analysis brought an interesting scenario to light that in *Mycobacterium tuberculosis*, low numbers of drug-tolerant persisters are present from the lag and early exponential phases, which increase sharply at late exponential and stationary phases roughly accounting for 1% of the total population. This further established a new understanding that dormancy is not an all or none phenomenon, and it is collectively governed by both deterministic and stochastic mechanisms (Keren et al., 2011).There are several models to study the phenomenon but one recent model based on multiple stress dormancy, that generates a lipid loaded drug tolerant dormant pathogen looks quite promising (Deb et al., 2009). A recent study using same model by Daniel *et.al* mimicking the microenvironment inside the human granuloma by incubating mycobacterium infected macrophages under hypoxic phase revealed that under these conditions macrophages produce lipid droplets containing Triacyl glycerol (TAG) which is smartly utilized by these bacteria too and exhibit dormancy like phenotype (Daniel et al., 2011). Pandey *et.al* from their work also demonstrated that Mycobacterium can effectively degrade cholesterol derived from host and use it for their carbon and energy source thus maintaining chronic infections in murine models and establishing persistence (Pandey & Sassetti, 2008).

1.3 Scheme of the process

Latent tuberculosis is characterized by a plethora of converging events; on one hand the immunological modulators govern the process in dynamic fashion, while on the other hand metabolic plasticity is at its best. The core strategy is to undergo a downshift in the needs in order to survive with minimal metabolic activity (Wayne & Hayes, 1996). Looking from a metabolomics perspective a variety of genes undergo fluctuations in their expression profile falling mainly under three major groups; respiratory enzymes, stress related proteins and proteins involved in fatty acid metabolism (Honer zu Bentrup & Russell, 2001). The shift to anaerobiosis leads to a metabolic shuffle triggering alternative pathway of glyoxylate shunt to meet the challenges ahead.

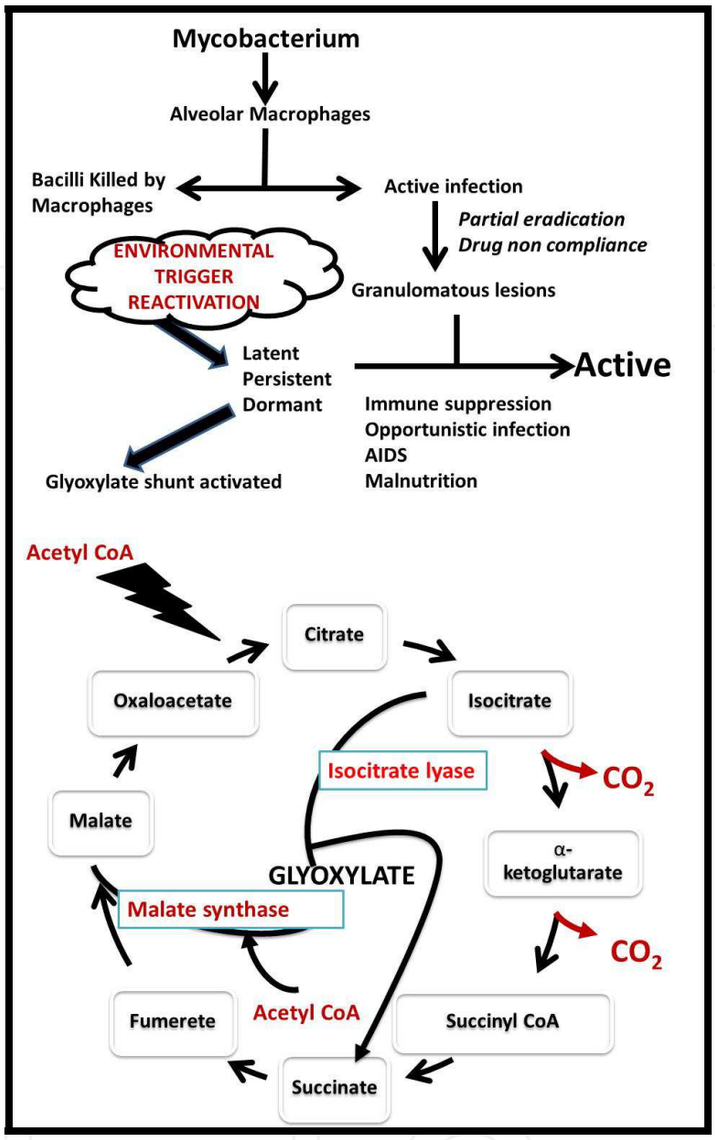


Fig. 1. Mycobacterium survival: the lipid lunch through glyoxylate shunt.

2. Glyoxylate pathway: The saviour of the anarchist

Tuberculosis is characterized by periods in which the disease may be non-obvious or even clinically inapparent but even in these regimens mycobacteria persist with the potential to reactivate the disease opportunistically. Persistence may be defined as a stage where the metabolic downshift to anaerobiosis brings about a nutritionally suspended condition. In the granulomas the bacterium does not replicate and becomes inert yet surviving in occult forms to get activated in immune-compromised situations. In persistent phase the *ala carte* is changed from glucose to delicious lipids, glycolysis is decreased and the glyoxylate shunt is upregulated allowing anaplerotic maintenance of the tricarboxylic acid (TCA) cycle (McKinney et al., 2000). The glyoxylate shunt converts isocitrate to succinate and glyoxylate, catalyzed by the enzyme isocitrate lyase (ICL), followed by the addition of Acetyl-CoA to

glyoxylate to form malate by malate synthase (MS) (Sharma et al., 2000). The glyoxylate shunt allows the bacteria to avoid the carbon dioxide generating steps of the Krebs cycle, enabling them to shunt carbons from fats to carbohydrate synthesis (Wayne, 1994). The drugs used today in combination therapy for treating tuberculosis were discovered 40 years ago and none of them has been effective against these robust persisters (Reddy et al., 2009). The global mandate today is focused to reduce the treatment time line which is six to nine months at present. Targeting pathways that get triggered during persistence phase can yield potential leads. As humans do not have functional glyoxylate pathway the enzymes of the pathway are promising drug target (Kumar & Bhakuni, 2008). The relative abundance of genes pertaining to fatty acid degradation (more than 150) in mycobacterial genome highlights the importance of survival on lipids derived from host *in vivo* (Cole, 1999).

2.1 The arsenal of survival

Enzymes of the glyoxylate shunt operate by diverting carbon from beta-oxidation of fatty acids into the glyoxylate pathway to sustain a nutrient starved intracellular infection they have been implicated for their roles in both virulence and persistence in candida (Lorenz & Fink, 2001) and salmonella (Allen et al., 2000) too. One protein present at significantly higher level in the phagocytized population was identified by microsequencing as isocitrate lyase, a key enzyme of the glyoxylate cycle (Manabe et al., 1999). Furthermore, cDNA selection technique to identify genes upregulated upon phagocytosis revealed Isocitrate lyase as one of the 11 genes identified and was the only metabolic gene in the set (Graham & Clark-Curtiss, 1999).Expression of ICL is upregulated under certain challenged growth conditions (Honer Zu Bentrup et al., 1999) and during infection of macrophages by Mycobacterium spp. It has also been demonstrated that ICL is important for survival of *M. tuberculosis* in the lungs of mice during the persistent phase of infection (2–16 weeks), but is not essential during the acute phase (0–2 weeks) of infection (McKinney et al., 2000). Recent report suggests that suppressing the apoptosis of host macrophage may be one of the important mechanisms for their increased intracellular survival (LI Jun-ming, 2008). Common to all ICL is a signature sequence 'KKCGH'. It has the nucleophilic cysteine residue that sits on a flexible loop which undergoes large conformational change after binding of substrate resulting in complete closing of the active site from the bulk solvent (Smith et al., 2004). Interestingly it is quite important to note that although the genome of *M. tuberculosis* encodes orthologues of two of the three enzymes of the methylcitrate cycle, methylcitrate synthase and methylcitrate dehydratase, it does not appear to contain a distinct 2-methyl isocitrate lyase (MCL). ICL from *M. tuberculosis* can clearly function as a MCL thus metabolizing both Acetyl and Propionyl CoA generated by β-oxidation of even and odd chain fatty acids facilitating the lipid lunch (Gould et al., 2006).

The other enzyme downstream in the pathway is Malate Synthase G (MtbMS) that drives the reaction ahead. A single malate synthase gene called *glcB* (Rv1837c) has been identified in *M. tuberculosis* encoding MtbMS (Smith et al., 2004). MtbMS is a very important housekeeping enzyme involved in persistence of the bacteria. Its intracellular/extracellular localization acting as adhesion and virulence factor together with persistence is quite enigmatic. A recent study further showed the existence of active dimeric form of the enzyme that may add to multiplicity of function that the enzyme exhibits (Kumar & Bhakuni, 2010). It was further revealed in a recent study that the enzyme in mycobacterium differ substantially as compared to *Escherichia coli* by having differences in molecular assembly

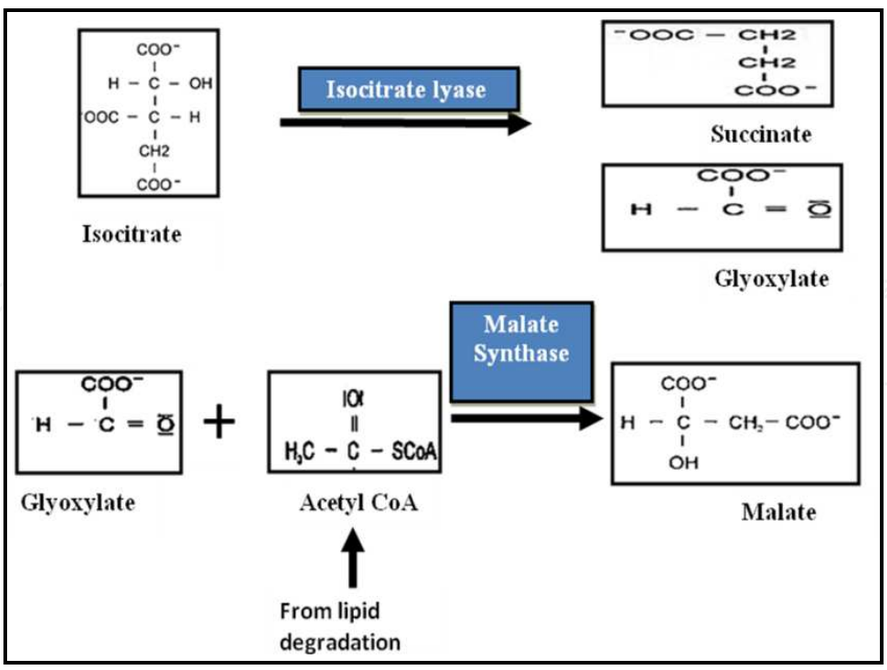
governed by subtle ionic interactions (Kumar & Bhakuni, 2011). Antibodies to MS have been discovered in 90% of patients during incipient subclinical tuberculosis (Singh et al., 2003; Singh et al., 2005). Bishai (Gyanu Lamichhane, 2003) reported the random insertion of transposons into the MtbMS gene (*glcB*, annotated Rv1837c) resulted in a non-viability under normal growth conditions; in contrast, Sassetti (Sassetti & Rubin, 2003) determined that MtbMS is not required for tuberculosis infection in mice using a similar transposon insertion method. However, attempts to knockout Mtb *glcB* have been unsuccessful (J. D. McKinney, personal communication). This consolidates the role of MS in metabolic up keeping of the bacterium when hostile environment prevails.

2.2 The rescue saga

In enzymology, an isocitrate lyase (EC 4.1.3.1) is an enzyme that catalyzes the reversible aldol cleavage of threo-DS(+)-isocitric acid to succinic and glyoxylic acids (Rua et al., 1989)

$$\text{Isocitrate} \rightleftharpoons \text{Succinate} + \text{Glyoxylate}$$

Hence, this enzyme has one substrate, isocitrate, and two products, succinate and glyoxylate. This enzyme belongs to the family of lyases, specifically the oxo-acid-lyases, which cleave carbon-carbon bonds. The systematic name of this enzyme class is isocitrate glyoxylate-lyase (succinate-forming). Isocitrate lyase is the first enzyme unique to the metabolic pathway known as the glyoxylate cycle which is required for the assimilation of fatty acids and acetate (Kelly et al., 2002). Recent reports have alluded to an additional role for this enzyme in *M. tuberculosis* metabolism, specifically for growth on propionate. A product of beta-oxidation of odd-chain fatty acids is propionyl-CoA. Clearance of propionyl-CoA and the



Acetyl-CoA + H_2O + Glyoxylate $\rightleftharpoons$ (S)-Malate + CoA

Fig. 2. Reaction scheme of glyoxylate shunt.

by-products of its metabolism via the methylcitrate cycle are vital due to their potentially toxic effects but no homolog of this enzyme has been found in the mycobacterium genome. This unique phenomenon points out on the dual role of isocitrate lyase in the glyoxylate and methylcitrate cycles in *M. tuberculosis* (Gould et al., 2006). ICL-deficient bacteria could not grow on propionate, suggesting that ICL might function as ICLs in the glyoxylate cycle and as MCLs in the methylcitrate cycle (Munoz-Elias et al., 2006).

The enzyme malate synthase (EC 4.1.3.2) catalyses the condensation reaction between the carbonyl group of glyoxylate and the methyl group of acetyl-CoA to form a thio-ester which, after hydrolysis, generates L-malate and CoA (Dixon et al., 1960).

Catalysis by ICL and MS ensures the bypass of two oxidative steps of the tricarboxylic acid cycle, permitting net incorporation of carbon during growth of most microorganisms on acetate or fatty acids as the primary carbon source. Thus, the glyoxylate bypass conserves carbon and ensures an adequate supply of tricarboxylic acid cycle intermediates for biosynthetic purposes when cells convert lipids to carbohydrates (Sharma et al., 2000).

2.3 Magnesium the magic molecule in metabolic recession

Magnesium serves as key ingredient in the recipe of lipid lunch as both the pivotal enzymes are activated in the presence of magnesium as a cofactor. There have been two schools of thoughts as far as role of magnesium on these enzymes are concerned the one thinks that magnesium is activating substrate by binding to it (magnesium-substrate complex) while other view rests on the idea that magnesium binds to the active site inducing conformational change and having catalytic role for efficient catalysis (2007).

The plant and bacterial ICL have an absolute requirement of Mg^{2+} ions for functional activity. Mg^{2+} ions were postulated to be necessary for catalysis on the active site of ICL however, a higher concentration of these ions has been found to have inhibitory effect on the enzyme (Beeckmans et al., 1997). Later on, Giachetti et al. (Giachetti & Vanni, 1991) performed detailed kinetic studies with *Pinus pinea* ICL and concluded that the Mg^{2+}-isocitrate complex and not isocitrate is the true substrate of enzyme. This conclusion has been supported by studies on ICL from several other sources. In the absence of divalent cations, only negligible activity was measured for the purified ICL, whereas addition of Mg^{2+} or Mn^{2+} supported enzyme activity. Mn^{2+} was able to replace Mg^{2+}, yielding 39% of the activity obtained with Mg^{2+}. Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} were not able to support significant ICL activity (Honer Zu Bentrup et al., 1999). A variety of metal ion combinations were studied for their ability to inhibit ICL activity. It is known for the isocitrate lyases of *Corynebacterium glutamicum* and *Acinetobacter calcoaceticus* that Mn^{2+} can partially substitute for Mg^{2+} (Hoyt et al., 1988; Hoyt et al., 1991).In the absence of divalent cations only negligible activity was measured for the purified MS. Mg^{2+} at 5 mM was found to be the most effective cation. Mn^{2+} was able to replace Mg^{2+}, yielding 40% of the activity obtained with Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} were not able to support significant MS activity (Smith et al., 2003). Zn^{2+} and Cd^{2+} were found to bring about structural alterations thereby inhibiting the function of enzyme in case of MtbIcl (Kumar & Bhakuni, 2008).

3. The shunt and the hunt

The structural intricacies have been deciphered for both the key enzymes successfully and all the information is at our disposal (Sharma et al., 2000; Smith et al., 2003; Anstrom & Remington, 2006). In the era of structure based drug design where high throughput screening, molecular modelling, *in silico* docking strategies have accelerated drug development timeline promising rescue of the hijacked host from the persistent mycobacteria. The TB Alliance has been strategically focusing to combat these molecular targets. ICL is a tetrameric protein with four subunits of 428 amino acids each. The high-resolution structure of ICL from *M. tuberculosis* has been solved to 2.0 Å resolution (Sharma et al., 2000). The enzyme structure in complex with inhibitors, 3-nitropropionate with glyoxylate and 3-bromopyruvate has also been resolved. 3-bromopyruvate inhibits ICL-activity by forming a covalent adduct with the nucleophilic Cys191 (Sharma et al., 2000). The inhibitor bound ICL structures, on one hand, provide crucial information regarding the active site microenvironment, and on the other hand, produce valuable information on the type of interactions prevalent at those localized site adding momentum strength to the drug discovery process. Several ICL inhibitors are being tested, which mainly include 3-nitropropionate (McFadden & Purohit, 1977), 3-bromopyruvate (Ko & McFadden, 1990), 3-phosphoglycerate (Ko et al., 1989), mycenon (Hautzel et al., 1990) and itaconate (McFadden & Purohit, 1977). However, *in vivo* application of these inhibitors is yet a dream because of their potent toxicity and low activity. Sesterterpene sulphate, which has recently been shown to effectively inhibit ICL in *Candida albicans* (Lee et al., 2008) is also promising.

The second enzyme of the glyoxylate shunt is encoded by a single gene identified in TB called *glcB* and encoding a 741 amino acid 80 kDa protein malate synthase (Smith et al., 2003). The enzyme catalyzes the Mg^{2+}-dependent condensation of glyoxylate and acetyl-coenzyme A and hydrolysis of the intermediate to yield malate and coenzyme A (Anstrom & Remington, 2006). The structure of MS from *M. tuberculosis* in complex with the substrate glyoxylate has been solved to 2.1 Å resolution structural analysis indicated that malate synthase is a much more druggable target by virtue of its deeper and more hydrophobic binding domain (Smith et al., 2003). Screening against this target will have a better chance of identifying tractable inhibitors as lead molecules (www.tballiance.org). Further refinement in understanding the mechanistic implications were brought forth by revised position of bound malate which is consistent with a reaction mechanism that does not require reorientation of the electrophilic substrate during the catalytic cycle(Anstrom & Remington, 2006) . These insights have been crucial in the inhibitor ergonomics. High throughput screening has been completed with a 1.4 million compound library and hits have been identified. The endeavour ahead is to confirm the potential hits and efficiently evaluate these, thus paving the pathway for identification of analogues and series for future optimization. It's also important to mention that high throughput screening initiatives for identifying inhibitors has not yielded very promising outcomes reason being the druggability of these potential targets. The challenge here is to design inhibitors that are equipped with permeability parameters that can reach the action targets.

Evolutionarily enzymes of glyoxylate shunt are highly conserved and have unique signature active site sequences which offer leverage to rational drug design approach thereby coming up with a broad spectrum more pharmacologically attractive target relevant to the treatment

of not only tuberculosis, but also candidiasis and melioidosis and many other pathogens (Kumar, 2009). The rationing mechanism for supply of the precursor amino acid and nucleic acids required for growth can be halted and paused by inhibitors of metabolic pathways and thus making it tough for the bug to proliferate and persist. The search for elixir of life continues with the effort to evolve a drug that has bactericidal activity both on the active and persistent form and a robust delivery mechanism that has ability to penetrate diverse metabolic niche to eradicate these killer bugs.

3.1 Sporulation speculations and beyond

The best form of dormancy has been studied in gram positive bacteria forming spores. As far as mycobacterium is concerned the existence of spore is debatable. A recent study by a group led jointly by Leif Kirsebom and Santanu Dasgupta in Uppsala University put forward the evidence for existence of spores in mycobacterium (Ghosh et al., 2009). The study revealed existence of endospores in very late stationary phase cultures of *Mycobacterium marinum*, a common model for acute mycobacterial infections. Utilizing transmission electron microscopy the distinct outer coat and cortex of the spores could be identified. Furthermore, heat tolerance, malachite green staining, and the presence of dipicolinic acid was also shown. They were able to isolate rRNA from these sporulating cells and identified it as *M. marinum* rRNA. Extensive bioinformatics analysis identified possible homologues of spore forming genes in mycobacterial genome. Thus they suggested that mycobacteria can form spores under stress and presented the hypothesis that sporulation might be one of the adaptations causing dormancy. When genes encoding GFP or mCherry were introduced on plasmids or integrated in to mycobacterium genome fluorescent spore were seen confirming their mycobacterial origin.

However, a rebuttal to this work by several US labs challenging the concept of endospore formation in mycobacteria was published (Traag et al., 2010) which was responded in to a subsequent rebuttal (Singh et al., 2010). The controversies continue with more supporting evidences yet to be deciphered about a phenomenon not so common to many other species.

4. Understanding the pathophysiology in light of protein synthesis

Mycobacterium can maintain itself in the fluctuating environment inside the host and the diversity of its survival mechanism makes it one of the most successful pathogens in the world. The ability of the bacteria to enter a stealth mode and exhibit occult form in combination with its starvation strategies suggests that there exists a complex series of events that is modulated by differential gene expression and protein synthesis. When mycobacterium switches its lifestyle from luxury to mere survival the DNA replication goes to a standby mode (Wayne, 1994) and the protein synthesis process undergoes drastic changes (Hu et al., 1998). Though the knowledge in this research area is limited, understanding the bacterium in the light of protein synthesis and protein turnover opens new avenues to understand this 'culprit in disguise' better.

4.1 Intricacies of macromolecular orchestration

The protein synthesis process is a hotspot for action of antibiotic drugs, which interact directly with mycobacterial ribosome and sabotage 'protein synthesis', the most vital

process of the cells. The mechanism of translation and the function of the ribosome in mycobacteria can thus be visited collectively in the light of action of the drugs which include aminoglycosides, macrolides, tetracycline and peptide antibiotics etc. (Inderlied, 1991). At the same time the study of the resistance mechanism in mycobacteria against these drugs, often involving certain mutations on rRNA or r-proteins, would shed light on the process.

In a study where several independent streptomycin-resistant mutants of *M. tuberculosis* H37Rv were isolated, it was shown that while in the wild-type streptomycin inhibited the incorporation of ^{14}C-amino acids into proteins, very little or no inhibition was observed in either high-level or low-level streptomycin-resistant strains (Shaila et al., 1973). This result on one hand points towards the activity of the drug against mycobacteria and on the other hand brings forward the potential problems arising from the development of resistance phenotypes. The Bottger-group has made very important contribution in the field in identifying potential drug candidates against mycobacteria (Hobbie et al., 2005; Hobbie et al., 2006a; Hobbie et al., 2006b; Hobbie et al., 2007). In addition, they have also identified potential hotspots for mutations leading to resistance against these drugs. Thus, these studies all together call for a continuous quest for identification of new and potential drug candidates for treatment of mycobacterial infections.

Protein synthesis involves a coordinated action of the ribosome, mRNA, tRNAs and translation factors; thus it needs an intelligent setup and precise orchestration between different components. The process, also known as translation, is divided into four distinct steps; namely initiation, elongation, termination and ribosome-recycling. The process initiates by association of large ribosomal subunit with an mRNA programmed small subunit bound with initiator tRNA and initiation factors. Once a 70S initiation complex is successfully formed, the initiation factors dissociate and the process proceeds to elongation. In the first step of elongation the elongation factor–Tu (EF-Tu) brings tRNA loaded with respective amino acids to the decoding center (DC) of the ribosome. This site includes highly conserved regions of 16S rRNA arranged in a highly ordered structure. Correct codon anticodon pairing between the tRNA and mRNA through initial selection and tRNA accommodation leads the process towards the most significant step of protein synthesis, i.e. peptide bond formation. A primarily rRNA (23S rRNA) constituted region of the large subunit, called the Peptidyl-transferase site, catalyses this step. After formation of the peptide bond the tRNA carrying the peptide chain moves one codon along the mRNA, a process called 'tRNA translocation', conducted by elongation factor-G (EF-G). Then the elongation steps repeat and the cycle continues until a stop codon on mRNA reaches the decoding center. The stop codons signal release factors to bind on the mRNA and release the peptide from the tRNA. The process ends by splitting of the ribosomal subunits, which recycle for the next round of protein synthesis. The detailed mechanism of these steps although studied mainly with components from gram-negative bacteria *E. coli*, can be extrapolated to gram-positive mycobacteria due to high degree of sequence conservation in the translation components in all eubacteria. It should be mentioned that although the protein synthesis machinery is highly conserved in general, the fine differences that exist in this system between the pathogenic bacteria and the host organisms provide ample opportunity for targeting the bacterial translation system in a rather specific manner.

Although in recent years, we have acquired a lot of knowledge on the mechanism of bacterial protein synthesis from high resolution crystal structure and fine-tuned biochemical

experiments, very little information specific to mycobacteria have been available. Generally it is believed that bacterial growth rate is highly dependent on the rate of protein synthesis and varies according to the number of translationally active ribosomes in the bacterial cells (Dennis & Bremer, 1974). Thus, mycobacterium, a remarkably slow growing pathogenic bacterium, provides a very interesting system for studying the rate of protein synthesis in different stages of its life.

In 2008, a strong step was taken to study the basic components of mycobacterial protein synthesis. It is known that although very similar, mycobacterial ribosomes differ from the well characterized ribosomes from *E. coli* to some extent. Firstly, the mutations on rRNA lead to somewhat different phenotype in these two bacteria. Secondly, the ribosomal stalk, a visible protuberance on the large subunit of the ribosome composed of the multicopy protein L12, differs in its composition and structural conformation between the two. In *E. coli,* the ribosomal stalk is composed of two dimers of L12 protein in contrast to three dimers in *M. smegmatis.* Also in *E. coli,* the stalk changes its conformation from compact to extended form depending on its state of interaction with the translation factors. In comparison, the stalk in mycobacteria seems to be extended most of the time, the functional significance of such behaviour is not known. It should also be mentioned that mycobacterial species often harbour only one or two rRNA operons in contrast to seven rRNA operons in *E. coli.* Thus mycobacteria provide a very good system to test the effect of mutations in the significant positions of rRNA towards the viability of the bacteria (Hobbie et al., 2007; Long et al., 2009). Also, homogeneous populations of the ribosomes carrying mutations in the rRNA could be isolated and characterized relatively easily from the mutated mycobacterial strains.

The study, based on translation components from *M. smegmatis* enriched the knowledge about the translation machinery in mycobacteria. A combined effort by the research groups of Erik C. Böttger and Marina Rodnina led the study where the genes for various translation factors were mapped, cloned, and expressed in heterologous (*E. coli*) expression system. Further, these factors were used together with mycobacterial ribosomes in a complete *in vitro* translation system complemented with some components from *E. coli*. In fast kinetics assay, the rate of initiation and elongation was measured and compared with those obtained from purely *E. coli* origin. Under these conditions, the rates of fundamental reactions of initiation and elongation of protein synthesis were found remarkably similar in the two systems. Thus, these results suggested that under *in* vitro conditions the basic mechanisms of protein synthesis are highly conserved in these two widely separated species of bacteria (Bruell et al., 2008). At the same time, this study provides an alternative system to identify and test the action of the antibiotics. However, it fails to provide any insight about the rate of protein synthesis in slow growing phases of mycobacteria and calls for detailed investigation of the same under controlled *in vivo* conditions using methods such as radio-labelled amino acid incorporation or beta-galactosidase synthesis assay.

4.2 Combat targets and troubleshooting

There are basically three main hotspots that can confer resistance to inhibitors against protein synthesis. The first is by mutations in the rRNA, the second being by mutations in the ribosomal proteins, and finally by post-transcriptional modification, especially methylation of the ribosomal RNA. Concerted efforts have revealed in the recent past that antibiotics might interfere with chemical probe binding to specific nucleotides in the rRNA

(Moazed & Noller, 1987). These studies suggested that antibiotics could act by interacting with highly conserved hotspots of the bacterial rRNA and thereby interfering with their natural functions. There are also evidences that antibiotics inhibit enzymes essential for creating modifications on rRNA vital for its function. Identification of the sites for resistant mutation often sheds light on the antibiotic action. Resistance to streptomycin which acts by causing misreading in the genetic code followed by inhibition of translation initiation and dubious proofreading (Moazed & Noller, 1987) is attributed to mutations in the aminoglycoside modifying enzyme (Benveniste & Davies, 1973) as well as in *rpsL* gene coding for r-protein S12 (Funatsu & Wittmann, 1972; Allen & Noller, 1989). Specific to mycobacteria, it has been shown that mutations in the *rpsL* gene that replaces Lys 43 or Lys 88 by arginine are associated with streptomycin resistance (Honore & Cole, 1994). There have been also evidences of mutations in 16S rRNA clustered in two regions in the 16SrRNA leading to resistance to streptomycin (Douglass & Steyn, 1993).

Macrolides are bacteriostatic in nature and inhibit the petidyl transferase function of the 50S ribosomal subunit by blocking the peptidyl exit-tunnel. The clinically acquired resistance against macrolides is described as the MLS phenotype (resistant to Macrolide, Lincomycin and Stretogramin B) which is thought to be collectively mediated by methylases coded by *erm* genes (Leclercq & Courvalin, 1991). These methylases brings about changes in the conserved loop of domain V of 23S rRNA (A2058 equivalent of *E.coli*) which is implicated to have definitive role in peptidyl transfer. Recent studies have shown paromomycin, an aminoglycoside, to be effective both *in vitro* and *in vivo* against MDR-TB (Kanyok et al., 1994). A study in *E.coli* has mapped the changes in rRNA that confer resistance by transfecting mutated rRNA operons in case of paromomycin(De Stasio et al., 1989). It would be interesting to see if similar mutations in mycobacteria would also confer resistance for this antibiotic.

In case of tetracycline studies it was revealed that the drug does not inhibit the growth of *M. tuberculosis* but inhibits protein synthesis *in vitro* suggesting that intact cells are impermeable to the drug (Bottger, 1994). Ribosomal mutations that confer tetracycline resistance are difficult to map because the mutations occur frequently in the system which pumps the drugs out.

Protein synthesis: The portfolio for inhibition and innovating novel combat strategies

The contemporary treatment of tuberculosis includes aminoglycosides (streptomycin, amikacin, kanamycin, and capreomycin) and oxazolidinones (linezolid).

- **Initiation:** Streptomycin / Rifampicin
- **Elongation and translocation:** Fusidic acid
- **Amino acyl tRNA inhibitors:** Tetracyclines
- **Proofreading inhibitors:** Aminoglycosides
- **Peptidyl transfer inhibitor:** Chloroamphenicol, Macrolides
- **EFG inhibitors:** Fusidic acid
- Inhibitors binding to ribosome

50S: Chloroamphenicol, Oxazolidinones, Macrolides
30S: Aminoglycosides and Tetracyclines

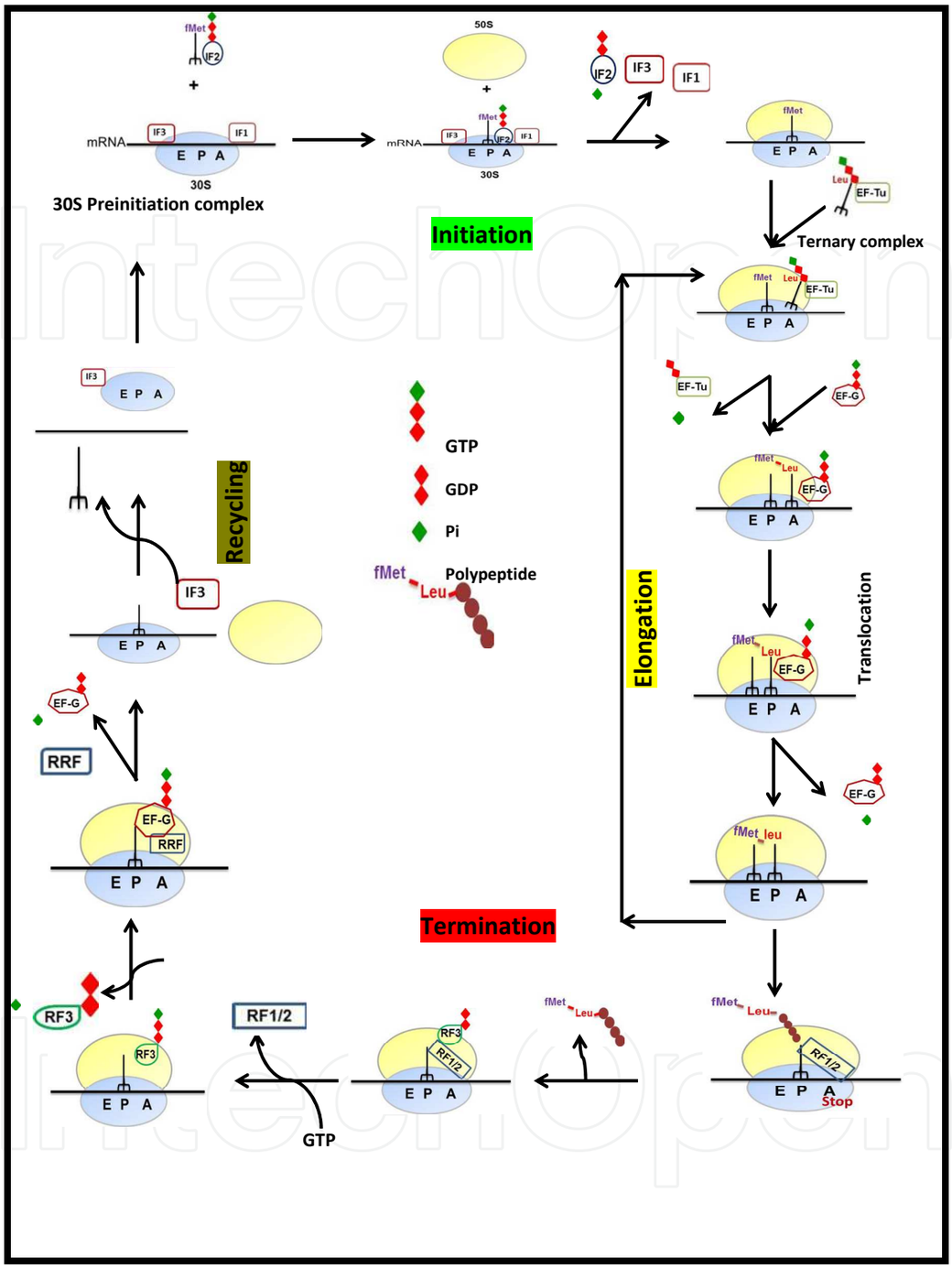


Fig. 3. Translation scheme showing various steps that can be targets of therapeutic intervention. (Adopted from Mandava CS, Ribosomal Stalk Protein L12 Structure Function and Application, thesis submitted to Uppsala University, Sweden 2011)

Nevertheless two important resistant determinants have been found in *E. coli TetO* and *TetM* genes. TetM acts by protecting the ribosome from antibiotic attack and the resistance to tetracycline encoded by these genes are located on plasmids or transposable elements

(Roberts, 1994). A detailed account of the process in slow growing mycobacteria is still not available, which would yield valuable insight about the antibiotic in treatment of mycobacteria.

4.3 The Vendetta in light of protein turnover

The complex milieu, in which the mycobacterium survives, necessitates dynamic adjustments and fine-tuning in protein synthesis and turn-over. During the transfer from active to dormant stage a change in the nature of the proteins expressed is bound to occur. It remains an open question whether protein synthesis continues in a slow rate in the dormant stage or stops completely following the change of the growth rate in mycobacteria. It is very important to understand how protein synthesis machinery work in the dormant or persistent stage since under favourable conditions this dormant form can again get triggered and reactivated leading to a full blown infection in the immune compromised host; thus posing a big threat. One recent report suggested that the protein synthesis is almost shut down in dormant mycobacterium and the conditions can be reversed by providing stimuli like oxygen or heat shock to anaerobic stationary cultures (Hu et al., 1998). It also illustrates the fact that the mycobacteria are quite responsive to stimuli and can trigger protein synthesis quite rapidly even in the dormant state. Hu *et al*. showed a 98% decrease in protein synthesis using ^{35}S-met pulse labeling experiments with microaerophilic cultures of mycobacterium tuberculosis. However, it is still not understood fully whether there is complete shutdown of protein synthesis or a minimal protein synthesis continues in this state to facilitate survival. Another recent report by Murphy *et.al*, suggests that during dormancy *M. tuberculosis* performs limited protein synthesis and the pathogen spends its available resources in maintaining cell wall, membrane potential, genome integrity as well as resisting host defence systems (Murphy & Brown, 2007). The transcriptiome analysis further revealed five times more downregulated genes in persisters than upregulated ones (1,408 versus 282) under dormant condition suggesting that the growth and energy metabolism pathways are significantly downregulated (Keren et al., 2011). Even the synthesis of the ribosomal proteins tends to get down regulated in the state of dormancy as revealed in several different models (Wayne & Hayes, 1996; Betts et al., 2002; Keren et al., 2011). Although these studies are insufficient to resolve whether proteins synthesis comes to a complete halt or not in the dormant stage, these also open up a new question regarding the availability of the vital proteins in this stage. In other words, these studies indicate towards a longer turn-over of the proteins in this stage.

Continuous translation is not the only crucial process that defines the abundance of a protein or enzyme in the cell; instead it is often regulated by the parameters controlling its propensity to degradation and stabilization. Thus, regulation of protein amounts in a non-favourable and fluctuating environment can be more dependent on turnover of proteins rather than the synthesis (Neher et al., 2003). Our knowledge regarding protein turnover in dormant phases in mycobacteria is still in infancy. However research in the last decade has thrown some light on it by attempting to examine the protein turnover at global level. Two seminal papers (Rao & Li, 2009; Rao et al., 2009) have helped in understanding the molecular basis of persistence in mycobacterium. Rao et.al showed that the protein turnover was much more effective to maintain relative protein abundance in the dormant phases of mycobacteria. These studies also help explain how the bacteria manage to exist as a submissive pathogen in the host for a prolonged period. The molecular detail behind the

prolonged protein-turnover mechanism in mycobacteria is not available yet. We speculate that under the dormant and stress condition, the protein degradation systems also slow down significantly as the protein synthesis system, thereby maintaining a good-enough concentration of the vital proteins in the cell and ensure survival of the bacteria.

Using the two-dimensional electrophoresis-based proteomics approach, a protein has been identified in *Escherichia coli* (Almiron et al., 1992), which expresses preferentially under starvation conditions. This protein is homologous to a family of proteins called Dps (DNA binding Protein from Starved cells) that are known to protect DNA under various kinds of environmental stresses. Although Dps kind of proteins have, so far, not been yet reported in mycobacteria, it is highly possible that similar system exists. Using comparative modelling it was possible to demonstrate that Dps from *Mycobacterium smegmatis* could form a dodecamer structure similar to the Dps from *Escherichia coli* (Gupta et al., 2002). The intriguing properties related to protein stability, DNA binding property and protection was further revisited to consolidate the structure function attribute of this unique protein (Ceci et al., 2005). These studies put emphasis on the need of global proteome analysis not only for actively growing cells but also for cells under dormant conditions. The proteomics can also be useful to identify the proteins related to starvation response of the pathogen.

Applying a systems biology approach the contemporary knowledge reveals approximately 4000 genes in mycobacterium genome and proteomic studies on the persistence reveals only very few which are differentially expressed during dormancy. A big proportion of these correspond to stress response proteins and metabolic enzymes (Cho et al., 2006). Unfortunately, there seems to be no exact correlation between the transcriptiome and the proteome data, which would otherwise be very useful (Mattow et al., 2006). The research group led by KVS Rao at ICGEB, New Delhi came up with an innovative strategy to comprehend the host key players that are involved in the onset and persistence of mycobacterium. They employed exhaustive genome-wide small interfering RNA (Si RNA) and identified 275 key molecules that are intricately associated in a network. This has led to a better understanding of host intracellular adaptation as result of bacterial diversification and host-pathogen interactions (Kumar et al., 2010). A bird's eye view from this aspect is bound to yield innovative insights in the pathophysiology of host pathogen interactions.

5. Future perspective and therapeutic interventions

There has been a great interest in looking for better alternatives and treatment modules throughout the globe to address the issue of this deadly pathogen. The focus has further intensified because of its proven role in immunodeficiency syndromes such as AIDS. The Global TB Alliance estimates that the disease will be a financial burden with expenditure ranging from $ 1-3 trillion in coming decades for poor countries (www.tballiance.org). Developing countries are at the hotspots where 94% of TB cases and 98% of death takes place. The present treatment employs the strategies being used since decades with longer treatment regimen. The standard treatment regimen of six to nine month was based on clinical practices dating almost half a century back. The puzzle is that the exact molecular mechanism still needs to be deciphered with evidence that Isoniazid one of the key cocktail medicine only acts on actively growing form (Fox et al., 1999) and not against the anaerobic occult form (Thadepalli et al., 1979) makes situation draconian. The first real rescue for tuberculosis came in the form of Streptomycin in 1943 that was found to bind to 16S rRNA

and block the initiation of translation (Waksman & Schatz, 1943). As early as in 1948 reports of streptomycin resistance hit the scene where streptomycin seems no more effective at all (1948). It was in 1950's that the field had many new drugs hitting the market and the situation looked quite under control. The most recent drug to hit the space is still 35 years old. Modern therapy relies on a combination of potent bactericidal agents, such as isoniazid, rifampicin and pyrazinamide, in a treatment with six month duration. Isoniazid and Rifampicin are the two most effective drugs in the treatment bouquet that is administered throughout the treatment (Zhang, 2005). These are complemented with Ethambutol and Pyrazinamide in the early phase of treatment. Since there has been no new drugs developed in the last four decades and with excessive use of antibiotics there has emerged a new family of multidrug and extremely drug resistance strains leading to what we call MDR and XDR-TB. MDR refers to a resistance against the frontline drugs i.e. Isoniazid and Rifampicin whereas XDR is a complex form which is coupled to MDR and resistant against second line of potent antibiotics like Amikacin, Kanamycin and Capreomycin etc. (Organization, 2010). The perennial problem of non-compliance led the WHO to come up with universal treatment adherence programs, through a process currently known as the directly observed treatment short-course (DOTS).

The complex milieu of *Mycobacterium tuberculosis* and its intelligent survival instinct by virtue of adaptive diversification in tuberculosis disease have been a major obstacle that hinders the development of shorter treatment regimens to eradicate the disease. Currently the TB Alliance, a global initiative in newer TB drug development is trying to come up with affordable, shorter, safer and effective alternatives to target tuberculosis. At present they have 20 promising projects in the pipeline which includes three crucial drug candidates and a novel treatment regimen under phase three clinical trials (REMOX TB) that would considerably shorten the treatment span by at least two months without compromising efficacy. Another blue- eyed boy is a potential compound TMC207 that promises to be active on both drug sensitive and drug resistance form is under phase two trials. A novel cocktail NC001 is also under clinical trial which would shorten treatment regimen and alternatively provide affordable treatment without compromise (Alliance, 2010).

On the vaccination front BCG still occupies the central position being one of the most widely used vaccine against TB to be administered globally without any serious side effects. Its low cost of production ability to confer lifelong protection without booster dose in single immunization, availability of oral alternatives makes it popular in infant immunization programs. BCG has been effective to prevent meningeal TB in children, but it does not confer immunity to pulmonary TB in adults (Orme, 2001b). An interesting investigation in India during the 1960's by WHO in order to establish the efficacy of the BCG vaccine on two separate groups comprising 375,000 people in the province of Madras where one group was vaccinated against TB and the other group was not finally concluded that: "The efficacy of the TB vaccine is 0%" (www.whale.to/a/tb_q.html). Since then in last decade most of the European countries has removed it from their immunization programme. The result is further substantiated by the argument that contracting TB doesn't provide any immunity against a second infection; and if a natural infection doesn't provide protection then a vaccination certainly won't provide protection either. As of now no promising vaccine has come in to the scene so far for tuberculosis in spite of various attempts in coming up with a DNA vaccine (Young et al., 1988; Lowrie et al., 1999; Orme, 2001b). The vaccine development has again come to picture after lacunae of several decades where the

development pipeline now includes seven vaccine candidates that are being tested in humans. Two non- replicating viral vector vaccines have very recently entered the first phase efficacy trial in infants (the first such trial in 80 years) and in human immunodeficiency virus–infected adults (Beresford & Sadoff, 2010). Yet we have a long way to go for an effective vaccine which would take care of all the thriving forms of this enigmatic bacterium.

6. The odyssey ahead

The geography of tuberculosis has achieved a global dimension with transmigration across the boundaries as a result TB pandemic has taken the world in its clutches thus making it a true global concern. The TB Alliance, a global initiative has embarked an integrated innovative approach to combat and sabotage one of the oldest, deadly, and most resilient enemies of the mankind. At the level of individual it impacts them in the most productive phase of their life thereby sucking their income and decreasing the productivity and thus inflicting a great loss in human capital. On average one person infects fifteen others before finally getting successfully treated; the death statistics is alarming with one death every twenty second. The right to health and hope is one of the fundamental rights that is robbed by this enemy of the mankind. Bill Gates propounded at the World Health Assembly in 2005: "Today, we have tuberculosis drugs you have to take for 9 months. Why can't we find one that works in 3 days?" this is still a dream.

The research arena has also received momentum with the deciphering of the Mycobacterial genome in 1998 coupled with advanced molecular biology tools, structural genomics, target based drug design, high throughput screening, *in silico* experiments, whole cell screening and advanced imaging technologies to study real time changes and system biology platforms coming under one umbrella. The idea now is to leverage on the existing portfolio having more than two dozens of potent molecules and drug regimen in pipeline at various stages of clinical trials. The TB development has galvanized further by the coming together of WHO, TB Alliance and DNDi (Drug for Neglected Disease initiative) sharing a common podium. TDR for research on diseases of poverty has been working under the joint conglomeration of WHO, UNICEF, WORLD BANK and UNDP to address this concern globally. Nelson Mandela commented that "we cannot win the battle against AIDS, if we do not also fight TB; TB is too often a death sentence for people with AIDS". The WHO targets to treat 80% of the MDR-TB patients by 2015 with an estimated cost of $15 billion. With initiatives at full bloom the scenario looks promising and hopeful in global attempts to address tuberculosis.

The conquest of the Mycobacterium to the mankind needs to be the priority of the synergistic efforts by the scientific community. In nutshell the enemy of humanity needs to be taken in to clutches by innovative approaches from drug development that includes quest for effective molecular scaffolds and their derivatives both old and new as well as reengineering delivery strategies for the drug to penetrate the recalcitrant stubborn microbes. On one hand there are challenges to finding safer, cheap, less toxic, shorter regimen and compatible drugs quickly while on the other the socio economic feasibilities that can deliver these magic bullets to the neediest ones have to be ensured. Revamping the health care system with Government, business houses and NGO's is the need of the hour to combat these perpetrators of human misery from the globe. Wave of optimism exists as we

attempt to streamline the drug discovery process together with policy issues, social engineering and outreach initiatives.

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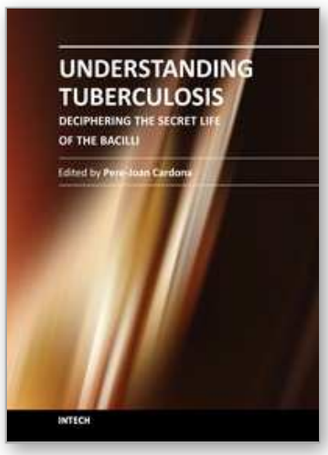

Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli

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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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