

# The Cord Factor: Structure, Biosynthesis and Application in Drug Research – Achilles Heel of *Mycobacterium tuberculosis*?

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## 1. Introduction

Recently, it was reported that the tuberculosis mortality in 2009 has dropped to 35% since 1990 (WHO, 2010). Nevertheless, the disease caused by the facultative intracellular bacterial pathogen *Mycobacterium tuberculosis* still remains the leading cause of death from a single bacterial species (Coker, 2004; Russell et al., 2010). The emergence of Multi-Drug Resistant (MDR) and Extreme Drug Resistant (XDR) strains of *M. tuberculosis* leads to prolonged treatment which drastically increases the therapy costs.

*M. tuberculosis* shows a remarkable property of existing in different states of invasion (infection), colonization and persistence (Casadevall & Pirofski, 2000). It also has outstanding mechanisms to escape from elimination and has a high degree of intrinsic resistance to most antibiotics, chemotherapeutic agents and immune eradication (Brennan & Nikaido, 1995; Coker, 2004). The major obstacle for host defence mechanisms and therapeutic intervention is the unusual robust cell wall which is unique among prokaryotes, and is a major determinant of virulence of the bacterium. The cell wall is critical for long-term persistence of *M. tuberculosis* in the hostile environment of the host's cells and for progression of tuberculosis (Barry et al., 1998). Approximately one-half of the cell wall mass is comprised of mycolic acids (Brennan & Nikaido, 1995). In the cell envelope, mycolic acids are esterified to the terminal pentaarabinofuranosyl unit of arabinogalactan, which is a peptidoglycan-linked polysaccharide. The outer envelope consists of trehalose 6,6'-dimycolate (TDM; cord factor) and TMM (trehalose 6,6'-monomycolate, the biosynthetic precursor of TDM), where the mycolic acids of TDM interact with the mycolyl-residues from the layer beneath (Brennan & Nikaido, 1995). The mycolic acid-containing layers have width of ~10 nm and limit the penetration of hydrophilic substances, whereas the inner saccharide layer inhibits the penetration of lipophilic substances. The high abundance of mycolic acids in the outer cell envelope is the main barrier for water soluble antibiotics (Brennan, 2003; Coker, 2004).

The purpose of this review is to highlight the importance of the cord factor as one of the most unique determinant for *Mycobacterium tuberculosis* virulence. This article will especially focus on the steps of the cord factor biosynthesis, i.e., the transfer of mycolic acid from a

TMM to another TMM to form TDM by Ag85 complex enzymes. The Ag85 complex is one of the promising targets for novel antimycobacterial drugs and vaccines. We present our recently developed high throughput screening (HTS) assays suitable for the identification of potential inhibitors against Ag85.

## 2. Discovery of the cord factor (Trehalose 6,6'-di-mycolate; TDM)

In 1884, Robert Koch described *M. tuberculosis* bacilli grown in culture as rope-like structures (Koch, 1884). More than half a century later, in 1947 Middlebrook found that the ability to form cords under specific conditions is an “essential accompaniment of virulence” (Middlebrook et al., 1947). In 1950 Bloch extracted the substance responsible for cord formation from virulent organisms and identified it as a “toxic substance” (Bloch, 1950).

The removal of the substance with petroleum ether resulted in somehow avirulent organisms but did not affect the growth of the bacilli. This suggested that the substance was located at the surface and since it was obtained only from “cordforming” organisms it was called “cord factor” (Bloch, 1950; Middlebrook et al., 1947). Six years later the cord factor was finally identified as trehalose 6,6'-di-mycolate (TDM) by Noll (Behling et al., 1993a; Noll, 1956). TDM is the most abundant glycolipid produced by virulent *M. tuberculosis* (Hunter et al., 2006a). TDM molecules consist of trehalose (TDM glycan-head). Trehalose is abundant in mycobacteria as a free component (Elbein & Mitchell, 1973; Elbein et al., 2003). In the cord factor trehalose is esterified to two mycolic acid residues and the residues length is variable from species to species (Fig. 1). Mycobacterial mycolic acids contain generally 20–80 carbons (Spargo et al., 1991).

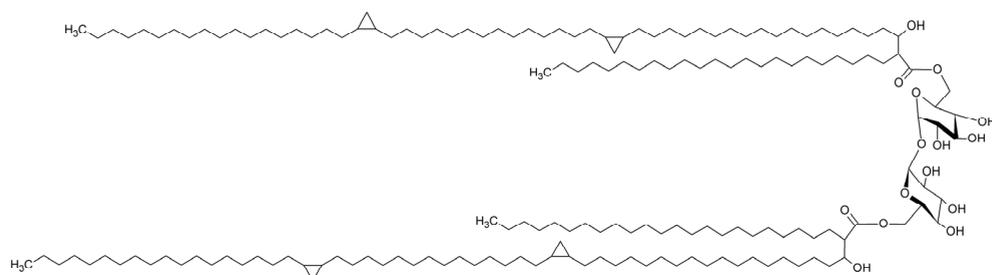


Fig. 1. Structure of trehalose 6,6'-di-mycolate (TDM, cord factor).

The cord factor is comprised of trehalose which esterified to two mycolic acid residues through their 6- and 6'-hydroxyl groups.

### 2.1 Effects of the cord factor on the immune system

TDM inhibits the process of phagosome-lysosome fusion and is thus a key compound for the survival of the bacillus inside the host's phagosomes (Indrigo et al., 2002). TDM induces a broad range of cytokine secretion in the host's immune system, especially production of IL-1 $\beta$ , IL-6, and TNF in macrophages (Matsunaga & Moody, 2009). In the recent years it has been shown that TDM is a key driver of secondary and cavitary disease type of tuberculosis (Hunter et al., 2006b). Despite the various severe effects on the host immune system the host

receptor could not be identified. In 2009 Ishikawa et al. could demonstrate that macrophage inducible C-type lectin (Mincle) is an essential receptor for TDM (Ishikawa et al., 2009).

## 2.2 TDM as vaccine adjuvant

In the last years TDM has been used intensively as immunomodulatory and vaccine adjuvant (Behling et al., 1993b; Noll, 1956). TDM can reproduce several pathophysiologic properties of *M. tuberculosis* infection including granuloma formation and induction of proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF in macrophages (Matsunaga & Moody, 2009). TNF (cachexin) has several toxic effects on host physiology, including sepsis, fever syndromes and cachexia (Silva & Faccioli, 1988).

Doses as little as 1–5  $\mu$ g are granulomagenic in the lungs of mice (Bekierkunst et al., 1969). Moreover TDM increases the production of antibodies (Behling et al., 1993b; Perez et al., 1994; Perez et al., 2000) and it up-regulates the expression of MHCII on macrophages (Ryll et al., 2001). TDM also induces *in vivo* production of IL-12 (Oswald et al., 1997). Injected as mineral oil solution (Silva & Faccioli, 1988) TDM forms monolayers, where the mycolic acids are exposed. In the monolayer form TDM is highly toxic (lethal dose in mice: LD<sub>50</sub> ~30  $\mu$ g) and kills macrophages in minutes (Hunter et al., 2006b). In aqueous suspension TDM forms micelles, where the mycolic acid groups are completely covered and TDM is non-toxic (lethal dose in mice: LD<sub>50</sub> >50,000  $\mu$ g). Micellar TDM prevents phagosome/lysosome fusion and thus promotes the survival of mycobacteria in the macrophage. Nevertheless, until now there is no experimental evidence for the existence or formation of TDM micelles or monolayers *in vivo*. Due to its strong immunostimulatory effect, several studies have used TDM as a potential adjuvant in different vaccination models. In 1976 Saito et al. were the first who described the cord factor as good adjuvant in mice and rats but with only low adjuvant effect in guinea pigs (Saito et al., 1976). Lima et al. could show that microspheres, containing TDM with a Hsp65-encoding DNA plasmid, were able to protect vaccinated mice against virulent *M. tuberculosis* (Lima et al., 2003) and against *Leishmania major* infection (Coelho et al., 2006). The major problem using TDM as adjuvant is the relatively high toxicity of the mycolic acids and the accompanying contaminants during the preparation of TDM. A synthetic analog of the cord factor, trehalose-6,6-dibehenate (TDB), was shown to be an effective and safe alternative (Davidsen et al., 2005). TDB is less toxic compared with TDM and easier to produce, making it a potent candidate in the field of vaccine development.

## 3. Drug targets in the biosynthesis of the cord factor

The cord factor (trehalose 6,6'-di-mycolate) is composed of a sugar and a mycolic acid component. In the following section we present the trehalose and mycolic acid biosynthesis steps and the target enzymes in their biochemical context. Especially enzymes of mycolic acid biosynthesis, such as methyl transferase (PcaA) (Glickman et al., 2000),  $\beta$ -ketoacyl-acyl carrier protein synthase (KasAB and FabH) (Bhatt et al., 2007), acyl-AMP ligase (Fad32) (Portevin et al., 2005) and polyketide synthase (Psk13) (Portevin et al., 2004), are regarded as promising targets for anti-TB drug development.

### 3.1 Biosynthesis of trehalose

Mycobacteria possess three pathways for trehalose synthesis (Kaur et al., 2009). Trehalose can be synthesized from glucose-6-phosphate catalyzed by trehalose-6-phosphate synthase (OtsA, *Rv3490*) (Pan et al., 2002) and trehalose-6-phosphate phosphatase (OtsB2, *Rv3372*) (Pan et al., 2002). The second pathway generates trehalose from glycogen involving the maltooligosyltrehalose synthase (TreY, *Rv1653c*) and the maltooligosyltrehalose trehalohydrolase (TreZ, *Rv1562c*). In the third pathway maltose is converted to trehalose by the trehalose synthase (TreS, *Rv0126*). While all the three pathways are functional and essential for the proliferation of *M. smegmatis* (Woodruff et al., 2004), the OtsAB pathway is predominant and strictly essential in *M. tuberculosis* (Fig. 2). In the genome sequence of *M. tuberculosis* exist two *otsB* homologues, but only OtsB2 (*Rv3372*) has a functional role in the pathway. OtsB2 has been suggested as an attractive target for novel drugs due to absence of trehalose in mammalian cell (Murphy et al., 2005).

### 3.2 Trehalose transporters

The LpqY-SugA-SugB-SugCATP-binding cassette transporter is highly specific for uptake of the disaccharide trehalose. Since trehalose is not present in mammals, it is unlikely that this system is used for sugar acquisition from the host. Trehalose release is known to occur as a byproduct of the biosynthesis of the mycolic acid cell envelope by *M. tuberculosis* antigen 85 complex. The mycolyltransferases of the antigen 85 complex transfer the lipid moiety of the glycolipid trehalose monomycolate (TMM) to arabinogalactan or another molecule of TMM, yielding trehalose dimycolate. These reactions lead to a constant release of trehalose from the cell. The LpqY-SugA-SugB-SugC ATP-binding cassette has been suggested as transporter system (Fig. 2), recycling the released trehalose. Perturbations in trehalose recycling strongly impaired virulence of *M. tuberculosis*. (Kalscheuer et al., 2010). These sugar transporters are thought to play an important role in bacterial pathogenesis and have been suggested as target for tuberculosis chemotherapy (Kalscheuer et al., 2010).

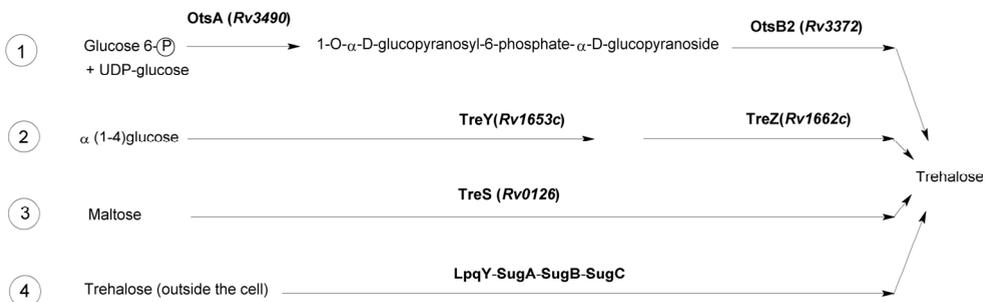


Fig. 2. Trehalose biosynthesis in *Mycobacterium tuberculosis*. 1, OtsAB pathway. 2, TreY-TreZ pathway. 3, TreS-pathway. 4, Trehalose import by an ATP-binding cassette transporter system.

### 3.3 Mycolic acid biosynthesis

Mycolic acids are  $\beta$ -hydroxy fatty acids with a long  $\alpha$ -alkyl side chain. They are homologous series of fatty acids differing by a two-carbon unit (Asselineau & Lederer, 1950). The mycolic acids are composed of an  $\alpha$  branch at the alpha position in respect to the carboxylic group and a meromycolate branch. The "short"  $\alpha$  branch contains species dependent 20-26 saturated carbon atoms. The "long" meromycolate branch has 50-60 carbon atoms and its chemical composition is highly variable, containing cyclopropyl or unsaturated bonds ( $\alpha$ -mycolates), methoxy (methoxymycolates) and keto (ketomycolates) groups (Alahari et al., 2007). The confusing denotation " $\alpha$ -mycolates" refers not to their position in the molecule but to their position on thin layer chromatography. The " $\alpha$ -mycolates" (cis, cis-cyclopropyl fatty acids) are the most abundant mycolic acids in *M. tuberculosis* (~57%), followed by methoxymycolates (32%) and ketomycolates (11%). The methoxy- and ketomycolates can have either the cis or trans configuration on the proximal cyclopropane ring. In summary there are five main classes of mycolic acids in *M. tuberculosis* (Schroeder et al., 2002; Takayama et al., 2005).

In *M. tuberculosis* mycolic acids are essentially provided via conventional fatty acid biosynthesis. Mycobacteria contain both type I and type II FAS fatty acid biosynthesis systems. Fatty acid biosynthesis is initiated by the multifunctional FAS I enzyme (*Rv2524c*), catalyzing the de novo synthesis of long-chain acyl-CoAs (C16:0 and C18:0) from acetyl-CoA and using malonyl-CoA as an extender unit. The domains of the FAS-I multienzyme-complex are organized in the following order: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein (ACP),  $\beta$ -keto reductase,  $\beta$ -ketoacyl synthase (Fernandes & Kolattukudy, 1996). In *M. tuberculosis* the C16:0- and C18:0-S-ACP adducts, are converted either to the CoA derivatives or further elongated by FAS I to produce C26:0 (Kikuchi et al., 1992). In mycobacteria the de novo fatty acid biosynthesis is exclusively carried out by FAS-I, whereas the FAS-II system performs only the elongation of the fatty acids, generated by FAS-I. The FAS I and FAS II systems are connected by a key condensing enzyme, the  $\beta$ -ketoacyl ACP synthase III or FabH, which catalyzes a decarboxylative condensation of malonyl-ACP with the acyl-CoA (C16:0-C20:0) products of the FAS-I system (Fig. 3). The resulting 3-ketoacyl-ACP product is reduced to an acyl-ACP (extended by two carbons) and shuffled into the FAS II cycle.

The ACP cycles the growing acyl chain between four enzymes MabA ( $\beta$ -ketoacyl reductase),  $\beta$ -hydroxyacyl dehydrase, InhA (enoyl reductase) and KasA/B ( $\beta$ -ketoacyl synthase). *M. tuberculosis* contains two  $\beta$ -ketoacyl synthases, KasA and KasB, which share 67% identity. KasA seems to be essential for growth, while KasB is not essential but produces longer carbon chains (Bhatt et al., 2005; Slayden & Barry, 2002; Swanson et al., 2009). The deletion of *KasB* in *M. tuberculosis* leads to mycolic acids that are 2-6 carbons shorter in length and a defect in trans-cyclopropanation of oxygenated mycolic acids. Phenotypically leads a deletion of *KasB* to a loss of acid-fastness (Bhatt et al., 2007). The most potent inhibitor for mycolic acid biosynthesis is isoniazid (INH). INH is a prodrug which is converted to the isonicotinoyl radical by KatG. INH forms a covalent adduct with NAD. This INH-NAD adduct inhibits FAS-II enoyl-ACP reductase InhA, which in consequence leads to inhibition of mycolic acid biosynthesis, and ultimately to cell death (Mdluli et al., 1998; Takayama et al., 1972; 1975; Wilming & Johnsson, 1999). In *M. tuberculosis*, the C26:0

fatty acids synthesized by FAS I will become the substrate of a dedicated acyl-CoA carboxylase (ACCase) to generate the  $\alpha$ -carboxy C26:0 fatty acid used as one of the substrate by the Pks 13 in the biosynthesis of mycolic acids (Gavalda et al., 2009). The last steps of the biosynthesis of mycolic acids are catalyzed by proteins encoded by the *fadD32-pks13-accD4* cluster. Pks13 ultimately condenses the two loaded fatty acyl chains to produce  $\alpha$ -alkyl  $\beta$ -ketoacids, the precursors of mycolic acids (Gavalda et al., 2009). FadD32 has been shown essential for growth (Carroll et al., 2011). Double bonds at specific sites on mycolic acid precursors are modified by the action of cyclopropane mycolic acid synthases (CMASs) such as MmaA1-A4, PcaA and CmaA2, which are S-adenosyl-methionine-dependent methyl transferases (Alahari et al., 2007). The antitubercular drug, thiacetazone (TAC) and its chemical analogues acts on CMASs, inhibiting mycolic acid cyclopropanation (Alahari et al., 2007; Alahari et al., 2009).

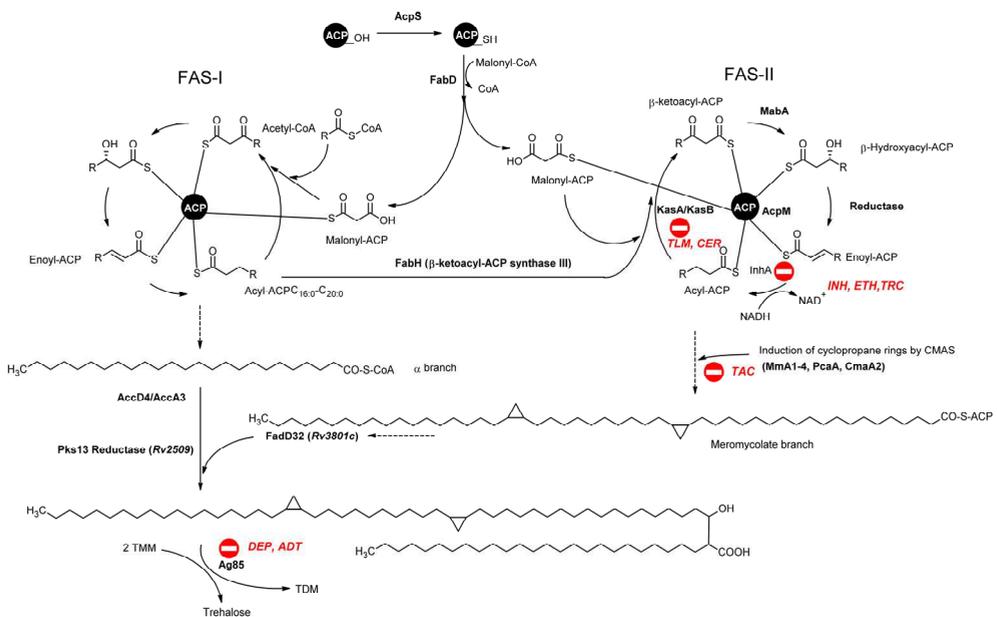


Fig. 3. Biosynthesis of mycolic acids for cord factor synthesis. Enzymes are in bold letters. Selected inhibitors are depicted in red bold letters. TLM, thiolactomycin. CER, cerulenin. ETH, ethionamide. INH, isoniazid. TRC, triclosan. TAC, thiacetazone. DEP, diethyl phosphate. ADT, 6-azido-6-deoxy- $\alpha,\alpha'$ -trehalose (See text for details).

### 3.4 TMM biosynthesis

TDM is thought to be synthesized exclusively outside the cell and its precursor TMM is transported outside the cell. In addition TMM has to be exported from the cytoplasm, to prevent the degradation of TMM inside the cell by the ubiquitously present Ag85/Fbp (Kilburn et al., 1982; Sathyamoorthy & Takayama, 1987). The mycolyl group is first transferred from mycolyl-S-Pks13 (mycolyl-S-PPB) to D-mannopyranosyl-1-phosphoheptaprenol by a proposed cytoplasmic mycolyltransferase I to yield Myc-PL 6-O-

mycolyl- $\beta$ -D-mannopyranosyl -1-phosphoheptaprenol (Myc-PL) (Besra et al., 1994). Myc-PL migrates to the inner surface of the cell membrane and docks next to an ABC transporter, with its hydrophobic heptaprenol tail. The mycolyl group is transferred to trehalose 6-phosphate by a proposed membrane-associated mycolyltransferase II to form TMM-phosphate, and the phosphate group is removed by the membrane-associated trehalose 6-phosphate phosphatase, yielding TMM. TMM is transported outside the cell by the ABC transporter (Fig. 4). There should be virtually no accumulation of TMM in the cytoplasm (Takayama et al., 2005).

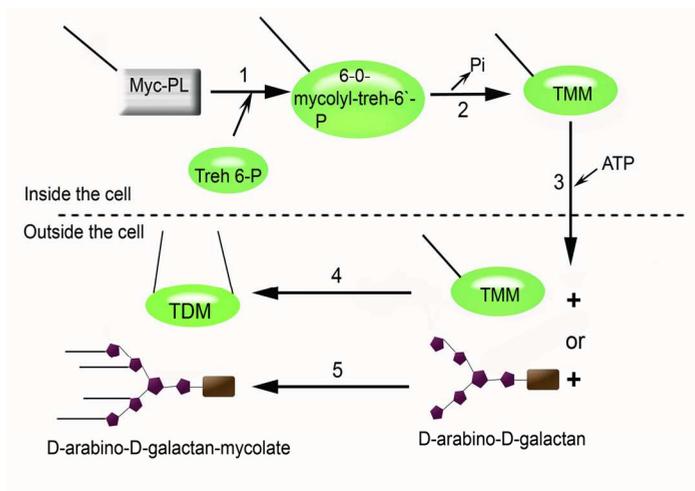


Fig. 4. The proposed process of incorporation of newly synthesized mycolic acids into major cell wall components. The process starts inside the cell. Newly synthesized mycolic acids are transferred to man-P-heptaprenol to produce 6-O-mycolyl- $\beta$ -D-mannopyranosyl-1-phosphoheptaprenol (Myc-PL) and after that to trehalose 6-phosphate to yield TMM-P by the proposed membrane-associated mycolyltransferase II (reaction 1). TMM is produced by dephosphorylation of TMM-P by the membrane-bound TMM-P phosphatase (reaction 2). The transportation of TMM to the outside is catalyzed by a proposed ABC transporter cassette (TMM transporter) (reaction 3). Outside the cell the Ag85 complex catalyzes the transfer of mycolate to another TMM and arabinogalactan to yield TDM (reaction 4) or arabinogalactan-mycolate (reaction 5) (Takayama et al., 2005).

### 3.5 TDM biosynthesis by Ag85

The antigen 85 complex is composed of Ag85A (FbpA), Ag85B (FbpB), and Ag85C (FbpC) as the predominant secreted proteins in *M. tuberculosis*. The corresponding genes are *fbpA* (*Rv3804c*), *fbpB* (*Rv1886c*), and *fbpC* (*Rv0129c*) (Belisle et al., 1997; Wiker & Harboe, 1992). The 85 complex proteins share 68–80% sequence identity (Belisle et al., 1997; Ronning et al., 2004). The mycolyltransferases of the antigen 85 complex are located outside the cell membrane and transfer the lipid moiety of the glycolipid trehalose monomycolate (TMM) to another molecule of TMM yielding trehalose dimycolate or to arabinogalactan to form cell wall arabinogalactan-mycolate (Fig. 4) (Sanki et al., 2009a).

There is also evidence, the Ag85 complex proteins bind to fibronectin and the fibronectin-binding property of the Ag85 complex is important for mycobacterium life cycle in the host and macrophages (Klegerman et al., 1994; Ronning et al., 2004). The crystal structures of the three 30–32 kDa proteins (Ag85A, B and C) have been determined (Anderson et al., 2001; Ronning et al., 2004). These proteins contain a carboxylesterase domain bearing the highly-conserved consensus sequence GXXSXXG. The interaction between Ag85 and fibronectin is mediated by the sequence homologous to residues 56–66 (FEEYYQSGLSV) of the recombinant *M. tuberculosis* Ag85C (Ronning et al., 2004). Up to date, the question remains open why *Mycobacterium tuberculosis* has three antigen 85 enzymes sharing the similar sequence and substrate specificity (Daffe, 2000; Ronning et al., 2004).

Ag85 complex members from *M. tuberculosis* belong to the  $\alpha/\beta$  hydrolase superfamily and catalyze the hydrolysis of ester and amide bonds using a catalytic triad comprised of Ser126, Glu230 and His262 in Ag85A/B and Ser124, Glu228 and His260 in Ag85C. All three enzymes contain two carbohydrate binding sites. The active site carbohydrate binding pocket binds TMM to form a temporary mycolate ester with the catalytic serine. The second carbohydrate binding site binds the incoming trehalose monomycolate, which “swings over” to the active site to displace the mycolate from its serine ester (Anderson et al., 2001; Ronning et al., 2004). The second trehalose binding site is separated from the acyl binding pocket by a bulky phenylalanine in Ag85A/B or a smaller leucine in Ag85C. All residues that form the active site carbohydrate binding pocket are 100% conserved in the *M. tuberculosis* antigen 85 proteins, while the surface of the acyl binding pocket, which is supposed to bind the long mycolate chains of TMM, exhibits slight differences. The conserved Leu152 in Ag85A and B is replaced by the bulky Phe150 in Ag85C, which in consequence leads to changes of surface topology in the mycolate binding portion (Fig. 5). The differences may alter substrate specificity and thus Ag85A, B and C might prefer different mycolic acids (Ronning et al., 2000; 2004).

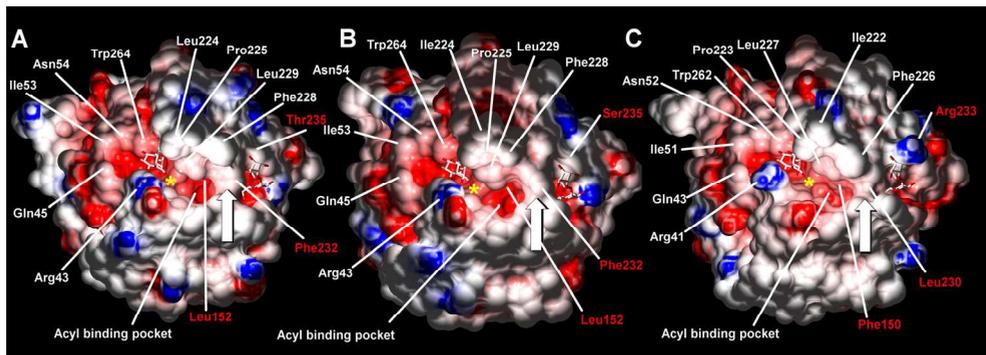


Fig. 5. Surface representation of Ag85A, B and C with two bound trehalose molecules. The trehalose molecules are depicted as ball-and-stick model. The position of the catalytic serine is indicated by a yellow asterisk. The carbohydrate binding pocket of all three proteins is 100% conserved (Arg43, Gln45, Ile53, Asn54, Trp264 in Ag85A and B) and Arg41, Gln43, Ile51, Asn52, Trp264 in Ag85C. Corresponding residues, which differ among the three proteins are shown with red labels. The separation of the second carbohydrate binding pocket from the acyl binding pocket by the Phe232 in Ag85A/B and the corresponding

smaller Leu230 in Ag85C is highlighted by an arrow. Also shown: Arginine 233, which covers the second carbohydrate binding pocket in Ag85C is replaced by smaller polar amino acids Thr235 and Ser235 in Ag85A and B, respectively. The surface is colored by electrostatic potential: The red and blue coloring represent negative and positive electrostatic potential, respectively. For Ag85A, B and C the coordinates from 1SFR, 1F0P and 1DQZ were used, respectively. The position of the trehalose molecules in Ag85A and Ag85C were modeled using the coordinates from 1F0P. The figure was prepared using GRASP (Nicholls et al., 1991).

### 3.6 Ag85 as a putative drug target for tuberculosis treatment

The ongoing treatment battle of tuberculosis is worsened by the emergence of new strains of *M. tuberculosis* which are resistant to standard antibiotics. In the urgent need of new targets the biogenesis of fatty acids, mycolic acids and glycolipids stay as hotspots. There is hope that the crystal structure of antigen 85A, 85B and 85C shall help in rational drug development for TB (Ronning et al., 2000; 2004).

The treatment by a trehalose analogue, 6-azido-6-deoxy- $\alpha,\alpha'$ -trehalose (ADT) inhibited the activity of all members of Ag85 complex *in vitro* and the growth of *Mycobacterium aurum*, and it also increased the efficacy of various antibiotics, supporting the importance of TDM (Belisle et al., 1997; Mizuguchi et al., 1983). *M. tuberculosis* strain lacking Ag85C has a 40% decrease in the amount of cell wall linked mycolic acid, but with no change in the relative amounts of TMM and TDM (Jackson et al., 1999; Sanki et al., 2009a). Furthermore, an Ag85A knockout strain lost the ability to grow in macrophage-like cell-lines and poor media which highlights the role of Ag85A in virulence and survival of the organism (Armitige et al., 2000). In the last decades several antitubercular drugs have focused on targets in the mycobacterial cell wall (Johnson et al., 2006). Most commonly, ethambutol targets the synthesis of arabinogalactan. Isoniazid and ethionamide inhibit biosynthesis of mycolic acids (Johnson et al., 2006). Obviously, the crystal structure of antigen 85 complex is expected to accelerate the design of new drugs against Ag85 activity and cord factor biosynthesis (Table 1) (Gobec et al., 2004; Sanki et al., 2009b; Wang et al., 2004).

## 4. Drug development: Novel high-throughput screening assays for mycolyltransferase 85A

Since the protein/substrate interactions and co-crystal structure of Ag85 are now known, the search for rapid assays for high-throughput screening (HTS) of large substance libraries has increased considerably. Most of the mycolyltransferase assays previously published are not suitable for HTS, due to their complexity or use of radioactive substances. The first one is a widely used radioassay which monitors enzymatic transfer of mycolic acids from a lipid-soluble TMM molecule to a radioactive water-soluble trehalose. Manipulation of the radioactive products in a two-phase reaction, extraction and thin layer chromatography allows visualization of the products (Kremer et al., 2002; Sathyamoorthy & Takayama, 1987). Another test published uses the substrate analogue p-nitrophenyl-6-O-octanoyl-H-D-glucopyranoside that functions as the acyl donor but it may not represent the natural enzymatic activity (Boucau et al., 2009). Also an excess of D-glucose is added to the reaction to function as an acyl acceptor and to promote turnover of the enzyme. Recently new assay

for Ag85 was developed based on the use of mono and dihexanoyl trehalose substrates, followed by quantitation of the acyl-transfer to the unnatural trehalose by mass spectrometry (Backus et al., 2011).

Synthesis step	Enzyme	Compound / class	References
<b>FAS-I and FAS-II</b>	KasA/KasB	Cerulenin (2R,3S-epoxy-4-oxo-7,10-trans,trans-dodecanoic acid amide)	(Schroeder et al., 2002) (Johansson et al., 2008)
<b>FAS-II</b>	KasA/KasB	TLM (Thiolactomycin)	(Douglas et al., 2002; Kremer et al., 2000; Luckner et al., 2010)
<b>FAS-II</b>	KasA/KasB	Platensimycin	(Brown et al., 2009)
	InhA	INH (Isoniazid)	(Slayden et al., 2000)
	InhA	ETH (Ethionamide)	(Kremer et al., 2003)
	InhA	TRC (Triclosan)	(McMurry et al., 1999)
	InhA	alkyl diphenyl ethers (Triclosan derivatives)	(Sullivan et al., 2006)
<b>Mycolic acid biosynthesis</b>	Unknown	2-(o-Tolyloxy)-5-hexylphenol (PT70)	(Luckner et al., 2010)
		N-octanesulfonylacetamide	(Parrish et al., 2001)
<b>Cyclopropanation</b>	CMA5s (cmaA2, mmaA2 or pcaA)	TAC (Thiacetazone)	(Alahari et al., 2007)
	MmaA4	TAC (Thiacetazone)	(Alahari et al., 2007)
<b>Cord-factor biosynthesis</b>	Ag85C	Methyl $\beta$ -D-arabinofuranoside	(Sanki et al., 2009b)
	Ag85C	5-S-alkyl-5-thio-arabinofuranoside analogues	(Sanki et al., 2008)
	Ag85 complex	Trehalose analogues	(Wang et al., 2004)
	Ag85 complex	ADT (6-azido-6-deoxy- $\alpha$ , $\alpha'$ -trehalose)	(Belisle et al., 1997; Mizuguchi et al., 1983)
	Ag85 complex	Phosphonate compounds	(Gobec et al., 2004; Gobec et al., 2007)
	Ag85C	DEP (Diethyl phosphate)	(Ronning et al., 2000)

Table 1. Inhibitors of cord factor biosynthesis

The mycobacterial glycolipids and TMM levels in the cell wall might give an indirect indication of the fitness of the cell inside the host cells specially in macrophages. Thus the quantitation of the mycobacterial TMM status after drug treatment may allow the estimation of drug effectiveness. Unfortunately, there is no method for measurement of the amount of glycolipids that is suitable for HTS. We designed an assay (Elamin et al., 2009) based on the use of natural substrate, and this mycolyltransferase assay offers a novel means to determine the TMM status of the mycobacterium cell wall and reflects the natural activity of mycolyltransferase enzyme based on simple steps. The new assay uses the natural substrate TMM, which can be easily purified from mycobacteria (Fujita et al.,

2005) and reflects natural activity, allowing to get the most accurate kinetic parameters. In the reaction Ag85A produces one molecule of trehalose as product per reaction cycle and by adding trehalase the trehalose converted to glucose, which can be easily measured (Fig. 6).

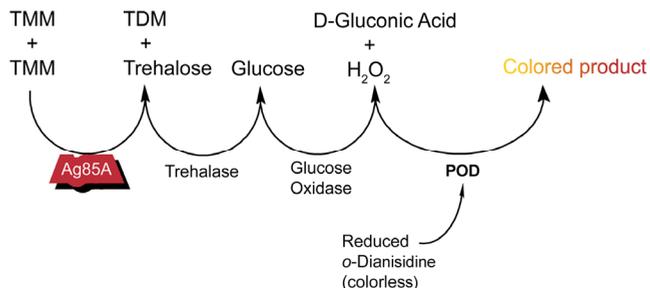


Fig. 6. Scheme for the new mycolyltransferase activity assay. Using trehalose that is produced as one of final products of mycolyltransferase reaction by trehalase to produce glucose, which is oxidized to gluconic acid and hydrogen peroxidase by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to produce a colored product (oxidized o-dianisidine), which will be converted to a stable colored product by sulfuric acid. The colored product is measured at 540 nm (Elamin et al., 2009).

Quantification of glucose is finally achieved by the glucose oxidase assay (Washko & Rice, 1961). The amount of glucose is proportional to the TMM concentration. The assay showed that the antigen 85A can be assayed in the presence of methanol or mixture of chloroform/methanol, which is usually used to extract and purify the glycolipids from mycobacterium cell wall fractions. The results from substrate/solvent experiments showed that the enzyme activity was reduced in the presence of organic solvents than in standard buffer reaction alone. This indicates and proves that this method is useful to quantify the TMM from the total lipid of mycobacterium cells.

One molecule of trehalose produced from TMM processed by Ag85 complex, which by our method is converted to two molecules of glucose. We can calculate the original concentration of TMM and concentration of TDM and trehalose in the reaction by the following equations:

$$\text{Concentration of [TMM]} = \text{concentration of [glucose]} \quad (1)$$

$$\text{Concentration of [TDM]} = \text{concentration of [glucose]}/2 \quad (2)$$

$$\text{Concentration of [trehalose]} = \text{concentration of [glucose]}/2 \quad (3)$$

One has to keep in mind that the extracted total lipids contain free trehalose and glucose and will affect the final calculations. In this case and to calculate the TMM concentration in total lipids one should run different negative controls. The Z' factor (Zhang et al., 1999) measurement of the current assay in different volumes indicates an excellent signal/ noise (S/N) ratio for the assay and its high potential for HTS applications (Table 2).

Reaction volume	200 $\mu$ l	300 $\mu$ l	350 $\mu$ l
Z' factor	0.67 $\pm$ 0.021	0.72 $\pm$ 0.014	0.73 $\pm$ 0.012

Table 2. The calculated Z' factor at different volumes from 96-well plate format assays.

## 5. Concluding remarks

Large gaps remain in our understanding of mycobacterium pathogenesis and persistence including the critical questions how bacteria survive in host cells and escape from the therapy. Future work on mycobacterial cell wall biosynthesis especially glycolipids and related pathways is expected to reveal *in vivo* drug-resistance mechanism. Perhaps more notably, the described new and low-cost colorimetric method based on use of TMM as natural substrate could bring flexibility and convenience in HT-screening of substance libraries and help in the development of novel drugs against tuberculosis.

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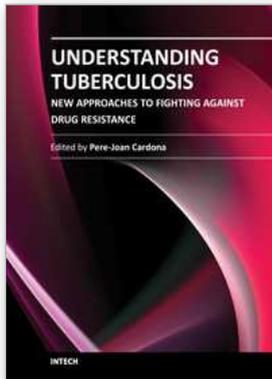
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## **Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance**

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-948-6

Hard cover, 376 pages

**Publisher** InTech

**Published online** 15, February, 2012

**Published in print edition** February, 2012

In 1957, a Streptomyces strain, the ME/83 (*S.mediterranei*), was isolated in the Lepetit Research Laboratories from a soil sample collected at a pine arboretum near Saint Raphael, France. This drug was the base for the chemotherapy with Streptomycin. The euphoria generated by the success of this regimen led to the idea that TB eradication would be possible by the year 2000. Thus, any further drug development against TB was stopped. Unfortunately, the lack of an accurate administration of these drugs originated the irruption of the drug resistance in *Mycobacterium tuberculosis*. Once the global emergency was declared in 1993, seeking out new drugs became urgent. In this book, diverse authors focus on the development and the activity of the new drug families.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ayssar A. Elamin, Matthias Stehr and Mahavir Singh (2012). The Cord Factor: Structure, Biosynthesis and Application in Drug Research – Achilles Heel of *Mycobacterium tuberculosis*?, *Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance*, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-948-6, InTech, Available from: <http://www.intechopen.com/books/understanding-tuberculosis-new-approaches-to-fighting-against-drug-resistance/the-cord-factor-structure-biosynthesis-and-application-in-drug-research-achilles-heel-of-mycobacteri>

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