Mamadou Daffé, Annaïk Quémard, and Hedia Marrakchi

Abstract

Mycolic acids are exceptionally long-chain fatty acids that are major and specific lipid components of the cell envelope of members of the *Corynebacteriales* order, which includes the causative agents of both tuberculosis and leprosy. These acids participate to the composition of the recently discovered “outer membrane,” a component unexpected for these Gram-positive microorganisms. Many proteins involved in mycolic acid biosynthesis and transport are essential for the mycobacterial survival and represent both validated targets and highly relevant candidates for the development of novel antimycobacterial agents, in the alarming context of multidrug-resistant tuberculosis.

Contents

1 Introduction ................................................................................... 2
2 Structures of Mycolic Acids ............................................................. 2
3 Mycolic Acid Conformational Packing into the Cell Wall ......................... 6
4 Mycolic Acid Biosynthetic Machinery and Regulation .............................. 7
  4.1 Biosynthesis of the Mycolic Acid Precursors .................................... 7
  4.2 Meromycolic Chain-Modifying Enzymes ........................................... 9
  4.3 Mycolic Acid Elongation Complexes ............................................. 12
  4.4 Mycolic Condensation and Transfer Onto Trehalose .......................... 13
  4.5 Translocation, Formation of the Mycolate-Containing Compounds, and Recycling . 14
  4.6 Mycolic Acid Biosynthesis Regulation ........................................... 15
5 Mycolic Acid Biogenesis as a Target for TB Drug Discovery .................... 17
  5.1 Isoniazid and Ethionamide .......................................................... 17
  5.2 Thiacetazone and Isoxyl ................................................................ 18
  5.3 Thiolactomycin (TLM) .................................................................... 19
1 Introduction

Originally, mycolic acids (MAs) were defined as C_{60}–C_{90} 2-alkyl 3-hydroxy fatty acids typifying the cell envelope of mycobacteria (Marrakchi et al. 2014; Quemard 2016), a group of microorganisms that may cause severe health-threatening diseases such as tuberculosis and leprosy. Structurally similar molecules with varying chain lengths have been later found in phylogenetically close microorganisms grouped in the Corynebacteriales order. MAs are found either linked to the cell wall arabinogalactan, the polysaccharide which, together with peptidoglycan, forms the insoluble cell-wall skeleton (Daffé and Draper 1998), or as esters of trehalose (or other polyols that may be present in the bacterial environment). Both forms are assumed to participate to the remarkable architecture and impermeability of the mycobacterial cell envelope as major lipid components of the two leaflets of the outer membrane, also called mycomembrane, unexpectedly found in these Gram-positive bacteria (Hoffmann et al. 2008; Sani et al. 2010; Zuber et al. 2008) (Fig. 1). In addition, MA-containing compounds have been associated with many physiological properties of mycobacteria such as their characteristic serpentine-like growth, called “cording,” biofilm formation, foamy macrophage formation in TB granulomas, and more largely in the pathogenicity process (Daffé and Draper 1998; Goren and Brennan 1979; Verschoor et al. 2012).

MA synthesis inhibition is one of the primary effects of the frontline and most efficient antitubercular drug isoniazid (INH) and several other inhibitors of the mycobacterial growth (Marrakchi et al. 2014; Quemard 2016). Accordingly, this metabolic pathway represents a niche of potential targets for the development of new antimycobacterial drugs. As such, their study has attracted much interest, especially in the alarming context of the emergence of drug-resistant tuberculosis. This chapter summarizes the current knowledge in the chemistry of mycolic acids, the biosynthetic pathways generating these compounds and their regulation, as well as their traffic through the cell envelope. It also describes some key biological roles played by these unique molecules.

2 Structures of Mycolic Acids

MAs are the longest fatty acids (FA) ever found in nature. They occur in all members of the Corynebacteriales order examined to date, with a very few exceptions (e.g., Corynebacterium amycolatum and C. kroppenstedtii). Mycobacteria produce
Fig. 1 Model, constituents and ultrastructure of the mycobacterial cell envelope. The outermost layer of the cell envelope, also called capsule in the case of pathogenic mycobacteria (Daffé and Draper 1998), is mainly composed of polysaccharides (a glycogen-like glucan, arabinomannan, and mannan) and proteins. Transmission electron microscopy (EM) micrographs of mycobacteria whole cell: upper panel: cryoEM showing native capsule, adapted from Sani et al. (2010); lower panel: cryo-electron microscopy of vitreous sections (CEMOVIS), adapted from Zuber et al. (2008), where the bilayer aspect of both the plasma membrane (PM) and the outer membrane (OM) is clearly visible. Scale bars: 20 nm. The OM, also called “mycomembrane,” represents a permeability barrier. Its inner leaflet is formed by a parallel arrangement of mycoloyl chains (in green) linked to arabinogalactan (AG), which in turn is covalently attached to peptidoglycan (PG), thus forming the mycoloyl-arabinogalactan-peptidoglycan complex (mAGP). The outer leaflet of the OM (in blue) is presumably composed of free lipids (noncovalently linked to the mAGP), which include trehalose dimycolate (TDM). Chemical representations (left) of TDM and mAGP show the very long-chains of mycolic acids that pack upon folding into “W-shape” to fit in a conventional membrane thickness of 7–8 nm. The periplasm (Peri) above the PM contains a granular layer (GL) composed of proteins.
specimens among the longest ones (C_{60}–C_{90}) (Fig. 2) but even longer molecules, called “ultra/extra-long-chain mycolic acids” (XL-MAs), were recently discovered in the *Segniliparus* genus and certain mycobacterial strains (Hong et al. 2012; Laneelle et al. 2013; Slama et al. 2016; Watanabe et al. 2001). MAs have been regarded as genus- and species-specific compounds and, consequently, were largely used as taxonomic markers (Daffé et al. 1983; Marrakchi et al. 2014); they consist in chains of 22 to 38 carbon atoms in *Corynebacterium*, 30 to 36 in *Hoyosella* and *Amycolicicoccus*, 34 to 38C in *Dietzia*, 34 to 52C in *Rhodococcus*, 46 to 60C in *Nocardia*, 46 to 66C in *Gordonia*, 64 to 78C in *Tsukamurella*, 60 to 90C in *Mycobacterium*, and up to 100C in *Segniliparus* (Marrakchi et al. 2014).

The first structure of mycolic acids has been described in 1950 (Asselineau and Lederer 1950) as α-branched, β-hydroxylated long-chain fatty acids, a feature that confers to the molecule the property to be cleaved at high temperature into a “mero”aldehyde main chain, also called “meromycolic” chain, and a “fatty acid,” a reaction similar to a reverse Claisen type condensation. The lengths of the released fatty acids range from C_{8} to C_{18} in *Corynebacterium* and up to C_{26} in the *M. tuberculosis* complex and *Mycobacterium xenopi* (Daffé et al. 1983). The stereochemistry of the centers at positions 2 and 3 has been shown to be conserved in all mycolic acid-containing genera, as 2R, 3R (Fig. 2) (Asselineau and Asselineau 1966; Asselineau et al. 1970a).

Structural elucidation of MAs has been addressed through the application of combined analytical techniques, notably thin-layer chromatography (TLC), gas chromatography, high pressure liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy. The structures of MAs of genera other than mycobacteria are relatively simple, being composed only of homologous series with various numbers of double bonds, up to six for some *Tsukamurella* species, borne by the meromycolic chain (Marrakchi et al. 2014; Tominay and Yano 1984). In contrast, MAs of mycobacteria display a large diversity of chain lengths and chemical functions, located at defined positions, the so-called proximal and distal (relative to the carboxyl group of MA), that define different classes, leading to complex TLC patterns (Daffé et al. 1983; Laneelle et al. 2015). The major homologues of the most apolar mycolic acids, referred to as α-MAs, contain 75–83 carbon atoms and generally two cis cyclopropyl groups, as found in *M. tuberculosis*, or two double bonds (of cis or trans configuration) encountered in many nontuberculous mycobacterial species such as *M. smegmatis*, and possibly a mixture of double bond and cyclopropyl group, located in the meromycolic chain (Fig. 2). A small fraction of α-MAs, XL-MAs, contains an extra unsaturated hydrocarbon segment, as observed in some strains of the *M. tuberculosis* complex (Watanabe et al. 2001; Slama et al. 2016), as well as in the *Segniliparus* genus (Laneelle et al. 2013). Polyunsaturated α-MAs were shown to represent a significant portion of the MAs in *M. fallax* (Rafidinarivo et al. 1985). MAs with 60–62 carbon atoms, known as α’-mycolic acids, and typical of *M. smegmatis*, contain one cis double bond (Fig. 2).

Moreover, in addition to the hydroxyl and carboxylic groups of the mycolic motif, MAs from most mycobacteria examined so far contain oxygen functions located in the distal part of the meromycolic chain, and defining the keto-,
**Non-oxygenated** (alpha) Mycolates

<table>
<thead>
<tr>
<th>Mycobacterium species</th>
<th>Type of mycolate: Functional groups</th>
<th>Structure</th>
<th>Carbon number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td>α CP cis / CP cis</td>
<td><img src="image1" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 1 CP cis / CP cis</td>
<td><img src="image2" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 1 CP trans / CP cis</td>
<td><img src="image3" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 2 CP cis / DB trans</td>
<td><img src="image4" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 3 CP cis / DB cis</td>
<td><img src="image5" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 4 DB cis / DB cis</td>
<td><img src="image6" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α 5 DB cis / DB cis</td>
<td><img src="image7" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>α / DB cis</td>
<td><img src="image8" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
</tbody>
</table>

**Oxygenated** Mycolates

<table>
<thead>
<tr>
<th>Mycobacterium species</th>
<th>Type of mycolate: Functional groups</th>
<th>Structure</th>
<th>Carbon number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td>Methoxy / CP cis</td>
<td><img src="image9" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>Methoxy / CP trans</td>
<td><img src="image10" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>Keto / CP cis</td>
<td><img src="image11" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>Keto / CP trans</td>
<td><img src="image12" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>Hydrox / CP cis</td>
<td><img src="image13" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>Wax / CP cis</td>
<td><img src="image14" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td><strong>M. smegmatis</strong></td>
<td>Epoxy trans / DB trans</td>
<td><img src="image15" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
<tr>
<td></td>
<td>1 Methoxy / DB cis / DB trans</td>
<td><img src="image16" alt="Structure" /></td>
<td>C76-C83</td>
</tr>
</tbody>
</table>

**Fig. 2** Structural features of mycobacterial mycolic acids (MAs). Detailed structures of known types of α-MAs from *M. tuberculosis* and *M. smegmatis* and representative types of “oxygenated” MAs from various mycobacterial species. The proximal and distal positions (relative to the carboxyl group of MA) on the various functions are indicated. The configuration of double bonds (DB) and cyclopropanes (CP) is either trans (with an adjacent methyl branch) or cis. The established functions of the MA methyl transferases (MA-MTs) are indicated. The stereochemistry of the asymmetric carbon atoms (S or R) is indicated. PcaA: proximal cyclopropane mycolic acid methyl transferase (MA-MT); MmaA1: *trans* cyclopropane MA-MT; MmaA2: *cis* cyclopropane MA-MT; CmaA2: cyclopropane MA-MT; MmaA3: methoxy MA-MT; MmaA4 (Hma): hydroxy MA-MT; UmaA1 (“unidentified MA-MT”): proximal *trans* double bond MA-MT. For each mycolate type, the carbon numbers of the major homologues, as determined by MALDI-MS and NMR (Laval et al. 2001, 2008), are indicated.
methoxy-, wax ester-, epoxy-, and hydroxy-types of MAs (Fig. 2); these so-called oxygenated MAs are absent from a few nontuberculous mycobacterial species such as *M. chelonae* and *M. abscessus* (Daffé et al. 1983). The additional oxygen functions occurring in mycobacterial MAs are typified by an adjacent methyl branch. It is noteworthy that in *M. tuberculosis*, the oxygenated MAs, i.e., keto-, methoxy-, and hydroxy-MAs, contain around 84–88 carbon atoms, 4–6 carbons longer than the α-MAs from the same strains (Laval et al. 2001; Watanabe et al. 2001). In sharp contrast, the chain lengths of epoxy- and wax ester-MAs (Fig. 2) found in nontuberculous mycobacterial species, in which keto-, methoxy-, and/or hydroxy-MAs have never been detected, are similar to those of α-MAs of the same strains (Laval et al. 2001), suggesting a biosynthetic filiation between α- and oxygenated MAs in the latter species.

### 3 Mycolic Acid Conformational Packing into the Cell Wall

The implication of the MAs in the cell wall permeability barrier was known long ago. Mycobacteria are extremely impermeable to small molecules, including nutrients, such as glucose and glycerol, and antibiotics, contributing to the intrinsic resistance of mycobacteria to hydrophobic drugs (Jarlier and Nikaido 1994; Liu et al. 1996). This low permeability was correlated with the extremely hydrophobic surface attributed mainly to the high amount of MAs in the cell wall (Fig. 1). Indeed, analyses of mutants in which specific genes that encode proteins involved in the biosynthesis and transfer of MAs show correlation between the permeability of mycobacterial strains with the amounts and nature of their MAs (Draper 1998; Jackson et al. 1999).

The structural features of mycobacterial MAs at distal and proximal positions of the meromycolic chains, e.g., double bonds, cyclopropyl groups, and oxygenated functions (see Sect. 2) (Fig. 2), are essential for their physiological roles in maintaining the cell wall architecture. The importance of the fine structure of MAs was illustrated by studying the conformational behavior of different types of MAs by Langmuir monolayers experiments. According to the MA type, α or oxygenated, folded or extended conformations were adopted, depending on temperature (Villeneuve et al. 2005). Double bonds or cyclopropane rings are regularly spaced, around 14–17 methylene units, and methyl branches are found at both proximal and distal positions adjacent to the transunsaturations (Fig. 2). As demonstrated in monolayer studies (Villeneuve et al. 2005, 2007), the regular spacing facilitates folding and packing. Furthermore, the *trans* cyclopropanes, which cause a decrease in the cell wall fluidity (Liu et al. 1996), facilitate the folding of the oxygenated MAs into W-form conformations with the four hydrocarbon chains in parallel, allowing tight packing of the MAs in *M. tuberculosis* H37Rv (Villeneuve et al. 2013). In the *M. tuberculosis* avirulent strain H37Ra, the absence of *trans*-cyclopropane rings prevents the oxygenated MAs from adopting stable fully folded W-form conformations. This folding ability is critical in allowing MAs to be accommodated within the dimensions of the mycobacterial outer membrane (Fig. 1), whose thickness is
surprisingly conventional as revealed by cryo-electron microscopy (Hoffmann et al. 2008; Zuber et al. 2008).

The overall MA chain length is also a major determinant of cell wall fluidity (Liu et al. 1996). Thus, the presence of XL-MAs in M. tuberculosis might be one of the factors contributing to the extremely low permeability of its mycomembrane. It has been proposed that the XL-MAs might adopt a “tripleU-form” conformation where the additional segment of the meromycolic chain folds parallel to the other hydrocarbon chains, facilitating MA packing (Slama et al. 2016).

4 Mycolic Acid Biosynthetic Machinery and Regulation

4.1 Biosynthesis of the Mycolic Acid Precursors

MAs are the products of a mixed fatty acid synthase (FAS)/polyketide synthase (PKS) biosynthesis pathway (Fig. 3). Mycobacteria have the unique property of possessing two FAS systems (Bloch 1977), the “eukaryotic-type” FAS-I for de novo synthesis and the “bacterial-type” FAS-II for fatty acid (FA) elongation. A single gene (fas, Rv2524c) encodes the multidomain FAS-I protein required for the de novo synthesis cycle, where the FAs produced are long-chain acyl-CoAs with a bimodal distribution, C_{16}–C_{18} and C_{24}–C_{26}, which is a unique feature of the mycobacterial FAS-I among those of Corynebacteriales (Bloch and Vance 1977). The mycobacterial FAS-II elongates the C_{16}–C_{18} FAs to yield C_{18}–C_{30} acyl-ACPs in vitro and is incapable of de novo FA synthesis from acetyl-CoA, unlike the type II synthases of other bacteria (Odriozola et al. 1977). It is believed that the mycobacterial FAS-II system produces full-size meromycolic chains in vivo. The synthesis proceeds through the elongation of enzyme-bound intermediates, covalently linked to the mycobacterial acyl carrier protein AcpM (Rv2244), by iterative cycles, each comprising four main steps (Bloch 1977) (Fig. 3) to yield the precursors of the meromycolic chains. On the other hand, the long-chain C_{24}–C_{26} acyl-CoAs made by FAS-I subsequently constitute, after carboxylation by an acyl-CoA carboxylase (ACCase), the α-chain of MAs. The decarboxylative condensation of the latter with the activated meromycolic chain generates mature MAs (see Sect. 4.4) (Fig. 3).

Within FAS-II, InhA, MabA, HadB, and KasA are essential proteins (Bhatt et al. 2005; Parish et al. 2007; Sacco et al. 2007; Vilcheze et al. 2000), consistent with the essentiality of MA biosynthesis. In contrast, neither HadC nor KasB are essential (Bhatt et al. 2007a; Gao et al. 2003; Slama et al. 2016). Despite the high sequence similarity between KasA and KasB on the one hand, and HadA and HadC subunits of HadAB and HadBC enzymes on the other hand, they show different chain-length specificities (Kremer et al. 2002; Schaeffer et al. 2001). Several lines of evidence have indicated that HadAB and KasA enzymes are involved in the initial extension steps catalyzed by FAS-II, while HadBC and KasB enzymes are implicated primarily in the late extension steps leading to the formation of full length meromycolic chains (Bhatt et al. 2007a; Gao et al. 2003; Sacco et al. 2007; Slama et al. 2016). This biosynthetic scheme is supported by the differential distribution of the proteins
Fig. 3 Biosynthesis, inhibition, and regulation of mycobacterial mycolic acids (MAs). The biosynthetic pathway of MAs is initiated with the de novo synthesis of fatty acids operated by the mycobacterial fatty acid synthase (FAS)-I, followed by their elongation catalyzed by FAS-II system. The \(\beta\)-ketoacyl-ACP synthase III (MtFabH) links FAS-I to FAS-II by catalyzing the first condensation between acyl-CoA precursors (produced by FAS-I) and the extender unit malonyl-ACP to form \(\beta\)-ketoacyl-ACPs. The iterative FAS-II elongation cycles include four main steps. The meromycolic chains are differentiated through the introduction of functional groups by a family of dedicated methyltransferases, most likely from \(cis\) double bonds first introduced by unknown mechanisms (possibly desaturations, dehydration-isomerization, or simple isomerization). The carboxylation of acyl-CoAs (the FAS-I products) provides the alkylmalonate at the origin of the \(\alpha\)-branch of MAs. Activation of both substrates (meromycolic acid and acyl-CoA) occurs prior to their condensation that yields \(\alpha\)-alkyl \(\beta\)-ketoester of trehalose, itself likely reduced to form the mature mycolic acid under the form of a trehalose monomycolate (TMM). The latter is transported through the plasma membrane by MmpL3. The mycoloyltransferases (Ag85 complex) associated to the mycomembrane synthesize biologically active mycolate-containing compounds, the mAGP
among the members of the Corynebacteriales order (Sacco et al. 2007). For genera producing long meromycolic chains, like Hoyosella, Gordonia, orthologues of kasA, kasB, and hadABC genes are detected in these organisms, while kasB and hadC orthologues are absent from genera producing shorter meromycolic chains like Rhodococcus and Nocardia (Laneelle et al. 2012; Sacco et al. 2007). Interestingly, in Segniliparus, a genus in which very long MAs are synthesized, two copies of hadB, the gene encoding the catalytic domain of the dehydratases, exist in the genome (Laneelle et al. 2013). Consistent with this scheme, the deletion of the hadC gene of M. tuberculosis strongly inhibited the synthesis of the oxygenated MAs and blocked the formation of the XL-MAs bearing a fourth cyclopropanated meromycolic segment (Slama et al. 2016). Interestingly, mutants in putative additional mycobacterial dehydratases of M. smegmatis and M. abscessus have been constructed and shown to be affected in their MA profiles, suggesting that extra dehydratases of FAS-II might exist (Carrere-Kremer et al. 2015; Halloum et al. 2016).

4.2 Meromycolic Chain-Modifying Enzymes

4.2.1 Possible Origins of Double Bonds Present in the Meromycolic Chain

The presence of double bonds is observed in all MAs, from the simplest C32–C34 corynomycolic acids to the most complex mycobacterial MAs. In corynebacteria, which are devoid of FAS-II system, unsaturated MAs result from the condensation of two FAs with at least one molecule of oleic acid (C18:1Δ9). The mechanisms by which the other Corynebacteriales introduce double bonds in their longer chain MAs are still under investigation. Several mechanisms have been described for the formation of unsaturated FAs in bacteria. First, the aerobic desaturation performed on a yet-synthesized long chain and requiring molecular oxygen leads to the formation of oleic acid (C18:1Δ9) from stearic acid (C18) through the action of the Δ9 desaturase, reaction which is performed by DesA3 (Rv3329c) desaturase in mycobacteria (Phetsuksiri et al. 2003). Such a mechanism has been proposed for the formation of the cis double bond at the distal position in α-MAs (Asselineau et al.)
Consistent with this, a recent survey suggests the involvement of the putative desaturase DesA1 in the introduction of one double bond at an as yet undefined position in the α-MAs of *M. smegmatis* (Singh et al. 2016). The function of the third mycobacterial putative desaturase DesA2 remains unknown.

An alternative mechanism is the anaerobic desaturation reaction performed by the 3-hydroxydecanoyl-ACP dehydratase (FabA) of *Escherichia coli* FAS-II system. This dehydratase/isomerase enzyme generates cis-3-enoyl intermediates (Fig. 3), which are subsequently not reduced but further elongated by the β-ketoacyl-ACP synthase I (FabB) to long-chain monounsaturated FAs, thus diverting the nascent acyl-chain into the unsaturated fatty acid synthetic pathway (Rock and Cronan 1996). Several groups of bacteria, including mycobacteria, lack both FabA and FabB enzymes, although they synthesize unsaturated fatty acids and/or have an anaerobic metabolism. In *Streptococcus pneumoniae*, a novel enzyme was discovered, FabM, a trans-2-cis-3-enoyl-ACP isomerase, able to divert fatty acid synthesis to the unsaturated mode (Marrakchi et al. 2002). Candidate FabM-like proteins exist in the *M. tuberculosis* genome and may function in a FabM-like manner for the formation of the cis double bond of the meromycolic chain, followed by the elongation of the cis-3-enoyl FA (Fig. 3) (Bloch 1969; Etemadi 1967).

In the case of the mycolic acids produced by the *Nocardia* genus, the "nocardomycolic acids," a mechanism of head-to-tail condensation of palmitic acid after an ω-oxidation step, has been proposed based on structural studies and labeling experiments, consistent with the double bond location at ω16 (Bordet and Michel 1969; Tarnok and Rohrscheidt 1976). Similarly, the structural relationship between different types of mycolic acids from mycobacteria and the location of the motifs at similar positions in the meromycolic chains led to the hypothesis that the building of the latter could be the result of condensation between three common fatty acids (C₂₀, C₁₆, C₂₂). The position of double bonds or oxygenated groups would be determined by the length of the FA precursors and by the presence or absence of a methyl group (Asselineau et al. 2002). Is it noteworthy that among the cis double bond-containing mycolates, the shortest α'-MAs are remarkable in their invariable chain lengths of C₆₂:₁–C₆₄:₁ (Fig. 2) regardless of the species where they are found, e.g., *M. smegmatis*, *M. abscessus*, or the new genus *Segniliparus* (Laneelle et al. 2013). The fact that no further modifications (cyclopropane, methyl branch, oxygenated functions) are observed in the α'-MAs suggests a distinct pathway for their synthesis.

### 4.2.2 Insertion of Cyclopropane Functions and Methyl Groups
It is not clear yet at which precise step of the MA biosynthesis pathway the chemical functions are introduced in the meromycolic chain, although an array of data suggest that at least some of them are formed during the FAS-II elongation cycles (Asselineau et al. 1970b; Qureshi et al. 1984; Takayama and Qureshi 1978). These modifications are operated by a family of paralogous S-adenosylmethionine (SAM)-dependent MA methyltransferases (MTs), which have been thoroughly reviewed (Marrakchi et al. 2014). It is believed that these enzymes adopt a catalytic mechanism similar to the classical scheme described for the introduction of cyclopropanes.
catalyzed by the *E. coli* cyclopropane synthase (CPS) and of methyl branches and which is based on the modification of a *cis* double bond by the methyl groups derived from SAM (Lederer 1969). Indeed, among the eight homologous SAM-dependent MTs present in the genome of *M. tuberculosis* (Cole et al. 1998), four MTs, namely CmaA1, CmaA2, PcaA (proximal cyclopropanation of alpha-MAs, formerly UmaA2), and MmaA2, display very strong sequence similarities with the *E. coli* CPS, suggesting a common reaction mechanism. Inactivation experiments of the *M. tuberculosis* MT genes have established their respective functions (Fig. 2) and have corrected some false interpretations concluded from protein overexpression experiments (reviewed in Marrakchi et al. 2014).

Nevertheless, some questions are still pending, especially regarding the specific MA-MTs responsible for introducing methyl groups at the proximal position and adjacent to *trans* cyclopropanes in the oxygenated MAs of the *M. tuberculosis* complex species and to double bonds in the α- and epoxy-MAs of *M. smegmatis*. While the *umaA1* orthologue in *M. smegmatis*, the MSMEG0913 protein, has been shown to catalyze the formation of a *trans* cyclopropyl group or double bond with an adjacent methyl branch at the proximal position of both α- and epoxy-MAs (Fig. 2), the *umaA1* (Rv0469) knock-out mutant exhibits no change in the MA profile in *M. tuberculosis* although the mutant strain exhibited a hypervirulence phenotype in the SCID mouse infection model (Laval et al. 2008).

### 4.2.3 Introduction of Oxygenated Functions

The biosynthesis of both keto- and methoxy-MAs (Fig. 2), two classes of MAs found in members of the *M. tuberculosis* complex and selective slow-growers, is intimately linked to that of the common hydroxymycolate precursor, which is catalyzed by the MT Hma (MmaA4, Rv0642c), a SAM-dependent enzyme. The proposed mechanism is the transfer of a methyl branch onto a *cis* double bond followed by the addition of an adjacent hydroxy group from a water molecule (Dubnau et al. 1997; Yuan and Barry 1996). The *M. tuberculosis* hma mutant no longer produces keto- or methoxy-MAs or their hydroxylated precursor (Dubnau et al. 2000), while still able to synthesize an unusual α-MAs bearing a *cis* double bond in the distal position (Dinadayala et al. 2003). The occurrence and precise position of this distal double bond puts forth this ethylenic mycolate as the possible substrate of Hma. A recent study has proposed the *M. tuberculosis* Rv0132c protein as the missing hydroxyl-MA dehydrogenase (HMAD) that would convert the hydroxyl-MAs synthesized by Hma into keto-MAs (Fig. 2) (Purwantini and Mukhopadhyay 2013). However, this work has been performed using the heterologous co-expression of *M. tuberculosis* hma and Rv0132c genes in *M. smegmatis*, a species that has oxygenated MAs distinct from those of *M. tuberculosis* (Fig. 2). As heterologous expression of MA biosynthesis genes may lead to misinterpretation of their function (Glickman et al. 2001), the validation of Rv0132c function awaits the phenotypic analysis of a *M. tuberculosis* knock-out mutant. The hydroxyl-MAs or their meromycolic precursors are the substrates of MmaA3 (Rv0643c) to yield methoxy-MAs. Interestingly, the gene that encodes the MmA3 protein is either
truncated or mutated in mycobacterial strains devoid of methoxy-MAs (e.g., *M. leprae*, or the Pasteur strain of *M. bovis* BCG) (Dubnau et al. 1998).

The proteins involved in the biosynthesis of other oxygenated functions such as wax-, epoxy-, and ω-1 methoxylated MAs (Fig. 2) remain to be discovered. Nevertheless, from structural data, it is possible to formulate a few hypotheses. The wax-mycolates have been shown to derive from the keto-MAs by Baeyer-Villiger oxidation, which consists in the insertion of molecular oxygen that converts the ketone to the corresponding ester (Toriyama et al. 1982), as postulated earlier (Etemadi and Gasche 1965; Laneelle and Laneelle 1970). Flavin-containing Baeyer-Villiger monoxygenases are present in the *M. tuberculosis* and other mycobacterial genomes.

Based on structural analyses, notably investigating the global chain length as well as the stereochemistry of the asymmetric carbon bearing the methyl branch adjacent to the oxygenated function (Fig. 2), it appears that no structural relationship can be established between the epoxy- and the keto-MAs. In contrast, it is reasonable to postulate that the formation of epoxy-MAs could result from the transformation of an α-mycolate-type (ethylenic) intermediate having a trans double bond at the distal position with an adjacent methyl branch (Dinadayala et al. 2003).

The ω-1 position of the methoxy group found in a class of MAs of some rapid grower mycobacterial species (Fig. 2) raises a new question. As no methyl branch adjacent to the oxygen function is found in this compound, it is tempting to speculate that the precursor of such a product could be an intermediate with a terminal double bond. In this respect, it is interesting to mention that a careful analysis of MAs from *M. smegmatis* had shown the presence of minor amounts of MAs with a terminal double bond (Wong and Gray 1979). Furthermore, *M. smegmatis* was shown to perform ω-1 oxidation of hydrocarbons (Rehm and Reiff 1981).

### 4.3 Mycolic Acid Elongation Complexes

Based on genetic and biochemical interaction experiments between the various FAS-II components, a working model has been proposed in which different specialized FAS-II complexes would be interconnected (Veyron-Churlet et al. 2004) and interact with MA-MTs (Cantaloube et al. 2011). This model predicts the occurrence of at least three specialized FAS-II complexes involved in MA synthesis, each complex being constituted by a “FAS-II core” (formed by InhA, MabA, MtFabD) associated to specific condensing (Kas) enzyme and dehydratase (Had) heterodimer, thus defining their substrate specificity. An “initiation” FAS-II complex (I-FAS-II), containing MtFabH, might allow the channeling of acyl-CoA from FAS-I to FAS-II during their condensation with malonyl-ACP (Fig. 3). This step would be followed by a type-I “elongation” complex (E1-FAS-II) formed by the core proteins and KasA plus HadAB, which would elongate medium-length acyl-ACP chains that could serve as substrates for a type-II elongation complex (E2-FAS-II). The latter, comprising the core and KasB plus HadBC, could end the synthesis of the meromycolic chain. This end-product of E2-FAS-II might be used in the terminal condensation
reaction catalyzed by Pks13, which is assumed to be part of a “termination” FAS-II (T-FAS-II) complex. Interestingly, these complexes also colocalized with cell division enzymes at the poles and septa of the growing mycobacteria (Carel et al. 2014).

4.4 Mycolic Condensation and Transfer Onto Trehalose

The ultimate biosynthesis steps of the MA biosynthesis are performed by the mycolic condensation system (Fig. 3). The latter catalyzes the condensation between a meromycolic chain provided by the FAS-II system and a fatty acyl chain provided by the FAS-I system (Gavalda et al. 2009), generating a C_{60–C_{100}} α-alkyl β-ketoacyl intermediate. This highly insoluble molecule is transferred onto a polar carrier, trehalose (Gavalda et al. 2014). The reduction of the β-ketone function of the lipid moiety (Lea-Smith et al. 2007) results in the formation of the α-alkyl β-hydroxy mycolic motif, generating a mature mycoloyl chain (Fig. 3). A candidate gene cluster, fadD32-pks13-accD4, was identified for these steps, highly conserved in Corynebacteriales (Portevin et al. 2004). Phenotypic studies of corynebacterial deletion mutants and mycobacterial conditional mutants have demonstrated the requirement of the three genes for mycolic acid production and their essentiality in M. smegmatis (Portevin et al. 2004, 2005). The different reaction steps catalyzed by the type-I polyketide synthase Pks13 (Rv3800c) and its partner protein FadD32 have been thoroughly deciphered (Gavalda et al. 2009; Leger et al. 2009). In contrast to known PKSs, which universally use short building blocks such as malonyl-CoA and methyl-malonyl-CoA, Pks13 appears as an atypical PKS performing a single Claisen-type condensation cycle between exceptionally long starter units (meromycolic chains) and extender units (carboxylated C_{22–C_{26}} acyl chain) (Fig. 3) (Gavalda et al. 2009).

Regarding the role of FadD32 (FAAL32, Rv3801c), the protein first catalyzes the activation of the very long-chain mero-MAAs produced by FAS-II into meromycoloyl-AMPs (Fig. 3) (Leger et al. 2009; Trivedi et al. 2004), a feature consistent with the protein crystal structures showing the long fatty acyl chain of the co-crystallized substrate analog buried in a hydrophobic tunnel close to the enzyme active site (Guillet et al. 2016). Then, FadD32 loads the meromycoloyl chain onto the P-pant arm of the N-terminal ACP domain of Pks13, thereby displaying a unique acyl-ACP ligase function, where the final acceptor is the P-pant arm of an ACP unit (Gavalda et al. 2009; Leger et al. 2009), in agreement with the identification of a putative P-pant binding site (Li et al. 2015).

Pks13 was shown to load the extender unit onto its C-terminal ACP domain via its acyltransferase (AT) domain (Gavalda et al. 2009). The latter plays the gatekeeper role, selecting the appropriate long-chain 2-carboxyacyl-CoA substrates using a dedicated channel evidenced by crystallography (Bergeret et al. 2012). Data strongly suggest that the long chain acyl-CoA carboxylase (ACCase) that provides the extender units to Pks13 includes at least AccA3 and AccD4 (Fig. 3) (Gande et al. 2004; Oh et al. 2006; Portevin et al. 2005). Yet the question of a putative involvement of additional subunits, AccD5 and AccE5, has been raised (Bazet Lyonnet et al. 2014).
After transfer of the carboxyacyl chain onto the C-terminal ACP domain of Pks13, the keto synthase domain achieves its condensation with the meromycoloyl chain to produce an \( \alpha \)-alkyl \( \beta \)-ketoacyl chain (Fig. 3) (Gavalda et al. 2009). Given the high hydrophobicity of Pks13 products, it was hypothesized that they would not be released as free acids but directly transferred onto a hydrophilic acceptor unit by discrete unknown mycoloyltransferases (Takayama et al. 2005). In contrast to this model, we have discovered that the thioesterase (TE)-like domain of Pks13 itself catalyzes the cleavage of the thioester bond with the condensation products. Following an unprecedented mechanism, the TE-like domain subsequently performs the intermolecular transfer of these chains onto trehalose, leading to the formation of \( \alpha \)-alkyl \( \beta \)-ketoacyl trehalose (Fig. 3) (Gavalda et al. 2014). Consistently, a putative trehalose-binding pocket was identified in the catalytic site of the TE-like domain (Gavalda et al. 2014). The mature MA chains are formed after the reduction of Pks13 products by the CmrA protein (Lea-Smith et al. 2007). Corynebacterium and \( M. \) smegmatis \( \text{cmrA} \) deletion mutants produce \( \alpha \)-alkyl \( \beta \)-ketoacyl trehalose (Bhatt et al. 2008; Lea-Smith et al. 2007), suggesting that the reduction step occurs on this trehalose derivative, leading to the synthesis of trehalose monomycolate (TMM) (Fig. 3).

4.5 Translocation, Formation of the Mycolate-Containing Compounds, and Recycling

Independent surveys have recently indicated that the inhibition of mycobacterial MmpL3 protein (mycobacterial membrane protein large 3, \( \text{Rv0206c} \)) activity and the conditional mutation of the \( \text{mmpL3} \) essential gene abolish the translocation of MAs, resulting in a decrease in trehalose dimycolate (TDM) and cell-wall-bound MAs, both anchored in the outer membrane and in intracellular accumulation of TMM (Grzegorzewicz et al. 2012a; Tahlan et al. 2012; Varela et al. 2012). The MmpL3 transporter, which belongs to the resistance-nodulation-division (RND) superfamily, is responsible for the export of mycoloyl chains, most likely under the form of TMM (Fig. 3). Interestingly, the nonessential MmpL11 (\( \text{Rv0202c} \)) transporter, whose gene is located in the vicinity of \( \text{mmpL3} \), would also play a role in the translocation of MA- or mero-MA-containing compounds, the monomeromycoloyl diacylglycerol (MMDAG) and a newly identified mycolate ester wax (Pacheco et al. 2013). MmpL3 and MmpL11 must be involved in two independent transport apparatuses since \( \text{mmpL11} \) inactivation has no impact on the TDM and cell-wall-linked MA contents. Its essentiality, both in vitro and during infection in mice (Li et al. 2016), underlines the preponderant role of the MmpL3-dependent export pathway over that of MmpL11 for the physiology of mycobacteria. The three active mycoloyltransferases of the antigen 85 complex (Belisle et al. 1997; Puech et al. 2002), Ag85A, Ag85B, and Ag85C (FbpABC, \( \text{Rv3804c} \), \( \text{Rv1886c} \), \( \text{Rv0129c} \)), whose respective specific roles remain unclear, perform the ultimate steps of the mycolate-containing compound production (for review, see Tang et al. 2012). In corynebacteria, part of the mycoloyltransferases (De Sousa-D’Auria et al. 2003)
presumably remains associated to the outer membrane (Marchand et al. 2012). Ag85ABC complex uses the TMM as a donor of mycoloyl chains, which they transfer onto selected acceptors for the biosynthesis of the mAGP complex and the mycolate-containing lipids, including TDM, used for the outer membrane biogenesis (Fig. 3) (see Sect. 3). In M. tuberculosis, the extracellular trehalase released during these reactions might be recycled by the LpqY-SugABC ATP-binding cassette (ABC) transporter that mediates trehalase retrograde transport (Kalscheuer et al. 2010) (Fig. 3). Thus, the latter step important for M. tuberculosis virulence might be needed for further MA biosynthesis specially during the infectious process since the trehalase is absent in the mammal hosts. The newly synthesized TDM can be hydrolyzed by a serine esterase (MSMEG_1529, TDM hydrolase) discovered in M. smegmatis, thus releasing free MAs (Ojha et al. 2010). This enzymatic activity, also detected in M. tuberculosis, is necessary for an efficient biofilm growth, which requires the formation of a free MA-rich extracellular matrix (see Sect. 6.3 and Fig. 4). Free MAs can also be released from the mAGP complex by the action of Lysin B (LysB) esterase produced by mycobacteriophage D29 in M. smegmatis (Payne et al. 2009). Importantly, the mutation of the mce1 operon in M. tuberculosis produces an accumulation of MAs in the cell wall or the culture supernatant, with a concomitant upregulation of genes involved in MA transport and metabolism (Cantrell et al. 2013; Forrellad et al. 2014; Queiroz et al. 2015). As mce1 operon is negatively regulated during mouse infection (Uchida et al. 2007), a model is emerging where free MAs would also be recycled by the cell wall putative ABC lipid importer Mce1 to be used as substrates of lipid biosynthesis and/or as carbon sources under starvation conditions, according to the concept that pathogenic mycobacteria switch their metabolism from carbohydrate to lipid pathways during their intracellular life (Forrellad et al. 2014). The free MAs might also serve as a barrier against host aggression or contribute to the organization of the bacilli in biofilms (Cantrell et al. 2013).

4.6 Mycolic Acid Biosynthesis Regulation

Mycobacterial lipids are essential structural constituents of the cell wall and their biosynthetic pathways are rigorously regulated at both genetic and biochemical levels. In M. tuberculosis, transcriptional regulators of MA metabolism have been identified, FadRmt (Rv0494) and MabR (Rv2242), both functioning as negative regulators of FAS-II genes (Biswas et al. 2013; Mondino et al. 2013; Salzman et al. 2010). MabR functions as a repressor of fabD-acpM-kasA-kasB-accD6 gene cluster essential for MA synthesis, while also affecting the expression of fas, which encodes the multifunctional FAS-I enzyme that supports phospholipid and triglyceride synthesis and provides the FAS-II system with precursors.

On the other hand, protein phosphorylation by serine/threonine protein kinases (STPKs), which can be reversed by protein phosphatases, is emerging as a major post-translational regulatory mechanism of fundamental biological processes in mycobacteria, including MA biosynthesis (Av-Gay and Everett 2000; Greenstein...
Under stress conditions, mycobacterial cells sense various signals and cues which promote their adaptation to environmental changes through numerous regulation processes. Amongst these, MA metabolism is down-regulated by transcriptional regulators (FadR, MabR) acting on genes encoding FAS-II proteins. On the other hand, post-translational modifications mediated by Ser/Thr Protein Kinase (STPK)-dependent phosphorylation of MA biosynthesis enzymes allow a direct and tight control of MA biosynthesis. Auto-phosphorylation of the mycobacterial STPKs (Pkn) and subsequent phosphorylation of their substrates are reversible; the dephosphorylation, which adds another level of regulation, is operated by the Ser/Thr protein phosphatase (PstP). Mycobacteria have the capacity to assemble into serpentine-like structures called “cords.” They are also able to form biofilms, where the bacilli are embedded in a mycolic acid-rich matrix. These structures of mycobacterial populations would play a role during the infectious process. During the latter, the ability of pathogenic mycobacteria to potentiate the host immune response is critical for the virulence and persistence of the pathogen. The lipid components of the mycobacterial envelope, and specifically the MA-containing compounds, play determinant immunomodulator roles.
et al. 2005; Wehenkel et al. 2008), with repercussions on the physiology and virulence of *M. tuberculosis*. In MA metabolism, many proteins have been identified as substrates for the STPKs (Figs. 3 and 4) and their phosphorylation was found to modulate their enzymatic activity to tightly control MA synthesis (Bhatt et al. 2007a; Molle and Kremer 2010). Phosphorylation of enzymes of the *M. tuberculosis* FAS-II system (Figs. 3 and 4), KasA, KasB, InhA, MabA, and the dehydratases HadAB and HadBC were found to down-regulate their respective activities (Molle and Kremer 2010; Slama et al. 2011; Vilcheze et al. 2014). Similarly, phosphorylation of the methyl transferase PcaA inhibits MA cyclopropanation, thus modulating intracellular survival of mycobacteria (Corrales et al. 2012). The proportion of phosphorylated HadAB and HadBC clearly increases at the stationary growth phase, suggesting that mycobacteria use this regulatory mechanism to tightly control mycolic acid production under nonreplicating conditions (Slama et al. 2011). It was recently shown that the activity of FadD32, a key enzyme of the mycolic condensation complex (see Sect. 4.4, Fig. 3), is also down-regulated by phosphorylation (Le et al. 2016). Identification of numerous STPK substrates brings to light the various combinations by which the bacillus can regulate its MA synthesis to promote adaptation to environmental changes. In a recent survey, our group has shown that when mycobacteria encounter starvation conditions during infection, this leads to a global down-regulation, partially mediated by the stringent response, of genes required for MA biosynthesis and transport, likely to slow down this highly energy-consuming process during nutrient scarcity (Jamet et al. 2015a). Therefore, at least two levels of regulation put forth a sophisticated manner in which MA metabolism is tightly regulated in order to efficiently adapt lipid composition to the different conditions that the microorganism faces during the complex life cycle within the host (Fig. 4).

5 Mycolic Acid Biogenesis as a Target for TB Drug Discovery

The MA biosynthesis and transport pathways represent one of the “Achilles’ heels” of the tubercle bacillus. Indeed, several drugs, specifically used in the chemotherapy of tuberculosis as well as novel candidates which are in the tuberculosis drug development pipeline, target these pathways. We report below the modes of action of some of these molecules.

5.1 Isoniazid and Ethionamide

Isoniazid (INH) is a first-line drug introduced in the TB chemotherapy in the early 1950s. The drug has been shown to directly inhibit the mycobacterial MA production (Quemard et al. 1991; Takayama et al. 1972), resulting in an alteration of the bacterial poles and subsequently amounting to profound morphologic changes (Bardou et al. 1996; Vilcheze et al. 2000). INH is activated by the catalase-peroxydase KatG leading to the formation of intermediate and highly reactive radicals (Johnsson and Shultz 1994; Quémard et al. 1996; Sinha 1983; Zhang et al. 1992). Ethionamide (ETH), a second-line
TB prodrug structurally related to INH, is activated by a distinct enzyme, the NADPH-specific flavin adenine dinucleotide–containing monooxygenase EthA (or EtaA) (DeBarber et al. 2000; Hanoulle et al. 2006). The overexpression or mutation of the \textit{inhA} gene confers resistance to both INH and ETH (Banerjee et al. 1994), strongly suggesting that the InhA enzyme is the primary target of both drugs. InhA was shown to be involved in the mycolic acid biosynthesis (Banerjee et al. 1994) and to catalyze the 2-\textit{trans}-enoyl-ACP reduction step of the FAS-II system (Marrakchi et al. 2000; Quemard et al. 1995). Mutations in InhA conferring resistance to INH and ETH map to the NADH-binding pocket and induce a loss of affinity of InhA for its cofactor (Basso et al. 1998; Desse et al. 1995; Quémard et al. 1995), consistent with the requirement of NADH for an efficient binding of INH and ETH to InhA (Quémard et al. 1996). Crystallographic studies showed that activated INH and ETH covalently bind to the nicotinamide ring of NADH within the InhA active site, generating inhibitory adducts (Rozwarski et al. 1998; Wang et al. 2007). The KasA enzyme of the FAS-II system had also been proposed as being the primary target of INH (Mdluli et al. 1996), but a thorough study has clearly shown that this was not the case (Kremer et al. 2003).

5.2 Thiacetazone and Isoxyl

Thiacetazone (TAC) and isoxyl (ISO, thiocarlide) are structurally related thiourea prodrugs. TAC differs from other TB drugs in being entirely bacteriostatic (Mitchison 1998). Introduced in 1946, it was progressively replaced by other molecules because of its relatively high toxicity. ISO was also successfully used for the clinical treatment of tuberculosis during the 1960s but fell from clinical use due to low bioavailability and untoward absorption kinetics. Both TAC and ISO must be activated via \textit{S}-oxidation of their thiocarbonyl moiety by the monooxygenase EthA that also activates ETH (Dover et al. 2007; Kordulakova et al. 2007). They possess multiple targets within mycobacteria, such as MA methyltransferases, epoxide hydrolases, and the stearoyl-CoA \textit{Δ}9 desaturase (Alahari et al. 2007; Brown et al. 2011; Phetsuksiri et al. 2003). These enzymes, however, unlikely constitute primary targets because of their dispensability for growth. In contrast, ISO and TAC treatments induce a complete inhibition of the essential MA biosynthesis, suggesting that their primary target (s) reside within this pathway (Winder 1982). Yet their precise mode of action has long remained unknown. The recent observation of the accumulation of long chain 3-hydroxyacids in TAC- or ISO-treated \textit{M. tuberculosis} pointed to the (3R)-hydroxyacetyl-ACP dehydration step of the FAS-II cycle as the possible target (Grzegorzewicz et al. 2012b). Consistent with this, overexpression of \textit{hadABC} genes encoding the FAS-II dehydratases or certain missense mutations in \textit{hadA} or \textit{hadC} conferred resistance to both drugs (Belardinelli and Morbidoni 2012; Gannoun-Zaki et al. 2013; Grzegorzewicz et al. 2012b). It was later shown that both ISO and TAC active forms covalently react with a specific cysteine residue (Cys61) of the HadA subunit, resulting in the inhibition of HadAB enzyme activity (Grzegorzewicz et al. 2015). Frameshift and missense mutations affecting Hma and MmaA2 were also found in \textit{M. tuberculosis} ISO and TAC spontaneous resistant mutants. The favored
hypothesis is that these MA-MTs would play a role in the action of these antibiotics via protein-protein interactions with FAS-II dehydratases (Grzegorzewicz et al. 2012b).

5.3 Thiolactomycin (TLM)

Most of the interest was driven towards the natural molecule produced by *Nocardiia*, thiolactomycin (TLM), which is specific for type-II synthases and exhibit broad activity towards Gram-negative and Gram-positive bacteria, and towards important pathogens, including *Plasmodium falciparum* (Kremer et al. 2000). TLM was shown to inhibit MA biosynthesis in vitro and retains good antitycobacterial activity in vivo (Douglas et al. 2002). TLM, composed of a thiolactone ring, competes malonate in binding in the same region of the active site of the condensing enzymes. The drug targets both *M. tuberculosis* KasA and KasB, the former being the more sensitive. Yet analogs of TLM may represent better inhibitors of the mycobacterial KasA enzyme (Kapilashrami et al. 2013).

5.4 Novel Molecules Targeting MA Biosynthesis and Translocation

In the context of MDR and XDR tuberculosis, targeting the MA pathway targeted by a frontline antibiotic (INH) is an effective strategy for anti-TB drug discovery by potentially bypassing existing resistances to current pathway inhibitors. This is particularly illustrated by the recent identification of inhibitors of the essential mycolic condensation enzymes Pks13/FadD32 and MA translocation and transfer enzymes MmpL3/Ag85 (Fig. 3).

The operon *fadD32-ks13-accD4* is essential for the viability of mycobacteria (Portevin et al. 2004, 2005) and was established both as a vulnerable target (Carroll et al. 2011) and “druggable” (Galandrin et al. 2013). Recently, the 4,6-diaryl-5,7-dimethyl coumarins, with the most potent CCA34 compound, were shown to kill *M. tuberculosis* by inhibiting FadD32 activity and effectively blocking bacterial replication in vitro and in animal models of TB (Stanley et al. 2013). Thiophene (TP) compounds bind to the N-ACP domain of Pk13 interfering with the activity of the enzyme and MA synthesis, thus leading to mycobacterial cell death (Wilson et al. 2013). A benzofuran compound, killing mycobacterial cells and discovered by combining high-throughput screening (HTS) with whole-genome sequencing (WGS) of resistant isolates, interferes with the function of the thioesterase domain of Pks13 (Ioerger et al. 2013).

The above HTS campaign with WGS has also identified a compound that inhibits the inner membrane transporter MmpL3 (Rv0206c). The latter is also targeted by the adamantyl urea derivative AU1235, the pyrrole derivative BM112 and the TB drug candidate SQ109, which all have potent bactericidal activities against *M. tuberculosis* (Grzegorzewicz et al. 2012a). These molecules abolish the translocation of trehalose monomycolates (TMM) to the envelope, resulting in a decrease in
TDM and cell-wall-bound MAs (see Sect. 4.5, Fig. 3) (Grzegorzewicz et al. 2012a; La Rosa et al. 2012; Tahlan et al. 2012). These compounds, like others, inhibit MmpL3 in replicating *M. tuberculosis* bacilli most likely by dissipating the transmembrane electrochemical proton gradient of the inner membrane where MmpL3 is located, which explains why such a large diversity of pharmacophores display inhibitory activities against MmpL3 (Li et al. 2014). Recently, optimized analogs of rimonabant, a cannabinoid receptor modulator structurally related to BM112, were also found to be potent inhibitors of *M. tuberculosis* growth (Ramesh et al. 2016). Moreover, a phenotypic screening against the pathogen *M. abscessus* revealed a piperidinol derivative as a potentially MmpL3-binding inhibitor (Dupont et al. 2016). Importantly, a new optimized indolecarboxamide likely targeting MmpL3 shows excellent activities against sensitive, MDR and XDR *M. tuberculosis* strains and in the TB aerosol lung infection model, and works in synergy with rifampin (Stec et al. 2016).

The mycoloyltransferase Ag85C protein was validated as a drug target by detailed characterization of an Ag85C-binding molecule, a benzothiophene derivative (I3-AG85), that exhibits an antibacterial activity towards *M. tuberculosis* growing both in vitro and within macrophages (Warrier et al. 2012) by blocking Ag85-mediated TDM synthesis with no effect on cell wall-bound MAs.

EthR (Rv3855) is a TetR-type repressor of the EthA enzyme, the common activator of ETH, ISO, and TAC (see above). Thus, counteracting EthR function would potentially result in an increased sensitivity of *M. tuberculosis* to these drugs. Thus, different series of ETH boosters were successfully developed (for review, see North et al. 2014), like for example N-phenylphenoxyacetamide derivatives and a family of 1,2,4-oxadiazole compounds (Flipo et al. 2012). More recently, a fragment-based rational drug design approach was used to develop small inhibitors filling the entire binding pocket of EthR (Nikiforov et al. 2016).

### 6 Some Biological Functions of Mycolic Acids

Several aspects of the biological activities of MAs have been reviewed (Glickman 2008; Vergne and Daffe 1998; Vander Beken et al. 2011; Verschoor et al. 2012). This chapter will thus recall some of them and focus on two aspects, namely the immunostimulatory activity of MA-containing compounds and their contribution to mycobacterial virulence.

#### 6.1 Immunostimulatory Activity of Mycolic Acid-Containing Compounds

Infection with *M. tuberculosis* gives rise to granulomatous inflammation at infection sites and a powerful induction of T-cell responses. Immunostimulatory activity of lipids associated with the mycobacterial cell wall has been recognized for several decades and exploited in a large variety of different adjuvant preparations. Several
compounds from the mycobacterial cell wall have been implicated in mediating host cell immune activation, among which trehalose dimycolate or TDM (cord factor). Numerous roles have been associated with TDM, greatly depending on the ways of mixing the glycolipid with other lipids (Vergne and Daffe 1998). These include the inhibition of phagosome-lysosome fusion and acidification of phagosomes (Indrigo et al. 2002, 2003), tissue damage and necrosis (Hunter et al. 2009) by inducing a high level of the proinflammatory cytokines TNF-alpha, IL-6, and IL-12 when used for stimulation of bone marrow dendritic cells.

Glycerol monomycolate (GroMM), delivered in cationic liposomes, was shown to be particularly efficient in TH1-inducing adjuvant formulation effective against tuberculosis. This mycobacterial antigen stimulates CD1b-stimulated CD4+ T cells, and both the hydroxyl group of glycerol and the MA lengths were shown to be critical for triggering the T-cell responses. The stereochemistry of the molecule, e.g., the (R)-1-O-mycoloyl-glycerol was more stimulatory than the (S)-1-O-mycoloyl-glycerol, as well as the chain lengths of MA were found to play an important role in T-cell responses (Layre et al. 2009). Similarly, in glucose monononycolate (GMM) presented on CD1b-restricted T cells, the precise structures of natural GMM, including the glucose, the linkage of the glucose to the MA and the R,R-stereochemistry of the hydroxyl part of the mycolate, have been shown to dictate the T-cell recognition (Moody et al. 1997, 2000).

Immunological properties have also been reported for chemical structures related to the arabinogalactan termini (Rombouts et al. 2012), but these substances may occur only after the killing and degradation of the bacilli.

### 6.2 Mycolic Acid Types and Virulence

*M. tuberculosis* synthesizes α-, keto-, and methoxy-MAs as the main classes of MA (Daffe et al. 1983) (Fig. 2). Both the quantity and fine structure of MAs are critical for the survival and pathogenicity of the tubercle bacilli and related pathogenic mycobacterial species. For instance, the deletion of the in vitro most active mycoloyltransferase Ag85C (Belisle et al. 1997), resulting in a strain producing 40%-less MA compared to its isogenic parental *M. tuberculosis* strain, was less virulent in mice and more permeable (Jackson et al. 1999). The deletion of the *hma* gene, which led to abolishing the production of the oxygenated keto- and methoxy-MAs, although not affecting the total amount of MAs, resulted in a less virulent and less permeable strain of *M. tuberculosis* (Dubnau et al. 2000). Similarly, cyclopropanation of α-MA at the proximal position was shown to impact both the formation of “cords” and the virulence of the tubercle bacilli (Glickman et al. 2000). Trans-cyclopropanation of MAs on trehalose dimycolate has been shown to suppress *M. tuberculosis*-induced inflammation and virulence (Rao et al. 2006). Moreover, kasB mutants of both *M. marinum* and *M. tuberculosis*, which produce MAs shorter by up to six carbon atoms, exhibit an increased permeability of their cell walls and a severe defect in resisting host defense mechanisms and antibiotic action (Gao et al. 2003).
M. tuberculosis is an obligate human parasite able to develop in alveolar macrophages. Accumulation of lipid droplets in the macrophages of individuals developing a post-primary infection, gives a foamy aspect to these macrophages. In alveolar foamy macrophages, the bacilli were mainly found within lipid droplets. The formation of foamy macrophages (FM), a granuloma-specific population characterized by its high lipid content, was studied comparatively in mycobacteria with different MA composition, i.e., in M. tuberculosis, M. smegmatis, and recombinant strains of the latter species overexpressing the hma gene responsible for the introduction of ketomycolic acids. Only bacteria containing oxygenated mycolic acids induced the formation of FMs. Oxygenated MAs triggered the differentiation of human monocyte-derived macrophages into FMs, which might constitute a shelter for persisting bacilli (Peyron et al. 2008).

Taking advantage from the nonessentiality of the hadC gene of M. tuberculosis (Jamet et al. 2015b; Slama et al. 2016), we have addressed its contribution to the virulence of the tubercle bacillus, through the knock-out of the gene in the virulent H37Rv variant. This resulted in a MA profile similar to that of the avirulent strain H37Ra that has a point mutation in this gene, i.e., a strong reduction of the global content of the oxygenated MAs (methoxy- and keto-MAs, Fig. 2), reminiscent of M. tuberculosis ΔkasB phenotype (Bhatt et al. 2007b), and inhibition of the formation of extra-long MAs bearing a fourth cyclopropanated meromycolic segment (Quemard 2016; Slama et al. 2016). This strongly affected the virulence in mice, the cording capacity, and the sensitivity to rifampicin. Furthermore, the deletion of hadA and hadC genes of M. smegmatis, which also triggered an alteration of the MA profile, had a dramatic effect on the bacterial physiology and fitness (Jamet et al. 2015b). Similarly, in M. abscessus the deletion of MAB_4780, a dehydratase that is distinct from the HadBC enzyme, led to a decreased level of α-MA with a concomitant lack of cording and an increased susceptibility to the drug thiacetazone compared with the wild type strain (Halloum et al. 2016). The mutant had also an attenuated virulence in macrophages as well as in zebrafish embryos, with only a few infectious foci and no induction of the formation of granulomas (Halloum et al. 2016). The mutation has a role in preventing phagosome-lysosome fusion, a key strategy that is used by pathogenic mycobacteria to avoid exposure to lysosomal hydrolases, thus promoting survival.

6.3 Mycolic Acids with Respect to Cording and Biofilm Formation

In a liquid medium without detergent, M. tuberculosis cells form microscopic structures that resemble cords, a phenomenon known as cord formation, or cording, and considered as a virulence factor in the M. tuberculosis complex. In the 1950s, cording was related to TDM that, consequently, was named the “cord factor.” The fine structure of the mycoloyl chains in the TDM plays a determinant role in the cording capacity, as illustrated by phenotypic analyses of mutants (see some examples in Sect. 6.2) (Glickman 2008). However, the subsequent demonstration of the occurrence of TDM in all the mycobacterial species studied so far, except M. leprae
where only TMM was clearly identified, suggested that it was not the only factor required for cording. Consistent with this, modern techniques of microbial genetics have revealed that cording can be affected by mutations in genes not directly involved in TDM biosynthesis. Natural or laboratory mutants of the *M. tuberculosis* complex that were unable to form microscopic cords showed impaired virulence compared to the original cording strains (Ferrer et al. 2009; Glickman 2008). A strong correlation exists between microscopic cords, rough colonial morphology, and increased persistence of mycobacteria inside macrophages (Julian et al. 2010).

Many bacterial cells have the ability to form biofilms that enable them to exist as communities and to adhere tightly to surfaces. Biofilm formation requires a switch from a motile to a sessile life style, and generation of an extracellular matrix. The biofilms of pathogenic microorganisms, including *M. tuberculosis*, are a major medical issue, because they are difficult to eradicate due to the presence in these structures of drug-tolerant bacteria (Ojha et al. 2008). The discovery that virulent tubercle bacilli grew on the surface of a liquid medium, forming veils that spread uniformly over the entire surface of the liquid medium, and climbed up the sides of the glass container, was first described by Koch in 1884 and later by others as a typical characteristic of *M. tuberculosis* complex strains. The natural tendency of *M. tuberculosis* to form biofilms has been exploited long ago for the production of large amounts of cell biomasses that are necessary for chemical characterization of the bacterial cell constituents. Indeed, historically, *M. tuberculosis* was grown as a surface pellicle, a biofilm-like structure, at the liquid-air interface of some synthetic media, like the Sauton’s medium. These in vitro biofilms were shown to be rich in free MAs released by enzymatic hydrolysis of TDM or cell lysis due to the long-time culture needed for biomass production (Ojha et al. 2010). A relationship between MA biosynthesis and biofilm formation has been demonstrated by inactivation of the gene GroEL1, a dedicated chaperone involved in MA biosynthesis. GroEL1 modulates synthesis of MAs specifically during biofilm formation and physically associates with KasA. Biofilm is associated with elevated synthesis of C56–C58 fatty acids (Ojha et al. 2005). In the same context, the deletion of groEL (also known as *cpn60.1* or *hsp60*) in *M. bovis* BCG led to the loss of its capacity to form biofilm, accompanied by changes in lipid composition, mainly in MAs (with 2–4 carbon atoms shorter) and phthiocerol dimyccerosates (Wang et al. 2011).

The types of mycolic acids also play a role in the biofilm formation, as typified by the essentiality of the keto-MAs (see Fig. 2) in these structures. By deletion of the *mmaA4* (or *hma*) gene, the resulting mutant devoid of keto-MAs was shown to be both pellicle-defective and highly sensitive to rifampicin under planktonic growth. It was proposed that when incorporated within the wild type pellicle biofilm, the cells were protected from the bactericidal activity of the antibiotic (Sambandan et al. 2013). The FAS-II enzymes KasA/KasB and HadBC are also important in biofilm formation, consistent with their roles in the elongation of the meromycolic chain of MAs (Gao et al. 2003; Jamet et al. 2015b; Slama et al. 2016).

During the past decade, considerable progress has been made in recognizing the importance of biofilms in chronic infections and understanding the biochemical and
cellular processes that lead to biofilm formation in vitro, although many questions remain. In the particular case of tuberculosis, the relationship between biofilm formation and pathogenesis has not been clearly established. Progress will require technologies that will enable sophisticated analyses of the biochemistry and cell biology of biofilm-forming microorganisms in vivo, as well as ongoing development of animal models that faithfully mimic chronic infections.

7 Research Needs

Despite the progress in the past decades in deciphering the biosynthesis pathway leading to mycolic acids, the whole biosynthesis is not fully known. Many relevant questions remain to be solved. Among these:

(i) In mycobacteria, all mycolates contain two positions, i.e., distal and proximal, that initially contain double bonds, subsequently modified into cis cyclopropane, trans double bond, transcyclopropane, or oxygenated function with an adjacent methyl branch. A very recent survey suggests the involvement of the desaturase DesA1 in the formation of one double bond in α-MAs of *M. smegmatis* (Singh et al. 2016). However, the exact mechanisms underlying the introduction of both double bonds in all types of meromycolic chain and the proteins catalyzing these desaturation/dehydration-isomerization steps remain to be discovered.

(ii) In *M. tuberculosis*, the characterization of monounsaturated fatty acids ranging from C_{24} to C_{30} with a double bond localized exactly at the position expected for the elongation of a Δ5 tetracosenoic acid precursor as well as other data suggest that at least some of the modifications are introduced during the growth of the meromycolic chain (Asselineau et al. 1970b; Qureshi et al. 1984; Takayama and Qureshi 1978; Yuan et al. 1998). Yet conclusive data are still lacking.

(iii) Several enzymes of the *M. tuberculosis* FAS-II system have been shown to be down-regulated by STPK-mediated phosphorylation (Molle and Kremer 2010), a growth phase-dependent phenomenon (Slama et al. 2011). However, the precise STPKs regulating this process, the timing, and its consequences on the bacterial physiology are unclear. A few transcriptional regulators of MA metabolism have also been identified. To face this fragmented information, a complete picture of the mechanisms of regulation of the MA metabolism involved in adapting the production level and the fine structure of these envelope components in response to environmental conditions must be established. The physiological advantages gained by post-translational modifications or transcriptional regulation and how the different regulatory mechanisms may be coordinated, in a synergistic or antagonistic way, need future exploration.

(iv) Recently, MmpL3 was identified as the transporter of the TMM, product of the mycolic condensation system, through the plasma membrane (Grzegorzewicz...
et al. 2012a), while MmpL11 was shown to export distinct MA derivatives (Pacheco et al. 2013). Thus, MA biosynthesis is very likely to occur on the cytoplasmic side of the plasma membrane. The mechanisms by which the MA derivatives are routed from the periplasmic side of the inner membrane transporters MmpL3 and MmpL11 to the envelope’s outer layers (like the mycomembrane) or the bacterial surface have not been investigated yet.

(v) The existence of protein-protein interactions between known FAS-II enzymes (Veyron-Churlet et al. 2004, 2005) and MA methyl transferases led to propose a model with interconnected specialized complexes (Cantaloube et al. 2011). The atomic structures of many individual proteins of MA biosynthesis and transfer have been solved, but besides that of the FAS-I system (Boehringer et al. 2013; Ciccarelli et al. 2013), the 3D structures of the MA biosynthesis and export machineries remain unknown. This should represent a future field of investigation that may open new avenues for antituberculosis inhibition strategies like the development of molecules blocking the assembly of protein complexes.

(vi) The role of biofilm growth during the colonization of the host by *M. tuberculosis* and other pathogenic mycobacteria is another outstanding question. For example, the *M. tuberculosis* pilus (MTP), which contributes to biofilm formation, has an impact on lesion architecture in infected lungs (Mann et al. 2016). Moreover, it has been shown that *M. tuberculosis* biofilms harbor an important drug-tolerant population that persists despite exposure to high antibiotic levels (Ojha et al. 2008). The mechanisms that enable these MA-rich mycobacterial biofilms to resist the action of antibiotics constitute a fundamental issue both in terms of antimycobacterial chemotherapies and prevention against nosocomial infections.

The interdisciplinary efforts to understand both the basic biology and pathogenesis of *M. tuberculosis* will enable substantial progress in developing new avenues for therapeutic interventions in the coming years.

**Acknowledgments** The authors are grateful to their colleagues for fruitful collaborations and discussions, and for sharing unpublished material. We acknowledge funding from the European Union (NM4TB, grant LSHP-CT-2005-018923; TB-Drug grant LSHP-CT-2006-037217; SysteMTb HEALTH-2009-2.1.2-1 241587), the Agence Nationale de la Recherche (XPKS-MYCO, grant 09-BLAN-0298-03; FASMY, grant ANR-14-CE16-0012), the Région Midi-Pyrénées (MYCA, FEDER grant 34249), the France-Argentina ECOS-MINCyT cooperation program (grant A11B04) and the “Vaincre la Mucoviscidose” association (IC0716, France).

**References**


Rock CO, Cronan JE (1996) Escherichia coli as a model for the regulation of dissociable (type II) fatty acid biosynthesis. Biochim Biophys Acta 1302:1–16


Tamok I, Rohrscheidt E (1976) Biochemical background of some enzymatic tests used for the differentiation of mycobacteria. Tubercle 57:145–150


