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Mycolic Acids: Structures, Biosynthesis, and Beyond

Hedia Marrakchi,[1,2] Marie-Antoinette Lanéelle,[1,2] and Mamadou Daffé[1,2,*]
[1]Unité Mixte de Recherche 5089, Département Mécanismes Moléculaires des Infections Mycobactériennes, Centre National de la Recherche Scientifique (CNRS), Institut de Pharmacologie et de Biologie Structurale (IPBS), 205 Route de Narbonne, BP 64182, 31077 Toulouse, France
[2]Université de Toulouse, Université Paul Sabatier, Toulouse III, IPBS, 205 Route de Narbonne, BP 64182, 31077 Toulouse, France
*Correspondence: mamadou.daffe@ipbs.fr

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Mycolic acids are major and specific lipid components of the mycobacterial cell envelope and are essential for the survival of members of the genus *Mycobacterium* that contains the causative agents of both tuberculosis and leprosy. In the alarming context of the emergence of multidrug-resistant, extremely drug-resistant, and totally drug-resistant tuberculosis, understanding the biosynthesis of these critical determinants of the mycobacterial physiology is an important goal to achieve, because it may open an avenue for the development of novel antimycobacterial agents. This review focuses on the chemistry, structures, and known inhibitors of mycolic acids and describes progress in deciphering the mycolic acid biosynthetic pathway. The functional and key biological roles of these molecules are also discussed, providing a historical perspective in this dynamic area.

Introduction

Mycolic acids (MAs), 2-alkyl, 3-hydroxy long-chain fatty acids (FAs), are the hallmark of the cell envelope of *Mycobacterium tuberculosis* and related species and genera (Daffé and Draper, 1998). They are found either unbound, extractable with organic solvents such as esters of trehalose or glycerol, or esterifying the terminal pentaarabinofuranosyl units of arabinogalactan (AG), the polysaccharide that, together with peptidoglycan, forms the insoluble cell wall skeleton (Daffé, 1996; Daffé and Draper, 1998; McNeil et al., 1991). Both forms presumably play a crucial role in the remarkable architecture and impermeability of the cell envelope, participating in the two leaflets of the mycobacterial outer membrane, also called the mycomembrane (Figure 1), recently visualized by electron microscopy (Hoffmann et al., 2008; Sani et al., 2010; Zuber et al., 2008). This concept has been established in *Corynebacterium glutamicum*, a species belonging to a genus that exhibits many features of the mycobacterial cell envelope (Daffé, 2005), notably the presence of an MA-containing outer membrane. Because *Corynebacterium* strains are viable in the absence of MAs (Portevin et al., 2004, 2005), it has been proven that the outer membrane was no longer observed in *C. glutamicum* mutant strains devoid of MAs (Hoffmann et al., 2008; Zuber et al., 2008). Because the inhibition of MA synthesis is one of the primary effects of the frontline and most efficient antitubercular drug isoniazid (INH) (Takayama et al., 1972), much interest has been devoted to deciphering the chemistry and biosynthesis of MAs. This metabolic pathway represents a valuable source for recruiting potential targets for the development of new antimycobacterial drugs in the alarming context of the emergence of *m*ulti*d*rug-*r*esistant (MDR), e*x*tremely *d*rug-*r*esistant (XDR), and *t*otally *d*rug-*r*esistant (TDR) tuberculosis (TB). In addition, MA-containing compounds have been associated in the past not only to many physiological properties of mycobacteria, such as their characteristic serpentine-like growing and "cord-forming," but also to numerous biological properties, such as adjuvant and antineoplastic capacity of purified and crude cell wall fractions (Daffé and Draper, 1998; Goren and Brennan, 1979; Verschoor et al., 2012).

The last decade has seen impressive progress in the development of the biochemistry, genetics, regulation, and structures of MAs. Novel roles have also been attributed to MAs and their subfamilies in various phenomena, such as biofilm formation and foamy macrophage (FM) formation in TB granulomas. This review summarizes these studies, focusing on the biosynthetic pathway and known inhibitors. It also describes some of the biological roles of these unique molecules.

Structure and Diversity of MAs

The first structures of MAs were described in 1950 (Asselineau and Lederer, 1950) as long-chain FAs, two-branched, three-hydroxylated, a feature that confers to the molecule the property to be cleaved at high temperature, by a reaction similar to a reverse Claisen-type condensation, into a "mero"aldehyde main chain, also called a "meromycolic chain," and a "fatty acid" (Table 1). MAs occur in all mycobacterial species examined to date. Structurally similar substances have been found, with very few exceptions (e.g., *Corynebacterium amycolatum* and *Corynebacterium kroppenstedtii*), in members of the order *Corynebacteriales*. The stereochemistry of the centers at positions 2 and 3 has been shown to be conserved in all MA-containing genera, as 2*R*-, 3*R*- (Figure 2) (Asselineau and Asselineau, 1966; Asselineau et al., 1970b).

Elucidation of the MA structure 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. Based on their structural variability and complexity, MAs have been regarded as genus- and species-specific compounds; consequently, they were largely used as taxonomic markers. They consist of chains of 22–38 carbon atoms in *Corynebacterium*, 30–36 in *Hoyosella* and *Amycolicicoccus*, 34–38 in *Dietzia*, 34–52 in *Rhodococcus*, 46–60 in *Nocardia*, 46–66 in *Gordonia*, 64–78 in *Tsukamurella*, 60–90 in *Mycobacterium*, and up to 100 in *Segniliparus* (Table 1). In addition, studies on the nature of the FAs released by pyrolysis have been of a great interest in clarifying

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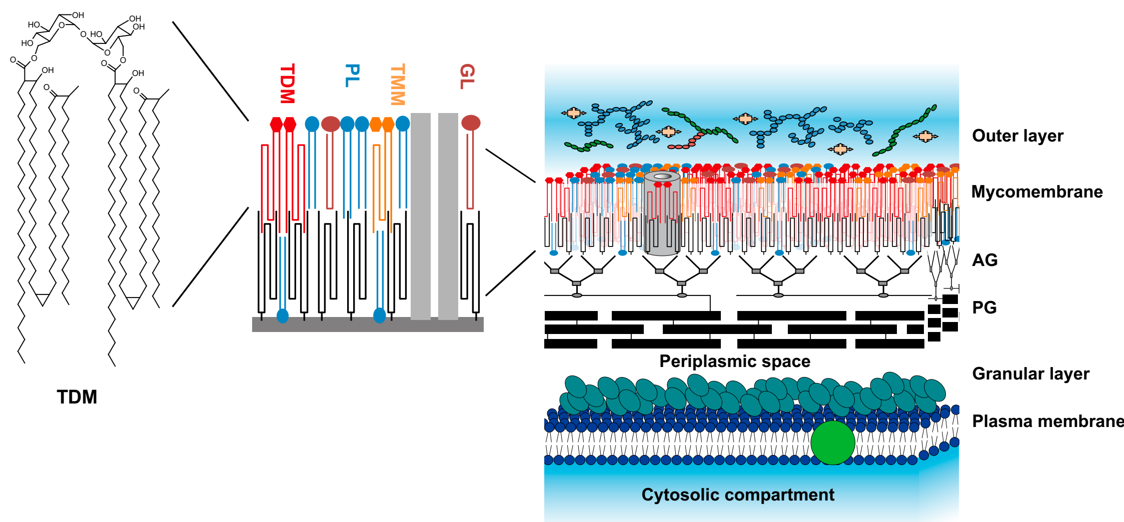

Figure 1. Model of the Mycobacterial Cell Wall
The outer layer is mainly composed of glucan and proteins, with only a tiny amount of lipid. The mycomembrane corresponds to the permeability barrier. Its inner leaflet is formed by a parallel arrangement of MA chains (in black) linked to AG that in turn is covalently attached to peptidoglycan (PG); the inner leaflet of the mycomembrane is presumably composed of free lipids that include TDM (in red), TMM (in orange), various glycolipids (GL, in brown), and phospholipid (PL, in blue). Adapted from Zuber et al. (2008). A representation of TDM shows the very-long chain of MAs that have to pack after folding at the site of the motifs (here mycolic unit, cyclopropane, and keto group) to fit in a conventional membrane of 7–8 nm in thickness. The granular layer above the plasma membrane is composed of proteins; these proteins may precipitate upon treatments of bacteria before the observation by transmission electron microscopy and yield a thicker appearance to the outer leaflet of the plasma membrane (see Zuber et al., 2008).

the phylogeny of members of the order *Corynebacteriales*. The lengths of these FAs consist in C_8–C_{18} in *Corynebacterium*, C_{12}–C_{18} in *Nocardia*, and C_{22}–C_{24} for most species of the genus *Mycobacterium*, with the notable exception of the *M. tuberculosis* complex and *Mycobacterium xenopi* where a C_{26} occurs as the main homolog in their mycolates (Daffé et al., 1983) (Table 1).

Until recently, the structures of MAs of genera other than that of mycobacteria were considered to be relatively simple in terms of chemical functions, being composed only of a homologous series with various numbers of double bonds, with up to six for some *Tsukamurella* species (Tomiyasu and Yano, 1984) (Table 1). In contrast, MAs of mycobacteria display a large diversity of chain lengths and chemical functions that define the different classes of MAs, leading to complex TLC patterns (Barry et al., 1998; Daffé et al., 1983; Minnikin et al., 1983). The most apolar MAs, referred to as α-MAs, contain 74–80 carbon atoms and generally two double bonds (of *cis*- or *trans*-configuration) or two *cis*-cyclopropyl groups located in the meromycolic chain (Figure 2). A small fraction of α-MAs may contain more unsaturations, as observed in some strains of the *M. tuberculosis* complex, with three unsaturations and longer chains (more than 6 to eight carbons) (Watanabe et al., 2001). Polyunsaturated α-MAs were shown to represent a significant portion of the MAs in *Mycobacterium fallax* (Rafidinarivo et al., 1985). MAs with 60–62 carbon atoms, known as α'-MAs, contain one *cis* double bond. Interestingly, the genus *Segniliparus* was recently defined with a large array of MAs with chain lengths similar to those of mycobacteria and ranging from 60 to 100 carbon atoms, composed of the C_{60}–C_{62} α'- and C_{77}–C_{79} α-MAs, and the novel, extremely long-chain C_{90}–C_{98} MAs, named α^+-MAs (Butler et al., 2005; Hong et al., 2012; Lanéelle et al., 2013).

In addition, MAs from most mycobacteria examined so far contain supplementary oxygen functions located in the distal part of the meromycolic chain, defining the keto-, methoxy-, wax ester-, epoxy-, and hydroxy-types of MA (Figure 2), absent from a few mycobacterial species (Daffé et al., 1983) and from genera of the order *Corynebacteriales* other than mycobacteria, including the genus *Segniliparus*. The oxygen functions occurring in mycobacterial MAs are typified by the occurrence of an adjacent methyl branch. It is noteworthy that in *M. tuberculosis*, the oxygenated MAs, i.e., keto-, methoxy-, and hydroxy-MAs, contain ~84–88 carbon atoms, and are thus four to six carbons (even greater in minor components) longer than the α-MAs from the same strains (Laval et al., 2001; Watanabe et al., 2001). In contrast, the chain lengths of epoxy- and wax ester-MAs found in nontuberculous mycobacterial species are similar to those of α-mycolates of the same strains in which keto-, methoxy-, and/or hydroxy-MAs have never been detected (Laval et al., 2001).

MA Biosynthesis

The biosynthesis of mycobacterial FAs, precursors of MAs, is achieved by at least two fatty acid synthases (FASs): (1) the "eukaryotic-like" multifunctional FAS-I produces FAs with a bimodal distribution, C_{16}–C_{18} and C_{24}–C_{26} acids, with the latter corresponding to the "α branch" found in MAs; and (2) the "bacterial-like" "dissociated" FAS-II, composed of a series of discrete soluble enzymes (type-II system); this FAS is responsible for the elongation of FAs at the origin of the very long meromycolic chains. The Claisen condensation of the latter with the α branch yields the characteristic 2-alkyl, 3-hydroxy ("mycolic motif") of MAs. Although the two FAS systems differ in their molecular organizations, substrates, and carrier specificities,

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Table 1. MA Features in Different Genera of the Order *Corynebacteriales*

Mycolic acid
π
R
CH—CH—COOH
OH
R'
(α branch)
R—CHO + R'—CH_2—COOH
Meroaldehyde (Mero-chain)
Fatty acid

Genus or Species	Total Carbon	Mero-Chain	FA	Unsaturation Degree[a]	Reference	Kas	Had
Corynebacterium	22–36	8–18	8–18	0–2	Collins et al., 1982	–	–
Dietzia	34–38[b]	19:1	15–17[b]	0,1	Nishiuchi et al., 2000		
Hoyosella	30–35[b]	20–25[c]	9–12[b]	0,1	Lanéelle et al., 2012	A	A,B
Amycolicicoccus	30–36[b]	20–25[c]	9–12[b]	0,1	Lanéelle et al., 2012	A	A,B
Rhodococcus	30–54		12–16	0–2	Nishiuchi et al., 2000	A	A,B
Rhodococcus equi	30–50	14–34	14,16	0–2	Hsu et al., 2011	A	A,B
Tomitella	42–52				Katayama et al., 2010		
Smaragdicoccus	43–49				Adachi et al., 2007		
Millisia	44–52				Soddell et al., 2006		
Williamsia	50–56		16,18		Kämpfer et al., 1999		
Nocardia	46–60	32–40	12–18	0–3	Nishiuchi et al., 1999	A	A,B fused
Skermania	58–64	42–48	16–20	2–6	Chun et al., 1997		
Gordonia	46–66		16,18	1–4	Nishiuchi et al., 2000	A	A,B fused
Tsukamurella	64–78	44–58	20:1,22:1	1–6	Tomiyasu and Yano, 1984	A	A,B
Mycobacterium	60–90	42–62	22–26	1,2	(Barry et al., 1998	A,B	A,B,C
Segniliparus	60–100	40–76	22,24	1–3	Hong et al., 2012; Lanéelle et al., 2013	A,B	A,B1,B2,C

MAs are characterized by their overall chain lengths and their pyrolysis products. Pyrolytic cleavage (π) at the C_2–C_3 carbon atoms releases a meroaldehyde and a FA; R and R′ indicate long hydrocarbon chains.
[a]Unsaturation degree refers to the number of double bonds and/or cyclopropanes.
[b]MAs containing even and odd carbons were detected in these genera.
[c]The (24:1), corresponding to C_{24} with one unsaturation, was identified as the major homolog. The occurrence of the FAS-II genes involved in the early and late elongation steps of the mero-chain were searched in the available genomes (right): genes encoding the KasA and KasB (β-ketoacyl-ACP synthase) and Had (β-hydroxyacyl-ACP dehydratase) activities are indicated; A, B and C are subunits of Had.

they share similar reaction sequences with an iterative series of reactions built on successive additions of a two-carbon (acetate) unit from malonyl-coenzyme A (CoA) to a nascent acyl group (Figure 3).

Malonyl-CoA is produced by the carboxylation of acetyl-CoA, catalyzed by the acetyl-CoA carboxylase, a key enzyme in most living organisms (Cronan and Waldrop, 2002). In mycobacteria, these enzymes display broad substrate specificities (acetyl-CoA, propionyl-CoA, butyryl-CoA …) and are therefore referred to as acyl-CoA carboxylases (ACCases) consisting of multiple subunits containing at least a biotinylated α subunit (AccA, including biotin carboxylase BC and biotin carboxyl carrier protein BCCP domains) and a β subunit (AccD, constituted by the carboxyltransferase domain) (Diacovich et al., 2002; Tong, 2005), the latter being responsible for the substrate selectivity. *M. tuberculosis* displays an unusual number of ACCases in its genome, with three α subunit genes (*accA1–accA3*) and six ACCase carboxyltransferase domain genes (*accD1–accD6*) and an epsilon subunit (*accE*, *Rv3281*) (Gago et al., 2006; Lin et al., 2006; Oh et al., 2006). Each ACCase, which always comprises AccA3 (Rv3285), presumably serves a different physiological role and provides various extender units for the biosynthesis of the rich diversity of mycobacterial FAs. The main physiological role of AccA3-AccD5 (Rv3280) (epsilon dependent) would be to generate methylmalonyl-CoA for the biosynthesis of multimethyl-branched FAs of the cell envelope (Daffé and Draper, 1998) and to provide (accessory role) malonyl-CoA as elongation unit for FA cycles (Oh et al., 2006). This latter function would be preferentially achieved by the AccA3-AccD6 (Rv2247) activity (epsilon inhibited) and could thus play an important role in MA biosynthesis by providing malonyl-CoA to the FAS-II complex (Gago et al., 2011). The AccA3-AccD4 (Rv3799c) ACCase would be rather dedicated to synthesize the carboxylated substrate (Portevin et al., 2005) involved in MA condensation (Figure 3). Interestingly, the *accD4* (*Rv3799c*) gene is in operon with the genes involved in the condensation reaction, *pks13* (*Rv3800c*) and *fadD32* (*Rv3801c*).

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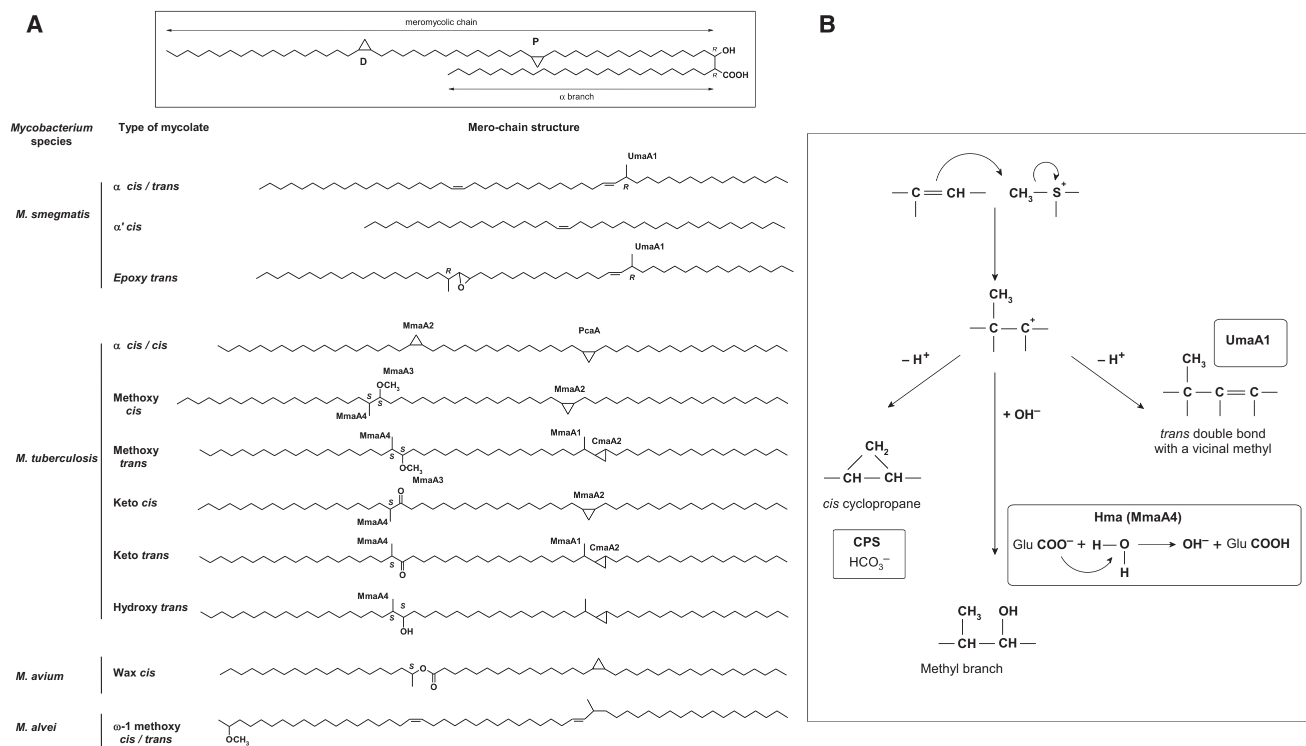

Figure 2. Structural Characteristics of the MA Mero-Chains

(A) Structure of the α-dicyclopanated MA from *M. tuberculosis*. P and D indicate the proximal and distal positions, respectively, on the mero-chain where the various motifs are found. The stereochemistry of the two conserved chiral centers, carbons 2 and 3, are *R*,*R*. Below are shown structures of the mero-chains of representative types of MAs from mycobacteria. The configuration of double bonds and cyclopropanes is either *cis* or *trans* (with an adjacent methyl branch). The established functions of the MTs are indicated. The stereochemistry of the asymmetric carbon atoms in the mero-chain is noted (*S* or *R*), i.e., carbon bearing methyl, methoxyl, or hydroxyl groups.

(B) Reactions catalyzed by mycobacterial MTs and proposed reaction mechanisms (adapted from Lederer, 1969). The electrons π of the *cis* double bond attacks the methyl group of *S*-adenosyl L-methionine to give a carbocation intermediate. Deprotonation steps catalyzed by a bicarbonate ion HCO_3^- leads to the formation of *cis* cyclopropane or to the methyl branch adjacent to the *trans* double bond. In the case of Hma, the bicarbonate ion is replaced by the side-chain carboxylate of E146 that induced the deprotonation of a molecule of water. The activated water molecule OH^- in presence of the MT leads to the simultaneous addition of the hydroxyl and the methyl on the carbocation intermediate. CPS, cylopropane synthase; Hma, hydroxy MA MT; UmaA1, unidentified MA MT.

Synthesis of the Alpha-Alkyl Branch

The mycobacterial FAS-I protein displays the seven distinct domains corresponding to the catalytic activities required for the synthesis cycle (Bloch and Vance, 1977; Fernandes and Kolattukudy, 1996). Mycobacteria have a single FAS-I-encoding gene (*fas*, *Rv2524c*), shown to be essential in *Mycobacterium smegmatis* (Zimhony et al., 2004) and suggested to be essential for *M. tuberculosis* growth based on high-density transposon mutagenesis (Griffin et al., 2011). The FAs produced by FAS-I are long-chain acyl-CoAs with a bimodal distribution, C_{16}–C_{18} and C_{24}–C_{26}, a unique feature of the mycobacterial FAS-I among those of the order *Corynebacteriales* (Bloch and Vance, 1977; Fernandes and Kolattukudy, 1996). The long-chain C_{24}–C_{26} acyl-CoAs subsequently participate, after carboxylation by ACCase, in MA biosynthesis (Figure 3).

Synthesis of the Meromycolic Backbone

Unlike the type-II synthases of other bacteria, the mycobacterial FAS-II elongates C_{12}–C_{16} FAs to yield C_{18}–C_{30} acyl-acyl carrier proteins (ACPs) in vitro, but it is incapable of de novo FA synthesis from acetyl-CoA (Odriozola et al., 1977). The synthesis proceeds through the elongation of enzyme-bound intermediates, covalently linked to mycobacterial acyl carrier protein (AcpM, Rv2244), by several iterative cycles, each comprising four steps (Bloch, 1977) (Figure 3).

The malonate group is transferred to ACP by malonyl-CoA:ACP transacylase (MtFabD, Rv2243) to form malonyl-ACP. FA synthesis is initiated by the Claisen condensation of malonyl-ACP with acyl-CoA, catalyzed by β-ketoacyl-ACP synthase III (MtFabH, Rv0533c), to form β-ketoacyl-ACP. Four enzymes catalyze each cycle of elongation: the β-keto group is reduced by the nicotinamide adenine dinucleotide phosphate-dependent β-ketoacyl-ACP reductase (MabA, Rv1483), and the resulting β-hydroxyl intermediate is dehydrated by the heterodimer β-hydroxyacyl-ACP dehydratases (HadAB and HadBC, Rv0635-Rv0636 and Rv0636-Rv0637, respectively) to an enoyl-ACP. Next, the reduction of the enoyl chain by the nicotinamide adenine dinucleotide hydrogen (NADH)-dependent *trans*-2-enoyl-ACP reductase (InhA, Rv1484) produces an acyl-ACP. Additional cycles of elongation are initiated by the β-ketoacyl-ACP synthase (KasA or KasB, Rv2245 or Rv2246) that elongates the acyl-ACP by two carbons to form a β-ketoacyl-ACP. Elongation likely ceases when the acyl-ACP attains the chain length required for meromycolic chain modification or condensation. Although AcpM is hypothesized to be

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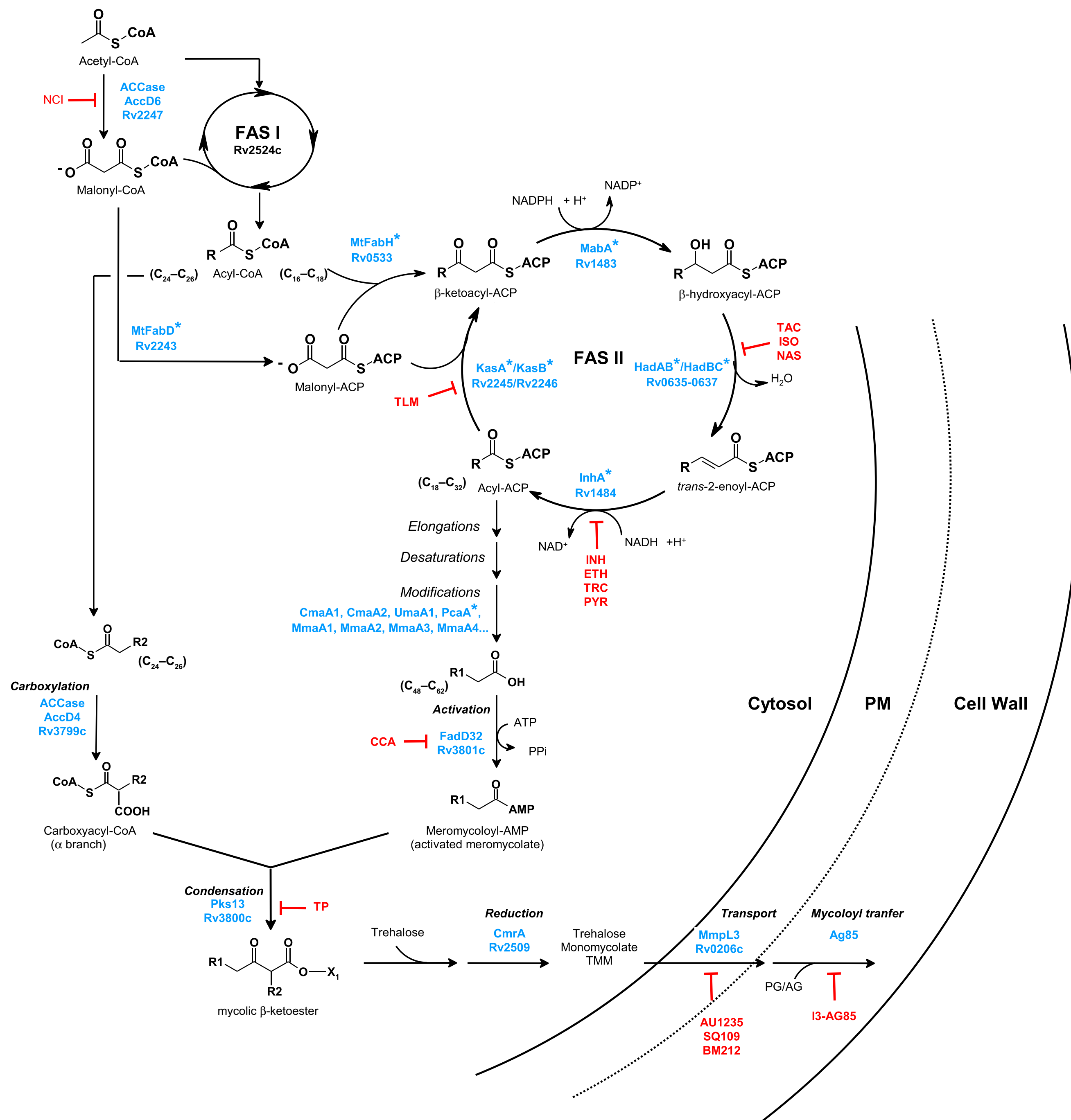

Figure 3. Biosynthesis, Inhibition, and Regulation of Mycobacterial MAs

The biosynthetic pathway of MA starts with the de novo synthesis and elongation of FAs operated by the mycobacterial FAS-I and FAS-II synthases, respectively. The FAS-II synthase products undergo further elongation and modifications/decorations to produce the long mero-chain precursors. The carboxylation of acyl-CoA (the FAS-I products) provides the alkylmalonate at the origin of the α branch. Condensation of the activated mero-chain with the alkylmalonate yields the β-ketoester that, upon reduction, leads to the mature MA. The proteins shown to be regulated by Ser/Thr protein kinases are indicated by an asterisk (*). Known inhibitors and the steps inhibited in the pathway are indicated (red): PYR, pyridomycin; NAS, NAS-21/NAS-91).

the carrier of the very-long meromycolic chain, no experimental evidence supports this hypothesis.

As expected from the essentiality of MA biosynthesis, InhA, MabA, HadB, and KasA are essential proteins (Bhatt et al., 2005; Brown et al., 2007; Parish et al., 2007; Sacco et al., 2007; Vilchèze et al., 2000). Neither HadA and HadC (our unpublished results) nor KasB (Bhatt et al., 2007; Gao et al., 2003) is essential. Even though KasA and KasB share significant sequence similarity, they show different chain-length specificities (Kremer et al., 2002; Schaeffer et al., 2001), suggesting a

distinct role for each enzyme. It has been shown that cell lysates of *M. smegmatis* expressing *M. tuberculosis kasA* could elongate FAs up to ~40 carbons, whereas the expression of both *kasA* and *kasB* resulted in the production of longer chain FAs (~54 carbons), equivalent to those of the meromycolate chains of MAs (Slayden and Barry, 2002). Based on these in vitro biosynthesis studies, it has been proposed that KasA is involved in the initial elongation of MAs that are extended to their full lengths by KasB (Kremer et al., 2002; Slayden and Barry, 2002). The *kasB* mutants of both *Mycobacterium marinum* and *M. tuberculosis* produce mycolates shorter by approximately two to four carbon units, by up to six carbon atoms in the latter species, and they are impaired in their growth, while exhibiting an increased permeability of their cell walls and a severe defect in resisting host defense mechanisms and antibiotic action (Bhatt et al., 2007; Gao et al., 2003). Interestingly, the deletion of *kasB* in *M. tuberculosis* is also accompanied by the loss of acid-fastness—the hallmark trait of the tubercle bacilli. Moreover, this *M. tuberculosis* mutant enters into a symbiotic state with immunocompetent mice, causing a latent infection that may be important for the persistence phenotype of tubercle bacilli (Bhatt et al., 2007). These studies support the hypothesis that KasA is involved in initial extension of the FAS-II-mediated growth of the MA chains, whereas the dispensable KasB is implicated primarily in extension to full lengths. In addition to the elongation condensing enzymes, chain-length specificity is very likely dictated as well by the two heterodimers of the β-hydroxyacyl-ACP dehydratases, HadAB and HadBC, with HadB being the catalytic subunit and HadA and HadC defining substrate selectivity for chain elongation. Thus, similarly to KasA, HadAB is most likely involved in the early FA elongation cycles, whereas the HadBC heterodimer is implicated in the late biosynthetic steps of the meromycolic chain of mycobacteria (Marrakchi et al., 2008; Sacco et al., 2007). This reasoning is supported by the distribution of the latter proteins in members of the order *Corynebacteriales* (Table 1). In genera producing very long-chain MAs, i.e., *Amycolicicoccus*, *Rhodococcus*, *Nocardia*, and *Gordonia*, where the synthesis involves elongation steps of the meromycolic chains, orthologs of *kasA* and *kasB* and *hadABC* genes are detected in the genomes (Lanéelle et al., 2012). Interestingly, in *Segniliparus*, a genus in which the longest MAs are synthesized, the organizations of both *kasA*/*kasB* and *hadABC* genes are different from those of mycobacteria (Lanéelle et al., 2013). More surprising is the presence of two copies of *hadB*, the gene encoding the catalytic domain of the dehydratase (Lanéelle et al., 2013). These observations reinforce the concept of implication of *kas* and *had* genes in the elongation of the mero-chains of MAs.

Modification of the Meromycolic Chain

Introduction of Double Bonds

The presence of double bonds is observed in all MAs, from the simplest C_{32}–C_{34} corynomycolic acids to the most complex mycobacterial MAs, and in the longest MAs of *Segniliparus*. Little is known about *cis* double-bond formation in MAs, except for corynebacteria where unsaturated MAs are the result of the condensation of two FAs with at least one molecule of oleic acid ($C_{18:1}\Delta9$).

Several mechanisms have been proposed for the formation of unsaturated FAs in other genera. Among these is the anaerobic desaturation exemplified by *Escherichia coli* enzymes with the β-hydroxydecanoyl ACP dehydratase (FabA) of FAS-II, a dehydratase/isomerase enzyme able to catalyze not only the dehydration of β-hydroxydecanoyl-ACP intermediate to give a *trans* double bond but also the isomerization of the *trans*-2 double bond into the *cis*-3 isomer. The *cis*-3-enoyl intermediate is 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 FA synthetic pathway (Marrakchi et al., 2002b; Rock and Cronan, 1996). Surprisingly, several groups of bacteria (including mycobacteria) lack both FabA and FabB enzymes, although they synthesize unsaturated FAs and/or have an anaerobic metabolism. This has led to the discovery in *Streptococcus pneumoniae* of the novel enzyme FabM, a *trans*-2-*cis*-3-enoyl-ACP isomerase, in addition to the dehydratase (FabZ), able to divert FA synthesis to the unsaturated mode (Marrakchi et al., 2002a). Candidate proteins belonging to this superfamily exist in the *M. tuberculosis* genome, namely, EchA10 and EchA11 (Takayama et al., 2005), and they may function in a FabM-like manner, ensuring the isomerization of the *trans*-2 intermediate produced in the FAS-II cycle (Figure 3) to the hypothetical *cis*-3-enoyl FA, leading ultimately, after several elongation cycles, to the formation of the *cis* double bond at the distal position of the meromycolic chain (Bloch, 1969; Etemadi, 1967). However, EchA10 and EchA11 are predicted to be nonessential (Griffin et al., 2011). Because MAs are essential in mycobacteria, the isomerization activity should be essential. Consequently, the mycobacterial FabM-like candidates remain to be discovered.

As an alternative, an aerobic desaturation performed on a yet-synthesized long chain and requiring both molecular oxygen and NADH has been proposed. This is the process that leads to the formation of oleic acid ($C_{18:1}\Delta9$) from stearic acid ($C_{18:0}$) through the action of the Δ9 desaturase. In mycobacteria this hypothesis has been postulated for the formation of the *cis* double bond at the distal position of the mero-chain of α-MAs. Considering the structural similarity between the methyl terminus of the diethylenic α-MAs and the C_{24} ethylenic FAs present in the bacteria, a Δ5 desaturase operating on $C_{24:0}$ to yield $C_{24:1}$, followed by elongation, was proposed (Asselineau et al., 1970a). This would place the double bond at the expected distal position for the α-mero-chain (Figure 2). Indeed, in various strains of mycobacteria, apart from the abundant oleic and palmitoleic ($C_{16:1}\Delta9$) acids, the most frequently encountered FAs (such as C_{24} and C_{26}) are accompanied by their analogs with a *cis* double bond in position 5,6 (Asselineau et al., 1970a). Analysis of the *M. tuberculosis* genome reveals three potential aerobic desaturases encoded by *desA1*, *desA2*, and *desA3* (Cole et al., 1998). Overexpression of DesA3 in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and biochemical analysis of the purified enzyme showed that DesA3 (Rv3329c) is responsible for the synthesis of oleic acid (Phetsuksiri et al., 2003). No function has been yet defined for the proteins encoded by the two putative desaturases DesA1 and DesA2. Among the possible desaturase candidates is Rv1371. The protein displays expected characteristics of membrane-bound acyl-lipid or acyl-CoA desaturases and has been identified in culture filtrates of *M. tuberculosis* and cell membrane fractions, but it exhibits only a weak similarity to Δ5 desaturases.

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Putative candidates for desaturation may be FabQ-like proteins, functionally similar to that recently identified in the group of Cronan. FabQ possesses a dual dehydratase and isomerase function and was identified in the saprophytic bacterium *Aerococcus viridans*. The protein allows the chain elongation by progressive isomerization reactions to form polyunsaturated FAs (Bi et al., 2013).

The possibility of the intervention of condensation enzymes catalyzing the assembly of precursors by a series of successive decarboxylation/condensation steps, as described for polyketide synthases, also exists. The latter enzymes occur in mycobacteria, and their implication in the biosynthesis of specific lipids of *M. tuberculosis* other than MAs, e.g., "phthiocerol and companions," has been demonstrated previously (Guilhot et al., 2008; Kolattukudy et al., 1997). Based on structural and labeling experiments, a head-to-tail condensation of palmitic acid after an ω-oxidation step has been proposed for nocardomycolic acid, consistent with the double-bond location at ω16 (Bordet and Michel, 1969; Tarnok and Röhrscheidt, 1976). The relationship between different types of MAs from mycobacteria and the position of the motifs at similar intervals has led to the hypothesis that the building of the mero-chain could be the result of condensation between three common FAs (C_{20}, C_{16}, and C_{22}). The position of double bonds or oxygenated groups would be determined by the length of the FA precursor and by the facultative methylation (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 $C_{62:1}$–$C_{64:1}$ (Figure 2), regardless of the species where they are found, e.g., *M. smegmatis*, *Mycobacterium abscessus*, or the new genus *Segniliparus* (Lanéelle et al., 2013). The fact that no further modifications (cyclopropyl, methyl branch, oxygenated functions) are observed in the α'-MAs suggests a distinct pathway for their synthesis.

Insertion of Cyclopropyl and Methyl Groups

The introduction of all cyclopropanes and methyl branches derives from methionine via *S*-adenosyl-methionine (SAM) involving a *cis* double bond (Lederer, 1969). A family of homologous (SAM)-dependent methyltransferases (MTs) that share 50%–75% identity are present in the genome of *M. tuberculosis* (Cole et al., 1998) and of sequenced mycobacterial species, e.g., *M. bovis* BCG, *M. smegmatis*, and *Mycobacterium leprae*. Among these MTs, the cyclopropane synthases (CPSs) of *M. tuberculosis* share 30%–35% identity with that of *E. coli* that catalyzes the addition of a methyl group to a double bond. Four of the eight MTs identified in *M. tuberculosis*, namely, CmaA1, CmaA2, PcaA (formerly UmaA2), and MmaA2, display strong sequence similarities with the *E. coli* CPS, suggesting a common reaction mechanism (Figure 2B). Inactivation experiments of the *M. tuberculosis* MT genes have established their respective functions (Figure 2) and corrected some false interpretations concluded from protein overexpression experiments. For instance, deletion of the *umaA2* (for *u*nknown *m*ycolic *a*cid MT) gene has shown that the encoded protein is responsible for the formation of the proximal *cis*-cyclopropane of *M. tuberculosis* α-MA. This gene was thus renamed *pcaA* (for *p*roximal *c*yclopropanation of *a*lpha-MAs) (Glickman et al., 2000) (Figure 2). This function was previously attributed to *cmaA2* (for *c*yclopropanation of *m*ycolic *a*cids) by overexpression of the encoded protein in *M. smegmatis* (George et al., 1995). Knockout (KO) of *cmaA2* in *M. tuberculosis* has impacted only the structures of oxygenated MAs (methoxy- and keto-MAs) of the strain that contained double bonds instead of the proximal *trans*-cyclopropane rings (Glickman et al., 2001). Similarly, the overexpression of *M. tuberculosis* CmaA1 led to the conclusion that this MT introduces the *cis*-cyclopropane at the distal position in α-MAs (Yuan et al., 1995), a conclusion subsequently contradicted by the analysis of a *cmaA1* null mutant, indicating that CmaA1 is not required for the distal *cis*-cyclopropanation of the α-MAs (Glickman, 2003). These genetic and biochemical studies have led to important advances in the understanding of how cyclopropanes are introduced on the meromycolic chain and enabled to assign a role to many, if not all, of the different CPS-type MA-MTs (Figure 2).

Numerous 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* (Figure 2). For instance, the *M. tuberculosis* *umaA1* (*umaA*, *Rv0469*) KO mutant exhibited no change in the MA profile despite the hypervirulence phenotype observed in the SCID mouse infection model, pointing to a specific impact of the mutation in an in vivo context (Laval et al., 2008). In contrast, the inactivation of the orthologous gene in *M. smegmatis* (*MSMEG_0913*) resulted in the abolition of the synthesis of subtypes of MAs containing a methyl branch adjacent to either *trans*-cyclopropyl group or *trans* double bond at the proximal position of both α- and epoxy-MAs (Laval et al., 2008) (Figure 2).

The crystal structures of several MTs from *M. tuberculosis* have been solved and compared with one another (Huang et al., 2002). Interestingly, a bicarbonate ion is conserved in all proteins that catalyze the cyclopropanation reaction (CmaA1, CmaA2, MmaA2, and PcaA), in contrast to the MTs involved in the formation of oxygen-containing groups (MmaA3 and MmaA4). The three-dimensional structures thus support the hypothesis that these CPS enzymes catalyze methyl transfer via a carbocation mechanism in which the bicarbonate ion acts as a general base (Figure 2B), as demonstrated for *E. coli* CPS (Courtois and Ploux, 2005; Huang et al., 2002). What defines the action of the MT enzymes on distal versus proximal parts of the acyl chain is still enigmatic, but the idea of substrate specificity dictated by different lengths of the substrate-binding cavity is an attractive hypothesis.

Introduction of Oxygenated Functions

The biosynthesis of both keto- and methoxy-MAs, 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 that is catalyzed by the MT Hma (MmaA4), a SAM-dependent enzyme. The *hma* mutant no longer produced keto- or methoxy-MAs (Dubnau et al., 2000), while still able to synthesize α-MAs having two cyclopropanes (as found in the parent strain), and displaying a novel α-mycolate type bearing a *cis* double bond in the distal position and a *cis*-cyclopropane in the proximal part of the molecule (Dinadayala et al., 2003). The presence and precise position of this distal double bond have led to postulate this ethylenic mycolate as the

substrate of Hma. Like other MA-MTs, Hma, specifically in the presence of a water molecule, would methylate the *cis* double bond and catalyze a concerted hydroxylation. The hydroxylated intermediate at the branch point would then give either the keto- or the methoxy-MAs, the latter resulting from the action of the "methoxylase" MmaA3 (Dinadayala et al., 2003; Dubnau et al., 1997, 1998, 2000; Yuan and Barry, 1996; Yuan et al., 1998) (Figure 2). Because the newly synthesized α-mycolate in the *hma* mutant is shorter by four to six carbon atoms than the keto- and methoxy-MAs of the wild-type strain, the key reaction on the ethylenic molecule would occur earlier, at the "meromycolate" stage.

The three-dimensional structures of Hma (Boissier et al., 2006), interestingly, point to some differences with those of the three CPS-type MA SAM-MTs previously crystallized (Huang et al., 2002). The bicarbonate ion found in the active site of the CPSs is absent in Hma. Instead, in the superimposed structures, the side-chain carboxylate of glutamate 146 (E146) is placed at exactly the same position as the bicarbonate in CPSs. It was thus tentatively proposed that the carboxylate of the E146 might function as a general base to activate a water molecule for a nucleophilic attack (Figure 2B) (Boissier et al., 2006). As originally proposed by Lederer (1969), it is tempting to state that the catalysis by MA-MTs would follow the same route to the carbocation intermediate and then differ in the second step, leading to either cyclopropaned, methyl-branched, or hydroxylated MAs with an adjacent methyl branch (Figure 2B). As an alternative, Hma would catalyze the introduction of the methyl group on a strongly nucleophilic site, such as a β-ketoester generated from a condensation reaction (Asselineau et al., 2002; Dinadayala et al., 2003; Lederer, 1969). The characterization of small amounts of an epoxymycolic acid devoid of an adjacent methyl branch in the *hma*-deleted mutant strain would represent a putative candidate for such methylation (Dinadayala et al., 2003).

The genes encoding the proteins involved in the biosynthesis of other oxygenated functions such as wax-, epoxy-, and ω-1 methoxylated MAs (Figure 2A) 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 that involves the insertion of molecular oxygen, thus converting the ketone to the corresponding ester (Toriyama et al., 1982), as postulated previously (Etemadi and Gasche, 1965; Lanéelle and Lanéelle, 1970). Flavin-containing Baeyer-Villiger monooxygenases (BVMOs) are known for using NADH/NADPH and molecular oxygen for degradation of toxic ketones. Such enzymes are present in mycobacteria, e.g., EthA (Rv3854c) that accepts a ketone as substrate and has been shown to activate the anti-TB prodrug ethionamide (ETH) to its active form (Fraaije et al., 2004). Six additional genes encoding BVMOs are present in the *M. tuberculosis* genome. Although ETH has been shown to inhibit the synthesis of oxygenated MAs in *Mycobacterium aurum*, a keto- and wax-MA-containing species (Quémard et al., 1992), the annotated genes are not likely to encode the enzymes involved in the oxidation of keto- into wax-MAs by a process of Baeyer-Villiger oxidation because *M. tuberculosis* strains are devoid of wax-MAs.

From structural analyses, notably the chain length and stereochemistry of the asymmetric carbons bearing both the methyl branches adjacent to the oxygenated functions (Daffé et al., 1981; Lacave et al., 1987; Minnikin et al., 1982), it clearly 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 intermediate having a *trans* double bond at the distal position with an adjacent methyl branch (Figure 2).

The ω-1 position of the methoxy group found in a class of MAs (Figure 2) raises a new question. Because no methyl branch adjacent to the oxygen function is found in this compound, it is tempting to speculate that the precursor of such product could be an intermediate with a terminal double bond rather than the result of a MmaA3/MmaA4-like process. 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).

MA Condensation and Transfer

The MA synthesis involves, in addition to FAS-I and FAS-II, a condensation reaction between two activated FAs, a penultimate and key step leading to the formation of the "mycolic motif." The formation of a new carbon-carbon bond by a Claisen condensation is a reaction shared by both FA and polyketide synthase biosyntheses and is catalyzed by condensing enzymes or condensases (Heath and Rock, 2002).

Condensation Enzymes

Despite extensive efforts in several laboratories, the condensase responsible for the synthesis of MAs has remained a mystery for more than 50 years. Given the importance and specificity of this final step, a condensase candidate must be present in all *Corynebacteriales* and conserved in *M. leprae*, the genome of which has a large number of pseudogenes. The polyketide synthase 13 (Pks13), a member of the type-I Pks family, was identified as the enzyme responsible for the final construction of MAs in both *C. glutamicum* and *M. smegmatis* (Portevin et al., 2004). The discovery of the condensase for MA synthesis probably illustrates the remarkable benefit of using the *Corynebacterium* model for exploring the more complex *Mycobacterium* MA biosynthetic pathway. More than 18 type-I *pks* genes are present in the *M. tuberculosis* genome (Cole et al., 1998). However, the search of the *Corynebacterium diphtheriae* and *C. glutamicum* genomes revealed the occurrence of a single *pks* gene conserved in all *Corynebacteriales* species analyzed, leading to the identification of Pks13 as the condensing enzyme (Portevin et al., 2004). In *C. glutamicum*, the inactivation of the *pks13* gene abolishes the production of MAs, thus altering the mycomembrane (Portevin et al., 2004) and the structure of the envelope (Hoffmann et al., 2008; Zuber et al., 2008). Pks13 is a protein displaying five distinct domains corresponding to the catalytic activities required for condensation: peptidyl carrier protein (PCP)-like domain, ketoacyl synthase (KS), acyl transferase (AT), ACP domain, and thioesterase (TE). To be activated, Pks13 needs a posttranslational modification, catalyzed by the mycobacterial 4′-phosphopantetheinyl transferase PptT, essential for activation of the ACP and PCP domains (Chalut et al., 2006).

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The final ''mycolic condensation'' involves not only Pks13 but also several enzymes involved in the activation of the two condensase substrates. Specifically, activation of the ''meromycolic'' chain (through the action of an acyl-adenylate [acyl-AMP] ligase) and carboxylation of the α branch (by an acyl-CoA carboxylase) are essential for the formation of MAs in *Corynebacterium* and *Mycobacterium* (Gande et al., 2004; Portevin et al., 2004; Portevin et al., 2005) (Figure 3). The *fadD32* gene (*Rv3801c*) is adjacent to *pks13* (*Rv3800c*), and this genetic organization *fadD32-pks13* is conserved in *M. tuberculosis* and *M. leprae* and was among the hints suggesting the involvement of the *fadD32*-encoded protein in the condensation reaction. FadD32 catalyzes the production from free FA of acyl-AMP (Trivedi et al., 2004) (Figure 3). Like *fadD32*, some of the 34 *fadD* are adjacent to *pks* genes on the *M. tuberculosis* genome and, interestingly, were found to encode fatty acyl-AMP ligases rather than having a fatty acyl-CoA ligase activity (Trivedi et al., 2004). These proteins activate long-chain FAs as acyl-adenylates that are then transferred to the multifunctional Pks for further chain extension. FadD32 acts in concert with Pks13 and activates the very-long meromycolic acid (C_{50}–C_{60}) before its condensation with a C_{24}–C_{26} FA (Gavalda et al., 2009; Léger et al., 2009).

The involvement of the AccD4 carboxyltransferase in MA synthesis (Gande et al., 2004; Portevin et al., 2005) and its essentiality for mycobacterial survival have been demonstrated by inactivation of the *fadD32* gene, resulting in the lack of production of MA (Portevin et al., 2005). The fact that a *fadD32*-KO mutation in *C. glutamicum*, which abolishes the synthesis of one of the activated partners for condensation (acyl-AMP), also led to the accumulation of the second condensation substrate (tetradecyl malonic acid) (Portevin et al., 2005) clearly proves that a carboxylation step, leading to the formation of a malonyl derivative, takes place before the condensation reaction occurs in MA biosynthesis. An alkyl-malonyl intermediate is also formed in a *C. glutamicum* mutant deficient in the synthesis of Pks13, indicating that carboxylation of the α branch chain occurs most likely before the transfer onto the condensase Pks13 (Portevin et al., 2005). The unique specificity of AccD4 for the C_{24}–C_{26} long chains is in agreement with the α branch lengths being of 24–26 carbons in mycobacteria and confirms the role of AccD4 in MA condensation (Oh et al., 2006).

Formation of Mature MAs and Their Transfer

Upon condensation, the product of Pks13 is the mycolic β-ketoester that needs to be reduced to produce the mature mycolate (Figure 3). In corynebacteria, it has been shown that a β-ketoester of trehalose was detected in the first minute of corynomycolic acid synthesis, pointing to the importance of trehalose in the metabolism of these acids (Ahibo-Coffy et al., 1978; Tropis et al., 2005; Walker et al., 1973). The enzyme catalyzing the reduction of the β-ketoester was identified by inactivation experiments of the candidate *NCgl2385* gene, the ortholog of *Rv2509* (*cmrA*) in *M. tuberculosis*, in *C. glutamicum* (Lea-Smith et al., 2007). The resulting mutant still synthesizes β-ketoester of both trehalose and AG (in lower amounts) but was deficient in AG-linked mature mycolates. Likewise, inactivation of the *M. smegmatis* ortholog of *cmrA* (*MSMEG_4722*) resulted in the loss of mature MAs linked to the cell wall AG, yet retained the production of β-ketoesters of AG and trehalose (Bhatt et al., 2008). The latter products are also synthesized by the purified Pks13 protein, provided that FadD32 and both free and carboxylated FAs are present in the in vitro reaction mixture (S. Gavalda, F. Bardou, M. Daffé, and A. Quémard, unpublished data). The transfer of the mycoloyl residues onto the cell wall AG acceptors occurs virtually by the mycobacterial fibronectin-binding proteins that have been shown to possess the mycoloyl transferase activity in vitro (Belisle et al., 1997) and in vivo (Puech et al., 2000, 2002).

MA Elongation Complexes

Based on the genetic and biochemical interaction experiments between the various FAS-II components, our group has proposed a working model in which different specialized FAS-II complexes would be interconnected (Veyron-Churlet et al., 2004, 2005) 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, with each complex being constituted by a ''FAS-II core'' (formed by InhA, MabA, and MtFabD) associated to a specific condensing (Kas) enzyme and dehydratase (Had) heterodimers, thus defining their substrate specificity. An ''initiation'' FAS-II complex, containing mtFabH, might allow the channeling of acyl-CoA from FAS-I to FAS-II during their condensation with malonyl-ACP (Figure 3). This step would be followed by a type-I ''elongation'' complex formed by the core proteins and KasA that 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, could end the synthesis of the meromycolic chain. This end product of E2-FAS-II may be used in the terminal condensation reaction catalyzed by Pks13, which is assumed to be part of a ''termination'' FAS-II complex.

MA Biosynthesis Regulation

Membrane lipid biogenesis is a vital facet of mycobacterial physiology that must be tightly regulated at both the genetic and biochemical level. *M. tuberculosis* also produces an impressive array of complex bioactive lipids that are intimately involved in pathogenesis and protective immunity. Mycobacterial survival depends on membrane lipid homeostasis and on the ability to adjust lipid composition to adapt the bacterial cell to optimize growth in the prevailing conditions it faces in vivo. FA and MA acyl chains determine the viscosity of the membrane that in turn influences many crucial membrane-associated functions. Many sophisticated regulatory strategies used by *M. tuberculosis* to finely control cell wall biosynthesis have now been elucidated (Dover et al., 2007b). In particular, mycobacteria control the expression of the genes responsible for the metabolism of MAs by adjusting the level and activity of biosynthetic enzymes to match the demand for new membrane synthesis and extension of its complex cell wall. These mechanisms are only starting to be understood.

The transcriptional regulator MabR (Rv2242) functions as a repressor in *M. tuberculosis* to control the expression of essential genes involved in MA biosynthesis (Salzman et al., 2010) by repressing the *fabD-acpM-kasA-kasB* gene cluster and affecting the expression of *fas* that encodes the multifunctional FA synthase FAS-I. Overexpression of MabR represses transcription and inhibits MA synthesis. The *mabR* gene is likely essential in

M. tuberculosis (Sassetti et al., 2001). This hypothesis is further supported by the demonstration that the chromosomal copy of *M. smegmatis mabR* could only be disrupted in the presence of a wild-type copy of the gene provided in *trans* (Salzman et al., 2010).

The activities of key enzymes in MA synthesis are also modulated at posttranslational levels, as are many metabolic processes in *M. tuberculosis* regulated by Ser/Thr protein kinases (STPKs)-mediated phosphorylation. In fact, a growing body of evidence suggests that Ser/Thr phosphorylation play important roles in the physiology and virulence of *M. tuberculosis.* Recently, the importance of protein phosphorylation networks in cell wall biosynthesis has attracted considerable interest. An array of two-component and eukaryotic-like STPK systems has been discovered, and several are implicated in central metabolic processes, cell division, and cell wall biogenesis. Comprehensive reviews on structure-function studies of Ser/Thr and Tyr protein phosphorylation have been published previously (Av-Gay and Everett, 2000; Greenstein et al., 2005; Wehenkel et al., 2008). The pathogen uses "eukaryotic-like" STPKs and phosphatases to regulate many intracellular metabolic processes, including MA biosynthesis. In this pathway, many proteins were identified as substrates for the STPKs (Figure 3), and their phosphorylation was found to modulate their enzymatic activity to control MA synthesis (Bhatt et al., 2007; Molle and Kremer, 2010). Phosphorylation of essential enzymes of the *M. tuberculosis* FAS-II system, KasA, InhA, and MabA, and the dehydratases HadAB and HadBC was found to downregulate their respective activities. Similarly, phosphorylation of the mycobacterial 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 shut down meromycolic acid chain production in the stationary phase (Slama et al., 2011). The differential expression of the mycobacterial STPKs in response to stress conditions might directly affect the phosphorylation profile of these substrates and as a consequence modulate MA biosynthesis to promote adaptation to environmental changes.

MA Inhibition and Antituberculous Drug Discovery

The rise in antibiotic-resistant *M. tuberculosis* and the relatively few candidates in the drug development pipeline for TB highlight the urgent need for novel anti-TB agents, ideally with mechanisms of action different from those of existing drugs and efficient on MDR, XDR, and TDR isolates. Recent years have seen an enormous increase in efforts to discover new lead compounds using both whole-cell screening and target-based biochemical approaches, although progress in validating new targets has been slower than expected. Many efforts currently focus on revisiting and optimizing existing inhibitors (of validated targets in FAS-II …) through rational drug design and structure-activity relationship studies.

The unique cell envelope lipids produced by *M. tuberculosis* play essential roles in the physiology and pathogenicity of these bacteria (Daffé and Draper, 1998). As such, the MA biosynthetic pathway is a validated site of action of many of the current first- and second-line anti-TB drugs (Figure 3) and therefore has significant potential as a target for the development of novel antimycobacterial drugs (Jackson et al., 2013), particularly because targeting new steps in well-validated biosynthetic pathways in antitubercular therapy is a powerful strategy that removes much of the usual uncertainty surrounding new targets and in vivo clinical efficacy.

FA Synthesis and Elongation Inhibitors

Acyl-CoA Carboxylase Inhibition. Acetyl-CoA carboxylases have crucial roles in FA metabolism (see above) and are attractive targets for drug discovery (Gago et al., 2011). In an effort to find inhibitors of the *M. tuberculosis* ACCases, the crystal structure of AccD5 (Lin et al., 2006) has been solved, and structure-based in silico screening has been performed as described previously (Chen et al., 2005). AccD5 inhibitors have been identified and assayed in vitro for inhibition of AccD5 and for the whole AccA3-AccD5 enzyme complex. Given the conserved structure of the AccD5 and AccD6 active sites, several inhibitors of AccD5 have been also screened as potential inhibitors of AccD6, leading to ligand NCI-172033 (NCI) that is capable of inhibiting AccD6 (Kurth et al., 2009). This compound also has shown bactericidal activity against several pathogenic mycobacteria by causing a strong inhibition of both FA and MA biosynthesis. Overexpression of AccD6 in *M. smegmatis* has conferred resistance to NCI, confirming that AccD6 is the main target of the inhibitor and a good target for the development of new antimycobacterial agents (Kurth et al., 2009) (Figure 3).

β-Ketoacyl-ACP Synthase Inhibition. The fact that several natural products target the elongation step in FA biosynthesis clearly emphasizes the condensing enzymes as desirable targets for drug development (Heath and Rock, 2004). Cerulenin (produced by *Cephalosporium caerulens*) irreversibly inhibits FAS from a variety of organisms (Price et al., 2001). It inhibits both KasA and KasB activity (Kremer et al., 2002; Schaeffer et al., 2001). However, although some therapeutic efficacy has been shown in models of yeast infection, cerulenin also inhibits eukaryotic FAS and has not been pursued as a lead for novel antimicrobials. More interest has been driven toward the natural molecule produced by *Nocardia*, thiolactomycin (TLM), that is specific for type-II synthases and exhibits broad activity toward Gram-negative and Gram-positive bacteria, and toward important pathogens, including *Plasmodium falciparum* and *M. tuberculosis* (Kremer et al., 2000). TLM, composed of a thiolactone ring, binds to the malonate portion of the active site of the condensing enzyme. The drug targets both *M. tuberculosis* KasA and KasB, binding preferentially to the KasA acyl-enzyme with low micromolar affinity (Machutta et al., 2010). Designed analogs of TLM with longer chains are better inhibitors of the mycobacterial KasA and KasB enzymes. TLM was shown to inhibit MA biosynthesis in vitro and retains good antimycobacterial activity in vivo (Douglas et al., 2002).

Recent efforts are being directed toward improving the affinity of TLM for KasA and developing chemical tools to investigate the substrate binding and catalytic mechanism of KasA through design and synthesis of classes of substituted TLM-derivatives with structural features reminiscent of either the acyl- or the malonyl-AcpM substrates of the enzyme (Kapilashrami et al., 2013).

Enoyl Reductase Inhibition. The *M. tuberculosis* enoyl-ACP reductase InhA has been demonstrated to be the target of both INH and ETH, validating it as a remarkable antitubercular

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drug target. INH, one of the oldest known anti-TB and structurally simplest drugs, was found to have an exquisite activity against *M. tuberculosis* more than 50 years ago, yet its precise mechanism of action could not be elucidated until genetic tools were developed in *M. tuberculosis*. The combination of genetic, biochemical, and X-ray crystallographic studies (Banerjee et al., 1994; Dessen et al., 1995; Rozwarski et al., 1998) provided a consistent model indicating that INH is activated by the catalase-peroxidase KatG to form an adduct with nicotinamide adenine dinucleotide (NAD) that inhibits the *inhA*-encoded NADH-dependent enoyl-ACP reductase of FAS-II (Figure 3). Likewise, ETH is a prodrug and is activated in *M. tuberculosis* by EthA, a flavin monooxygenase, to form a covalent ETH-NAD adduct that inhibits InhA (Morlock et al., 2003; Wang et al., 2007). Expectedly, inhibition of InhA leads to inhibition of MA biosynthesis, accumulation of long-chain FAs (Takayama et al., 1975; Winder and Collins, 1970), and ultimately to cell death (Vilchèze et al., 2000; Wang et al., 2007). INH and ETH are highly specific and effective, and being prodrugs, mutations in the activators KatG and EthA have been correlated to most of the clinical resistance in the cases of drug-resistant TB (Hazbón et al., 2006; Morlock et al., 2003).

The search for compounds that do not require activation and that directly target InhA represents a promising approach to circumvent this resistance mechanism (Freundlich et al., 2009). In this context, the broad-spectrum antimicrobial triclosan (TRC) inhibits InhA without needing activation, although its use as an antitubercular may be limited by its suboptimal bioavailability (Kuo et al., 2003). Using a structure-based drug design approach, a series of substituted triclosan derivatives have been developed, and compounds were identified with dramatically enhanced potency against purified InhA, with some up to 50-fold more potent than triclosan. X-ray crystal structures of InhA in complex with triclosan derivatives revealed the structural basis for the inhibitory activity, and selected triclosan derivatives have been tested against INH-sensitive and -resistant strains of *M. tuberculosis*. Among those, the best inhibitor had a bactericidal activity 10-fold lower than that of triclosan. A subset of these triclosan analogs were more potent than INH against INH-resistant *M. tuberculosis* strains, demonstrating the significant potential of structure-based design in the development of next generation antitubercular drugs (Freundlich et al., 2009).

The natural product pyridomycin (PYR), first described in 1953 with specificity toward mycobacteria (Maeda et al., 1953), was also found to target InhA and to inhibit MA synthesis in *M. tuberculosis* (Hartkoorn et al., 2012). Interestingly, the most frequently encountered INH-resistant clinical isolates are fully susceptible to PYR, thus opening promising avenues for the treatment of INH-resistant tubercle bacilli.

Dehydratase Inhibition. Isoxyl (ISO) and thiacetazone (TAC), once used in the clinical treatment of TB, are prodrugs that have to be activated by the mycobacterial monooxygenase EthA (Dover et al., 2007a). They have dramatic effects on MA biosynthesis, consistent with both drugs inhibiting an early and essential step of the pathway, e.g., the elongation of the main (meromycolate) chain by FAS-II (Figure 3). A combination of genetic and biochemical approaches identified the precise steps of the biosynthetic pathway interrupted by these two prodrugs to be the dehydratase step, catalyzed by the β-hydroxyacyl-ACP dehydratases (encoded by the *hadABC* genes) of the FAS-II elongation system (Belardinelli and Morbidoni, 2012; Coxon et al., 2013; Grzegorzewicz et al., 2012a). Both compounds are the first clinically used anti-TB drugs reported to act at the dehydration step. Consistent with their mode of action and similarly to INH, ISO and TAC display bactericidal activity on actively replicating *M. tuberculosis* in vitro.

The antimalarial phenylsulfanylmethyl-[1,4]-naphthoquinones NAS-21 and NAS-91, first identified as inhibitors of the β-hydroxyacyl-ACP dehydratase FabZ of *P. falciparum* (Sharma et al., 2013), were found to exhibit potent antimycobacterial activity, with NAS-91 being more active than NAS-21 (Gratraud et al., 2008). Synthesis of NAS analogs and evaluation of their whole-cell activity against *M. bovis* BCG allowed for determination of their activity against FAS-II but not FAS-I synthesis in vitro, implicating in particular Rv0636 (HadB) as the candidate target of these inhibitors (Bhowruth et al., 2008).

MA Condensation and Transport Inhibitors

Targeting unique steps in MA pathway already inhibited by a current antibiotic, e.g., INH, is an effective strategy for anti-TB drug discovery by potentially bypassing existing resistance to current pathway inhibitors, especially in the context of MDR, XDR, and TDR TB. This is particularly illustrated by the recent identification of inhibitors of the (essential and validated) mycolic condensation enzymes Pks13/FadD32 and the enzymes for MA translocation and transfer MmpL3/Ag85 (Figure 3).

The operon *fadD32-pks13-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 a "druggable" target (Galandrin et al., 2013). The 4,6-diaryl-5,7-dimethyl coumarins (CCAs), 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).

The discovery and validation of Pks13 as the key condensing enzyme required in MA synthesis (Gavalda et al., 2009; Portevin et al., 2004) have brought this protein to the forefront as an attractive and essential drug target. Thiophene (TP) compounds that interfere with the function of the N-terminal ACP domain of Pks13 have been identified; they inhibit both the activity of the protein and MA synthesis, leading to mycobacterial cell death (Wilson et al., 2013). "Compound 3," identified by combining high-throughput screening (HTS) with whole-genome sequencing (WGS) of resistant isolates, kills mycobacterial cells, likely through interference with the TE activity in the C-terminal domain of Pks13 (Ioerger et al., 2013).

The above-mentioned HTS campaign with WGS (Ioerger et al., 2013) has identified "compound 2" as a promising drug that inhibits the inner membrane transporter MmpL3 (Rv0206c). This protein is also targeted by several inhibitors, among which are the anti-TB pyrrole derivative BM212 (La Rosa et al., 2012); a novel urea derivative AU1235 [1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea] (Grzegorzewicz et al., 2012b); and the anti-TB drug SQ109 (Tahlan et al., 2012); the latter compounds abolished the translocation of trehalose monomycolates (TMMs) from their cytoplasmic or membrane-associated site of production to the periplasm where they can then be transferred onto arabinogalactan or used in the formation of the outer membrane trehalose dimycolates (TDMs, cord factor) (Figure 3). The

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mycoloyl transferase Ag85C protein was validated as a drug target by detailed characterization of an Ag85C-binding molecule, 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile (I3-AG85), that exhibits an antimycobacterial activity toward *M. tuberculosis* growing both in vitro and within macrophages (Warrier et al., 2012) by blocking Ag85-mediated TDM synthesis with no effect on covalently linked MAs.

It appears therefore that the chemical and biological validation as essential proteins and therapeutic targets of the key enzymes in the MA pathway (InhA, HadABC, FadD32, Pks13, MmpL3 …) opens an important avenue for the development of antitubercular drug discovery. Additional lead compounds need to be identified to compensate for high attrition rates in drug discovery pipelines and to counteract the continuing emergence of drug resistance. Also, design and synthesis of derivatives of validated drugs with improved anti-TB potency and pharmacokinetic properties represent additional promising approaches.

MA Patterns and Packing into the Cell Wall

MAs possess unique characteristics essential for their physiological roles in maintaining the cell wall structure of mycobacteria. The importance of the MAs in the formation of the cell wall permeability barrier was known long ago. Mycobacteria are extremely impermeable to hydrophilic molecules, including antibiotics and nutrients such as glucose and glycerol (Jarlier and Nikaido, 1990). This low permeability was correlated with the extremely hydrophobic surface attributed mainly to the high amounts of MAs in the cell wall (Figure 1).

In the early 1980s, Minnikin (1982) proposed that MAs are assembled as a monolayer with their very-long chains parallel to one another. The alkyl chains of other lipids would intercalate between the long mero-chain and α branch chain of mycolates, with the whole structure forming an asymmetrical bilayer that would form a barrier to hydrophilic molecules (Figure 1). Support of this hypothesis came from freeze fracture experiments that showed two fracture planes of weakness in the mycobacterial envelope: (1) the plasma membrane, as expected, and (2) the cell wall (Barksdale and Kim, 1977). Furthermore, X-ray diffraction measurements of purified envelopes showed a strong reflection at 4.2 Å, distinctive of ordered fatty acyl chains, and a diffuse reflection at 4.5 Å, indicating a less ordered region (Nikaido et al., 1993). That the molecules involved are mycolates was demonstrated by measuring the "melting point" of cell walls purified from mycobacterial species of various mycolate compositions by differential scanning calorimetry (George et al., 1995; Liu et al., 1995, 1996). The occurrence and configurations of the double bonds and cyclopropyl groups as well as the lengths of the MAs are important for the observed phase changes. Determination of the fine structure of how mycoloyl residues are attached to the pentaarabinosyl termini of AG (McNeil et al., 1991) further explained the existence of a mycolate monolayer despite their attachment to the polysaccharide.

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 types of MAs, α or oxygenated, folded or extended conformations were adopted, depending on temperature (Villeneuve et al., 2005). Double bonds or cylopropane rings are regularly spaced, around 14–17 methylene units, and methyl branches are found at both proximal and distal positions adjacent to the double bonds. It has been previously demonstrated that the regular spacing of functional groups in mycobacterial MAs facilitates folding and packing in monolayer studies (Villeneuve et al., 2005, 2007). This folding ability is important in allowing MAs to be accommodated within the dimensions of the mycobacterial outer membrane (Figure 1), whose thickness is surprisingly conventional as revealed by cryo-electron microscopy (Hoffmann et al., 2008; Zuber et al., 2008).

Some Biological Properties of MAs

M. tuberculosis is an obligate human parasite able to develop in alveolar macrophages. The persistence for decades in the human host, thus resisting to an active immune response, is a characteristic of the resulting disease. It takes 6 months and four drugs to treat uncomplicated TB. Infection with *M. tuberculosis* gives rise to granulomatous inflammation at infection site and a powerful induction of T cell responses. The existence of mycobacterial components with substantial immunostimulatory activity is furthermore reflected in the prominent activity of Freund's complete adjuvant based on mineral oil formulated with heat-killed mycobacteria. Immunostimulatory activity of lipids associated with the mycobacterial cell wall has been recognized for several decades and has been exploited in a large variety of different adjuvant preparations, such as the Ribi systems where the corynebacterial TDM analog represents one of the key constituents. Several compounds from the mycobacterial cell wall have been implicated in mediating host cell immune activation, including lipids that constitute up to 40% of the dry weight of the cell envelope. Several aspects of the biological activities of MAs have been reviewed previously (Barry et al., 1998; Vergne and Daffé, 1998; Verschoor et al., 2012).

Immunostimulatory Activity of MA-Containing Compounds

The first observation concerning the virulence of *M. tuberculosis* was the formation of "cords" under suitable conditions in vitro. By treating the living virulent bacilli with petroleum ether, this characteristic was lost and the virulence was reduced (Bloch, 1950); the solvent-extracted compound was identified as α-α', trehalose 6-6′ dimycolate or TDM (formerly known as "cord factor") as the principal biologically active lipid from mycobacteria (Noll et al., 1956). TDM protects the bacilli within macrophages by reducing antibiotic effectiveness and inhibiting the stimulation of protective immune responses. Numerous other roles have been associated with TDM, greatly depending on the ways of mixing the glycolipid with other lipids (Vergne and Daffé, 1998). These roles include the inhibition of phagosome-lysosome fusion and acidification of phagosomes (Indrigo et al., 2002, 2003) and tissue damage and necrosis (Hunter et al., 2009) by inducing a high level of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-6 (IL-6), and IL-12 when used for stimulation of bone marrow dendritic cells.

To achieve its survival inside macrophages, *M. tuberculosis* establishes chronic infection characterized by the formation of granulomas, whose production has been reproduced by purified TDM. Recently, this glycolipid has been shown to activate the Syk-Card9 signaling pathway in macrophages through binding

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to the C-type lectin receptor Mincle. The Mincle-Card9 pathway is required for activation of macrophages by TDM in vitro and for granuloma formation in vivo after injection of TDM (Lang, 2013). It is not known, however, whether this pathway is also exploited by *M. tuberculosis* to transform the macrophage into a comfortable niche.

The adjuvant activity of the mycobacterial lipid extract from *M. bovis* BCG lipid extract was shown to be also attributable to glycerol monomycolate, delivered in cationic liposomes, that was particularly efficient in T helper 1-inducing adjuvant formulation effective against TB. 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 free MAs and glucose monomononomycolate (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).

Although immunological properties have been described for chemical structures related to the AG termini (Rombouts et al., 2012; Watanabe et al., 1999), these may occur only after the killing and degradation of the bacilli.

MA Types and Virulence

In *M. tuberculosis* and species that form the complex α-, keto-, and methoxy-MAs are the main classes of MAs, except some *M. bovis* BCG strains where methoxymycolates may be absent (Daffé et al., 1982). The three classes of MA are found in all the MA-containing compounds. The different types of MAs may differ in their ability to attack neutrophils, induce FMs, or adopt an antigenic structure for antibody recognition, depending on the chemical functions decorating the meromycolic chain, *cis*/*trans*-cyclopropanes, and oxygenated groups.

TB is characterized by a tight interplay between *M. tuberculosis* bacilli and host cells within granulomas. Accumulation of lipid droplets in the macrophages of individuals developing a postprimary infection gives the foamy aspect to these macrophages. In alveolar FMs, the bacilli were mainly found within lipid droplets. These cellular aggregates restrict bacterial spreading but do not kill all the bacilli, and they can persist for years (Russell, 2007). The formation of FMs, a granuloma-specific population characterized by its high lipid content, was studied comparatively in mycobacteria with different MA composition, i.e., *M. tuberculosis*, *M. smegmatis*, and a transformant strain of the latter species overexpressing the *hma* gene and responsible for the introduction of keto-mycolic acids in the strain. Only bacteria containing oxygenated MAs induced the formation of FMs. Oxygenated MAs triggered the differentiation of human monocyte-derived macrophages into FMs that could constitute a shelter for persisting bacilli (Peyron et al., 2008). Using the *hma*-deleted mutant strain, oxygenated MAs have been shown to be necessary for the virulence of *M. tuberculosis* in mice (Dubnau et al., 2000). Similarly, cycolopropanation 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).

MAs and Biofilm Formation

A biofilm is defined as a structural community of bacteria enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. Biofilms are strongly implicated in chronicity and transmission of several infections. *M. tuberculosis* is able to form biofilms, and this property was exploited long ago for the production of cell biomasses necessary to chemically characterize the constituents of the bacterial cells. Indeed, historically, *M. tuberculosis* was grown as a surface pellicle, a biofilm-like structure, at the liquid-air interface in some synthetic media, notably on Sauton medium. Several other mycobacteria have been shown to form biofilms with implications for their survival (Nyka, 1974; Smeulders et al., 1999). These in vitro mycobacterial biofilms were shown to be rich in free MAs released either from enzymatic hydrolysis of TDM or after cell lysis due to long-time culture (Ojha et al., 2010). Biofilm formation is also associated with increased synthesis of C_{56}–C_{58} FAs (Ojha et al., 2005). A correlation between MA biosynthesis and biofilm formation has been demonstrated by inactivation of the *groEL1* gene, a dedicated chaperone involved in MA biosynthesis. GroEL1 modulates synthesis of MAs specifically during biofilm formation and physically associates with the ketoacyl-ACP synthase KasA. In the same context, the deletion of *cpn60.1* (also known as *hsp60* or *groEL*) in *M. bovis* BCG led to loss of the capacity to form a biofilm, accompanied by changes in lipid composition, mainly MAs (with two to four carbon atoms shorter) and phthiocerol dimycocerosates (Wang et al., 2011). Expectedly, the reduction in the mycolate chain length caused by the deletion of *kasB* also impacts biofilm formation (Gao et al., 2003).

The types of MAs also play a role in biofilm formation, as typified by the essentiality of the keto-mycolic acids in these structures. By deletion of *mmaA4* (or *hma*) gene, the resulting mutant devoid of keto-mycolic acid 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).

Concluding Remarks and Perspectives

In the past decades, extensive efforts have been devoted to deciphering the biosynthesis pathway leading to MAs, a reservoir of new targets urgently needed to fight MDR and XDR tubercle bacilli strains. Novel, promising drug candidates targeting the MA biosynthesis and other pathways are in the pipeline, especially with the current use of whole-cell screening in addition to the more conventional target-based HTS strategy. This does not mean, however, that the whole biosynthesis is fully known and in detail. Many relevant questions remain to be solved. Some of these questions are presented below.

(1) Is FAS-II the system that carries out the elongation of the acyl chains in vivo until reaching the full-length "meromycolates"? Actually, FAS-II has been shown to elongate C_{12}–C_{16} FAs to yield C_{18}–C_{30} acyl-ACPs in vitro (Odriozola et al., 1977), which are most likely the precursors of the very long-chain "meromycolic" acids. The in vitro

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observed preference of some of the type-II FAS components for longer chain acyl thioester substrates (KasA, KasB, MabA, InhA, and HadABC) supports this functional differentiation. Based on the in vitro studies, the FAS-II ketoacyl synthases (KasA and KasB) and the hydroxyacyl dehydratases (HadABC) are involved, with distinct chain length specificities, in the subsequent elongation of AcpM acyl thioesters to C_{40} and C_{54} carbon chains, respectively (Schaeffer et al., 2001; Slayden and Barry, 2002). Even though FAs of up to C_{50} have been shown to be synthesized in cell-free extracts (Qureshi et al., 1984), the extension of C_{18}–C_{30} to C_{50}–C_{60} carbon acyl chains by FAS-II have not been demonstrated formally.

(2) Are the elongation/desaturation/modifications that characterize the mature ''meromycolic'' acid occurring on the growing chain? In *M. tuberculosis*, the characterization of monounsaturated FAs ranging from C_{24} to C_{30} with a double bond localized exactly at the position expected for the elongation of a $\Delta 5$ tetracosenoic acid precursor (Asselineau et al., 1970a) suggests that at least some of the modifications are introduced during the growth of the meromycolic chain (Takayama and Qureshi, 1978); a report in favor of this hypothesis has been also published (Yuan et al., 1998). Nevertheless, conclusive data proving that these modifications are introduced either in the completed MAs or at an earlier stage in their synthesis are still lacking.

(3) Which genes encode the proteins dedicated to the desaturation/isomerization during the biosynthesis of MA? In mycobacteria, all mycolates have two positions, i.e., distal and proximal, that initially contain double bonds, subsequently modified into *cis*-cyclopropane, *trans* double bonds, cyclopropane, epoxide, or hydroxyl with an adjacent methyl branch. The proteins catalyzing these desaturation/isomerization steps, as well as the underlying mechanisms, remain to be discovered and studied.

(4) Is the biosynthesis of MAs a tightly regulated process, and if so, when and how? The activities of several enzymes of the *M. tuberculosis* FAS-II system, e.g., KasA, InhA, MabA, HadAB, and HadBC, have been shown to be downregulated by phosphorylation (Molle and Kremer, 2010), a growth phase-dependent phenomenon (Slama et al., 2011). However, the genes regulating this process, the timing and its consequences on the bacterial physiology are unknown.

(5) The discovery of the MA biosynthesis regulator MabR raises several important questions concerning how this regulator is used by bacterial cells for adaption to their environment. A key advance will be the identification of the ligand(s) that control DNA-binding properties of this factor. It is likely that MabR may control a broader set of genes in addition to those identified previously (Salzman et al., 2010). In addition, a global analysis of the MabR regulatory activity may provide the answers to why this transcriptional regulator is essential for mycobacterial survival.

(6) Is the production of MAs coordinated with its transport to the cell wall? To which compartment? Our group has provided data on protein-protein interactions between known FAS-II enzymes (Veyron-Churlet et al., 2004, 2005), and with MA MTs (Cantaloube et al., 2011). Accordingly, we have proposed a working model with different specialized FAS-II complexes that would be interconnected and interact. The resulting meromycolic acid will be condensed to yield the β-keto precursor of MAs. This synthesis is very likely to occur in the cytosol and MAs have to be transported to the mycomembrane and beyond. Recently, MmpL3 was identified as the transporter of TMM through the plasma membrane (Grzegorzewicz et al., 2012b). Where these proteins are localized and whether they are clustered have to be experimentally established. Our unpublished data (C. Carel, K. Nukdee, S. Cantaloube, M. Bonne, C.T. Diagne, F. Laval, M. Daffé, and D. Zerbib) suggest that the different players in the biosynthesis of MAs are located at the bacterial poles.

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