Mycolic acids are major and specific long-chain fatty acids that represent essential components of the Mycobacterium tuberculosis cell envelope. They play a crucial role in the cell wall architecture and impermeability, hence the natural resistance of mycobacteria to most antibiotics, and represent key factors in mycobacterial virulence. Biosynthesis of mycolic acid precursors requires two types of fatty acid synthases (FASs), the eukaryotic-like multifunctional enzyme FAS I and the acyl carrier protein (ACP)–dependent FAS II systems, which consists of a series of discrete mono-functional proteins, each catalyzing one reaction in the pathway. Unlike FAS II synthases of other bacteria, the mycobacterial FAS II is incapable of de novo fatty acid synthesis from acetyl-coenzyme A, but instead elongates medium-chain-length fatty acids previously synthesized by FAS I, leading to meromycolic acids. In addition, mycolic acid subspecies with defined biological properties can be distinguished according to the chemical modifications decorating the meromycolate. Nearly all the genetic components involved in both elongation and functionalization of the meromycolic acid have been identified and are generally clustered in distinct transcriptional units. A large body of information has been generated on the enzymology of the mycolic acid biosynthetic pathway and on their genetic and biochemical/structural characterization as targets of several antitubercular drugs. This chapter is a comprehensive overview of mycolic acid structure, function, and biosynthesis. Special emphasis is given to recent work addressing the regulation of mycolic acid biosynthesis, adding new insights to our understanding of how pathogenic mycobacteria adapt their cell wall composition in response to environmental changes.
attractive reservoir of targets for future chemotherapy, whose development is particularly urgent in the context of multidrug-resistant (MDR) tuberculosis and the nearly untreatable (10) extensively drug-resistant (XDR) strains of M. tuberculosis.

Through a combination of genetic and biochemical studies over the past 15 years, impressive progress has been made in understanding the metabolism, structure, and regulation of many cell envelope components including mycolic acids, whose biosynthesis remains an active field of research. This article focuses on the principal metabolic steps and enzymatic components in the biogenesis of mycolic acids, highlighting the key recent advances in this very dynamic area of research.

**STRUCTURE AND DIVERSITY OF MYCOLIC ACIDS**

**Mycolic Acid Structures**

It has been 75 years since R. J. Anderson demonstrated that the prolonged saponification of the wax fraction from the human tubercle bacillus yielded large amounts of very high molecular weight hydroxy fatty acids that were named “mycolic acids” (11). The first structural information came from heating the mycolic acid extract under reduced pressure to 250 to 300°C (Fig. 1), which generated hexacosanoic acid and an unidentified long-chain component that together showed an empirical formula of C_{88}H_{172}O_{4}. Intensive development of chromatography in the late 1950s and 1960s made it possible to define the general structure of mycolic acids as very...
long-chain, α-alkyl β-hydroxy fatty acids (Fig. 1). This definition explained the nature of the two products of mycolic acid pyrolytic cleavage: a short alkyl chain called the α branch (placed in the α position according to the carboxylic acid group) and a long-chain meromycolate called the meromycolic acid chain (the part of the molecule from the terminal methyl to the carbon atom bearing the hydroxyl group) (12). The two stereocenters in the α and β positions relative to the carboxylic group have both been found to be in the R configuration for all the mycolic acids examined, irrespective of the other functional groups (13).

The use of reversed-phase high-pressure liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy, and mass spectrometry permitted the identification of the variety of mycolic acids with respect to their length and chemical modifications. In contrast to the nonfunctionalized, fully saturated α branch, the meromycolate chain is usually composed of three polymethyleneic parts (Fig. 1), linked by two (sometimes three) carbon atoms with either a double bond, cyclopropane rings, or various oxygen functional groups (2). Structural modifications occur in two positions of the meromycolate chain: proximal and distal, with respect to the β-hydroxy group (2). Polar modifications are usually restricted to the distal position and include keto-, epoxy-, and methoxy-functional groups. Nonpolar modifications occur at both proximal and distal positions and include double bonds or cyclopropane rings in cis or trans conformation. Nonpolar modifications in trans conformation are always accompanied by an adjacent methyl group (2). Each mycobacterial species is characterized by a specific mycolic acid profile. M. tuberculosis possesses three major classes of mycolic acids: α-mycolic, keto-mycolic, and methoxy-mycolic acids (Fig. 1). The α-mycolic acid is a cis, cis-dicyclopentyl fatty acid that can occur in two variants, depending on the strain. These variations occur in the length of the terminal alkyl group and the number of methylene groups between the cyclopropane rings and the carboxyl group. Methoxy- and keto-mycolic acids can also contain either cis- or trans-cyclopropane rings at the proximal position.

In addition to mycobacteria, mycolic acids are also found in other genera named “mycolata” of the Corynebacterineae suborder: Corynebacterium, Dietzia, Rhodococcus, Nocardia, Gordonia, Tsukamurella, and Segniliparus. The common feature of the nonmycobacterial mycolic acids is the presence of a double bond as the only modification within the meromycolate chain. The clear distinctions in chain size between genera can be utilized in their differentiation (14). In Corynebacterium the mycolic acids are the smallest (C22 to C36) (15), and a recent finding dethroned M. tuberculosis as the bacteria producing the longest mycolate chains. The rapidly growing, acid-fast staining bacteria isolated from the human respiratory tract, assigned to the Segniliparus genera—Segniliparus rotundus—produces mycolates (termed segnilomycolates) that range from C58 to C100 (16). The overall chain length, the chain length diversity, and the degree of cis unsaturation of segnilomycolates are larger than previously described for other mycolic acid–producing organisms (16).

Originally, α-mycolic acid was the first fraction eluted from a column of adsorption chromatography during separation of structural classes of mycolic acids. This subspecies represents the most widespread class of mycolic acids, containing cyclopropane rings or double bonds in cis or trans with respect to the adjacent methyl group conformation. It is the major mycolic acid in most mycobacterial species and commonly possesses 76 to 86 carbons (17). Subsequently, the shorter α-mycolates with about 60 carbon atoms, designated α’-mycolic acids, were identified in mycobacterial species such as Mycobacterium smegmatis (C62 to C64), Mycobacterium chelonae (C64), Mycobacterium fortuitum (C68), and Mycobacterium vaccae (C58 to C60), but not in M. tuberculosis. Mycolic acids containing keto-, epoxy-, and methoxy-functional groups are eluted from a column after the α-mycolates. Like the α-mycolates, the keto-mycolates are also a widely distributed class of mycolic acids, being present in pathogenic and saprophytic strains regardless of their growth rate. Epoxy-mycolic acids contain a trans-epoxy ring in the meromycolate chain (18). Their presence is restricted to several slow- and fast-growing species such as Mycobacterium farcinogenes, Mycobacterium senegalense, Mycobacterium chitae, Mycobacterium aurum, and M. smegmatis. However, neither the growth rate nor the pathogenic status correlates with epoxy-mycolic acid distribution (2).

With only a few exceptions, the presence of methoxy-mycolates is restricted to pathogenic, slow-growing species. More than 20 years after the initial description of methoxylated mycolates (19), Luquin et al. identified a novel mycolic acid containing a methoxy group at the ω-1 position and two double bonds in the long meroricin hydrate chain (20). The last fraction of mycolates eluted from a chromatographic column contains highly adsorbed carboxy-mycolic acids and their oxygenated precursors—wax-mycolic acids. Carboxy-mycolic acids have a second carboxyl group and a lower molecular weight (C60 to C68) (21).
The variability of mycolic acids is not limited to the structural class. Heterogeneity is also observed within each class and can be described on three levels: the length of the α branch, the length of the meromycolate chain, and the internal position of the functional groups (2). Mycolic acid species- and strain-specific patterns of Mycobacterium and other Corynebacterineae provide specific fingerprints that have been valuable in chemo-taxonomic assignments. Because mycolates are bacteria-derived (not synthesized by humans), chemically inert, and directly involved in the host-pathogen interaction, they are attractive diagnostic markers for tuberculosis. Early methods for profiling the different classes of mycolates used one- and two-dimensional thin-layer chromatography (22), but as HPLC gained widespread application in biochemistry, this was proposed as an aid in mycobacterial classification and offered as a standard test for the identification of mycobacteria in clinical specimens. Although HPLC-based methods are constantly being improved (23), recent work suggests that the future of clinically useful methods of Mycobacterium speciation belongs to mass spectrometry (24). These authors used electrospray ionization/mass spectrometry to develop a selective and sensitive diagnostic strategy that involves targeted quantification of mycolates in poorly defined heterogeneous biological material such as sputum. The approach is safe and fast, and because no culturing or chemical derivatization is needed, sample preparation and handling are simplified.

Although useful in taxonomy and diagnostics, the complexity of natural mixtures of mycolic acids is problematic when attempting to characterize a single enantiomer molecule and elucidate its physical and chemical properties or function(s). For these reasons, extensive work was done recently in the field of chemical synthesis of mycolic acids. Alpha-mycolates were the first synthetic mycolate reported (25), but the synthesis of methoxy-, keto-, and epoxy-mycolic acids as well as single enantiomers of cis- and trans-alkenes-containing mycolates have also been reported and recently reviewed (26).

Mycolic Acid Biological Functions

Mycolic acids exist in mycobacteria in three forms: (i) covalently bound to the AG, a peptidoglycan-linked polysaccharide in the inner leaflet of cell wall lipid bilayer; (ii) esterified to a variety of carbohydrate-containing molecules (mostly as trehalose mono- and dimycolate) in the extractable lipid fraction of the cell wall outer leaflet; and (iii) secreted as free mycolic acids. Depending upon the organization in the cell envelope, mycolic acids give rise to important characteristics including resistance to chemical injury and dehydration, low permeability to hydrophobic antibiotics, the ability to form biofilms (4, 27, 28), and the capacity to persist within the host (28-30). In the mycobacterial cell wall, mycolic acid hydrocarbon chains of the inner leaflet are tightly packed in a parallel fashion, perpendicular to the cell surface (1, 3). They esterify hexaarabinose motifs of AG at the C-5 position, thereby limiting their lateral mobility. Simultaneously, 1,5-linked arabinofuranose units allow for exceptional conformational flexibility of the hexaarabinose “head groups” that likely facilitates closer packing of the mycolate chains (31). Functionalities localized in the proximal part of the meromycolate chain are generally responsible for maintaining the viscosity of the cell wall at an appropriate level, while the trans- proximal unsaturations or cyclopropane rings make this part of the meromycolate chain more rigid. Conversely, the distal part of the mycolate layer is the region that interacts with extractable lipids; thus, cis unsaturations and methyl groups at the distal position disrupt tight local packing of mycolates and allow the extractable lipids to intercalate their acyl chains into the inner leaflet, where they associate with AG-linked mycolates. As shown by Yuan et al., distal cis-cyclopropanation renders Mycobacterium more resistant to killing by hydrogen peroxide (32). Oxygenated mycolates (mainly keto-mycolates) are very active as hydrogen bond acceptors, which promotes the association of peripheral cell surface molecules (2).

Mycolic acids of the extractable lipid fraction exist predominantly as mono- and di-mycolyl trehalose (TMM, TDM). TDM represents the most abundant, granulomagenic, and significantly toxic lipid extractable from the cell surface of virulent M. tuberculosis (33). It has immunostimulating (34) and adjuvant properties (35) and potent antitumor activity (36) and actively participates in blocking mycobacterial phagosome maturation (37). It was recently shown that macrophage-inducible C-type lectin (Mincle) (38) on the macrophage surface recognizes M. tuberculosis TDM, and by working together with the Fcγ receptor transmembrane segment, it induces inflammation (39). In addition, mycolic acids can also be found in dimycolyl diarabinoglycerol (DMAG) (40), an extractable, cell wall-associated glycolipid capable of inducing the expression of proinflammatory cytokines and promoting the expression of the ICAM-1 and CD40 cell surface antigens through a TLR2-dependent mechanism (41).

There is a growing body of evidence of the significance of secreted free mycolates as potential players in
the host-pathogen interaction during *M. tuberculosis* infection. The presence of free mycolic acids in the extracellular matrix of *M. tuberculosis* biofilms was confirmed *in vitro* (4), and Ojha et al. provided evidence that newly synthesized TDM is one of the precursors of free mycolates. TDM is processed by a TDM-specific esterase releasing mycolic acids that are subsequently secreted to form the biofilm matrix (42). The role of mycolic acids in immune regulation was reported by Beckman et al., who showed that presentation of mycolates on CD1b of human dendritic cells stimulated CD4/CD8 double negative T cell lines (43). Administration of purified mycolic acids into mice airways elicited an acute neutrophilic airway inflammation that was accompanied by a moderate and chronic IL-12 production (44). Working on modified synthetic mycolic acids, Vander Beken et al. confirmed that the type of distal group oxygenation in the meromycolate chain is the main determinant of pulmonary inflammatory potency: oxygenated methoxy- and keto-mycolic acids with cyclopropane rings in the cis conformation exhibited strong and mild inflammatory responses, respectively, whereas α-mycolates did not cause any inflammation (45).

**MYCOLIC ACID BIOSYNTHESIS**

**Synthesis of Malonyl-CoA**

Malonyl-coenzyme A (malonyl-CoA) is the universal, two-carbon substrate for the synthesis of mycolic and other fatty acids in mycobacteria. It is generated by the carboxylation of acetyl-CoA in a biotin-dependent, two-step reaction catalyzed by acetyl-CoA carboxylase (ACC) (46) and incorporated into the growing acyl chain during the repetitive cycle of the fatty acid synthase I and II (FAS I/FAS II) reactions. Each half-reaction is catalyzed by a specific ACC subunit: the first step by biotin carboxylase and the second step by carboxyltransferase, each catalytic subunit being encoded by a separate gene (47). Three genes thought to encode the β subunit or biotin carboxylase (*accA1* to *accA3*) and six genes believed to encode the β subunit or carboxyltransferase (*accD1* to *accD6*) have been identified in the *M. tuberculosis* genome (48). Since the β subunits confer the substrate specificity of ACC, the large number of *accD* genes in mycobacterial genomes reflects the ability of mycobacteria to carboxylate not only acetyl-CoA but also several other distinct substrates. Among the six carboxyltransferase genes in *M. tuberculosis*, *accD4*, *accD5*, and *accD6* are essential for cell survival (49, 50) and are expressed at high levels during intensive mycolate biosynthesis (51). For several years, AccD4 and AccD5 were the only carboxyltransferase subunits purified from mycobacterial cell extracts, so they were initially considered to be major constituents of ACC complexes in tubercle bacilli, but *in vitro* analysis showed that neither of them can be considered the subunit dedicated exclusively to acetyl-CoA carboxylation (52–56).

Recent studies have focused attention on the third essential carboxyltransferase gene, *accD6* (Rv2247), which surprisingly remained the least characterized carboxyltransferase gene, despite being the only ACC candidate of the FAS II locus (Fig. 2). An *M. tuberculosis* mutant exhibiting 10-fold reduction of *accD6* expression presented restricted growth, inhibition of proper fatty and mycolic acid biosynthesis, and an altered cell morphology (50). Inhibition of fatty acid synthesis occurred at a very early stage, likely reflecting the impaired activity of acetyl-CoA carboxylase. These results provided the first *in vivo* evidence that AccD6 is a key player in mycolate biosynthesis and implicated AccD6 as the critical component of *M. tuberculosis* ACC, in agreement with *in vitro* findings demonstrating that the AccD6 (Rv2247) protein (β subunit), together with AccA3 (Rv3285) (α subunit), reconstitutes an enzyme that preferentially carboxylates acetyl-CoA (51). Strikingly, parallel studies demonstrated that although essential for *M. tuberculosis*, the *M. smegmatis* *accD6* homologue—MSMEG_4329—can be deleted without affecting the cell envelope integrity and permeability (50). This finding, contradicting that of Kurth et al. (57), suggests that another AccD subunit fulfills the function of AccD6 in *M. smegmatis*. In addition, although in both species *accD6* is a member of the FAS II transcriptional unit and its expression is controlled by the P*fasII* promoter, it was found that *accD6* of *M. tuberculosis*, but not *M. smegmatis*, possesses its own, additional promoter (P*acc*). This implies that in the pathogenic strain, *accD6* expression may be controlled by two regulatory sequences, P*fasII* and P*acc*. Although the additional promoter seems not to participate in supporting the physiological expression level of *accD6* in standard growth conditions, it is able to sustain the expression of this gene on a level allowing for cell survival in the absence of P*fasII* (50). The cause of the difference in *accD6* essentiality between pathogenic and nonpathogenic species as well as the role of possible differences in regulation of its expression remain to be investigated, but insights into the enzymatic mechanism of the *M. tuberculosis* AccD6 should be forthcoming thanks to the recently published crystallographic analysis (58).
**FAS I and Short-Chain Fatty Acid Biosynthesis**

The *fasI* gene (Rv2524c) encodes the multifunctional type I fatty acid synthase (FAS I) that carries all the functional domains required for *de novo* fatty acid synthesis (59). These domains are organized in the following order: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein, ketoacyl reductase, and ketoacyl synthase. All intermediates that are generated during the process of elongation remain enzyme-bound and undergo transacylation to other catalytic sites within the enzyme. FAS I catalyzes the *de novo* synthesis of C16:0 and C18:0 acyl-CoAs from acetyl-CoA using malonyl-CoA as the extender unit (Fig. 3). These acyl-CoAs either can be used for the synthesis of membrane phospholipids or can be further elongated by FAS I to produce C24:0-CoA in the fast-growing organism *M. smegmatis* (60) and C26:0-CoA products in the slow-growing *Mycobacterium bovis* and *M. tuberculosis* (61).

This bimodal product distribution characterizing FAS I has been further investigated by generating a recombinant *M. smegmatis* strain in which the native *fasI* gene was deleted and replaced with the *M. tuberculosis* *fasI* gene (62). Surprisingly, the *in vivo* elongation of C16:0 did not follow the *M. tuberculosis* profile, but this recombinant strain contained both C24:0 and C26:0 fatty acids, thus challenging the simple concept that the C16:0 elongation to C24:0 in *M. smegmatis* or C26:0 in *M. tuberculosis* is exclusively dependent on FAS I; this is likely to result from a more complex interaction between the FAS I enzyme and the FAS II cycle. The bimodal product distribution may also rely on intrinsic determinants of the FAS polypeptides and/or external factors affecting the elongation process. For example, studies on the product-regulation mechanisms for fatty acid biosynthesis catalyzed by *M. smegmatis* FAS I demonstrated that the formation of palmitic acid in the presence of synthetic O-alkyl polysaccharide is regulated by the removal of the end product via a tight stoichiometric complex between the C16:0 fatty acid and the O-alkyl polysaccharide (63). In *M. tuberculosis* the C26:0 fatty acids synthesized by FAS I, known as the α branch, will become the substrate of a dedicated acyl-CoA carboxylase to generate the α-carboxy C26:0 fatty acid used as one of the substrates by the polyketide synthase Pks13. The recent cryo-electron microscopy reconstruction of the 2-MDa *M. smegmatis* FAS I at 7.5 Å revealed that there is a high degree of conservation between FAS I multifunctional enzymes from different kingdoms not only in the amino acid sequence but also on the overall architectural level (64).

**FabH: the Pivotal Link Between FAS I and FAS II**

The FAS I and FAS II systems are interconnected by the β-ketoacyl-ACP synthase III or FabH (Rv0533c) (Fig. 3). This enzyme catalyzes a decarboxylative condensation of malonyl-ACP with the acyl-CoA (C16:0 to C18:0) products of the type I FAS. The resulting 3-ketoacyl-ACP-M derivatives are then reduced to an acyl-ACP, extended by two carbons, and shuffled into the FAS II cycle.
cycle. FabH represents the pivotal link between the two FAS systems and thus initiates meromycolic acid biosynthesis (65). This proposed function of FabH is supported by the presence of a conserved catalytic triad (Cys112, His244, and Asn274) characteristic of β-ketoacyl-ACP synthase III enzymes, by its ability to produce β-ketoacyl-AcpM derivatives (65), and by its marked preference for acyl-CoA as a substrate, rather than acyl-ACP primers. Compared with the general Escherichia coli FabF/FabH type of β-ketoacyl synthases, the M. tuberculosis FabD-AcpM, the building block used by the condensase involved in initial extension of the mycolate chain, is lined with numerous hydrophobic amino acids (66). It contains a CoA/malonyl-ACP binding channel that runs from the surface of the protein to the cysteine residue within the active site. FabH also contains a second hydrophobic pocket leading from the active site, which is blocked by Thr87. The difference in these amino acid residues is postulated to account for the difference in substrate specificities between the two enzymes; Phe87 constrains specificity to acyl-CoA in E. coli, while Thr87 allows binding of long-chain acyl-CoAs in FabH. This second hydrophobic pocket in FabH is capped by an α helix, which restricts the bound acyl chain length to 16 carbons, thus excluding longer-chain acyl-CoA products (C24 to C26) from chain elongation. In addition, several residues influencing catalysis and substrate specificity of FabH have been assigned through the combination of structural studies and site-directed mutagenesis (67). These observations are consistent with the proposed role of FabH as the initiator of mycolic acid elongation and clearly distinguish it from the chain extension steps catalyzed by KasA/KasB.

**FAS II and Long-Chain Fatty Acid Biosynthesis**

The genes encoding the FAS II enzymes lie in three independent transcription units (Fig. 2). The first is formed by fabD-AcpM-kasA-kasB-accD6, the second by fabA-inhA, and the third consists of three genes: hadA-hadB-hadC. The first open reading frame of the five-gene operon, fadD, encodes a malonyl-CoA:AcpM transacylase (MCAT) that catalyzes the formation of malonyl-AcpM, the building block used by the condensing enzymes for the extension of the fatty acid chain (Fig. 3). FabD catalyzes *in vitro* the transacylation of malonate from malonyl-CoA to phosphopantothenylated bolo-AcpM (68). The second open reading frame of this operon encodes the acyl carrier protein AcpM, a C-terminally extended homologue of bacterial ACPs that shuttles the growing acyl chain between the discrete monofunctional enzymes that catalyze the individual steps (68, 69). The elongation process that follows the FabH-dependent condensation reaction of the FAS II system is carried out by the β-ketoacyl-AcpM enzymes encoded by kasA (Rv2245) and kasB (Rv2246) that are also in the five-gene operon (Fig. 2 and Fig. 3).

Both KasA and KasB catalyze the condensation of acyl-AcpM and malonyl-AcpM, hence elongating the growing meromycolate chain by a further two carbon units (8, 70, 71). KasA and KasB share many similarities, including the specificity for long chain acyl-AcpM primers. The Kas enzymes of *E. coli*, ecFabB/ecFabF, and KasA/KasB of *M. tuberculosis* share substantial sequence similarity (70): KasA and KasB are 67% identical and are 92% and 91% identical with their respective homologues in *Mycobacterium leprae*. These enzymes first transfer the acyl chain to the active-site cysteine, resulting in an acylated KasA intermediate. Subsequently, the acyl chain is elongated by two more carbon atoms derived from the second substrate malonyl-AcpM in a condensation reaction with the KasA intermediate (72). Participation of Cys171, His311, Lys340, and His345 in KasA catalysis was subsequently confirmed by replacing these residues with alanine, which abolishes the overall elongation activity of KasA (72). Both the KasA and KasB crystal structures have been solved (73, 74), and by using polyethylene glycol to mimic a 40-carbon fatty acid substrate, it was possible to characterize the hydrophobic acyl-binding cavity of KasA, which is lined with numerous hydrophobic amino acids and perfectly accommodates the growing fatty acid chain (73). Although appearing highly redundant, KasA or KasB might catalyze the initial elongation reactions, while KasB extends the elongation to full-length mycolates. Cell lysates of *M. smegmatis* overproducing KasA generate C40-monounsaturated fatty acids, whereas cell lysates of *M. smegmatis* overproducing both KasA and KasB generate C54-multiprenylsaturated fatty acids (75). In addition, disruption of kasB in *M. tuberculosis* resulted in meromycolate chains that were four to six carbons shorter than wild-type mycolates and also in the loss of the trans-cyclopropane oxygennonate mycolates, which are usually produced by the trans-cyclopropane synthase CmaA2 (29).

These results indicate that KasA and KasB function independently on separate sets of substrates and that KasB is required for the full extension of the mycolic acids. In contrast to KasB, KasA is indispensable (76), representing the only essential β-ketoacyl-ACP synthase II. Overall, these findings suggest that KasA is very likely the condensase involved in initial extension of the mycolate chains, whereas KasB is primarily involved in the full-length extension of these molecules. Interestingly,
FIGURE 3 The FAS I and FAS II pathways in *M. tuberculosis*. In both systems, the chain elongation steps consist of an iterative series of reactions built on successive addition of a two-carbon unit to a nascent acyl group, and reaction intermediates are covalently linked to the acyl carrier protein AcpM. FAS I is capable of de novo synthesis from acetyl-CoA producing acyl-CoA either used to synthesize the α-branch or C16/C18-CoA that are directly shuttled into FAS II for the production of the meromycolic acid. FAS II is primed by the CoA-dependent β-ketoacyl-AcpM synthase FabH, which condenses the acyl-CoA with malonyl-AcpM to generate a β-ketoacyl-AcpM, subsequently converted into a saturated enoyl-AcpM by the sequential actions of a β-ketoacyl-AcpM reductase (MabA), a β-hydroxyacyl-AcpM dehydratase complex (HadABC), and a trans-2-enoxy-AcpM reductase (InhA). Subsequent rounds of elongation are initiated by either the KasA or KasB β-ketoacyl AcpM synthases. KasA is thought to be responsible for the early rounds of elongations, whereas KasB is involved in later stages. Also shown are the acetyl-CoA carboxylase (AccA3/AccD6) that produces malonyl-CoA, the malonyl-CoA:AcpM transacylase (FabD) responsible for the synthesis of malonyl-AcpM, as well as the set of SAM-dependent methyltransferases involved in functionalization of the meromycolic acid. doi:10.1128/microbiolspec.MGM2-0003-2013.f3
the *M. tuberculosis* kasB mutant lost its acid-fast staining and was unable to persist in immunocompetent infected mice (29). Together, these findings suggest that both KasA and KasB could be potent drug targets, which seems feasible because both enzymes have been shown to be inhibited by thiolactomycin and related analogues (70, 72).

KasA has recently been shown to link together the FAS II and the phthiocerol dimycocerosate (PDIM) biosynthesis pathways through a specific interaction between PpsB and PpsD. This raises the possibility that lipids could be transferred between the two pathways as a means of increasing mycobacterial lipid diversity (78).

After the condensation step, the β-ketoacyl-AcpM product undergoes a cycle of keto-reduction, dehydrogenation, and enoyl-reduction catalyzed by the β-ketoacyl-AcpM reductase (MabA, FabG1) (79), the β-hydroxyacyl-AcpM dehydratase complex HadABC (80, 81), and the enoyl-AcpM reductase InhA (82), respectively. The genes encoding the functionally and structurally related FAS II reductases, *mabA* (*Rv1483*) and *inhA* (*Rv1484*), are found in a cluster in the *M. tuberculosis* genome (Fig. 2). The *inhA* gene was identified as the putative target for both INH and ethionamide in *M. tuberculosis* (83), and the InhA protein was demonstrated to catalyze the 2-trans-enoyl-ACP reduction, with a preference for long-chain substrates (82) (Fig. 3). MabA encodes a β-ketoacyl-AcpM reductase with preference for long-chain substrates and was shown to be involved in the elongation activity of FAS II (79) (Fig. 3). In comparison with other bacterial reductases, MabA has unique functional and structural properties, such as a large hydrophobic substrate-binding pocket, which is consistent with its preference for long-chain substrates and its role in mycolic acid biosynthesis (79, 84). Despite intensive investigation over several years, there have been no reports of the successful generation of *mabA* or *inhA* gene knockout mutants, strongly suggesting that they are essential for mycobacterial viability.

Dehydration of the β-hydroxyacyl-ACP intermediate into 2-trans-enoyl-ACP is catalyzed by the β-hydroxyacyl-AcpM dehydratases encoded in the gene cluster *hadA-hadB-hadC* (*Rv0635, Rv0636, Rv0637*, respectively) (80) (Fig. 2 and Fig. 3). Heterologous expression of these proteins in *E. coli* led to the formation of two heterodimers, HadAB and HadBC, both showing the enzymatic properties expected for the mycobacterial FAS II dehydratases, with a marked specificity for both long-chain and AcpM-bound substrates. Interestingly, several recent studies indicated that mutations within the HadA or HadC components were associated with resistance to the antitubercular drugs thiacetazone (85–87) and isoxyl (87, 88). Future work defining the three-dimensional structures of HadAB and HadBC heterocomplexes should help in understanding how these mutations generate drug resistance and should also delineate new structural scaffolds for drug development.

**Functionalization of the Meromycolic Acid**

As mentioned above, the different mycolic acid species are determined by the chemical modifications of the unsaturations within the meromycolic chain and the nature of chemical groups introduced. *M. tuberculosis* carries three types of mycolic acids (Fig. 1) containing cyclopropane rings, methoxy and keto functions, or methyl branches (3, 89). The chemical modifications are performed by a series of eight S-adenosyl methionine (SAM)-dependent methyltransferases, encoded at four genetic loci (89) (Table 1). These enzymes have extensive sequence and structural similarities (90–92), but genetic studies have defined the distinct functional roles of each in the biosynthesis of mycolic acids, which were not revealed when the enzymes were overexpressed in *M. smegmatis* (32, 93, 94).

Deletion of *pcaA* greatly reduces synthesis of the proximal cyclopropane ring of the α-mycolates (28), whereas deletion of *mmaA2* reduces the distal cyclopropane of the same lipid and also causes a mild impairment of methoxy-mycolate, but not keto-mycolate, *cis*-cyclopropanation (95). Similar genetic approaches established *cmaA2* as the only *trans*-cyclopropane synthase of oxygenated mycolates, because inactivation of this gene was associated with double bonds in place of the proximal *trans*-cyclopropane rings (96). To examine potential redundancy between mycolic acid methyltransferases, *M. tuberculosis* mutants were generated lacking *mmaA2* and *cmaA2*, *mmaA2* and *cmaA1*, or *mmaA1* alone (97). The strain lacking both *cmaA2* and *mmaA2* failed to *cis*-cyclopropanate methoxy-mycolates or keto-mycolates, phenotypes not shared by the *mmaA2* and *cmaA2* single mutants. In contrast to the loss of *mmaA2* alone, disruption of both *cmaA1* and *mmaA2* had no effect on mycolic acid modification. Deletion of *mmaA1* from *M. tuberculosis* abolishes *trans*-cyclopropanation without accumulation of *trans*-unsaturated oxygenated mycolates, placing MmaA1 in the biosynthetic pathway for *trans*-cyclopropanated oxygenated mycolates before CmaA2. These results suggest a substantial redundancy of function for MmaA2 and CmaA2, the latter of which can function as both a *cis*- and *trans*-cyclopropane synthase for the oxygenated...
mycolates (97). No mycolic acid modification function has been elucidated for the umaA1 or cmaA1 gene products in M. tuberculosis (95, 97, 98), despite the cyclopropanating activity of the latter when expressed in M. smegmatis (32).

Deletion of mmaA4 abolishes synthesis of both methoxy- and keto-mycolates, while still able to synthesize α-mycolates (27, 99, 100). MmaA4 is capable of methylation of the cis double bond and, in the presence of a water molecule, catalyzes hydroxylation. The hydroxy-mycolate intermediates are at the branch point between keto- and methoxy-mycolates, the latter resulting from the action of the methoxylase MmaA3 (27, 94, 101–103). Many M. bovis BCG strains have point mutations within the mmaA3 gene that inactivate its activity and thus abolish the production of methoxy-mycolates (102, 104).

Several studies have implicated individual cyclopropanate modifications as important factors in the M. tuberculosis host-pathogen interactions. Inactivation of pcaA was associated with a loss of bacterial cording, defective persistence, less-severe granulomatous pathology, and attenuation in a mouse tuberculosis model (28, 105). Recent work has also highlighted the unexpected role of PcaA in intracellular survival and the inhibition of phagosome maturation in M. bovis BCG–infected human monocyte-derived macrophages (106). In contrast to pcaA, deletion of cmaA2 had no effect on bacterial loads during mouse infection but appeared to produce hypervirulence while stimulating a more severe granulomatous pathology (107). Inactivation of mmaA4, which leads to an absence of methoxy- and keto-mycolates, causes a severe growth defect during the first 3 weeks of infection (27, 99). Overall, these studies suggest that the fine structure of mycolic acids plays a role in the pathogenesis of M. tuberculosis. One mechanism by which cyclopropanation mediates virulence is through altered inflammatory activity of TDM. The cyclopropane content of TDM has been shown to be a major determinant of its inflammatory activity and M. tuberculosis virulence (99, 105, 107). From studies on mutant strains constructed to contain multiple gene deletions, it was found that M. tuberculosis is viable either without cyclopropanation or without

### TABLE 1: Genes involved in M. tuberculosis fatty/mycolic acid biosynthesis

<table>
<thead>
<tr>
<th>Activity</th>
<th>Gene</th>
<th>Designation</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>De novo synthesis of fatty acids</td>
<td>fadl</td>
<td>Rv2524c</td>
<td>Type I fatty acid synthase</td>
</tr>
<tr>
<td>Fatty acid elongation</td>
<td>fabD</td>
<td>Rv2243</td>
<td>Malonyl-CoA:AcpM transacylase</td>
</tr>
<tr>
<td></td>
<td>acpM</td>
<td>Rv2244</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td></td>
<td>kasA</td>
<td>Rv2245</td>
<td>β-Ketoacyl-AcpM synthase I</td>
</tr>
<tr>
<td></td>
<td>kasB</td>
<td>Rv2246</td>
<td>β-Ketoacyl-AcpM synthase II</td>
</tr>
<tr>
<td></td>
<td>mabA</td>
<td>Rv1483</td>
<td>β-Ketoacyl-AcpM reductase</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Rv1484</td>
<td>2-trans-Enoyl-AcpM reductase</td>
</tr>
<tr>
<td></td>
<td>hadA</td>
<td>Rv0635</td>
<td>β-Hydroxacyl-AcpM dehydratase</td>
</tr>
<tr>
<td></td>
<td>hadB</td>
<td>Rv0636</td>
<td>β-Hydroxacyl-AcpM dehydratase</td>
</tr>
<tr>
<td></td>
<td>hadC</td>
<td>Rv0637</td>
<td>β-Hydroxacyl-AcpM dehydratase</td>
</tr>
<tr>
<td></td>
<td>FabH</td>
<td>Rv0533</td>
<td>β-Ketoacyl-AcpM synthase III</td>
</tr>
<tr>
<td>Synthesis of fatty/mycolic acid precursors</td>
<td>accD6</td>
<td>Rv2247</td>
<td>Carboxyltransferase subunit of acetyl-CoA carboxylase</td>
</tr>
<tr>
<td></td>
<td>accD5</td>
<td>Rv3280</td>
<td>Carboxyltransferase subunit of propionyl-CoA carboxylase</td>
</tr>
<tr>
<td></td>
<td>accA3</td>
<td>Rv3285</td>
<td>Biotin carboxylase subunit of acetyl-,- propionyl-,- and fatty acyl-CoA carboxylase</td>
</tr>
<tr>
<td>Meromycolic acid functionalization^a</td>
<td>mmaA1</td>
<td>Rv0645c</td>
<td>Methyl branch in trans-cyclopropanated, oxygenated mycolates</td>
</tr>
<tr>
<td></td>
<td>mmaA2</td>
<td>Rv0644c</td>
<td>cis-Cyclopropane synthase of α and oxygenated mycolates</td>
</tr>
<tr>
<td></td>
<td>mmaA3</td>
<td>Rv0643c</td>
<td>Methoxy mycolic acid synthase</td>
</tr>
<tr>
<td></td>
<td>mmaA4</td>
<td>Rv0642c</td>
<td>Hydroxy mycolic acid synthase</td>
</tr>
<tr>
<td></td>
<td>cmaA1</td>
<td>Rv3392c</td>
<td>Methytransferase of unknown specificity</td>
</tr>
<tr>
<td></td>
<td>cmaA2</td>
<td>Rv0503c</td>
<td>trans-Cyclopropane synthase of oxygenated mycolates (redundant with mmaA2)</td>
</tr>
<tr>
<td></td>
<td>pcaA</td>
<td>Rv0470c</td>
<td>cis-Cyclopropane synthase of α-mycolates</td>
</tr>
<tr>
<td></td>
<td>umaA1</td>
<td>Rv0469</td>
<td>Methyl transferase of unknown specificity</td>
</tr>
<tr>
<td>Myolic acid condensation</td>
<td>accD4</td>
<td>Rv3799c</td>
<td>Carboxyltransferase subunit of fatty acyl-CoA carboxylase</td>
</tr>
<tr>
<td></td>
<td>Pks13</td>
<td>Rv3800c</td>
<td>Polyketide synthase</td>
</tr>
<tr>
<td></td>
<td>fadD32</td>
<td>Rv3801c</td>
<td>Fatty acyl-AMP ligase/fatty acyl-ACP synthetase</td>
</tr>
<tr>
<td></td>
<td>cmrA</td>
<td>Rv2509</td>
<td>β-Ketoacyl reductase</td>
</tr>
</tbody>
</table>

^aRoles of the various methyltransferases are depicted in Fig. 1.
both cyclopropanation and oxygenated mycolates (108). A complete lack of cyclopropanation confers severe attenuation during the first week after aerosol infection of the mouse, whereas complete loss of all methyltransferases confers attenuation in the second week of infection. Characterization of immune responses to the cyclopropane- and methyltransferase-deficient strains indicated that the net effect of mycolate cyclopropanation is to dampen host immunity.

These findings establish the immunomodulatory function of the mycolic acid modifications and their role in M. tuberculosis pathogenesis, which makes this enzyme family an attractive target for antitubercular drug development.

Unsaturated acyl-CoA substrates are activated to acyl-AMP by FadD32 (C24-26) and FadD35 (C26-28), respectively (53). The functional equivalence of FadD32 and FadD35 was shown in in vitro experiments by swapping the substrate specificities (52). These data establish the role of FadD32 in the activation of long-chain acyl-CoAs at the ends of mycolate chains.

In addition to catalyzing the production of the terminal acyl-CoAs, FadD32 functions as a fatty acyl-AMP ligase, binding to a 2-carboxyl-CoA moiety (52). This enzyme catalyzes the amidation reaction to produce a fatty acyl-AMP, forming a complex that activates the substrate-binding pocket of FadD32. This complex is essential for the activation of the substrate-binding pocket of FadD32.

The combined inhibition of mycolic acid cyclopropanation and oxygenation in M. tuberculosis knockouts (MSMEG_1351-accD4 and MSMEG_1351-accD5) confers attenuation in the second week after infection of the mouse, whereas complete loss of all methyltransferases confers attenuation in the second week of infection. Characterization of immune responses to the cyclopropane- and methyltransferase-deficient strains indicated that the net effect of mycolate cyclopropanation is to dampen host immunity.

These findings establish the immunomodulatory function of the mycolic acid modifications and their role in M. tuberculosis pathogenesis, which makes this enzyme family an attractive target for antitubercular drug development. Indeed, compounds that inhibit this class of enzyme have been shown to kill mycobacteria, thus emphasizing their importance for mycobacterial viability (92, 109). In addition, the activity of thiacetazone, a second-line antitubercular drug, was shown to inhibit mycolic acid cyclopropanation (110), and this activity was dependent on an intact mmaA4 gene. Several thiacetazone-resistant strains of M. bovis BCG or M. tuberculosis harboring mutations within mmaA4 were found to lack keto-mycolates (85, 86, 100). Overall, these results suggest that the combined inhibition of the whole family of methyltransferases involved in mycolic acid functionalization is highly detrimental to M. tuberculosis, thus supporting this enzyme family as a valid target for future antimycobacterial drug development.

Although all mycobacteria synthesize mycolic acids, it had been thought that only pathogenic mycobacteria produce significant quantities of mycolic acids with cyclopropane rings. Recently, however, the presence of cyclopropanated cell wall mycolates was demonstrated in the nonpathogenic species M. smegmatis (98, 111). MSMEG_1351 was identified as a gene encoding a pcaA homologue, and disruption of MSMEG_1351 produced a marked deficiency in cyclopropanation of α-mycocates. Unexpectedly, α-mycolic acid cyclopropanation in M. smegmatis was induced at low growing temperatures (111). The functional equivalence of PcaA and MSMEG_1351 was established by cross-complementation of the MSMEG_1351 knockout mutant with the M. tuberculosis gene. In addition, complementation of an M. bovis BCG pcaA mutant strain with MSMEG_1351 restored the wild-type mycolic acid profile and the cording phenotype. Although the biological significance of mycolic acid cyclopropanation in nonpathogenic mycobacteria remains obscure, it may represent a mechanism of adaptation of the cell wall structure and composition to cope with environmental stresses.

Mycolic Acid Condensation

The ultimate step in the synthesis of full-length, mature mycolic acid relies on the condensation of its two constituents—a C26-CoA acyl chain (α branch) released from FAS I and C52-monomycyl-AcpM, the FAS II end-product. It has been shown that this process requires at least three enzymes encoded within the putative fadD32-pks13-accD4 operon (Table 1) that is only present in mycolic-acid-producing bacterial species (53, 112, 113). Prior to condensation by the polyketide synthase Pks13, the two acyl chains must be activated by AccD4 and FadD32, respectively (53, 114, 115). AccD4 is a putative carboxyltransferase that associates with the AccA3 subunit to form a complex that activates the α branch (C26-CoA) through carboxylation, yielding 2-carboxyl-C26-CoA (53). The M. tuberculosis AccD4 substrate specificity for long-chain C24 to C26 acyl-CoAs was confirmed in vitro (52). It had been proposed that the AccD5 carboxyltransferase might also play a role in the activation of condensation substrates, but solving its crystal structure refuted this notion by showing that the substrate binding pocket of AccD5 is too small to accommodate even the 16-carbon palmitoyl-CoA (116).

FadD32, a fatty acyl-AMP ligase (FAAL) converts the second condensation substrate—meromycolyl-AcpM—to meromycolyl-AMP (53, 115). Following activation, both acyl substrates are loaded onto Pks13, a type I polyketide synthase (PKS) composed of several domains that allow completion of the mature mycolate synthesis (117) (Fig. 4). In addition to its fatty acyl-AMP ligase activity, FadD32 functions as a fatty acyl-ACP synthetase (FAAS) with the N-terminal ACP domain of Pks13 being the natural and most efficient acceptor (118). After meromycolyl-AMP/ACP transacylation, the meromycolyl chain is transferred onto the Pks13 ketoacyl synthase (KS) domain. Simultaneously, 2-carboxyl-C26-CoA is loaded onto the acyl transferase (AT) domain, which catalyzes its covalent attachment to the enzyme active site and then transfers it onto the Pks13 C-terminal ACP domain (117, 119). The ketoacyl synthase domain catalyzes the Claisen-type condensation between the meromycolyl and the carboxyacyl chains to produce α-alkyl, β-keto thioester, which remains bound to the C-terminal ACP domain. Subsequently, the thioesterase (TE) domain catalyzes the release of the α-alkyl, β-keto acyl chain from Pks13. A recent report identified a new class of thiophene compounds that efficiently kill M. tuberculosis by specifically inhibiting fatty acyl-AMP loading onto Pks13 (120), thus confirming the essential requirement of Pks13 in mycolic acid biosynthesis and validating Pks13 as a druggable target.
The final step of mycolic acid synthesis involves reduction of the β-ketoacyl product to yield the mature mycolic acid (Fig. 4). There are a large number of putative reductases in mycobacterial genomes, but comparative studies between C. glutamicum and M. tuberculosis led to the identification of the putative reductase encoding gene, Rv2509, which is highly homologous to cmrA of C. glutamicum (121). Deletion of this gene in Corynebacterium resulted in a slow-growing strain that was deficient in AG-linked mycolates and synthesized...
abnormal forms of the trehalose dicorynomycolate and trehalose monocorynomycolate. Analysis of the aberrant glycolipids by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry indicated that they contained an unreduced β-keto ester. Thus, these data confirmed the role of CmrA and its mycobacterial homologue in the final step of (coryno) mycolic acid maturation and demonstrated the importance of the β-keto ester reduction in the subsequent attachment of mycolic acids to AG in Corynebacterineae.

Because the genes belonging to the pks13 cluster are essential in mycobacteria, they represent attractive drug targets, and a recent screening identified novel classes of FadD32 inhibitors (122). Similarly, an assay was recently described to screen for inhibitors of PptT, the phosphopantetheinyl transferase that activates Pks13 and was shown to be essential for in vitro growth and persistence of M. tuberculosis (123). Following synthesis, MmpL3 transports the mycolic acids outside of the cell in the form of TMM (124–127), which is then used as a substrate by the mycolyltransferase enzymes of the Ag85 complex (128, 129) to transfer the mycolate residue either to the AG complex or to another TMM to form TDM.

Protein-Protein Interactions within the “Mycolome”

The EccA1 component of the ESX-1 protein secretion system has recently been shown to be required for optimal synthesis of mycolic acids (130). Increased mycolic acid synthesis defects were observed in an eccA1 mutant of M. marinum and correlated with decreased in vivo virulence and intracellular growth. This phenotype was proposed to be linked by the interaction between EccA1 with Pks13, KasA, KasB, and MmaA4. This prompted speculation that a normal function of EccA1 is to make mycolic acid biosynthesis more efficient, perhaps by shuffling relevant enzymes to sites of mycolate production (130). These findings indicate that two mycobacterial virulence hallmarks, ESX-1-dependent protein secretion and mycolic acid synthesis, are critically linked via EccA1. The concept of a “mycolome” has recently emerged from elegant work demonstrating that the FAS II system of M. tuberculosis is organized in specialized interconnected complexes composed of the condensing enzymes, dehydratase heterodimers, and methyltransferases (131–133). This led the authors to propose that because the heterotopic interactions among these enzymes are crucial and their disruption detrimental for M. tuberculosis survival, the protein interactions could represent attractive drug targets. In this model of interactome, three types of FAS II specialized complexes are interconnected together: (i) the “initiation FAS II” (I-FAS II) is formed by a core consisting of the reductases, FabD, and FabH, thus linking together FAS I and FAS II; (ii) two “elongation FAS II” (E-FAS II) complexes consisting of a core and either KasA (E1-FAS II) or KasB (E2-FAS II), which are capable of elongating acyl-AcpM to produce full-length meromycolyl-AcpM; (iii) the “termination FAS II” (T-FAS II) involving Pks13, which interacts with KasB and condenses the α branch with the meromycolic branch. The specialized and interconnected complexes of the “dissociated” FAS II system of M. tuberculosis may adopt a composition and architecture similar to a multifunctional FAS I protein.

REGULATION OF MYCOLIC ACID BIOSYNTHESIS

Although a large amount of information has been generated on the genetics, enzymology, and biochemical characterization of the FAS II components, little is known about the regulation of lipid homeostasis in mycobacteria. Since fatty/mycolic acids are not only essential but also energetically expensive for mycobacteria, it is very likely that these microorganisms have developed mechanisms that tightly regulate lipid concentrations. In this context, important advances have been made recently, allowing better understanding of how mycobacteria regulate their mycolic acid metabolism and composition. At least two levels of regulation have been proposed, at a transcriptional and at a posttranslational level, indicating the need for a high degree of control over the synthesis of mycolic acids, to efficiently adapt its composition to the various environmental conditions encountered during the complex lifestyle within the host and/or during the infection process.

Transcriptional Control by MabR

MabR (mycolic acid biosynthesis regulator) was originally identified as a putative transcriptional regulator encoded by Rv2242 and located immediately upstream of the main fasII operon (Fig. 2) (134). In vitro characterization indicated that MabR functions as a transcriptional repressor of the fasII genes by binding directly to the region upstream of fabD. Moreover, overexpression of MabR in M. smegmatis represses transcription of fabD, acpM, kasA, and kasB and was accompanied by reduced levels of mycolic acids and...
changes in the colony morphotype. Knockdown of mabR expression using antisense RNA increased transcription of both fasII genes and fasI. MabR does not directly regulate fasI expression, but the reduced fasI transcripts in a MabR-overexpressing strain illustrate the coordination of phospholipid, triglyceride, and mycolic acid synthesis in mycobacteria (134). These observations, along with the fact that a mabR knockout mutant could only be generated in a mero-diploid strain of M. smegmatis, confirm the predicted essential role of this repression in controlling mycobacterial mycolic acid metabolism. The discovery of MabR raises several important questions concerning how this regulator is integrated into mycobacterial physiology, and a key issue will be to identify the ligand(s) that modulate the DNA binding properties of this transcription factor.

Posttranslational Regulation by Ser/Thr Phosphorylation

Reversible protein phosphorylation is a key mechanism by which extracellular signals can be translated into cellular responses through the modulation of protein expression or activity. Signaling through Ser/Thr phosphorylation has emerged as a critical regulatory mechanism in various microorganisms and particularly in mycobacteria. M. tuberculosis possesses 11 Ser/Thr protein kinases (STPKs) (48, 135) that have been shown to regulate various biological functions ranging from central metabolism to environmental adaptive responses and pathogenicity. Recent studies have emphasized the importance of Ser/Thr phosphorylation in regulating mycobacterial cell shape/division and also the synthesis of major cell envelope components, including mycolic acids (136).

It was originally demonstrated that KasA and KasB were phosphorylated in vitro by multiple STPKs and that phosphorylation modulates their condensing activity (137). This suggests that environmental conditions might directly influence the phosphorylation profile of the condensing enzymes and thus modulate mycolic acid biosynthesis in order to adapt to various stresses. This view was further supported by the fact that, in addition to KasA and KasB, the other condensase, FabH, is also a substrate for mycobacterial STPKs and is efficiently phosphorylated at Thr45, by PknF and PknA (138). Interestingly, Thr45 is located at the entrance of the substrate channel on the crystal structure of FabH, suggesting that the phosphate group may affect FabH enzymatic activity by altering substrate accessibility. Importantly, a T45D variant of FabH, designed to mimic constitutive phosphorylation, exhibited markedly decreased transacylation, malonyl-AcpM decarboxylation, and condensing activities compared with the wild-type protein (138). MabA was also reported to be efficiently phosphorylated in vitro and in vivo, with Thr191 being the primary phosphor-acceptor (139). A MabA T191D mutant, designed to mimic constitutive phosphorylation, exhibited markedly decreased ketoacyl reductase activity compared to the wild-type protein, as well as impaired binding of the NADPH cofactor. The negative effect of phosphorylation on MabA enzymatic activity and the consequent effect on mycolic acid biosynthesis was also shown when constitutive overexpression of the mabA (T191D) allele strongly impaired mycobacterial growth, and conditional expression of the phosphomimetic MabA T191D led to a significant inhibition of de novo biosynthesis of mycolic acids. Studies on the reductases culminated with the recent demonstration that InhA, the primary target of INH, is also controlled via phosphorylation on Thr266 (140). The physiological relevance of Thr266 phosphorylation was demonstrated using an inhA phosphomimetic (T266D) mutant. The in vitro, enoyl reductase activity was severely impaired in the mimetic mutant, and the introduction of the inhA (T266D) allele failed to complement an inhA- thermosensitive M. smegmatis strain, demonstrating that the inhA phosphorylation inhibited mycolic acid in a manner similar to that seen with INH and growth inhibition (140). Furthermore, the activity of the HadAB and HadcBC dehydratases also decreased when these enzymes where phosphorylated (141).

Altogether, these studies strongly suggest that all essential enzymes forming the central core of type II fatty acid synthase are regulated by STPK phosphorylation, and M. tuberculosis may subtly control its FAS II system by regulating each step of the elongation cycle. Because phosphorylation of HadAB and HadcBC enzymes was found to be increased during stationary growth phase (141), it is tempting to speculate that mycobacteria shut down meromycolic acid chain production under nonreplicating conditions, a view that is supported by the fact that the mycolic acid biosynthesis is growth phase–dependent and abrogated during the stationary phase (142). However, whether the control of the meromycolic acid chain length by phosphorylation of FAS II components contributes to M. tuberculosis virulence and/or persistence remains to be investigated.

There is also some evidence that STPK-dependent phosphorylation may additionally regulate the activity of enzymes involved in functional modification of mycolic acids, such as cyclopropane synthases. The
phosphorylation of cyclopropane synthase PcaA on Thr168 and Thr183 by mycobacterial Ser/Thr kinases was reported both in vitro and in vivo (106), although phosphorylation of MmaA2 was not found. Phosphorylation of PcaA was associated with a significant decrease in the methyltransferase activity, and its physiological relevance was further assessed by generating the corresponding PcaA phosphoablative (T168A/T183A) or phosphomimetic (T168D/T183D) M. bovis BCG strains. In contrast to the wild-type and phosphoablative pcaA alleles, introduction of the phosphomimetic pcaA allele in a ΔpcaA mutant failed to restore the parental mycolic acid profile and cording morphotype. Importantly, the PcaA phosphomimetic mutant strain exhibited reduced survival in human macrophages and was unable to prevent phagosome maturation (106), thus providing a first link between a Ser/Thr kinase-dependent mechanism for modulating mycolic acid composition and intra-macrophage survival, a hallmark of mycobacterial virulence.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Considerable progress has been made over the past two decades in determining the role of mycolic acids in mycobacterial physiology and virulence, as well as in identifying the genes that participate in mycolic acid biosynthesis. Genetics has clearly established that several genes in this pathway are essential for mycobacterial growth, while others participate in the interaction with the host immune system and/or are required for persistence, thus playing a critical role in the physiopathology of the disease. Because these genes are absent from humans, they represent valuable targets for future drug development. Several independent laboratories are currently developing large panels of chemical analogues of INH, thiolactomycin, thiacetazole to specifically target the enzymes of the mycolic acid synthetic pathway. Combined with genetics to identify the mutations conferring drug resistance and structural studies of the target enzymes, these studies should foster the design of new lead compounds with potent antitubercular activity that could be particularly valuable against multidrug-resistant or extensively drug-resistant strains of M. tuberculosis. Recent work has also identified proteins that participate in the postbiosynthetic processing and transport of mycolic acids, which represent an additional source of potential drug targets, as exemplified by the discovery of inhibitors that target the mycolic acid transporter MmpL3 (124–127, 143).

The important breakthroughs that have begun to unravel the molecular mechanisms involved in the regulation of mycolic acid biosynthesis should also help in understanding how M. tuberculosis modulates its cell wall lipid composition to respond to the changes of environment encountered during infection. Among the key features that remain to be resolved are the signaling events that activate the different Ser/Thr protein kinases and how their phosphorylation of the different components modulates the activity of the FAS II enzymes and the transport of mycolates to the cell surface during the active and chronic phases of the disease. A distinguishing feature of M. tuberculosis is its acid-fast staining, and this property in Ziehl-Neelsen staining remains the cornerstone for diagnosing tuberculosis, particularly in poor countries where the infection is highly prevalent (144). However, dormant bacilli have distinct structural alterations in the cell wall and become Ziehl-Neelsen negative (145). This loss of acid-fastness during dormancy may be linked, at least partially, to changes within the mycolic acid profile, but this remains to be conclusively addressed experimentally. Therefore, investigating the role and contribution of regulatory mechanisms that tightly control mycolic acid biosynthesis under nonreplicating conditions where there is a loss of acid-fast staining could potentially affect our thinking on how to diagnose latent tuberculosis.

ACKNOWLEDGMENT

The authors wish to thank Howard Takiff for critically reading the manuscript.

JP is supported by a grant from the National Science Centre (contract 2012/05/N/NS2/00622) and European Regional Development Fund under the Operational Programme Innovative Economy, grant POIG.01.01.02-10-07/09.

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