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Minireview

MINIREVIEW

# Cholesterol and fatty acids grease the wheels of *Mycobacterium tuberculosis* pathogenesis

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**One sentence summary:** *Mycobacterium tuberculosis* uses cholesterol and fatty acids together to cause disease, we are just now beginning to understand how this process occurs.

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## ABSTRACT

Tuberculosis is a distinctive disease in which the causative agent, *Mycobacterium tuberculosis*, can persist in humans for decades by avoiding clearance from host immunity. During infection, *M. tuberculosis* maintains viability by extracting and utilizing essential nutrients from the host, and this is a prerequisite for all of the pathogenic activities that are deployed by the bacterium. In particular, *M. tuberculosis* preferentially acquires and metabolizes host-derived lipids (fatty acids and cholesterol), and the bacterium utilizes these substrates to cause and maintain disease. In this review, we discuss our current understanding of lipid utilization by *M. tuberculosis*, and we describe how these pathways promote pathogenesis to fuel metabolic processes in the bacillus. Finally, we highlight weaknesses in these pathways that potentially can be targeted for drug discovery.

**Keywords:** cholesterol; fatty acid; lipid; LucA; Mce1; Mce4

## INTRODUCTION

In 2015, *Mycobacterium tuberculosis* caused 1.8 million deaths and initiated ∼10.4 million new cases of tuberculosis (TB; World Health Organization 2016). TB is an insidious disease and ∼90% of infected individuals develop an asymptomatic or latent form of TB in which immunity contains, but does not eliminate, the bacteria from the host. These asymptomatic *M. tuberculosis* infections persist for the entire lifespan of the infected individual; meanwhile *M. tuberculosis* maintains a keen ability to cause debilitating or fatal disease. Due to factors that are not completely understood, roughly ∼10% of these infected individuals will develop active TB disease immediately following initial infection or sometime later in life (Pai *et al.* 2016). Without antibiotic intervention, active TB can be deadly with fatality rates of 53–86 with a weighted mean of 70% in HIV-negative individuals (Tiemersma *et al.* 2011) and this phase of the disease facilitates the aerosol transmission of the bacteria between humans. Thus, the bacterium's ability to persist for long periods of time ensures that *M. tuberculosis* will maintain a pandemic grip on the human population for many years to come.

Humans are the only known reservoir for *M. tuberculosis*, and it is likely that thousands of years of co-evolution in this host have uniquely shaped the bacterium's physiology and pathogenicity (Wirth *et al.* 2008; Comas *et al.* 2013). Many studies have determined that *M. tuberculosis* has a unique ability to assimilate and to utilize host lipids (fatty acids and cholesterol), and this is a defining characteristic of this pathogen (Cole *et al.* 1998). Host-derived lipids are important carbon sources for *M. tuberculosis*, which are catabolized to fuel central metabolic pathways to facilitate the bacterium's persistence (Russell *et al.* 2009). However, lipids are more than simple carbon sources and recent reports have revealed that lipid utilization by *M. tuberculosis* is more complex than was thought previously. *Mycobacterium*

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*tuberculosis* imports and utilizes fatty acids and cholesterol to convert both these lipids into bacterial end products that mediate bacterial pathogenesis. These bacterial lipid end products regulate bacterial replication, drug tolerance and virulence.

In this review, we focus on our understanding of the lipid assimilation and utilization pathways in *M. tuberculosis* with a special emphasis on how these pathways contribute to *M. tuberculosis* pathogenesis. Further, we highlight potential targets in these pathways that may be perturbed with drugs to enhance current and future TB antibiotic treatment(s).

## CHOLESTEROL UTILIZATION BY *M. TUBERCULOSIS*

Over the past 10 years, numerous labs have demonstrated that cholesterol is required for the optimal growth and persistence of *M. tuberculosis* in various animal models of infection (Chang *et al.* 2007; Van der Geize *et al.* 2007; Yam *et al.* 2009; Hu *et al.* 2010; Nesbitt *et al.* 2010; VanderVen *et al.* 2015) and that cholesterol accumulates in human granulomas (Kim *et al.* 2010). The complete degradation of cholesterol by bacteria or any organism is unusual, and only a relatively few gram-negative and *Actinomyces* species are known to do this (Yam *et al.* 2011; Wipperman, Sampson and Thomas 2014). The *M. tuberculosis* genome contains a cluster of ~80 genes (Van der Geize *et al.* 2007) that encode proteins dedicated to the complex processes of cholesterol import, degradation and regulation (Capyk *et al.* 2009, 2011; Yam *et al.* 2009; Dresen *et al.* 2010; Driscoll *et al.* 2010; Lack *et al.* 2010; Nesbitt *et al.* 2010; Ouellet *et al.* 2010; Griffin *et al.* 2011, 2012; Thomas *et al.* 2011; Casabon *et al.* 2013a,b; Casabon *et al.* 2014; Wipperman *et al.* 2013; Frank, Madrona and Ortiz de Montellano 2014; Yang *et al.* 2014, 2015; Lu, Schmitz and Sampson 2015; Ho *et al.* 2016; Crowe *et al.* 2017).

## CHOLESTEROL IMPORT

The mycobacterial cell envelope represents a significant physical barrier for the import of any molecule, and this structure is comprised of a hydrophobic cytoplasmic membrane bilayer, an aqueous pseudo-periplasmic space and an unusually thick layer of hydrophobic mycolic acids (Brennan 2003). To import cholesterol across this problematic atypical diderm cell envelope, *M. tuberculosis* relies on the multiprotein complex termed Mce4. Mutant studies with *M. tuberculosis* have confirmed that the Mce4 complex is required for cholesterol import (Pandey and Sassetti 2008; Nazarova *et al.* 2017) and for cholesterol utilization when *M. tuberculosis* is cultured on cholesterol as the sole carbon source (Pandey and Sassetti 2008; Griffin *et al.* 2011; Perkowski *et al.* 2016; Nazarova *et al.* 2017). The Mce4 complex is one of four closely related, ATP-binding cassette (ABC) transporter-like complexes that are encoded by the *mce1–4* operons in the *M. tuberculosis* genome (Casali and Riley 2007). The *mce4* operon spans the genes *rv3492c-rv3501c* and encodes 10 putative 'core' proteins that make up the Mce4 complex (Fig. 1). This core

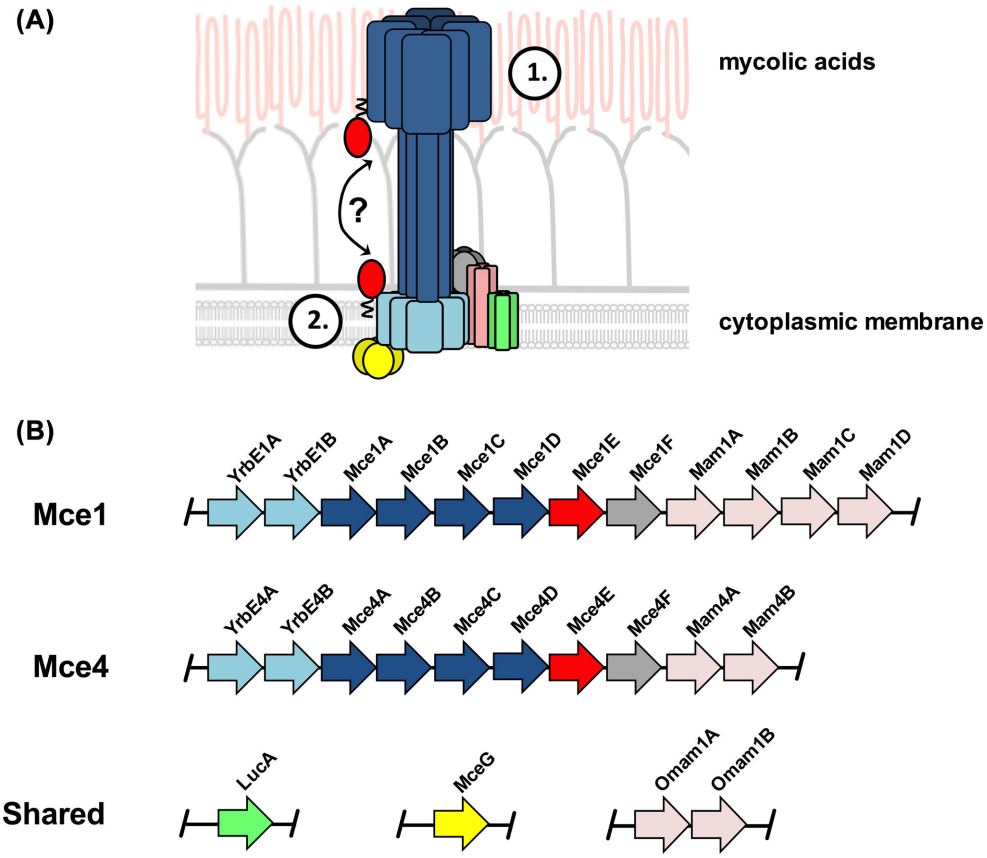


**Figure 1.** Hypothetical model for Mce-mediated lipid transport in *M. tuberculosis* and organization of the *mce1* and *mce4* operons. (**A**) Stage 1 depicts a process where Mce proteins bind and transport the lipid substrates across the exterior portion of the mycobacterial cell wall and pseudoperiplasmic space. Stage 2 illustrates the final translocation of lipid substrates across the cytoplasmic membrane by a putative permease complex. (**B**) The substrate-specific or 'core proteins' of the Mce1 and Mce4 complexes are encoded within the *mce1* and *mce4* operons. The putative subunits shared by the Mce1 and Mce4 complexes (LucA, MceG and OmamAB) are encoded by genes outside of the *mce1* and *mce4* operons.

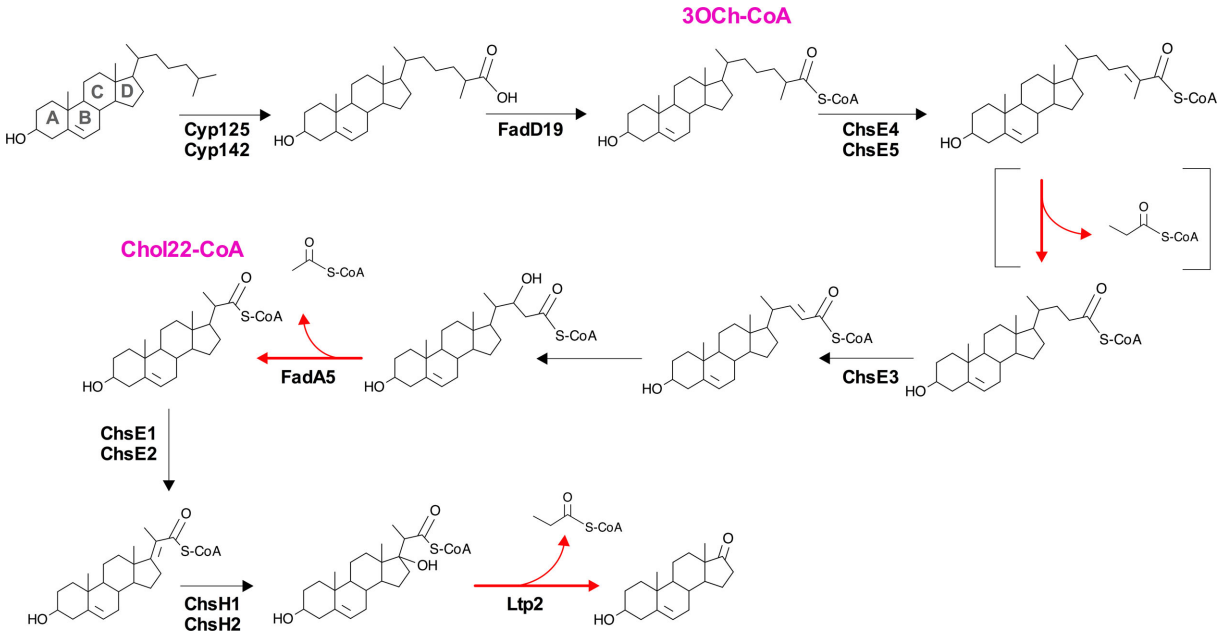


**Figure 2.** Cholesterol side chain degradation in *M. tuberculosis*. Confirmed enzymes are denoted with the enzyme name(s) next to the reaction arrows. Reactions that are indicated with red arrows indicate the release of either acetyl-CoA or propionyl-CoA. Brackets indicate stages in the pathway requiring multiple enzymatic reactions. Pink text denotes chemical name abbreviations of the catabolic intermediates.

complex is comprised of two putative, integral membrane permease subunits (Rv3501/YrbE4 and Rv3502/YrbE4B), which are thought to translocate cholesterol across the cytoplasmic membrane (Casali and Riley 2007). Additionally, the Mce4 complex is comprised of six putative cell wall proteins (Rv3499/Mce4A, Rv3498/Mce4B, Rv3497/Mce4C, Rv3496/Mce4D, Rv3495/Mce4E, Rv3494/Mce4F), all of which conserve distinct Mce domains that probably facilitate cholesterol transport across the mycolic acid layer and/or the pseudoperiplasmic space (Casali and Riley 2007). In addition, the *mce4* operon encodes two accessory proteins (Rv3493/Mam4A, Rv3492/Mam4B), which are required for cholesterol import (Casali and Riley 2007). These accessory proteins likely play a regulatory role to control stability or assembly of the Mce4 complex (Nazarova *et al.* 2017). Although a structural and mechanistic understanding of how the Mce4 complex functions is lacking, the collective data are consistent with the idea that Mce4 mediates cholesterol import. We propose a hypothetical model for the function of proteins in the Mce4 complex, which we describe in more detail below.

Notably, the *mce4* genes are required for optimal growth and persistence of *M. tuberculosis in vivo*. Early genetic screens predicted that the *mce4* genes were required for *M. tuberculosis*'s survival when passaged in mice during 2–4 weeks of infection (Sassetti and Rubin 2003). This observation was confirmed subsequently using an intravenous, competitive infection assay with a mutant lacking the putative Mce4 permease subunit (Rv3501/YrbE4A) (Pandey and Sassetti 2008). In this competition assay, the Mce4 mutant replicated slower in lung tissues relative to wild type beginning 4 weeks post-infection, and this growth defect worsened progressively through 14 weeks post-infection. Additionally, a *M. tuberculosis* mutant that lacks the entire *mce4* operon grows more slowly in a murine, low-dose, aerosol infection model, and this growth defect is most apparent during the persistent phase of infection (Senaratne *et al.* 2008). Thus, Mce4 is required for optimal bacterial growth and persistence in murine lung tissue regardless of the route of infection. The slow growth phenotype of the Mce4 mutants *in vivo* correlates with the onset of adaptive immunity and the generation of antigen-specific, interferon gamma (IFN-$\gamma$)-secreting CD4 + T cells. Consistent with this, *M. tuberculosis* Mce4 mutants replicate more poorly in IFN-$\gamma$ activated macrophages relative to wild-type bacteria (Pandey and Sassetti 2008). Given that *M. tuberculosis* resides within macrophages and the bacteria appear to preferentially rely on cholesterol in his host cell type (VanderVen *et al.* 2015) blocking cholesterol utilization in *M. tuberculosis* could target intracellular bacteria and augment current therapies.

## DEGRADATION OF CHOLESTEROL

The degradation of cholesterol by *M. tuberculosis* can be categorized into three main processes: (i) $\beta$-oxidation of the cholesterol side chain, (ii) oxygen-dependent cleavage of the A and B rings and (iii) oxidative degradation of the C and D rings. Our understanding of cholesterol degradation by *M. tuberculosis* is not complete, but the current model indicates that the cholesterol degradation principally yields acetyl-CoA, propionyl-CoA, succinyl-CoA and pyruvate that feed intobacterial central metabolism.

## $\beta$-OXIDATION OF THE SIDE CHAIN

Degradation of the cholesterol side chain results in the release of two propionyl-CoA units and one acetyl-CoA unit (Fig. 2), and this process begins with hydroxylation of the terminal C26 or C27 carbon of cholesterol by the monoxygenases, Rv3545/Cyp125 or Rv3518/Cyp142 (Capyk *et al.* 2009; Driscoll *et al.* 2010; Ouellet *et al.* 2010). After side chain hydroxylation, the acyl-CoA synthase, Rv3515/FadD19, ligates a coenzyme-A (CoA) onto the side chain (Casabon *et al.* 2014). This step is necessary to initiate the first cycle of $\beta$-oxidation and the subsequent release of a propionyl-CoA unit. Completion of the first cycle of $\beta$-oxidation requires the multimeric acyl-CoA dehydrogenase complex that is comprised of Rv3504/ChsE4 and Rv3505/ChsE5, but the additional enzymes that are involved in this cycle have

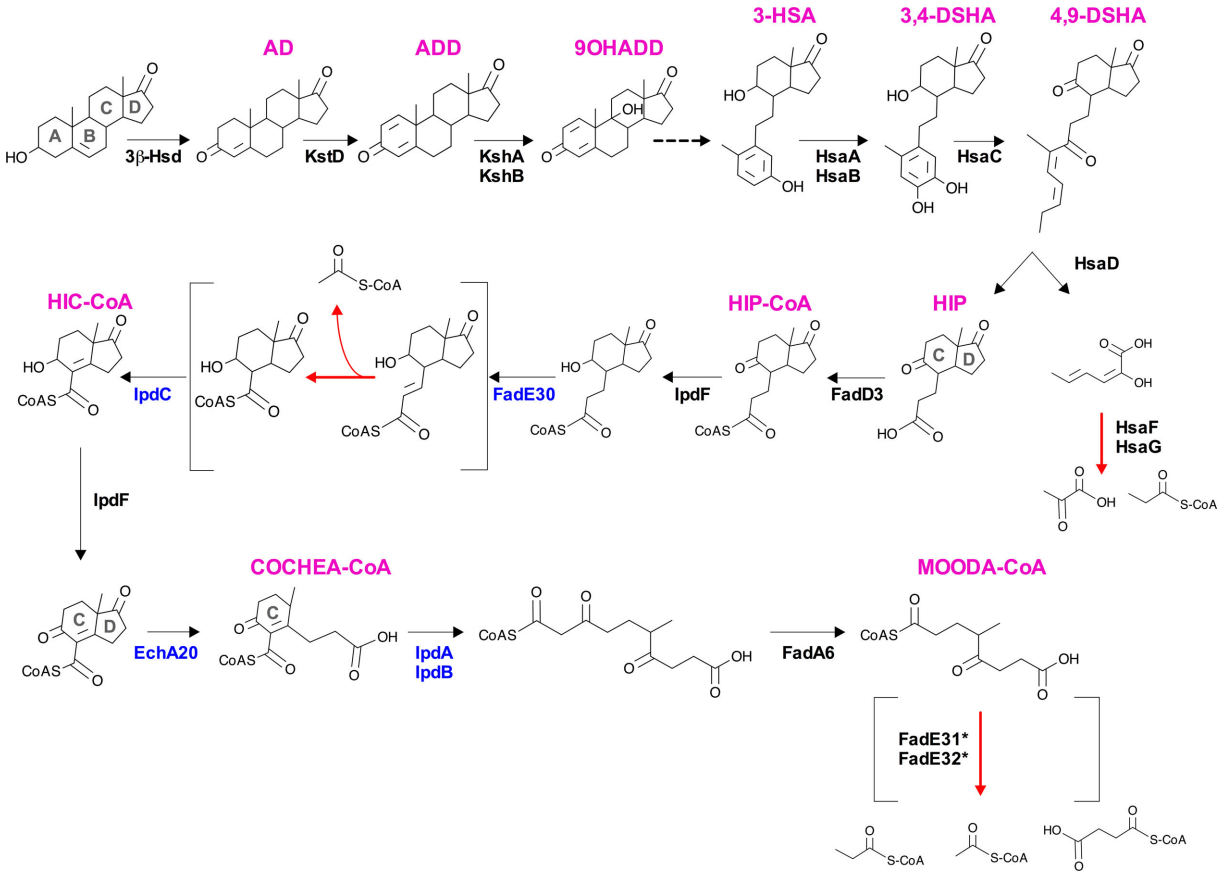


**Figure 3.** Degradation of the cholesterol rings in *M. tuberculosis*. Confirmed enzymes are denoted with the enzyme name(s) next to the reaction arrow. Pink text denotes the abbreviation of the catabolic intermediate chemical name. Black text indicates enzymes from *M. tuberculosis* that catalyze the specific reactions, and blue text indicates confirmed homologous enzymes from related *Actinomyces* spp. that catalyze the specific reactions. Asterisks indicate a tentative assignment, given that MOODA-CoA accumulates in a cholesterol-grown mutant of *M. smegmatis* that lacks FadE32 (Crowe *et al.* 2017). The dashed arrow indicates a spontaneous, non-enzymatic, ring cleavage event in 9OHADD. Reactions indicated with red arrows indicate the release of acetyl-CoA, propionyl-CoA, pyruvate or succinyl-CoA. Brackets highlight stages in the pathway that require multiple enzymatic reactions.

not been identified (Wipperman *et al.* 2013; Yang *et al.* 2015). The dehydrogenase Rv3572/ChsE3 (Yang *et al.* 2015) and the thiolase Rv3546/FadA5 (Nesbitt *et al.* 2010) participate in the second cycle of side chain $\beta$-oxidation to release a single acetyl-CoA unit. The final cycle of side chain $\beta$-oxidation releases a propionyl-CoA unit in three discrete stages. Dehydrogenation of the cholest-22-CoA (Chol22-CoA) is catalyzed by the multimeric dehydrogenase complex (Rv3543/ChsE1 and R3544/ChsE2) (Thomas and Sampson 2013). Next, the hydratase complex (Rv3541/ChsH1 and Rv3542/ChsH2) hydroxylates the C17 carbon in the D ring of the cholesterol (Yang *et al.* 2014). The last carbon–carbon bond between the side chain and the D ring is cleaved by the aldolase Rv3540/Ltp2 to release the final propionyl-CoA unit from the side chain (Gilbert, Hood and Seah 2017).

## DEGRADATION OF THE A AND B RINGS

Degradation of the cholesterol A and B rings results in the release of one propionyl-CoA unit and one pyruvate unit (Fig. 3) The enzymes responsible for degrading the A and B rings of cholesterol have been characterized over the past 10 years in a series of elegant studies. To simplify the names of the cholesterol degradation intermediates, this description assumes that the side chain has been degraded. It should be noted that degradation of the side chain and rings can occur simultaneously (Thomas *et al.* 2011). Catabolic degradation of the cholesterol A and B rings begins with a dehydrogenation reaction, which is catalyzed by Rv1106/3$\beta$-HSD to form 4-androstendione (AD) (Yang *et al.* 2007). Next, desaturation of the A ring by Rv3537/KstD produces 1,4-androstendione (ADD) (Knol *et al.* 2008). Subsequent hydroxylation by the monoxygenase/reductase complex Rv3526/KshA and Rv3571/KshB at the C9 position of the sterol forms 9-hydroxy-androsta-1,4-diene-3,17-dione (9OHADD) (Capyk *et al.* 2009, 2011). The precise order of the preceding desaturation and hydroxylation reactions is unclear. Irrespective of the reaction order, once formed, 9OHADD undergoes non-enzymatic cleavage in the B ring spontaneously, and the A ring is aromatized. Together, these reactions form 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3-HSA). Rv3570/HsaA and Rv3567/HsaB comprise an oxygenase/reductase complex that hydroxylates 3-HSA to form 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DSHA) (Dresen *et al.* 2010). Next, the A ring is cleaved through the activity of the dioxygenase, Rv3568/HsaC; this introduces two oxygen atoms into the molecule and opens the dihydroxylated A ring to generate 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) (Yam *et al.* 2009). 4,9-DSHA is cleaved further by the carbon–carbon hydrolase Rv3569/HsaD, which produces (3a$\alpha$-H-4$\alpha$(3'-propanate)-7a$\beta$-methylhexahydro-1,5-indanedione (HIP) and 2-hydroxyhexa-2,4-dienoic acid (HHD) (Lack *et al.* 2010). Finally, HHD is degraded by the aldolase/dehydrogenase complex Rv3534/HsaF

and Rv3535/HsaG, which produces pyruvate and propionyl-CoA (Carere *et al.* 2013).

## DEGRADATION OF C AND D RINGS

The final stages of cholesterol degradation release at least one acetyl-CoA unit, and probably a unit of propionyl-CoA, pyruvate or succinyl-CoA. Degradation of the sterol C and D rings commences when the acyl-CoA synthase, Rv3561/FadD3, ligates a CoA molecule onto degradation intermediate HIP, which forms Hip-CoA (Casabon *et al.* 2013b) (Fig. 3). Recent studies on Hip-CoA degradation in *M. tuberculosis* have made it possible to propose a model of C ring and D ring degradation (Crowe *et al.* 2017). Briefly, a cholesterol-grown mutant of *M. tuberculosis* that lacked Rv3551/IpdA and Rv3552/IpdB accumulated (R)-2-(2-carboxyethyl)-3-methyl-6-oxycyclohex-1-ene-1-carboxy-CoA (COCHEA-CoA). Accumulation of COCHEA-CoA indicates that the D ring is opened prior to the opening of the C ring (Fig. 3). The same study also established that recombinant enzymes from *M. tuberculosis* (Rv3559/IpdF and Rv3556/FadA6) and homologous enzymes from related *Actinomyces* spp. (IpdC, EchA20, IpdA and IpdB) transform 3a$\alpha$-H-4$\alpha$(3′-carboxyal-CoA)-5-hydroxy-7a$\beta$-methylhexahydro-1-indanedione (HIC-CoA) into COCHEA-CoA and 4-methyl-5-oxo-octanedioic acid (MOODA-CoA) (Crowe *et al.* 2017). Furthermore, a cholesterol-grown mutant of *M. smegmatis* lacking a FadE32 homolog accumulates MOODA-CoA, supporting the C and D ring degradation model in *M. tuberculosis* (Fig. 3).

Although the end products of MOODA-CoA degradation are unknown currently, it is likely that acetyl-CoA, propionyl-CoA and/or succinyl-CoA are released from this metabolic intermediate. Moreover, this model of cholesterol degradation is consistent with metabolomic analyses which demonstrated that propionyl-CoA pools increase when *M. tuberculosis* is grown on cholesterol (Jain *et al.* 2007; Yang *et al.* 2009; Griffin *et al.* 2012). Interestingly, cholesterol-grown *M. tuberculosis* also accumulates high levels of methyl-succinate (Griffin *et al.* 2012). It is tempting to speculate that methyl-succinate could be released from MOODA-CoA, but this remains to be determined.

## TRANSCRIPTIONAL REGULATION OF CHOLESTEROL DEGRADATION

The TetR-like transcriptional repressors Rv3574/KstR1 and Rv3557/KstR2 regulate cholesterol degradation in *M. tuberculosis*. These repressors are de-repressed in a 'feed forward' manner after binding with specific intermediates of cholesterol degradation. KstR1 is de-repressed by the catabolic intermediate 3-hydroxy-cholest-5-ene-26-oyl-CoA (3OCh-CoA) (Fig. 2), which promotes transcription of the genes encoding enzymes required for degrading the side chain and the A and B rings of cholesterol (Ho *et al.* 2016). KstR1 also binds putative promoter sequences throughout the *M. tuberculosis* genome (Galagan *et al.* 2013) and controls expression of numerous genes outside the main cholesterol catabolic gene cluster (Kendall *et al.* 2007; Wipperman, Sampson and Thomas 2014). This suggests that cholesterol utilization is integrated into a larger metabolic network, but how these genes/proteins participate in cholesterol utilization is unknown currently. Similarly, the KstR2 repressor is de-repressed by HIP-CoA (Fig. 3), which specifically activates transcription of genes in the KstR2 regulon that encode enzymes required for C and D ring degradation (Casabon et al. 2013).

## CHOLESTEROL FUELS CENTRAL METABOLIC PATHWAYS DURING INFECTION

Propionyl-CoA is a major metabolite that is released during cholesterol degradation, and this three-carbon intermediate lies at a critical axis in *M. tuberculosis* metabolism (Savvi *et al.* 2008). *Mycobacterium tuberculosis* can generate propionyl-CoA through $\beta$-oxidation of odd-chain fatty acids, degradation of branched-chain amino acids (leucine, isoleucine and valine) and cholesterol degradation (Savvi *et al.* 2008; Venugopal *et al.* 2011). The major lipids in mammalian cells are typically even chain and thus not likely a source of propionyl-CoA for *M. tuberculosis* during infection. Although *M. tuberculosis* can import and metabolize host-derived amino acids (Gouzy, Poquet and Neyrolles 2014), it is unknown how branched chain amino acids contribute to propionyl-CoA pools during infection. Recent studies indicate that cholesterol is the main source of propionyl-CoA for *M. tuberculosis* during infection. For example, the inhibitor 3-nitropropionate (3-NP) induces a propionyl-CoA-dependent toxicity in *M. tuberculosis* by inhibiting bacterial isocitrate lyase enzymes (Munoz-Elias and McKinney 2005; Eoh and Rhee 2013). Treatment with 3-NP reduces bacterial fitness during infection in macrophages (Griffin *et al.* 2012; Eoh and Rhee 2013), and this 3-NP induced toxicity can be partially reversed by deleting the Mce4 cholesterol transporter in *M. tuberculosis*. Additionally, we developed a reporter construct for *M. tuberculosis* that expresses GFP in response to increases in propionyl-CoA pools. Using this tool we found that mutants of *M. tuberculosis* lacking Mce4 and Rv3723/LucA poorly express GFP during infection in macrophages relative to wild-type bacteria (Nazarova *et al.* 2017).

The three metabolic fates of propionyl-CoA are as follows: (i) anaplerotic assimilation by the methylcitrate cycle (MCC) (Muñoz-Elías *et al.* 2006; Eoh and Rhee 2014), (ii) anaplerotic assimilation through the methylmalonyl pathway (MMP) (Savvi *et al.* 2008) and (iii) biosynthetic incorporation into methyl-branched, polyketide lipids (Fig. 4). The MCC condenses propionyl-CoA with oxaloacetate to ultimately generate succinate and pyruvate for central metabolism. In sequence, the methylcitrate synthase Rv1131/PrpC, methylcitrate dehydratase Rv1130/PrpD, aconitase Rv1475/Acn and isocitrate lyase Rv0467/Icl1 or Rv1915–16/Icl2 enzymes all make up the MCC (Munoz-Elias and McKinney 2005; Muñoz-Elías *et al.* 2006; Upton and McKinney 2007). The enzymes Icl1 and/or Icl2 catalyze the last reaction of the MCC in *M. tuberculosis* and these enzymes are bifunctional and necessary in the glyoxylate shunt for sustain bacterial growth on even chain fatty acids (McKinney *et al.* 2000; Munoz-Elias and McKinney 2005). Consequently, Icl1 and Icl2 are involved in two anaplerotic pathways that assimilate both propionyl-CoA and acetyl-CoA. When a *M. tuberculosis* Icl1/2 double mutant was provided acetate or propionate as sole carbon sources the bacteria were unable to assimilate neither carbon source, which depleted pools of central metabolic intermediates, disrupted membrane potential and perturbed pH homeostasis (Eoh and Rhee 2014). Based on this, it is likely that the severe attenuation of the Icl1/2 double mutant in animal models is multifactorial and could be partly due to this mutant's inability to assimilate propionyl-CoA into central metabolism (Munoz-Elias and McKinney 2005).

Inactivating Icl1 activity drives the accumulation of propionyl-CoA derived intermediates of the MCC that are also capable of intoxicating *M. tuberculosis* and inhibiting bacterial growth in rich media containing cholesterol or propionate (Lee *et al.* 2013; VanderVen *et al.* 2015). This metabolic toxicity is not a starvation response because the growth inhibition occurs

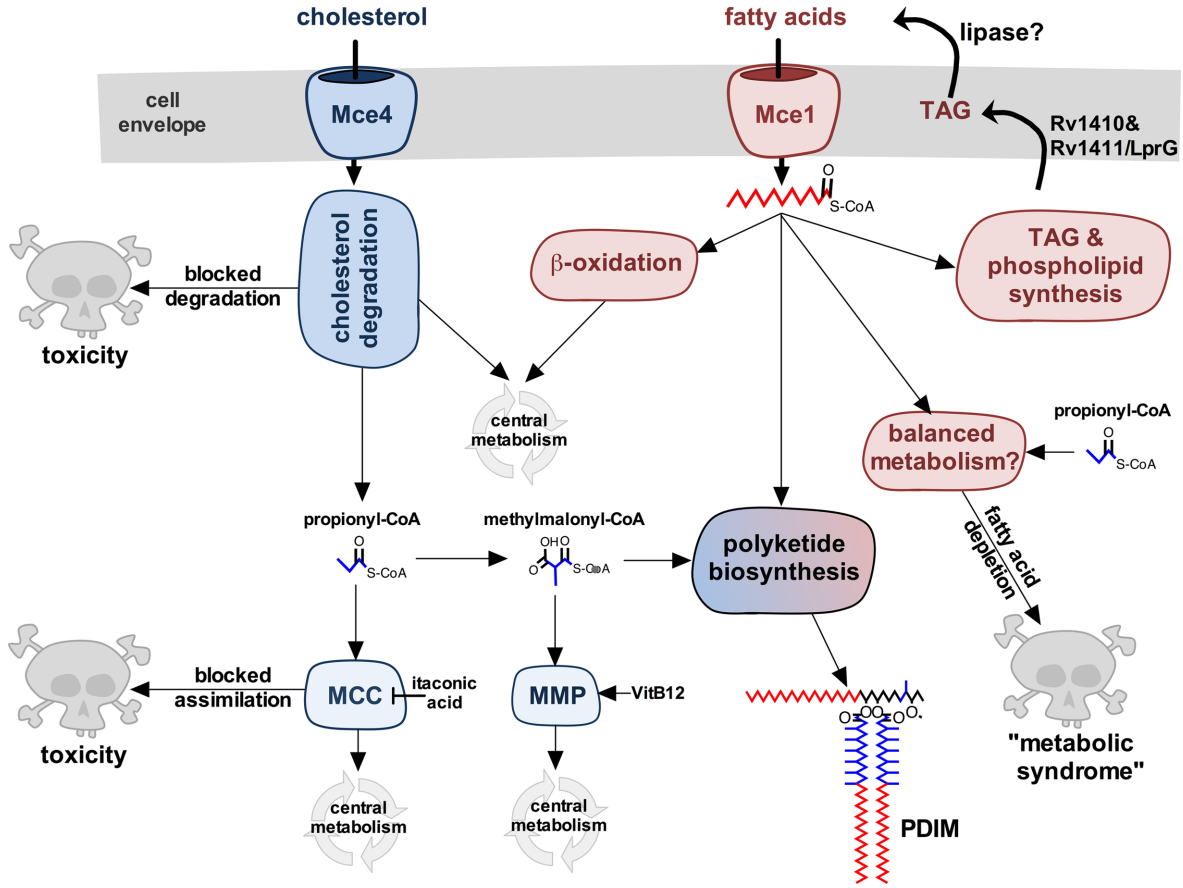


**Figure 4.** Cholesterol and fatty acid utilization in *M. tuberculosis*. Cholesterol and fatty acid degradation fuels central metabolism and supplies pathways required for pathogenesis. Cholesterol-derived propionyl-CoA can be assimilated by the MCC or converted into methylmalonyl-CoA. Methylmalonyl-CoA can be assimilated by the MMP or used for polyketide lipid synthesis. Inactivating cholesterol degradation enzymes or the MCC can induce metabolic intoxication that inhibits bacterial growth. Salvaged fatty acids can also be used in biosynthetic reactions to generate membrane lipids, TAG and polyketide lipids. Cholesterol-derived propionyl-CoA induces a 'metabolic syndrome' that is associated with an inability to export TAG and/or when fatty acid pools are depleted. Vitamin B12 is indicated as VitB12.

in the presence of other nutrients (glucose, glycerol and fatty acids), which together are capable of sustaining anaplerotic metabolism in *M. tuberculosis*. In an Icl1-deficient strain of *M. tuberculosis*, this cholesterol-dependent toxicity can be reversed by introducing secondary mutations in Rv1131/PrpC which indicates that the observed toxicity is associated with an inactive MCC (Nazarova *et al.* 2017). Furthermore, chemical inhibition of Rv1131/PrpC also reverses the cholesterol-dependent metabolic toxicity in an Icl1-deficient strain of *M. tuberculosis* (VanderVen *et al.* 2015). Similarly, chemical or genetic inactivation of enzymes in the cholesterol breakdown pathway also reverses the metabolic intoxication in an Icl1-deficient strain of *M. tuberculosis* when the bacteria are grown in cholesterol-containing media (VanderVen *et al.* 2015; Nazarova *et al.* 2017).

Propionyl-CoA can also be converted to methylmalonyl-CoA, which then feeds into central metabolism through the MMP (Fig. 4). The MMP assimilates methylmalonyl-CoA through the activity of the methylmalonyl-CoA mutase complex that is comprised of Rv1492/MutA and Rv1493/MutB. The MutAB complex requires the cofactor vitamin B12 for enzymatic activity (Savvi *et al.* 2008). In the presence of vitamin B12, propionyl-CoA can assimilated into anaplerotic metabolism. Importantly, the addition of vitamin B12 can reverse the cholesterol-dependent toxicity in a *M. tuberculosis* strain lacking Icl1 (Lee *et al.* 2013). Vitamin B12 also partially rescues growth of the Icl1 mutant during infection in macrophages following treatment with 3-NP (Griffin *et al.* 2012). Therefore, if cholesterol is a major source of propionyl-CoA for the bacterium during infection, there are also unfavorable consequences associated with cholesterol metabolism when *M. tuberculosis* is unable to efficiently assimilate propionyl-CoA into anaplerotic metabolism.

## CHOLESTEROL-DERIVED, PROPIONYL-COA FUELS VIRULENCE LIPID BIOSYNTHESIS

The propionyl-CoA that is converted to methylmalonyl-CoA can also be used for the synthesis of methyl-branched, polyketide virulence lipids in *M. tuberculosis* (Rainwater and Kolattukudy 1983; Jackson, Stadthagen and Gicquel 2007; Quadri 2014) (Fig. 4). Polyketide synthases incorporate methylmalonyl-CoA to generate the methyl-branched moieties of phthiocerol-dimycocerosate (PDIM), polyacylated trehalose and sulfolipid (SL) (Quadri 2014). It is well known that *M. tuberculosis* grown in axenic media without cholesterol still synthesizes methyl-branched polyketide lipids (Layre *et al.* 2011; Sartain *et al.* 2011). In axenic media lacking cholesterol, branched-chain amino acids probably serve as the source of propionyl-CoA (Venugopal *et al.* 2011). However, when *M. tuberculosis* is cultured in media that contains propionate or cholesterol, the absolute amount of PDIM and SL increases dramatically, reflecting increases in the bacterial propionyl-CoA pools (Jain *et al.* 2007; Yang *et al.*

2009; Griffin *et al.* 2012). Additionally, the methyl-branched acyl chains of PDIM and SL are longer when the bacteria are supplied with propionate or cholesterol (Jain *et al.* 2007; Yang *et al.* 2009; Griffin *et al.* 2012). These 'high mass forms' of PDIM and SL have additional methylmalonyl-CoA units incorporated into their acyl chains, resulting in more methyl-branched subunits in the lipids. Given the role of PDIM and SL in pathogenesis, these observations indicate that an expansion of propionyl-CoA pools in *M. tuberculosis* modulates important virulence factors of the bacterium (Lee *et al.* 2013). Importantly, these 'high mass forms' of PDIM and SL are also produced by *M. tuberculosis in vivo* (Jain *et al.* 2007), which further supports the idea that cholesterol is a dominant nutrient for *M. tuberculosis* which feeds propionyl-CoA pools during infection.

It has long been known that *M. tuberculosis* lipids in general, and PDIM in particular, play important roles in pathogenesis (Camacho *et al.* 1999; Cox *et al.* 1999), so the coupling of cholesterol degradation to the biosynthesis of methyl-branched lipids such as PDIM may have important consequences in pathogenesis. PDIM has been implicated in macrophage invasion and recruitment (Astarie-Dequeker *et al.* 2009; Cambier *et al.* 2014), resistance to immune-mediated stress (Rousseau *et al.* 2004; Kirksey *et al.* 2011; Day *et al.* 2014), masking cell wall antigens (Cambier *et al.* 2014) and facilitating bacterial escape from macrophage phagosomes (Barczak *et al.* 2017; Quigley *et al.* 2017). All of these activities could be influenced by fluctuating abundances or structural alterations of PDIM or other polyketide virulence lipids in the bacterial cell wall.

## DEGRADATION AND METABOLISM OF FATTY ACIDS

In addition to cholesterol, fatty acids are also a highly abundant lipid found in human granulomas (Kim *et al.* 2010). Seminal work by Segal and Bloch published in 1956 demonstrated that fatty acids, but not carbohydrates, specifically stimulated *ex vivo* respiration rates of *M. tuberculosis* harvested from murine lung tissue (Segal and Bloch 1956). Although *M. tuberculosis's* ability to assimilate and to metabolize fatty acids has long been appreciated, genome sequencing revealed that the bacterium has an expanded set of putative fatty acid $\beta$-oxidation genes (Cole *et al.* 1998). Initially, it was thought that *M. tuberculosis* contained roughly ~250 enzymes dedicated to fatty acid $\beta$-oxidation (Cole *et al.* 1998). Subsequent work demonstrated that many of these putative lipid oxidation enzymes are involved in lipid biosynthesis (Trivedi *et al.* 2004; Krithika *et al.* 2006), function as multimeric complexes or are not involved in fatty acid $\beta$-oxidation at all (Casabon et al. 2013; Thomas and Sampson 2013; Yang *et al.* 2014, 2015). Nonetheless, *M. tuberculosis* still possesses a remarkable number of proteins that are involved in the breakdown of lipids, but the precise functions/substrates for most of these putative enzymes remain uncharacterized.

Although cholesterol appears only to be degraded by *M. tuberculosis* to generate metabolic intermediates, fatty acids can either be degraded to fuel metabolism or used for biosynthesis directly (Fig. 4). During infection, *M. tuberculosis* likely scavenges fatty acids but can also synthesize $C_{16–22}$ fatty acids through the fatty acid synthase-I (FAS-I) enzyme (Takayama, Wang and Besra 2005). However, given the energetic cost associated with *de novo* fatty acid synthesis, the bacteria likely $\beta$-oxidizes scavenged fatty acids or incorporates these lipids directly into biosynthetic pathways (Fig. 4). Fatty acids in the form of acyl-AMP intermediates are donated to polyketide synthases which iteratively incorporate malonyl-CoA and/or methylmalonyl-CoA to synthesize polyketide lipids (Quadri 2014). Full-length mycolic acids are synthesized by elongating fatty acids through the specialized fatty acid synthase-II complex (FAS-II) to a length of $C_{22–62}$ (Marrakchi, Laneelle and Daffe 2014). The FAS-II complex elongates either salvaged or *de novo* synthesized fatty acids to generate the full-length acyl-chains of mycolic acids (Odriozola, Ramos and Bloch 1977; Portevin *et al.* 2004). Fatty acids can also be assimilated directly into membrane phospholipids or converted into TAG. Phospholipids are required to maintain integrity of cytoplasmic membranes, and TAG functions as a carbon reserve that can be catabolized when nutrients are limiting (Daniel *et al.* 2004, 2011). While these pathways for utilizing fatty acids in *M. tuberculosis* are relatively well known, recent evidence indicates that utilization of fatty acids and cholesterol is coordinated, and the co-utilization of these substrates influences pathogenesis, summarized in more detail below.

## FATTY ACID IMPORT

The mechanism responsible for fatty acid import across the *M. tuberculosis* cell envelope has remained enigmatic until recently. Using a series of unbiased approaches, we discovered that the Mce1 complex imports fatty acids into *M. tuberculosis* (Nazarova *et al.* 2017). Similar to the *mce4* operon, the *mce1* operon encodes two putative permease subunits (Rv0167/YrbE1A and Rv0168/YrbE1B), six Mce proteins (Rv0169/Mce1A, Rv0170/Mce1B, Rv0171/Mce1C, Rv0172/Mce1D, Rv0173/Mce1E and Rv0174/Mce1F) and four accessory subunits (Rv0175/Mam1A, Rv0176/Mam1B, Rv0177/Mam1C and Rv0178/Mam1D) (Fig. 1). In our studies, we found that in *M. tuberculosis* lacking Rv3723/LucA, the Mce1A, Mce1D and Mce1E proteins are degraded, and the Rv3723/LucA mutant is unable to import fatty acids during infection and in axenic media. Based on this observation, we next confirmed that *M. tuberculosis* mutants lacking genes in the *mce1* operon are also unable to import fatty acids in axenic media and during infection in macrophages. Because of the similarities between the genes in the *mce1* and *mce4* operons, it was previously hypothesized that Mce1 imports fatty acids (Forrellad *et al.* 2014), and our data strongly support this idea. Added support that Mce1 functions as a fatty acid transporter is inferred from studies in *M. leprae*. The minimal genome of *M. leprae* conserves a single *mce* operon, which is most closely related to the *mce1* operon from *M. tuberculosis* (Wiker *et al.* 1999). Importantly, *M. leprae* is capable of importing and metabolizing fatty acids *ex vivo*, suggesting that Mce1 also imports fatty acids in related mycobacterial pathogens (Franzblau 1988).

## HOW DOES MCE1 CONTRIBUTE TO *M. TUBERCULOSIS* PATHOGENESIS?

Proteins of the Mce1 complex have long been regarded as virulence factors in *M. tuberculosis*, despite conflicting evidence regarding their specific functions. Early studies concluded that Mce1 proteins are virulence factors, which mediate bacterial entry into mammalian cells. Surface expression of the *M. tuberculosis* Mce1A protein in a non-pathogenic strain of *Escherichia coli* endows the bacterium with an ability to invade mammalian epithelial cells; consequently, proteins related to Mce1A were named *m*ammalian cell *e*ntry or Mce proteins (Arruda *et al.* 1993). The domain of Mce1A that interacts with mammalian cells was mapped to the Mce domain of Mce1A (amino acids 106–177), and this is the minimal region responsible for the *E. coli* invasion

phenotype (Casali *et al*. 2002). In *M. tuberculosis*, Mce1A is secreted and found on the outer surface of the bacterial cell envelope (Chitale *et al*. 2001). Since it is now understood that proteins conserving Mce domains bind lipids (Krachler, Ham and Orth 2011; Ekiert *et al*. 2017), it is plausable that surface Mce1A binds lipids for import into *M. tuberculosis*. Expressing Mce1A on the surface of *E. coli* may promote close interactions between the bacteria and mammalian cell membrane lipids to facilitate host cell invasion by non-pathogenic *E. coli*. Given the multitude of cell entry pathways that are exploited by *M. tuberculosis* (Russell 2011), we hypothesize that the principle function of Mce1 is to import fatty acids to promote *M. tuberculosis* pathogenesis.

Analysis of transposon mutants passaged within mice found that mutations throughout the *mce1* operon confer *in vivo* growth defects, primarily early in infection (Sassetti and Rubin 2003). Supporting this, both targeted and unbiased studies also reported that Mce1 mutants displayed fitness defects in mice and macrophages, in addition to causing less severe lung pathology in animals (Gioffre *et al*. 2005; Stewart *et al*. 2005; Joshi *et al*. 2006; McCann *et al*. 2011). In contrast, *M. tuberculosis* Mce1 mutants can display hyper-virulent phenotypes in mice. Additionally, macrophages infected with Mce1 mutants produced less TNF-$\alpha$ and nitric oxide, and these infected macrophages were unable to control intracellular growth of an Mce1 mutant relative to wild-type *M. tuberculosis* (Shimono *et al*. 2003). The reported anti-inflammatory phenotype of the Mce1 mutant in macrophage infection models has been attributed to free mycolic acids (Sequeira, Senaratne and Riley 2014) that accumulate in the cell envelope of a Mce1 mutant (Cantrell *et al*. 2013; Forrellad *et al*. 2014; Queiroz *et al*. 2015). The apparent contradictions of the Mce1 mutant phenotypes *in vivo* may be explained by differences in the genetic background of mice, routes of infection, location/type of genetic mutations or the duration of the experiments.

Other data have been reported that are consistent with Mce1 functioning as a *M. tuberculosis* fatty acid importer. For example, cell wall lipids and membrane phospholipid levels are reduced in an Mce1 mutant, and the mutant overexpresses the FAS-I enzyme (Queiroz *et al*. 2015). This indicates that fatty acid pools are depleted in the Mce1 mutant and the bacteria compensate for the fatty acid depletion by increasing *de novo* fatty acid synthesis. Free mycolic acids also accumulate in the cell envelope of Mce1 mutants; therefore, it was proposed that Mce1 functions to recycle mycolic acids (Cantrell *et al*. 2013; Forrellad *et al*. 2014). Importing mycolic acids through Mce1 seems unlikely given that a Mce1 mutant had no growth defect when the bacteria were supplied exogenous mycolic acids as a sole carbon source in axenic culture (Dunphy *et al*. 2010); however, the substrate specificity of Mce1 has yet to be defined. Together, these results are consistent with the idea that Mce1 functions as a fatty acid importer and fatty acids are required for *M. tuberculosis* to establish and maintain optimal infections in animals.

## EXPANDING MODEL FOR MCE1 AND MCE4 TRANSPORTERS

The Mce1 and Mce4 transporters are analogous to multisubunit, ABC transporters that are comprised of putative substrate binding proteins (SBP), permease proteins and an ATPase subunit that provides energy for the transport process (Wilkens 2015). Gram-negative bacteria shuttle lipids across their cell envelope, and these processes also rely on proteins that conserve Mce domains (Malinverni and Silhavy 2009; Thong *et al*. 2016; Isom *et al*. 2017; Nakayama and Zhang-Akiyama 2017). Mce proteins make up the MlaD superfamily (cl27420) and participate in trafficking lipids across double membrane structures (Malinverni and Silhavy 2009; Sutterlin *et al*. 2016; Thong *et al*. 2016; Ekiert *et al*. 2017; Isom *et al*. 2017; Nakayama and Zhang-Akiyama 2017). The Mce proteins in gram-negative bacteria form multimeric structures and are predicted to bind and facilitate lipid transport (Ekiert *et al*. 2017). In *M. tuberculosis,* the Mce or SBP proteins of the Mce1 and Mce4 complexes are Mce1A-F and Mce4A-F (Fig. 1). Mce1A-E and Mce4A-E conserve canonical, secretory signal sequences, and they are abundant in *M. tuberculosis* cell envelope protein fractions (Feltcher *et al*. 2015). In addition, Mce1A has been shown to localize on the cell surface of the bacteria (Chitale *et al*. 2001). Mce1F and Mce4F proteins conserve a putative N-terminal transmembrane domain and are probably inserted within the cytoplasmic membrane or cell wall.

We hypothesize that these Mce proteins bind and shuttle lipid substrates across the mycolic acid layer and the pseudoperiplasmic space of the *M. tuberculosis* cell envelope. The large number of putative Mce proteins in the Mce1/4 complexes (six each) suggests that these proteins form a pore or channel and allow substrate shuttling from the bacterial surface to deliver substrates to the permease subunits within the cytoplasmic membrane. The putative permease subunits (YrbE1A/B and YrbE4A/B) are probably embedded in the cytoplasmic membrane and complete lipid translocation across the cytoplasmic membrane. Finally, accessory proteins (Mam1A-D and Mam4A-B) encoded within the *mce1* and *mce4* operons are required for lipid import, but the function of these proteins is unclear. Our recent work demonstrated that the accessory protein Rv3492/Mam4B is required for cholesterol import by the Mce4 complex, but the Rv3492/Mam4B protein appears to be involved in regulating the activity or assembly of the Mce4 transporter (Nazarova *et al*. 2017).

It has been proposed that the Mce systems in *E. coli* use two distinct mechanisms for transporting lipid substrates across the aqueous periplasm. One system relies on a soluble protein that likely binds and shuttle lipids across the periplasm; the other system employs a closed channel that spans the periplasmic space, and lipids are likely transported internally within the protein complex (Ekiert *et al*. 2017). It will be exciting to learn how the Mce1 and Mce4 complexes function mechanically and structurally. Given the similarities between the putative subunits in Mce1 and Mce4 complexes, these transporters probably use very similar mechanisms to import substrates, and the differences between these complexes likely define the substrate specificities of these transporters. Finally, assembly and regulation of the Mce1 and Mce4 transporters in the bacterial cell envelope are poorly understood processes.

Our hypothetical model proposes that proteins encoded in *mce1* and *mce4* operons comprise the 'core proteins' of Mce1 and Mce4 transporters, respectively. However, it is now clear that unlinked genes that are located outside the *mce1* and *mce4* operons also encode proteins that are required for Mce1- and Mce4-mediated nutrient transport (Fig. 1). The putative ATPase subunit Rv0655/MceG is predicted to hydrolyze ATP and provide energy for substrate import, and MceG is required for cholesterol import (Mohn *et al*. 2008; Pandey and Sassetti 2008). It has been hypothesized that Rv0655/MceG may also function as the 'common' ATPase required for the Mce1, Mce2 and Mce3 complexes (Joshi *et al*. 2006). We have recently confirmed that Rv0655/MceG is involved in fatty acid uptake in *M. tuberculosis* (unpublished). It is currently unknown if Rv0655/MceG participates in Mce2- and Mce3-mediated lipid import, and the enzymatic activity of Rv0655/MceG has not yet been confirmed.

| Protein | Function |
|---|---|
| Rv0655/MceG | Putative ATPase |
| Rv3270/CtpC | Mn2+ ATPase |
| Rv1469/CtpD | $CO^{2+}$/$Ni^{2+}$ ATPase |
| Rv1410 | TAG export |
| Rv1411/LprG | TAG export |
| Rv2038 | Unknown |
| Rv3103 | Unknown |
| Rv1422 | Unknown |
| Rv2936DrrA | PDIM precursor transport |
| Rv2938/DrrC | PDIM precursor transport |
| Rv3540/Ltp2 | Cholesterol side chain degradation |
| Rv3541/ChsH1 | Cholesterol side chain degradation |
| Rv3542/ChsH2 | Cholesterol side chain degradation |
| Rv3543/ChsE1 | Cholesterol side chain degradation |
| Rv3723/LucA | Mce1 and Mce4 import coordinator |

**Figure 5.** Proteins involved in pathways linked to Mce1 and Mce4. Proteins identified by genetic epistasis mapping from *in vivo* attenuation phenotypes in mice.

Recently, it was reported that the orphaned Mce accessory protein Rv0199/OmamA plays a role in cholesterol utilization and that Rv0199/OmamA stabilizes the Mce1 complex (Perkowski *et al.* 2016). Another protein common to both transporters is Rv3723/LucA, which interacts with accessory subunits (Mam1C, Mam4B and OmamA) of the Mce1 and Mce4 transport complexes (Nazarova *et al.* 2017). Importantly, Rv3723/LucA is the first known *M. tuberculosis* protein that is required for both cholesterol and fatty acid import (Nazarova *et al.* 2017). Based on this observation, our hypothetical model for Mce1 and Mce4 transporters in *M. tuberculosis* also includes proteins that are shared by both complexes. Based on these findings, it is likely that additional unknown proteins mediate or coordinate nutrient import using Mce1 and Mce4. Our current hypothesis is that a network of proteins including the 'core proteins' of the Mce1 and Mce4 transporters coordinates fatty acid and cholesterol import. Characterizing the network of lipid import proteins will be important to understand *M. tuberculosis* pathogenesis and persistence fully.

## CONNECTIONS BETWEEN CHOLESTEROL AND FATTY ACID UTILIZATION

The first indication that cholesterol and fatty acid utilization is coordinated was gleaned from *in vivo* genetic screens that used *M. tuberculosis* mutants lacking Mce1 and Mce4 (Joshi *et al.* 2006). This work used attenuation phenotypes in mice as a readout to predict proteins that potentially function in the same pathways as Mce1, Mce4, or both Mce1 and Mce4 together (Fig. 5). Rv3723/LucA was among the proteins identified as being involved in the same pathway as Mce4 (Joshi *et al.* 2006). We independently discovered that Rv3723/LucA is required for Mce4-mediated cholesterol import. Surprisingly Rv3723/LucA is also required for Mce1-mediated fatty acid import (Nazarova *et al.* 2017). Rv0655/MceG was also identified as being in the same pathways as Mce4 and Mce1, and this observation has been subsequently confirmed (unpublished) (Joshi *et al.* 2006; Pandey and Sassetti 2008). These observations are consistent with our hypothesis that a network of proteins facilitates and coordinates cholesterol and fatty acid import in *M. tuberculosis*. Additionally, several proteins are involved in the downstream utilization of fatty acids and cholesterol and function in the same pathways as Mce1 and Mce4. Characterization of these common proteins involved in the utilization of fatty acids and cholesterol downstream of Mce1 and Mce4 have demonstrated a 'metabolic codependency' of these two substrates in *M. tuberculosis* metabolism.

## FATTY ACIDS BUFFER THE METABOLIC COSTS OF CHOLESTEROL UTILIZATION

Rv1410 and Rv1411/LprG are two proteins that function in the same pathways as both Mce1 and Mce4 and are involved in the downstream utilization of lipids in *M. tuberculosis*. Specifically, these two proteins facilitate the export of TAG from the bacterial cytosol (Martinot *et al.* 2016). These proteins are required for full virulence of *M. tuberculosis* (Gaur *et al.* 2014; Martinot *et al.* 2016) and, notably, these proteins have been linked to mycobacterial growth on cholesterol (Ramon-Garcia *et al.* 2015). Importantly, a cholesterol-grown *M. tuberculosis* mutant lacking Rv1410 and Rv1411/LprG experiences a cholesterol-derived, propionyl-CoA-dependent growth defect that can be reversed by supplying the bacteria exogenous free fatty acids, but not vitamin B12 (Martinot *et al.* 2016). Chemically blocking the release of free fatty acids from TAG exacerbated the growth defect in an Rv1410 and Rv1411/LprG-deficient mutant when the bacteria were grown in cholesterol media. Together, these data demonstrate that in the absence of Rv1410 and Rv1411/LprG *M. tuberculosis* can become intoxicated by cholesterol, and expanding the pool size of free fatty acids alleviates the intoxication or 'metabolic syndrome'. It remains to be determined precisely how Mce1 is involved in the 'metabolic syndrome', but it is conceivable that Mce1 is required to recycle fatty acids released from TAG stored in the cell envelope (Fig. 4).

Cholesterol degradation in *M. tuberculosis* increases the flux of methylmalonyl-CoA (derived from propionyl-CoA) into polyketide lipid biosynthesis (Jain *et al.* 2007; Yang *et al.* 2009; Griffin *et al.* 2012). Increased biosynthesis of polyketide lipids requires sufficient levels of free fatty acids to serve as fatty acid-AMP primers for polyketide synthase enzymes (Quadri 2014) (Fig. 4). Synchronizing fatty acid and cholesterol import by Mce1 and Mce4 may help to ensure that a balanced supply of biosynthetic precursors is maintained to efficiently synthesize polyketide lipids. Biosynthesis of polyketide lipids also serves as a sink to prevent accumulation of toxic metabolic intermediates

generated by the MCC (Lee *et al*. 2013). Metabolic detoxification requires the conversion of propionyl-CoA into methylmalonyl-CoA and excess fatty acid-AMP primers to synthesize polyketide lipids. For instance, growth inhibition of an *M. tuberculosis* mutant lacking Icl1 is reversed by supplying excess fatty acids during infection, and these fatty acids are preferentially incorporated into PDIM (Lee *et al*. 2013). This suggests that the amount of available free fatty acids impacts the flux of methylmalonyl-CoA (derived from propionyl-CoA) into polyketide lipid biosynthesis. During infection, fatty acids could also help to counter growth inhibition due to itaconic acid (Michelucci *et al*. 2013), which is produced by IFN-$\gamma$-activated macrophages cells and inhibits Icl1/2 in *M. tuberculosis* (Eoh and Rhee 2014) (Fig. 4).

Therefore, cholesterol metabolism is associated with numerous detrimental burdens on bacterial metabolism, but these can be buffered by increasing the pools of available fatty acids. Given that *M. tuberculosis* probably encounters fatty acids and cholesterol simultaneously *in vivo* (Kim *et al*. 2010), perhaps the metabolic pathways in the bacterium evolved to operate most efficiently when cholesterol and fatty acids are co-metabolized. Finally, coordinating the import of these lipid substrates through Mce1 and Mce4 may ensure that metabolism in the bacterium remains balanced.

## THERAPEUTIC POTENTIAL OF LIPID UTILIZATION PATHWAYS IN *M. TUBERCULOSIS*

TB antibiotic therapy will always require multidrug treatments to prevent drug resistance and to treat the different bacterial subpopulations that differentiate *in vivo* (Evangelopoulos, da Fonseca and Waddell 2015). Thus, new drugs capable of targeting bacterial subpopulations that are not effectively eliminated by current antibiotics could enhance current TB drug regimens and shorten therapy, prevent drug resistance and reduce relapse. Our recent drug discovery efforts to identify compounds that inhibit *M. tuberculosis* replication in macrophages have found a large number of compounds that inhibit processes related to cholesterol utilization in the bacterium (VanderVen *et al*. 2015). We predict that compounds capable of blocking cholesterol utilization in *M. tuberculosis* could specifically inhibit the growth of bacterial subpopulations within macrophages. Chemical perturbation of cholesterol utilization in *M. tuberculosis* can induce carbon starvation, metabolic intoxication and unbalanced central metabolism. Consequently, there are a large number of potential vulnerabilities in the cholesterol utilization pathways of *M. tuberculosis* that can be exploited for drug discovery.

## TARGETING CHOLESTEROL AND FATTY ACID UTILIZATION PATHWAYS

*Mycobacterium tuberculosis* can simultaneously metabolize cholesterol from 'both ends' to release metabolic intermediates from both the rings and side chain of the molecule (Thomas *et al*. 2011). This suggests that not all cholesterol degradation enzymes are equally good targets for drug development if the goal is to prevent the release of metabolic intermediates from the sterol. We have found that compounds that inhibit the cholesterol degradation enzymes (HsaAB) can block intracellular replication of the bacteria (VanderVen *et al*. 2015), indicating that these enzymes are a weakness in the pathway. Alternatively, inactivating the ability of *M. tuberculosis* to import cholesterol efficiently may induce a type of carbon starvation in the bacterium. For example, inhibiting the Mce4 cholesterol transporter may starve the bacteria by restricting bacterial access to cholesterol, particularly at specific stages of infection such as during persistence (Pandey and Sassetti 2008).

Inhibiting key enzymes in the cholesterol degradation pathways may also promote the accumulation of toxic cholesterol-derived degradation intermediates. For example, *M. tuberculosis* mutants lacking Cyp125, HsaC and IpdAB are intoxicated by cholesterol-derived degradation intermediates and exhibit growth defects even in the presence of additional carbon sources (Chang *et al*. 2009; Yam *et al*. 2009; Ouellet *et al*. 2010; Crowe *et al*. 2017). Thus, inhibiting proteins in cholesterol metabolism is compelling since inhibiting key steps in the cholesterol degradation pathway of *M. tuberculosis* could potentially transform a preferred nutrient of the bacterium into a growth-restricting toxin. *Mycobacterium tuberculosis* mutants lacking the TAG transporter suffer from a cholesterol-dependent 'metabolic syndrome' which is associated with unbalanced metabolism (Martinot *et al*. 2016) (Fig 5). This cholesterol-dependent metabolic intoxication could also be exploited with inhibitors of Rv1410/Rv1411/LprG.

The pathways for fatty acid degradation in *M. tuberculosis* are heavily redundant, which is a significant hurdle for drug development. However, chemical inhibition of Mce1 may limit the availability of free fatty acids and perturb *M. tuberculosis* metabolism. The encouraging realization that proteins such as Rv3723/LucA are required for the function of both Mce1 and Mce4 indicates that it may be possible to develop compounds that disable both transporters simultaneously.

## ROLE OF CAMP IN CHOLESTEROL UTILIZATION

We recently identified a novel class of compounds that stimulated 3′,5′-cyclic adenosine monophosphate (cAMP) production in *M. tuberculosis* by activating the bacterial adenylyl cyclase Rv1625c/Cya (VanderVen *et al*. 2015). Activating cAMP production with these compounds blocks cholesterol utilization in *M. tuberculosis* and inhibits bacterial replication in macrophages. Although the mechanism explaining how cAMP blocks cholesterol utilization in *M. tuberculosis* is still incomplete, our analyses indicate that cAMP blocks early stages in the cholesterol degradation pathway, and that this metabolic blockade is responsible for the inhibition of bacterial replication in macrophages (VanderVen *et al*. 2015).

*Mycobacterium tuberculosis* is capable of producing at least 10 active adenylyl cyclases, including Rv1625/Cya, all of which convert ATP into cAMP (Knapp and McDonough 2014). These adenylyl cyclases are diverse structurally, and each protein contains a unique-sensing domain. The putative-sensing domains of these proteins are thought to respond to environmental stimuli or ligands which control adenylyl cyclase activity (Agarwal and Bishai 2009; Bai, Knapp and McDonough 2011). The natural ligand or stimuli that activate Rv1625/Cya is unknown, but our model suggests that Rv1625/Cya can be activated by a small molecule that binds at one of two active sites in the enzyme complex (Johnson *et al*. 2017). Because elevated levels of cAMP can impact central metabolism (Xu, Hegde and Blanchard 2011; Lee *et al*. 2012; Knapp *et al*. 2015; VanderVen *et al*. 2015), transcription (Kahramanoglou *et al*. 2014), pathogenicity (Agarwal *et al*. 2009), dormancy (Shleeva *et al*. 2013) and stress responses (Choudhary, Bishai and Agarwal 2014) in *M. tuberculosis*, activating production of cAMP could be a novel approach to target bacterial subpopulations that reside within macrophages.

## CONCLUDING REMARKS

Recent insights into the lipid utilization pathways of *M. tuberculosis* have introduced some new lines of questioning. Given that Mce1 and Mce4 import fatty acids and cholesterol, respectively, it is likely that Mce2 and Mce3 also import hydrophobic lipid substrates that are yet unknown. The finding that the fatty acid and cholesterol importers rely on common proteins (Rv3723/LucA and Rv0655/MceG) suggests that additional bottlenecks of these two pathways exist, which could be targeted with drugs to disable multiple processes simultaneously. *Mycobacterium tuberculosis* causes a heterogeneous disease that involves various organs, cell types, immune responses and tissue pathology (Cadena, Fortune and Flynn 2017). It will be exciting to determine how *M. tuberculosis* utilizes lipid nutrients in different environments, cell type and time points during infection, ultimately to understand how the immune response constrains or modulates bacterial lipid metabolism and the available nutrients. This is an exciting time to continue investigating fundamental aspects of the physiology and pathogenesis of *M. tuberculosis*. With new insights and tools, we are optimistic that novel approaches and findings from current *M. tuberculosis* research will apply the brakes to slow or to stop the well-greased wheels of *M. tuberculosis* pathogenesis.

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