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Pathogen 'Roid Rage: Cholesterol Utilization by *Mycobacterium tuberculosis*

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Abstract

The ability of science and medicine to control the pathogen *Mycobacterium tuberculosis* (*Mtb*) requires an understanding of the complex host environment within which it resides. Pathological and biological evidence overwhelmingly demonstrate how the mammalian steroid cholesterol is present throughout the course of infection. Better understanding *Mtb* requires a more complete understanding of how it utilizes molecules like cholesterol in this environment to sustain the infection of the host. Cholesterol uptake, catabolism, and broader utilization are important for maintenance of the pathogen in the host and it has been experimentally validated to contribute to virulence and pathogenesis. Cholesterol is catabolized by at least three distinct sub-pathways, two for the ring system and one for the side chain, yielding dozens of steroid intermediates with varying biochemical properties. Our ability to control this worldwide infectious agent requires a greater knowledge of how *Mtb* uses cholesterol to its advantage throughout the course of infection. Herein, the current state of knowledge of cholesterol metabolism by *Mtb* is reviewed from a biochemical perspective with a focus on the metabolic genes and pathways responsible for cholesterol steroid catabolism.

Keyterms

catabolism; metabolism; enzyme; pathway; nutrition; persistence

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> The practical insolubility of cholesterol in water renders the obtaining of an insight into the mechanism of this breakdown process very difficult. In this respect cholesterol cannot be rightly compared with the fats, since a simple hydrolysis is able to convert these latter compounds into more or less water-soluble components. To the contrary the breakdown of cholesterol will ask for a direct action of the desmolytic catalysts of the cells, or in other words this compound must as such be subject to reactions of an oxido-reduction type (Tak, 1942).
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> J.D. Tak, On Bacteria Decomposing Cholesterol, 1942

One breath is all it takes to inhale and give shelter to the pathogen that has successfully infected more than one third of the global human population—*Mycobacterium tuberculosis*

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(*Mtb*) (2012). *Mtb* is the causative agent of tuberculosis (TB) disease, historically known as consumption, and is responsible for at least two million deaths each year. Today, anywhere from 5–15% of people infected with *Mtb* will go on to develop active TB disease, which is highly contagious and often deadly. The average person with active TB disease will spread it to 10–15 people. Half of all people diagnosed with TB in some developing countries will die, usually soon after diagnosis (Dye et al., 1999). Worldwide, TB is the leading killer of people infected with HIV or suffering from AIDS, and this disease disproportionally affects those with compromised immune systems, especially very young children and the elderly.

In the 20th century the hope for a remedy came to fruition for the first time in history when Selman Waksman discovered the aminoglycoside streptomycin and demonstrated its success in treating *Mtb* infection, and Merck brought it to market in the 1940s (Waksman, 1953). It seemed as if the long-awaited anti-mycobacterial cure for TB had finally arrived. Rates of TB infection, which were already in decline due to improved sanitation, continued to plummet into the 1950s and 1960s, and there was optimism that this disease could finally be cured once and for all. However, *Mtb* strains resistant to streptomycin were noted very soon after the drug entered the market, in the late1940s. This initial resistance to the first TB drug forebode the current situation more than half a century later.

In order to effectively cure TB today, treatments almost always include the use of multiple antibiotics taken simultaneously. During the initial phase of treatment, lasting two months, patients take a combination of two first-line drugs, rifampicin and isoniazid, and typically additional antibiotics like pyrazinamide and ethambutol. The continuation phase of treatment lasts for an extra four to seven months and includes second-line antibiotics, depending on the severity of disease progression. Despite combination drug therapy for a prolonged period of time, the emergence of drug resistance is increasingly on the rise (Reichman and Tanne, 2002). A major factor contributing to the global problem of drug-resistant TB is the patient's failure to complete a full antibiotic cycle.

First-line drugs isonizazid, rifampicin, and ethambutol are generally only bactericidal in patients with clinically active TB where *Mtb* is actively dividing. Isoniazid is a prodrug activated through ligation with NADH by the catalase-peroxidase KatG (*Rv1908c*). The active drug binds enoyl-acyl carrier protein reductase InhA (*Rv1484*), effectually inhibiting fatty acid biosynthesis. Rifampicin inhibits the function of the mycobacterial RNA polymerase through binding to the β-subunit of the enzyme, RpoB (Rv0667). Pyrazinamide is activated by the pyrazinamidase PncA (*Rv2043c*) to pyrazinoic acid, whose action is effective for both replicating and non-replicating *Mtb*. Ethambutol also inhibits cell wall biosynthesis by interfering with the arabinogalactan synthesis enzyme arabinosyltransferase, EmbB (*Rv3795*). Mutations in prodrug-activating enzymes or the drug target account for much of the resistance to anti-mycobacterial drugs worldwide.

In 2012, almost half a million people developed multi-drug resistant TB (MDR-TB), which is more deadly, more costly, and more difficult to cure than drug susceptible TB. An estimated 9.6% of those with MDR-TB have extensively drug resistant TB (XDR-TB), which responds to even fewer antibiotics than MDR-TB. Recently, clinical strains of TB that are totally drug resistant (TDR-TB) have been isolated in India (Udwadia et al., 2012). Drug

resistance has resulted in a modern day epidemic of disease whose intricate and elusive biology is as rich and complex as its extensive history. It is clear that science and medicine need to develop new drugs with novel targets to combat the rise in drug resistant strains of *Mtb*.

The remarkably slow pace of *Mtb* replication in the host cells is emblematic of the advantage that *Mtb* has to adapt to the nutrient-deprived environment of the macrophage. Although the link is not direct, the prolonged treatment with several different antibiotics necessary for effective treatment of *Mtb* is in part due to this non-replicating/slow growing state *in vivo*. Current anti-tuberculosis drugs target bacterial machinery that is utilized during cell replication. During the chronic phase of infection, *Mtb* doubles only once every several days.

Genes that are up-regulated during the chronic phase of infection and their corresponding proteins offer a unique avenue for drug design that would allow treatment of latent TB infections. Recently, mycobacterial-specific inhibitors of the *Mtb* proteasome, oxathiazol-2-one compounds, have been identified that kill non-replicating *Mtb* (Lin et al., 2009). These compounds act similarly to human proteasome drugs by acting as suicide-substrate inhibitors via cyclocarbonylation of the proteasome active site threonine. The nitroimidazopyran drug PA-824 currently in Phase II clinical trials shows promising anti-mycobacterial activity against this non-replicating population of bacteria (Stover et al., 2000). Finally, the diarylquinoline Bedaquiline (Sirturo), the first new TB drug approved in 40 years and marketed by Janssen Pharmaceuticals, targets ATP synthase, and is approved specifically for the treatment of MDR-TB (Villemagne et al., 2012). Understanding the environment in which *Mtb* sustains infection and the biological machinery necessary for the bacterium's survival is requisite for the rational development of new drugs with novel mechanisms of action targeting chronic infection. Mounting evidence suggests that cholesterol metabolism gene products are promising targets for further investigation, and these targets will be discussed in this review.

Pulmonary TB disease is caused by an *Mtb* infection of the respiratory system, where this pathogen resides in host alveolar macrophages. These tissue bound cells are involved in both the acute and chronic immune response intended to stifle foreign pathogens through various bactericidal mechanisms. *Mtb* has evolved the ability, through millennia of co-evolution with humans, to not only thwart this powerful immune response, but to also use it to its advantage. The immune system is directed by *Mtb* to form the granuloma, the clinical hallmark of TB infection, which is a chronic granulomatous inflammatory lesion composed of lymphocytes, macrophages, and multinucleated giant cells (Russell et al., 2009). Here, *Mtb* can reside for decades in a so-called latent state until the host immune system becomes compromised, often by HIV, old age, or poor nutrition. The infection can then progress to active disease where the caseous necrosis within the granulomatous lesion liquefies, extracellular bacteria are released, and the infection is rapidly spread from person to person (Scanga et al., 1999, Robertson, 1933).

The current paradigm asserts that during the latent phase of infection, *Mtb* is in a metabolically dormant and non-replicating state within the granuloma, sequestered away by

the immune system. This is based on the clinical observation that although a third of the world population is latently infected with TB, just under ten million each year will go on to develop active TB disease (Dye et al., 1999). Recent evidence, however, contradicts the classical school of thought, and this comatose metabolism might not be as real as was once thought. Convergent evidence suggests that an assortment of metabolic genes are in fact significantly up-regulated throughout the course of latent infection and disease. For example, in an *in vitro* human granuloma model of non-replicating *Mtb*, the metabolic isocitrate lyase (*icl*) genes were up-regulated (Peyron et al., 2008). Isocitrate lyase is an essential enzyme in the glyoxylate shunt cycle, which is one of two pathways that can be used for the metabolism of fatty acids, the second pathway being β-oxidation.

The intracellular phagocytic environment of *Mtb* is likely limited in energy resources since these immune cells are adept at killing foreign pathogens. However, *Mtb* infection of macrophages alters the intracellular environment of the macrophage, causing dysregulation of host lipid biosynthesis, uptake, and sequestration. A disproportionately high number of host lipid metabolism genes (compared to other metabolic genes) are up-regulated in caseous TB granulomas from patients with TB disease (Kim et al., 2010). Global expression profiling experiments in *Mtb* have shown that many hypothetical lipid-metabolizing genes are up-regulated during infection of both macrophages and mice (Camacho et al., 1999, Pandey, 2008, Fontan et al., 2008a).

Infected human pulmonary TB granulomas have an increased abundance of lipids compared to uninfected lung tissue. The abundance of lipid and cholesterol molecules within the granuloma is reflected in the accumulation of foamy macrophages resulting from excess lipid uptake by these cells through an imbalance between the export of low-density lipoprotein (LDL) through macrophage associated efflux pumps like ABCA-1 and the excess uptake of LDL through scavenger receptor A (SRA) and CD36, in addition to other mechanisms like pinocytosis (Russell et al., 2009). Mass spectral analysis of the lipid rich granuloma identified LDL particles composed of triacylglycerides, phospholipids, cholesterol, and cholesterol esters (Kim et al., 2010). This composition is very convenient for *Mtb*, since *Mycobacteria* and some of their bacterial relatives like Proteobacteria have an unusual ability to utilize steroids like the mammalian molecule cholesterol as a carbon and energy source.

In a laboratory setting, some *Mycobacteria* can grow on cholesterol as a sole carbon source. Cholesterol is obtained from the host via an ABC-like ATP-dependent cholesterol import system encoded by the *mce4* locus—the bacteria are unable to synthesize this molecule on their own (Mohn et al., 2008). It has been shown *in vivo* that cholesterol is necessary for persistence of *Mtb* during the latent stage of infection (Pandey, 2008, Nesbitt et al., 2010). Some evidence even points to the idea that high levels of cholesterol in the host's diet can have an adverse effect on the ability of the host immune system to respond to infection (Han, 2009, Schafer et al., 2009).

Gene expression studies performed *in vivo* highlight that *Mtb* relies on lipids, including steroids, as sources of carbon and energy, and that lipid metabolism is crucial for virulence. The mounting lines of evidence suggest that cholesterol metabolism offers this pathogen a

unique evolutionary advantage for deriving both energy and other valuable steroid intermediates. Here, we review what is known about the steroid metabolic pathway in *Mtb* to provide a comprehensive perspective. Sterol metabolism in bacteria has been studied for just over a century, and we highlight major findings in Actinobacteria that have contributed to the elucidation of the pathway in *Mtb*. Since these topics have been reviewed elsewhere (Donova, 2007, García et al., 2011), we focus primarily on new discoveries from the last decade. A more complete understanding of *Mtb* cholesterol metabolism is highly relevant for finding new approaches to treating TB. Thus we focus on the biochemistry that has been elucidated to date, and make note of areas in which there are gaps in our current understanding.

Early Actinobacterial Steroid Metabolism Studies

Actinobacteria are generally considered Gram-positive bacteria, and they can inhabit a wide range of environments. A large number of them are soil bacteria, but they can also live in water or as pathogens within a host organism. Most Actinobaceria have high G + C content in their genomes (>65%). However, some fresh water Actinobacteria can have low G + C content as well (<45%) (Ghai et al., 2012). Some Actinobacteria play an important role in the carbon cycle because of their ability to decompose a variety of complex organic compounds including many toxic byproducts of modern industry (Kobayashi and Rittmann, 1982). It is well established that Actinobacteria can metabolize sterols including cholesterol, which contains the familiar tetracycloalkane (gonane) steroid nucleus with an eight-carbon methyl branched side chain (Figure 1). Species of bacteria found throughout nature including *Arthrobacter*, *Mycobacteria*, *Nocardia*, *Rhodococcus*, *Gordonia*, and *Streptomyces* can all utilize cholesterol as a single carbon source for nutrition. Animals, plants, and fungi all biosynthesize and use steroids for various purposes, and although it has been reported that several bacteria might be able to biosynthesize steroids *de novo*, these results are few and controversial (Bode et al., 2003).

Investigation into the degradation of hydrocarbon substrates by microorganisms (mostly fungi, yeast, and bacteria) began in the late 19th century, and has been reviewed (Bushnell and Haas, 1941). The aerobic degradation of alkane substrates specifically by *Mycobacteria* was described in Germany for the first time just over a century ago (Söhngen, 1913). Both the steroid core and side chain portions of the molecule were found to be catabolized for energy through oxidation of the steroid framework and sidechain. Turfitt demonstrated that carbon C4 of the steroid core (Figure 1) was oxidized and the A-ring of cholesterol was opened in the process, yielding a product deemed Windaus' keto acid with loss of C4 and *iso*-caproic acid. Even though blocking oxidation of the 3-hydroxyl position with cholesterol acetate prevents oxidative cleavage of the A-ring, *iso*-caproic acid (C6H12O2) was still observed and presumed to be from side chain catabolism (Turfitt, 1947). Subsequent studies isolated the cholesterol dehydrogenase responsible for oxidation of C4 and determined that C4 and C26 were metabolized to CO2, although the metabolism of C26 was slower than C4 (Stadtman et al., 1954).

Bacterial steroid metabolism research was of particular interest during the late 1940s and 1950s due to the pharmaceutical usefulness of ring-intact sterols with partially metabolized

side chains (Malaviya and Gomes, 2008, Van Der Geize and Dijkhuizen, 2004). Androstenedione (AD) and other 17-keto steroids are key starting materials for the preparation of clinically useful steroids such as testosterone, estradiol, progesterone, cortisone, and cortisol (Hogg, 1992). This early work focused on the identification of small molecule metabolic intermediates in order to understand bacterial degradation pathways so that they could be exploited to obtain pharmaceutically valuable compounds. Of particular interest was the controlled enzymatic oxidation of various positions of the steroid framework. Chemical assays were limited to organic extraction of intermediates from bacterial cultures, and it was not until newer methods were developed for identifying intermediates that their structures were confirmed. Efforts focused on microbial fermentation methods to better optimize growth and yield. However, the enzymes that actually catalyzed the chemistry were for the most part not isolated since interest focused on large-scale fermatnation processes for drug development.

Hydroxylated steroids were by far some of the most valuable intermediates isolated during the late 1940s, since these were ultimately used as precursors for valuable steroid molecules and are often unavailable from nature as starting materials. H.C. Murray and D. H. Peterson of The Upjohn Company, who were a bacteriologist and an endocrine biologist, respectively, discovered and patented the microbial conversion of progesterone to C11-hydroxyprogesterone (Peterson, 1952). This coveted process was utilized to synthesize a number of steroids and steroid analogs, most notably the adrenocortical hormone hydrocortisone (Figure 2) (Hogg, 1992). Thus, early studies were focused on isolating ring-intact sterol metabolites with modified side-chains.

Elucidation of steroid catabolic pathways in bacteria

Nocardia was grown on cholesterol radiolabeled at C4 and C26 to help identify partially metabolized cholesterol intermediates (Sih et al., 1968a, Sih et al., 1968b). These studies demonstrated that cholesterol side chain metabolism to C17 keto-sterols proceeds through intermediates in which the side chain has been cleaved at C24 followed by C22 (Figure 1). Propionyl-CoA is lost from the side chain during the formation of the C24 intermediate, and acetyl-CoA is lost during the formation of the C22 intermediate. Identification of these partially degraded intermediates suggested that the side chain is metabolized via conventional fatty acid β-oxidation, with carbon-carbon cleavage reactions occurring at C24-C25 and C22-C23.

In bacteria, a final loss of C20-C21-C22 as propionyl-CoA results in a C17 keto steroid. Sih et al. proposed dehydrogenation, hydration, and aldolytic fission reactions based on studies performed with *Nocardia restrictus* (ATCC14887). These early studies proposed an atypical oxidation sequence that ends with the β-hydroxythioester, 3-oxo-17-hydroxy-pregna-4-ene-22-oyl-CoA undergoing a retro-aldol reaction because oxidation of the 17-hydroxyl to a ketone to form a β-ketoester is not possible at C17 (Figure 1).

The net yield of side-chain catabolism is two molecules of propionate (propionyl-CoA) and one molecule of acetate (acetyl-CoA) (Sih et al., 1968a). In contrast, human fatty acyl-CoA thioesters that are branched at the β position, like phytanic acid, undergo α-oxidation in

peroxisomes with the loss of a single carbon and the resultant chain is fed into a more typical β-oxidation pathway (Wanders et al., 2011).

A *Nature* report in the early 1950's described a Gram-negative bacterium able to use testosterone (which lacks a side chain) as a sole source of carbon. The amount of testosterone added to these cultures dictated the total oxygen consumption of the organism, with 40 – 60% of the theoretical consumption going to carbon dioxide or water (Talalay et al., 1952). Paul Talalay isolated *Pseudomonas testosteroni* (renamed *Comamonas testosteroni*) and identified its ability to grow on testosterone as a sole carbon source (Talalay et al., 1952). The *C. testosteroni* Δ^5-3-ketosteroid isomerase that converts Δ^5-3-ketosteroids to their corresponding Δ^4-3-ketosteroid enone products was isolated. By the early 1960s, a complete mechanism for this enzymatic transformation was elucidated using spectroscopy, as well as potential steroid inhibitors of this enzyme identified (Wang et al., 1963).

Likewise tracking the metabolic outcomes for some carbons and trying to harmonize these results with what was known about enzymes involved in degradative pathways was studied in *Nocardia*. It was found that radiolabeled C4 in the A ring of the steroid framework (cholesterol or cholestenone) was converted to CO2 about four times as rapidly as C26 located on the steroid side chain, indicating different metabolic fates for these two carbons (Stadtman et al., 1954).

A tendency for some bacteria to preferentially utilize one carbon source over another, known as carbon catabolite repression, was observed as early as the 1950s. *C. testosteroni* grown on testosterone as a sole carbon source was found to be much more readily cultured on acetate than on glucose when the source of carbon was switched, indicating that the enzymes involved in fatty acid metabolism differ from those of sugar metabolism (Santer et al., 1952).

Taken together, the results from the experimental work performed during the first half of the 20^{th} century helped to shape our understanding of how microorganisms like bacteria, and specifically *Mycobacteria*, are able to adapt to their environments.

Cholesterol metabolism gene annotation in Mtb

Recently, interest in identifying the enzymes involved in cholesterol metabolic pathways has been revived in part due to compounding evidence suggesting cholesterol metabolism by *Mtb* is important for pathogenesis. Much of what is known about sterol ring metabolism in *Mtb* was discovered in part based on similarities to better studied steroid-transforming bacteria like *C. testosteroni*, *Rhodococcus sp.*, and other steroid metabolizing Actinobacteria and Proteobacteria described above. Species of *Rhodococcus* are of great interest, and therefore, well studied, because of their potential as useful bioremediation agents and the ease of their study. However, many of the strains used are soil bacteria and have prodigious gene redundancy to adapt to many toxic environments. Moreover, most of these strains are not pathogenic. The *Mtb* genome sequence reported in 1998 opened the door to a better understanding of cholesterol metabolism in the pathogen itself, since preliminary assignments of function to genes could be undertaken (Cole et al., 1998).

Upon sequencing of the *Mtb* genome, the genes were computationally annotated with proposed biochemical functions (Cole et al., 1998). However, these annotations relied on the limited information in public databases at the time, were missing useful functional assignments, and in addition, contained many incorrect assignments (Schnoes et al., 2009). The *Mtb* genome contains around four thousand genes and the functions of about 45% were predicted based on similarity to known genes or proteins. Nevertheless, a large portion of the genome was not annotated, and 16% had no similarity to known proteins (Camus et al., 2002, Cole et al., 1998). Furthermore, many of the annotations that were initially ascribed based on sequence similarity were wrong due to an initial incorrect gene assignment that was propagated through early databases, a phenomenon known as genome rot (Schnoes et al., 2009). The genome of *Mtb* was annotated to contain approximately 250 genes involved in lipid and fatty acid metabolism (Cole et al., 1998). This large number greatly complicated any meaningful assignment of genes that are involved in cholesterol metabolism, and identification of what role they would play in the various cholesterol metabolic pathways in *Mtb*.

The cholesterol transcriptome

Comparison of transcriptional profiles of *Mtb* cultured with or without cholesterol identified over 200 genes that are regulated by cholesterol (Nesbitt et al., 2010). Many, but not all of these genes are a subset of the 250 lipid-metabolism genes identified through annotation. At least 52 cholesterol-regulated genes are within an 83-gene region referred to as the “Cho-region” of the *Mtb* genome (Table) (Nesbitt et al., 2010). The genes in the Cho-region encode primarily homologs of β-oxidation and biphenyl degradation genes from other organisms. A similar set of genes was found to be up-regulated in *Rhodococcus RHA1* as well, demonstrating a degree of conservation in this pathway between Actinobacteria (Van Der Geize et al., 2007). Phenotypic profiling has identified 96 genes important for growth on cholesterol (Griffin et al., 2011). This subset includes most of the annotated genes in the Cho-region predicted to degrade the side chain and catabolize the sterol rings, as well as genes in other regions of the genome. Most importantly for our understanding of *Mtb* pathogenesis, a large portion of these genes overlap with those that are up-regulated in a variety of *in vivo* models of infection.

The transcription of the Cho-region is controlled by at least two transcriptional regulators: KstR1 and KstR2 (Table) (Kendall et al., 2010, Kendall et al., 2007). KstR1 and KstR2 are members of the TetR family of transcriptional repressors. They each contain an N-terminal DNA binding region that recognizes a 14 base pair semi-palindromic sequence, binding of which efficiently blocks RNA polymerase binding (Uhía et al., 2011a, Kendall et al., 2007). The binding of a small molecule inducer, presumably a catabolite, alleviates transcriptional repression of TetR repressors. KstR1 and KstR2 are conserved within Actinobacteria and have been identified in *Rhodococcus*, *Nocardia*, and other *Mycobacteria* species, including *M. smegmatis* where they were first described (Kendall et al., 2010, Kendall et al., 2007).

The KstR1 regulon in *Mtb* contains 74 genes, including those involved in A & B ring degradation as well as side chain metabolism (Kendall et al., 2007). In *Mtb*, a subset of the KstR1 regulon is up-regulated by cholesterol; however, a distinct subset is up-regulated by

the fatty acid palmitate (Table). It is likely that KstR1 controls transcription of lipid metabolizing genes in addition to genes for cholesterol metabolism, consistent with the coexistence of lipids and cholesterol in LDL. It is not known if cholesterol or palmitate is the chemical inducer of KstR1. Many of the genes found within these regulons overlap with genes that have been shown to be essential *in vivo* in mouse or macrophage models of infection, including *fadA5*, *mce4*, and the *igr* operon (Nesbitt et al., 2010, Pandey, 2008, Schnappinger et al., 2003, Rengarajan et al., 2005, Sassetti and Rubin, 2003).

The KstR2 regulon comprises 15 genes in *Mtb* thought to be responsible for metabolizing the C & D rings of the steroid nucleus (Table). In the case of KstR2, the two-ring cholesterol catabolite 3aα-H-4α(3′-propanoyl-CoA)-7aβ-methylhexa-hydro-1,5-indanedione-CoA (HIP-CoA) has been identified as the inducer molecule and inhibits KstR2 DNA binding, de-repressing the KstR2 regulon, whereas HIP or CoA alone do not (Casabon et al., 2013c).

KstR1 and KstR2 regulate independently indicating separate functions for the genes involved in cholesterol metabolism. Their independence is consistent with the presence of two distinct sub-pathways: one for the degradation of the C & D rings and a second for the degradation of the A & B rings of the steroid nucleus and side chain, sometimes referred to as the higher and lower pathways of cholesterol metabolism. KstR1 and KstR2 have evolved separately, and are only distantly similar to each other (they are in different orthogroups); they have divergent functions (Mcguire et al., 2012).

In some *Mycobacteria*, *Rhodococcus*, and *Nocardia*, there is a single copy of KstR1. However, in more distantly related Actinobacteria often found in the environment like *M. ulcerans, M. avium, and M. vanbaalenii* there are additional copies of KstR1 that do not exist in *Mtb* (Mcguire et al., 2012) consistent with their more varied growth environments. TetR-like transcriptional regulators can also be found in Proteobacteria (Ramos et al., 2005). In *Mtb*, approximately 22 additional TetR-like repressors have been annotated, in addition to a host of other helix-turn-helix transcriptional repressors. At least one of these repressors, Mce3R (Santangelo et al., 2002), regulates genes that are also regulated by cholesterol.

Obtaining functional information for the large number of uncharacterized *Mtb* genes is a daunting task. Categorization through transcriptomics has helped to delineate genes into sub-pathways of cholesterol or lipid metabolism. Globally profiling genes required for growth under defined conditions can further identify genes required for cholesterol metabolism (Griffin et al., 2011). It should be noted that the latter type of studies do not distinguish between a gene disruption that blocks nutrition-generating catabolism, and a gene disruption that results in accumulation of a toxic metabolite.

To establish fully the biochemical functions of the cholesterol-regulated gene products, direct experimental validation is required. Typically, assignment of function relies on three types of experiments: mutation or deletion of the gene of interest and determination of phenotype, metabolite characterization of gene knockouts, and the expression and purification of recombinant enzyme and synthesis of putative chemical substrates for a biochemical assay.

ATP-dependent cholesterol import

Some of the best evidence substantiating cholesterol metabolism as important for *Mtb* growth and virulence came from studies performed on the *mce4* locus in both *R. jostii* RHA1 and *Mtb*. The 11 gene *mce4* regulon encodes an ABC-like ATP-dependent transport system that includes two permeases whose function is to actively import cholesterol into the bacterial cell. Deletion of the *mce4* locus compromises the uptake of steroids by *R. jostii* RHA1 (Van Der Geize et al., 2007), and the transporter was demonstrated to bind cholesterol (Mohn et al., 2008). In *Mtb*, mutation of the ATPase subunit, *mceG*, or deletion of the *mce4* operon fully inhibits *in vitro* growth on cholesterol, but not on glycerol (Pandey, 2008).

In *Mtb*, it was demonstrated that an *mce4* mutant was unable to grow in IFN-γ activated macrophages (which are a model for the phenotype of a latent or progressive infection), but was able to survive in resting macrophages (Pandey, 2008). An *Mtb* Δ *mce4* mutant was initially able to persist *in vivo* in a C57BL/6 mouse lung infection, but after four weeks of infection, growth defects became apparent as measured by decreasing cfu counts in the lungs of infected mice. These results clearly indicated a principal role for cholesterol utilization during *Mtb* infection and disease, especially during the chronic phase of infection.

Characterized enzymes in the *Mtb* cholesterol catabolic pathway

The bacterial metabolism of cholesterol is long and complicated, The MetaCyc (SRI) curated interactive pathway and database, provides an excellent graphical overview of the metabolite structures and enzymes involved (Caspi et al., 2010) (SRI). Please refer to the superpathway of cholesterol degradation II (cholesterol dehydrogenase) on the MetaCyc website [http://www.biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-6947&detail-level=4&detail-level=3] to follow the discussion below.

A & B ring metabolism

A & B ring degradation requires ten genes that perform eight enzymatic reactions, *kstD*, *kshAB*, and *hsaABCDEFG* (Figure 3). These enzymes are all EC 1 class oxidoreductases responsible for the sequential oxidation of the A & B carbon framework.

3β-hydroxysteroid dehydrogenase and cholesterol oxidase

In Actinobacteria the first reaction of ring metabolism is oxidation and isomerization of cholesterol to form cholest-4-ene-3-one. This involves the sequential oxidation of the 3-hydroxy position to a ketone and conversion of the resultant β,γ-unsaturated ketone to the α,β-conjugated enone product. In bacteria, this reaction is catalyzed either by a 3β-hydroxysteroid dehydrogenase (3β-HSD) or cholesterol oxidase (ChOX).

3β-HSD is a member of the short chain dehydrogenase superfamily and uses NAD^+ or $NADP^+$ as an electron acceptor. Bacterial cholesterol oxidase (ChOx) is a member of the glucose-methanol-choline (GMC) oxidoreductase family. It is expressed extracellularly as a monomer, binds flavin adenine dinucleotide (FAD) as a cofactor, and uses O_2 as an electron

acceptor, which gets reduced to hydrogen peroxide in order to regenerate FAD. Although ChOx and 3β-HSD use different reaction mechanisms, both enzymes catalyze the same transformation, and either or both are found within the genomes of all steroid-utilizing bacteria. *Nocardia sp*. (Horinouchi et al., 1991), *C. testosteroni* (Horinouchi et al., 2012), and *R. jostii* (Rosloniec et al., 2009) utilize 3β-HSD while *Streptomyces spp*. (Ishizaki et al., 1989), *Rhodococcus equi* (*R. equi*) (Machang'u and Prescott, 1991), and *Gordonia cholesterolivorans* (Drzyzga et al., 2011) utilize ChOx.

The genome of *Mtb* contains an annotated cholesterol oxidase, *choD* (*Rv3409c*) and 3β-hydroxysteroid dehydrogenase (*Rv1106c*). Recombinant 3β-HSD has been enzymatically characterized and is able to convert cholesterol to cholest-4-ene-3-one, and is also able to convert pregnenolone, and dehydroepiandrosterone to their respective 3-keto-4-ene steroid products (Yang et al., 2007). This conversion has not been demonstrated with recombinant ChoD. Knockout experiments indicate 3β-HSD is required for growth of *Mtb* on cholesterol while growth of a Δ*choD* knockout strain was not affected (Yang et al., 2007, Yang et al., 2011). In addition, culture supernatant lost the ability to oxidize cholesterol to cholest-4-ene-3-one in an *Rv1106c* mutant strain (CDC1551) indicating 3β-HSD is the sole cholesterol-oxidizing enzyme in *Mtb* (Yang et al., 2007). *M. smegmatis* culture supernatant from a *choD* (*msmeg_1604*) mutant strain was still able to convert cholesterol to cholest-4-ene-3-one (Yang et al., 2011, Uhía et al., 2012). Likewise in *Mycobacterium sp.* the Δ*choD* knockout strain was able to form 3-keto-4-ene steroids (Ivashina et al., 2012). Moreover, in global growth profiling experiments, *choD* was not required for catabolism of cholesterol, whereas mutation of *Rv1106c* resulted in reduced fitness (Griffin et al., 2011). These results suggest that 3β-HSD, and not ChoD, is responsible for 3β-hydroxy-5-ene steroid oxidation in *Mtb* and possibly in other mycobacterial species as well.

A role for ChoD in cholesterol metabolism has not been substantiated and its exact enzymatic function is still unknown. However, recent reports have suggested its importance in *Mtb* virulence. For example, an *Mtb* Δ*choD* strain showed attenuated virulence in both peritoneal macrophages and mouse models of infection (Klink et al., 2013, Brzostek et al., 2007). The *M. smegmatis* Δ*choD* knockout accumulates a hyperrhamnosylated polar glycopeptidolipid (GPL) called L1334, which lacks acetylation on the 6-deoxytalose moiety (Gao and Sampson, 2014). In *M. avium*, surface-exposed GPLs stimulate the host Toll-like receptor 2 (TLR-2) mediated inflammatory response, and acetylation of GPLs is obligatory for this activity (Pang et al., 2013, Schorey and Sweet, 2008). These results suggest ChoD is involved in tuning the TLR-2 mediated host inflammatory response and that annotation as a cholesterol oxidase is incorrect (Gao and Sampson, 2014).

Interestingly, *3β-hsd* is not regulated by KstR1 in *Mtb*. In other *Mycobacteria* including *M. smegmatis*, *3β-hsd* is under regulatory control of KstR1 (Uhía et al., 2011a, Uhía et al., 2011b). In *Nocardia*, when added to growth media cholesterol induces cholesterol dehydrogenase (ChoA) (Horinouchi et al., 1991, Kishi et al., 2000). This difference in regulation implies that sometime in recent evolutionary history, *Mtb* lost its ability to specifically up-regulate the gene responsible for the first step in cholesterol metabolism. Up-regulation is certainly not requisite to enzymatic activity since regardless of whether cholesterol is available, 3β-HSD enzymatic activity is always present.

In vitro studies with a CDC1551 mutant suggest that *Rv1106c* is important for cholesterol metabolism (Yang et al., 2011), but another study suggests that *Rv1106c* is dispensable for cholesterol metabolism in H37Rv. The reason for this discrepancy may be a result of differences between the strains as has been observed before with *cyp125* (Johnston et al., 2010). The possibility that there is an additional dehydrogenase that can compensate for 3β-HSD activity has been investigated. In *M. smegmatis*, there is at least one additional gene (*msmeg_5233*) that encodes a dehydrogenase that can convert cholesterol to cholest-4-en-3-one (Uhía et al., 2011b). However, there is not an ortholog of *msmeg_5233* in *Mtb*.

Moreover, a *3β-hsd* mutant strain replicated at a similar rate to wild type in macrophages, and infection studies in the guinea pig infection model showed identical cfus in the lungs of wild type, *3β-hsd* mutant, and *3β-hsd* complement. It was concluded that *3β-hsd* is not necessary for nutrition acquisition (Yang et al., 2011), likely because during infection *Mtb* has access to and utilizes multiple carbon sources (Beste et al., 2013). In most bacteria, carbon sources are catabolized in order of their ability to support growth. However, *Mtb* can catabolize multiple carbon sources simultaneously (De Carvalho et al., 2010). The essential or primary role of cholesterol metabolism in the pathogenesis of *Mtb* is unlikely to be solely carbon catabolism.

3-ketosteroid-Δ1-dehydrogenase

The second step of cholesterol A & B ring metabolism is 1,2-desaturation of cholest-4-ene-3-one to a di-α,β enone product, and this reaction is catalyzed by 3-ketosteroid-Δ1-dehydrogenase (KstD). Note, cholesterol side chain β-oxidation and A & B ring oxidation can happen concurrently *in vivo*, albeit with different kinetics. Thus, we present the order of the steps presented is for clarity of discussion and is not absolute. KstD enzymes involved in steroid metabolism have been identified in *Nocardia* (Sih, 1962), *Rhodococcus* (Kaufmann et al., 1992, Knol et al., 2008), and *M. smegmatis* (Brzostek et al., 2005). Interestingly, some bacteria like *R. erythropolis* SQ1, have been shown to encode more than one KstD enzyme and deletion of both were required to block 1,2-desaturation (Van Der Geize et al., 2002).

In *Mtb*, KstD is encoded by *Rv3537* (Knol et al., 2008). Its enzyme activity has been demonstrated *in vitro* with the substrates 5α-androstane-3,17-dione and 17β-hydroxy-5α-androstane-3-one. Genomic analysis has indicated that *Rv3537* is the sole KstD encoding gene in *Mtb*, since an *Rv3537* disrupted strain of *Mtb* accumulates 9-hydroxy-4-androstene-3,17-dione (9-OHAD) (Brzostek et al., 2009). KstD is required for growth of *M. tuberculosis* in minimal medium supplemented with cholesterol (Griffin et al., 2011). In resting THP-1 macrophages, growth of a Δ*kstD* knockout is attenuated compared to H37Rv wild-type strain (Klink et al., 2013).

KshA/B

Next in ring catabolism, a 3-ketosteroid-9α-hydroxylase catalyzes the addition of a hydroxyl group at C9, which leads to subsequent aromatization of the A ring and opening of ring B. This enzyme is a two-component Rieske monooxygenase made up of KshA, the oxygenase component, and KshB, the reductase component. While KshA/B activity has been observed in several Actinobacteria (Andor et al., 2006, Strijewski, 1982, Van Der Geize et al., 2002),

it was not until recently that this activity was demonstrated with recombinant enzyme from *Rhodococcus* for substrates AD and androstadienedione (ADD) (Petrusma et al., 2009). It has also been shown that the substrate specificity and transcriptional regulation of enzymes from *Rhodococcus* that are involved in steroid degradation differs from *Mtb* significantly (Petrusma et al., 2011).

The 3-ketosteroid-9α-hydroxylase homologs in *Mtb*, KshA (*Rv3526*) and KshB (*Rv3571*), have been recombinantly expressed in *E. coli* and purified (Capyk et al., 2009a). Activity was reconstituted *in vitro* with several substrates including AD, ADD, the CoA thioester of 3-oxo-4-pregnene-20-carboxylic acid, and the CoA thioester of 3-oxo-1,4-pregnadiene-20-carboxylic acid (Capyk et al., 2011).

An *Mtb* Δ*kshA*/Δ*kshB* double mutant was unable to grow on cholesterol, AD, or 5α-androstane-3,17-dione (Hu et al., 2010). It was also shown that the deletion of either *kshA* or *kshB* resulted in the rapid clearance of infection in an *in vitro* macrophage model, or an *in vivo* mouse infection model. In a mutant where only *kshA* was deleted there was no observed phenotypic change. Interestingly, the *Mtb* strain CDC1551 does not contain any *kshA* orthologs (Fleischmann et al., 2002). However, when *kshB* was deleted, there was a marked change in cellular wall physiology. This was attributed to an accumulation of tri-acyl-trehaloses (TAT) lipids, and a decrease in the synthesis of di-acyl-trehaloses (DAT), which was measured using radiolabeled ^{14}C-acetic acid and autoradiography. The involvement of the reductase KshB in fatty acid biosynthesis was attributed to its possible interaction with other Rieske-type oxygenase enzymes with structural homology to KshA that are involved in the synthesis of cell wall lipids.

HsaA/HsaB

C. testosteroni is able to utilize testosterone as a sole carbon source and studies elucidating this pathway were some of the first to provide insight into cholesterol ring metabolism in *Mtb*. For instance, gene disruption of the *tesA1* and *tesA2* genes of *C. testosteroni* grown with testosterone was shown to accumulate 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3-HSA) and *tesA1* and *tesA2* are required for the formation of 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DSHA) (Horinouchi et al., 2004). TesA1 and TesA2 are assigned as a flavin-dependent oxygenase/reductase pair responsible for converting 3-HSA to 3,4-DSHA.

In *Mtb* this reaction is assigned to *hsaA* (*Rv3570c*) and *hsaB* (*Rv3567c*). Conversion of 3-HSA to the catechol-secosteroid derivative 3,4-DSHA has been demonstrated *in vitro* with recombinant HsaA/B (Dresen et al., 2010). The crystal structure of HsaA demonstrated that there was an elongation of the substrate tunnel at C17, and this information in conjunction with the relatively weak substrate specificity constants of complete side chain degraded substrates suggests that *in vivo* partially degraded side chain substrates are utilized as well (Hu et al., 2010). Mutagenesis analysis showed that *Rv3570c* is required for growth of *Mtb* in macrophages (Rengarajan et al., 2005).

HsaC: 2,3-dehydroxyphenyl dioxygenase

Studies in *C. testosteroni* identified *tesB* as the iron-dependent extradiol dioxygenase, responsible for the meta-cleavage of the A ring of 3,4-DHSA to give 4,5-9,10-diseco- α3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) (Horinouchi et al., 2004, Horinouchi et al., 2001). In *Rhodococcus sp.* HsaC (*Rv3568c*) is homologous to TesB and a *hsaC*-disrupted strain grown on cholesterol accumulates 3,4-DHSA (Van Der Geize et al., 2007).

In *Mtb*, HsaC shares 98% sequence identity to TesB and encodes the dioxygenase responsible for the formation of 4,9-DSHA. Activity has been demonstrated *in vitro* with recombinant enzyme and substrate 3,4-DHSA (Yam et al., 2009, Van Der Geize et al., 2007). The *hsaC* gene is required for growth of *Mtb* on cholesterol, but not on glycerol. Knockout studies reveal that *hsaC* is important for pathogenesis. Immunocompromised mice and guinea pigs infected with Δ*hsaC* lived significantly longer than those infected with wild type (Yam et al., 2009). In addition, harvested lungs showed lower bacterial loads after 8 weeks of infection. These results established that *hsaC* is important for pathogenesis of *Mtb*.

4,9-DSHA hydrolase

In *C. testosteroni*, TesD was confirmed as the hydrolase that cleaves 4,9-DSHA to form HIP and 2-hydroxy-hexa-2,4-dienoic acid (HHD). In *Mtb*, HsaD (*Rv3569c*) catalyzes the same reaction (Lack et al., 2010, Van Der Geize et al., 2007). The hydrolase activity has been demonstrated with recombinant enzyme and substrate 4,9-DSHA. In addition, *hsaD* is important for *Mtb* survival within macrophages (Rengarajan et al., 2005). Like other enzymes that catalyze reactions earlier in the degradation pathway for rings A & B, an *hsaD* mutant shows significant growth attenuation when grown on minimal medium supplemented with cholesterol (Griffin et al., 2011).

Metabolism of HDD

The genes for the metabolism of HDD by *Mtb* have been proposed based on homology to enzymes in *C. testosteroni*. HDD is metabolized to 4-hydroxy-2-oxohexanoic acid in *C. testosteroni* by 2-hydroxypentadienoate hydratase, TesE (Horinouchi et al., 2005). Next *tesG*, encoding a 4-hydroxy-2-oxovalerate aldolase, forms pyruvate and propionaldehyde. Pyruvate can be converted to carbohydrates or fatty acids. TesF, an acetaldehyde dehydrogenase, converts propionaldehyde to propionyl-CoA.

In *Mtb*, HsaEFG (*Rv3534c*/*Rv3535c*/*Rv3536c*) are hypothesized to metabolize HDD (Van Der Geize et al., 2007). HsaE, HsaF, and HsaG share 41%, 47%, and 56% amino acid identity with TesE, TesG, and TesF from *C. testosteroni*, respectively. Recently HsaF and HsaG were shown to form a heterotetrameric complex, formation of which is required for activity (Carere et al., 2013). Aldolase HsaF catalyzes the cleavage of 4-hydroxy-2-oxohexanoate to propionaldehyde and pyruvate in the presence of NAD+ and CoA. Volatile propionaldehyde is channeled to dehydrogenase HsaG where is it converted to propionyl-CoA. The activity of HsaE has not been verified. Selection studies reveal that *hsaE*, *hsaF* and *hsaG* are not required for growth on cholesterol, although their deletion does result in slower growth on cholesterol as a carbon source (Griffin et al., 2011). Because an *hsaE*,

hsaF, or *hsaG* mutant still has the requisite genes required for catabolism of the side chain and the C & D rings of cholesterol, growth remains possible in minimal medium supplemented with cholesterol (Griffin et al., 2011). The reason for slower growth could be due to toxicity of accumulated metabolites, or other secondary effects.

Steroid C & D ring degradation

How extensively the C & D rings are degraded by *Mtb* is not known. After initial catabolism of the A & B rings, it is presumed that the C & D rings are catabolized in an oxidative manner and acetyl-CoA and/or propionyl-CoA are generated. This assumption is supported by the fact that, after a significant lag phase, *Mtb* is able to grow on HIP, a C & D ring derivative, as a sole carbon source, thus highlighting the fate of at least some of the C & D ring carbons (Casabon et al., 2013a). The precise fate of HIP (Figure 3) in *Mtb* has not yet been established.

The degradation of the hexahydroindanone C & D steroid ring intermediate begins through the thioesterification of the propionate moiety left over from A & B ring oxidation. FadD3, an acyl-CoA ligase, was recently demonstrated to perform this function on HIP (Casabon et al., 2013a). A *Rhodococcus jostii* RHA1 Δ*fadD3* knockout strain was impaired when grown on cholesterol, but when the *Mtb fadD3* (*Rv3561*) gene was knocked in, the control growth rate was restored. Steady-state kinetic analysis of recombinantly expressed FadD3 from *Mtb* demonstrated that the specificity constant for HIP, which contains a keto group at the 5 position of the indanone, is 165 times greater than the specificity for 5α-OH HIP, where this is a hydroxyl moiety.

Both cholesterol and cholate degradative genes converge at this point to a single, relatively conserved cluster in *Rhodococcus* species that are able to degrade both steroids (Mohn et al., 2012). In *Rhodococcus* spp., it was demonstrated that there is a gene cluster similar to those that exist for other Actinobacteria that is able to degrade bile acids like cholate. The regulation of this process is under the control of the TetR-like transcriptional repressor KstR2 in both *Mtb* (*Rv3557c*) and *Rhodococcus jostii* RHA1 (*RHA1_ro04598*). This regulon consists of 15 genes in *Mtb* and *M. smegmatis*, and 14 genes in RHA1. Contrary to what was proposed in Kendall, et. al, 2010, cholesterol does not relieve binding of the KstR2 repressor to its conserved binding sequence. Rather, it is the hexahydroindanone-CoA metabolite that acts as the chemical inducer to alleviate KstR2 repressor binding (Casabon et al., 2013a). These recent experimental results help to further demonstrate that there is a distinct pathway for the degradation of the C & D rings of the steroid nucleus the regulation of which is dependent on metabolites from A & B ring degradation degradation that occurs first.

Attempts to identify the Proteobacterial genes responsible for the degradation of the C & D rings of steroids have been pursued using gene knockout and metabolic profiling studies. For example, a gene-disrupted mutant of ORF18 in the testosterone degrading bacteria *C. testosteroni* TA441 was constructed and grown on ADD, chenodeoxycholic acid, and cholic acid in order to identify any buildup of metabolic intermediates (Horinouchi et al., 2006). When incubated with testosterone, no metabolic intermediates accumulated (Horinouchi et al., 2003). However, when the ORF18-disrupted mutant is grown on ADD, it accumulates

HIP. This study establishes the importance of this gene as encoding a probable CoA-ligase essential for further degradation of the C & D ring steroid nucleus. Additional substitutions like hydroxyl groups on the steroid ring system, as seen for cholic acid for example, did not preclude metabolism to the step encoded by ORF18.

It is hypothesized that FadE30 of *Rhodococcus equi* catalyzes the dehydrogenation of HIP because a Δ*fadE30* knockout strain of *Rhodococcus equi* accumulates 7aβ-methyl-hexahydro-5-indanone when cultured with cholesterol. *R. equi* FadE30 shares 68% amino acid identity with FadE30 (*Rv3560c*) from *Mtb*. However, this function has not yet been demonstrated with *Mtb* FadE30 (Van Der Geize et al., 2011). Expression of the gene triplet *fadE31-fadE32-fadE33* is controlled by KstR2 and has been proposed to be involved in C & D ring metabolism (Van Der Geize et al., 2007), although this awaits further biochemical characterization.

Until recently, it was not clear if side chain degradation was prerequisite to C & D ring catabolism. However, current evidence suggests that any partially catabolized bacterial substrate must have the side chain completely removed before any C & D oxidation enzymes are able to function. FadD3 was unable to ligate a CoA onto a side chain-containing metabolite similar to HIP, 1β(2′-propanoate)-3aα-H-4aα(3”-propanoate)-7aβ-methylhexahydro-5-indanone (Casabon et al., 2013a).

Furthermore, an *Mtb* mutant lacking the *igr* operon, which removes the last three carbons of the steroid side chain, accumulated a hexahydroindanone metabolite that contained a three-carbon side chain (Thomas et al., 2011). Therefore, the presence of a side chain precludes further catabolism of rings C & D and results in a buildup of metabolites (*vide infra*). In multiple metabolite profiling studies, AD and ADD were identified when *Mtb* is cultured with cholesterol (Nesbitt et al., 2010, Thomas et al., 2011). In addition, side chain metabolizing ChsE1-ChsE2 prefers ring intact substrates (Thomas and Sampson, 2013, Thomas et al., 2011), while the ring system metabolizing enzyme KstD prefers fully side chain metabolized 5α-AD to the partially side chain metabolized 5α-pregnane-3,20-dione or progesterone (Knol et al., 2008).

Cholesterol side chain metabolism

Cytochrome P450 125

The side chain of cholesterol was initially proposed to be degraded through a process akin to classical fatty acid β-oxidation (Figure 4). The β-oxidation cycle requires four sequential enzymatic steps catalyzed by an acyl-CoA dehydrogenase (FadE), an enoyl-CoA-hydratase (EchA), a 3-hydroxy-acyl-CoA-dehydrogenase (FadB), and a 3-keto-acyl-CoA thiolase (FadA), respectively (Figure 4). The genome of *Mtb* encodes a large number of β-oxidation genes, including 35 *fad*E, 21 *echA*, 5 *fadB*, and 6 *fadA* genes. This redundancy in the genome complicates assignment of function to individual steps in the multiple possible pathways. For example, *fadE14* from *Mtb* is annotated as a *fadE*, however, it was shown to participate in the synthesis of the iron-scavenging siderophore mycobactin, and is thus not involved in degradation at all (Rodriguez et al., 2002, Lamarca et al., 2004). Further

complicating gene assignments, the β-oxidation enzymes responsible for side chain metabolism in other Actinobacteria have not been identified.

Prior to entering the β-oxidation cycle, the hydrocarbon side chain must first be oxidized to the carboxylic acid and subsequently activated through formation of the CoA thioester (Figure 4). It has long been proposed that a cytochrome P450 is responsible. Actinobacteria have a large number of cytochrome P450s, again making it challenging to assign function—the *Mtb* genome encodes 20 cytochrome P450 enzymes. The enzyme responsible for catalysis was identified in *Rhodococcus jostii* (*R. jostii*) as *cyp125* from knockout studies demonstrating *cyp125* is required for growth on cholesterol, but not for growth on side chain-activated 5-cholestene-26-oic acid-3β-ol (Rosloniec et al., 2009). In *Mtb*, *cyp125* (*Rv3545c*), located in the intracellular growth (*igr*) operon, is the ortholog of *cyp125* from *R. jostii*.

Mtb cyp125 was recombinantly expressed, in separate accounts, in *R. jostii RHA1* or in *E. coli* with coexpression of folding chaperones, GroEL and GroES (Chang et al., 2009, Capyk et al., 2009b). Purified protein was shown to bind cholesterol and cholest-4-ene-3-one with sub-micromolar affinity (Capyk et al., 2009b). Cyp125, with reductase KshB (*Rv3571*) or spinach ferredoxin reductase, and NADH was able to transform cholesterol and cholest-4-ene-3-one *in vitro* to the C26 hydroxy and the C26 carboxylic acid products.

The crystal structure of Cyp125 has been reported without ligand as well as with AD and the antitubercular drug econazole (Mclean et al., 2009). These ligands were found to bind within the active-site cavity of the enzyme. Docking was used to incorporate cholesterol into the structure and it was found for the lowest energy structure that the C26 and C27 of cholesterol were 5.3 and 6.3 Å from the heme iron center, respectively. The position of these carbons relative to the active site of the enzyme substantiates that this is where the transformation occurs.

Knockout *cyp125* (Δ*cyp125*) *Mtb* CDC1551 cultures grown with cholesterol accumulate cholest-4-ene-3-one, suggesting that this is the physiological substrate for the enzyme *in vivo* (Ouellet et al., 2010). The *cyp125* gene is required for growth on cholesterol in CDC1551, but interestingly is not required in the H37Rv strain. When expressed recombinantly *in vitro*, Cyp142 in addition to Cyp125 catalyze the oxidation of C26 in the side chain of cholesterol. Expression of either *cyp125* or *cyp142* can support the growth of Δ*cyp125 Mtb* on cholesterol (Driscoll et al., 2011, Johnston et al., 2010). Therefore Cyp142 provides compensatory activity in *Mtb* H37Rv. The *cyp142* gene is not expressed in CDC1551 wild type due to a promoter mutation.

Acyl-CoA ligases

Following oxidative activation of the hydrocarbon side chain by Cyp125, an acyl-CoA ligase (FadD) forms an acyl-CoA thioester, which is required for the substrate to enter the β-oxidation pathway since these enzymes require CoA substrates (Figure 4). The *Mtb* H37Rv genome contains 36 genes annotated as *fadD*s, a functional redundancy that complicates their respective substrate assignments. *E. coli*, which is able to metabolize fatty acids as a sole source of carbon, encodes only one FadD that accepts fatty acids of varying length

(Kameda and Nunn, 1981). The large difference in *fadD* numbers between *Mtb* and *E. coli* suggests that FadD enzymes in *Mtb* have potentially many additional functional roles.

In *Mtb*, several *fadD* genes located adjacent to polyketide synthase (PKS) genes in the genome, have been shown to encode fatty acyl-AMP ligases with important roles in mycolic acid, DIM, and PGL synthesis (Cox et al., 1999, Khare et al., 2009, Pethe et al., 2010, Simeone et al., 2010, Trivedi et al., 2004). Interestingly, in the opportunistic pathogenic bacterium responsible for many deaths from Cystic fibrosis, *Pseudomonas aeruginosa*, the presence of a plurality of acyl-CoA ligases was attributed to virulence, since these enzymes are responsible for 'activating' all carbon substrates destined to enter a variety of modification pathways (Kang et al., 2010).

Four acyl-CoA synthetase *fadD* genes are up-regulated during growth of *Mtb* on cholesterol: *fadD3*, *fadD17*, *fadD18*, and *fadD19* (Nesbitt et al., 2010). FadD3 was recently biochemically characterized as being involved in C & D ring metabolism by initiating HIP degradation (Casabon et al., 2013a). FadD19 in *Rhodococcus rhodochrous* (*R. rhodochrous*) catalyzes thioesterification of C24-branched steroid-C26-oic acid substrates, and is therefore a sterol-CoA ligase important for degradation of the C24 sterols β-sitosterol and campesterol (Wilbrink et al., 2011). Recombinant *R. rhodochrous* FadD19 (67% amino acid identity with *Mtb* FadD19 (*Rv3515c*) also catalyzes thioesterification of steroid-C26-oic acid substrates. A recent report demonstrated that the ortholog of this protein in *Mtb* catalyzes thioesterification of 3-oxo-4-cholesten-26-oate with a k_{cat}/K_m of $0.33\pm0.03\times105\ M^{-1}s^{-1}$, thus validating this enzyme as the eight-carbon side chain steroid-CoA ligase (Casabon et al., 2013b).

Casabon et al. also demonstrated that FadD17 (*Rv3506*) is the steroid-24-oyl-CoA synthetase that ligates CoA onto the 5-carbon side chain of the intermediate metabolite (Casabon et al., 2013b). This activity is required for salvaging cholesterol-24-oic acids that are formed as a result of CoA ester hydrolysis during β-oxidation or that are formed through an alternate pathway (*vide infra*) (Holert et al., 2013a).

The FadD18 (*Rv3513c*) sequence is almost identical to the C-terminal half of FadD19 as well as the C-terminal portion of other fatty acid-CoA synthases. No information is available about the function of FadD18 (Lechat et al., 2008). However, it appears that *fadD18* could be the result of partial gene duplication (Lew et al., 2011).

Phenotypic profiling experiments using transposon mutant libraries and deep sequencing performed by Griffin et al (2011) suggested a possible role for FadD36 (*Rv1193*) in *Mtb* in performing the first step in *Mtb* steroid side chain thioesterification. However, FadD19 has been shown to perform this function (Casabon et al., 2013b). Moreover, the *fadD36* gene is not regulated by KstR1. Therefore, it is unlikely that this functional assignment is correct (Griffin et al., 2011).

Acyl-CoA dehydrogenases

CoA thioesters of lipids including steroids are substrates for β-oxidation, and the first enzyme in this four step catabolic cycle is catalyzed by an acyl-CoA dehydrogenase

(ACAD). Degradation of the cholesterol side chain requires three cycles and therefore, three ACADs (Figure 4). Of the 35 *acad* genes, 13 are up-regulated and 4 are down-regulated by cholesterol (Nesbitt et al., 2010). Eight *fadE* genes, including *chsE1* (*fadE28*) and *chsE2* (*fadE29*) of the *igr* operon, and *fadE5*, *fadE25*, *fadE30*, *fadE31*, *fadE32*, and *fadE33* are required for growth on cholesterol, validating their importance in cholesterol metabolism (Griffin et al., 2011). This functional redundancy of *fadE* genes is at first perplexing—although Mtb has 35 *fadE* genes, E. *coli* only has one (*yafH*) (Campbell and Cronan, 2002) — and steroid metabolism accounts for about one third of this redundancy.

Recently, our laboratory has identified several heteromeric enzymes encoded by polycistronically expressed *fadE* genes in *Mtb* (Wipperman et al., 2013). Interestingly these genes are all regulated by cholesterol, suggesting they are involved in cholesterol transformation and/or metabolism. We have characterized six heteromeric ACADs encoded by cistronic genes in *Mtb* including *chsE1(fadE28)-chsE2(fadE29)*, *fadE17-fadE18*, *fadE23-fadE24*, *fadE26-fadE27*, *fadE31-fadE32*, and *fadE31-fadE33*. All of the identified heteromeric enzymes contain two active sites and bind two FAD cofactors, unlike a traditional homotetrameric ACAD, which has four active sites and binds four FAD cofactors. Each polypeptide of the complex is either insoluble or inactive when recombinantly expressed alone, and both chains are required to form intact FAD cofactor binding sites. The α and β chains are not interchangeable between heterotetramers.

Heterotetrameric ChsE1(FadE28)-ChsE2(FadE29) is encoded by two adjacent genes in the *igr* operon, *Rv3544c* and *Rv3543c* (Figure 5). The *igr* operon is up-regulated by cholesterol and is located within the cholesterol regulon in the *Mtb* genome (Nesbitt et al., 2010). The *igr* operon knockout (Δ*igr*) strain does not grow on cholesterol as a sole carbon source *in vitro*. However, Δ*igr* can grow just as well as wild-type *Mtb* on pyruvate, valerate, isovalerate, propionate, palmitate, dodecanoate, glycerol, Tween, and dextrose confirming its role in cholesterol metabolism and not fatty acid metabolism (Chang et al., 2009). The *igr* operon is conserved in pathogenic and nonpathogenic *Mycobacteria*, including *M. smegmatis*, *M. avium*, and *M. bovis*. In other Actinobacteria including *Rhodococcus*, *Streptomyces*, and *Gordonia*, the operon is only partially conserved and lacks *cyp125*.

In a Δ*igr* knockout strain of *Mtb*, growth was attenuated in resting macrophages and early in the infection process in immunocompetent mice. The cfu levels in the lungs and spleen were greatly reduced compared to wild type. The growth attenuation observed in the *Mtb* Δ*igr* knockout demonstrates that this operon is important for intracellular growth, which is where *igr* derives its name (Chang et al., 2007). The *in vitro* phenotype of Δ*igr* requires cholesterol containing media and the genes encoding Mce4, an ATP-dependent cholesterol transporter (Chang et al., 2009). These studies suggest that the observed attenuation in Δ*igr* is not due to lack of cholesterol, but rather the inability to fully metabolize cholesterol and the accumulation of a toxic intermediate.

Metabolic tracing studies with isotopically labeled [1,7,15,22,26-^{13}C]-LDL cholesterol led to the accumulation of methyl 1β-(2′-propanoate)-3aα-H-4α(3′-propanoic acid)-7aβ-methylhexahydro-5-indanone in the Δ*igr* knockout strain. This metabolite is derived from

cholesterol and contains partially metabolized A & B rings, intact C & D rings and a partially metabolized three-carbon side chain (Thomas et al., 2011).

Based on the metabolite studies, the substrate for ChsE1-ChsE2 was hypothesized to be 3-oxo-23,24-bisnorchol-4-en-22-oyl-CoA or 1β-(2′-propanoyl-CoA)-3aα-H-4α(3′-propanoic acid)-7aβ-methylhexahydro-5-indanone. Both substrates were tested as well as short and branched fatty acyl-CoAs. Only the steroid thioesters were oxidized by ChsE1-ChsE2. The four ring 3-oxo-23,24-bisnorchol-4-en-22-oyl-CoA was a significantly better substrate with a k_{cat}/K_m that was 255-fold better than for the two-ring indanone. The regiochemistry of dehydrogenation was confirmed by NMR spectroscopy to be at C17-C20 (Thomas and Sampson, 2013) (Figure 5); the double bond that is introduced during the final cycle of cholesterol side chain β-oxidation. This heterotetrameric enzyme was the first characterized ACAD involved in sterol side chain metabolism (Thomas et al., 2011).

We proposed, based on its sequence similarity to ChsE1-ChsE2 and transcriptional regulation by KstR1, that FadE26-FadE27 catalyzes dehydrogenation in the first and/or second cycles of cholesterol side chain β-oxidation. Preliminary activity data demonstrated that FadE26-FadE27 introduced a double bond into the 3-keto-4-cholene-24-oyl-CoA substrate, the cholesterol metabolite with a 5-carbon side chain (Wipperman et al., 2013). With this preliminary activity data for the five-carbon substrate, and the fact that expression of both ChsE1-ChsE2 and FadE26-FadE27 are regulated by KstR1, it is probable that the only other KstR1 regulated ACAD, FadE34, is responsible for the first dehydrogenation step in cholesterol metabolism during the first cycle of β-oxidation. Interestingly, FadE34 has high protein sequence homology with the human very long chain ACAD enzymes, which are rare examples of ACAD dimers rather than tetramers. This protein structure evolved to bind very long chain fatty acids, and it is possible that FadE34 is a dimer with a large enough binding cavity to bind a partially oxidized C27-CoA steroid, although this activity remains to be assessed.

Enoyl-CoA hydratases

The second step of β-oxidation is the hydration of enoyl-CoA thioesters to β-hydroxy-acyl-CoA products by an enoyl-CoA hydratase (Figure 4). In *Mtb*, 21 genes are computationally annotated as (*S*)-specific enoyl-CoA hydratases, (Cole et al., 1998). (*S*)-hydratases, such as the classic example of crotonase, have been well studied, and are typically involved in fatty acid β-oxidation (Stern and Del Campillo, 1956). One or possibly more enoyl-CoA hydratases are required for catabolism of the cholesterol side chain. Of these 21 genes, two are found in the cholesterol degradation cluster, *echA19* (*Rv3516*) and *echA20* (*Rv3550*), though neither is required for *Mtb* growth on cholesterol (Griffin et al., 2011, Nesbitt et al., 2010). Using a promoter trap screen, *echA19* was found to be essential for infection in THP-1 human macrophages (Dubnau et al., 2002).

The *echA19* gene is located adjacent to FadD19 in the genome, although it is transcribed in the opposite direction. Its location suggests that EchA19 catalyzes the hydration of the steroid-26-enoyl-CoA, which has an 8-carbon side chain.

The *echA20* gene is transcriptionally regulated by KstR2, and was found to be up-regulated significantly in *R. jostii* RHA1 when the strain is grown on cholesterol or HIP (Casabon et al., 2013c). This regulation suggests that EchA20 plays a role in C & D ring catabolism. It should be noted that *R. jostii* RHA1 contains an additional hydratase in the KstR2 regulon that is not present in *Mycobacteria* again highlighting the differences in metabolic capabilities between *Mycobacteria* and *Rhodococcus*.

Two additional (*S*)-enoyl-CoA hydratases have been implicated in cholesterol metabolism. The *echA9* gene is required for growth on cholesterol, although its expression is not regulated by cholesterol. The expression of *echA9* is under the control of the transcriptional regulator SigE (Rohde et al., 2012). The *echA13* gene is in the same operon as *fadE17* and *fadE18*; an operon that is up-regulated by cholesterol and under the control of the transcriptional repressor Mce3R (De La Paz Santangelo et al., 2009). Their role in cholesterol metabolism has yet to be determined.

Two genes of the *igr* operon, *Rv3541c* and *Rv3542c* encode MaoC-like (*R*)-enoyl-CoA hydratases, which in bacteria are often involved in carbon storage through polyhydroxyalkanoate biosynthesis. The gene products of *Rv3541c* and *Rv3542c* form a heteromeric complex, just like ChsE1-ChsE2 (Thomas et al., 2011) also encoded in the *igr* operon. Based on the metabolite accumulated in the Δ*igr* knockout and the enzymatic activity profile of ChsE1-ChsE2, we proposed that the heteromeric Rv3541c-Rv3542c enzyme catalyzes hydration of 3-oxo-23,24-bisnorchol-4,20-dien-22-oyl-CoA (Thomas et al., 2011). Addition of the (*R*)-hydratase family to the 21 *echA* enoyl-coA hydratases encoded in the *Mtb* genome increases the number of candidate enoyl-CoA hydratases involved in lipid or sterol metabolism.

Characterized (*R*)-hydratases in bacteria, including those in *Aeromonas caviae* (*A. caviae*) (Fukui et al., 1998), *Pseudomonas aeruginosa* (Tsuge et al., 2000), *Rhodospirillum rubrum* (Reiser et al., 2000), and *E. coli* (Park and Lee, 2003) are involved in the biosynthesis of a class of carbon storage polyesters known as polyhydroxyalkanoates (PHA). (*3R*)-hydroxyacyl-CoA monomers produced by (*R*)-hydratases are shunted towards PHA synthesis and polymerized by PHA synthase (Fukui et al., 1998, Tsuge et al., 2000). To the best of our knowledge, *Mtb* does not synthesize PHAs and lacks an obvious PHA synthase, a key enzyme in this pathway.

In mammals, (*R*)-hydratases are part of multifunctional enzyme 2 (MFE-2), which contains three functional domains with (3*R*)-hydroxyacyl-CoA dehydrogenase, (*R*)-hydratase, and sterol carrier protein activities. MFE-2 has been shown to be important for β-oxidation of very-long-chain and α-methyl-branched fatty acids, as well as bile acid synthesis (Koski et al., 2005). For example, MFE-2 in rat liver peroxisomes has been shown to be involved in cholic acid synthesis (Qin et al., 1997). The biosynthesis of cholic acid requires shortening of the side chain of C27 steroids by β-oxidation. Recombinant (*R*)-hydratase is able to catalyze the hydration of (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA, an intermediate in bile acid synthesis. In rats, ΔMFE-2 knockout mice accumulate very long chain fatty acids, branched chain fatty acids, and bile acid intermediates (Baes et al., 2000).

The *Mtb* gene *hsd4B* (*Rv3538*) is annotated as a probable (*R*)-enoyl-CoA hydratase with a conserved hydratase motif including important catalytic residues. The *hsd4B* gene is located in the cholesterol-regulated region of the genome and is regulated by KstR1. Furthermore, it is adjacent to *kstD*, which is known to be involved in the A-ring metabolism of cholesterol by *Mtb*. However, its substrate has not been identified. Likewise, the enoyl-CoA hydratase(s) responsible for the first two cycles of β-oxidation of the cholesterol side chain have not yet been identified.

β-hydroxy-acyl-CoA dehydrogenases

No β-hydroxy-acyl-CoA dehydrogenase encoding genes are located in the cholesterol regulon, and it is not clear which genes are required for this step in the β-oxidation pathway. *Mtb* FadB (*Rv0860*) has been shown to be part of the trifunctional enzyme (TFE) complex required for fatty acid metabolism (Venkatesan and Wierenga, 2013). Recombinantly expressed *Mtb* FadB2 (*Rv0468*) can turn over β-hydroxybutyryl-CoA *in vitro* in the presence of NAD+ (Taylor et al., 2010). The *M. smegmatis fadB2* mutant, which shares 84% identity at the amino acid level to *Mtb* FadB2, was not impaired in growth using various carbon sources including glycerol, glucose, acetate, propionate, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, oleic acid, linoleic acid, or cholesterol as the sole carbon source (Taylor et al., 2010). The *fadB2* gene is located adjacent to *icl1* (*Rv0467*) in the genome and is conserved among *Mycobacteria*. FadB3 (*Rv1715*) is not conserved in *Mycobacteria* and conflicting reports make its importance for survival questionable (Lamichhane et al., 2003, Williams et al., 2011, Sassetti and Rubin, 2003). Recombinant FadB3 was not active with β-hydroxybutyryl-CoA with NAD+ or NADP+ (Taylor, 2011).

The *Mtb* genes encoding FadB4 (*Rv3141*) and FadB5 (*Rv1912c*) are annotated as probable quinone oxidoreductases and are unlikely to encode the β-hydroxy-acyl-CoA dehydrogenases involved in side chain oxidation. Coincidentally, the *fadB4* gene is located directly upstream of *fadE23 and fadE24* (which are down-regulated by cholesterol, but up-regulated in a human THP-1 macrophage infection model) and is transcribed in the same direction, although there is no evidence to show that they share an operon (Fontan et al., 2008a).

Mtb Hsd4A (*Rv3502c*) has been proposed to be the β-hydroxy-acyl-CoA dehydrogenase involved in side chain β-oxidation because it is required for growth on cholesterol and is similar to 17β-hydroxysteroid dehydrogenase (Griffin et al., 2011). The biochemical function of Hsd4A has not been demonstrated, but it is a likely candidate for catalysis of oxidation of β-hydroxy acyl-CoAs in the sterol side chain.

3-ketoacyl-CoA thiolases

Two acyl-CoA thiolases are found in the cholesterol regulon, *fadA5* (*Rv3546*) and *fadA6* (*Rv3556c*). Only fadA5 is required for growth of *Mtb* on cholesterol, suggesting it is involved in cholesterol metabolism (Nesbitt et al., 2010). Transcription of *fadA5* is up-regulated in macrophages and the mouse lung, whereas *fadA6* is not (Dubnau et al., 2005, Dubnau et al., 2002). An *Mtb* Δ*fadA5* knockout strain shows growth attenuation in the

mouse model of infection; cfu in the lungs showed a marked decrease after 8 weeks of infection compared to wild type and complement strains (Nesbitt et al., 2010).

Nesbitt *et al.* demonstrated *in vitro* that this enzyme, which contains an active-site cysteine nucleophile and a general acid/general base histidine and cysteine can catalyze thiolysis of acetoacetyl-CoA and is therefore a thiolase (Nesbitt et al., 2010). Wild-type *Mtb* cultured with cholesterol accumulates AD and ADD and these oxidized steroids are secreted in small amounts, while these two steroid metabolites are not detected in the Δ*fadA5* knockout strain, thus demonstrating that FadA5 is required for their production and likely the thiolase that catalyzes carbon-carbon cleavage in the steroid side chain (Figure 4). This Δ*fadA5* knockout strain blocked cholesterol metabolism since it was unable to grow on cholesterol as a sole carbon source, but it did not accumulate any toxic metabolites and was able to grow in the presence of cholesterol. These experiments demonstrate that FadA5 is required for full metabolism of the side chain of cholesterol. However, it is unclear if an additional thiolase like FadA6 is required for β-oxidation of the cholesterol side chain or if two cycles are catalyzed by FadA5.

Lipid transfer proteins and alternative side chain metabolism

What is known about the role of the lipid transfer protein genes (*ltp*) in *Mtb* is limited. However, three genes annotated as lipid transfer proteins are regulated by cholesterol: *ltp2* (*Rv3540c*), *ltp3* (*Rv3523*), and *ltp4* (*Rv3522*). We proposed on the basis of the *igr* operon metabolite profiling and enzymatic activities that Ltp2 is responsible for the thermodynamically downhill retroaldol C17-C20 cleavage in the last step of steroid side chain degradation (Thomas et al., 2011). This catalytic step would provide the C17 ketone, deviating from the classical β-oxidation pathway in this respect (Figure 4). Sih had originally proposed in the 1960s that this step occurred non-enzymatically (Sih et al., 1967, Sih et al., 1968a). However, the genomic and bioinformatic evidence suggest that an enzymatic catalyst that shares some active site similarities with thiolases (FadAs) is responsible. Recombinantly expressed Ltp2 in *E. coli* was found to be insoluble (Thomas et al., 2011) and biochemical verification of this activity remains to be established.

In *Pseudomonas* sp. strain Chol1, it has been proposed on the basis of gene knockout and metabolite studies, and assays in cell lysates that cleavage of the 24-oyl-CoA intermediate produces an aldehyde metabolite rather than a thioester via a classical β-oxidation pathway. This cleavage is catalyzed by an aldolase (shy) that is homolgous to Ltp3 from *R. rhodochrous* DSM 43269 (Holert et al., 2013a, Holert et al., 2013b). The aldehyde is then oxidized to the carboxylic acid by an aldehyde dehydrogenase (sad) and esterified by a CoA ligase. There are Ltp orthologs in *Mtb* that are both cholesterol and KstR1 regulated (*ltp2*, *ltp3*, and *ltp4*) and acyl-CoA ligases, e.g. FadD17. One apparent aldehyde dehydrogenase ortholog, Rv0223c, is in the KstR1 regulon and regulated by cholesterol. However, it is remote from the Cho region of the genome. The most similar gene to *sad* in *Mtb* is *Rv0147*, but it is also remotely located from the Cho region of the genome, and not regulated by cholesterol or KstR1; thus less likely to be required for side-chain metabolism. The presence in the *Mtb* genome of both thiolases (FadAs) and aldolases (Ltps) points to the possibility that cholesterol metabolites can be processed through multiple competing pathways in *Mtb*.

However, in *Mtb*, steroid side chain aldehyde formation and dehydrogenase-catalyzed oxidation activities remain to be established.

Beyond cholesterol degradation

Linkage of Mtb cholesterol degradation to the metabolic pool

Mtb metabolism in the intracellular environment of the host and during conditions of stress, like hypoxia, *in vitro*, depends upon the up-regulation of oxidative genes that are able to break down cholesterol, fatty acid and complex lipids into C_2 (acetate) and C_3 (propionate) carbon units (Galagan et al., 2013). Acetate and propionate are then fed into other metabolic pathways to produce ATP and biosynthetic intermediates.

Propionate can be generated as a product of cholesterol, methyl- and odd-branched fatty acid, and amino acid metabolism. Propionate is toxic to bacterial cells and therefore needs to be channeled into appropriate detoxification pathways to prevent accumulation. *Mtb* incorporates propionate into the biosynthesis of new lipids, some of which contribute to sustaining infection and virulence, like sulfolipid (SL-1) and phthiocerol dimycocerosates (PDIM) (Jain et al., 2007, Yang et al., 2009, Cox et al., 1999, Rousseau et al., 2004). In the absence of exogenous sources of carbon (carbohydrates or sugar), there is an increase in the molecular weight of PDIM for *Mtb* grown *in vitro* on cholesterol, and these data in conjunction with our understanding of the lipid diet of *Mtb in vivo* support the hypothesis that this is an adaptive strategy to deal with a toxic catabolic product in a way that might enhance virulence (Yang et al., 2009). Likewise, phenolic glycolipid (PGL), which is structurally related to PDIM, is hypothesized to cause hypervirulence in certain *Mtb* isolates, as well as down-regulate the host immune response, specifically the release of important pro-inflammatory cytokines like TNFα, IL-6, and IL-12 (Reed et al., 2004).

Requisite to the utilization of propionate is its conversion from propionyl-CoA to methylmalonyl-CoA through the methylmalonyl pathway or succinate through the methylcitrate cycle (Savvi et al., 2008) (Figure 6). (*S*)-Methylmalonyl-CoA is generated from propionyl-CoA and bicarbonate in a condensation reaction catalyzed by propionyl-CoA carboxylase (EC 6.4.1.3). In the TCA cycle, condensation of oxaloacetate with acetyl-CoA to generate citrate is catalyzed by citrate (*Si*)-synthase (EC 2.3.3.1). In the methylcitrate cycle, the enzyme 2-methylcitrate synthase (EC 2.3.3.5) catalyzes the condensation of oxaloacetate with propionyl-CoA resulting in the formation of methylcitrate instead of citrate. This molecule is isomerized to methylisocitrate, which can go on to form succinate and pyruvate that feed into other metabolic pathways.

Acetate generated during cholesterol, fatty acid, and lipid metabolism can condense with oxaloacetate to form citrate, which is isomerized to isocitrate. Isocitrate is then converted to glyoxylate and succinate by isocitrate lyase (Icl), an essential enzyme in the glyoxylate shunt. This shunt avoids two decarboxylation steps and allows the formation of four carbon subunits from acetate, a necessary process for an organism to obtain carbon and energy from acetate.

The isocitrate lyase genes (*icl1* and *icl2*) are up-regulated when *Mtb* is grown on fatty acids *in vitro*, as well as *in vivo* when harvested from infected mouse lungs (Timm et al., 2003, Munoz-Elias et al., 2006). In knockout models, each *icl* gene is dispensable and deletion does not affect bacterial viability for infection. However, deletion of both genes (Δ*icl1*/Δ*icl2*) results in fully attenuated virulence in an *in vivo* mouse infection model (Munoz-Elias et al., 2006).

The Δ*icl1*/Δ*icl2 Mtb* strain is completely unable to grow on propionate or acetate as a carbon source, and this was attributed to the fact that they can act as both isocitrate lyase and methylisocitrate lyase genes. However, because of their dual activity, it was initially unclear whether or not these genes were essential *in vivo* because they participated in the glyoxylate cycle or the methylcitrate cycle. An *Mtb* double-mutant deficient in the methylcitrate cycle genes methylcitrate synthase (*prpC*) and methylcitrate dehydratase (*prpD*) is completely unable to grow on propionate as a carbon source *in vitro* or in murine bone marrow-derived macrophages. This phenotype implies that the methyl citrate cycle plays an important role in managing the toxic propionate. It was therefore puzzling that the Δ*prpDC* knockout was totally viable in a mouse infection model up to 20 weeks post infection, with spleen and lung pathology nearly identical to the wild-type (Munoz-Elias et al., 2006). The researchers concluded that the methylcitrate cycle is dispensable *in vivo* during infection. The corollary being that propionate flux is managed differently *in vivo*.

Cholesterol-associated genes remaining to be annotated

In addition to the significant number of oxidation and regulatory genes involved in cholesterol utilization discussed so far, there are still many others that remain to be studied in order to validate their role in the cholesterol utilization pathway. The regulation of cholesterol metabolic genes is complex, and likely reflects an underlying set of sub-pathways, all of which make use of steroid degradation intermediates for various reasons.

In the Cho region, nine genes encode PE (Pro–Glu) or PPE (Pro–Pro–Glu) glycine-rich proteins, part of a family with highly conserved N-terminal proline and glutamine motifs of unclear function (Cole et al., 1998). Of the remaining 74 genes in this region of the *Mtb* genome, probable functions have been assigned to approximately 60% (Table). These assignments account for the cholesterol metabolism pathway described in Figures 3 and 4. *In vitro* biochemical assays have been performed to establish function for 27 of the corresponding 43 gene products. Putative enzyme class has been assigned to about half of the 31 (40% of the Cho region) genes for which no preliminary biochemical evidence of function exists.

Outside the Cho region, there are an additional 150 *Mtb* genes significantly up-regulated in the presence of cholesterol as compared to glycerol (Nesbitt et al., 2010). A large number of genes identified through forward genetics as essential for *Mtb* pathogenesis and virulence (Sassetti and Rubin, 2003) or essential for growth on cholesterol (Griffin et al., 2011) overlap with these cholesterol-regulated genes that do not have functions within any known cholesterol utilization pathway. Their regulation could be a secondary effect from a metabolite produced during the cholesterol degradation process (propionate, for example),

or it could be that these genes are feeding cholesterol metabolites into other yet unknown pathways.

Mce3R

There are three additional mammalian cell entry (*mce*) regulons in *Mtb* proposed to be involved in host-TB interactions, which include lipid transport and virulence: *mce1*, *mce2*, and *mce3* (Santangelo et al., 2002, Mitra et al., 2005, Uchida et al., 2007, Gioffre et al., 2005, Arruda et al., 1993, Mohn et al., 2008). Like *Mtb* Δ*mce4*, *Mtb* Δ*mce3* has an attenuated phenotype *in vivo* when used to infect C57BL/6 mice (Senaratne et al., 2008). The survival times of the mice, the cfu count in the lungs, and lung post-infection pathology suggest that this repressor is important for virulence. Likewise, BALB/c immunocompetent mice infected with Δ*mce1*, Δ*mce2*, and Δ*mce3* gene knockouts of *Mtb* H37Rv all survived, whereas mice infected with the wild-type strain begin to die within five weeks, and after nine weeks were no longer living (Gioffre et al., 2005).

The Mce3 regulon consists of three operons near each other in the genome, including the *mce3* operon, whose expression is self-regulated (Table) (Santangelo et al., 2008, Santangelo et al., 2002). The Mce3 regulon is under control of the Mce3R TetR-like transcriptional repressor (*Rv1963*), as was demonstrated using transcriptional profiling of the Δ*mce3R* knockout strain (De La Paz Santangelo et al., 2009). The bioinformatically-annotated functions of these genes include general oxidoreductases, β-oxidation, and lipid transport genes (De La Paz Santangelo et al., 2009).

Interestingly, one of the three operons that is part of the *mce3* regulon is also regulated by cholesterol in *Mtb*, *Rv1933c–Rv1935c* (Table) (Nesbitt et al., 2010). These three genes encode an ACAD α subunit and an ACAD β subunit proposed to form a heteterotetrameric ACAD (Wipperman et al., 2013) and an enoyl-CoA hydratase. The substrates for these enzymes are unknown, but a recent finding might offer a clue to their putative function.

Mycobacterium bovis (*M. bovis*) strain Type 17 is the fastest spreading strain of *M. bovis* in the United Kingdom, and it costs farmers about 3 billion dollars in lost revenue annually. The only difference between this strain and other types of *M. bovis* is the absence of seven genes, the orthologs of *Mtb Rv1928c-Rv1936*, which include the cholesterol-regulated operon in the Mce3 regulon. Lipid radiolabeling studies were performed to compare the lipid profiles of *M. bovis* wild type to the Type 17. *M. bovis* Type 17 has a low flux of propionate into acetyl-CoA, which is used for the biosynthesis of mycolic acids (Wheeler et al., 2008). Instead, propionate labeling accumulates in pyruvate, which *M. bovis* naturally lacks the ability to biosynthesize, since it does not have functional *ald* or *pykA* genes. The absence of these β-oxidation genes might therefore offer *M. bovis* Type 17 a compensatory mechanism to generate an intracellular pool of pyruvate, which could offer an interesting evolutionary advantage for this organism. In the case of *Mtb*, cholesterol metabolism is known to increase the metabolic pool of propionate (Yang et al., 2009) and the *mce3*-regulon may be required to distribute the propionate. The fact that the *mce3* virulence regulon encodes a heterotetrameric ACAD that appears unique to cholesterol-metabolism pathways (Wipperman et al., 2013) strengthens the linkage between the *mce3* regulon and

cholesterol metabolism. The direct connection between cholesterol metabolism and *mce3*-operon encoded enzyme function remains to be elucidated.

SigE

SigE, a stress-induced extracytoplasmic function sigma factor, is a transcriptional activator whose 16-gene regulon includes *fadE23* and *fadE24* (Fontan et al., 2008b). SigE is regulated by SigH under oxidative stress (Raman et al., 2001) and is predicted to be involved in expression and control of genes involved in cell envelope maintenance and lipid recycling. SigE regulon expression is up-regulated post-phagocytosis, indicating that it may be involved in virulence or evasion of the host's immune system (Schnappinger et al., 2003). Deletion of *sigE* in *Mtb* resulted in an attenuated ability for *Mtb* to kill its murine host (Ando et al., 2003). Interestingly, transcriptional profiling experiments have demonstrated that *fadE23-fadE24* is down-regulated by cholesterol (Nesbitt et al., 2010) but up-regulated during THP-1 human macrophage infection (Fontan et al., 2008a). The distinctive heterotetrameric FadE23-FadE24 ACAD structure suggests that the substrate or allosteric regulator contains a steroid framework (Wipperman et al., 2013). The connection between cholesterol metabolism, the predicted structure of FadE23-FadE24, and SigE remains to be established, and is an example of the complexity of cholesterol metabolism in *Mtb*.

Envoi

Understanding the environment within which an intracellular pathogen resides throughout different stages of its lifecycle is complex, since it is by nature multifactorial and temporal. Evidence continues to accumulate that cholesterol utilization and metabolism contribute to pathogenesis and virulence of *Mtb* throughout the course of clinical infection and disease. Why *Mtb* uses this ubiquitous steroid is still a fertile area of enquiry just over a century after the first report of cholesterol metabolism by *Mycobacteria* (Söhngen, 1913).

Cholesterol is just one molecule present in the intracellular host environment, but it is an important molecule. We have presented what is known of the cholesterol metabolic pathway for which gene and enzyme functions have been elucidated. There are an additional ∼150 genes out of 230-250 genes postulated to have a role involving cholesterol metabolism which have yet to have their function experimentally identified.

The important role that cholesterol plays in the lifecycle of this pathogen is evident, and the need for new antibiotic therapies in the face of bacterial drug resistance is more urgent than ever. A more complete understanding of cholesterol utilizing enzymes coupled with small-molecule inhibitors of their function could be used to either eliminate *Mtb* outright if these inhibitors were to act as bactericidal antibiotics, or to alter *Mtb* metabolism to make it more susceptible to current anti-mycobacterial therapies. Future work in this field should continue to focus on elucidating the specific function(s) of recombinantly expressed genes that are found to be important during infection and how these functions contribute to the virulence and pathogenesis of *Mtb* in order to support strategic development of new treatments for *Mtb* infection.

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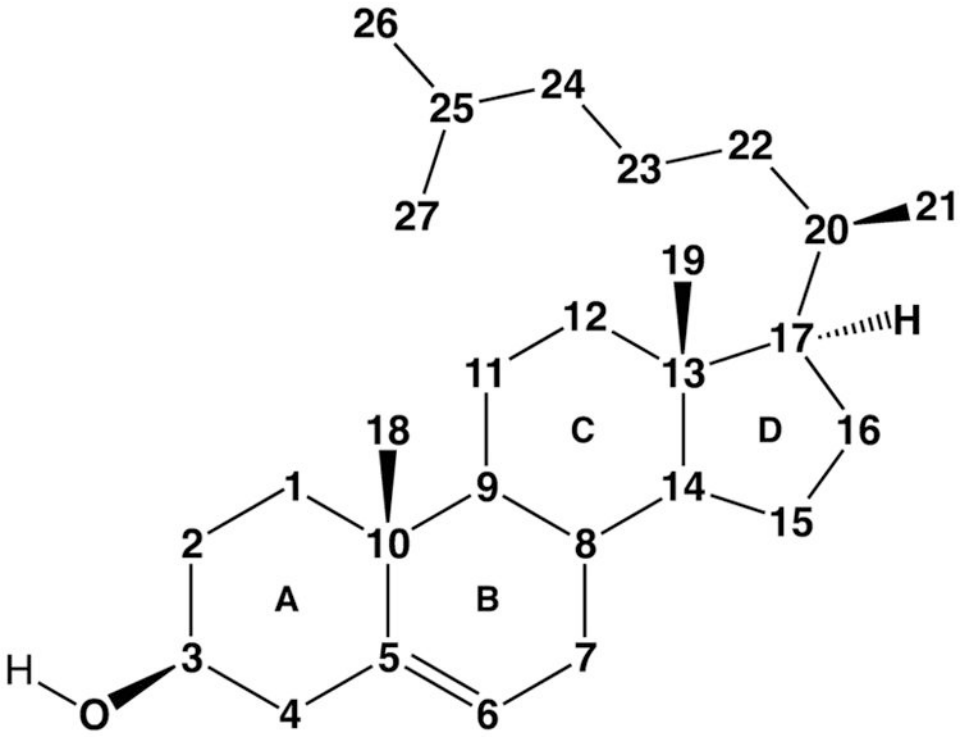

Figure 1.
Cholesterol numbering scheme.

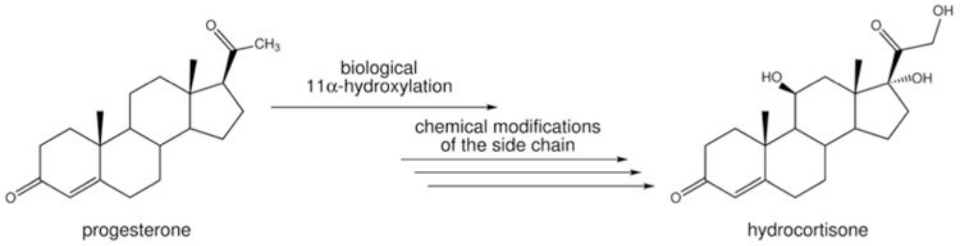

Figure 2.

The commerically developed biological/chemical conversion of progesterone to hydrocortisone, pioneered by The Upjohn Company. The biological 11α-hydroxylation of progesterone was carried out by the fungus *Rhizopus nigricans*, followed by several chemical steps to ultimately give hydrocortisone.

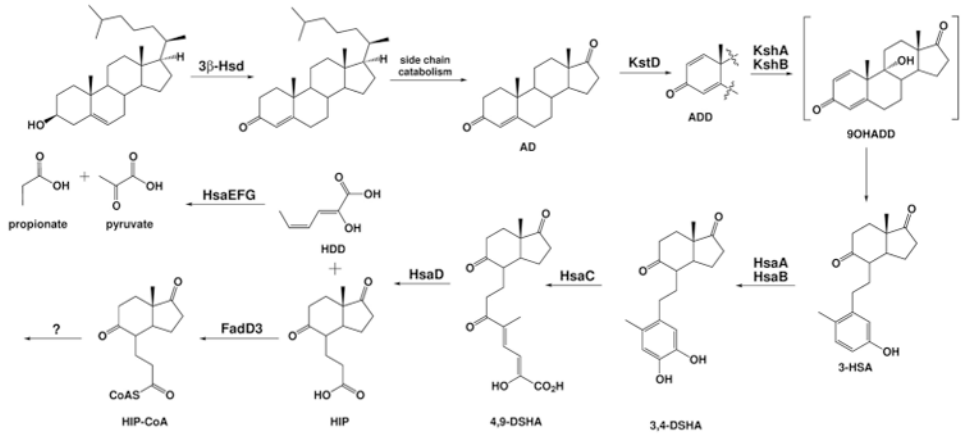

Figure 3. Steroid ring degradation in bacteria

HSD: 3β-hydroxysteroid dehydrogenase; **ChoE**: cholesterol oxidase; **KstD**: ketosteroid dehydrogenase; **KshA/B**: 3-ketosteroid 9α-hydroxylase; **HsaA/B**: 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione 4-monooxygenase; **HsaC**: 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase; **HsaD**: 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oate hydrolase; **HsaE**: 2-hydroxyhexa-2,4-dienoate hydratase; **HsaF**: 4-hydroxy-2-oxohexanoate aldolase; **HsaG**: propanal dehydrogenase. Abbreviations of compound names: **AD**: 4-androstenedione; **ADD**: 1,4-androstenedione; **9OHADD**: 9-hydroxy-androsta-1,4-diene-3,17-dione; **3-HSA**: 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; **3,4-DSHA**: 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; **4,9-DSHA**: 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid; **HIP**: 3aα-H-4α(3′-propanoyl-CoA)-7aβ-methylhexa-hydroindane-1,5-dione; **HDD**: 2-hydroxy-hexa-2,4-dienoic acid.

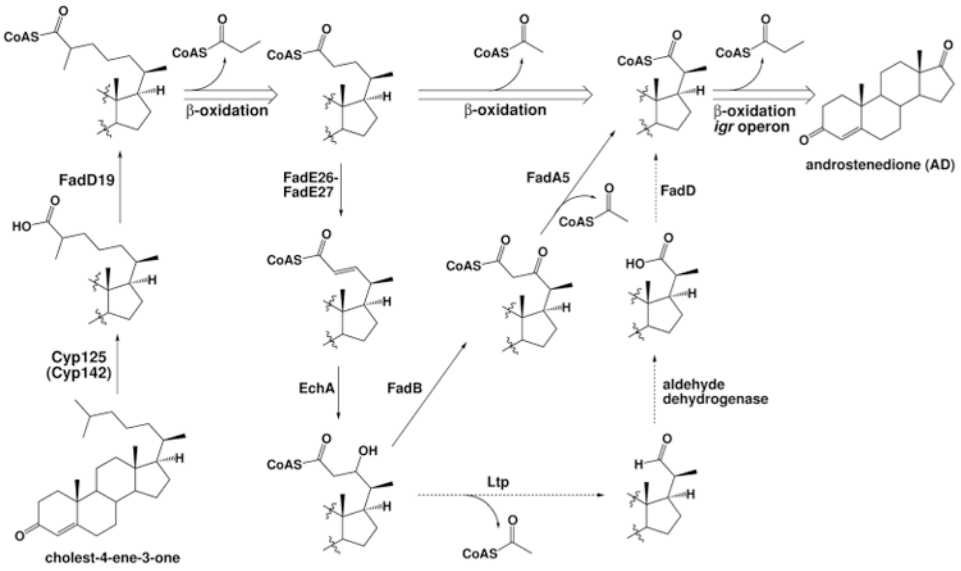

Figure 4.

Steroid side chain degradation is initiated by Cyp125, and alternatively Cyp142. A FadD acyl-CoA ligase activates the resultant steroid carboxylic acid through esterification with CoA. The steroid side chain is truncated via three cycles of β-oxidation to yield acetyl-CoA, two units of propionyl-CoA, and a 3,17-dione steroid. The individual steps in the classical β-oxidation pathway are shown for the second cycle with solid arrows. Recently proposed alternate steps via an aldehyde (Holert et al., 2013a, Holert et al., 2013b) are shown as dashed arrows. The individual steps catalyzed in the third cycle are shown in Figure 5. Steps are labeled with specific enzyme names (see Table), if known.

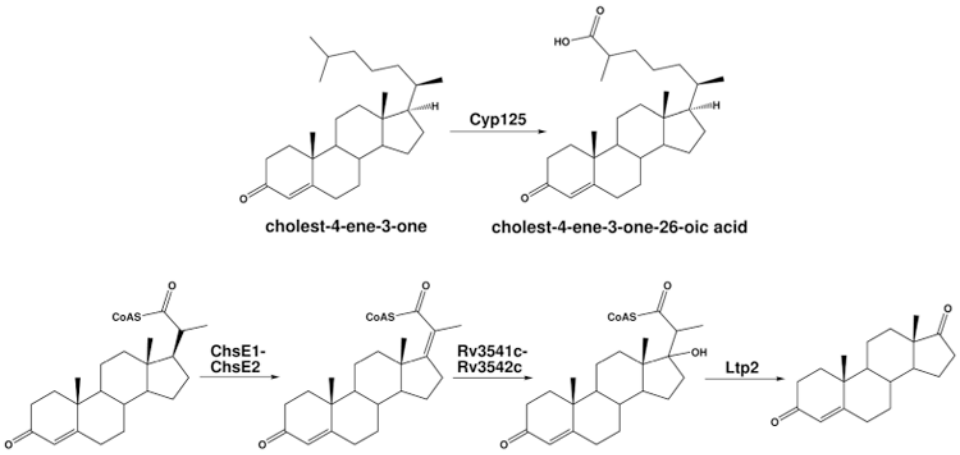

Figure 5.

The *igr* operon in *Mtb*. The β-oxidation chemistry catalyzed by the *igr*-operon encoded enzymes.

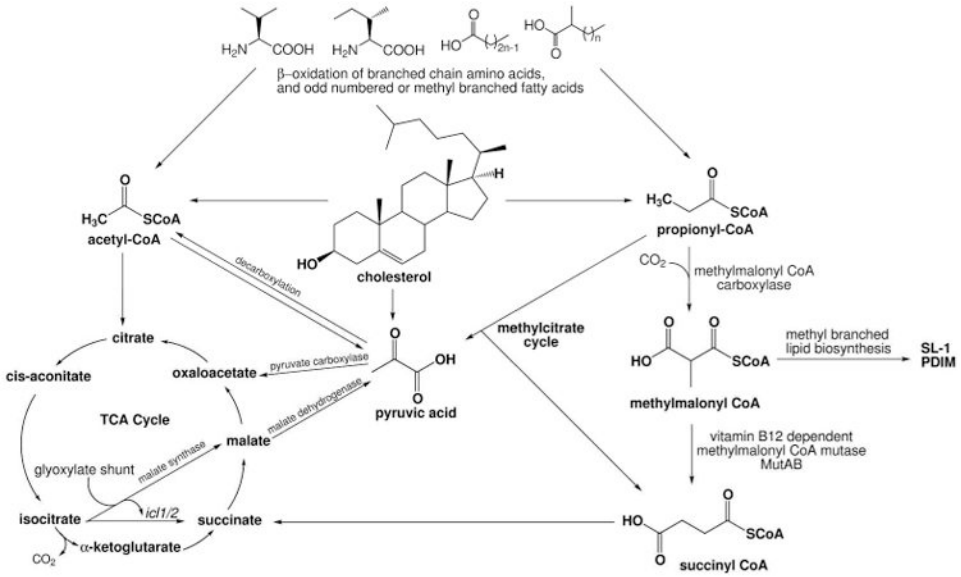

Figure 6.

Cholesterol catabolism generates acetyl-CoA, propionyl-CoA, and pyruvate, which are fed into the metabolic pathways shown. **PDIM:** phthiocerol dimycoserate; **SL-1**: sulfolipid-1.

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Table

Mtb genes associated with cholesterol metabolism or its regulons.[a]

Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (☐) Computation Annotated (?)	Gene Regulation and Essentiality				
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]
Rv0138	MSMEG_6475		conserved hypothetical protein	?					KstR1
Rv0139	MSMEG_6474		oxidoreductase	?					KstR1
Rv0162c	MSMEG_0217	*adhE1*	alcohol dehydrogenase	?					KstR1
Rv0223c	MSMEG_0309		Aldehyde dehydrogenase	?	✓				KstR1
Rv0244c	MSMEG_0406	*fadE5*	acyl-CoA dehydrogenase	?	✓	✓	Induced		
Rv0468	MSMEG_0912	*fadB2*	3-hydroxyacyl-CoA dehydrogenase	☑	✓		Induced		
Rv0551c	MSMEG_1098	*fadD8*	fatty acyl-CoA synthetase	?	✓				KstR1
Rv0687	MSMEG_1410			?	✓			Essential	KstR1
Rv0926c	MSMEG_5586		conserved hypothetical protein	?					KstR1
Rv0927c	MSMEG_5584		short-chain type dehydrogenase/reductase	?					KstR1
Rv0940c	MSMEG_5555		oxidoreductase	?	✓				KstR1
Rv0953c	MSMEG_5520		oxidoreductase	?	✓				KstR1
Rv1059	MSMEG_5286	*dapB*	dihydrodipicolinate reductase	?					KstR1
Rv1071c	MSMEG_5276	*echA9*	enoyl-CoA hydratase	?		✓	Induced		
Rv1106c	MSMEG_5228	*3β-hsd*	3β-hydroxy steroid dehydrogenase	☑					KstR1 (only in M. smeg)
Rv1132	MSMEG_5202		conserved membrane protein	?			Induced		KstR1
Rv1193	MSMEG_5114	*fadD36*	fatty acyl-CoA synthetase	?		✓			
Rv1323	MSMEG_4920	*fadA4*	thiolase	?			Essential	Essential	
Rv1426c	MSMEG_0302	*lipO*	esterase	?					KstR1
Rv1427c	MSMEG_0304	*fadD12*	fatty acyl-CoA synthetase	?					KstR1
Rv1428c	MSMEG_0305		conserved hypothetical protein	?					KstR1
Rv1627c	MSMEG_3844		non-specific lipid transfer protein	?	✓				KstR1
Rv1628c	MSMEG_3843		conserved hypothetical protein	?	✓		Induced		KstR1

Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (□) Computation Annotated (?)	Gene Regulation and Essentiality				
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]
Rv1715	MSMEG_6791	*fadB3*	3-hydroxyacyl-CoA dehydrogenase	?			Induced		
Rv1894c	MSMEG_3519		conserved hypothetical protein	?	✓		Induced		KstR1
Rv1912c	-	*fadB5*	3-hydroxyacyl-CoA dehydrogenase	?			Induced		
Rv1930c	MSMEG_1107		conserved hypothetical protein	?				Essential	Mce3R
Rv1931c	MSMEG_1106		transcriptional regulator	?				Essential	Mce3R
Rv1933c	-	*fadE18*	steroid acyl-CoA dehydrogenase β subunit	□					Mce3R
Rv1934c	-	*fadE17*	steroid acyl-CoA dehydrogenase α subunit	□	✓		Induced		Mce3R
Rv1935c	-	*echA13*	enoyl-CoA hydratase	?	✓		Induced		Mce3R
Rv1936	-		monooxygenase	?	✓		Induced		Mce3R
Rv1937	-		electron transfer oxygenase	?	✓				Mce3R
Rv1938	-	*ephB*	epoxide hydrolase	?	✓				Mce3R
Rv1939	-		FMN oxidoreductase	?	✓		Induced	Essential	Mce3R
Rv1940	-	*ribA1*	bifunctional enzyme: GTP cyclohydrolase II and	?	✓				Mce3R
Rv1941	-		short-chain dehydrogenase	?	✓				Mce3R
Rv1942c	-		conserved hypothetical protein,	?			Induced		Mce3R
Rv1943c	-		conserved hypothetical protein,	?					Mce3R
Rv1963c	-	*mce3R*	transcriptional repressor (TetR-like)	☑		✓			
Rv1964	-	*yrbE3A*	Mce-family	?	✓				Mce3R
Rv1965	MSMEG_0345	*yrbE3B*	Mce-family	?	✓				Mce3R
Rv1966	MSMEG_0346	*mce3A*	Mce-family	?	✓				Mce3R
Rv1967	MSMEG_0347	*mce3B*	Mce-family	?					Mce3R
Rv1970	MSMEG_0350	*lprM*	Mce-family lipoprotein LprM (Mce3E)	?			Induced		Mce3R
Rv1971	MSMEG_0351	*mce3F*	Mce-family	?					Mce3R
Rv1972	MSMEG_0352, MSMEG_5208		Mce-associated	?			Induced		Mce3R
Rv1973	MSMEG_0353		Mce-associated	?			Induced		Mce3R

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Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (□) Computation Annotated (?)	Gene Regulation and Essentiality				
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]
Rv1975	MSMEG_0355		protein expressed in hypoxia	?			Induced		Mce3R
Rv2668	MSMEG_2790		conserved hypothetical protein	?	✓	✓			KstR1
Rv2669	MSMEG_2789		conserved hypothetical protein	?					KstR1
Rv2799	MSMEG_2645		membrane protein	?		✓			KstR1
Rv2800	MSMEG_2644		conserved hypothetical protein	?					KstR1
Rv3139	MSMEG_2081	*fadE24*	steroid acyl-CoA dehydrogenase β subunit	□	✓		Induced		sigE
Rv3140	MSMEG_2080	*fadE23*	steroid acyl-CoA dehydrogenase α subunit	□	✓		Induced		sigE
Rv3141	MSMEG_2033	*fadB4*	3-hydroxyacyl-CoA dehydrogenase	?					
Rv3492c	MSMEG_5893		conserved hypothetical protein	?		✓			KstR1
Rv3493c	MSMEG_5894		conserved hypothetical protein	?		✓			KstR1
Rv3494c	MSMEG_5895	*mce4F*	mce4 operon: lipid transfer	☑		✓			KstR1
Rv3495c	MSMEG_5896	*mce4E*	mce4 operon: lipid transfer	☑		✓		Essential	KstR1
Rv3496c	MSMEG_5897	*mce4D*	mce4 operon: lipid transfer	☑		✓			KstR1
Rv3497c	MSMEG_5898	*mce4C*	mce4 operon: lipid transfer	☑		✓		Essential	KstR1
Rv3498c	MSMEG_5899	*mce4B*	mce4 operon: lipid transfer	☑		✓			KstR1
Rv3499c	MSMEG_5900	*mce4A*	mce4 operon: lipid transfer	☑		✓		Essential	KstR1
Rv3500c	MSMEG_5901	*supBh*	mce4 operon: lipid transfer	☑		✓			KstR1
Rv3501c	MSMEG_5902	*supAh*	mce4 operon: lipid transfer	☑		✓		Essential	KstR1
Rv3502c	MSMEG_5903	*hsd4A*	steroid β-hydroxy-acyl-CoA dehydrogenase	□	✓	✓		Essential	KstR1
Rv3503c	MSMEG_5904	*fdxD*	ferredoxin	?	✓		Induced		KstR1
Rv3504	MSMEG_5906	*fadE26*	3-keto-4-cholene-24-oyl-CoA dehydrogenase α subunit	☑	✓			Essential	KstR1
Rv3505	MSMEG_5907	*fadE27*	3-keto-4-cholene-24-oyl-CoA dehydrogenase β subunit	☑	✓		Induced		KstR1
Rv3506	MSMEG_5908	*fadD17*	3-keto-chol-4-ene-24-oyl-CoA ligase	☑					KstR1
Rv3507	-	*PE_PGRS53*	PE family of glycine rich proteins	?					
Rv3508	MSMEG_2146	*PE_PGRS54*	PE family of glycine rich proteins	?					

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Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (□) Computation Annotated (?)	Gene Regulation and Essentiality				
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]
Rv3509c	MSMEG_4348	*ilvX*	acetohydroxyacid synthase	?					
Rv3510c	-	*Rv3510c*	conserved hypothetical protein	?					
Rv3511	-	*PE_PGRS55*	PE family of glycine rich proteins	?					
Rv3512	-	*PE_PGRS56*	PE family of glycine rich proteins	?					
Rv3513c	-	*fadD18*	acyl-CoA ligase (fragment)	?					
Rv3514	-	*PE_PGRS57*	PE family of glycine rich proteins	?					
Rv3515c	MSMEG_5914	*fadD19*	3-keto-4-cholest-4-ene-26-oyl-CoA ligase	☑	✓	✓			KstR1
Rv3516	MSMEG_5915	*echA19*	enoyl-CoA hydratase	?	✓		Induced		KstR1
Rv3517	-		conserved hypothetical protein	?					
Rv3518c	MSMEG_5918	*cyp142*	cholest-4-en-3-one 26-monooxygenase	☑	✓		Induced		KstR1
Rv3519	MSMEG_5919		conserved hypothetical protein	?				Essential	KstR1
Rv3520c	MSMEG_5920		coenzyme F420-dependent oxidoreductase	□	✓				KstR1
Rv3521	MSMEG_5921		conserved hypothetical protein	?	✓		Induced		KstR1
Rv3522	MSMEG_5922	*ltp4*	3-hydroxy-acyl-CoA aldolase	?	✓				KstR1
Rv3523	MSMEG_5923	*ltp3*	3-hydroxy-acyl-CoA aldolase	□	✓			Essential	KstR1
Rv3524	-		conserved membrane protein	?			Induced		
Rv3525c	-		siderophore binding protein	?					
Rv3526	MSMEG_5925	*kshA*	3-ketosteroid 9α-hydroxylase oxygenase component	☑	✓	✓			KstR1
Rv3527	MSMEG_5927		conserved hypothetical protein	?	✓				KstR1
Rv3528c	-		hypothetical protein	?					
Rv3529c	MSMEG_5930		conserved hypothetical protein	?	✓				KstR1
Rv3530c	MSMEG_5931		oxidoreductase	?	✓				KstR1
Rv3531c	MSMEG_5932		conserved hypothetical protein	?	✓	✓			KstR1
Rv3532	-	*PPE61*	PPE family of glycine rich proteins	?					
Rv3533c	-	*PPE62*	PPE family of glycine rich proteins	?					

Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (□) Computation Annotated (?)	Gene Regulation and Essentiality				
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]
Rv3534c	MSMEG_5937	*hsaF*	4-hydroxy-2-oxohexanoate aldolase	□	✓	✓	Induced	Essential	KstR1
Rv3535c	MSMEG_5939	*hsaG*	propanal dehydrogenase	□	✓		Induced		KstR1
Rv3536c	MSMEG_5940	*hsaE*	2-hydroxyhexa-2,4-dienoate hydratase	□	✓	✓	Induced		KstR1
Rv3537	MSMEG_5941	*kstD*	3-keto-A4-steroid A1-dehydrogenase	☑	✓	✓	Induced, Essential		KstR1
Rv3538	MSMEG_5943	*hsd4B*	steroid 2-enoyl-CoA hydratase	□	✓		Induced		KstR1
Rv3539	-	*PPE63*	PE family of glycine rich proteins	?					
Rv3540c	MSMEG_5990	*ltp2*	3-oxo-17-hydroxy-pregna-4-ene-22-oyl-CoA aldolase	□	✓	✓	Induced	Essential	KstR1
Rv3541c	MSMEG_5991		3-oxo-pregna-4,17-diene-22-oyl-CoA hydratase α subunit	□	✓	✓	Induced, Essential	Essential	KstR1
Rv3542c	MSMEG_5992		3-oxo-pregna-4,17-diene-22-oyl-CoA hydratase β subunit	□	✓	✓	Induced, Essential	Essential	KstR1
Rv3543c	MSMEG_5993	*chsE1 (fadE28)*	3-oxo-pregn-4-ene-22-oyl-CoA dehydrogenase β subunit	☑	✓	✓	Essential		KstR1
Rv3544c	MSMEG_5994	*chsE2 (fadE29)*	3-oxo-pregn-4-ene-22-oyl-CoA dehydrogenase α subunit	☑	✓	✓	Induced	Essential	KstR1
Rv3545c	MSMEG_5995	*cyp125*	cholest-4-en-3-one 26-monooxygenase	☑	✓	✓	Induced	Essential	KstR1
Rv3546	MSMEG_5996	*fadA5*	steroid β-keto-acyl-CoA thiolase	☑	✓	✓	Induced		KstR1
Rv3547	MSMEG_5998		conserved hypothetical protein	?					KstR1
Rv3548c	MSMEG_5999		short-chain-type dehydrogenase/reductase	?	✓				KstR2
Rv3549c	MSMEG_6000		short-chain-type dehydrogenase/reductase	?	✓				KstR2
Rv3550	MSMEG_6001		enoyl-CoA hydratase	?	✓				KstR2
Rv3551	MSMEG_6002		CoA-transferase α subunit	?	✓	✓	Essential	Essential	KstR2
Rv3552	MSMEG_6003		CoA-transferase β subunit	?	✓		Induced, Essential		KstR2
Rv3553	MSMEG_6004		oxidoreductase	?		✓	Induced, Essential		KstR2
Rv3554	-	*fdxB*	ferridoxin B	?			Induced	Induced	
Rv3555c	MSMEG_5314		conserved hypothetical protein		✓		Induced	Induced	
Rv3556c	MSMEG_6008	*fadA6*	thiolase	?	✓		Essential	Induced, Essential	KstR2
Rv3557	MSMEG_6009	*kstR2*	transcriptional repressor (TetR-like)	☑	✓		Induced		KstR2

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Mtb H37Rv Gene Ref[b]	*M. smegmatis* ortholog gene number	Gene Name	Enzyme Function	Function Biochemically Validated (☑) Biochemically Predicted (□) Computation Annotated (?)	Gene Regulation and Essentiality					Wipperman et al.
					Mtb Transcript Up-Regulated by Chol[c]	Gene Required for Growth on Chol[d]	Transcript Up-Regulated[e,f] or Required for Growth[g] In Macroφ	Requiredfor Survival in Mice[h]	Transcript Repressor[i]	
Rv3558	-	*PPE64*	PPE family of glycine rich proteins	?						
Rv3559c	MSMEG_6011		oxidoreductase	?	✓	✓			KstR2	
Rv3560c	MSMEG_6012	*fadE30*	acyl-CoA dehydrogenase	□	✓	✓	Induced, Essential	Essential	KstR2	
Rv3561	MSMEG_6013	*fadD3*	3aα-H-4α(3'-propanoate)-7aβ- methylhexahydro-1,5-indanedione-CoA synthase	☑	✓	✓	Induced		KstR2	
Rv3562	MSMEG_6014	*fadE31*	steroid acyl-CoA dehydrogenase α subunit	□	✓		Induced		KstR2	
Rv3563	MSMEG_6015	*fadE32*	steroid acyl-CoA dehydrogenase β subunit	□	✓	✓	Essential	Essential	KstR2	
Rv3564	MSMEG_6016	*fadE33*	steroid acyl-CoA dehydrogenase β subunit	□	✓	✓	Induced		KstR2	
Rv3565	MSMEG_6017	*aspB*	aspartate aminotransferase	?	✓				KstR2	
Rv3566c	MSMEG_0306	*nat*	arylamine N-acetyltransferase	□	✓		Induced	Induced		
Rv3567c	MSMEG_6035	*hsaB*	3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione 4-hydroxylase reductase component	☑	✓				KstR1	
Rv3568c	MSMEG_6036	*hsaC*	3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase	☑	✓	✓			KstR1	
Rv3569c	MSMEG_6037	*hsaD*	4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oate hydrolase	☑	✓	✓	Essential		KstR1	
Rv3570c	MSMEG_6038	*hsaA*	3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione 4-monooxygenase component	☑	✓	✓	Essential		KstR1	
Rv3571	MSMEG_6039	*kshB*	3-ketosteroid 9α-hydroxylase reductase component	☑	✓	✓	Induced		KstR1	
Rv3572	MSMEG_6040		conserved hypothetical protein	?					KstR1	
Rv3573c	MSMEG_6041	*fadE34*	cholest-4-en-3-one-26-oyl-CoA dehydrogenase	□	✓	✓			KstR1	
Rv3574	MSMEG_6042	*kstR1*	transcriptional repressor (TetR-like)	☑	✓		Induced	Essential	KstR1	

[a] *Mtb* genes in the Cho region, KstR1, KstR2, and Mce3R regulons, and selected cholesterol-regulated genes or potential cholesterol metabolizing genes discussed in the text are included.

[b] Genes in the Cho region are shaded with a gray background: (Van Der Geize et al., 2007;Nesbitt et al, 2010).

[c] Transcriptionally up-regulated by cholesterol after 3 or 24 h: (Nesbitt et al, 2010).

[d] Required for growth in cholesterol media: (Griffin et al, 2011).

[e] Upregulated 24 h after THP-1 macrophage infection: (Fontán et al, 2007).

[f] Induced in macrophages: (Schappinger et al, 2003).

[g] Essential in macrophages using TraSH: (Rengaragan et al, 2005).

[h] Essential in mice using TraSH: (Sassetti and Rubin, 2003).

[i] Under control of the transcriptional repressors, KstR1: (Kendall et al., 2007); KstR2: (Kendall et al., 2010); Mce3R: (De La Paz Santangelo et al., 2009).