A Thiolase of *Mycobacterium tuberculosis* Is Required for Virulence and Production of Androstenedione and Androstadienedione from Cholesterol^{∇†}

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Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular pathogen that shifts to a lipid-based metabolism in the host. Moreover, metabolism of the host lipid cholesterol plays an important role in *M. tuberculosis* infection. We used transcriptional profiling to identify genes transcriptionally regulated by cholesterol and KstR (Rv3574), a TetR-like repressor. The *fadA5* (Rv3546) gene, annotated as a lipid-metabolizing thiolase, the expression of which is upregulated by cholesterol and repressed by KstR, was deleted in *M. tuberculosis* H37Rv. We demonstrated that *fadA5* is required for utilization of cholesterol as a sole carbon source in vitro and for full virulence of *M. tuberculosis* in the chronic stage of mouse lung infection. Cholesterol is not toxic to the *fadA5* mutant strain, and, therefore, toxicity does not account for its attenuation. We show that the wild-type strain, H37Rv, metabolizes cholesterol to androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) and exports these metabolites into the medium, whereas the *fadA5* mutant strain is defective for this activity. We demonstrate that FadA5 catalyzes the thiolysis of acetoacetyl-coenzyme A (CoA). This catalytic activity is consistent with a β -ketoacyl-CoA thiolase function in cholesterol β -oxidation that is required for the production of androsterones. We conclude that the attenuated phenotype of the *fadA5* mutant is a consequence of disrupted cholesterol metabolism that is essential only in the persistent stage of *M. tuberculosis* infection and may be caused by the inability to produce AD/ADD from cholesterol.

Circumstantial evidence implicates a role for cholesterol in *M. tuberculosis* infection. How this host lipid affects infection and the role of its metabolism by the bacteria is not clear. Foamy macrophages, laden with lipid droplets, are known to accumulate in lung granulomas in human infection (23) as well as in infected mouse lungs (4). Electron microscopy revealed that *M. tuberculosis* is in close apposition to lipid bodies in foamy macrophages and that the bacteria eventually merge with these lipid bodies and even accumulate intracellular lipids (22). In fact, since foamy macrophages contain high levels of cholesterol esters (19, 32), it is possible that the lipid bodies observed to merge with *M. tuberculosis* bacteria are composed of cholesterol esters. Macrophages infected with *Mycobacterium leprae* also accumulate cholesterol esters (17) that are thought to be responsible for the conversion of macrophages into foam cells. Significantly, bacteria from the sputum of human tuberculosis (TB) patients were observed to contain lipid bodies, and transcriptional profiling of bacteria from these

samples demonstrated the induction of genes proposed to encode enzymes required for cholesterol utilization (13). These studies implicate host lipids and possibly cholesterol in the development of mycobacterial disease.

The first direct evidence suggesting that bacterial cholesterol metabolism is required for *M. tuberculosis* virulence came from studies on an *mce4* mutant that is defective in cholesterol transport and attenuated for virulence in the mouse model (21). Cholesterol was shown to colocalize with bacteria in bone marrow macrophages, and metabolic labeling studies with *M. tuberculosis* demonstrated that the bacteria can convert cholesterol to both CO₂ and phthiocerol dimycoserolates (PDIM) (21). Cholesterol degradation yields propionyl-coenzyme A (CoA) (35) which, in combination with CO₂, is converted to methyl-malonyl-CoA, a metabolite used in methyl branched-lipid biosynthesis. Propionyl-CoA is also shunted to acetyl-CoA through the methylcitrate pathway. Thus, cholesterol is degraded by *M. tuberculosis*, and its metabolites contribute to flux through both catabolic and anabolic pathways.

A group of genes encoding enzymes annotated as involved in lipid metabolism was shown to be upregulated during incubation with cholesterol in the actinomycete *Rhodococcus* RHA1 and were proposed to be responsible for cholesterol catabolism in that organism (30). Some of these genes have orthologs in the *M. tuberculosis* genome, suggesting that a similar pathway functions in *M. tuberculosis* (Fig. 1), and the genes required for cholesterol A- and B-ring metabolism have been identified. The enzymatic activities of recombinant Hsd (34), KstD (16), HsaC (30), HsaD (30), and KshA/B (3) from *M. tuberculosis* are consistent with their predicted functions, as expected due

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FIG. 1. Role of Hsd, KstD, KshA/KshB and HsaA-HsaD in *M. tuberculosis* cholesterol degradation. KstD, KshA/KshB, and HsaA-HsaD most likely accept side chain metabolites as substrates. Only the 17-keto (AD) metabolite is shown for clarity.

to their high levels of similarity to other actinomycete orthologs, and their substrate specificities are consistent with cholesterol-derived metabolites being the physiological substrates. Recently *hsaC*, which encodes a cholesterol ring-cleaving dioxygenase, was shown to be required for optimal growth on cholesterol as a sole carbon source (33). Cholesterol was toxic to the mutant strain due to the accumulation of catechols, and it was somewhat attenuated in SCID mice and in guinea pigs, possibly due to cholesterol toxicity (33). In addition, the *igr* operon, which encodes putative β -oxidation enzymes, was found to be required for growth in the presence of cholesterol (6). Cholesterol was toxic to the mutant strain (6), which grew more slowly in mice from the time of initial infection (5). The precise function of *igr*-encoded enzymes has yet to be established.

As a first step to identify additional *M. tuberculosis* genes and enzymes involved in cholesterol metabolism, we describe the *M. tuberculosis* cholesterol regulon by transcriptional profiling. We show that a major portion of this regulon is under the control of the KstR transcriptional regulator, initially described in *Mycobacterium smegmatis* (15). *fadA5* (Rv3546) is one of the genes regulated by cholesterol and by KstR. We demonstrate that a *fadA5* mutant strain of *M. tuberculosis* does not grow on cholesterol as a sole carbon source in broth, thus establishing *fadA5*'s involvement in cholesterol metabolism. We show that this gene is also required for persistence in the mouse model of *M. tuberculosis* infection. We demonstrate that cholesterol is not toxic in broth culture to the *fadA5* mutant, in contrast to the *hsaC* and *igr* mutants, which showed cholesterol toxicity (6, 33). We provide the first evidence that cholesterol is converted to androst-4-ene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) by *M. tuberculosis* H37Rv and that these metabolites accumulate in the supernatant. We show that *fadA5* is required for this activity and that FadA5 is a thiolase whose activity is presumed to be required for β -oxidation of the cholesterol side chain. These results suggest that one role of cholesterol metabolism in *M. tuberculosis* is to provide metabolites, possibly the prohormones AD and ADD, that allow persistence in the lung.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *M. tuberculosis* cultures were grown at 37°C in Middlebrook 7H9 liquid medium (Becton Dickinson), supplemented with 0.05% Tween-80, 10% albumin-dextrose-NaCl complex (ADN) (1),

and 0.2% glycerol, or on Middlebrook 7H10 plates supplemented the same way. Kanamycin was added at 20 $\mu\text{g ml}^{-1}$, and hygromycin was added at 100 $\mu\text{g ml}^{-1}$. Growth on cholesterol as a sole carbon source was done by supplementing 7H9 medium with 1 mg ml^{-1} cholesterol made up in the nonionic surfactant tyloxapol (Sigma). The stock solution was 20 mg ml^{-1} cholesterol made in pure tyloxapol, autoclaved, and then boiled to dissolve the cholesterol completely. When this is diluted 20-fold into 7H9 medium, it must be boiled again to enable the formation of a uniform medium. Under these conditions, the cholesterol does not precipitate, and the growth of the culture can be followed by measuring optical density (OD) at 540 nm. When cholesterol was used as an inducer for transcriptional profiling, cultures were grown to mid-log phase in supplemented 7H9 medium, and then cholesterol (1 mg ml^{-1} final concentration) in Tween-80 (1%, wt/vol, final concentration) was added, and the cultures were incubated for an additional 24 h. Cholesterol stock solutions (20 mg ml^{-1}) were prepared in aqueous Tween-80 (20%, wt/vol). Treatment with 1% Tween-80 only was used as the control.

Cloning and construction of *M. tuberculosis* mutant strains. DNA recombinant techniques were performed following standard procedures (24). All restriction enzymes and modifying enzymes were commercially obtained from Promega Laboratories or New England Biolabs and used according to the manufacturer's recommendations. Electroporation of *M. tuberculosis* and DNA Southern blot analysis from the *M. tuberculosis* chromosome were performed as previously described (18). The *fadA5* mutation in *M. tuberculosis* was constructed using pSM270, as described previously (18), with two primers: 5'-GTGATGGAGTTGCGGGTGG-3' (forward primer) and 5'-CTTGGTGGTATGGCTGGGC-3' (reverse primer). The plasmid construct contained a deletion of the *fadA5* gene, which was replaced with a kanamycin cassette. This plasmid was used to electroporate H37Rv, with selection for kanamycin and streptomycin, to obtain the first crossover recombinants. The second crossover recombinants were selected by resistance to sucrose and sensitivity to streptomycin. Southern blot analysis (data not shown) of presumptive recombinants indicated that strains had the predicted disruptions in *fadA5* (strain ST76). Complementation of the *fadA5* mutant was accomplished by cloning the entire open reading frame with 166 bp flanking at the N terminus into the integrative plasmid pMV306 (MedImmune, Inc.) that confers hygromycin resistance with two primers: 5'-TGGGCACGGTAGTTCTCCTAACTG-3' (forward primer) and 5'-CTAGGTTAAATCCGCTCGATGATGG-3' (reverse primer). This construct was electroporated into ST76, and selection for Hyg^r transformants resulted in strain ST93.

Infection of mice with *M. tuberculosis*. All mouse protocols were approved by the University of Medicine and Dentistry of New Jersey (UMDNJ) IACUC. Six- to eight week-old female C57BL/6 mice were infected by the respiratory route, as previously described (31), using a nose-only aerosolization apparatus (In-Tox Products) (29). Cultures of the various strains of *M. tuberculosis* were grown in Middlebrook 7H9 liquid medium (described above) to the logarithmic stage and diluted to 1×10^5 CFU ml^{-1} in phosphate-buffered saline (PBS)-Tween. This dilution (10 ml) was used in the nebulizer, resulting in the initial deposit of about 100 to 300 CFU/lung. Three mice were sacrificed at each time point, and the lungs, homogenized in PBS-Tween, were plated on 7H10 solid medium with serial dilutions. After 3 weeks of incubation at 37°C, the number of colonies was enumerated.

Preparation of RNA and qRT-PCR. Preparation of RNA from *M. tuberculosis* grown in liquid cultures for microarray and quantitative reverse transcription-PCR (qRT-PCR) analysis using SYBR green was done as described previously

(31). PCR primers, synthesized by Integrated DNA Technologies, are listed in Table S1 in the supplemental material. PCR conditions were as follows: a 10-min denaturation step at 95°C followed by 30 cycles of denaturation at 95°C for 0.2 min and annealing and extension at 67°C for 0.45 min. The extent of amplification was determined by generating a standard curve for each primer containing 0, 1,000, 10,000, and 100,000 theoretical copies of H37Rv cDNA. These reactions were run in tandem with the experimental samples. All values were normalized to the amount of 16S mRNA.

DNA microarrays. Microarray analysis was carried out at the Center for Applied Genomics (CAG) at UMDNJ. cDNA was labeled with Cy5 or Cy3 and hybridized to DNA chips containing oligomers of 70 bp representing all *M. tuberculosis* open reading frames. The hybridized chips were read using an Affymetrix Integrated GeneChip Instrument System equipped with a Fluidics Station and GeneChip Scanner 3000 TG. The data were filtered by removing all spots that were below the background noise or that were flagged as "bad." Spots were considered to be below the background noise if the sum of the median intensities of the two channels was less than twice the highest average background of the chip. The chips were normalized by the print-tip Lowess method (11). Ratios were calculated for expression of genes in cultures with cholesterol treatment versus cultures without cholesterol and in the wild-type versus the *kstR* mutant; genes for which the ratios were greater than 1.5 were considered up-regulated.

Lipid extractions and LC/MS analysis. Three independent cultures of *M. tuberculosis* H37Rv, *fadA5*, and complemented *fadA5* were grown as described for transcriptional profiling and were induced with cholesterol (1 mg ml⁻¹) for 48 h. Both cell pellets and culture supernatants were autoclaved. Cell pellets were extracted by the method of Bligh and Dyer (2), concentrated to dryness, and resuspended in ethyl acetate (EtOAc). The culture supernatants were extracted with EtOAc twice. The aqueous layers were acidified to pH 5 and extracted with EtOAc twice. For all samples, the EtOAc extracts were washed with H₂O three times and concentrated, and the concentrates were analyzed by liquid chromatography-mass spectrometry-UV detection (LC/MS/UV). A Waters Acquity Ultra Performance LC system (Milford, MA), equipped with a photodiode array (PDA) detector, and a single quadrupole (SQ) detector was used for identification of metabolites. Chromatography was performed with a C₁₈ reverse-phase column (2.1-mm internal diameter by 100 mm; 1.7-μm particle size; Waters Acquity UPLC BEH) maintained at 55°C. The elution solvents were solvent A, consisting of 10% MeOH in H₂O, and solvent B, consisting of MeOH, and the flow rate was 0.5 ml min⁻¹. The eluting solvent was isocratic for 0.02 min with 100% solvent A, followed by a linear gradient to 44% solvent A in 0.3 min, a second linear gradient to 0% solvent A in 2 min, isocratic for 7 min with 0% solvent A, a third linear gradient to 100% solvent A in 0.5 min, and isocratic with 100% solvent A for 0.5 min. The mass spectrometer was operated with an atmospheric pressure chemical ionization source in positive ion mode with the source temperature set to 150°C; the desolvation temperature was set to 450°C with a corona voltage of 1.5 kV. The UV detection wavelength range was 200 to 400 nm. Radioactive samples were analyzed only by UV detection. Fractions were collected every 10 s and analyzed by liquid scintillation counting after the addition of 4 ml of Scintiverse II LSC cocktail.

FadA5 purification and assay. *M. tuberculosis fadA5* (Rv3546) was cloned into the BamHI site of the *E. coli-M. smegmatis* shuttle vector pSD31 (7) by PCR with *Pfu* Turbo (Stratagene) using primers NN78 (5'-GCC GCC GGA TCC ATG GGT TAC CCG GTC ATC-3') and NN82 (5'-GGC GGC GGC GGA TCC TTA AAT CCG CTC GAT GAT G-3') to introduce an N-terminal hexahistidine tag. The pSD31(*fadA5*) construct was electroporated into *M. smegmatis* mc²155, a single colony was selected on solid medium, and liquid cultures were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80, 50 μg ml⁻¹ hygromycin, 10 μg ml⁻¹ cycloheximide, and 200 μg ml⁻¹ ampicillin to an OD₆₀₀ of ~1.7 at 35.5°C. Expression of *fadA5* was induced by the addition of 0.2% acetamide at 35.5°C. After 24 h, cells were harvested and resuspended in 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, and 10 mM imidazole. Cells were disrupted by sonication, and cellular debris was removed by centrifuging at 15,000 rpm for 1 h. FadA5 was purified by immobilized metal affinity chromatography (IMAC) using Hisbind resin (Novagen, Madison, WI) following the manufacturer's protocol. Pure protein was exchanged into buffer lacking imidazole using an Amicon stirred cell (Millipore, Bedford, MA) with a YM-30 membrane (30,000-molecular-mass cutoff). Glycerol was added to FadA5 to a final concentration of 25%, and the enzyme was stored at -20°C. The identity of the purified protein was confirmed by tryptic digestion and matrix-assisted laser desorption ionization (MALDI) mass fingerprinting. FadA5 (~15 μg of pure protein) was denatured in 50 mM Tris-HCl-5 mM dithiothreitol (DTT), pH 8.0, containing 2 M urea. After a 1-h incubation at 37°C, 10 mM iodoacetamide was added to the reaction mixture to alkylate cysteines present on the protein.

Alkylation was allowed to proceed for 1 h at room temperature in the dark. The product was digested with trypsin (300 ng) in 20 μl of 50 mM NH₄HCO₃ for 17 h. The tryptic fragments were extracted with 50% CH₃CN/H₂O-0.1% trifluoroacetic acid (TFA; 50 μl) three times. The combined extracts were dried and redissolved in 5 μl of 0.1% TFA-H₂O. MALDI-time of flight (TOF) mass spectra were acquired in positive ion mode using a saturated solution of α-cyano-4-hydroxy cinnamic acid as the matrix. MALDI mass spectra were acquired on a Bruker Autoflex II TOF/TOF instrument. Data were analyzed using Flex-Analysis software and MS-Bridge (<http://prospector.ucsf.edu/cgi-bin/msform.cgi?form=msbridgestandard>).

Thiolase activity was measured in the degradative direction following the method of Thompson et al. (28) and Middleton (20). The acetoacetyl-CoA (AcAcCoA) contained less than 0.1% free CoA as determined by LC/MS/UV. Briefly, assay mixtures containing 100 mM Tris-HCl, pH 8.1, 25 mM MgCl₂, 0 to 50 μM CoA, 0 to 200 μM acetoacetyl-CoA, and 5 mM Tris(2-carboxyethyl)phosphine were preincubated at 30°C for 5 min. A background rate of thiolysis was measured. Reactions were initiated by the addition of FadA5 (250 nM) to the assay mixture. Loss of the acetoacetyl-CoA-Mg²⁺-enolate was followed at 303 nm for 5 min in the scanning mode using a UV2550 UV/visible light spectrophotometer (Shimadzu Scientific Instruments) and its associated software, and the initial velocity was determined from the linear portion, at approximately 2 min. An extinction coefficient of 16.9 mM⁻¹ cm⁻¹ at 303 nm was used to determine the concentration of consumed acetoacetyl-CoA-Mg²⁺-enolate (20). The background rate of thiolysis in the absence of FadA5 was subtracted from the measured enzymatic rate. Steady-state kinetic parameters were obtained as apparent (app) rate constants at a fixed concentration of the second substrate (S) from hyperbolic fits of the apparent Michaelis-Menten equation (equation 1) to the data by using Grafit:

$$v = V_m^{\text{app}}[S]/(K_m^{\text{app}} + [S]) \quad (1)$$

in which V_m^{app} is the apparent maximal velocity.

Microarray accession number. Transcriptional profiling data acquired as described above were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE13978.

RESULTS

Regulation of *M. tuberculosis* genes by cholesterol and KstR.

To determine the effect of cholesterol on the expression of *M. tuberculosis* genes, mid-log-phase cultures of H37Rv growing in standard medium were incubated with 0.1% (wt/vol) cholesterol made up in 20% Tween-80 or with only Tween-80. RNA was isolated at 3 h and 24 h after addition of cholesterol, and gene expression was analyzed using microarrays. Fifty-two of the *M. tuberculosis* genes which were upregulated are found in an 83-gene region of the chromosome that corresponds to a 223-gene region in *Rhodococcus* RHA1 (30) proposed to encode proteins that catabolize cholesterol, and we will refer to this 83-gene region as the "Cho-region" (Fig. 2). For full results, see the GEO database (GSE13978). We validated the microarray results by analyzing the expression levels of 16 genes by qRT-PCR. In all cases, the values were normalized to levels of 16S RNA. The genes chosen included 14 that were upregulated by cholesterol according to microarray analysis and 2 that were not upregulated but were of interest for functional considerations. The qRT-PCR results (see Table S2 in the supplemental material) were all consistent with the microarray results.

KstR is a transcriptional repressor of many genes annotated as involved in lipid metabolism in *M. smegmatis* (15), orthologs of which are found in the 223-gene region of *Rhodococcus* corresponding to the Cho-region. To determine the genes under the control of KstR in *M. tuberculosis*, a transposon-disrupted mutant in Rv3574, encoding *kstR*, as well as its parent strain, CDC1551, were grown to mid-log phase, and RNA was isolated 24 h later for gene expression analysis by microar-

Rv number	Gene name	Function	c3	c24	k	Rv number	Gene name	Function	c3	c24	k
Rv3492c*		CHP MCE associated protein				Rv3534c*	<i>hsaF</i>	4-Hydroxy-2-oxovalerate aldolase			
Rv3493c*		CHP MCE associated protein				Rv3535c*	<i>hsaG</i>	Acetaldehyde dehydrogenase			
Rv3494c*	<i>mce4F</i>	Mce4 operon: lipid transfer				Rv3536c*	<i>hsaE</i>	2-Hydroxypentadienoate hydratase			
Rv3495c*	<i>lprN</i>	Mce4 operon: lipid transfer				Rv3537*	<i>kstD</i>	3-Ketosteroid- Δ 1-dehydrogenase			
Rv3496c*	<i>mce4D</i>	Mce4 operon: lipid transfer				Rv3538*	<i>hsd4B</i>	2-Enoyl acyl-CoA hydratase			
Rv3497c*	<i>mce4C</i>	Mce4 operon: lipid transfer				Rv3539		PE			
Rv3498c*	<i>mce4B</i>	Mce4 operon: lipid transfer				Rv3540c*	<i>ltp2</i>	Branched-chain 3-ketoacyl-CoA thiolase			
Rv3499c*	<i>mce4A</i>	Mce4 operon: lipid transfer				Rv3541c*		CHP			
Rv3500c*	<i>YrbE4B/supB</i>	Mce4 operon: lipid transfer				Rv3542c*		CHP			
Rv3501c*	<i>YrbE4A/supA</i>	Mce4 operon: lipid transfer				Rv3543c*	<i>fadE29</i>	Acyl-CoA dehydrogenase			
Rv3502c*	<i>hsd4A</i>	3-Ketoacyl-ACP reductase				Rv3544c*	<i>fadE28</i>	Short/branched chain acyl-CoA dehydrogenase			
Rv3503c*	<i>fdxD</i>	Ferredoxin				Rv3545c*	<i>cyp125</i>	Cytochrome P450 125			
Rv3504*	<i>fadE26</i>	Acyl-CoA dehydrogenase				Rv3546*	<i>fadA5</i>	Acetyl-CoA acetyltransferase			
Rv3505*	<i>fadE27</i>	Acyl-CoA dehydrogenase				Rv3547*		CHP			
Rv3506*	<i>fadD17</i>	Fatty acid CoA synthetase				Rv3548c*		Short-chain dehydrogenase/reductase			
Rv3507		PE_PGRS				Rv3549c*		Short-chain dehydrogenase/reductase			
Rv3508		PE_PGRS				Rv3550*	<i>echA20</i>	Enoyl-CoA hydratase			
Rv3509c	<i>ilvX</i>	Acetohydroxyacid synthase				Rv3551*		ATP-dependent CoA transferase α subunit			
Rv3510c		CHP				Rv3552*		ATP-dependent CoA transferase β subunit			
Rv3511		PE_PGRS				Rv3553*		2-Nitropropane dioxygenase			
Rv3512		PE_PGRS				Rv3554	<i>fdxB</i>	Ferredoxin			
Rv3513c	<i>fadD18</i>	Probable fatty acid CoA ligase				Rv3555c		CHP			
Rv3514		PE_PGRS				Rv3556c*	<i>fadA6</i>	Acetyl-CoA acetyltransferase			
Rv3515c*	<i>fadD19</i>	Fatty acid CoA synthetase				Rv3557c*		TetR-type transcriptional regulator			
Rv3516*	<i>echA19</i>	Enoyl-CoA hydratase				Rv3558		PPE			
Rv3517	<i>whiB3</i>	Transcription factor				Rv3559c*		Short chain dehydrogenase/reductase			
Rv3518c*	<i>cyp142</i>	Cytochrome P450				Rv3560c*	<i>fadE30</i>	Acyl-CoA dehydrogenase			
Rv3519*		CHP				Rv3561*	<i>fadD3</i>	Acyl-CoA synthetase [AMP forming]			
Rv3520c*		FMN-dependent tetrahydromethanopterin reductase				Rv3562*	<i>fadE31</i>	Acyl-CoA dehydrogenase			
Rv3521*		CHP				Rv3563*	<i>fadE32</i>	Short/branched chain acyl-CoA dehydrogenase			
Rv3522*	<i>ltp4</i>	3-Ketoacyl-CoA thiolase				Rv3564*	<i>fadE33</i>	Short/branched chain acyl-CoA dehydrogenase			
Rv3523*	<i>ltp3</i>	SCPx related 3-ketoacyl-CoA thiolase				Rv3565	<i>aspB</i>	Possible aspartate aminotransferase			
Rv3524		Probable conserved membrane protein				Rv3566c	<i>nhoA</i>	Arylamine N-acetyltransferase			
Rv3525c		Possible siderophore binding protein				Rv3567c*	<i>hsaB</i>	3-HSA hydroxylase, reductase			
Rv3526*	<i>kshA</i>	Ketosteroid-9 α -hydroxylase, oxygenase				Rv3568c*	<i>hsaC</i>	2,3-Dihydroxybiphenyl 1,2-dioxygenase			
Rv3527*		CHP				Rv3569c*	<i>hsaD</i>	4,9-DSHA-hydrolase			
Rv3528c		HP				Rv3570c*	<i>hsaA</i>	3-HSA-hydroxylase, oxygenase			
Rv3529c		CHP				Rv3571*	<i>kshB</i>	Ketosteroid-9 α -hydroxylase, reductase			
Rv3530c		Oxidoreductase				Rv3572		CHP			
Rv3531c		CHP				Rv3573c*	<i>fadE34</i>	Acyl-CoA dehydrogenase			
Rv3532		PPE				Rv3574*	<i>kstR</i>	TetR regulator			
Rv3533c		PPE									

FIG. 2. The Cho-region of the *M. tuberculosis* genome. The region of the *M. tuberculosis* genome (Rv3492c to Rv3574) identified by microarray analysis that includes a large cluster of genes regulated by cholesterol and KstR. In the shaded columns, black shading indicates at least 1.5-fold induction, and light gray shading indicates no change in induction: c3, induced by cholesterol in *M. tuberculosis* after 3 h; c24, induced by cholesterol in *M. tuberculosis* after 24 h; k, induced by mutation of *kstR* in *M. tuberculosis*. White indicates missing data. CoA, coenzyme A; CHP, conserved hypothetical protein; HP, hypothetical protein. *, genes that have orthologs in the *Rhodococcus* RHA1 Cho-region.

FIG. 3. *fadA5* is required for growth on cholesterol as the sole carbon source. The strains were grown in 7H9 medium containing 1 mg ml⁻¹ (2.6 mM) cholesterol in tyloxapol or 7H9 medium containing only tyloxapol at 37°C. The wild-type H37Rv (Rv), *fadA5* mutant, and complemented *fadA5* (*fadA5comp*) strains were grown with and without cholesterol as indicated on the graph. Data represent results of each experiment run in duplicate.

ray. We obtained this transposon mutant strain from the Tuberculosis Animal Research and Gene Evaluation Taskforce (TARGET; NIH/NIAID contract NO1-AI30036). Many of the genes in the Cho-region were upregulated in the mutant strain, as predicted (15) (Fig. 2). For full results, see the GEO database (GSE13978). We did not perform qRT-PCR on the RNA used to analyze the KstR regulon since the expression of 16S RNA, used for normalization, was affected in the *kstR* mutant (data not shown).

Growth on cholesterol as a sole carbon source. We reasoned that some of the β -oxidation genes induced by cholesterol might encode enzymes required for cholesterol catabolism. *fadA5* is adjacent to, but transcribed in the reverse direction of, the *igr* operon (Rv3545c-Rv3540c). FadA5 has been proposed to function in β -oxidation of the propionyl side chain of metabolite 5 shown in Fig. 1 (30). We prepared a strain of H37Rv carrying a mutation in *fadA5* (Rv3546), a putative thiolase and one of the genes upregulated by cholesterol and repressed by KstR, and tested its ability to grow on cholesterol as a sole carbon source. In these experiments, we dissolved cholesterol in pure tyloxapol. Unlike Tween-80, tyloxapol cannot be catabolized by *M. tuberculosis*, and no growth was observed in control 7H9-tyloxapol medium (Fig. 3). Inclusion of tyloxapol prevented bacterial clumping and enabled us to monitor cell growth by light scattering at 540 nm. Wild-type H37Rv grew on cholesterol as a sole carbon source, but the *fadA5* mutant was unable to grow (Fig. 3). The ability to grow on cholesterol was restored in the complemented strain. The *fadA5* mutant grew normally in standard 7H9 medium supplemented with glycerol and ADC (10% albumin-dextrose-catalase).

Attenuation in the mouse model of infection. Wild-type H37Rv and the *fadA5* mutant as well as the complemented strain were used to infect mice by the aerosol route. The *fadA5* mutant initially grew as rapidly as the wild-type strain, and then at about 8 weeks, the number of CFU in the mouse lungs decreased about 10-fold compared with the level in the wild-type strain (Fig. 4). The virulence of the *fadA5* mutant was

FIG. 4. *fadA5* is required for virulence in the mouse model of infection. Mice were infected with 100 to 300 CFU/lung of the respective strain of *M. tuberculosis* wild-type H37Rv, *fadA5* mutant, or *fadA5* complement, as indicated. Data represent the results of three mice at each time point. This profile is representative of three independent time courses performed.

restored when the strain was complemented with the wild-type gene integrated at the *att* site. The fact that the *fadA5* mutant strain grew normally for the first 4 to 5 weeks in mouse lungs strongly implies that cholesterol utilization as a carbon source is not required, at least during the stage of infection prior to the onset of the immune response.

Cholesterol toxicity assays. We considered the possibility that attenuation of the *fadA5* mutant strain was due to cholesterol toxicity. We grew the wild-type and mutant strains in complete medium in the presence and absence of cholesterol, using the same medium as used for the transcriptional profiling assays. Strains were grown to the exponential phase and diluted into medium with and without cholesterol (2.6 mM in 20% Tween), and growth curves were determined using assays for CFU. The concentration of cholesterol used is typical of concentrations employed for induction of actinomycete cultures and higher than used in studies of *hsaC* (0.5 mM) (33) and *igr* (0.1 mM) (6) toxicity. The results show that cholesterol does not inhibit the growth of H37Rv and its *fadA5* mutant (Fig. 5), and, in fact, the strains grew slightly better in the presence of cholesterol. We conclude that cholesterol, or the accumulation of a cholesterol-derived metabolite, is not toxic for *fadA5*.

FadA5 is a thiolase. The FadA5 sequence is predicted with 100% probability to fold into a thiolase structure (27). In addition, the two sequence motifs (PF00108 and PF02803 [12]) that are signature motifs for thiolases are conserved in FadA5 (14). These motifs contain the active-site cysteine nucleophile and a general acid and a general base, histidine and cysteine, that catalyze the thiolase reaction.

FadA5 was heterologously expressed as an N-terminally His₆-tagged protein in *M. smegmatis* and purified to homogeneity using immobilized-metal ion affinity chromatography

FIG. 5. Mutation of *fadA5* does not cause cholesterol toxicity. Strains were grown to exponential phase in 7H9 medium supplemented with glycerol and dextrose. Cultures were diluted into 7H9 medium containing glycerol and dextrose with or without cholesterol (1 mg ml⁻¹ in 20% Tween). Values are for the wild-type H37Rv strain without cholesterol, wild-type H37Rv with cholesterol, the *fadA5* mutant without cholesterol, and *fadA5* mutant with cholesterol, as indicated. Data represent the results of the experiment done in triplicate.

(Fig. 6). The protein identity was confirmed by MALDI-TOF mass analysis of a peptide mixture generated by trypsin digestion. Eighty-six percent of the protein sequence was covered, confirming that the protein was expressed correctly in frame. The purified enzyme was assayed in the degradative direction with acetoacetyl-CoA (AcAcCoA) and coenzyme A as the substrates to yield acetyl-CoA at pH 8.1. The apparent rate constants for AcAcCoA were measured at a fixed concentration of CoA (50 μ M). The apparent rate constants for CoA were measured at a fixed concentration of AcAcCoA (150 μ M). Fully saturating concentrations were not used due to substrate inhibition, as has been previously observed with many thiolases. The steady-state kinetic parameters were the following: K_M^{app} (CoA), 15 ± 1 μ M; $k_{\text{cat}}^{\text{app}}$ (CoA), 0.018 ± 0.001 s⁻¹; K_M^{app} (AcAcCoA), 464 ± 207 μ M; and $k_{\text{cat}}^{\text{app}}$

FIG. 6. SDS-PAGE analysis of purified FadA5. Lane 1, Ni²⁺-IMAC-purified FadA5; lane 2, molecular mass markers. Numbers represent the mass in kDa.

FIG. 7. Two-substrate steady-state kinetics of FadA5. Initial velocities were measured over a range of CoA and AcAcCoA concentrations in a mixture of 100 mM Tris-HCl, pH 8.1, 25 mM MgCl₂, and 5 mM Tris(2-carboxyethyl)phosphine at 30°C. The data were fit to equation 1. The data shown are the average of two independent experiments, and the errors are the standard deviation of the measurements. (A) Hyperbolic plot of initial velocity versus [CoA] at a fixed [AcAcCoA] of 150 μ M. (B) Hyperbolic plot of initial velocity versus [AcAcCoA] at a fixed [CoA] of 50 μ M.

(AcAcCoA), 0.076 ± 0.002 s⁻¹ (Fig. 7). Thus, FadA5 is a thiolase. However, the activity with these substrates is slow relative to known thiolases (28), and, thus, the physiological substrate is most likely not acetoacetyl CoA.

***M. tuberculosis* H37Rv metabolizes cholesterol to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione and the *fadA5* mutant is deficient in this activity.** We grew cultures of H37Rv, the *fadA5* mutant, and the complemented *fadA5* mutant in complete medium to the logarithmic stage of growth. Cholesterol was added to the cultures, and an aliquot was removed from each to which [4-¹⁴C]cholesterol was added. Samples were withdrawn from the radioactive cultures, and at 48 h, more than 90% of the starting cholesterol was consumed in both wild-type and mutant cultures. The cultures with no [4-¹⁴C]cholesterol were collected 48 h after the addition of cholesterol, lipids of both cell pellets and culture supernatants were extracted, and the extracts were analyzed by LC with MS and UV detection. The extracts of the cell pellets of both wild-type and mutant contained the same metabolites. In contrast, the wild-type strain secreted two metabolites into the culture supernatants that were absent in the *fadA5* mutant cultures (Fig. 8; see also Fig. S1 in the supplemental material). The two metabolites retained the ¹⁴C label from cholesterol and accounted for 2% of the ¹⁴C label in the cultures. They had maximum wavelengths (λ_{max} s) of 245 and 243 nm and had MH⁺ parent ions at 285.2 and 287.2, respectively (see Fig. S2

FIG. 8. *fadA5* is required for the production of AD and ADD from cholesterol by *M. tuberculosis*. Data are from liquid chromatography with detection at 240 nm for analysis of H37Rv, *fadA5*, and complemented *fadA5* culture supernatants that were prepared as described in Materials and Methods. The chromatographic profile from 1.3 to 2.0 min is shown. The full profile and mass spectral analysis are shown in Fig. S1 and S2 in the supplemental material. These profiles are representative of three independent biological replicates. AU, arbitrary units.

in the supplemental material). These spectroscopic data as well as the retention times were consistent with assignment to predicted metabolites ADD and AD (Fig. 1 and 9). Coinjection of the samples with authentic AD confirmed the identity of the second metabolite. Complementation of the *fadA5* mutant strain restored production of ADD and AD (Fig. 8). Several metabolites unique to *fadA5* were observed in the culture supernatants. These metabolites had lost the $4\text{-}^{14}\text{C}$ label, suggesting that the A ring of cholesterol is still degraded in *fadA5*. However, the structures of these metabolites were not further elucidated due to the small quantities isolated. This observation is consistent with metabolism of cholesterol in other actinomycetes for which it has been found that side chain and ring degradation occur concomitantly (8).

DISCUSSION

Cholesterol is clearly important in many aspects of *M. tuberculosis* infection (13, 17, 19, 21, 32). However, the role of *M. tuberculosis* cholesterol metabolism is not yet clear. Cholesterol may serve as a nutritional source or as a starting material for the production of secondary metabolites that regulate the host-bacteria relationship. Elucidation of the essential *in vivo* function(s) of cholesterol metabolism genes has been difficult

to deconvolute because of accumulation of toxic metabolites in cholesterol metabolism mutants studied to date (6, 33). In this work, deletion of the *fadA5* gene blocked cholesterol metabolism without toxicity effects and allowed us to delineate when cholesterol metabolism is essential to bacterial survival in the mammalian host.

We present evidence that an important role of cholesterol metabolism by *M. tuberculosis* is to provide steroid metabolites during the chronic phase of infection. Moreover, metabolism of cholesterol as a primary carbon source is not required during the growth phase of infection. This conclusion is based on the observation that the *fadA5* mutant grows normally in mouse lungs at first, but the number of bacteria in the lungs declines after induction of the cellular immune response, demonstrating a classical “persistence” phenotype. If cholesterol were the sole carbon source available in the mouse lung, the *fadA5* mutant, which cannot utilize cholesterol as a sole carbon source, should not be able to grow. The proposed role for *fadA5* in the persistence phenotype is consistent with the observation of Pandey and Sasseti (21) that disruption of *mce4*, encoding a cholesterol transporter, has an effect on the chronic stage of mouse infection and not the initial stages. The fact that *fadA5*, which is upregulated by incubation with cholesterol, is also upregulated during growth of *M. tuberculosis* in both human macrophages (10) and in mouse lungs (9) is consistent with the idea that the bacteria are in contact with cholesterol during *in vivo* growth.

Furthermore, we show that *M. tuberculosis* H37Rv metabolizes cholesterol to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, which are found in the culture supernatant, and that the attenuated *fadA5* mutant lacks this activity, which is restored, together with virulence, in the complemented strain (Fig. 4). Growth of the *fadA5* mutant is not inhibited by incubation with cholesterol (Fig. 5), and therefore toxicity does not account for its attenuation as has been proposed for *hsaC* (33) and the *igr* operon (6). We conclude that the function of *FadA5* is required for the production of androsterone metabolites. These metabolites may play a role in the host immune response that results in persistence of *M. tuberculosis* infection. Alternatively, the attenuated phenotype of the *fadA5* mutant may be a consequence of disrupted cholesterol catabolism that is only essential in the persistent stage of *M. tuberculosis* infection.

Our data confirm that *fadA5* encodes a β -ketoacyl-CoA thiolase as annotated. Based on the identities of isolated metabo-

FIG. 9. Proposed catalytic role or roles for *FadA5* in the pathway for metabolism of the cholesterol side chain.

lites in other actinomycetes (25, 26), two β -oxidation cycles have been proposed in the pathway for cholesterol side chain degradation that ultimately yields the 17-keto androsterooids, e.g., AD and ADD. However, the specific gene products responsible for catalyzing these reactions have not been identified. In *M. tuberculosis*, we propose FadA5 as a candidate enzyme for thiolytic cleavage of the β -ketoacyl intermediate(s) in cholesterol side chain metabolism (Fig. 9). Thiolytic cleavage of these intermediates is necessary for the production of the androsterooids. This function is more consistent with the data than a catalytic role in β -oxidation of the propionyl side chain of metabolite 5. Disruption of enzymes required for the metabolism of intermediate 5 would be expected to result in an increased accumulation of AD and ADD because the intermediate is downstream of AD and ADD in the pathway. Confirmation of the precise step or steps catalyzed by FadA5 requires further mechanistic investigation of the enzyme and the cholesterol metabolism pathway, which we are pursuing.

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