Actinobacterial Acyl Coenzyme A Synthetases Involved in Steroid Side-Chain Catabolism

Israël Casabon,^a Kendra Swain,^a Adam M. Crowe,^b Lindsay D. Eltis,^{a,b} William W. Mohn^a

Departments of Microbiology and Immunology^a and Biochemistry and Molecular Biology,^b Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Bacterial steroid catabolism is an important component of the global carbon cycle and has applications in drug synthesis. Pathways for this catabolism involve multiple acyl coenzyme A (CoA) synthetases, which activate alkanoate substituents for β -oxidation. The functions of these synthetases are poorly understood. We enzymatically characterized four distinct acyl-CoA synthetases from the cholate catabolic pathway of *Rhodococcus jostii* RHA1 and the cholesterol catabolic pathway of *Mycobacterium tuberculosis*. Phylogenetic analysis of 70 acyl-CoA synthetases predicted to be involved in steroid metabolism revealed that the characterized synthetases each represent an orthologous class with a distinct function in steroid side-chain degradation. The synthetases were specific for the length of alkanoate substituent. FadD19 from *M. tuberculosis* H37Rv (FadD19_{Mtb}) transformed 3-oxo-4-cholesten-26-oate ($k_{\text{cat}}/K_m = 0.33 \times 10^5 \pm 0.03 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and represents orthologs that activate the C₈ side chain of cholesterol. Both CasG_{RHA1} and FadD17_{Mtb} are steroid-24-oyl-CoA synthetases. CasG and its orthologs activate the C₅ side chain of cholate, while FadD17 and its orthologs appear to activate the C₅ side chain of one or more cholesterol metabolites. CasI_{RHA1} is a steroid-22-oyl-CoA synthetase, representing orthologs that activate metabolites with a C₃ side chain, which accumulate during cholate catabolism. CasI had similar apparent specificities for substrates with intact or extensively degraded steroid nuclei, exemplified by 3-oxo-23,24-bisnorcholesterol-4-en-22-oate and 1 β -(2'-propanoate)-3 α -H-4 α -(3"-propanoate)-7 α -methylhexahydro-5-indanone ($k_{\text{cat}}/K_m = 2.4 \times 10^5 \pm 0.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $3.2 \times 10^5 \pm 0.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively). Acyl-CoA synthetase classes involved in cholate catabolism were found in both *Actinobacteria* and *Proteobacteria*. Overall, this study provides insight into the physiological roles of acyl-CoA synthetases in steroid catabolism and a phylogenetic classification enabling prediction of specific functions of related enzymes.

Cholesterol and bile salts are the most abundant steroids produced in animals. Cholesterol is an essential constituent of animal cells, as it modulates membrane fluidity (1). Although bacteria usually do not synthesize cholesterol, many actinobacterial species can efficiently degrade cholesterol (2). Recently, cholesterol catabolism has been shown to be critical for the pathogenesis of *Mycobacterium tuberculosis* (3–5). Bile salts are surface-active steroids that aid in the metabolism of dietary fats in vertebrates. Synthesized in the liver from cholesterol, bile salts, including cholate and chenodeoxycholate (6), are eventually excreted in feces and urine. Humans excrete up to 600 mg of bile salts per person per day (7). Thus, biodegradation of steroids is an important facet of global carbon cycling (8). Additionally, the processes involved in microbial transformation of steroids are potent biotechnological tools for generating steroid-based drugs and intermediates (2).

To date, the majority of work on microbial cholate degradation has focused on the *Proteobacteria*, including *Comamonas testasteroni* TA441 (9) and *Pseudomonas* sp. strain Chol1 (10–12). Conversely, cholesterol catabolism has been extensively studied in *Actinobacteria*, especially *M. tuberculosis* and *Rhodococcus* species (13–17). During microbial degradation of steroids, the side chains are degraded via a process similar to the β -oxidation of fatty acids. Catabolism of the cholesterol alkyl side chain initially requires oxidation by a cytochrome P450 to form cholestenoate, with a carboxylate group at position C-26 (Fig. 1) (18–21). Cholestenoate and cholate side-chain degradation then proceeds via activation of the free terminal carboxylate by an AMP-forming acyl coenzyme A (CoA) synthetase (22). Acyl-CoA synthetases belong to a large family of acyl-adenylate/thioester-forming enzymes (23). These enzymes use ATP and coenzyme A (CoASH), in a

two-step mechanism with an acyl-adenylate intermediate, to CoA thioesterify substrates containing a free carboxylate (Fig. 2) (22). The resulting CoA thioesters can then be degraded via β -oxidation.

Holert et al. recently showed evidence for degradation of the cholate side chain in *Proteobacteria* via a variation of β -oxidation, in which an acetyl-CoA moiety is removed by aldolytic, rather than thiolytic, cleavage (12, 24). The resulting aldehyde is proposed to be oxidized to an acid and reactivated by CoA thioesterification for another round of β -oxidation. It is unclear whether this variation of β -oxidation occurs in *Actinobacteria*.

Recent studies of *Rhodococcus jostii* RHA1 have begun to shed light on cholate degradation in *Actinobacteria*. In RHA1, separate gene clusters encode cholate catabolism (25) and cholesterol catabolism (26) (see Fig. S1 in the supplemental material). The cholate catabolic pathway includes two membrane transporters required for the uptake of cholate metabolites as well as homologs of many cholesterol catabolic enzymes. During growth of RHA1 on cholate, two metabolites retaining an isopropanoate side chain

Received 27 August 2013 Accepted 8 November 2013

Published ahead of print 15 November 2013

Address correspondence to Lindsay D. Eltis, eltis@mail.ubc.ca, or William W. Mohn, wmohn@mail.ubc.ca.

I.C. and K.S. contributed equally.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.01012-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.01012-13

FIG 1 Chemical structure of cholesterol. Rings are labeled A to D, and atoms are numbered 1 to 27, according to IUPAC-recommended nomenclature.

at position C-17 were structurally characterized and shown to be excreted and then reassimilated at different growth stages (27). Upon reassimilation, the acidic side chain of these metabolites presumably requires activation by an acyl-CoA synthetase. In the cholate cluster, two genes encoding acyl-CoA synthetases were recently described: *casG* and *casI*. Purified CasG was demonstrated to be a cholyl-CoA synthetase capable of forming CoA thioesters with steroids harboring a C₅ side chain, while CasI transformed isopropanoate-bearing steroid metabolites (25). These enzymes were not further characterized.

Along with *casG* and *casI* from the cholate cluster, RHA1 harbors three genes encoding acyl-CoA synthetases in the cholesterol gene cluster: *fadD3* (*ro04595*), *fadD19* (*ro04689*), and *fadD17* (*ro04691*) (see Fig. S1 in the supplemental material) (26). Although there is no report about FadD17 or FadD19 of RHA1, their orthologs from *M. tuberculosis* have been shown to catalyze the CoA thioesterification of long-chain fatty acids (LCFAs) (28), while FadD19 from *Rhodococcus rhodochrous* DSM 43269 is involved in degradation of C₂₄ branched-chain sterols (29). Additionally, FadD3 was recently identified as being essential for the catabolism of cholesterol and cholate rings C and D in RHA1 (14), and disruption of *orf18*, encoding a homolog of FadD3 in *C. testosteronei* TA441, abrogates degradation of bile acid rings C and D (30). Finally, *fadD19* and *fadD3* were among those genes predicted by transposon mapping to be essential for growth of *M. tuberculosis* on cholesterol (31).

In the present study, we used bioinformatic and biochemical approaches to characterize four divergent acyl-CoA synthetases involved in steroid side-chain degradation in RHA1 and *M. tuberculosis*. A phylogeny was established for these and related enzymes. A range of predicted substrates, differing in side-chain length and steroid nucleus configuration, were tested with the enzymes, and three of the enzymes were characterized by using steady-state kinetic analysis.

MATERIALS AND METHODS

Chemicals and reagents. ATP, phosphoenolpyruvate (PEP), CoASH, NADH, cholic acid, deoxycholic acid, chenodeoxycholic acid, 2-hydroxypropyl-β-cyclodextrin, L-lactic dehydrogenase, adenylate kinase, pyruvate kinase, and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO). Sodium pyruvate and sodium benzoate were purchased from MG Scientific (Pleasant Prairie, WI) and Fisher Scientific (Ottawa, ON, Canada), respectively. 3-Oxo-23,24-bisnorchol-4-en-22-oic acid (4-BNC) and 3-oxo-4-cholesten-26-oic acid were purchased from Steraloids, Inc.

FIG 2 Reactions catalyzed by acyl-CoA synthetases.

(Newport, RI). 3β-Hydroxy-5-cholesten-26-oic acid was kindly provided by Robert van der Geize (University of Groningen, The Netherlands). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (Ipswich, MA). The Expand High Fidelity PCR system and *Taq* polymerase were purchased from Roche (Laval, QC, Canada) and Qiagen (Valencia, CA), respectively. Oligonucleotides were purchased from Integrated DNA Technologies (San Diego, CA) through the Nucleic Acid Protein Service Unit (NAPS) at The University of British Columbia (UBC). Poly-His-tagged tobacco etch virus proteinase (TEV^{Pro}) was obtained as described previously (32). All other reagents were of high-performance liquid chromatography (HPLC) or analytical grade. Water for buffers was purified by using a Barnstead Nanopure Diamond system (Dubuque, IA) to a resistivity of at least 18 MΩ.

Culturing procedures. Strains and plasmids used in this study are described in Table S1 in the supplemental material. RHA1 (33) derivative strains were cultivated as previously described (14, 27). *Escherichia coli* DH5α was used for cloning purposes, and *E. coli* BL21(DE3) (34), Rosetta(DE3)/pLysS, and Rosetta 2(DE3)/pLysS (EMD Millipore) were used for heterologous expression. *E. coli* strains were grown at 37°C (200 rpm) in Luria-Bertani (LB) broth. Bacto agar (1.5%, wt/vol; Difco) was used for solid medium. Kanamycin (25 μg ml⁻¹), chloramphenicol (34 μg ml⁻¹), or carbenicillin (50 μg ml⁻¹) was added to LB medium when appropriate.

Cloning of CasI, FadD17, and FadD19. DNA was propagated, digested, ligated, and transformed by using standard protocols (35). Plasmid DNA was purified and transformed into *E. coli* by electroporation using 0.1-cm GenePulser cuvettes with a MicroPulser from Bio-Rad (Hercules, CA). The *casI* gene was amplified from plasmid pETCasI (25) by using forward primer 5'-GCTGAACCATATGCATCACCATCACCATC ACGAGAACCCTGTACTTCCAGTCGGAGTTGGAACGCACACGATC GC-3' and reverse primer 5'-GTCAAGCTTTCACACGTGCAGGAATC GTTGCC-3'. NdeI (forward primer) and HindIII (reverse primer) restriction sites were introduced (underlined) and used to clone *casI* into pET-41b(+) (EMD Millipore) to yield pCSI822. CasI produced from this construct carries an N-terminal hexahistidine tag that can be proteolytically removed by using TEV^{Pro}. The proteolytically treated CasI is identical in amino acid sequence to the wild-type protein except that the N-terminal methionine is replaced by a serine. The *fadD17* and *fadD19* genes were amplified from *M. tuberculosis* H37Rv genomic DNA by using forward primers 5'-CTGTACCATGGGCCATCACCATCACCATCACATC GAAGGTAGGACTCCCACTACCCGACCGTCAC-3' and 5'-CTGTA CCATGGGCCATCACCATCACCATCACATCGAAGGTAGGGCCGTG GCCCTGAATATTGCC-3' and reverse primers 5'-GTCAAGCTTCTAC CGGCGAATCGGCCACAC-3' and 5'-GTCAAGCTTTCACCCACCGC TCGTCACGTG-3', respectively. NcoI (forward primer) and HindIII (reverse primer) restriction sites were introduced (underlined) and used to clone *fadD17* and *fadD19* into pET-28a(+) (EMD Millipore) to yield pETFD17 and pETFD19, respectively. Both enzymes produced from these constructs carry an N-terminal hexahistidine tag followed by a factor Xa endoprotease cleavage site. The nucleotide sequences of these constructs were confirmed.

Protein production and purification. For expression of CasI, *E. coli* BL21(DE3) was freshly transformed with pCSI822 and grown at 37°C (200 rpm) in LB broth supplemented with 25 μg ml⁻¹ kanamycin. When the culture reached mid-log phase (optical density at 600 nm [OD₆₀₀] of 0.6), 0.5 mM isopropyl-β-D-1-thiogalactopyranoside was added to induce *casI* expression. Cells were transferred to 16°C, incubated for an additional 16 h, harvested by centrifugation at 4°C (4,000 × g for 10 min), and frozen until further use. The cell pellet from 4 liters of culture was sus-

pendent in 20 ml of lysis buffer consisting of 9 mM sodium phosphate (pH 8.0), 10% glycerol, 50 $\mu\text{g ml}^{-1}$ DNase I, and the Complete Mini Protease Inhibitor cocktail (Roche, Laval, QC, Canada). Cells were subjected to five rounds of 30 s of bead beating by using an MP Biomedicals FastPrep-24 bead beater (Solon, OH) set to 4.5, with 5 min of incubation on ice between rounds. Cell debris was removed by centrifugation at 4°C (17,000 $\times g$ for 20 min), and the supernatant was further clarified by ultracentrifugation at 4°C (255,000 $\times g$ for 1 h). The cytoplasmic extract was recovered, supplemented with 10 mM imidazole, and loaded onto 2.5 ml Ni-Sepharose 6 Fast Flow resin (GE Healthcare). The tagged protein was eluted with ice-cold 20 mM sodium phosphate (pH 8.0)–50 mM NaCl–500 mM imidazole, dialyzed overnight at 4°C against 2 liters of 25 mM HEPES (pH 7.5)–50 mM KCl, and digested with TEV^{pro}. Briefly, 15 mg of poly-His-tagged CasI was incubated at 22°C for 22 h in the presence of 0.2 mg TEV^{pro} in a total volume of 7.5 ml containing 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM dithiothreitol (DTT). Cleaved CasI was separated from cleaved tag and TEV^{pro} by passing the mixture through a 0.4-ml Ni-Sepharose column equilibrated with ice-cold 20 mM sodium phosphate (pH 8.0)–300 mM NaCl–10 mM imidazole. CasI was recovered in the flowthrough and dialyzed overnight at 4°C against 2 liters of 25 mM HEPES (pH 7.5)–50 mM KCl, concentrated at 4°C by ultrafiltration to a final concentration of 3.0 mg ml⁻¹, flash-frozen in liquid nitrogen, and stored at -80°C.

For expression of FadD17 from *M. tuberculosis* H37Rv (FadD17_{Mtb}), *E. coli* Rosetta(DE3)/pLysS was freshly transformed with pETFD17 and grown at 37°C (200 rpm) in LB broth supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol. *fadD17* expression was induced as described above. Cells were transferred to 16°C for 18 h, harvested, and frozen until further use. The cell pellet from 0.2 liters of culture was suspended in 1 ml lysis buffer, and cells were disrupted as described above. Crude extract was clarified by centrifugation at 4°C (16,100 $\times g$ for 20 min), and the supernatant was loaded onto 1 ml Ni-nitrilotriacetic acid (NTA) resin (Qiagen). The protein was eluted with ice-cold 20 mM sodium phosphate (pH 8.0)–50 mM NaCl–300 mM imidazole and then desalted on Sephadex G25 resin (GE Healthcare) equilibrated with ice-cold 25 mM HEPES (pH 7.5)–50 mM KCl. FadD17 was concentrated by ultrafiltration at 4°C to a final concentration of 2.6 mg ml⁻¹, flash-frozen in liquid nitrogen, and stored at -80°C.

For expression of FadD19_{Mtb}, *E. coli* Rosetta 2(DE3)/pLysS was freshly transformed with pETFD19 and grown at 37°C (200 rpm) in LB broth supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol. Expression of *fadD19* was induced as described above. Cells were transferred to 16°C for 23 h, harvested, and frozen until further use. The cell pellet from 1 liter was suspended in 20 ml of a solution containing 25 mM sodium phosphate (pH 8.0), 100 mM NaCl, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 50 $\mu\text{g ml}^{-1}$ DNase I, and 50 $\mu\text{g ml}^{-1}$ lysozyme, and cells were disrupted as described above. Crude extract was clarified by ultracentrifugation, and FadD19 was purified from the supernatant on Ni-Sepharose as described above for CasI. Tagged FadD19 was concentrated by ultrafiltration at 4°C to a final concentration of 29 mg ml⁻¹, flash-frozen in liquid nitrogen, and stored at -80°C. Attempts to remove the affinity tag by using factor Xa (Boehringer) resulted in FadD19 degradation. CasG was purified as previously described (25).

Preparation of steroid substrates. 3-Oxo-23,24-bisnorchola-1,4-dien-22-oate (1,4-BNC), 3 α -H-4 α -(3'-propanoate)-7 α -methylhexahydro-1,5-indanone (HIP), 1 β -(2'-propanoate)-3 α -H-4 α -(3''-(R)-hydroxy-3'-propanoate)-7 α -methylhexahydro-5-indanone (HHIDP), and 1 β -(2'-propanoate)-3 α -H-4 α -(3''-propanoate)-7 α -methylhexahydro-5-indanone (HIDP) were obtained as previously described (14, 27, 36). 3-Hydroxy-9-oxo-9,10-*seco*-23,24-bisnorchola-1,3,5(10)-trien-22-oate (HSBNC), 3-hydroxy-9-oxo-9,10-*seco*-23,24-bisnorchola-1,3,5(10),17-tetren-22-oate (Δ^{17} -HSBNC), 3,4-dihydroxy-9-oxo-9,10-*seco*-23,24-bisnorchola-1,3,5(10)-trien-22-oate (DSHBNC), and 4,5-9,10-*diseco*-3-hydroxy-5,9-dioxo-23,24-bisnorchola-1(10),2-dien-4,22-dioate (DSHBNC) were obtained

as described elsewhere (A. M. Crowe, I. Casabon, and L. D. Eltis, unpublished data).

HIP, HSBNC, Δ^{17} -HSBNC, DSHBNC, HHIDP, and HIDP were dissolved in methanol to concentrations ranging from 10 to 50 mM. 4-BNC was dissolved to 50 mM in 94% ethanol containing 60 mM sodium hydroxide. 1,4-BNC was dissolved in ethanol to a concentration of 15 mM. Cholic acid, deoxycholic acid, and chenodeoxycholic acid solutions (100 mM) were prepared in dimethyl sulfoxide (DMSO). 3 β -Hydroxy-5-cholesten-26-oate (1 mM) and 3-oxo-4-cholesten-26-oate (10 mM) solutions were prepared in 10 and 25% 2-hydroxypropyl- β -cyclodextrin, respectively.

HPLC-based activity assay. Reactions were performed in 100 μl of 100 mM HEPES (pH 7.3) with 5 mM MgCl₂, 2.5 mM ATP, and 1 mM CoASH. CasI, CasG, FadD17, or FadD19 was added at 2 μM . The tested substrate was added at 1 mM (Table 1; see also Fig. S2 in the supplemental material), except for 3 β -hydroxy-5-cholesten-26-oate (0.2 mM), 3-oxo-4-cholesten-26-oate (0.5 mM), and DSHBNC (0.5 mM). Reaction mixtures were incubated at 22°C $\pm$ 1°C for 4 h, and both substrate depletion and product formation were analyzed by HPLC as described previously (14).

Purification and analysis of CoA thioesters. 1 β -(2'-Propanoyl-CoA)-3 α -H-4 α -(3''-(R)-hydroxy-3'-propanoate)-7 α -methylhexahydro-5-indanone (HHIDP-CoA) was generated by incubating HHIDP (1 mM) with CasI (2 μM), and 3-oxo-4-cholesten-26-oyl-CoA was generated by incubating 3-oxo-4-cholesten-26-oate (0.5 mM) with FadD19 (2 μM). Both CoA thioesters were in a final volume of 1 ml buffer (0.1 M HEPES, pH 7.3), containing ATP (2.5 mM), CoASH (1 mM), and Mg²⁺ (5 mM), for 4 h at 22°C $\pm$ 1°C. Both CoA thioesters were purified by HPLC as previously described (14). Under these conditions, the retention times for HHIDP-CoA and 3-oxo-4-cholesten-26-oyl-CoA were 10.5 and 19.7 min, respectively. Purified samples of HHIDP-CoA and 3-oxo-4-cholesten-26-oyl-CoA were diluted to 0.1 mM in methanol-water-acetic acid (99.5:99.5:1, vol/vol/vol) and subjected to electron spray ionization tandem mass spectrometry (ESI-MS/MS) in the positive-ion mode. The scanned mass range was m/z 50 to 2,000 Da.

Steady-state kinetics. Steady-state kinetic studies of CasI, CasG, and FadD19 were performed as described previously (14), using a spectrophotometric assay that couples AMP formation to NADH oxidation (37). Reaction mixtures contained various concentrations of HIDP (0 to 300 μM), 4-BNC (0 to 1,000 μM), or HSBNC (0 to 600 μM) for CasI kinetics; cholate (0 to 1 mM) for CasG kinetics; or 3-oxo-4-cholesten-26-oate (0 to 500 μM) for FadD19 kinetics. The mixtures were incubated for 5 min at 22°C $\pm$ 1°C, and the reaction was initiated by the addition of 5, 10, or 300 nM CasI for HIDP, 4-BNC, and HSBNC kinetics, respectively; 100 nM CasG; or 50 nM FadD19. The initial velocity was recorded at 340 nm over 1 min. Kinetic parameters were evaluated by using the least-squares fitting and dynamic weighting options of LEONORA (38), by fitting the Michaelis-Menten equation to the data for CasI and FadD19 and the substrate inhibition equation to the data for CasG.

Bioinformatic analyses. Protein sequences were obtained from the NCBI databases, except for three sequences from *Pseudomonas putida* DOC21 (E. R. Olivera and J. M. Luengo, unpublished data). Multiple-sequence alignments were performed with ClustalW, using the default parameters (39). Phylogenetic trees were built by using the maximum likelihood model (ProML, with default parameters and 22 jumbles) of the Phylip 3.69 package. Bootstrap values were obtained by using Seqboot (default parameters and 100 bootstraps), ProML (default parameters and 3 jumbles), and Consense (default parameters) of the Phylip package.

RESULTS

Phylogenetic analysis. To assess the occurrence of acyl-CoA synthetases involved in steroid degradation in *Actinobacteria* and *Proteobacteria*, we performed a phylogenetic analysis, using as query sequences CasG and CasI, from the cholate catabolic pathway of RHA1, as well as FadD3, FadD17, and FadD19, from the cholest-

TABLE 1 Substrate preferences of acyl-CoA synthetases from the cholate (Cas) and cholesterol (FadD) pathways of RHA1 and *M. tuberculosis*^a

Substrate	Structure	Acyl-CoA synthetase				
		FadD19	CasG ^b	FadD17	CasI ^c	FadD3 ^d
3β-Hydroxy-5-cholesten-26-oate		+	ND	—	ND	—
3-Oxo-4-cholesten-26-oate		+	—	ND	—	ND
Cholate		—	+	+	—	—
Chenodeoxycholate		—	+	+	—	ND
4-BNC		—	—	—	+	—
HSBNC		—	—	ND	+	ND
HIDP		—	—	ND	+	—
HIP		—	—	—	—	+

^a +, substrate depletion and product formation were detected; —, neither substrate depletion nor product formation was detected; ND, not determined.
^b CasG also transformed deoxycholate (this study) and lithocholate and 3β-hydroxy-5-cholenate (25) (see Fig. S2 in the supplemental material for chemical structures).
^c CasI also transformed 1,4-BNC, Δ¹⁷-HSBNC, DHSBNC, DSHBNC, and HHIDP (this study) and 3β-hydroxy-23,24-bisnor-5-cholen-22-oate (25) (see Fig. S2 in the supplemental material for chemical structures).
^d Data from reference 14.

terol catabolic pathway of *M. tuberculosis*. A predicted bile acid: CoA ligase (BaiB) from *Saccharopolyspora erythraea* NRRL 2338, a homolog of the characterized BaiB from *Eubacterium* sp. strain VPI 12708 (40), was used as a sixth query sequence. As subject sequences, we used the proteins encoded in the genomes of 21 different bacteria (Fig. 3) known or predicted to catabolize ste-

roids. Orthologs of the query proteins were identified via reciprocal best BLAST hits and verified by gene synteny. This included verifying the presence of steroid catabolic genes, such as those encoding ketosteroid dehydrogenases, oxygenases, acyl-CoA dehydrogenases, and thiolases, in the vicinity of the acyl-CoA synthetase gene. These sequences were culled for the final phyloge-

FIG 3 Dendrogram showing the phylogeny of bacterial acyl-CoA synthetases. Coding sequences are identified by name or number and the strain in which they occur. CNB2, *Comamonas testosteroni* CNB-2; TA441, *Comamonas testosteroni* TA441; Gbro, *Gordonia bronchialis* DSM 43247; KTR9, *Gordonia* sp. strain KTR9; HNE, *Hyphomonas neptunium* ATCC 15444; Mps, *Mycobacterium parascrofulaceum* ATCC BAA-614; MSMEG, *Mycobacterium smegmatis* MC²155; Mtb, *Mycobacterium tuberculosis* H37Rv; MYST1, *Myxococcus stipitatus* DSM 14675; nfa, *Nocardia farcinica* IFM 10152; Choll1, *Pseudomonas* sp. strain Choll1; DOC21, *Pseudomonas putida* DOC21; P1, *Pseudonocardia* sp. strain P1; PSHA, *Pseudoalteromonas haloplanktis* TAC125; RHA1, *Rhodococcus jostii* RHA1; REQ, *Rhodococcus equi* 1035; RER, *Rhodococcus erythropolis* PR4; SACE, *Saccharopolyspora erythraea* NRRL 2338; Strop, *Salinispora tropica* CNB-440; Sulb, *Sulfobacter* sp. strain EE-36; Tcur, *Thermomonospora curvata* DSM 43183; BaiB, bile acid:CoA ligase. The conservation of the C-terminal P_XGK motif required for acetylation is represented on the tree by an "a" in superscript. Dark- and light-shaded groups contain only actinobacterial and proteobacterial sequences, respectively.

netic analysis such that no two retained proteins shared >72% amino acid sequence identity. In culling, characterized enzymes were preferentially retained, as were enzymes encoded by genes linked to characterized steroid degradation pathways. In addition, sequences from *Thermomonospora curvata* were included, since this strain harbors a cholate gene cluster (25). Similarly, the chromosome of *Salinispora tropica* contains an *hsa* operon and a *kshA* ortholog in the vicinity of the *fadD3* and *fadD19* homologs. Two additional sequences were added to the analysis: FadD36_{Mtb}, predicted to be essential for growth on cholesterol *in vitro* (31), and Ro02518, encoded within a cluster of putative steroid catabolism genes in RHA1 (25). The final analysis was performed by using 53 sequences, sharing at least 15% amino acid sequence identity, from 21 organisms (Fig. 3). The 17 acyl-CoA synthetases that were culled due to the 72% amino acid sequence identity cutoff are listed in the supplemental material.

The phylogenetic tree calculated from an alignment of the 53 sequences revealed five major classes that appear to correspond to enzyme function (Fig. 3). FadD19, CasG, BaiB, and FadD3 belong to classes I, II, IV, and V, respectively, while CasI and FadD17 both belong to class III. Classes II, III, and V are symmetrical inasmuch

as each class contains similarly structured subclasses with sequences from *Actinobacteria* and *Proteobacteria* (Fig. 3, dark and light shading, respectively). These subclasses are most divergent in class III, whose members share as little as 21% amino acid sequence identity, and least divergent in class V, whose members share at least 44% identity. Two sequences that did not clearly fit into subclasses were Gbro_3835 and MSMEG_4952. These share greater amino acid sequence identity with FadD17_{RHA1} (50%) than with CasI (45%). However, *Gordonia bronchialis* and *Mycobacterium smegmatis* both have other enzymes which are FadD17 orthologs, Gbro_3957 and MSMEG_5908, respectively. Accordingly, Gbro_3835 and MSMEG_4952 are predicted to be CasI orthologs. Synteny around the corresponding genes further supported this prediction.

Some acyl-CoA synthetases are known to be controlled by acetylation (41, 42). Acetylation occurs at a strictly conserved lysyl residue, which is essential for adenylation of the substrate (i.e., the first half of the reaction) (Fig. 2) (42). Recent bioinformatic analyses identified four motifs in the C-terminal part of the acetylated FadDs (43), including the PX₄GK motif, which is required but not sufficient for acetylation (43, 44). We thus searched for the pres-

TABLE 2 Apparent steady-state kinetic parameters for AMP formation by acyl-CoA synthetases from the cholate (Cas) and cholesterol (FadD) pathways of RHA1 and *M. tuberculosis*^a

Enzyme and substrate	Mean K_m (μM) (SE)	Mean k_{cat} (s^{-1}) (SE)	Mean k_{cat}/K_m ($10^5 \text{ M}^{-1} \text{ s}^{-1}$) (SE)
FadD19 _{Mtb} 3-Oxo-4-cholesten-26-oate	23 (3)	0.75 (0.04)	0.33 (0.03)
CasI			
4-BNC	75 (5)	18 (1)	2.4 (0.1)
HSBNC	500 (80)	1.4 (0.2)	0.030 (0.002)
HIDP	190 (50)	60 (10)	3.2 (0.3)
CasG			
Cholate ^b	160 (20)	2.4 (0.2)	0.15 (0.01)

^a Values in parentheses represent standard errors.

^b CasG showed substrate inhibition with cholate ($K_i = 1.2 \pm 0.3 \text{ mM}$), so the corresponding parameters are K_S and k_{cat}/K_S , respectively.

ence of the latter motif in the above-mentioned 53 sequences to identify enzymes that are not acetylated. Members of class II all harbor this motif, while members of class I and class III do not (Fig. 3). Two exceptions were in class IIb, Tcur_3484 and nfa32990. While these two sequences harbor the PX₄GK motif, they do not contain any of the three other motifs (43), suggesting that these enzymes are not acetylated. Finally, the actinobacterial FadD3 members of class Va harbor the PX₄GK motif, while the proteobacterial members of class Vb do not.

Acyl-CoA synthetase activities. The activities of poly-His-tagged FadD17, FadD19, and CasG as well as untagged CasI were assessed by using a variety of cholesterol and cholate metabolites. These included several structures with two-, three-, and four-membered nuclei harboring either a C₃, C₅, or C₈ side chain or a keto group at C-17 (Table 1; see also Fig. S2 in the supplemental material). The four enzymes showed clear preferences for particular side-chain lengths. CasI detectably transformed only compounds with a C₃ isopropanoate side chain at C-17 (Table 1; see also Fig. S2 in the supplemental material). For instance, when the reaction was performed with HHIDP, the HPLC peaks for ATP and CoASH were depleted, and two new peaks appeared (see Fig. S3 in the supplemental material). The first new peak had a retention time (2.8 min) and an electronic absorption spectrum that corresponded with AMP. The second new peak eluted at 10.5 min and had spectrum features consistent with those of a CoA thioester. Alkaline hydrolysis of this second product yielded HHIDP plus CoASH, as determined by gas chromatography-mass spectrometry (GC-MS) and HPLC analyses, which indicated that the peak eluting at 10.5 min was HHIDP-CoA. Further confirmation of the identity as HHIDP-CoA was obtained by ESI-MS/MS (see Fig. S4 in the supplemental material). CasI failed to form CoA thioesters from substrates with C₅ and C₈ side chains. In contrast, CasG and FadD17 detectably transformed only compounds with a C₅ side chain at C-17, while FadD19 detectably transformed only compounds with a C₈ side chain at C-17 (Table 1; see also Fig. S2 in the supplemental material). The previously unidentified product of FadD19 eluted at 19.7 min under the HPLC conditions described above, and its identity was confirmed to be 3-oxo-4-cholesten-26-oyl-CoA by ESI-MS/MS (see Fig. S5 in the supplemental material). Control experiments performed without substrate, cosubstrate, or enzyme indicated that all CoA thioester formation was well coupled with ATP hydrolysis.

In general, the configuration of the steroid nucleus was much

less important than side-chain length in affecting enzyme substrate preferences. For example, CoA thioester formation by CasI was detected with compounds representing different stages of steroid nucleus degradation: 4-BNC, having intact A and B rings; HSBNC, having an aromatized A ring and an opened B ring; and HIDP, having only rings C and D (Table 1; see also Fig. S2 in the supplemental material). Furthermore, among all the compounds tested, the presence of hydroxyl groups at C-3, C-7, and C-12 did not prevent the respective enzyme activities.

Steady-state kinetics. Kinetic parameters for FadD19, CasI, and CasG were determined under steady-state conditions, using the coupled spectrophotometric assay. In all cases, full coupling between AMP and CoA thioester formation was confirmed via control reactions. The CasI-catalyzed CoA thioesterification of 4-BNC, HSBNC, and HIDP displayed Michaelis-Menten kinetics at pH 7.3 and 22°C $\pm$ 1°C (see Fig. S6 in the supplemental material). CasI turned over HIDP roughly three times faster than did 4-BNC (i.e., a k_{cat} $\sim 3\times$ higher), even though the apparent K_m value for the former was twice as high, resulting in similar apparent specificity constants (k_{cat}/K_m) for HIDP and 4-BNC (Table 2). In contrast, the specificity constant of CasI for HIDP was more than 100 times higher than that for HSBNC, consistent with CasI turning over HIDP roughly 40 times faster than HSBNC while possessing an apparent K_m value about 3 times lower for HIDP.

The FadD19 transformation of 3-oxo-4-cholesten-26-oate to 3-oxo-4-cholesten-26-oyl-CoA also displayed Michaelis-Menten kinetics at pH 7.3 and 22°C $\pm$ 1°C (see Fig. S6 in the supplemental material). Of the three enzymes and five substrates tested, the conversion of 3-oxo-4-cholesten-26-oate by FadD19 showed the slowest turnover, with a k_{cat} of 0.75 s⁻¹. In contrast, under the same conditions, CasG activity with cholate as the substrate showed a fitted curve characteristic of substrate inhibition (Fig. 4), but the inhibition constant, K_i , was high, around 1.2 mM (Table 2).

DISCUSSION

Steroid side-chain degradation has been studied in both *Actinobacteria* and *Proteobacteria* (9, 12, 13). Nevertheless, the physiological substrates of the enzymes involved in this catabolism are largely unknown. The current study establishes the physiological

FIG 4 Steady-state kinetic analysis of CasG_{RHA1} for cholate. Shown is the dependence of the initial velocity (V_o) of AMP formation on the cholate concentration. The solid line represents a best fit of the nonhyperbolic substrate inhibition equation to the data ($K_S = 160 \pm 20 \mu\text{M}$; $V_{\text{max}} = 14 \pm 1 \mu\text{M min}^{-1}$; $K_i = 1,200 \pm 300 \mu\text{M}$; $R^2 \geq 0.97$).

roles of several acyl-CoA synthetases that activate steroid side chains for subsequent degradation. The acyl-CoA synthetases showed strong preferences for particular chain lengths while tolerating variable steroid nucleus structures (Table 1; see also Fig. S2 in the supplemental material). This pattern is consistent with concurrent side-chain and nucleus degradation by *Actinobacteria*, as recently established for cholesterol degradation (17, 36) and bile acid degradation (27). In contrast, bile acid degradation by *Proteobacteria* involves complete degradation of the side chain to a keto group, prior to the initial ring cleavage reaction, which opens ring B (9, 12). The phylogenetic analysis permits extrapolation of the enzymological data to a broad range of bacteria, since the enzyme classes identified correspond to orthologous groups predicted to have common functions (Fig. 3). Gene synteny further supports the predicted functions. Accordingly, the groups represented by FadD19 (class I) and FadD17 (class IIIa) contain enzymes from *Actinobacteria* known to share a cholesterol degradation pathway, while the groups represented by CasG (class II) and CasI (classes IIIb and IIIc) contain enzymes from both *Actinobacteria* and *Proteobacteria* that grow on bile acids.

The kinetic data establish that CasG is a steroid-24-oyl-CoA synthetase (Tables 1 and 2) and support the previous proposal that it initiates degradation of the C₅ cholate side chain (25). This study is consistent with previous qualitative assays indicating that CasG can transform other bile acids as well as cholate (25). While RHA1 cannot use those other bile acids as sole growth substrates, it might cometabolize them in the presence of cholate. Based on the high K_i value, the substrate inhibition observed for CasG (Table 2) appears to be too weak to occur at physiological substrate concentrations.

The data further establish that CasI is a steroid-22-oyl-CoA synthetase, able to activate steroids with a C₃ isopropanoate side chain and a variety of nucleus structures (Tables 1 and 2), consistent with previous qualitative assays (25). CasI likely functions in reassimilation of the metabolites which accumulate when RHA1 grows on cholate (27). CasI orthologs likely function in further degradation of an intermediate resulting from the above-mentioned aldolytic cleavage of the cholate side chain by *Proteobacteria* (12, 24). It is unclear whether such aldolytic cleavage occurs in *Actinobacteria*. Based on the data described above, CasI would be essential for cholate catabolism in *Proteobacteria* but may not be essential in *Actinobacteria*. Since the cholate metabolites known to be reassimilated by RHA1 have two or three rings (27), the preference of CasI for a four-ringed substrate over a three-ringed one is surprising.

As predicted, the proteobacterial enzymes StdA1 and StdA2 each act upon the same substrates as their respective actinobacterial orthologs, CasG and CasI (Fig. 3) (Á. Barrientos, E. Merino, I. Casabon, J. Rodríguez, A. M. Crowe, J. Holert, B. Philipp, L. D. Eltis, E. R. Olivera, and J. M. Luengo, unpublished data). These observations confirm the correlation between phylogeny and enzymatic function across distant taxa. Furthermore, this indicates a common origin for at least some of the enzymes in the bile acid degradation pathways in these two phyla.

FadD19 is a cholesten-26-oyl-CoA synthetase (Tables 1 and 2) that catalyzes the thioesterification of the C₈ alkanolate side chain of cholestenolate generated during cholesterol catabolism. FadD19 occurs in all actinobacterial cholesterol degradation pathways characterized to date (25, 26, 29, 45). So far, *Proteobacteria* have not been reported to catabolize cholesterol, and accordingly, they

lack orthologs of FadD19. One exception to this pattern is *Comamonas testosteroni* CNB-2, which has a gene of unknown function encoding a protein that shares 37% amino acid sequence identity with FadD19_{RHA1}. FadD19 of *R. rhodochrous* DSM 43269 was also shown to transform cholestenolates, but it is not essential for cholesterol degradation (29). On the other hand, FadD19 of *M. tuberculosis* is essential for cholesterol degradation (31). This difference might be due to the presence of FadD19 paralogs in rhodococci, exemplified by the occurrence of Ro04675 in the same class as FadD19_{RHA1} (48% amino acid sequence identity) (Fig. 3) (46). Although both ro04675 and fadD19 are located in the cholesterol cluster, only the latter was upregulated during growth on cholesterol (26). Thus, FadD19 paralogs may have diverged in function while retaining the capacity to complement one another.

Finally, the data establish that FadD17 is a steroid-24-oyl-CoA synthetase (Tables 1 and 2). Based on its occurrence in the actinobacterial cholesterol catabolic cluster, FadD17 appears to function in activating cholesterol metabolites with a C₅ side chain, analogous to the role of CasI in cholate catabolism. Interestingly, the orthologs of CasI and FadD17 cluster in the phylogenetic tree (class III), indicating a common origin of these enzymes. The conservation of FadD17 in cholesterol-degrading bacteria suggests that it is important, but the reason for this is unclear. Metabolites with a C₅ side chain have not been reported to accumulate during growth on cholesterol. Furthermore, degradation of the cholesterol side chain is predicted to proceed via thiolytic cleavage, yielding a C₅ CoA thioester intermediate not requiring activation (47). Consistent with this prediction, FadD17 does not appear to be essential for growth of *M. tuberculosis* on cholesterol (31). It may be that cholesterol metabolites accumulate under certain conditions, when any CoA thioester intermediates would likely be rapidly hydrolyzed to recycle the CoA. FadD17 may thus enable salvaging such metabolites possessing a C₅ side chain. Although FadD17 is isofunctional with CasG (Table 1), the latter is absent in many cholesterol-degrading *Actinobacteria* and is probably not highly expressed in RHA1 or other rhodococci growing on cholesterol (26). In contrast, fadD17 appears to be upregulated during growth of RHA1 on cholate (25), so FadD17 might contribute to cholate degradation in RHA1 and other rhodococci.

In *M. tuberculosis*, FadD17 may have an alternate or additional function. The sequence of FadD17_{Mtb} includes a fatty acid transport protein (FATP) signature motif (48), which is not present in the orthologous enzyme from RHA1. FATPs are conserved proteins found in humans, mammals, primates, vertebrates, insects, nematodes, fungi, yeasts, and mycobacteria (49, 50). These proteins facilitate the transport of LCFAs across biological membranes (50), and some members of this family also possess acyl-CoA synthetase activities with specificity toward LCFAs and bile acids (51, 52). An intriguing possibility is that FadD17_{Mtb} could be a membrane-associated protein involved in transport and/or CoA thioesterification of fatty acids and/or steroids. Consistent with this hypothesis, this enzyme was previously shown to catalyze the CoA thioesterification of LCFAs (28). Moreover, fadD17 was among the genes recently identified by microarray as potentially being involved in regulating the intracellular level of acetyl-CoA in *M. tuberculosis* via processing of LCFAs (53). The postulated membrane association of FadD17_{Mtb} could explain the very low yields of soluble protein that we observed when trying to produce it heterologously in *E. coli* (not shown).

FadD3 was previously shown to transform HIP or its deriva-

tives (14). The *fadD3* gene is part of the KstR2 regulon (54, 55), which was proposed to encode degradation of rings C and D, the final phase of steroid catabolism (54). This was further proposed to be a convergent pathway in *Actinobacteria*, serving to complete the degradation of cholesterol, cholate, and likely other steroids (25). In accordance with this universal role of FadD3 in steroid degradation, orthologs (class V) (Fig. 3) were found in all bacteria known to catabolize steroids. The function of this class in a proteobacterium is confirmed by the essentiality of the orthologous gene, *orf18*, for catabolism of steroid rings C and D in *C. testosteroni* TA441 (30).

A distinct class (class IVb) of putative steroid-24-oyl CoA synthetases is represented by BaiB. However, none of the bacteria containing a BaiB ortholog are known to grow on bile acids, so another physiological function for these enzymes, such as detoxification, is possible. BaiB of the intestinal bacterium *Eubacterium* sp. is required for 7 α -dehydroxylation of bile acids, which is a major fate of bile acids in the intestine, accounting for up to 25% of the biliary pool in humans (40). Overall, classes IIa, IIIa, and IVb appear to be isofunctional but to contribute to distinct metabolic processes.

An increasing body of evidence indicates that protein lysine acetylation plays a dominant role in several aspects of bacterial metabolism (56, 57). Recently, eight FadDs of *M. tuberculosis* were shown to be acetylated *in vitro*, and it was proposed that acetylation might play an important role in the bacterium's pathogenesis (43). Our sequence analysis indicates that *Actinobacteria* and *Proteobacteria* might regulate cholate catabolism via acetylation of the PX₄GK motif of CasG. In contrast, it is not possible for *Actinobacteria* to regulate cholesterol side chain catabolism via such acetylation of FadD19. Finally, catabolism of steroid rings C and D might be regulated by such acetylation of FadD3 in *Actinobacteria*, but this is not possible in *Proteobacteria*.

This study established the functions of several classes of acyl-CoA synthetases and yielded new insights into the evolution of steroid catabolic pathways. An unexpected outcome was the broad occurrence of acyl-CoA synthetases that appear to activate steroid intermediates or metabolites with shortened carboxyl side chains. However, the physiological significance of such metabolites remains unclear. The results also indicate important similarities and differences between actinobacterial and proteobacterial bile acid catabolic pathways, which require further elucidation.

ACKNOWLEDGMENTS

This research was supported by a Natural Sciences and Engineering Research Council discovery grant to W.W.M. and a Canadian Institutes for Health Research operating grant to L.D.E. I.C. is a recipient of a postdoctoral fellowship from the Fonds de Recherche en Santé du Québec and the Michael Smith Foundation for Health Research.

We thank Robert van der Geize for providing 3 β -hydroxy-5-cholesten-26-oic acid. Jason Rogalski helped analyze the MS data.

REFERENCES

- Gimpl G, Burger K, Fahrenholz F. 1997. Cholesterol as modulator of receptor function. *Biochemistry* 36:10959–10974. <http://dx.doi.org/10.1021/bi963138w>.
- Donova MV, Egorova OV. 2012. Microbial steroid transformations: current state and prospects. *Appl. Microbiol. Biotechnol.* 94:1423–1447. <http://dx.doi.org/10.1007/s00253-012-4078-0>.
- Ouellet H, Johnston JB, Ortiz de Montellano PR. 2011. Cholesterol catabolism as a therapeutic target in *Mycobacterium tuberculosis*. *Trends Microbiol.* 19:530–539. <http://dx.doi.org/10.1016/j.tim.2011.07.009>.
- Pandey AK, Sasseti CM. 2008. Mycobacterial persistence requires the utilization of host cholesterol. *Proc. Natl. Acad. Sci. U. S. A.* 105:4376–4380. <http://dx.doi.org/10.1073/pnas.0711159105>.
- Yam KC, D'Angelo I, Kalscheuer R, Zhu H, Wang JX, Snieckus V, Ly LH, Converse PJ, Jacobs WR, Jr, Strynadka N, Eltis LD. 2009. Studies of a ring-cleaving dioxygenase illuminate the role of cholesterol metabolism in the pathogenesis of *Mycobacterium tuberculosis*. *PLoS Pathog.* 5:e1000344. <http://dx.doi.org/10.1371/journal.ppat.1000344>.
- Hofmann AF, Mysels KJ. 1988. Bile salts as biological surfactants. *Colloids Surf.* 30:145–173.
- Fernandes P, Cruz A, Angelova B, Pinheiro HM, Cabral JMS. 2003. Microbial conversion of steroid compounds: recent developments. *Enzyme Microb. Technol.* 32:688–705. [http://dx.doi.org/10.1016/S0141-0229\(03\)00029-2](http://dx.doi.org/10.1016/S0141-0229(03)00029-2).
- Philipp B. 2011. Bacterial degradation of bile salts. *Appl. Microbiol. Biotechnol.* 89:903–915. <http://dx.doi.org/10.1007/s00253-010-2998-0>.
- Horinouchi M, Hayashi T, Kudo T. 2012. Steroid degradation in *Comamonas testosteroni*. *J. Steroid Biochem. Mol. Biol.* 129:4–14. <http://dx.doi.org/10.1016/j.jsmb.2010.10.008>.
- Birkenmaier A, Holert J, Erdbrink H, Moeller HM, Friemel A, Schoenenberger R, Suter MJ, Klebensberger J, Philipp B. 2007. Biochemical and genetic investigation of initial reactions in aerobic degradation of the bile acid cholate in *Pseudomonas* sp. strain Choll. *J. Bacteriol.* 189:7165–7173. <http://dx.doi.org/10.1128/JB.00665-07>.
- Birkenmaier A, Möller HM, Philipp B. 2011. Identification of a thiolase gene essential for β -oxidation of the acyl side chain of the steroid compound cholate in *Pseudomonas* sp. strain Choll. *FEMS Microbiol. Lett.* 318:123–130. <http://dx.doi.org/10.1111/j.1574-6968.2011.02250.x>.
- Holert J, Kulic Z, Yucel O, Suvekbala V, Suter MJ, Moller HM, Philipp B. 2013. Degradation of the acyl side chain of the steroid compound cholate in *Pseudomonas* sp. strain Choll proceeds via an aldehyde intermediate. *J. Bacteriol.* 195:585–595. <http://dx.doi.org/10.1128/JB.01961-12>.
- Yam KC, Okamoto S, Roberts JN, Eltis LD. 2011. Adventures in *Rhodococcus*—from steroids to explosives. *Can. J. Microbiol.* 57:155–168. <http://dx.doi.org/10.1139/W10-115>.
- Casabon I, Crowe AM, Liu J, Eltis LD. 2013. FadD3 is an acyl-CoA synthetase that initiates catabolism of cholesterol rings C and D in actinobacteria. *Mol. Microbiol.* 87:269–283. <http://dx.doi.org/10.1111/mmi.12095>.
- Petrusma M, Hessels G, Dijkhuizen L, van der Geize R. 2011. Multiplicity of 3-ketosteroid-9 α -hydroxylase enzymes in *Rhodococcus rhodochrous* DSM43269 for the specific degradation of different classes of steroids. *J. Bacteriol.* 193:3931–3940. <http://dx.doi.org/10.1128/JB.00274-11>.
- Thomas ST, Sampson NS. 2013. *Mycobacterium tuberculosis* utilizes a unique heterotetrameric structure for dehydrogenation of the cholesterol side chain. *Biochemistry* 52:2895–2904. <http://dx.doi.org/10.1021/bi4002979>.
- Thomas ST, VanderVen BC, Sherman DR, Russell DG, Sampson NS. 2011. Pathway profiling in *Mycobacterium tuberculosis*: elucidation of a cholesterol-derived catabolite and the enzymes that catalyze its metabolism. *J. Biol. Chem.* 286:43668–43678. <http://dx.doi.org/10.1074/jbc.M111.313643>.
- Capyk JK, Kalscheuer R, Stewart GR, Liu J, Kwon H, Zhao R, Okamoto S, Jacobs WR, Jr, Eltis LD, Mohn WW. 2009. Mycobacterial cytochrome P450 125 (cyp125) catalyzes the terminal hydroxylation of C27 steroids. *J. Biol. Chem.* 284:35534–35542. <http://dx.doi.org/10.1074/jbc.M109.072132>.
- Rosloniec KZ, Wilbrink MH, Capyk JK, Mohn WW, Ostendorf M, van der Geize R, Dijkhuizen L, Eltis LD. 2009. Cytochrome P450 125 (CYP125) catalyzes C26-hydroxylation to initiate sterol side-chain degradation in *Rhodococcus jostii* RHA1. *Mol. Microbiol.* 74:1031–1043. <http://dx.doi.org/10.1111/j.1365-2958.2009.06915.x>.
- Johnston JB, Ouellet H, Ortiz de Montellano PR. 2010. Functional redundancy of steroid C26-monooxygenase activity in *Mycobacterium tuberculosis* revealed by biochemical and genetic analyses. *J. Biol. Chem.* 285:36352–36360. <http://dx.doi.org/10.1074/jbc.M110.161117>.
- Ouellet H, Guan S, Johnston JB, Chow ED, Kells PM, Burlingame AL, Cox JS, Podust LM, Ortiz de Montellano PR. 2010. *Mycobacterium tuberculosis* CYP125A1, a steroid C27 monooxygenase that detoxifies intracellularly generated cholest-4-en-3-one. *Mol. Microbiol.* 77:730–742. <http://dx.doi.org/10.1111/j.1365-2958.2010.07243.x>.

22. Groot PHE, Scholte HR, Hulsmann WC. 1976. Fatty acid activation: specificity, localization, and function. *Adv. Lipid Res.* 14:75–126.
23. Babbitt PC, Kenyon GL, Martin BM, Charest H, Slyvestre M, Scholten JD, Chang KH, Liang PH, Dunaway-Mariano D. 1992. Ancestry of the 4-chlorobenzoate dehalogenase: analysis of amino acid sequence identities among families of acyl:adenyl ligases, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases. *Biochemistry* 31:5594–5604. <http://dx.doi.org/10.1021/bi00139a024>.
24. Holert J, Jagmann N, Philipp B. 2013. The essential function of genes for a hydratase and an aldehyde dehydrogenase for growth of *Pseudomonas* sp. strain Chol1 with the steroid compound cholate indicates an aldolytic reaction step for deacetylation of the side chain. *J. Bacteriol.* 195:3371–3380. <http://dx.doi.org/10.1128/JB.00410-13>.
25. Mohn WW, Wilbrink MH, Casabon I, Stewart GR, Liu J, van der Geize R, Eltis LD. 2012. Gene cluster encoding cholate catabolism in *Rhodococcus* spp. *J. Bacteriol.* 194:6712–6719. <http://dx.doi.org/10.1128/JB.01169-12>.
26. van der Geize R, Yam K, Heuser T, Wilbrink MH, Hara H, Anderton MC, Sim E, Dijkhuizen L, Davies JE, Mohn WW, Eltis LD. 2007. A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 104:1947–1952. <http://dx.doi.org/10.1073/pnas.0605728104>.
27. Swain K, Casabon I, Eltis LD, Mohn WW. 2012. Two transporters essential for reassimilation of novel cholate metabolites by *Rhodococcus jostii* RHA1. *J. Bacteriol.* 194:6720–6727. <http://dx.doi.org/10.1128/JB.01167-12>.
28. Trivedi OA, Arora P, Sridharan V, Tickoo R, Mohanty D, Gokhale RS. 2004. Enzymic activation and transfer of fatty acids as acyl-adenylates in mycobacteria. *Nature* 428:441–445. <http://dx.doi.org/10.1038/nature02384>.
29. Wilbrink MH, Petrusma M, Dijkhuizen L, van der Geize R. 2011. FadD19 of *Rhodococcus rhodochrous* DSM43269: a steroid-coenzyme A ligase essential for degradation of C-24 branched sterol side chains. *Appl. Environ. Microbiol.* 77:4455–4464. <http://dx.doi.org/10.1128/AEM.00380-11>.
30. Horinouchi M, Hayashi T, Koshino H, Kudo T. 2006. ORF18-disrupted mutant of *Comamonas testosteroni* TA441 accumulates significant amounts of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostane-5-oic acid and its derivatives after incubation with steroids. *J. Steroid Biochem. Mol. Biol.* 101:78–84. <http://dx.doi.org/10.1016/j.jsmb.2006.06.006>.
31. Griffin JE, Gawronski JD, DeJesus MA, Ioerger TR, Akerley BJ, Sassetti CM. 2011. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog.* 7:e1002251. <http://dx.doi.org/10.1371/journal.ppat.1002251>.
32. Kapust RB, Tózsér J, Fox JD, Anderson DE, Cherry S, Copeland TD, Waugh DS. 2001. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. *Protein Eng.* 14:993–1000. <http://dx.doi.org/10.1093/protein/14.12.993>.
33. Seto M, Kimbara K, Shimura M, Hatta T, Fukuda M, Yano K. 1995. A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. *Appl. Environ. Microbiol.* 61:3353–3358.
34. Studier FW, Moffatt BA. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189:113–130. [http://dx.doi.org/10.1016/0022-2836\(86\)90385-2](http://dx.doi.org/10.1016/0022-2836(86)90385-2).
35. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36. Capyk JK, Casabon I, Gruninger R, Strynadka NC, Eltis LD. 2011. Activity of 3-ketosteroid 9 α -hydroxylase (KshAB) indicates cholesterol side chain and ring degradation occur simultaneously in *Mycobacterium tuberculosis*. *J. Biol. Chem.* 286:40717–40724. <http://dx.doi.org/10.1074/jbc.M111.289975>.
37. Horswill AR, Escalante-Semerena JC. 2002. Characterization of the propionyl-CoA synthetase (PrpE) enzyme of *Salmonella enterica*: residue Lys592 is required for propionyl-AMP synthesis. *Biochemistry* 41:2379–2387. <http://dx.doi.org/10.1021/bi015647q>.
38. Cornish-Bowden A. 1994. Analysis of enzyme kinetic data. Oxford University Press, New York, NY.
39. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882. <http://dx.doi.org/10.1093/nar/25.24.4876>.
40. Mallonee DH, Adams JH, Hylemon PB. 1992. The bile acid-inducible *baiB* gene from *Eubacterium* sp. strain VPI 12708 encodes a bile acid-coenzyme A ligase. *J. Bacteriol.* 174:2065–2071.
41. Starai VJ, Celic I, Cole RN, Boeke JD, Escalante-Semerena JC. 2002. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science* 298:2390–2392. <http://dx.doi.org/10.1126/science.1077650>.
42. Xu H, Hegde SS, Blanchard JS. 2011. The reversible acetylation and inactivation of *Mycobacterium tuberculosis* acetyl-CoA synthetase is dependent on cAMP. *Biochemistry* 50:5883–5892. <http://dx.doi.org/10.1021/bi200156t>.
43. Nambi S, Gupta K, Bhattacharyya M, Ramakrishnan P, Ravikumar V, Siddiqui N, Thomas AT, Visweswariah SS. 2013. Cyclic AMP-dependent protein lysine acylation in mycobacteria regulates fatty acid and propionate metabolism. *J. Biol. Chem.* 288:14114–14124. <http://dx.doi.org/10.1074/jbc.M113.463992>.
44. Crosby HA, Rank KC, Rayment I, Escalante-Semerena JC. 2012. Structural insights into the substrate specificity of the *Rhodopseudomonas palustris* protein acetyltransferase RpPat: identification of a loop critical for recognition by RpPat. *J. Biol. Chem.* 287:41392–41404. <http://dx.doi.org/10.1074/jbc.M112.417360>.
45. Chen HP, Zhu SH, Casabon I, Hallam SJ, Crocker FH, Mohn WW, Indest KJ, Eltis LD. 2012. Genomic and transcriptomic studies of an RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)-degrading actinobacterium. *Appl. Environ. Microbiol.* 78:7798–7800. <http://dx.doi.org/10.1128/AEM.02120-12>.
46. McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dossanj M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD. 2006. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc. Natl. Acad. Sci. U. S. A.* 103:15582–15587. <http://dx.doi.org/10.1073/pnas.0607048103>.
47. Nesbitt NM, Yang X, Fontán P, Kolesnikova I, Smith I, Sampson NS, Dubnau E. 2010. A thiolase of *Mycobacterium tuberculosis* is required for virulence and production of androstenedione and androstadienedione from cholesterol. *Infect. Immun.* 78:275–282. <http://dx.doi.org/10.1128/IAI.00893-09>.
48. Zou Z, DiRusso CC, Ctrnacta V, Black PN. 2002. Fatty acid transport in *Saccharomyces cerevisiae*. Directed mutagenesis of FAT1 distinguishes the biochemical activities associated with Fat1p. *J. Biol. Chem.* 277:31062–31071. <http://dx.doi.org/10.1074/jbc.M205034200>.
49. Hirsch D, Stahl A, Lodish HF. 1998. A family of fatty acid transporters conserved from mycobacterium to man. *Proc. Natl. Acad. Sci. U. S. A.* 95:8625–8629. <http://dx.doi.org/10.1073/pnas.95.15.8625>.
50. Stahl A, Gimeno RE, Tartaglia LA, Lodish HF. 2001. Fatty acid transport proteins: a current view of a growing family. *Trends Endocrinol. Metab.* 12:266–273. [http://dx.doi.org/10.1016/S1043-2760\(01\)00427-1](http://dx.doi.org/10.1016/S1043-2760(01)00427-1).
51. Zou Z, Tong F, Faergeman NJ, Borsting C, Black PN, DiRusso CC. 2003. Vectorial acylation in *Saccharomyces cerevisiae*. Fat1p and fatty acyl-CoA synthetase are interacting components of a fatty acid import complex. *J. Biol. Chem.* 278:16414–16422. <http://dx.doi.org/10.1074/jbc.M210557200>.
52. DiRusso CC, Darwis D, Obermeyer T, Black PN. 2008. Functional domains of the fatty acid transport proteins: studies using protein chimeras. *Biochim. Biophys. Acta* 1781:135–143. <http://dx.doi.org/10.1016/j.bbalip.2008.01.002>.
53. Lee W, Vandervan BC, Fahey RJ, Russell DG. 2013. Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *J. Biol. Chem.* 288:6788–6800. <http://dx.doi.org/10.1074/jbc.M112.445056>.
54. Casabon I, Zhu SH, Otani H, Liu J, Mohn WW, Eltis LD. 2013. Regulation of the KstR2 regulon of *Mycobacterium tuberculosis* by a cholesterol catabolite. *Mol. Microbiol.* 89:1201–1212. <http://dx.doi.org/10.1111/mmi.12340>.
55. Kendall SL, Burgess P, Balhana R, Withers M, ten Bokum A, Lott JS, Gao C, Uhia-Castro I, Stoker NG. 2010. Cholesterol utilization in mycobacteria is controlled by two TetR-type transcriptional regulators: *kstR* and *kstR2*. *Microbiology* 156:1362–1371. <http://dx.doi.org/10.1099/mic.0.034538-0>.
56. Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y, Ning ZB, Zeng R, Xiong Y, Guan KL, Zhao S, Zhao GP. 2010. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327:1004–1007. <http://dx.doi.org/10.1126/science.1179687>.
57. Zhang J, Sprung R, Pei J, Tan X, Kim S, Zhu H, Liu CF, Grishin NV, Zhao Y. 2009. Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*. *Mol. Cell. Proteomics* 8:215–225. <http://dx.doi.org/10.1074/mcp.M800187-MCP200>.