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Activity of 3-Ketosteroid 9α-Hydroxylase (KshAB) Indicates Cholesterol Side Chain and Ring Degradation Occur Simultaneously in *Mycobacterium tuberculosis*[*] [S]

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Background: *Mycobacterium tuberculosis* (*Mtb*) degrades cholesterol throughout its infection cycle.
Results: Cholesterol ring-degrading enzymes have higher activities with side chain degradation intermediates than with compounds with fully degraded side chains.
Conclusion: Cholesterol side chain and ring degradation occur concurrently.
Significance: Understanding bacterial cholesterol catabolism facilitates the design of novel therapeutics and the production of high value steroids.

***Mycobacterium tuberculosis* (*Mtb*), a significant global pathogen, contains a cholesterol catabolic pathway. Although the precise role of cholesterol catabolism in *Mtb* remains unclear, the Rieske monooxygenase in this pathway, 3-ketosteroid 9α-hydroxylase (KshAB), has been identified as a virulence factor. To investigate the physiological substrate of KshAB, a rhodococcal acyl-CoA synthetase was used to produce the coenzyme A thioesters of two cholesterol derivatives: 3-oxo-23,24-bisnorchol-4-en-22-oic acid (forming 4-BNC-CoA) and 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid (forming 1,4-BNC-CoA). The apparent specificity constant (k_{cat}/K_m) of KshAB for the CoA thioester substrates was 20–30 times that for the corresponding 17-keto compounds previously proposed as physiological substrates. The apparent $K_{m_{O_2}}$ was 90 ± 10 μM in the presence of 1,4-BNC-CoA, consistent with the value for two other cholesterol catabolic oxygenases. The Δ^1 ketosteroid dehydrogenase KstD acted with KshAB to cleave steroid ring B with a specific activity eight times greater for a CoA thioester than the corresponding ketone. Finally, modeling 1,4-BNC-CoA into the KshA crystal structure suggested that the CoA moiety binds in a pocket at the mouth of the active site channel and could contribute to substrate specificity. These results indicate that the physiological substrates of KshAB are CoA thioester intermediates of cholesterol side chain degradation and that side chain and ring degradation occur concurrently in *Mtb*. This finding has implications for steroid metabolites potentially released by the pathogen during infection and for the design of inhibitors for cholesterol-degrading enzymes. The methodologies and rhodococcal enzymes used to generate thioesters will facilitate the further study of cholesterol catabolism.**

Mycobacterium tuberculosis (*Mtb*)[4] infects about one-third of all people and causes nine million new cases of pulmonary disease each year (1). The success of the bacterium as a pathogen is due in part to its remarkable ability to persist and replicate inside human macrophages (2). Although the mechanisms enabling *Mtb* to evade sterilization by the human immune system are not well understood, recent studies have revealed that the bacterium has a lipid-biased metabolic profile uniquely adapted to its intracellular lifestyle (3). Studies further suggest that genes necessary for lipid catabolism are preferentially expressed during intra-macrophage growth (4, 5). Furthermore, *Mtb* has been shown to direct carbon flux away from the tricarboxylic acid cycle (6), consistent with the hypothesis that growth on fatty acids via the glyoxylate cycle is an important component of *Mtb* virulence (7).

A cholesterol catabolic pathway (8) (Fig. 1) enabling the bacterium to grow *in vitro* using this steroid as a sole source of carbon (9) is proposed as a key component of *Mtb* lipid-biased metabolic strategy. Analogous to cholic acid degradation in *Pseudomonas* sp. Chol1 (10) and *Rhodococcus jostii* RHA1,[5] cholesterol side chain degradation in *Mtb* is expected to proceed via CoA thioester intermediates in three cycles of β-oxidation (11, 12). Degradation of the steroid rings A and B include three successive oxygenase reactions (13–15) (Fig. 1). The catalytic rates measured for these oxygenases using the predicted

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[4] The abbreviations used are: *Mtb*, *M. tuberculosis*; 4-AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; 4-BNC, 3-oxo-23,24-bisnorchol-4-en-22-oic acid; 1,4-BNC, 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid; 4-BNC-CoA, 3-oxo-23,24-bisnorchol-4-en-22-oyl-coenzyme A thioester; 1,4-BNC-CoA, 3-oxo-23,24-bisnorchola-1,4-dien-22-oyl-coenzyme A thioester; 3-HSBNC, 3-hydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oic acid; 3-HSBNC-CoA, 3-hydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oyl-CoA thioester.
[5] W. W. Mohn, M. H. Wilbrink, I. Casabon, G. R. Stewart, J. Liu, L. D. Eltis, and R. van der Geize, in preparation.

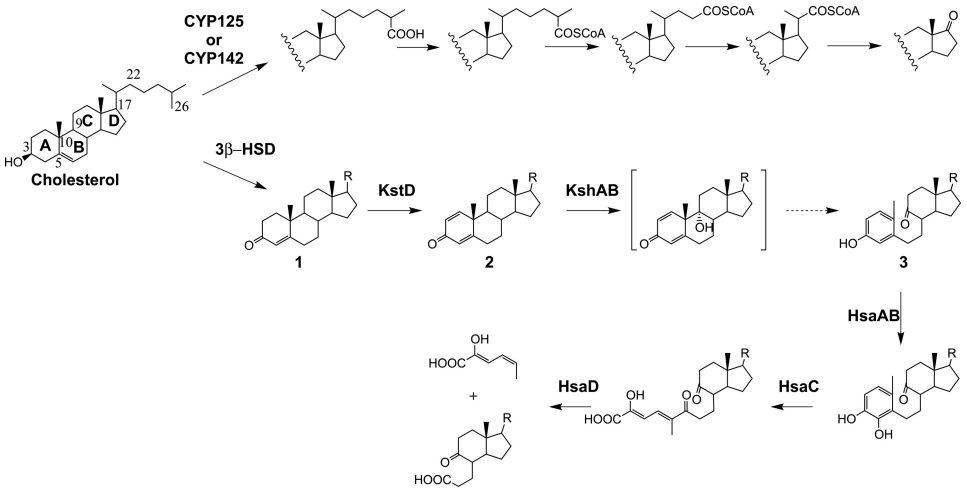

FIGURE 1. **Schematic of the proposed cholesterol degradation pathway of *Mtb*.** Key carbons of cholesterol are numbered using the steroid numbering convention. The processes of cholesterol side chain and partial ring degradation are shown as parallel pathways. Enzymes known to catalyze specific reactions in *Mtb* are indicated. Non-enzymatic reaction is shown as a *dashed arrow*. *Compounds 1*, *2*, and *3* represent 4-AD, ADD, and 3-hydroxy-9,10-seconandrost-1,3,5(10)-trien-9,17-dione (*3-HSA*) (R = ketone); 4-BNC, 1,4-BNC, and 3-HSBNC, (R = isopropionate); 4-BNC-CoA, 1,4-BNC-CoA, and 3-HSBNC-CoA, (R = isopropionyl-CoA).

17-keto metabolites (Fig. 1) were less than their non-steroid-transforming homologues (13–15). Genetic knockouts of several of the cholesterol degradation genes have resulted not only in failure to grow on cholesterol *in vitro* but in attenuated pathogenicity in various infection models (9, 11, 14, 16, 17).

One important aspect of cholesterol catabolism in *Mtb* that has yet to be established is the sequence of the side chain and ring degradation reactions with respect to each other. Metabolite accumulation studies for knockouts of *kshA* and *kstD*, encoding the ring-degrading enzymes 3-ketosteroid 9α-hydroxylase and Δ^1 3-ketosteroid dehydrogenase, respectively, have suggested that the side chain is completely degraded to the 17-ketone before any significant ring degradation occurs (18–20). This conclusion was further supported by the accumulation of the 17-keto metabolites 4-androstene-3,17-dione (4-AD) and 1,4-androstadiene-3,17-dione (ADD) in the supernatant of *Mtb* cultures (11). Interestingly, the order of particular reactions seems to be species-dependent. For example, 3β-hydroxy-steroid dehydrogenase (3β-HSD) of *Mtb* CDC1551 and *Mycobacterium bovis* BCG are able to transform cholesterol (21, 22), whereas the corresponding ring-degrading enzyme of *R. jostii* RHA1 requires the activity of the first side chain-transforming enzyme, 26-cholesterol hydroxylase (Cyp125) (23).

3-Ketosteroid 9α-hydroxylase comprises a Rieske oxygenase, encoded by *kshA*, and a reductase, encoded by *kshB* (24). KshAB utilizes O_2 and NADH and acts in concert with KstD to effect opening of ring B and aromatization of ring A in the *Mtb* cholesterol degradation pathway (15, 25) (Fig. 1). Studies of KshAB from *Mtb* (15) have probed its substrate specificity, suggesting that ADD is its physiological substrate. Nonetheless, KshAB exhibits specificity constants for the 17-keto steroids 4-AD and ADD that are 2–3 orders of magnitude less than those of other characterized Rieske oxygenases (15). Additionally, this enzyme exhibits an apparent $K_{m_{O_2}}$ in excess of 1.2 mM in the presence of ADD. The relatively low reaction rates observed for KshAB have prompted speculation that the true physiological substrate for this enzyme may not be a 17-keto steroid but an intermediate of cholesterol side chain degradation (15). Deleting either *kshA* or *kshB* completely abrogated the virulence of *Mtb* in several mouse and macrophage infection models, leading to the suggestion that the enzyme reaction product has an immunomodulatory function (17).

Herein we explored the hypothesis that the physiological substrates for cholesterol ring-degrading enzymes are intermediates of side chain β-oxidation bearing a CoA thioester. CoA thioester derivatives of the 17-isopropionate 3-keto steroids 3-oxo-23,24-bisnorchol-4-en-22-oic acid (4-BNC) and 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid (1,4-BNC) (Fig. 2) were synthesized using a rhodococcal acyl-CoA synthetase (CasI) and KstD from *Mtb*. The BNCs and their CoA thioesters were used to elucidate the substrate specificity of KshAB. The enzyme substrate binding pocket was investigated using molecular modeling. The substrate preference of KstD was also investigated. The results are discussed in terms of microbial steroid catabolism and the pathogenesis of *Mtb*.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—ADD and 4-BNC were purchased from Steraloids, Inc. (Newport, RI). 4-AD was purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes and the Expand High Fidelity PCR System were purchased from New England Biolabs (Ipswich, MA) and Roche Applied Science, respectively. Oligonucleotides for amplifying *kstD* were purchased from Integrated DNA Technologies (San Diego, CA) through the Nucleic Acid Protein Service Unit at the University of British Columbia. All other reagents were of HPLC or analytical grade. Water for buffers was purified using a Barnstead Nanopure Diamond^{TM} system (Dubuque, IA) to a resistivity of at least 18 megaohms.

Cloning of kstD—DNA was propagated, digested, ligated, and transformed using standard protocols (26). Plasmid DNA was purified as described previously (27) and was used to transform *Escherichia coli* by electroporation using a MicroPulser from

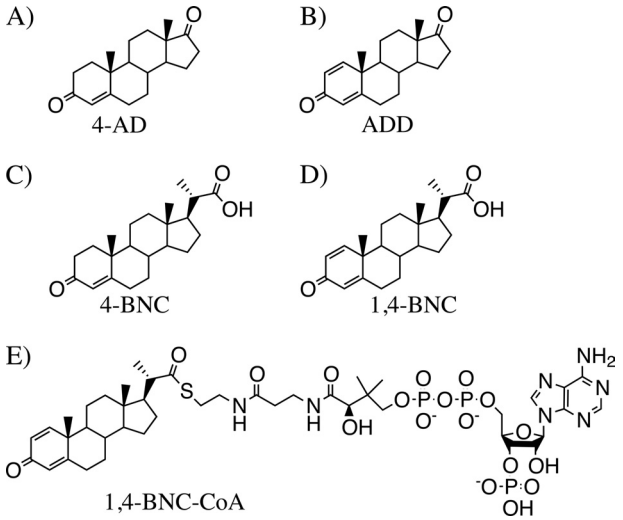

FIGURE 2. **Compounds investigated as KshAB substrates.** Shown are 4-AD (*A*), ADD (*B*), 4-BNC (*C*), 1,4-BNC (*D*), and 1,4-BNC-CoA (*E*). Not shown is 4-BNC-CoA, which differs from *E* by a 1–2 saturated bond on ring A.

Bio-Rad with Bio-Rad 0.1-cm GenePulser Cuvettes. The *kstD* gene was amplified from *Mtb* H37Rv genomic DNA using forward and reverse primers with the sequences 5′-TACGCTAGCACTGTGCAGGAGTTCGACGTCG-3′ (the NheI site is underlined) and 5′-CAGAATTCTCAGCGCTTTCCCGCCTG-3′ (the EcoRI site is underlined), respectively. Polymerase chain reactions contained 20 ng of template DNA, 2 units of the Expand High Fidelity PCR System polymerase, 20 μM each of dNTP, and 30 pmol each of oligonucleotide in a volume of 100 μl. Reactions were subject to 22 temperature cycles using a Stratagene Robocycler Gradient 96 instrument (La Jolla, CA) as follows: 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 60 s. The *kstD* amplicon was digested with NheI and EcoRI and ligated into pET-28a(+) (EMD) to yield pETKD1 such that the expressed protein was produced with an N-terminal thrombin-cleavable His_6 tag. Nucleotide sequences were confirmed by the Nucleic Acid Protein Service Unit.

Protein Production—KstD was produced in *E. coli* BL21(DE3) cells grown in LB with 25 μg/ml kanamycin at 20 °C and 200 rpm. Expression of *kstD* was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside when cells reached an A_{600} of 0.5. After 20 h, cells were harvested and washed by centrifugation. The cell pellet from 0.3 liters of culture was suspended in 0.5 ml of 50 mM Tris/HCl, pH 7.4, containing 0.175 mg/ml phenylmethanesulfonyl fluoride and DNase I and subjected to five rounds of bead beating using an MP Biomedicals FastPrep-24 bead beater (Solon, OH) set to 5.0 for 20 s with 4 min on ice between rounds. Cell debris was pelleted by centrifugation (16,100 $g \times 10$ min) at 4 °C. The supernatant, referred to hereafter as KstD-lysate, was separated from the pellet and kept on ice until use.

CasI (Ro05822) and CasG (Ro05820) of *R. jostii* RHA1 were produced in *E. coli* Rosetta™ (DE3)pLysS containing pETCasI and pETCasG.[5] Cells were grown at 37 °C with shaking at 200 rpm in 100 ml of LB supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Expression of the *cas* genes was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside when the culture attained an A_{600} of 0.6. The cells were incubated for a further 20 h at 16 °C and were then harvested by centrifugation. Pellets from 100 ml of culture were resuspended in 1 ml of 9 mM sodium phosphate, pH 8.0, containing 10% glycerol, the Complete Mini Protease Inhibitor mixture, and DNase I (Roche Applied Science) and then were lysed using a bead beater and centrifuged. Proteins were purified from the supernatant using a standard nickel-nitrilotriacetic acid protocol, exchanged into 25 mM HEPES, pH 7.5, with 50 mM potassium chloride by dialysis at 4 °C, and concentrated to ~5 mg/ml by ultrafiltration. Proteins were flash-frozen in liquid nitrogen and stored at −80 °C. KshA and KshB were produced and purified as previously described (15).

Preparation of Substrates—4-BNC was dissolved to concentrations of 50 mM in 94% ethanol containing 60 mM NaOH. 4-AD, ADD, and 1,4-BNC were dissolved in ethanol up to 15 and 25 mM. CoA thioesters were stored and used as aqueous solutions up to 6 mM. To produce 3-oxo-23,24-bisnorchol-4-en-22-oyl-coenzyme A thioester (4-BNC-CoA), 5.52 μmol of 4-BNC was incubated with 0.75 mg of purified CasI for 7 h at 22 °C in 6 ml of 100 mM HEPES buffer, pH 7.5, containing 6.77 μmol of CoASH, 30 μmol of $MgCl_2$, and 15 μmol of ATP. To produce 3-oxo-23,24-bisnorchola-1,4-dien-22-oyl-coenzyme A thioester (1,4-BNC-CoA), 3.02 μmol of 4-BNC-CoA was incubated with 0.6 mg (total protein) of KstD lysate for 2 h at 22 °C in 15 ml 50 mM Tris/HCl, pH 7.4, containing 6 μmol of NAD^+. To produce 1,4-BNC, 4.06 μmol of 4-BNC was incubated with 5 mg (total protein) of KstD lysate for 23 h at 22 °C in 4 ml of 50 mM Tris/HCl, pH 7.4, containing 8 μmol of NAD^+. The pH of the reaction solutions was adjusted to 7.4 with NaOH or HCl (for reactions containing 4-BNC-CoA and 4-BNC, respectively) before the addition of KstD lysate. After incubation, reaction mixtures were diluted 1:1 with methanol and incubated on ice for 10 min. Methanol was evaporated at room temperature under low pressure using a Savant SC110A SpeedVac concentrator (Thermo Electron Corp.). The mixture was centrifuged at 4 °C (16,000 $g \times 10$ min). The supernatant was recovered, treated a second time with methanol as described above, filtered using a 0.2 μm PTFE filter unit (Millipore), and stored on ice. Compounds were chromatographically purified using a Waters 2695 Separations HPLC module (Milford, MA) equipped with a Waters 2996 photodiode array detector and a LUNA 3 μm PFP(2) 50 × 4.6-mm column (Phenomenex, Torrance, CA) using a solvent system of 0.1 M ammonium acetate, pH 4.5 (Buffer A), and 90% methanol in 0.1 M ammonium acetate, pH 4.5 (Buffer B). 500 μl (for 1,4-BNC) or 1 ml (for the CoA thioesters) of the reaction mixture was injected onto the column, which was equilibrated with Buffer A. Eluates were monitored at 248 nm. 4-BNC-CoA was eluted using a gradient of 0 to 100% Buffer B over 20 min at 1 ml/min (Gradient A). Under these conditions, the retention times for 4-BNC and 4-BNC-CoA were 18.7 and 16.7 min, respectively. 1,4-BNC and 1,4-BNC-CoA were eluted using a biphasic gradient consisting of 0 to 70% Buffer B at 1 ml/min over 14 min followed by 70 to 100% Buffer B at 0.5 ml/min over 12 min (Gradient B). Under these conditions, the retention times for 4-BNC, 1,4-BNC, 4-BNC-CoA, and 1,4-BNC-CoA were 24.3, 22.7, 19.8, and 18.0 min, respectively. Methanol was removed under nitrogen flow, and

CoA thioesters were stored at −80 °C. Solutions of 1,4-BNC were lyophilized and stored at −80 °C.

Analysis of Substrates and Products—Reaction substrates and products were routinely verified by HPLC using the conditions described above. Reactions containing 4-AD and ADD were resolved using a gradient similar to Gradient A but at pH 5.0. Under these conditions the retention times for 4-AD and ADD were 17.1 and 16.2 min, respectively. CoA thioesters, including 3-hydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oyl-CoA thioester (3-HSBNC-CoA) were identified by subjecting them to alkaline hydrolysis at pH 13 for 1 h at 50 °C. The solutions were then acidified to pH ~ 4.5 with 6 M HCl and examined by HPLC to verify the generation of the corresponding steroid carboxylic acid and CoASH. Products from the hydrolyzed CoA thioester mixes were also extracted with ethyl acetate and dried. These compounds as well as purified 1,4-BNC were derivatized with trimethylsilyldiazomethane and subjected to gas chromatography-coupled mass spectrometry (GC-MS) performed using an HP 6890 series GC system fitted with an HP-5MS 30-m × 250-μm column (Hewlett-Packard, Palo Alto, CA) and an HP 5973 mass-selective detector. Additionally, purified samples of 4-BNC-CoA and 1,4-BNC-CoA were subjected to electrospray ionization-mass spectrometry (ESI-MS) using a Bruker Esquire-LC ion trap mass spectrometer equipped with an ESI ion source, which was operated in the positive ion mode. Samples were dissolved 1:9 in methanol to a final concentration of 20–30 pmol/μl. Five μl were injected at a flow rate of 200 μl/min in 1:9 water:methanol. The scanned mass range was m/z 50–2000 Da.

Steroid concentrations were measured spectrophotometrically using the following extinction coefficients for aqueous solutions at 22 °C: ϵ_{248} = 8,700 M^{-1} cm^{-1} for 4-BNC, ϵ_{248} = 8,500 M^{-1} cm^{-1} for 1,4-BNC, ϵ_{248} = 17,200 M^{-1} cm^{-1} for 4-BNC-CoA, and ϵ_{248} = 16,900 M^{-1} cm^{-1} for 1,4-BNC-CoA. The extinction coefficient for 4-BNC was measured using a solution containing a gravimetrically determined amount of the compound. That for 1,4-BNC was calculated from the ratios of values for 4-AD and ADD and for 4-AD and 4-BNC assuming a linear relationship for the contributions of the Δ^1 bond and isopropionate group. The values for 4-BNC-CoA and 1,4-BNC-CoA were calculated from linear sums of the values for the steroid and CoA components. The extinction coefficients were validated in an oxygraph assay as described below.

Steady-state Kinetics—Steady-state kinetic analysis was performed for KshA using an oxygraph system as described previously (15). Activity assays were performed at 22 °C in a total volume of 1 ml of air-saturated 0.1 M potassium phosphate, pH 7.0, containing 430 μM NADH, 1.1 μM KshB, and 0.2 μM KshA. The reaction was initiated by adding substrate after equilibration of all other components for 3 min. The apparent steady-state kinetic parameters for O_2 were measured in the presence of 57 μM 1,4-BNC-CoA. Kinetic parameters were evaluated by fitting the Michaelis-Menten equation to the data using the least-squares fitting and dynamic weighting options of LEONORA (28).

Measuring KstD Reaction—Reactions were performed at 22 °C in a 1-ml volume containing 200 μM 4-AD or 4-BNC-CoA, 400 μM NAD^+, and 0.04 mg (total protein) KstD-lysate in 50 mM Tris/HCl, pH 7.4. For reactions containing 4-BNC-CoA, the reaction pH was readjusted to 7.4 with NaOH before the addition of the KstD-lysate. At various time points, 200 μl of each reaction was stopped by rapid dilution into 200 μl of methanol, evaporated to remove methanol, filtered, and analyzed by HPLC.

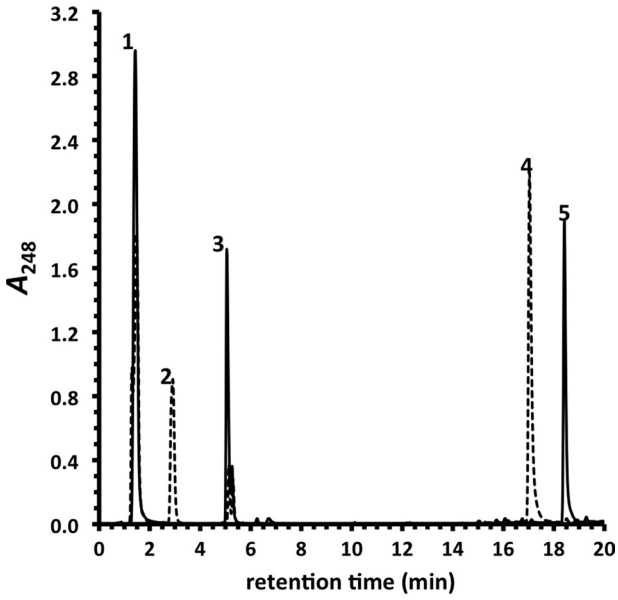

FIGURE 3. **Transformation of 4-BNC to 4-BNC-CoA by CasI.** HPLC chromatograms are shown of 50 μl of a CasI reaction (2 μM, *dashed line*) and no-enzyme control (*solid line*) containing 0.9 mM 4-BNC, 1.1 mM CoASH, 5 mM $MgCl_2$, and 2.5 mM ATP after 7 h. Peaks were resolved using a linear gradient of 0 to 90% methanol in 0.1 M ammonium acetate, pH 4.5, over 20 min. Substrate peaks are numbered: *1*, ATP; *3*, CoASH; *5*, 4-BNC. Product peaks are numbered: *2*, AMP; *4*, 4-BNC-CoA.

Modeling of Substrates into KshA Crystal Structure—Models of 1,4-BNC and 1,4-BNC-CoA were generated using PRODRG (29) and were docked to KshA (PDB 2ZYL) (15) with AutoDock Vina (30) using default parameters. A grid of 20 × 24 × 20 Å centered about the active site was defined as the searchable area for placement of the ligand. The top 10 docked ligand conformations were output, and the one with the lowest binding energy was chosen as the best structure.

RESULTS

Production and Purification of Steroid CoA Thioesters—Two potential CoA thioester side chain degradation intermediates were generated to test the hypothesis that such compounds are the physiological substrates of KshAB. To generate the CoA thioesters, two acyl-CoA synthetases, CasI and CasG, from *R. jostii* RHA1 were tested for their ability to esterify 4-BNC.

Transformation of 4-BNC and CoASH in the presence of ATP and Mg^{2+} by CasI resulted in the time-dependent depletion of all substrate peaks, and the formation of two new peaks resolved by HPLC using Gradient A (Fig. 3). The first product peak had the same retention time (2.8 min) and spectrum as an AMP standard. The second product peak eluted at 16.7 min and had a spectrum with features characteristic of both the steroid and CoA components. This product was purified and further characterized. Alkaline hydrolysis yielded 4-BNC and CoASH as determined by HPLC. The product identity of 4-BNC-CoA

was confirmed by electrospray ionization-MS (supplemental Fig. 1*A*). Under the described reaction conditions, the product yield was typically 75%. By contrast, CasG did not detectably catalyze the thioesterification of 4-BNC.

To generate 1,4-BNC-CoA, purified 4-BNC-CoA was incubated with lysate from cells containing KstD of *Mtb.* KstD has previously been shown to catalyze the Δ^1-dehydrogenation of 4-AD,[6] 5α-androstane-3,17-dione, 5α-testosterone, and progesterone (31). Monitoring of the reaction by HPLC revealed a decrease in the 4-BNC-CoA peak and appearance of a new peak eluting at 18 min of Gradient B. This compound was analyzed as described above and identified as 1,4-BNC-CoA (supplemental Fig. 1*B*). Transformation of 4-BNC-CoA to 1,4-BNC-CoA by incubation with KstD-lysate resulted in 87% conversion after 2 h. A greater total yield of 1,4-BNC-CoA could be obtained by adding KstD-lysate and NAD^+ directly to the CasI reaction mixture after 3 h, omitting the intermediate purification step. Transformation of 4-BNC to 1,4-BNC by incubation with KstD-lysate using a similar procedure resulted in a >90% turnover after 23 h. The product identity was confirmed by GC-MS analysis (supplemental Fig. 1*C*).

KshAB Transforms CoA Thioester Steroids—Consistent with KshAB of *Mtb* catalyzing the 9α-hydroxylation of 4-AD and ADD (15), each of 4-BNC, 1,4-BNC, 4-BNC-CoA, and 1,4-BNC-CoA stimulated the depletion of oxygen in the presence of KshA, KshB, and NADH in the standard oxygen electrode activity assay. For each steroid substrate, the amount of oxygen consumed corresponded with the calculated amount of substrate added. These data are consistent with full coupling between oxygen and steroid substrate consumption and indicate that the extinction coefficients calculated for each of 1,4-BNC, 4-BNC-CoA, and 1,4-BNC-CoA are accurate.

To confirm that KshAB catalyzes the 9α-hydroxylation of the thioester substrates, a reaction mixture of KshAB with 1,4-BNC-CoA was examined by HPLC. The product peak had a retention time (16.8 min using Gradient B) and a spectrum that was identical to those of 3-HSBNC-CoA.[7] Moreover, alkaline hydrolysis yielded 3-HSBNC and CoASH, as determined by GC-MS (supplemental Fig. 1*D*) and HPLC analyses, confirming the product identity.

Steady-state Kinetic Parameters for KshAB—The specificity of KshAB for each of the substrates discussed above was investigated using the oxygen electrode assay at ambient oxygen concentration (Fig. 4) (Table 1). Under these conditions, the apparent specificity constants were 1,4-BNC-CoA > 4-BNC-CoA > 4-BNC > 1,4-BNC. Significantly, apparent k_{cat}/K_m values for all compounds except 1,4-BNC were greater than for either previously proposed 17-keto substrates, 4-AD and ADD (15). In fact, the value for 1,4-BNC-CoA (160,000 ± 20,000 M^{-1} s^{-1}) is more than 20 times greater than that for ADD, the previously proposed physiological substrate.

The turnover numbers were consistently greater for substrates with $\Delta^{1,4}$ desaturated A rings: k_{cat} = 0.25 ± 0.03 s^{-1} for 1,4-BNC *versus* 0.08 ± 0.01 s^{-1} for 4-BNC and 2.7 ± 0.2 s^{-1} for 1,4-BNC-CoA *versus* 0.61 ± 0.03 s^{-1} for 4-BNC-CoA. Significantly, the turnover numbers for the CoA thioester substrates were approximately nine and three times greater than for the corresponding 17-keto steroids 4-AD and ADD, respectively. The K_m values for 4-BNC-CoA and 1,4-BNC-CoA were also 3.5 and 6.5 times less than those for their 17-keto derivatives.

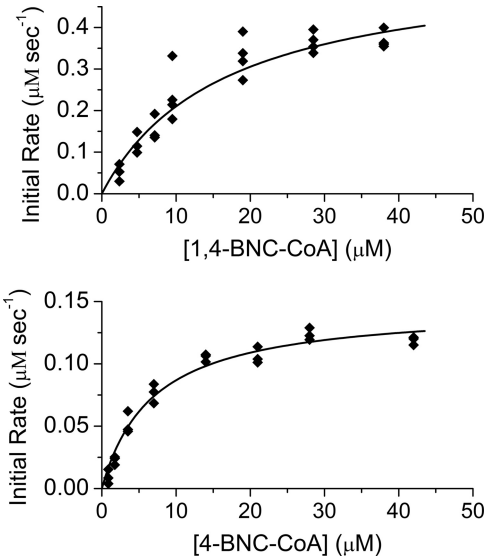

FIGURE 4. **Steady-state kinetic analyses of KshAB with CoA thioesters.** Shown is the dependence of the initial velocity of O_2 consumption on 1,4-BNC-CoA (*A*) and 4-BNC-CoA (*B*) concentrations in air-saturated buffer with fitted parameters K_m = 17 ± 3 and 6.8 ± 0.1 μM, and V_{max} = 0.56 ± 0.04 and 0.146 ± 0.007 μM s^{-1}, respectively. The best fit of the Michaelis-Menten equation to the data using the least squares dynamic weighting options of LEONORA is represented as a *solid line*.

TABLE 1

Apparent steady-state kinetic parameters of KshAB for various steroids

Experiments were performed using 0.1 M potassium phosphate, pH 7.0, at 22 °C. Values reported in parentheses represent S.E.

Substrate	K_m	k_{cat}	K_{cat}/K_m
	μM	s^{-1}	$M^{-1}s^{-1}$
4-AD[a]	24 (16)	0.07 (0.01)	3,000 (2,000)
ADD[a]	110 (20)	0.80 (0.05)	7,600 (700)
4-BNC	3 (2)	0.08 (0.01)	30,000 (10,000)
1,4-BNC	70 (10)	0.25 (0.02)	3,500 (400)
4-BNC-CoA	6.8 (0.1)	0.61 (0.03)	90,000 (10,000)
1,4-BNC-CoA	17 (3)	2.7 (0.2)	160,000 (20,000)

[a] Values presented in Capyk *et al.* (15).

The reactivity of KshAB with O_2 was investigated in the presence of 1,4-BNC-CoA. In the presence of 57 μM 1,4-BNC-CoA, the apparent steady-state kinetic parameters for O_2 were K_m = 90 ± 10 μM, k_{cat} = 2.5 ± 0.1 s^{-1}, and k_{cat}/K_m = 29,000 ± 2,000 M^{-1} s^{-1}.

To further probe the role of the CoA moiety in KshAB reactivity, we tested the ability of CoASH to inhibit enzymatic turnover of either ADD or 1,4-BNC-CoA. These assays were carried out under the conditions of the standard assay with the steroid substrates at concentrations corresponding to their respective K_m values. No inhibition was observed at CoASH concentrations up to 10-fold greater than substrate concentration (results not shown).

Reaction of KstD with 4-AD and 4-BNC-CoA—To explore further the possibility that CoA thioesters are the relevant substrates for cholesterol ring catabolic enzymes in *Mtb*, we investigated the reaction rates of KstD with each of 4-AD and

[6] J. K. Capyk and L. D. Eltis, unpublished observations.
[7] I. Casabon and L. D. Eltis, unpublished observations.

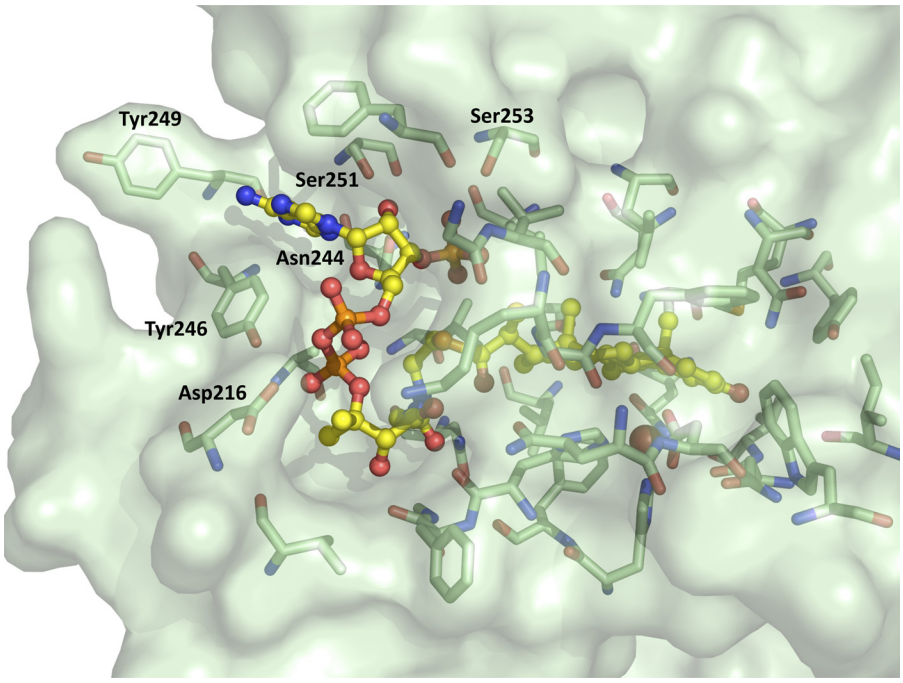

FIGURE 5. **Docking of 1,4-BNC-CoA in active site of KshA.** 1,4-BNC-CoA is shown as a *ball and stick representation*. KshA (PDB ID 2ZYL (15)) is shown as a *green*, semitransparent surface. KshA amino acids within 4 Å of the CoA group of 1,4-BNC-CoA are shown as *sticks* and labeled. Carbon atoms of amino acid residues and the substrate are colored *green* and *yellow*, respectively. Oxygen, phosphorus, and nitrogen atoms are shown in *red*, *orange*, and *blue*, respectively. The catalytic iron is shown as a rust-colored sphere. Residues mentioned in the text are labeled.

4-BNC-CoA (supplemental Fig. 2). Each compound was incubated with 40 μg (total protein) of KstD-lysate, and reaction progress was followed by HPLC analysis at various time points. After 2 h, 87% of 4-BNC-CoA had been converted to 1,4-BNC-CoA, whereas only 30% of 4-AD had been turned over. Complete conversion was not observed over extended periods without the addition of fresh enzyme, suggesting that there is a time-dependent decrease in enzyme activity. Under these conditions, the specific activity of 1,4-BNC-CoA was estimated at 195 μM mg^{-1} min^{-1}, whereas that for 4-AD was 25 μM mg^{-1} min^{-1}. Similar results were obtained using each of three different concentrations of KstD-lysate.

Modeling of 1,4-BNC-CoA in Active Site of KshA—Attempts to produce high quality KshA crystals with bound substrate by either co-crystallization or soaking experiments were not successful. To investigate the likely spatial implications of KshA CoA thioester substrates, docking simulations were performed using the published crystal structure of KshA (PDB ID 2ZYL) (15) and a structural model of 1,4-BNC-CoA.

In a previous experiment ADD docked to KshA in two orientations within the enzyme active site. One of these orientations was conducive to 9α-hydroxylation of the molecule, albeit orienting the C17 ketone group toward the interior of the enzyme rather than pointing out of the active site channel (15). Initial docking experiments of 1,4-BNC revealed a positioning of the substrate that is inconsistent with the known outcome of the reaction; C5 and C6 of ring A, rather than C9, were positioned close to the catalytic iron. This orientation had a calculated binding energy of -8.6 kcal mol^{-1}. By contrast, simulations using 1,4-BNC-CoA resulted in a single, well defined conformation of the steroid moiety with a docking energy of -11.1 kcal mol^{-1} (Fig. 5). This conformation was conducive to 9α-hydroxylation. The CoA moiety adopted more than one conformation in different simulation experiments, all of which had similar binding energies. These conformations show significantly different potential binding positions for CoA at the mouth of the KshA active site channel. The conformation with the lowest binding energy is shown.

The docked model shows the CoA group fitting snuggly into a pocket at the mouth of the active site channel. It also shows several predicted polar contacts including between the adenine moiety, Ser^{251}, and the carboxylate of Tyr^{249}; the ribose hydroxyl group and Asn^{244}; the ribose phosphate, Ser^{253}, and Asn^{244}; the phosphate proximal to the steroid group, Asp^{216}, and Tyr^{246}. Most of the above-mentioned residues are not conserved in KshAs from *Rhodococcus erythropolis* SQ1, *Rhodococcus rhodochrous* DSM43269, and *R. jostii* RHA1, all of which are predicted to be involved in steroid catabolism (20, 32, 33). However, the substrate specificities of the rhodococcal enzymes have not been defined, and it is likely that many do not act on CoA thioester substrates.

DISCUSSION

The results we present suggest that steroid CoA thioesters are the likely physiological substrates for *Mtb* KshAB. The steady-state kinetic analyses demonstrate that KshAB transforms the CoA thioesters more efficiently than 17-keto steroids, the previously proposed physiological substrates (15). This finding is especially dramatic in the enzyme reactivity with oxygen. The k_{cat}/K_{mO_2} in the presence of 1,4-BNC-CoA is an order of magnitude greater than that measured in the presence of ADD (15). Perhaps more significantly, the K_{mO_2} of 90 ± 10 μM is similar to that of other oxygenases in the cholesterol degradation pathway (13, 14) as well as in the lung tissues of healthy

rabbits (34). This is compared with the value in excess of 1.2 mM previously measured in the presence of ADD (15). The apparent specificity constants for steroid metabolites were measured in air-equilibrated buffer, so the high $K_{m\mathrm{O}_2}$ for the keto-substituted steroid means O_2 was well below saturating levels. In contrast, the lower $K_{m\mathrm{O}_2}$ in the presence of a CoA thioester substrate means that in the presence of the latter, KshAB is closer to being saturated with O_2. Thus, the increased reactivity of KshAB for O_2 in the presence of the CoA thioester substrates could account for the greater apparent substrate specificity compared with the 17-keto substrates.

The steady-state kinetic parameters reveal that the turnover numbers and K_m values for 4-BNC-CoA and 1,4-BNC-CoA are significantly different but that the specificity constants for the two compounds differ by less than 2-fold. The kinetic differences between the Δ^4 and $\Delta^{1,4}$ thioesters mirror what was found previously for 4-AD and ADD (15). This trend of greater k_{cat} and K_m for $\Delta^{1,4}$ compounds than for their Δ^4 counterparts is also evident in the comparison of 4-BNC and 1,4-BNC notwithstanding the relatively low specificity constant for the latter. The mechanistic determinants for the KshAB low turnover number for 1,4-BNC compared with other $\Delta^{1,4}$ substrates are not clear from the available data. The relative substrate specificities of KshAB for Δ^4 and $\Delta^{1,4}$ compounds appear to differ between species, with KshAB from *Mycobacterium smegmatis* exhibiting a much lower capacity to transform Δ^4 steroids than *Mtb* KshAB, based on metabolite accumulation studies in Δ*kstD* knock-out cultures (18, 19).

This study suggests 4-BNC-CoA and 1,4-BNC-CoA as candidate physiological substrates for KshAB but does not address the possibility of other CoA thioester substrates. Our molecular docking experiment demonstrates that there is room for this large molecule in the enzyme active site channel and that favorable electrostatic interactions with the CoA moiety may exist and could contribute to KshA substrate specificity. Nonetheless, KshAB inhibition by CoASH was not observed, suggesting the significantly greater apparent specificity constants for the CoA thioester substrates is not solely attributable to KshA affinity for the CoA moiety. The side chain degradation pathway proposed for *Mtb* (11) includes CoA thioester steroids with 8-carbon and 5-carbon side chains in addition to the 3-carbon isopropionyl-CoA thioester steroids we tested. Additionally, compounds with oxidized and hydrated side chains are produced during each round of β-oxidation. Further studies are needed to determine whether KshAB preferentially hydroxylates a particular thioester.

A major implication of CoA thioesters as physiological substrates for KshAB is concurrent cholesterol side chain and ring degradation in *Mtb.* All previous biochemical experiments on purified *Mtb* cholesterol ring-degrading enzymes have used compounds representing end products of side chain degradation (13–15, 35). The CoA thioesters examined in this study represent hypothetical intermediates in cholesterol side chain degradation, suggesting that the true physiological substrates for some of these enzymes may also be side chain degradation intermediates, and countering the current paradigm that cholesterol side chain degradation in *Mtb* is completed before breakdown of the ring structure. This has implications for the probable steroid molecules produced during cholesterol degradation in *Mtb* as well as for the design of small molecule inhibitors for the cholesterol ring-degrading enzymes. KshAB has been implicated in the management of potentially immunomodulatory steroids suggested to play a role in early infection (17), and our results broaden the range of potential compounds in this category. Additionally, these results expand the potential substrate range and industrial potential of steroid ring-degrading enzymes from other species. It remains unclear what degree of side chain degradation is completed before the activity of each ring-degrading enzyme *in vivo* or whether physiologically relevant parallel pathways exist. This study, in combination with previous investigations (15, 17), strongly suggests the existence of more than one functional pathway in *Mtb*.

The rhodococcal acyl-CoA synthetases provide useful tools for further study of the cholesterol degradation pathway in *Mtb*. None of the FadDs in the *Mtb* cholesterol degradation cluster (*fadD17*, *Rv3506*; *fadD19*, *Rv3515c*; *fadD3*, *Rv3561*) was able to catalyze CoA esterification of 4-BNC. This result is not unexpected as the proposed side chain degradation pathway is predicted to involve a single acyl-CoA synthetase activity that forms the C26 CoA thioester; further CoA groups are incorporated by thiolase enzymes. Although the physiological substrates for *R. jostii* RHA1 CasI and CasG have not been determined, the ability of enzymes from *R. jostii* RHA1 to transform steroids of intermediate side chain length coupled with the methodology we developed to purify and manipulate these compounds could be exploited for research in a variety of contexts. Such compounds can be used to study potential cholesterol catabolic intermediates as substrates for a range of enzymes in *Mtb* and other bacteria and as standards to interrogate the occurrence of bacterial and mammalian *in vivo* metabolites.

Our knowledge of the *Mtb* cholesterol degradation pathway comprises a list of catabolic "limits" defining the biochemical capacities of specific enzymes rather than their physiological roles. Both the *in vitro* nature of experiments exploring this pathway and a level of facultative promiscuity suggested by current data for some of the biochemically investigated enzymes contribute to the limitations inherent in our understanding of the pathway. Thus, it is established that 3β-hydroxysteroid dehydrogenase (3β-HSD) but not KstD can transform cholesterol before Cyp125 catalyzes the first side chain reaction, and that Cyp125 is capable of transforming cholesterol or 4-cholesten-3-one (21, 22, 36). Likewise, side chain degradation can proceed to its conclusion before the action of either KstD or KshA (11), but both of these enzymes can transform intermediates of side chain degradation.

The current view of the metabolites occurring in the cholesterol degradation pathway derives from *in vitro* or gene knockout studies that do not faithfully recapitulate the physiological conditions for *Mtb* in the human host. Indeed, even wild type *Mtb* grown under laboratory conditions cannot be expected to be representative of a naturally occurring physiological state. This discrepancy in growth conditions may explain why 4-AD and ADD were observed to accumulate in *Mtb* culture supernatants (11), whereas our results suggest that the KstD and KshA reactions cleaving ring B likely take place before forma-

tion of the 17-ketone. Similarly, a previous study was not able to detect the transformation of 4-AD by KstD (31), whereas we are able to measure a reaction rate. Although the substrate specificities of purified enzymes are suggestive of physiologically relevant reactions, the reaction conditions used for these assays are far removed from those in the bacterial cytoplasm. Full understanding of the cholesterol catabolites in *Mtb*, therefore, requires us to probe these compounds in *Mtb* growing under conditions representative of human infection.

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REFERENCES

1. WHO (2010) *Global Tuberculosis Control: WHO Report* 2010, pp. 2–42, World Health Organization, Geneva, Switzerland
2. Russell, D. G. (2007) *Nat. Rev. Microbiol* **5,** 39–47
3. Russell, D. G., VanderVen, B. C., Lee, W., Abramovitch, R. B., Kim, M. J., Homolka, S., Niemann, S., and Rohde, K. H. (2010) *Cell Host Microbe* **8,** 68–76
4. Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C., and Schoolnik, G. K. (2003) *J. Exp. Med.* **198,** 693–704
5. Homolka, S., Niemann, S., Russell, D. G., and Rohde, K. H. (2010) *PLoS Pathog.* **6,** e1000988
6. Baek, S. H., Li, A. H., and Sassetti, C. M. (2011) *PLoS Biol.* **9,** e1001065
7. McKinney, J. D., Höner zu Bentrup, K., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr., and Russell, D. G. (2000) *Nature* **406,** 735–738
8. Van der Geize, R., Yam, K., Heuser, T., Wilbrink, M. H., Hara, H., Anderton, M. C., Sim, E., Dijkhuizen, L., Davies, J. E., Mohn, W. W., and Eltis, L. D. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 1947–1952
9. Pandey, A. K., and Sassetti, C. M. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 4376–4380
10. Birkenmaier, A., Holert, J., Erdbrink, H., Moeller, H. M., Friemel, A., Schoenenberger, R., Suter, M. J., Klebensberger, J., and Philipp, B. (2007) *J. Bacteriol* **189,** 7165–7173
11. Nesbitt, N. M., Yang, X., Fontán, P., Kolesnikova, I., Smith, I., Sampson, N. S., and Dubnau, E. (2010) *Infect. Immun.* **78,** 275–282
12. Wilbrink, M. H., Petrusma, M., Dijkhuizen, L., and van der Geize, R. (2011) *Appl. Environ. Microbiol.* **77,** 4455–4464
13. Dresen, C., Lin, L. Y., D'Angelo, I., Tocheva, E. I., Strynadka, N., and Eltis, L. D. (2010) *J. Biol. Chem.* **285,** 22264–22275
14. Yam, K. C., D'Angelo, I., Kalscheuer, R., Zhu, H., Wang, J. X., Snieckus, V., Ly, L. H., Converse, P. J., Jacobs, W. R., Jr., Strynadka, N., and Eltis, L. D. (2009) *PLoS Pathog.* **5,** e1000344
15. Capyk, J. K., D'Angelo, I., Strynadka, N. C., and Eltis, L. D. (2009) *J. Biol. Chem.* **284,** 9937–9946
16. Chang, J. C., Harik, N. S., Liao, R. P., and Sherman, D. R. (2007) *J. Infect Dis.* **196,** 788–795
17. Hu, Y., van der Geize, R., Besra, G. S., Gurcha, S. S., Liu, A., Rohde, M., Singh, M., and Coates, A. (2010) *Mol. Microbiol.* **75,** 107–121
18. Brzostek, A., Sliwiński, T., Rumijowska-Galewicz, A., Korycka-Machała, M., and Dziadek, J. (2005) *Microbiology* **151,** 2393–2402
19. Brzostek, A., Pawelczyk, J., Rumijowska-Galewicz, A., Dziadek, B., and Dziadek, J. (2009) *J. Bacteriol.* **191,** 6584–6591
20. Petrusma, M., Hessels, G., Dijkhuizen, L., and van der Geize, R. (2011) *J. Bacteriol* **193,** 3931–3940
21. Ouellet, H., Guan, S., Johnston, J. B., Chow, E. D., Kells, P. M., Burlingame, A. L., Cox, J. S., Podust, L. M., and de Montellano, P. R. (2010) *Mol. Microbiol.* **77,** 730–742
22. Capyk, J. K., Kalscheuer, R., Stewart, G. R., Liu, J., Kwon, H., Zhao, R., Okamoto, S., Jacobs, W. R., Jr., Eltis, L. D., and Mohn, W. W. (2009) *J. Biol. Chem.* **284,** 35534–35542
23. Rosłoniec, K. Z., Wilbrink, M. H., Capyk, J. K., Mohn, W. W., Ostendorf, M., van der Geize, R., Dijkhuizen, L., and Eltis, L. D. (2009) *Mol. Microbiol.* **74,** 1031–1043
24. van der Geize, R., Hessels, G. I., van Gerwen, R., van der Meijden, P., and Dijkhuizen, L. (2002) *Mol. Microbiol.* **45,** 1007–1018
25. Petrusma, M., Dijkhuizen, L., and van der Geize, R. (2009) *Appl. Environ. Microbiol.* **75,** 5300–5307
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Pulleyblank, D., Michalak, M., Daisley, S. L., and Glick, R. (1983) *Mol. Biol. Rep.* **9,** 191–195
28. Cornish-Bowden, A. (1994) *Analysis of Enzyme Kinetic Data*, Oxford University Press, Oxford, New York
29. Schüttelkopf, A. W., and van Aalten, D. M. (2004) *Acta Crystallogr. D Biol. Crystallogr.* **60,** 1355–1363
30. Trott, O., and Olson, A. J. (2010) *J. Comput. Chem.* **31,** 455–461
31. Knol, J., Bodewits, K., Hessels, G. I., Dijkhuizen, L., and van der Geize, R. (2008) *Biochem. J.* **410,** 339–346
32. Yam, K. C., van der Geize, R., and Eltis, L. D. (2010) in *Biology of Rhodococcus* (Alvarez, H. M., ed.) pp. 133–170, Springer-Verlag New York Inc., New York
33. van der Geize, R., Hessels, G. I., Nienhuis-Kuiper, M., and Dijkhuizen, L. (2008) *Appl. Environ. Microbiol.* **74,** 7197–7203
34. Via, L. E., Lin, P. L., Ray, S. M., Carrillo, J., Allen, S. S., Eum, S. Y., Taylor, K., Klein, E., Manjunatha, U., Gonzales, J., Lee, E. G., Park, S. K., Raleigh, J. A., Cho, S. N., McMurray, D. N., Flynn, J. L., and Barry, C. E., 3rd (2008) *Infect. Immun.* **76,** 2333–2340
35. Lack, N. A., Yam, K. C., Lowe, E. D., Horsman, G. P., Owen, R. L., Sim, E., and Eltis, L. D. (2010) *J. Biol. Chem.* **285,** 434–443
36. Yang, X., Dubnau, E., Smith, I., and Sampson, N. S. (2007) *Biochemistry* **46,** 9058–9067