Supplemental Information for:

Enzymatic β-oxidation of the cholesterol side chain in *Mycobacterium tuberculosis* bifurcates stereospecifically at hydration of 3-oxo-cholest-4,22-dien-24-oyl-CoA

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Table S1. Expression constructs used in this work.

| Construct name | Genes    | Restriction sites | Purified enzyme     | Source/Reference |
|----------------|----------|-------------------|---------------------|------------------|
| pTipQC1        | -        | -                 | -                   | 1                |
| pchsB1         | Rv3502c  | NdeI/HindIII      | N-His6-ChsHAD       | This work<sup>a</sup> |
| pchsH3         | Rv3538   | NdeI/HindIII      | N-His6-ChsH3        | This work<sup>a</sup> |

<sup>a</sup>Genes were cloned from H37Rv genomic DNA and ligated into pTipQC1 with the indicated restriction sites to include an N-terminal His<sub>6</sub> fusion tag.
Figure S1. SDS-PAGE analysis of isolated ChsB1 (A) and ChsH3 (B).
Figure S2. ChsB1 and ChsH3 both exist as homodimers in solution. (A) Sedimentation velocity analytical ultracentrifugation (SV-AUC) experiments of ChsB1 shows it sediments as a dimer. (B) SV-AUC experiments of ChsH3 shows it sediments as a dimer. (C) Representative sedimentation velocity AUC profile of ChsB1. (D) Representative sedimentation velocity AUC profile of ChsH3. The absorbance of the sample at 280 nm and the residuals are plotted against the radial position in the cell. One in every ten scans is plotted. (E) Estimated molecular weights from AUC analysis and theoretical molecular weights.

|       | Sedimentation Coefficient (S) | Estimated MW (kDa) | Theoretical MW for homodimer (kDa) | RMSD   |
|-------|-------------------------------|--------------------|-------------------------------------|--------|
| ChsB1 | 4.18                          | 61.4               | 65.4                                | 0.003925 |
| ChsH3 | 3.77                          | 57.9               | 60.4                                | 0.004254 |
Figure S3. Protein sequence alignment of ChsH3 and its homologs across species. The active site housing segment of ChsH3 is aligned with other MaoC-like enoyl-CoA hydratases across species using Clustal Omega. The conserved active site residues are highlighted. The protein sequence aligned are: ChsH1 (Rv3541c) from Mtb; peroxisomal hydratase-dehydrogenase-epimerase, Chain A, pdb 1PN2, from Candida tropicalis; 2-enoyl-CoA hydratase 2 domain of peroxisomal multifunctional enzyme type 2, pdb 1S9C, from Homo sapiens; putative MaoC-like dehydratase, BAC73582.1, from Streptomyces avermitilis; ChsH3 (Rv3538) from Mtb; protein NFA_4620, BAD55304.1, from Nocardia farcinica.
Figure S4. Phylogenetic trees for ChsH3 or EchA19 and its homologues across Actinobacteria. Homologues of ChsH3 and EchA19 were identified from BLAST. All of the ChsH3 and EchA19 homologues with over 60% identities to the Mtb proteins are found to be present simultaneously in the same organism. Phylogenetic trees are generated by Clustal Omega.
Figure S5. ChsB1 dimer interaction site of alpha helix 10 of monomer 1 with alpha helix 9 of monomer 2.
Figure S6. Structural alignment of ChsB1 and its homologs across species. The apoprotein structure of ChsB1 in cyan is aligned with its homologues from rat (PDB: 1GZ6) in yellow, human (PDB: 1ZBQ) in blue, and *Candida tropicalis* (PDB: 2ET6) in silver. Strand β8 from the ChsB1 structure overlaid with the homologous β strands is enlarged from the box.
Figure S7. Predicted CoA binding residues in ChsB1 by CoABind. The acetoacetyl-CoA in the structure was modeled based on the similarity between ChsB1 and (R)-3-hydroxybutyryl-CoA dehydrogenase from *Ralstonia eutropha* (PDB: 4N5M).
Reference

1. Nakashima, N.; Tamura, T., Isolation and characterization of a rolling-circle-type plasmid from Rhodococcus erythropolis and application of the plasmid to multiple-recombinant-protein expression. *Appl Environ Microbiol* **2004**, *70* (9), 5557-68.