Appendix for

Cholesterol Is Not an Essential Source of Nutrition for *Mycobacterium tuberculosis* During Infection

Xinxin Yang,[1,2] Jin Gao,[1,3] Issar Smith,[4] Eugenie Dubnau[4] and Nicole S. Sampson[1,*]

[1]Department of Chemistry, Stony Brook University, NY, 11794-3400

[2]Present Address: Tuberculosis Research Section, LIG/NIAID/NIH, Bethesda, MD, 20892

[3]Present Address: Department of Chemistry, Boston University, Boston, MA, 02215

[4]Public Health Research Institute and [3]Department of Medicine, University of Medicine and Dentistry New Jersey-New Jersey Medical School, Newark, NJ

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Experimental Methods

Bacterial strains, media, growth conditions, and buffers. 7H9 Middlebrook broth supplemented with 0.2% (w/v) glucose and 0.05% (v/v) Tween-80, supplemented with 50 μg mL^{-1} hygromycin was used as the protein expression medium for *M. smegmatis* cultures. Agar growth media for carbon source comparison were prepared in 7H10 agar +1.5% agar as follows: medium A: supplemented with 5 mL L^{-1} glycerol; medium B: supplemented with 2 mM cholesterol and 1.5% 2-propanol (v/v); medium C: supplemented with 1.5% 2-propanol.

M. tb cultures were grown at 37 °C in Middlebrook 7H9 liquid media (Becton Dickinson), supplemented with 0.05% Tween-80, 10% albumin-dextrose-NaCl complex (ADN) (2) and 0.2% glycerol, or on Middlebrook 7H10 plates supplemented the same way. Kanamycin was added at 20 μg mL^{-1} and hygromycin at 100 μg mL^{-1}. Growth on cholesterol as a sole carbon source in liquid media was done by supplementing 7H9 media with 1 mg mL^{-1} cholesterol made up in the nonionic surfactant tyloxapol (Sigma). The stock solution was 20 mg mL^{-1} cholesterol made in pure tyloxapol, autoclaved, and then boiled to dissolve the cholesterol completely. When this is diluted 20-fold into 7H9, it must be boiled again to enable the formation of a uniform medium. Under these conditions, the cholesterol does not precipitate and the growth of the culture can be followed by measuring optical density (OD) at 540 nm. When *M. tb* cholesterol metabolism was investigated, cultures were grown to mid-log phase in 7H9 supplemented with 0.05% Tween-80, 10% albumin-dextrose-NaCl complex (ADN) (2) and 0.2% glycerol and then cholesterol (1 mg mL^{-1} final concentration) in Tween-80 (1% w/v final concentration) was added. The cultures were incubated for an additional 5 h. Cholesterol stock solutions (20 mg mL^{-1}) for metabolic analysis were prepared in aqueous Tween-80 (20% w/v).

The buffers used include: binding buffer: 20 mM TrisHCl, 10 mM imidazole, 0.5 M NaCl, 10% (v/v) glycerol, pH 7.4; washing buffer: 20 mM TrisHCl, 5 mM imidazole, 0.5 M NaCl, 10% (v/v) glycerol,

pH 7.4; elution buffer: 20 mM TrisHCl, 150 mM imidazole, 0.5 M NaCl, 10% (v/v) glycerol, pH 7.4; assay buffer: 50 mM sodium phosphate, pH 7.0. Cholesterol (3 mM) in 2-propanol. FAD (5 mM) in DDI water. Horseradish peroxidase (1000 unit/mL) in sodium phosphate buffer, pH 7.0, stock. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (10 nM) in 50 mM sodium phosphate pH 7.0.

Cloning and construction of M. tb and M. smegmatis mutant strains. We obtained *M. tb* transposon mutant strains with inserts into *hsd* (*Rv1106c*) (ST144) and *choD (Rv3409c)* from TARGET (Tuberculosis Animal Research and Gene Evaluation Taskforce, NIH/NIAID NO1-AI30036). These transposon mutants were all in CDC1551. We obtained the previously described *M. smegmatis* mutant *myc11* with a tranpsoson insert in *MSMEG1604*, which is the ortholog of *choD* from Dr. Jean-Marc Reyart (5). Complementation of the *hsd* mutant (strain ST144) was carried out as previously described (7) by cloning the entire open reading frame of *Rv1106c* with 999 bp upstream of the N-terminus into pMV306. This construct was electroporated into ST144 and selection for Hyg-r transformants yielded strain ST160.

Construction of Rv3409c expression plasmids. The entire open reading frame of *Rv3409c* was cloned into pET28b(+) using the NdeI and HindIII restriction sites in the MCS of the plasmid using cassette PCR to provide an N-terminally His_6-tagged construct. Then the *Rv3409c* gene and the N-terminal His_6-tag open reading frames were subcloned into pVV16.hygro between the BamHI and HindIII sites using PCR to provide pVV16.hygro.N-Rv3409c.

Expression and purification of N-terminal-His_6-tagged Rv3409c (N-Rv3409c). Starting cultures of pVV16.hygro.N-Rv3409c were grown at 37 °C until OD_{600} = 0.6-0.8. The cell pellets were collected and inoculated into 1 L protein expression medium in a 4 L flask which was grown at 35.5 °C. The temperature was raised to 45.0 °C to induce protein expression when the OD_{600} of the culture was ~1.0. After 24 h, the cells were harvested and re-suspended in 15 mL binding buffer for cell lysis. After

passage through a French press at 10,000 psi three times, the cell lysate supernatant was collected by ultracentrifugation at 135, 000 g for 90 min, 4 °C. The cell lysate supernatant was loaded onto a pre-equilibrated Ni^{2+} resin column (Invitrogen, 1X5 mL) and the column was further washed with binding buffer and washing buffer to remove impurity proteins. The protein was eluted with elution buffer and the fractions were collected and analyzed by 10% SDS-PAGE. Fractions containing N-Rv3409c were combined for size-exclusion chromatography. They were concentrated by ultrafiltration (Centricon, NMWCO=10,000). A Superdex 200 16/60 column (Pharmacia Biotech) was pre-equilibrated with 20 mM sodium phosphate, pH 7.4, 4 °C. After loading N-Rv3409c, buffer (20 mM sodium phosphate buffer, pH 7.4) was applied at a flow rate of 0.5 mL/min, with UV detection at 280 nm. All fractions absorbing at 280 nm were further analyzed by 10% SDS-PAGE. N-Rv3409c containing fractions were collected and combined. A Mono Q anion exchange column (Pharmacia, 5/5) was pre-equilibrated with starting buffer (20 mM sodium phosphate, 10% glycerol, pH 7.4). The N-Rv3409c sample was loaded onto the Mono Q 5/5 column and 5 column volumes (CV) starting buffer were applied to wash the column. A slow linear gradient from starting buffer to high salt buffer (20 mM sodium phosphate, 10% glycerol, 1 M sodium chloride, pH 7.4) over 10 CV at a flow rate of 0.5 mL/min was used for elution with UV detection at 280 nm. All fractions absorbing at 280 nm were analyzed further by 10% SDS-PAGE. The fractions containing N-Rv3409c were combined for further analysis.

Reconstitution of N-Rv3409c. Denaturing buffer: 0.05 M, potassium phosphate, 6 M urea, 3.5 M KBr, pH 7.4; Elution buffer: 0.03 M potassium phosphate, 6 M urea, pH 7.4; 4 mM FAD in Elution buffer; Dialysis Buffer: 0.03 M potassium phosphate, 0.01 M KCl, pH 7.4; and a G25 column (1×9cm). Purified enzyme was concentrated by Ultrafiltration (Centricon, NMWCO=10,000) to about 1.5 mg/mL into Denaturing buffer and incubated at 25 °C for 4 h in the dark. 4 mM FAD was loaded onto a G25 column pre-equilibrated with Elution buffer and allowed to migrate 3 cm before loading the denatured

N-Rv3409c sample. Fractions from the G25 column were collected and the N-Rv3409c containing fractions were combined and dialyzed (NMWCO=10,000). Dialysis was performed against 2 L Dialysis buffer.

In-gel trypsin digestion verification of Rv3409c identity. In-gel trypsin digestion combined with MALDI-TOF/MS was applied to confirm N-Rv3409c's identity. About 5 μg N-Rv3409c from the SDS-PAGE gel was treated with trypsin and the digested fragments were extracted three times with 60% CH_3CN/0.1% trifluoroacetic acid (TFA) in water. The total extracts were lyophilized and the residue was dissolved in 5 μL 0.1% TFA. A saturated α-cyano-4-hydroxycinnamic acid solution was freshly prepared as the matrix. MALDI-TOF/mass spectra were acquired in positive ion mode.

Kinetic Assays. Cholesterol was prepared as a 3 mM stock solution in 2-propanol. Assays were performed at 37 °C. The standard assay mixture contained 1 μM N-Rv3409c enzyme. Assay mixtures were extracted with ethyl acetate and analyzed by thin layer chromatography.

1) UV-based cholestenone detection assay. The production of cholest-4-en-3-one was monitored by an increase in UV absorbance using ε_{240} = 12,100 $M^{-1}cm^{-1}$. The standard assay mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.05% (w/v) Triton X-100, and 150 μM cholesterol, which was added as a cholesterol stock dissolved in 2-propanol.

2) UV-based ABTS-HRP coupled Assay. The standard assay mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.05% (w/v) Triton X-100, 0.32 nM ABTS and 1 unit/ mL Horseradish peroxidase (HRP), 150 μM cholesterol, which was added as a cholesterol stock dissolved in 2-propanol. The formation of oxidized ABTS was monitored at 600 nm.

3) UV-based DCIP/PMS electron transfer assay (*110*). The standard assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.05 % (w/v) Triton X-100, and 150 μM cholesterol, which was added as a 2-propanol solution. Either 0.048 mM 2,6-dichloroindophenol (DCIP) or 0.9 mM phenazine

methosulfate (PMS) was added as the electron acceptor. Formation of the DCIP or PMS oxidized form was monitored at 600 nm.

*Lipid extractions and LC/MS Analysis***.** Three independent cultures of *M. tb* CDC1551, *hsd*, and complemented *hsd* were grown as described and either 0.2 μCi of [4-^{14}C]-cholesterol or 1 mg mL^{-1} cholesterol was added. After 5 h, both cell pellets and culture supernatants were autoclaved. Cell pellets were extracted by the Bligh-Dyer method (3), concentrated to dryness and resuspended in EtOAc. The culture supernatants were extracted with EtOAc twice. For all samples, the EtOAc extracts were washed with H_2O three times, concentrated, and the concentrates were analyzed by LC/MS/UV. A Waters ACQUITY Ultra Performance LC system (Milford, MA), equipped with a PDA detector and a SQD detector was used for identification of metabolites. Chromatography was performed with a C18 reversed-phase column (2.1mm x 100mm, i.d., 1.7 μm particle size, Waters ACQUITY UPLC BEH) maintained at 55 °C. The elution solvents were A: 10% MeOH in H_2O and B: MeOH, and the flow rate was 0.5 mL min^{-1}. The eluting solvent was isocratic for 0.02 min with 100% A, followed by a linear gradient to 44% A in 0.3 min, a second linear gradient to 0% A in 2 min, isocratic for 7 min with 0% A, a third linear gradient to 100% A in 0.5 min and isocratic with 100% A for 0.5 min. The mass spectrometer was operated with an atmosphere pressure chemical ionization source in positive ion mode with the source temperature set to 150 °C, the desolvation temperature was set to 450 °C with a corona voltage of 1.5 kV. The UV detection wavelength range was 200 to 400 nm. Radioactive samples were only analyzed by UV detection. Fractions were collected every 10 sec and analyzed by liquid scintillation counting after addition of 4 mL of Scintiverse II LSC cocktail.

Activation and infection of THP-1 macrophages. Activated THP-1 cells (40 nM 12-O-tetradecanoylphorbol-13-acetate (PMA) (6)) were infected with wild-type *M. tb* and *hsd* mutant in triplicate for 4 h at a multiplicity of infection of about 1, followed by washing with PBS, and

replacement of the medium with fresh medium. Intracellular bacteria were isolated at the indicated time points after lysis of the macrophages with 0.05% sodium dodecyl sulfate and collection of the bacteria by centrifugation and the number of colony forming units enumerated.

Guinea pig plot/histology. The *hsd* and *Rv3409c* mutants were tested in the guinea pig infection model by NIH/NIAID under the TARGET contract. All guinea pig protocols were approved by the Texas A&M IACUC. Hartley strain guinea pigs (310-340 g) were infected by the respiratory route with a nebulizer concentration of 10^4 CFU/mL of the CDC1551, *hsd (ST144)*, *Rv3409c,* and *hsd* complemented (ST160) strains (n= 14 guinea pigs per group). At days 1 and 21, 4 guinea pigs were sacrificed for each time point; 6 guinea pigs were sacrificed at day 42. At each time point, the lungs and spleen were removed and weighed after which, the lungs were homogenized in sterile saline, and were plated on 7H10 solid media with serial dilutions. After three weeks incubation at 37 °C, the number of colonies was enumerated. At days 21 and 42, a small fragment of lung lobe was used for histopathology.

Results

M. smegmatis myc11 can utilize cholesterol as a carbon source. In previous studies, Andor et al. demonstrated that *M. smegmatis* can utilize cholesterol as a carbon source (1). *Myc11* and wild-type growth rates were compared on agar plates supplemented with cholesterol solubilized in 2-propanol. Neither strain grew on control plates containing only 2-propanol (medium C). Both strains grew at identical rates and to identical sizes on plates containing cholesterol as the only carbon source (medium B). Likewise, no growth differences were observed when cultures on plates containing glycerol as the only carbon source (medium A).

Heterologous expression of M. tb Rv3409c. Plasmids carrying the *Rv3409c* gene behind the T7

and lac-T7 promoters were constructed with both N- and C-terminal tags for affinity purification. Heterologous expression of *Rv3409c* was attempted in *E. coli* using a variety of strain types, i.e., BL21(DE3), BL21(DE3)pLysS, C41(DE3), and Rosetta(DE3), at temperatures ranging from 10 °C to 25 °C. All conditions surveyed resulted in the formation of inclusion bodies with no FAD cofactor incorporated. Therefore, plasmids for expression in *M. smegmatis* were prepared with the *Rv3409c* gene behind the acetamidase and the heat shock *hsp60* promoters with both N- and C-terminal His_6 tags. The N-terminally tagged expression vectors produced soluble cofactor with cofactor bound. Expression yields were highest with the *hsp* promoter (~3 mg L^{-1}).

Purification of M. tb N-Rv3409c. N-Rv3409c was purified by immobilized-metal ion affinity chromatography. Further purification by size exclusion chromatography and anion exchange chromatography provided protein that was > 95% pure (Figure S3). The identity of the purified protein was confirmed by tryptic digest and MALDI-TOF spectroscopy.

Spectroscopic analysis and mass spectrometric analysis of the cofactor released by denaturation revealed that the FAD cofactor had formed an adduct. Therefore, the holo protein was prepared by partial denaturation in urea and KBr ((4). The apo protein was reconstituted on a G25 column preloaded with FAD in about 25% yield as judged by UV/vis spectroscopy (Figure S4). .

Assay of M. tb N-Rv3409c. Three assays were used to test for cholesterol oxidase activity using 150 μM cholesterol and up to 1 μM enzyme (60 μg/mL). The first assay monitored the formation of cholestenone. The second assay monitored the formation of hydrogen peroxide, which is formed upon reoxidation of the reduced cofactor by O_2. The third assay used a dye as the reduced cofactor oxidant and monitored the formation of reduced dye. No cholest-4-en-3-one formation was detected under any of these initial velocity assay conditions by UV detection. Likewise, no cholest-4-en-3-one or cholest-5-en-3-one was detected by extraction and TLC analysis after 17 h incubation of the assay mixtures.

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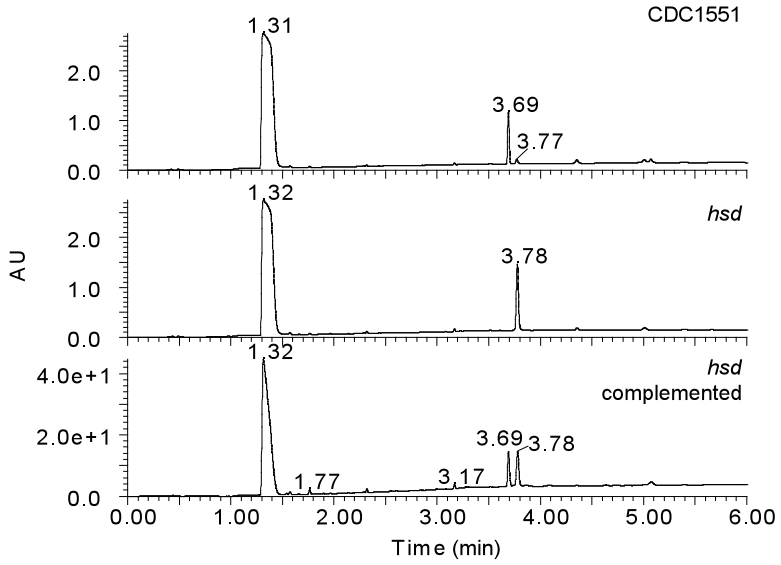

Figure S1. *hsd* is required for the conversion of cholesterol to cholest-4-en-3-one by *M. tb*. Entire UV chromatogram from UPLC/UV/MS analysis of CDC, *hsd* (ST144), and complemented *hsd* (ST160) *M. tb.* *M. tb* strains were extracted by the Bligh-Dyer method (2). Chloroform:methanol extracts were analyzed on a Waters Acquity BEH C18 column. The flow rate was 0.5 mL/min with a gradient of 10% methanol/water to 100% methanol/water. These profiles are representative of three independent biological replicates.

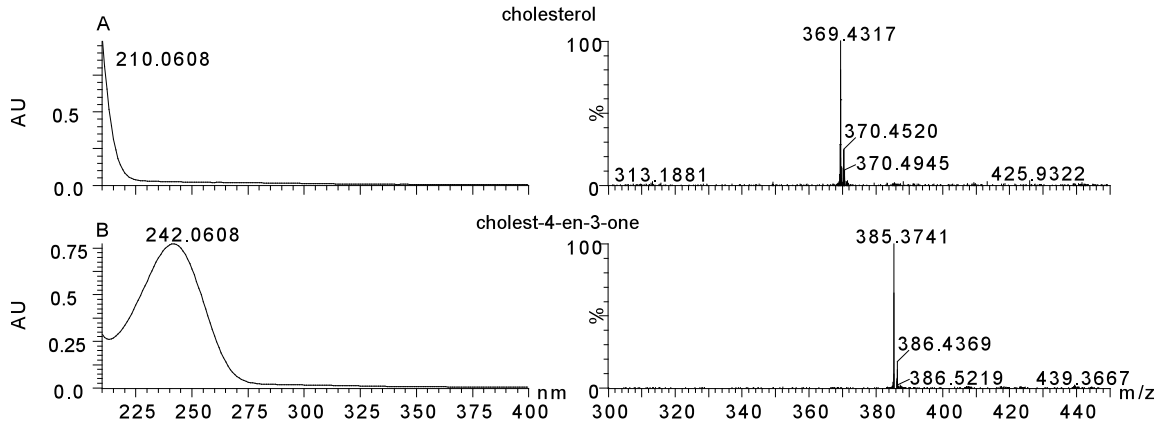

Figure S2. MS/UV analysis of peaks observed in Figure S1 and Figure 2 at (A) 3.8 min and (B) 3.7 min. The mass spectra were obtained with APCi ionization in positive ion mode. The UV spectra were acquired with a photodiode array detector.

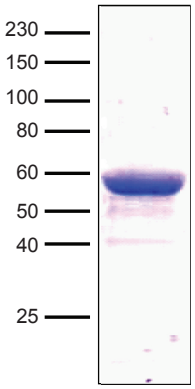

Figure S3. SDS-PAGE of purified N-Rv3409c.

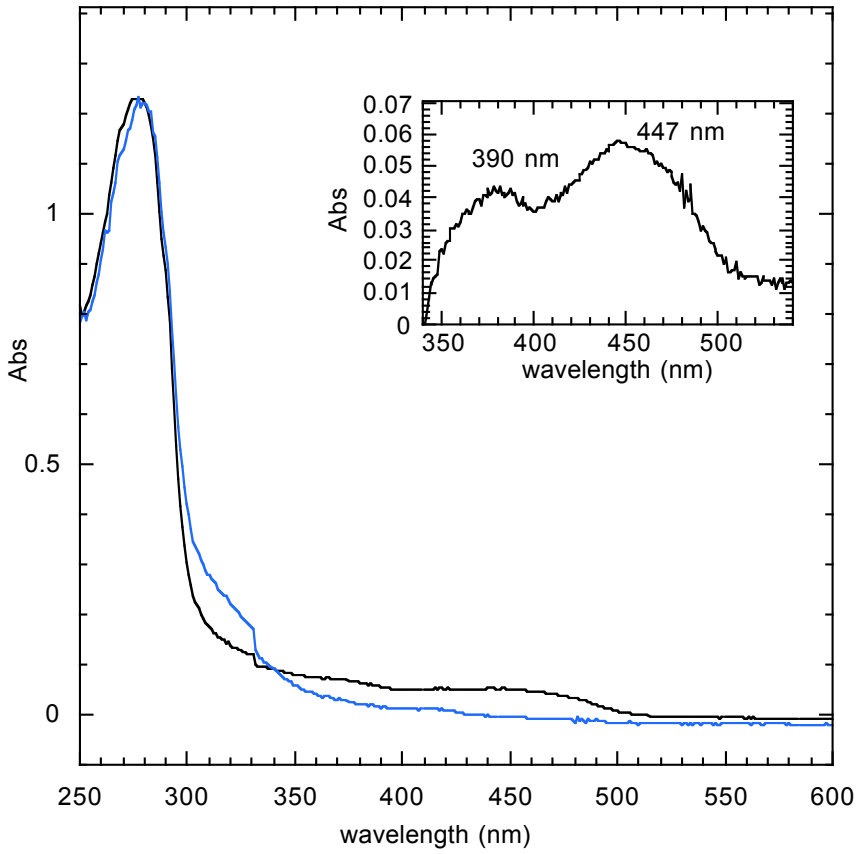

Figure S4. UV/vis spectra of the holo-N-Rv3409c (blue line) and reconstituted apo-N-Rv3409c (black line). Inset: difference spectrum in the flavin region.

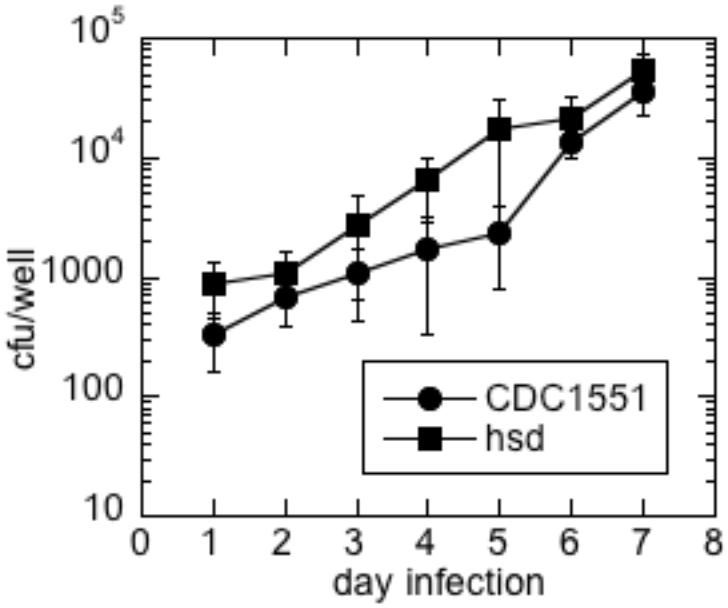

Figure S5. Intracellular growth of wild-type *M.* tb and *hsd* mutant (ST144) in macrophages. Activated THP-1 cells were infected with wild-type CDC1551 *M. tb* and *hsd* mutant in triplicate for 4 h at a multiplicity of infection of about 1. Intracellular bacteria were isolated at the indicated time points and enumerated.