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NOTES

Cholesterol Is Not an Essential Source of Nutrition for *Mycobacterium tuberculosis* during Infection▽†

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Rv1106c (*hsd*; 3β-hydroxysteroid dehydrogenase) is required by *Mycobacterium tuberculosis* for growth on cholesterol as a sole carbon source, whereas Rv3409c is not. Mutation of Rv1106c does not reduce *Mycobacterium tuberculosis* growth in infected macrophages or guinea pigs. We conclude that cholesterol is not required as a nutritional source during infection.

Mycobacterium tuberculosis is a nocardioform actinomycete and is a facultative intracellular bacterium that usually infects the host macrophage. *M. tuberculosis* has coevolved with humans and persists despite the actions of the immune system. Survival of *M. tuberculosis* requires adaptation to the host microenvironment (14). In the intracellular environment, *M. tuberculosis* shifts from a carbohydrate-based to a fatty acid-based metabolism (3, 15, 21), and in culture, *M. tuberculosis* will grow on cholesterol as the sole carbon source (18). One role for cholesterol in the intracellular environment could be as a source of carbon, e.g., catabolism to acetate and propionate (5, 24, 25). Additionally, cholesterol can serve as a building block for complex structures, e.g., lipids and hormones through anabolism.

Through transcriptional profiling (16, 24), bioinformatic analysis, and metabolic analysis of other actinomycetes (9), a partial metabolic pathway for cholesterol metabolism in *M. tuberculosis* has been sketched. The first step is the conversion of cholesterol to cholest-4-en-3-one (17) (Fig. 1). In *Streptomyces* spp. and *Rhodococcus equi*, this step is catalyzed by cholesterol oxidases, which share 60% amino acid identity and have structures and mechanisms that are nearly identical (13, 20). The closest *M. tuberculosis* homolog, Rv3409c, shares only 24% amino acid identity with the well-characterized cholesterol oxidases from *Streptomyces* and *Rhodococcus.* Although *Mycobacterium smegmatis* cellular lysates overexpressing Rv3409c were reported to contain cholesterol oxidase activity, characterization of the purified enzyme was not reported (4).

Nocardia spp. (10, 12), proteobacteria (7), and most likely *Rhodococcus jostii* (19) utilize a 3β-hydroxysteroid dehydrogenase to catalyze the conversion of cholesterol to cholest-4-en-3-one. In *M. tuberculosis*, Rv1106c (*hsd*) is the closest homolog (75% identity with the *Nocardia* enzyme, UniProtKB ID Q03704). Indeed, we demonstrated in earlier work that Rv1106c encodes a functional 3β-hydroxysteroid dehydrogenase (HSD) that can utilize cholesterol, pregnenolone, and dehydroepiandosterone as substrates (26). Here, we investigate the essentiality of these genes for growth of *M. tuberculosis in vitro* and *in vivo*.

First, we tested whether *in vitro* growth with cholesterol as the carbon source required either Rv3409c or *hsd*. (Detailed experimental protocols may be found in the supplemental material.) We found that *hsd* is required for growth on cholesterol as a sole carbon source in broth culture, whereas the Rv3409c mutant grew as well as the wild type (Fig. 2). To further confirm the nonessentiality of Rv3409c for growth on cholesterol, we tested an *M. smegmatis* Rv3409c transposon mutant (*myc11*) (22) for growth on cholesterol as a sole carbon source on agar plates. The *myc11* mutant formed colonies as readily as the mc^2155 wild-type strain (data not shown).

Complementation of the *hsd* mutant with the wild-type gene and 1,000 bases upstream of the open reading frame (26) completely restored growth on cholesterol (Fig. 2). All the strains grew normally in standard 7H9 medium supplemented with glycerol and 10% albumin-dextrose-NaCl complex (ADN) (data not shown). We conclude that *hsd*, but not Rv3409c, is required for growth on cholesterol as a sole carbon source.

Previously, we demonstrated that *hsd* is required for cholesterol oxidation activity in cell lysates (26). To investigate whether *hsd* is required for 3β-hydroxysterol oxidation in intact cells, the strains were grown in standard medium (7H9 liquid medium [Becton Dickinson], supplemented with 0.05% Tween 80, ADN [1], and 0.2% glycerol). After the cells reached log phase, 0.2 μCi of [4-^{14}C]cholesterol was added. Five hours after cholesterol addition, lipids were extracted (2) and analyzed by liquid chromatography with scintillation counting and UV detection. Analysis of the wild-type cells revealed that

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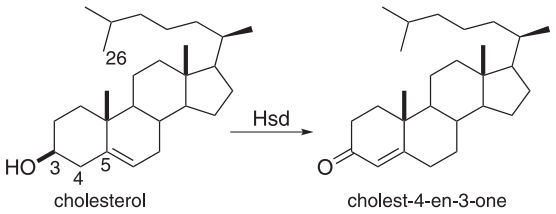

FIG. 1. The reaction catalyzed by *M. tuberculosis* 3β-hydroxysteroid dehydrogenase (HSD).

>99% of the [^{14}C]cholesterol was consumed within 5 h (Fig. 3A). At the same time point, large amounts [^{14}C]cholesterol (>40% of total counts) remained in the *hsd* mutant (Fig. 3A).

Nonradioactive samples were prepared in an analogous fashion (final concentration of cholesterol, 1 mg ml^{-1}) for mass spectrometric analysis, which confirmed that the HSD reaction product, cholest-4-en-3-one, was formed in the wild-type cells (Fig. 3B; see also Fig. S1 and S2 in the supplemental material). However, no cholest-4-en-3-one could be detected in the *hsd* mutant by absorbance at 240 nm or single ion monitoring mass spectrometry (Fig. 3B; see also Fig. S1 and S2). Complementation of the *hsd* mutant strain restored production of cholest-4-en-3-one (Fig. 3; see also Fig. S1 and S2). As an additional control, Rv3409c was heterologously expressed to determine whether it was a cholesterol oxidase. Expression behind the acetamidase or heat shock *hsp60* promoters in *M. smegmatis* mc^{2}155 provided soluble protein upon induction (see Fig. S3 and S4 in the supplemental material). Despite assessment of the purified protein as both an oxidase (electron acceptor, O_2) and a dehydrogenase (electron acceptor, phenazine methosulfate or 2,6-dichloroindophenol), no oxidation of cholesterol could be detected. On the basis of these complementary experiments, we concluded that HSD is required for conversion of cholesterol to cholest-4-en-3-one and that a second cholesterol oxidase activity is not present in *M. tuberculosis*.

Next, the role of *hsd* in *M. tuberculosis* growth in macrophages was assessed. Wild-type and mutant cultures were used to infect THP-1 cells that had been made to differentiate into macrophage-like cells with 40 nM 12-*O*-tetradecanoylphorbol-13-acetate (PMA) (23). No difference in the intracellular growth rate was detected (see Fig. S5 in the supplemental material). Therefore, disruption of *hsd* does not limit *M. tuberculosis* replication in the macrophage.

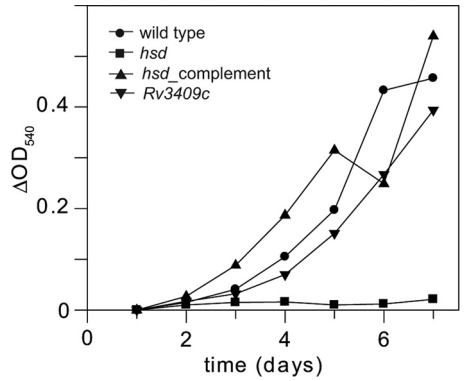

FIG. 2. *hsd*, but not Rv3409c, is required for growth on cholesterol as the sole carbon source. The strains were grown in 7H9 medium containing 1 mg ml^{-1} cholesterol (in tyloxapol) at 37°C. Data represent results of each experiment run in duplicate.

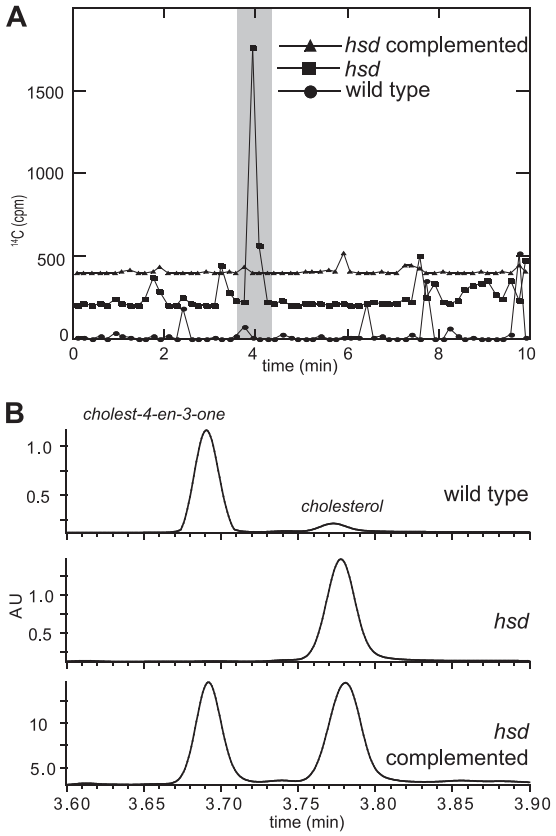

FIG. 3. *hsd* is required for the conversion of cholesterol to cholest-4-en-3-one by *M. tuberculosis.* Ultrahigh-performance liquid chromatography–mass spectrometry–UV analysis results are shown for the wild type, *hsd* mutant, and complemented *hsd* mutant. (A) *M. tuberculosis* was incubated for 5 h with [4-^{14}C]cholesterol and analyzed by scintillation counting and UV absorbance. The cpm reflect the relative mass balance between samples. (B) *M. tuberculosis* was incubated for 5 h with cholesterol. The UV chromatographic profile from 3.6 to 3.9 min (shaded portion in panel A) is shown. The absorbance intensities do not reflect the relative mass balance between samples, which were concentrated to different extents for analysis. For the full profile and mass spectral analysis, see Fig. S1 and S2 in the supplemental material.

M. tuberculosis-infected guinea pigs develop granulomas similar to those seen in human disease. Therefore, the guinea pig model was employed to assess the *in vivo* role of *hsd*. The *in vivo* growth rate, lung weight, lung morphology, and lung histology were determined over a 6-week time course. No reduction in growth was observed in the mutant strain (Fig. 4). The number of granulomas in the lungs of animals infected by the *hsd* mutant and the complemented strain appeared to be higher than in the wild type (Fig. 4B and C). This difference may be the result of differing immune responses. Regardless, the *hsd* gene is not required for growth or survival of *M. tuberculosis* in the guinea pig. Moreover, if the buildup of cholesterol in the *hsd* mutant occurs during infection, as it does *in vitro*, the high level of cholesterol is not toxic to the bacterium. This result is in contrast to the toxicity of accumulated

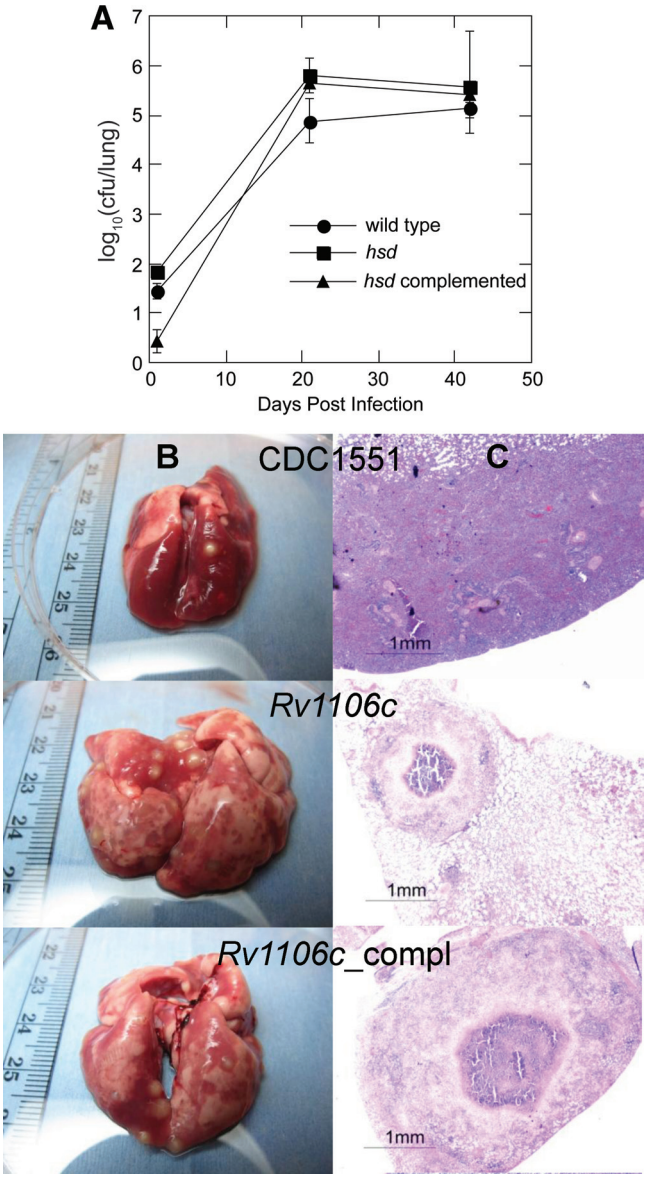

FIG. 4. Mutation of *hsd* does not affect granuloma formation in the guinea pig model of infection. Fourteen guinea pigs were infected with ~10^2 CFU/lung of each *M. tuberculosis* strain. At the indicated time points, four to six guinea pigs per strain were sacrificed, and lungs were weighed, a portion was excised for histology, and the remainder was homogenized for CFU titration. (A) *M. tuberculosis* growth rates in the lungs of aerosol-infected guinea pigs. Error bars are the standard deviations. (B) Gross pathology of lungs 42 days after infection. (C) Histopathology of the lungs shown in panel B.

metabolites that is observed upon disruption of genes encoding ring-metabolizing enzymes later in the *M. tuberculosis* cholesterol pathway (6, 17, 25).

In conclusion, we have established that the 3β-hydroxysteroid dehydrogenase encoded by Rv1106c (*hsd*) is required for growth on cholesterol as a sole carbon source, whereas the putative cholesterol oxidase, Rv3409c, is not. Lipidomics experiments have revealed that methyl-branched lipid carbon sources from the host are the primary source of nutrition *in vivo* for *M. tuberculosis* (11, 27). Our observation that *hsd* is not required for growth in the activated macrophage or in the guinea pig model of *M. tuberculosis* infection suggests that cholesterol is not a sole nutrition source *in vivo*. Moreover, *fadA5*, tentatively annotated as encoding a side chain-cleaving enzyme, is required for cholesterol metabolism and growth on cholesterol as a sole carbon source *in vitro*. Although FadA5 is not required for growth of *M. tuberculosis* in mice, it is required for maintenance in the host (16). These combined observations suggest that *M. tuberculosis* does not rely on cholesterol as a sole energy source in the host. Our results are consistent with the availability of multiple lipid energy sources in the host and with the recent work of Rhee and coworkers demonstrating that *M. tuberculosis* cocatabolizes multiple carbon sources (8).

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ADDENDUM IN PROOF

Garcia and coworkers recently reported that *M. smegmatis* Rv3409c is not required for cholesterol mineralization (I. Uhia, B. Galán, V. Morales, and J. K. García, Environ. Microbiol., doi:10.1111/j.1462-2920.2010.02398x, 2011).

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