Lipid Droplet-associated Proteins Are Involved in the Biosynthesis and Hydrolysis of Triacylglycerol in *Mycobacterium bovis* Bacillus Calmette-Guérin*§*

Kai Leng Low†, Guanghou Shui§, Klaus Natter†, Wee Kiang Yeo†, Sepp D. Kohlwein§, Thomas Dick‖, Srinivasa P. S. Rao‡,‡,‡ and Markus R. Wenk‡,‡,‡,‡,‡

From the †NUS Graduate School for Integrative Sciences and Engineering, the ‡Department of Biochemistry, Yong Loo Lin School of Medicine, and the §Department of Biological Sciences, Faculty of Science, National University of Singapore (NUS), Singapore 117456, the ¶Novartis Institute for Tropical Diseases, Singapore 138670, and the ‖Institute of Molecular Biosciences, University of Graz, A8010 Graz, Austria

Mycobacteria store triacylglycerols (TGs) in the form of intracellular lipid droplets (LDs) during hypoxia-induced nonreplicating persistence. These bacteria are phenotypically drug-resistant and therefore believed to be the cause for prolonged tuberculosis treatment. LDs are also associated with bacilli in tuberculosis patient sputum and hypervirulent strains. Although proteins bound to LDs are well characterized in eukaryotes, the identities and functions of such proteins have not been described in mycobacteria. Here, we have identified five proteins: Tgs1 (BCG3153c), Tgs2 (BCG3794c), BCG1169c, BCG1489c, and BCG1721, which are exclusively associated with LDs purified from hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Disruption of genes *tgs1*, *tgs2*, *BCG1169c*, and *BCG1489c* in *M. bovis* BCG revealed that they are indeed involved in TG metabolism. We also characterized BCG1721, an essential bi-functional enzyme capable of promoting buildup and hydrolysis of TGs, depending on the metabolic state. Nonreplicating mycobacteria overexpressing a BCG1721 construct with an inactive lipase domain displayed a phenotype of attenuated TG breakdown and regrowth upon resuscitation. In addition, by heterologous expression in baker’s yeast, these mycobacterial proteins also co-localized with LDs and complemented a lipase-deficient yeast strain, indicating that neutral lipid deposition and homeostasis in eukaryotic and prokaryotic microorganisms are functionally related. The demonstrated functional role of BCG1721 to support growth upon resuscitation makes this novel LD-associated factor a potential new target for therapeutic intervention.

*Mycobacterium tuberculosis*, the etiologic agent of tuberculosis (TB) infects more than one-third of the world population, resulting in two million deaths per year. The success of this pathogen hinges on its ability to enter into a nonreplicating persisting (NRP) state upon infecting the human host. In this state, the mycobacteria survive quiescently for decades (1) and reactivate opportunistically, such as during co-infection with human immunodeficiency virus, to cause disease (2). However, metabolic processes and enzymes necessary for the tubercle bacilli to transit into and out of the dormant state are not well characterized yet.

It is widely recognized that *M. tuberculosis* utilizes fatty acids (FAs) as a carbon source during persistent infection (3). Mycobacteria store FAs in the form of triacylglycerols (TGs) during dormancy (4, 5) and in TB patient sputum (6, 7). Moreover, a direct correlation between the buildup of TGs and phenotypic drug resistance in *M. tuberculosis* was established (5), highlighting the importance of these lipids during latency and pathogenicity. TG utilization occurs when *M. tuberculosis* is subjected to a nutrient-deprived environment (8) and when *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is resuscitated from a hypoxic, nongrowing state (9), which underscores the important functional role of intracellular lipid storage during NRP and pathogen reactivation in the host.

Despite the attention given to the roles of intracellular TGs in mycobacteria, the metabolic genes involved in the buildup and breakdown of TG are poorly understood. To date, four proteins, encoded by TG synthase-encoding genes (*tgs1*—*tgs4*), have been shown to contain diacylglycerol acyltransferase activity, catalyzing the final step of TG biosynthesis (4). Among these, *Tgs1* (Rv3130c) is the most active TG-degrading enzyme under carbon-limiting conditions (8). However, the deletion of *lipY* in *M. bovis* BCG does not

---

*This work was supported in part by Singapore National Research Foundation Competitive Research Programme Award 2007-04, Academic Research Fund Grant R-183-000-160-112, Novartis Institute for Tropical Diseases Grant R-183-000-166-592, and Austrian Science Fund Project SFB LIPOTOX (to K. N. and S. D. K.).

The on-line version of this article is available at http://www.jbc.org. It contains supplemental text, Table S1, and Figs. S1–S5.

†Recipient of a scholarship from the NUS Graduate School for Integrative Sciences and Engineering.

‡Both authors contributed equally to this work.

§To whom correspondence may be addressed: 28 Medical Dr., Level 04-21, Singapore 117456. Tel.: 65-65163624; E-mail: bchmrw@nus.edu.sg.

The abbreviations used are: TB, tuberculosis; TG, triacylglycerols; LD, lipid droplet; BCG, bacillus Calmette-Guérin; Tgs, TG synthase; NRP, nonreplicating persisting; FA, fatty acid; AC5L, long chain acyl-CoA synthase; GFP, green fluorescent protein; WT, wild type; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
not prevent the hydrolysis of intracellular neutral lipid inclusions during regrowth (9), demonstrating that other lipases must exist. These findings indicate that the understanding of TG metabolism in mycobacteria is still rather limited.

TGs are typically stored as cytoplasmic lipid droplets (LDs) in pro- and eukaryotes and have been extensively studied using classical biochemical and proteomics approaches in the latter (11–18). Although the mechanisms of prokaryotic LD formation are potentially different from those in eukaryotes (19), proteins associated with mammalian LDs can be localized to the bacterial LDs when expressed in this heterologous host (20). Moreover, a bacterial diacylglycerol acyltransferase is able to functionally complement a yeast mutant devoid of TG biosynthetic genes (21). To date, however, no proteins are known to be physically associated with bacterial LDs (19). Identification and characterization of these factors is expected to have a major impact on our understanding of TG homeostasis, LD biogenesis, and turnover in prokaryotic systems and processes, which are critical to cellular maintenance and survival.

In this study, we have identified five novel proteins (BCG1169c, BCG1489c, Tgs1 (BCG3153c), Tgs2 (BCG3794c), and BCG1721) that are exclusively bound to an LD-enriched fraction from hypoxic NRP M. bovis BCG. We show that these proteins are involved in the metabolism of TG and specifically characterize BCG1721 as an essential and presumably bi-functional enzyme with lipase and potential long chain acyl-CoA synthase (ACSL) activities on a single polypeptide chain. The identification of LD-associated key proteins involved in TG metabolism provides new insights into the mechanisms of prokaryotic neutral lipid storage and may lead to novel targets for TB therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Media, and Chemicals—**M. bovis BCG Pasteur strain (ATCC 35734) and knock-out mutants were grown in Dubos liquid medium (Difco) or on Middlebrook 7H11 agar (Difco). When required, antibiotics were added to the culture media at the following concentrations: kanamycin (Sigma-Aldrich), 25 μg/ml; hygromycin (Roche Applied Science), 80 μg/ml. Culturing conditions including logarithmic, hypoxia-induced NRP, and regrowth from dormancy were carried out as described previously (9).

**Isolation of Intracellular LDS and Protein Analysis from Mycobacteria—**Cells of M. bovis BCG were cultured in a hypoxia-induced NRP state, harvested by centrifugation (20 min, 6,000 × g, 4 °C), and resuspended in a hypotonic lysis medium containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and protease inhibitor mixture (Roche Applied Science). After 4-fold passage through a French pressure cell, crude extracts were obtained and subjected to discontinuous sucrose gradient ultracentrifugation as described (14). Floating lipid fraction and cytosolic components (bottom phase) were collected. All of the preparations were delipidated by extracting with chloroform/methanol (1:1; v/v). The interphase containing protein was washed with methanol, air-dried, and solubilized in 10 mM Tris-HCl (pH 7.4) with 8 M urea. The proteins were quantified by BCA assay kit (Pierce), and equal amounts of proteins from each preparation were premixed with sample buffer, separated by 4–12% NuPAGE Bis-Tris gel electrophoresis in a MES buffer system (Invitrogen), and silver-stained using a glutaraldehyde-free system (22).

**MS Analysis and Database Searching—**Prior to analysis by MS, the dried tryptic-digested peptides were dissolved in 1 μl of matrix solution (5 mg/ml of α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile) followed by spotting onto the matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry target plate. MS analysis of generated peptide mixtures was performed by the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems), operating in a result reflector positive MS and tandem MS mode.

For peptide and protein identification, GPS Explorer™ software version 3.6 (Applied Biosystems) was used to create and search files with Mascot search engine (version 2.1; Matrix Science). The NCBI nonredundant protein sequence database was used for the search and was restricted to tryptic peptides. Peptide mass tolerance and fragment mass tolerance were set to 100 ppm and 0.2 Da, respectively. The proteins were identified based on their scores, expectation values, sequence coverage, and number of unique matching peptides. Protein scores greater than 81 (p < 0.05), low expectation values, larger sequence coverage, and higher numbers of unique matching peptides correspond to confident identifications. Respective accession numbers and their functional information were obtained from the BGCList and Tuberculent databases.

**Generation of Knock-out Mutants and Complemented Strains of M. bovis BCG—**The disrupted mutants of various mycobacterial genes (tgs1, tgs2, BCG1169c, and BCG1489c) were constructed by performing allelic exchange, as described (23). A sacB-lacZ cassette was excised from the pGOAL17 (24) and ligated into the PacI site of pYUB854 containing 5′- and 3′-flanking sequences of the gene of interest locus. The final plasmids were UV-irradiated prior to electroporation into M. bovis BCG. Deletion mutants were confirmed by Southern blotting (supplemental Fig. S5). For complementation, the coding sequences of the mycobacterial genes, including ~400 bp upstream of the start codon, were amplified from genomic DNA of M. bovis BCG and cloned into promoterless Escherichia coli-Mycobacterium shuttle vector pMV306 (25). The constructs were electroporated into their respective gene-null mutants, and transformants were selected on 7H11 plates containing hygromycin and kanamycin.

**Construction of Comparative Model Structure of BCG1721—**pDomThreader (26) was used to identify domains that may be present in the amino acid sequence of BCG1721 and thereafter align the predicted domains to the corresponding sequence segments of the x-ray crystal structures with resemblance to the BCG1721 protein in terms of secondary structure features. Based on the alignments generated by pDomThreader, a comparative model of the BCG1721 lipase domain was built using the x-ray crystal structure of a mammalian gastric lipase (Protein Data Bank code 1k8q) as the template. The pDomThreader-generated sequence alignment of the predicted lipase domain of BCG1721 with the template was used as input for MODELLER9v3 (27), which generated three-dimensional model structures of proteins by satisfaction of spa-
Lipid Droplet-associated Proteins in Mycobacteria

Tissue restraints. For the model building procedure, default parameters included in the “automodel” class were used. A comparative three-dimensional model structure of the BCG1721 lipase domain was thus obtained. The quality of the model structure was assessed using PROCHECK (28).

Overexpression of BCG1721 and BCG1721(S150A) in M. bovis BCG—For cloning of BCG1721 and BCG1721(S150A) into plasmid pMV262, the genes were amplified by PCR using pCR2.1 vectors containing BCG1721 or BCG1721(S150A) as templates. The constructs were electroporated into wild type M. bovis BCG, and transformants were selected on 7H11 plates with kanamycin. Overexpression of BCG1721 and BCG1721(S150A) in isolated colonies from the plates were assessed using quantitative reverse transcription-PCR (see Fig. 4D).

Lipid Analysis—Various methods such as Nile red staining, TLC, liquid chromatography-tandem MS, and TG lipase activity were performed as described (9, 29).

Co-localization Studies of Mycobacterial Proteins in Yeast—LD-associated proteins from M. bovis BCG were amplified and expressed in GFP episomal fusion plasmid and transformed into yeast as described earlier (29). Microscopic analysis was performed using a Zeiss LSM 510 Meta confocal microscope equipped with a 488-nm, 30-milliwatt argon laser as the excitation source. The images were viewed using EC Plan-Neofluar 100×/1.30 oil objective lens (Zeiss) and captured using a photomultiplier tube.

Site-directed Mutagenesis, Quantitative Reverse Transcription-PCR, and Lipid Analysis of BCG1721 in Yeast—For details, please refer to the supplemental “Experimental Procedures.”

Statistical Analysis—Data were obtained from at least three independent experiments performed in triplicate. Statistical significance was analyzed using Student’s t test; p < 0.05 was considered to be significant.

RESULTS

Identification of Proteins in the LD-enriched Fraction from Hypoxic NRP M. bovis BCG—An LD-enriched fraction from hypoxia-induced M. bovis BCG in the NRP state was isolated using sucrose gradient ultracentrifugation. TLC analysis showed that most of the TGs were located in the top fraction (supplemental Fig. S1). The LD layer and also the total lysate and cytosolic fraction were delipidated, precipitated, and separated by SDS-PAGE to compare their protein patterns. The protein composition of the LD fraction was distinctly different from that of crude lysate and cytosolic preparations, suggesting significant enrichment of LD-specific proteins (Fig. 1). All of the protein bands present in the LD fraction and the corresponding bands from other fractions were subjected to trypsin digestion, and the proteins were identified using MALDI-MS (supplemental Table S1). Notably, band 4 of the LD fraction was thicker in comparison with other preparations, which is in line with the finding that it contained three protein hits instead of one (supplemental Table S1). Protein hits such as S-adenosyl-L-homocysteine hydrolase and elongation factor Tu were found in all three preparations, which is consistent with the view that the LD-enriched preparation may contain some contamination of highly abundant cytosolic proteins. We classified LD-associated proteins to be polypeptides that are found either exclusively or in higher amounts in the LD fraction, compared with the crude extract. Based on the above criteria, gene products of BCG1721, tgs1, tgs2, BCG1489c, BCG1169c, and hspX (α-cystallin; BCG2050c) belong to the LD proteome of M. bovis BCG. The identified mycobacterial LD-associated proteins and their known or putative functions are summarized in Table 1. Notably, the LD-associated proteins identified in M. bovis BCG are well conserved in M. tuberculosis H37Rv.

Mycobacterial LD-associated Proteins Localize to Lipid Particles in Baker’s Yeast—Heterologous expression has been successfully used to study localization and enzymatic properties of lipid metabolizing enzymes. To determine the specificity of LD association of the mycobacterial proteins in a heterologous sys-

**TABLE 1**

Lipid droplet-associated proteins in hypoxia-induced NRP M. bovis BCG

| Band No. | Gene name  | M. bovis BCG | M. tuberculosis H37Rv | Molecular mass | Function* |
|----------|------------|--------------|-----------------------|----------------|----------|
| 1        | BCG1721    | BCG1721      | Rv1683                | 107.4          | [Long chain acyl-CoA synthase and lipase] |
| 4        | tgs1       | BCG3153c     | Rv3130c               | 50.7           | Triacylglycerol synthase |
| 4        | tgs2       | BCG3794c     | Rv3734c               | 49.3           | Triacylglycerol synthase |
| 6        | BCG1489c   | BCG1489c     | Rv1428c               | 29.9           | [Acylglycerol-phosphate acyltransferase] |
| 7        | BCG1169c   | BCG1169c     | Rv1109c               | 23.0           | [Unknown] |
| 8        | hspX; acr  | BCG2050c     | Rv2031c               | 16.2           | Heat shock protein |

* Accession number in BCGList database.

† Accession number in Tuberculist database.

‡ Functions are based on amino acid sequence similarity analysis and as annotated in Tuberculist database. Square brackets indicate hypothetical functions.
tem, they were expressed as fusion proteins to the GFP tag in yeast. As revealed in Fig. 2, BCG1721-GFP, Tgs1-GFP, Tgs2-GFP, BCG1489c-GFP, and BCG1169c-GFP localized exclusively to yeast LDs, as demonstrated by their co-localization with Nile red. On the other hand, GroES-GFP and GFP alone (empty pUG35 vector), respectively, were co-stained with lipophilic dye Nile red, revealing localization of the tagged protein to either lipid droplets or cytosol. All of the GFP fusion proteins were expressed under control of the MET25 promoter in yeast. The panels (from left to right) display fluorescent images of GFP signals, Nile red signals, dual signals (Merge), and transmission microscope (Trans) images of baker’s yeast. The scale bar is 5 μm. The images are representative of the cells in at least 50 different microscopic fields.

Lipid Droplet-associated Proteins in Mycobacteria

Mycobacterial LD-associated Proteins Regulate TG Buildup—The majority of LD-associated proteins has functions related to lipid metabolism (11–18). To evaluate the role of the identified proteins in TG metabolism, we generated single gene deletion mutants of tgs1, tgs2, BCG1489c, and BCG1169c and their respective complemented strains in M. bovis BCG. Several attempts to disrupt gene BCG1721 failed, suggesting that it encodes an essential activity (see below). The strains were grown under hypoxia-induced NRP conditions. Neutral lipids were extracted and analyzed by TLC. Densitometry showed that TG storage was moderately diminished in all gene deletion mutants, and it was fully restored in their complemented strains expressing the plasmid-borne wild type (WT) genes (Fig. 3A). Liquid chromatography-tandem MS analysis of lipid extracts from Δ-tgs1 and Δ-BCG1169c mutants revealed that TG species containing very long chain FAs (>C22, e.g. TG(60:1)) were more significantly affected than those with long chain FAs (C12–C22, e.g. TG(52:3)) in these strains (Fig. 3B and supplemental Fig. S2). In contrast, the Δ-tgs2 and Δ-BCG1489c mutants displayed an overall reduction of all measured TG species (Fig. 3B and supplemental Fig. S2). These data demonstrate that the identified LD proteins indeed participate in TG homeostasis but are distinct with respect to their contribution to TG molecular species.

BCG1721 Promotes the Biogenesis of TGs in Mycobacteria—Domain alignment analysis of BCG1721 indicated that the protein contains a lipase and an ACSL domain at the N- and C-terminal regions, respectively (Fig. 4A). Based on these observations, a comparative model structure of the lipase domain was computed (27) using a mammalian TG lipase (Protein Data Bank code 1k8q) as a template (Fig. 4B). By superimposing these structures, the putative catalytic triad consisting of Ser150, Asp309, and His338 was deduced (Fig. 4C). The model also indicates that the lipase domain contains a “lid” (31) that may regulate access of the substrate to the active site (Fig. 4B). To ascertain the putative lipase activity of BCG1721, serine 150 of the catalytic triad was mutated to an alanine residue (S150A)
Lipid Droplet-associated Proteins in Mycobacteria

by site-directed mutagenesis. BCG1721 and BCG1721(S150A) were expressed as glutathione S-transferase (GST) fusion proteins in a baker's yeast, Δ-tg3Δ-tg4 double mutant that is devoid of TG hydrolysis (29) and tested for lipase activity. Transformed yeast mutants overexpressing BCG1721-GST or Tgl4-GST, the endogenous LD-associated lipase (11), indeed had reduced TG content as compared with Δ-tg3Δ-tg4 mutants expressing BCG1721(S150A)-GST or GST alone (supplemental Fig. S3). In agreement with its localization to yeast LDs, these data confirm that BCG1721 is indeed a TG lipase in vivo and that serine 150 is essential for catalysis.

Because repeated efforts to generate a gene deletion mutant of BCG1721 failed, indicating that the gene might be essential for growth (32), we attempted overexpression of the gene in M. bovis BCG to characterize its potential role in TG metabolism. BCG1721 and BCG1721(S150A) were cloned into plasmid pMV262 under a constitutive hsp60 promoter and introduced into M. bovis BCG to generate strains pBCG1721 and pBCG1721(S150A), respectively. Quantitative reverse transcription-PCR indeed confirmed elevated levels of BCG1721 transcripts in both strains (Fig. 4D).

LD formation was assessed in vivo in logarithmically growing BCG WT and pBCG1721 or pBCG1721(S150A) mutant strains by confocal microscopy after staining with Nile red (Fig. 4D). LD were absent from the WT, as expected under these experimental conditions of stimulated TG catabolism, but were observed in both pBCG1721 and pBCG1721(S150A). These data suggest that the putative ACSL, rather than the lipase function, of the gene might be dominant under this experimental condition (Fig. 4D). Indeed, TLC and liquid chromatography–tandem MS analysis revealed complex qualitative and quantitative changes in TG levels (Fig. 4, E and F, and supplemental Fig. S4); TGs containing long chain FAs (e.g. TG(52:3)) were significantly enriched in both pBCG1721 and pBCG1721(S150A) strains; however, TGs with very long chain FAs (e.g. TG(60:1)) were significantly reduced only in pBCG1721 but not in pBCG1721(S150A), compared with the BCG WT (Fig. 4F and supplemental Fig. S4). These results demonstrate that BCG1721 is involved in both anabolism and catabolism of TGs in M. bovis BCG.

Overexpression of BCG1721(S150A) Leads to Reduced TG Hydrolysis and Cell Viability during Regrowth from NRP—To investigate the physiological role of BCG1721 during hypoxic NRP and regrowth phases, cultures of WT, pBCG1721, or pBCG1721(S150A) were grown in the hypoxia-induced NRP model, before reactivation with oxygen (air). TLC analysis showed elevated levels of TGs in hypoxic NRP strain pBCG1721 and the pBCG1721(S150A) mutant as compared with the WT (Fig. 5A). No TG hydrolase activity was observed in pBCG1721 during the NRP state (Fig. 5B), suggesting that the lipase is likely to be inactive under this physiological condition. Whereas the TG content of WT dropped during exposure to oxygen, consistent with stimulated TG hydrolysis, overexpression of BCG1721(S150A) led to a marked reduction of TG breakdown during regrowth (Fig. 5A). This observation suggests a dominant negative effect of the inactive lipase under this condition (Fig. 5B).

Because TGs are required for the regrowth of mycobacteria from hypoxic NRP (9) and TG breakdown was attenuated in pBCG1721(S150A), we evaluated the viability of the strain during regrowth. Indeed, bacilli overexpressing BCG1721(S150A) showed significantly reduced regrowth compared with cells without plasmid or with an additional WT copy of BCG1721. These results further demonstrate that TG breakdown is required for the regrowth of M. bovis BCG from the hypoxic NRP state (Fig. 5C).

DISCUSSION

M. tuberculosis is known to accumulate LDs during NRP states such as hypoxia-induced dormancy (6, 7). These bacilli are phenotypically drug-resistant (5), which is believed to be a cause for lengthy TB treatment and latent infection. Virulent W-Beijing family strains of M. tuberculosis also store up large amounts of TGs (33). Garton et al. (6, 7) reported the presence of intracellular LDs in M. tuberculosis isolated from the sputum of TB patients, highlighting the importance of TGs during infection. Our earlier findings emphasized the role of LD formation and utilization during dormancy (NRP) and regrowth of M. bovis BCG (9). To obtain functional insight into the role of TG metabolism in mycobacteria, we attempted the identification and characterization of proteins involved in this process, which would greatly assist in developing novel antibacterials to
treat TB. Here we decipher the mycobacterial LD proteome and its role in lipid biosynthesis and metabolism.

Our work identified five LD-associated proteins involved in TG metabolism, and their presumed positions in the TG metabolic pathway are indicated in Fig. 6. The TG synthases, Tgs1 and Tgs2, which are orthologs of mammalian LD-associated diacylglycerol acyltransferase 2 (34, 35), are abundant proteins in the mycobacterial LD-enriched fraction, thus validating the isolation method. Moreover, given the substrate specificity for very long chain FAs, our results are consistent with the analyses obtained for Tgs1 (10). TG profiling of Tgs2-null mutant cells implied that Tgs2 may have a broader preference for FA substrates as compared with that of Tgs1.

Identification of different distinct TG molecular species supports the concept that bacterial LDs are synthesized initially as small diacylglycerol acyltransferase-associated pre-LDs, before coalescing to become mature LDs, as shown in Rhodococcus opacus and Acinetobacter calcoaceticus (19). In our analysis of LD-associated proteins, we have identified the putative 1-acylglycerol-3-phosphate acyltransferase, BCG1489c. This enzyme catalyzes the formation of phosphatidic acid, which is an important precursor for the synthesis of diacylglycerol that is subsequently acylated to TG, by Tgs. In addition, or alternatively, BCG1489c may also function to generate phosphatic acid as a precursor for the synthesis of phospholipids. Because deletion of the BCG1489c gene resulted in an overall reduction of TG levels, it rather suggests a more prominent role in providing precursors for TG synthesis, in mycobacteria. Interestingly, we did not detect any proteins with homologies to phosphatidic acid phosphatases to be associated with LDs; presumably such proteins, like their yeast and mammalian orthologs, are cytoplasmic and/or membrane-bound (36, 37).

A novel, presumably bi-functional enzyme BCG1721 containing an N-terminal lipase and a C-terminal ACSL domain was found to be associated with LDs. We demonstrate that this enzyme has TG lipase activity also in the heterologous yeast domain and that this activity is abolished by a single point muta-

![Figure 4](image-url)
tion (S150A) in the predicted catalytic consensus site (GXSXG). Moreover, the ACSL domain at the C terminus appears to promote the biosynthesis of TG and LD accumulation, in agreement with reports that ACSL orthologs in human myocytes (38) and HEK293 cells (39) increase TG content rather than phospholipid production.

The twin functions of the protein allow us to postulate that, during reactivation from the hypoxic NRP state, BCG1721 is able to hydrolyze TGs via the lipase domain to yield nonesterified FAs, which are next derivatized to acyl-CoA with the help of the ACSL domain (FA activation). The final product, fatty acyl-CoA, may subsequently be used in a number of metabolic pathways such as β-oxidation (40) or synthesis of phospholipids for membrane turnover (41) or diacylglycerol re-esterification to TG. In eukaryotes, ACSL and TG lipase domains have never been described on a single polypeptide, but rather both enzymes may indeed exist independently of each other on LDs (11, 14). We found that the ACSL domain was active, independent of the lipase function of the protein, thus providing fatty acyl-CoA substrates for the Tgs-catalyzed reactions, specifically during the logarithmic phase and the hypoxia-induced NRP state.

In the absence of active BCG1721 lipase (S150A mutant), TG breakdown was efficiently blocked in M. bovis BCG during regrowth. Consequently, the TG-loaded bacteria were unable to reactivate from the NRP state. This finding is consistent with our previous observation that utilization of TG is required for regrowth (9) and further highlights the importance of the intracellular TG pools in mycobacteria. Although the pBCG1721(S150A) mutant strain harbors a WT copy of the gene, the observed TG phenotype is presumably due to a dominant negative effect, as has previously been observed in the case of a kinase-dead mutant of PknG in M. bovis BCG (42).

The highly abundant LD-associated protein HspX, which is ubiquitously present in mycobacteria (30), acts as a chaperone enhancing the stability of protein by preventing misfolding (43). Similar chaperone proteins were also identified on eukaryotic LDs (11, 13, 14), although their functional relevance in LD biology still remains elusive. Because LDs have hydrophobic
cores, HspX could either associate nonspecifically to the LD, or it may be functionally involved in stabilizing LD-associated proteins in their native conformations.

The LD-associated protein BCG1169c is unique to the Mycobacterium family (44) and is not found in other prokaryotes and eukaryotes. Although BCG1169c has no functional annotation, it also appears to play a role in TG biogenesis: mutants deleted of BCG1169c displayed a TG profile similar to Δ-tgs1 bacilli. Because of its relatively small size of 23 kDa and the absence of any conserved motifs indicative of known lipases (8) or acyltransferases (4), we propose that BCG1169c may be a co-activator or other regulator of TG metabolizing enzymes; its specific functional role in TG homeostasis remains to be determined.

TGs are considered important pathogenicity factors, based on the occurrence of M. tuberculosis containing intracellular LDs in human sputum (6, 7). This raises the possibility that genes encoding LD-associated proteins could be overexpressed during virulence. Indeed, it was shown that the DosR operon in hypervirulent strains of the W-Beijing M. tuberculosis family is involved in TG buildup (33). In particular, hspX and tgs1 genes, which comprise this dormancy regulon, are constitutively overexpressed in W-Beijing strains (33) and TG-loaded M. tuberculosis isolated from human sputum (7). Moreover, HspX is required for the growth of mycobacteria in macrophages, establishing a role for this chaperone during initial infection (45). An additional regulator, KstR (Rv3574), that is induced in vivo (46) and is required for infection in mice (47), links virulence to lipid homeostasis. Genomic phenotyping of the kstR-null mutant in M. smegmatis revealed that the Tet-R regulator controls expression of a large number of lipid metabolizing genes, including an ortholog of BCG1489c (48). These results further highlight the functional link between LD-associated proteins and M. tuberculosis virulence.

Results from many independent studies converge in the view that intracellular LDs typically share a general structure, consisting of a core of TGs and/or steryl esters, surrounded by a monolayer of phospholipids with a few embedded proteins (49). Subcellular localization of mycobacterial LD-associating proteins to the yeast LDs demonstrates that targeting and anchorage of such polypeptides are largely independent of the host organism. These results also strongly imply that the identified proteins are indeed LD-associated in mycobacteria and are not mere contaminations from other cellular fractions or the isolation procedure. These data are furthermore in agreement with other reports that (i) murine TG lipase localizes to yeast LDs (29) and (ii) eukaryotic PAT (termed after the first three identified members: perilipin, adipophilin, and tail-interacting protein of 47 kDa) family proteins are correctly targeted to the LDs of prokaryotes (20).

Interestingly, mycobacteria and yeast lack genes that encode for orthologs of PAT proteins (50), suggesting that this family of proteins may not be essential to the integrity of LD structure in these microorganisms. Obviously, regulatory mechanisms involved in TG storage and utilization between various cell types are quite diverse. On the other hand, a comparison of our results and a similar study in yeast (11) revealed that proteins with certain functions are conserved in both microorganisms and include 1-acylglycerol-3-phosphate acyltransferase, TG lipase, and ACSL. In fact, enzymes with the latter two functions are also conserved in LDs of mammalian cells (14, 16). This suggests functional conservation of these lipid metabolizing enzymes in a wide variety of prokaryotic and eukaryotic species. It furthermore demonstrates that these are presumably the core enzymatic functions required for LD biogenesis and degradation, conserved from mycobacteria to mammals. The occurrence of dual function activities on single polypeptides, such as the TG lipase and acyltransferase activities on yeast LD-associated proteins Tgl3 and Tgl5 (51) or the lipase and putative ACSL activity on the mycobacterial BCG1721, is intriguing and further highlights the complex metabolic and regulatory interplay to establish and maintain TG homeostasis and the importance of this process for cellular maintenance and survival.

Acknowledgments—We thank Nurhidaya Binte Shadan and Fui Leng Yan for technical assistance with confocal microscopy; William Jacobs for the gift of the pYUB854, pMV262, and pMV306 plasmids; Tanya Parish for the gift of the pGOAL17 plasmids; and Julia Petschogg for helpful discussions.

REFERENCES

1. Wayne, L. G., and Sohaskey, C. D. (2001) Annu. Rev. Microbiol. 55, 139–163
2. Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., and Dye, C. (2003) Arch. Intern. Med. 163, 1009–1021
3. Russell, D. G. (2003) Nat. Cell Biol. 5, 776–778
4. Daniel, J., Deb, C., Dubey, V. S., Sirakova, T. D., Abomoelak, B., Morbidoni, H. R., and Kolattukudy, P. E. (2004) J. Bacteriol. 186, 5017–5030
5. Deb, C., Lee, C. M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., Pawar, S., Rogers, L., and Kolattukudy, P. E. (2009) PLoS ONE 4, e6077
6. Garton, N. J., Christensen, H., Minnikin, D. E., Adegbona, R. A., and Barer, M. R. (2002) Microbiology 148, 2951–2958
7. Garton, N. J., Waddell, S. J., Sherratt, A. L., Lee, S. M., Smith, R. J., Senner, C., Hinds, J., Rajakumar, K., Adegbona, R. A., Besra, G. S., Butcher, P. D., and Barer, M. R. (2008) PLoS Med. 5, 675
8. Deb, C., Daniel, J., Sirakova, T. D., Abomoelak, B., Dubey, V. S., and Kolattukudy, P. E. (2006) J. Biol. Chem. 281, 3866–3875
9. Low, K. L., Rao, P. S., Shui, G., Bendt, A. K., Pethe, K., Dick, T., and Wenk, M. R. (2009) J. Bacteriol. 191, 5037–5043
10. Sirakova, T. D., Dubey, V. S., Deb, C., Daniel, J., Korotkova, T. A., Abomoelak, B., and Kolattukudy, P. E. (2006) Microbiology 152, 2717–2725
11. Arensmaa, T., Jaakkola, M., Lintula, J., Heikkinen, M. J., and Kuokkanen, M. (2009) J. Lipid Res. 50, 1600–1609
12. Rats, T., Zheng, Z., Chen, Y., Serrero, G., Zhao, Y., and Liu, P. (2007) J. Proteome. Res. 6, 3256–3265
13. Beller, M., Riedel, D., Jansch, L., Dieterich, G., Wehland, J., Jaehne, H., and Kühnlein, R. P. (2006) J. Lipid. Res. 47, 1079–1089
14. Brasaemle, D. L., Dobos, G., Shapiro, L., and Wang, R. (2004) J. Biol. Chem. 279, 46835–46842
15. Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Moriz, M., Higashi, Y., Kojima, S., and Takano, T. (2004) Biochem. Biophys. Acta 1647, 47–59
16. Liu, P., Ying, Y., Zhao, Y., Mundy, D. I., Zhu, M., and Anderson, R. G. (2004) J. Biol. Chem. 279, 3737–3792
17. Sato, S., Fukasawa, M., Yamakawa, Y., Natsume, T., Suzuki, T., Shoji, I., Azai, A., Miyamura, T., and Nishijima, M. (2006) J. Bacteriol. 189, 921–930
18. Yan for technical assistance with confocal microscopy; William Jacobs for the gift of the pYUB854, pMV262, and pMV306 plasmids; Tanya Parish for the gift of the pGOAL17 plasmids; and Julia Petschogg for helpful discussions.

"Lipid Droplet-associated Proteins in Mycobacteria"
Lipid Droplet-associated Proteins in Mycobacteria

20. Hänsch, J., Wäldermann, M., Robenek, H., and Steinbüchel, A. (2006) *Appl. Environ. Microbiol.* **72**, 6743–6750
21. Kalscheuer, R., Luftmann, H., and Steinbüchel, A. (2004) *Appl. Environ. Microbiol.* **70**, 7119–7125
22. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68**, 850–858
23. Bardarov, S., Bardarov, S., Jr., Pavelka, M. S., Jr., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W. R., Jr. (2002) *Microbiology* **148**, 3007–3017
24. Parish, T., and Stoker, N. G. (2000) *Microbiology* **146**, 1969–1975
25. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, G. F., Snapper, S. B., Barletta, R. G., Jacobs, W. R., and Bloom, B. R. (1991) *Nature* **351**, 456–460
26. Lobley, A., Sadowski, M. I., and Jones, D. T. (2009) *Bioinformatics* **25**, 1761–1767
27. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2007) *Current Protocols in Protein Science*, Chapter 2, Unit 2.9, John Wiley & Sons, New York
28. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 283–291
29. Kurat, C. F., Natter, K., Petschnigg, J., Wolinski, H., Scheuringer, K., Scholz, H., Zimmermann, R., Leber, R., Zechner, R., and Kohlwein, S. D. (2006) *J. Biol. Chem.* **281**, 491–500
30. Cunningham, A. F., and Spreadbury, C. L. (1998) *J. Bacteriol.* **180**, 801–808
31. Secundo, F., Carrea, G., Tarabiono, C., Gatti-Lafranconi, P., Brocca, S., Lotti, M., Jaeger, K. E., Puls, M., and Eggert, T. (2006) *J. Mol. Catal. B Enzym.* **39**, 166–170
32. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) *Mol. Microbiol.* **48**, 77–84
33. Reed, M. B., Gagneux, S., Deriemer, K., Small, P. M., and Barry, C. E., 3rd (2007) *J. Bacteriol.* **189**, 2583–2589
34. Cases, S., Stone, S. J., Zhou, P., Yen, E., Tow, B., Lardizabal, K. D., Voelker, T., and Farese, R. V., Jr. (2001) *J. Biol. Chem.* **276**, 38870–38876
35. Kueschner, L., Moessinger, C., and Thiele, C. (2008) *Traffic* **9**, 338–352
36. Lin, Y. P., and Carman, G. M. (1989) *J. Biol. Chem.* **264**, 8641–8645
37. Han, G. S., Wu, W. I., and Carman, G. M. (2006) *J. Biol. Chem.* **281**, 9210–9218
38. García-Martínez, C., Marotta, M., Moore-Carrasco, R., Guitart, M., Camps, M., Busquets, S., Montell, E., and Gómez-Fox, A. M. (2005) *Am. J. Physiol. Cell Physiol.* **288**, C1264–C1272