



Published in final edited form as:

Microbiology (Reading). 2006 September ; 152(Pt 9): 2717–2725. doi:10.1099/mic.0.28993-0.

Identification of a diacylglycerol acyltransferase gene involved in accumulation of triacylglycerol in *Mycobacterium tuberculosis* under stress

Tatiana D. Sirakova[†], Vinod S. Dubey[†], Chirajyoti Deb, Jaiyanth Daniel, Tatiana A. Korotkova, Bassam Abomoelak, Pappachan E. Kolattukudy

Biomolecular Science Center, and Department of Molecular Biology and Microbiology, Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL 32816-2364, USA

Abstract

Mycobacterium tuberculosis under stress stores triacylglycerol (TG). There are 15 genes in *M. tuberculosis* that belong to a novel family of TG synthase genes (*tgs*), but it is not known which of them is responsible for this accumulation of TG. In this paper, it is reported that *M. tuberculosis* H37Rv accumulated TG under acidic, static or hypoxic growth conditions, or upon treatment with NO, whereas TG accumulation was drastically reduced in the *tgsI* (Rv3130c) disrupted mutant. Complementation with *tgsI* restored this TG accumulation. C₂₆ was a major fatty acid in this TG, indicating that the TGS1 gene product uses C₂₆ fatty acid, which is known to be produced by the mycobacterial fatty acid synthase. TGS1 expressed in *Escherichia coli* preferred C₂₆:0-CoA for TG synthesis. If TG storage is needed for the long-term survival of *M. tuberculosis* under dormant conditions, the *tgsI* product could be a suitable target for antilateness drugs.

INTRODUCTION

Tuberculosis (TB), one of the biggest killers among the infectious diseases, poses a major public health problem mostly in the developing world with 8 million new cases and 2 million deaths a year (World Health Organization, 2005; Zhang, 2004). *Mycobacterium tuberculosis*, the causative agent of TB, is inhaled by people as an aerosol emanating from active TB patients. Usually, only a small fraction of the people (~5 %) thus infected develop active TB, while the rest carry a latent infection for the rest of their lives until reactivation of the pathogen occurs upon weakening of the immune system. Thus, AIDS causes reactivation of the latent tubercle bacillus leading to rapid bacillary growth and dissemination, resulting in the development of active TB (Cosma et al., 2003; Zahrt, 2003). The ability of the pathogen to go into a latent/dormant non-replicating state, with very low metabolic activity and phenotypic resistance to all of the currently available antituberculosis drugs (Zhang, 2004; Zahrt, 2003), makes TB eradication an extremely difficult challenge.

The metabolic and physiological state of the dormant pathogen that allows it to survive inside the host for decades remains unclear. There is strong evidence that fatty acids are the

Correspondence Pappachan E. Kolattukudy pk@mail.ucf.edu.

[†]These authors contributed equally to this work.

energy source used by the pathogen for its long-term survival during the persistence phase of infection (Gomez & McKinney, 2004; Russell, 2003). In *M. tuberculosis*, two genes, *icl1* and *icl2*, encode isocitrate lyase (ICL), a key metabolic enzyme involved in fatty acid utilization. It has recently been shown that deletion of both *icl1* and *icl2*, but not deletion of either one alone, results in complete impairment of intracellular replication in macrophages and rapid elimination from mouse lungs (Munoz-Elias & McKinney, 2005). Chemical inhibition of both ICL1 and ICL2 also blocks the growth of *M. tuberculosis* on fatty acids and in macrophages. However, the source of the fatty acids remains unknown (Munoz-Elias & McKinney, 2005; Russell, 2003). Recently it has been shown that *M. tuberculosis* can synthesize and store triacylglycerol (TG) as an energy reserve under hypoxia and NO treatment, stress conditions among others that lead to a dormancy-like state in culture (Daniel et al., 2004). Intracellular TG inclusion bodies found in pathogenic *M. tuberculosis* obtained from organ lesions (Garton et al., 2002), and decreased host-cell TG levels induced by growth of *Mycobacterium bovis* BCG in macrophages (Jackson et al., 1989), suggest that TG is used as an energy source for intracellular growth of the pathogen.

Fifteen members of a novel class of diacylglycerol acyltransferase genes designated *tgs* (triacylglycerol synthase) have been identified (Daniel et al., 2004), based on homology to a gene found in *Acinetobacter calcoaceticus* (Kalscheuer & Steinbuechel, 2003). Which, if any, of these genes encodes the enzyme(s) actually involved in TG synthesis is not known. In this report, we show that *tgs1* deficiency abolishes TG accumulation under several stress conditions, and that complementation with *tgs1* restores TG accumulation. We also show that TGS1 expressed in *Escherichia coli* preferentially uses C₂₆ fatty acyl-CoA for TG synthesis, and that C₂₆ fatty acid, which is known to be produced by the fatty acid synthase of *M. tuberculosis* (Kikuchi et al., 1992), is a major long-chain fatty acid in the TG accumulated under stress conditions.

METHODS

Bacterial strains, growth media and chemicals

M. tuberculosis H37Rv (ATCC 25618), the *tgs1* mutant and the complemented strain were grown in Middlebrook 7H9 (Difco), Dubos–Tween–albumin medium broth (Difco) and Sauton medium, as previously described (Daniel et al., 2004). *E. coli* DH5 α and HB101 strains (Life Technologies) were used for cloning and propagation of plasmids and phasmids. For selection of transformants, *E. coli* was grown in Luria–Bertani (LB) broth or on agar. *Mycobacterium smegmatis* mc²155 was grown in liquid LB medium with 0.5 % Tween 80 for competent cell preparation, and in Middlebrook 7H9 broth with 0.05 % Tween 80 for transduction. When required, antibiotics were added to the culture media at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$ for *E. coli*; hygromycin B, 150 $\mu\text{g ml}^{-1}$ for *E. coli* or 75 $\mu\text{g ml}^{-1}$ for *M. tuberculosis*; and kanamycin, 50 $\mu\text{g ml}^{-1}$ for *E. coli* or 20 $\mu\text{g ml}^{-1}$ for *M. tuberculosis*. The NO donor (spermine NONOate) and its reference compound spermine tetrahydrochloride were purchased from Alexis Corporation. Other chemicals and antibiotics were from Sigma and Fisher Scientific. DNA restriction and modifying enzymes were obtained from New England Biolabs. AcylCoA substrates were purchased from American Radiolabelled Chemicals.

Growth conditions for *M. tuberculosis*

We subjected *M. tuberculosis* to several *in vitro* stress conditions, such as hypoxia, NO treatment, low pH and static growth. For hypoxia and NO treatment, experiments were done as previously described (Daniel et al., 2004). For hypoxia experiments, the initial OD₆₀₀ of the starting culture was 0.06, and, at each time point, an equal volume of cell culture (~200 ml) from each strain was harvested, and the cells were used for lipid extraction. At each time point, an equal proportion of lipids from each strain was used for (TLC) separation for TG analysis. Resistance to isoniazid (0.4 µg ml⁻¹) and sensitivity to metronidazole (12 µg ml⁻¹) of hypoxic cultures were also tested (Daniel et al., 2004). The culture conditions for the NO treatment experiment were the same as those previously described (Daniel et al., 2004), except that the cells from each strain were collected after 6 h of NO treatment, and used for RNA isolation and TG analysis. For low-pH growth, *M. tuberculosis* cells were initially grown in Middlebrook 7H9 media to OD₆₀₀ 0.6–0.8, and the cells were washed and suspended in Middlebrook 7H9 media, pH 5.0, and grown for 3 weeks in a roller bottle at 37 °C. For growth under static conditions, *M. tuberculosis* cells were grown for 2 weeks in Middlebrook 7H9 media to OD₆₀₀ 1.6, and then the culture was left to stand for 1 week at 37 °C. Cells collected at different time periods were used for RNA isolation and TG analysis.

Generation of *tgs1*-disrupted mutant of *M. tuberculosis*

The general strategy used for gene disruption with the specialized transducing phage system was similar to that used previously by Bardarov et al. (2002) and Sirakova et al. (2001). *tgs1* was disrupted by allelic exchange via specialized transduction using conditionally replicating mycobacteriophage phAE87. The disrupted copy of the gene was constructed by PCR amplification of the 5′- and 3′-flanking regions of the gene using *M. tuberculosis* DNA as a template, and primer pairs introducing *Bsp*HI and *Pst*I sites, and *Xho*I and *Bsp*HI sites, at the 5′ and 3′ ends of the products, respectively (Table 1). Both products were then sequentially inserted on either side of the hygromycin-resistance gene (*hyg*) cloned in vector pUC19. The 5′-flanking region consisted of the first 10 bp of *tgs1* and the 784 bp sequence upstream of the gene. The 3′-flanking region contained the last 122 bp of the *tgs1* ORF and the 667 bp downstream sequence. The disrupted copy of the gene was released by *Bsp*HI digestion, and cloned into pYUB572, which contains a bacteriophage lambda cos site and a *Pac*I site. The recombinant cosmid was digested with *Pac*I, ligated into the *Pac*I site of phAE87, and the resultant recombinant phage was used to transduce wild-type *M. tuberculosis*. Individual hygromycin-resistant colonies were analysed by PCR and Southern blot analysis to identify clones in which allelic exchange had occurred at the *tgs1* locus. Several *tgs1* mutants were screened by PCR analysis using specific primers located inside the deleted fragment of the gene (F and R), as shown in Table 1. The allelic exchange by double crossover was confirmed with two sets of primers, each representing a *hyg* primer (primers H1 and H2) and a primer (primers E and F) in the mycobacterial genome outside the gene sequence used for making the disruption construct (Fig. 1A).

Complementation of *M. tuberculosis tgs1* mutant

For complementation experiments, the *tgs1* coding sequence was amplified from genomic *M. tuberculosis* H37Rv DNA using Pfu Turbo Hotstart DNA Polymerase (Stratagene) and primers that introduced a *KpnI* site at both ends. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen). After sequencing to confirm the absence of any mutations, the insert was excised from this vector by *KpnI* digestion, and subcloned into *KpnI*-digested replicative shuttle vector pMIP12 for expression under the *pBlaf⁺* promoter (LeDantec et al., 1991). The construct was electroporated into the *tgs1*-disrupted mutant, and transformants were selected on 7H11 plates with both kanamycin and hygromycin. Isolated colonies from the plates were checked for the expression of *tgs1* by RT-PCR using the primers shown in Table 1. RNA isolation and RT-PCR analysis were done as described previously (Daniel et al., 2004).

Genomic DNA isolation and Southern blotting

M. tuberculosis H37Rv genomic DNA was isolated by the guanidine thiocyanate (GTC) method using a solution of GTC, Tris/HCl and sarcosyl (Derbyshire & Bardarov, 2000). DNA samples were digested with *EcoRI* and *EcoRI/BamHI* restriction enzymes, subjected to 1 % agarose gel electrophoresis, transferred to nylon membranes (Nytran Plus; Schleicher and Schuell), and hybridized with [α -³²P]dCTP-labelled probes using the random prime labelling system Rediprime II (Amersham Pharmacia).

TG analysis

M. tuberculosis wild-type, the *tgs1* mutant and the complemented strain of *tgs1* cells were subjected to the desired stress at defined experimental time points, recovered by centrifugation, autoclaved and subjected to lipid extraction in chloroform/methanol (2 : 1, v/v) (Sirakova et al., 2001). In a separate set of hypoxia experiments, aliquots of the culture of *M. tuberculosis* wild-type and the *tgs1* mutant, taken at various time points, were incubated with 50 μ M oleic acid in 0.5 % BSA solution, and after 6 h incubation, lipids were extracted. At each defined time point, under various stress conditions, an equal proportion of the total extracted lipids (~5–10 mg) from each strain was subjected to Silica-G TLC for TG analysis, using n-hexane/diethylether (9 : 1, v/v) as a solvent system. Dichromate/sulfuric acid charring of TLC plates was used to detect lipids. The charred TLC plates were also scanned for quantification of TG accumulation using the AlphaImager 2200 Gel Doc system (Alpha Innotech). Fatty acid methyl esters were prepared from TG by transesterification with 14 % BF₃ in methanol, and analysed by (GC) using the flame-ionization detector response; the GC conditions for fatty acid methyl esters were the same as those previously described (Sirakova et al., 2001). All stress growth experiments were repeated at least three times, and similar patterns were seen in all cases. Since details such as cell density were not absolutely identical in all repetitions, we present the mean values and results from a typical experiment.

Enzyme activity of TGS1 expressed in *E. coli*

TGS1 was expressed in *E. coli* grown in Dual Media (Zymo Research) for 12 h at 37 °C, and total cell lysates were assayed for TGS activity. The assays were performed in 0.1 M citrate/

phosphate buffer, pH 6.5, containing 1 mg BSA, 10 mM MgCl₂, 14.5 μM [¹⁴C]diolein (specific activity 55 Ci mol⁻¹, 2.04 TBq mol⁻¹, American Radiolabelled Chemicals), 500 μM diolein, indicated concentrations of oleoyl (C_{18:1})-CoA or hexacosanoyl (C_{26:0})-CoA and 100–200 μg protein in a total volume of 250 μl for 2 h at 37 °C. Following the assay, the reaction mixture was extracted and the TG product was quantified as described previously (Daniel et al., 2004).

RESULTS

Disruption of *tgs1* in *M. tuberculosis* and complementation of the disrupted mutant

On the basis of the enzymic activities of expressed *tgs* gene products and the level of their induction as the pathogen was subjected to hypoxia and NO stress conditions, *tgs1* appeared to have the greatest potential for involvement in TG synthesis (Daniel et al., 2004). To test this possibility, we generated a *tgs1*-disrupted mutant of *M. tuberculosis*. *tgs1* was disrupted by allelic exchange via specialized transduction using conditionally replicating mycobacteriophage phAE87 (Bardarov et al., 2002). To prepare the *tgs1* disruption construct, a 1260 bp *tgs1* ORF, out of the total 1392 bp, was replaced with the hygromycin-resistance gene, and was used as the substrate for allelic exchange by double crossover (Fig. 1A). PCR screening of the hygromycin-resistant transductants with a set of primers (F and R) specific for the deleted segment identified several mutants that failed to amplify the expected 662 bp fragment (data not shown). Disruption of *tgs1* by homologous recombination was confirmed by further PCR analysis of the flanking regions (primer pairs E/H1 and H2/F), which yielded the expected-size products. Southern blot analysis of *M. tuberculosis* wild-type and three *tgs1* mutants is shown in Fig. 1(B). Genomic DNA from *M. tuberculosis* digested with *EcoRI* and *BamHI/EcoRI* showed a 5.2 kb and a 2.5 kb hybridization band, respectively, when the 5'-flanking region of the construct was used as the probe. DNA from the mutants, under the same conditions, showed a 1.7 kb band from both digestions, due to the presence of an *EcoRI* site in the *hyg* gene sequence and the absence of a *BamHI* site. RT-PCR analysis showed that the mutant did not produce any *tgs1* transcripts (Fig. 1C).

We used a replicative shuttle vector, pMIP12, to complement the disrupted mutant by expressing the *tgs1* ORF under the control of the β-lactamase promoter (*pblaF**) of *Mycobacterium fortuitum*, one of the strongest mycobacterial promoters (LeDantec et al., 1991). After electroporation of the expression construct into the *tgs1*-disrupted mutant, transformants were selected on 7H11 plates supplemented with both kanamycin and hygromycin. Isolated colonies were checked for the restored expression of *tgs1*. RT-PCR analysis of the complemented strain showed that *tgs1* was expressed (Fig. 1C).

TG analysis of *M. tuberculosis*, *tgs1* mutant and complemented mutant under hypoxia

Previously we have shown that *M. tuberculosis* accumulates TG under hypoxia (Daniel et al., 2004). To test whether *tgs1* deficiency affected the ability to accumulate TG under such conditions, we cultured the cells under hypoxia. Under these conditions, the pathogen developed isoniazid resistance and metronidazole susceptibility by 16 days. The samples from both the wild-type and *tgs1* mutant were collected at 0, 6, 11 and 16 days, and

extracted lipids were separated by TLC and subjected to dichromate/sulfuric acid charring to visualize TG. The *tgsI* mutant showed no detectable level of TG at any time points under hypoxic growth conditions, whereas the wild-type started accumulating TG from day 6 and reached a maximum at day 16 (Fig. 2A). Lipids were isolated from the silica gel region corresponding to TG, and fatty acid analysis was done by GC. This analysis showed that TG from the wild-type contained all fatty acids ranging from C₁₆ to C₃₀, with C₂₆ as the major fatty acid among the longer-chain fatty acids, whereas C₂₆ fatty acid in the *tgsI* mutant was virtually absent (Fig. 3). To determine whether TG accumulation could be restored in the *tgsI* mutant by providing exogenous fatty acids, we added oleic acid as a BSA complex at days 6, 11 and 16 to both wild-type and *tgsI* mutant cell cultures. Charring of lipids extracted from these cultures showed that the *tgsI* mutant at days 11 and 16 contained a drastically reduced level of TG compared to the wild-type (Fig. 2B). Even the trace amounts of TG present in the *tgsI* mutant contained small amounts of the fatty acids present in the wild-type TG, but C₂₆ was absent (data not shown). The small amount of TG produced by the oleic acid-supplemented cultures of the *tgsI* mutant contained oleic acid as a major component and some C₁₆–C₂₄ fatty acids in similar proportions to those found in the wild-type, but C₂₆ was barely detectable (data not shown).

In a separate set of experiments, we also subjected the complemented strain of the *tgsI* mutant to the same hypoxic conditions to see if it could restore TG accumulation. Cultures of the wild-type, *tgsI* mutant and complemented *tgsI* strain were grown under hypoxia for 16 days, the lipids extracted from the cells were separated by TLC, and the plates were subjected to dichromate/sulfuric acid charring. The results showed that the *tgsI* mutant was unable to accumulate TG, whereas its complemented strain accumulated TG just as in the wild-type (Fig. 4). TG isolated from both the wild-type and the complemented strain showed a similar fatty acid profile, with C₂₆ as the major fatty acid among the longer-chain fatty acids, whereas C₂₆ fatty acid was virtually absent in the *tgsI* mutant (Fig. 3).

Acyl chain length preference of recombinant TGS1

The fatty acid analysis of the TG produced by the wild-type and *tgsI* mutant suggested that TGS1 may prefer C₂₆ fatty acyl-CoA as a substrate. To test for this possibility, we expressed TGS1 in *E. coli* and assayed the TGS activity in the total cell lysate using both C_{18:1}- and C_{26:0}-CoA as substrates. Results clearly showed that TGS1 had a preference for C_{26:0}-CoA over C_{18:1}-CoA (Fig. 5). The K_m values for C_{26:0}-CoA and C_{18:1}-CoA were calculated to be 306 and 540 μM , respectively, from linear double-reciprocal plots. The respective V_{max} values for C_{26:0}-CoA and C_{18:1}-CoA were calculated to be 2.4 and 1.1 $\text{nmol mg}^{-1} \text{min}^{-1}$.

Role of *tgs1* in TG synthesis under acidic and static growth conditions and upon NO treatment

Besides hypoxic stress, bacilli are also believed to encounter other stress factors, such as acidic pH in caseous granuloma, slow growth in the static phase, and NO production inside the host macrophages (Chan et al., 2001; Florczyk et al., 2003; Nathan, 2002; Saviola et al., 2003). Therefore, we tested whether the *tgsI* mutant was able to accumulate TG under such stress conditions. *M. tuberculosis* wild-type, the *tgsI* mutant and the *tgsI* complemented strain were grown under acidic stress conditions, and TG accumulation was analysed. After

3 weeks growth in acidic medium, the wild-type accumulated TG, but it was not detected in the *tgsI* mutant, whereas the *tgsI* complemented strain accumulated a level of TG similar to that found in the wild-type (Fig. 6A). Fatty acid analysis of the TG accumulated under acidic conditions showed that the major fatty acids were C₁₆–C₂₈, with C₂₆ as the major component among the longer-chain fatty acids. The *tgsI* mutant showed no detectable level of C₂₆ fatty acid, even in the trace amount of TG that was found. The complemented *tgsI* mutant showed a TG fatty acid profile identical to that of the wild-type (data not shown).

tgsI is reported to be induced in *M. tuberculosis* grown under static conditions (Florczyk et al., 2003). To test whether TG accumulates, and to ascertain whether the *tgsI* product is involved in TG synthesis under such conditions, we tested the wild-type, *tgsI* mutant and the complemented mutant. Cell cultures grown for 2 weeks in Middlebrook 7H9 medium in a roller incubator were allowed to settle, and were further incubated for 1 week as standing cultures. Total lipids were extracted from these cultures and analysed by TLC. Dichromate/sulfuric acid charring results showed that the *tgsI* mutant lost the ability to accumulate TG. The complemented strain accumulated TG to a level similar to that observed in the wild-type (Fig. 6B). Fatty acid analysis of the TG from both the wild-type and complemented mutant strain showed a similar fatty acid profile, with C₁₆ and C₁₈ being the major species, along with a very low level of longer-chain fatty acids (C₂₀–C₂₆), whereas in the *tgsI* mutant that showed the same proportion of smaller-chain fatty acids, C₂₆ was not even detectable (data not shown).

Treatment of *M. tuberculosis* with NO is known to trigger induction of the hypoxia regulon, including *tgsI* (Ohno et al., 2003; Voskuil et al., 2003), and to cause accumulation of TG (Daniel et al., 2004). To test for the role of *tgsI* in TG accumulation, we subjected the *tgsI* mutant and its complemented strain to NO treatment. After 6 h NO treatment, cells were harvested for total lipid extraction. The lipids were separated by TLC, and dichromate/sulfuric acid charring of the TLC plate showed that the TG level in the *tgsI* mutant was much less (30 %) than that in the wild-type, whereas the complemented strain accumulated TG to a level comparable to that found in the wild-type (Fig. 6C). Fatty acid analysis of TG isolated from both the wild-type and the *tgsI*-complemented strain showed C₁₆–C₂₈ fatty acids, with C₂₆ as a major component; however, in the *tgsI* mutant, the level of C₂₆ fatty acid was drastically reduced (data not shown).

RT-PCR analyses of *tgs* transcript levels under various stress culture conditions

Induction levels of 15 *tgs* genes were assessed by semi-quantitative RT-PCR analyses of mRNA isolated from cells of the wild-type, *tgsI* mutant and *tgsI*-complemented strains grown under different stress conditions. The *tgsI* transcript was completely absent in the *tgsI* mutant but it was induced in the wild-type and *tgsI*-complemented strains under all the stress conditions, as expected. Transcript levels of the other *tgs* genes under hypoxic, static and acidic growth conditions were similar in all three strains; however, in NO-treated cells of the *tgsI* mutant, transcript levels of a few *tgs* genes (Rv3371, Rv1760, Rv2285 and Rv1425) were slightly higher than those in the wild-type and *tgsI*-complemented strains (data not shown).

DISCUSSION

In spite of the strong evidence for the use of fatty acids as the major source of energy for growth in the persistent stage (Munoz-Elias & McKinney, 2005; Russell, 2003; Segal & Bloch, 1956; Wayne & Sohaskey, 2001), the source of fatty acids has not been identified. TG is widely used as a storage form of energy in many living organisms. Being a completely reduced form of substrate, TG can generate much higher levels of energy than other types of storage materials such as carbohydrates. For long-term survival with very low metabolic rates, TG is used by many organisms, including animals in hibernation, dormant oil seeds and fungal spores. We postulated that the fatty acids used during the long-term survival of *M. tuberculosis* in the persistent/dormant state are stored in the form of TG (Daniel et al., 2004). This hypothesis is supported by the finding that TG-containing inclusion bodies appear soon after the provision of fatty acids, and under nutrient starvation (Garton et al., 2002). Growth of *M. bovis* BCG in macrophages causes decreased TG levels in the host cells (Jackson et al., 1989), suggesting that TG is used as an energy source for the intracellular growth of the pathogen. These findings suggest that lipid body formation and accumulation of TG play an important role in the metabolism of pathogenic *M. tuberculosis*.

We have previously identified 15 genes in the *M. tuberculosis* genome which could potentially encode TGS; all 15 *tgs* genes are expressed in this pathogen in culture, and their products expressed in *E. coli* show TGS activity when tested with dioleoylglycerol and oleoyl-CoA as substrates (Daniel et al., 2004). Some of these *tgs* genes have been found to be highly induced as the pathogen enters a non-replicating state in culture, upon slow withdrawal of oxygen and upon treatment with NO (Daniel et al., 2004), two conditions known to trigger induction of some of the genes potentially involved in latency/dormancy (Ohno et al., 2003; Voskuil et al., 2003). Based on the enzymic activities of the expressed *tgs* gene products and the degree of their induction, *tgsI* appears to have the potential to be a major contributor to TG synthesis induced by hypoxia and NO (Daniel et al., 2004). To assess the role of the *tgsI* locus in TG accumulation, *tgsI* was disrupted by allelic exchange using a phage-mediated delivery system. We subjected the pathogen to various *in vitro* stress conditions, and found that all of these resulted in accumulation of TG in the wild-type, but in the *tgsI* mutant, TG accumulation was drastically reduced under such conditions. To confirm that the inability of the mutant to accumulate TG was due to inactivation of the *tgsI* gene, and not to any secondary effects, we complemented the mutant with a copy of the *tgsI* gene expressed under the strong *pBlaF** promoter on the replicative plasmid pMIP12. Under similar *in vitro* stress conditions, where the *tgsI* mutant showed drastically reduced levels of TG accumulation, complementation of the *tgsI* gene restored the ability of the pathogen to accumulate TG.

Oxygen limitation (hypoxic conditions) during granuloma development has been proposed as one of the main signals that can change the metabolic status of the bacilli so that they enter the dormant state (Wayne & Sohaskey, 2001). *tgsI* has been found to be part of the dormancy regulon (Park et al., 2003), and its promoter region contains a motif characteristic of the promoters of the α -crystalline homologue protein (*acr*) gene family, which is consistent with the gene being upregulated under hypoxic conditions (Florczyk et al., 2003). It is noteworthy that *tgsI* shows high induction *in vivo* within macrophages upon IFN- γ

activation (Schnappinger et al., 2003), under both hypoxia and NO treatment (Daniel et al., 2004; Ohno et al., 2003; Park et al., 2003; Voskuil et al., 2003), and in static culture (Florczyk et al., 2003). Along with several other stress factors, such as hypoxia, nutrient deprivation, and production of NO and IFN- γ by host cells (Chan et al., 2001; Nathan, 2002; Wayne & Sohaskey, 2001), acidic pH is also considered to be one of the major stress factors which bacteria may encounter inside the host granuloma (Saviola et al., 2003). Previously, when *M. tuberculosis* was subjected to 15 min acidic shock, changes in *tgsI* expression were not reported (Fisher et al., 2002). However, in our experimental conditions, where *M. tuberculosis* was grown under acidic conditions for 3 weeks, we observed accumulation of TG in the wild-type pathogen, whereas the *tgsI* mutant did not accumulate TG, implicating an important role for *tgsI* in acidic growth conditions.

Complementation restored TG storage with similar fatty acids, with C₂₆ as the major long-chain fatty acid component. The C₂₆ preference of TGS1, suggested by these results, was further supported by the finding that recombinant TGS1 showed significantly higher TGS activity with C_{26:0}-CoA as a substrate than with C_{18:1}-CoA. Since C₂₆ fatty acid is exclusively produced by *M. tuberculosis* and not synthesized by its host, the preference of TGS1 protein for C_{26:0}-CoA over C_{18:1}-CoA implies its central role in utilizing the endogenously synthesized long-chain C₂₆ fatty acid. *M. tuberculosis* is known to have a multifunctional fatty acid synthase that generates fatty acids with a bimodal distribution centred at C₁₆ and C₂₆ (Kikuchi et al., 1992), as previously reported for *Mycobacterium phlei* (Brindley et al., 1969). The present results show that C₂₆ is a major fatty acid in the TG stored by the pathogen under stress. When exogenous [¹⁴C]oleic acid is provided to the pathogen under stress, ¹⁴C is incorporated into C₂₆, as well as the C_{18:1} found in the stored TG (Daniel et al., 2004), indicating that exogenous fatty acids are catabolized, and the C₂ units generated are used for the synthesis of C₂₆ by the fatty acid synthase within the pathogen. We found that C₂₆ was absent even in the very small amount of TG that was made by the *tgsI*-deficient organism.

We have already demonstrated the impaired ability of the *tgsI* mutant in storing TG under *in vitro* stress conditions; however, it is possible that different sets of *tgs* genes are induced in response to the different stress factors encountered in the host by the pathogen, in order to enable the organism to synthesize TG with maximum efficiency, if TG is indeed used *in vivo* as the main form of energy storage in dormancy. Elevated expression of other *tgs* genes has also been reported in various *in vitro* growth conditions. Microarray analysis has shown that, under nutrient starvation conditions, Rv3734c, Rv3087 and Rv3088 are upregulated, whereas Rv3233c and Rv3234c are repressed (Betts et al., 2002). Rv3087 and Rv3088 are also upregulated after 15 min of acidic shock to *M. tuberculosis* (Fisher et al., 2002), and Rv3087 and Rv3371 are predicted to be required for *in vivo* survival in mice (Sasseti & Rubin, 2003). It is possible that under *in vivo* conditions, different *tgs* genes are induced under the influence of the different host factors.

ACKNOWLEDGEMENTS

This work was supported in part by grants AI46582 and AI35272 from the National Institutes of Health.

Abbreviations

ICL	isocitrate lyase
TB	tuberculosis
TG	triacylglycerol

REFERENCES

- Bardarov S, Bardarov S Jr, Pavelka MS Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs WR Jr. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology*. 2002; 148:3007–3017. [PubMed: 12368434]
- Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol*. 2002; 43:717–731. [PubMed: 11929527]
- Brindley DN, Matsumura S, Bloch K. *Mycobacterium phlei* fatty acid synthase – a bacterial multienzyme complex. *Nature*. 1969; 224:666–669.
- Chan ED, Chan J, Schluger NW. What is the role of nitric oxide in murine and human host defense against tuberculosis? Current knowledge. *J Respir Cell Mol Biol*. 2001; 25:606–612.
- Cosma CL, Sherman DR, Ramakrishnan L. The secret lives of the pathogenic mycobacteria. *Annu Rev Microbiol*. 2003; 57:641–676. [PubMed: 14527294]
- Daniel J, Deb C, Dubey VS, Sirakova TD, Abomoelak B, Morbidoni HR, Kolattukudy PE. Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. *J Bacteriol*. 2004; 186:5017–5030. [PubMed: 15262939]
- Derbyshire, KM, Bardarov, S. DNA transfer in mycobacteria: conjugation and transduction. In: Hatfull, GF, Jacobs, WR, Jr, editors. *Molecular Genetics of Mycobacteria*. American Society for Microbiology; Washington, DC: 2000. 93–107.
- Fisher MA, Plikaytis BB, Shinnick TM. Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes. *J Bacteriol*. 2002; 184:4025–4032. [PubMed: 12081975]
- Florczyk MA, McCue LA, Purkayastha A, Currenti E, Wolin MJ, McDonough KA. A family of *acracr*-coregulated *Mycobacterium tuberculosis* genes shares a common DNA motif and requires Rv3133c (dosR or devR) for expression. *Infect Immun*. 2003; 71:5332–5343. [PubMed: 12933881]
- Garton NJ, Christensen H, Minnikin DE, Adegbola RA, Barer MR. Intracellular lipophilic inclusions of mycobacteria *in vitro* and in sputum. *Microbiology*. 2002; 148:2951–2958. [PubMed: 12368428]
- Gomez JE, McKinney JD. *M. tuberculosis* persistence, latency, and drug tolerance. *Tuberculosis*. 2004; 84:29–44. [PubMed: 14670344]
- Jackson SK, Stark JM, Taylor S, Harwood JL. Changes in phospholipids fatty acid composition and triacylglycerol content in mouse tissues after infection with bacilli Calmette–Guerin. *Br J Exp Pathol*. 1989; 70:435–441. [PubMed: 2669926]
- Kalscheuer R, Steinbuechel A. A novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *J Biol Chem*. 2003; 278:8075–8082. [PubMed: 12502715]
- Kikuchi S, Rainwater DL, Kolattukudy PE. Purification and characterization of an unusually large fatty acid synthase from *Mycobacterium tuberculosis* var. bovis BCG. *Arch Biochem Biophys*. 1992; 295:318–326. [PubMed: 1586161]
- LeDantec C, Winter N, Gicquel B, Vincent V, Picardeau M. Genomic sequence and transcriptional analysis of a 23-kilobase mycobacterial linear plasmid:evidence for horizontal transfer and identification of plasmid maintenance systems. *J Bacteriology*. 1991; 183:2157–2164.

- Munoz-Elias EJ, McKinney JD. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nat Med.* 2005; 11:638–644. [PubMed: 15895072]
- Nathan C. Inducible nitric oxide synthase in the tuberculous human lung. *Am J Respir Crit Care Med.* 2002; 166:130–131. [PubMed: 12119220]
- Ohno H, Zhu G, Mohan VP, Chu D, Kohno S, Jacobs WR Jr, Chan J. The effects of reactive nitrogen intermediates on gene expression in *Mycobacterium tuberculosis*. *Cell Microbiol.* 2003; 5:637–648. [PubMed: 12925133]
- Park HD, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik GK, Sherman DR. Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol Microbiol.* 2003; 48:833–843. [PubMed: 12694625]
- Russell DG. Phagosomes, fatty acids and tuberculosis. *Nat Cell Biol.* 2003; 5:776–778. [PubMed: 12951608]
- Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A.* 2003; 100:12989–12994. [PubMed: 14569030]
- Saviola B, Woolwine SC, Bishai WR. Isolation of acid-inducible genes of *Mycobacterium tuberculosis* with the use of recombinase-based *in vivo* expression technology. *Infect Immun.* 2003; 71:1379–1388. [PubMed: 12595455]
- Schnappinger D, Ehrt S, Voskuil MI. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med.* 2003; 198:693–704. [PubMed: 12953091]
- Segal W, Bloch H. Biochemical differentiation of *Mycobacterium tuberculosis* grown *in vivo* and *in vitro*. *J Bacteriol.* 1956; 72:132–141. [PubMed: 13366889]
- Sirakova TD, Thirumala AK, Dubey VS, Sprecher H, Kolattukudy PE. The *Mycobacterium tuberculosis* *pkS2* gene encodes the synthase for the hepta- and octamethyl-branched fatty acids required for sulfolipid synthesis. *J Biol Chem.* 2001; 276:16833–16839. [PubMed: 11278910]
- Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med.* 2003; 198:705–713. [PubMed: 12953092]
- Wayne LG, Sohaskey CD. Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol.* 2001; 55:139–163. [PubMed: 11544352]
- World Health Organization. Global tuberculosis control. 2005 <http://www.who.int/tb/publications/2005/en/index.html>
- Zahrt TC. Molecular mechanisms regulating persistent *Mycobacterium tuberculosis* infection. *Microbes Infect.* 2003; 5:159–167. [PubMed: 12650774]
- Zhang Y. Persistent and dormant bacilli and latent tuberculosis. *Front Biosci.* 2004; 9:1136–1156. [PubMed: 14977534]

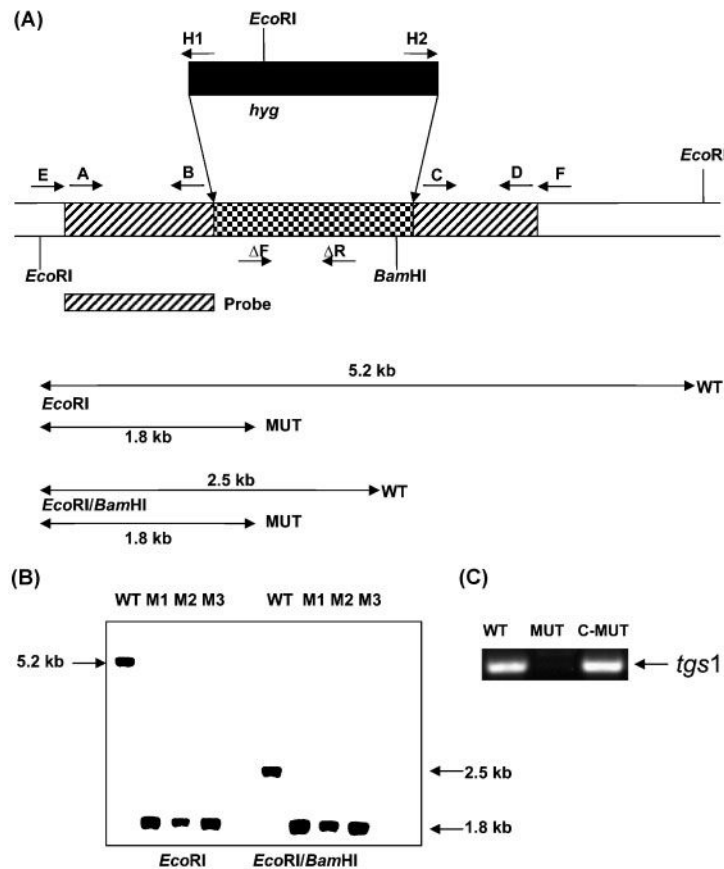


Fig. 1. Generation of a *tgs1* mutant of *M. tuberculosis*. (A) Schematic representation of the disruption construct for *tgs1*. Hatched and checked segments represent the regions used to make the disruption construct. The checked segment was replaced with the *hyg* gene cassette (black box). Primer pairs E/H1, H2/F and F/R were used for PCR analysis of homologous recombination as described in the text. (B) Southern blot hybridization of the wild-type (WT) and three mutant clones (M1, M2 and M3). (C) RT-PCR analysis showing the *tgs1* transcript in the WT and *tgs1* complemented strains (C-MUT), but not in the *tgs1* mutant (MUT).

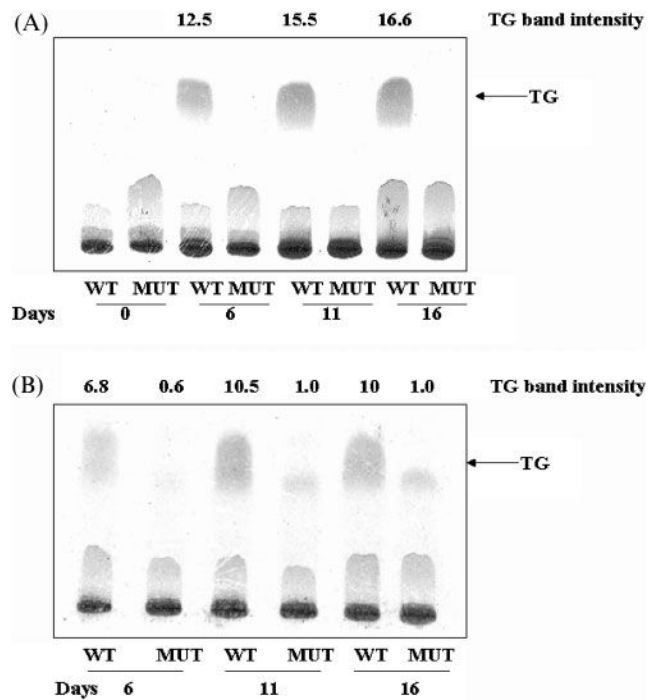


Fig. 2. Dichromate/sulfuric acid charring of lipids showing TG accumulation in *M. tuberculosis* under hypoxia. At each time point total lipids were extracted from *M. tuberculosis* WT and MUT (A), and after 6 h incubation with 50 μ M oleic acid/0.5% BSA (B). In each case, the same proportion of lipids was separated by TLC using n-hexane/diethyl ether (9 : 1, v/v). The intensity of the TG band determined in arbitrary units by the AlphaImager 2200 Gel Doc system is shown at the top of each panel. For strain abbreviations, see legend to Fig. 1.

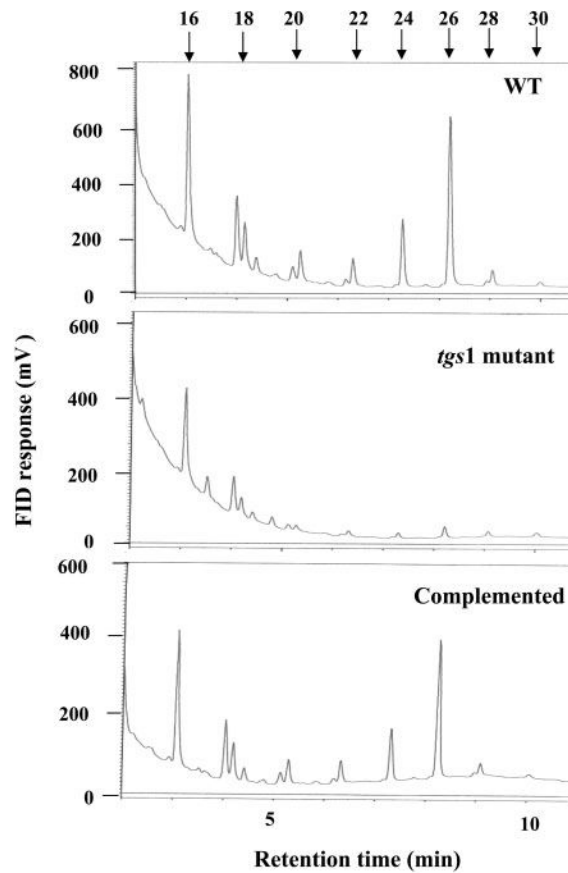


Fig. 3. GC analysis of fatty acids in TG from *M. tuberculosis* cells after 16 days under hypoxia. Fatty acid methyl esters were prepared from TG recovered from TLC plates from the wild-type (WT; top panel), *tgs1* mutant (middle panel) and complemented *tgs1* mutant (bottom panel). Retention times of n-fatty acids are indicated at the top.

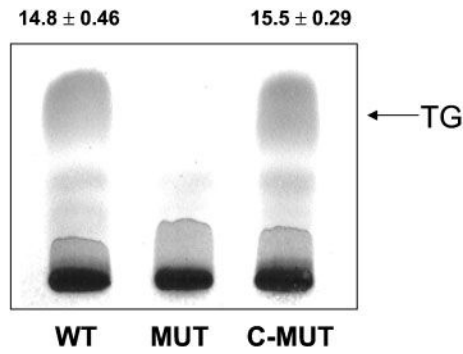


Fig. 4. TG accumulation in *M. tuberculosis* WT, MUT and C-MUT after 16 days under hypoxia. The same proportion of lipids was separated by TLC using n-hexane/diethyl ether (9 : 1, v/v), and lipids were visualized by dichromate/sulfuric acid charring. A charred TLC chromatogram is shown from a typical experiment, and the intensity of the TG band was determined in arbitrary units by the AlphaImager 2200 Gel Doc system. The values are given as the mean \pm SEM of three independent measurements. For strain abbreviations, see legend to Fig. 1.

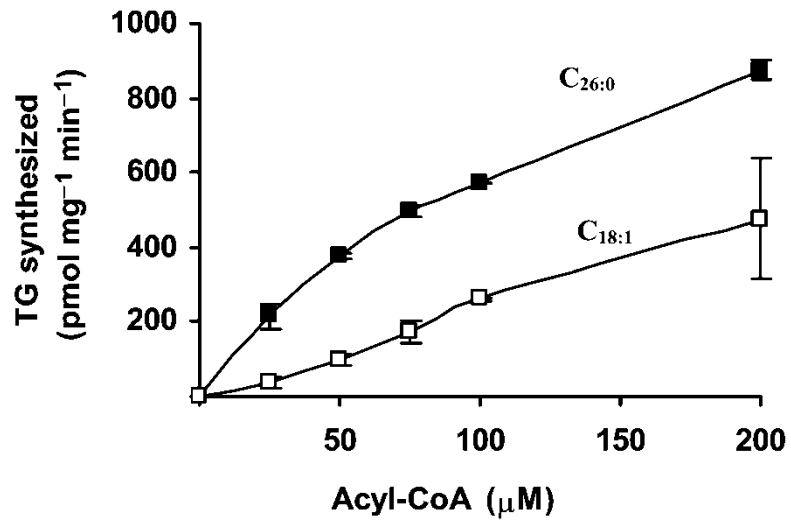


Fig. 5. Effects of acyl chain length on TG synthesis by recombinant TGS1. Total cell lysates of *E. coli* expressing TGS1 were assayed for TGS activity using C_{26:0}-CoA (■) and C_{18:1}-CoA (□).

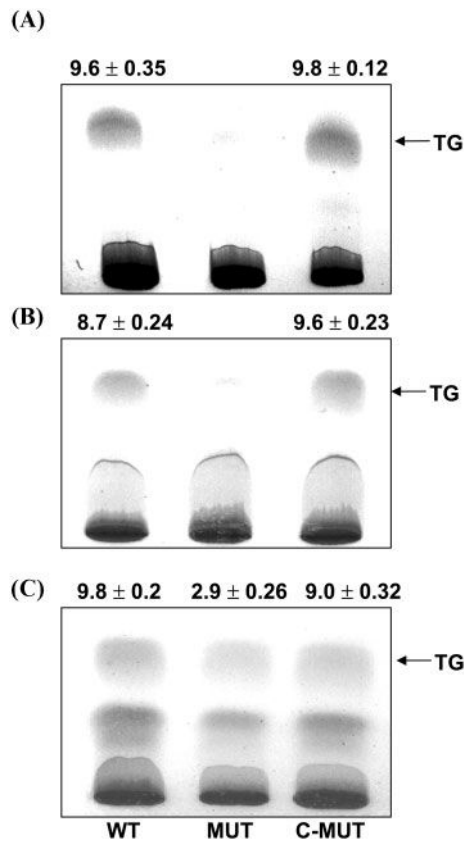


Fig. 6. Dichromate/sulfuric acid charring of lipids showing TG accumulation in *M. tuberculosis* grown under stress conditions. Total lipids extracted from WT, MUT and C-MUT under low pH (A) or static growth conditions (B), or with NO treatment (C) are shown. In each case, the same proportion of lipids was separated by TLC using n-hexane/diethyl ether (9:1, v/v). Charred TLC chromatograms are shown from a typical experiment, and the intensity of the TG band was determined in arbitrary units by the Alpha Imager 2200 Gel Doc system. The values are given as the mean \pm SEM of three independent measurements. For strain abbreviations, see legend to Fig. 1.

Table 1Primers used for making the *tgs1* disruption construct in *M. tuberculosis*

Primer pair	Primer	Sequence
Primer pairs used to generate 5' and 3' flanks of <i>tgs1</i> genes		
5' flank	A	5'-tcatgatcttgccgatccagc-3'
	B	5'-ctgcagggtgattcatggtcagc-3'
3' flank	C	5'-ctcgagactggttagcagatgcc-3'
	D	5'-tcatgatactcccgactgcc-3'
Primer pairs inside the deleted segment		
	F	5'-ttatgctcctcctcaacgc-3'
	R	5'-ttccgtaatcggcaagcgc-3'
Primer pairs used for genomic flanks		
5' flank	E	5'-cggcattgatcgggtcccaacc-3'
	H1	5'-tgagcggatggtgtcgtatct-3'
3' flank	H2	5'-ggaactggcgcagttcctctggg-3'
	F	5'-tcacgccaactccaacacaccg-3'
Primer pairs used for RT-PCR		
<i>tgs1</i>	F	5'-tgggtcgtcgacatgggtggcgag-3'
	R	5'-cgtgctaagcccgcgcgtc-3'
23S rRNA	F	5'-gtggcgtttctggaccggaagcg-3'
	R	5'-gtccatcgactacgcctgtcgcc-3'