Targeting of long chain triacylglycerol hydrolase gene for tuberculosis treatment (CON)

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TARGETING OF LONG CHAIN TRIACYLGLYCEROL HYDROLASE GENE FOR TUBERCULOSIS TREATMENT

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Related U.S. Application Data
Continuation of application No. 11/561,460, filed on Nov. 20, 2006, now Pat. No. 7,601,357.
Provisional application No. 60/748,284, filed on Dec. 7, 2005.

References Cited
U.S. PATENT DOCUMENTS

OTHER PUBLICATIONS

* cited by examiner

Disclosed herein are novel methods for screening for compounds useful in treating or preventing tuberculosis. In exemplary embodiments, screening methods are based on the implementation or manipulation of triacylglycerol hydrolase like polypeptides or polynucleotides encoding the same. The methods are useful in identifying agents active against TB infection.

ABSTRACT

7 Claims, 8 Drawing Sheets
FIGURE 1

A

5.9 ± 0.4  3.3 ± 0.2

0 hr  6 hr in PBS

B

16.3 ± 0.7  8.1 ± 0.6

0 hr  6 hr in PBS

C

18.4 ± 0.7  18.0 ± 0.8  7.4 ± 0.7  19.3 ± 1.3  18.5 ± 0.5  17.1 ± 1.2

0 h  7H9  PBS  0 h  7H9  PBS

Wild type  Δ-lipY mutant
FIGURE 3

A

Rv3096

Rv3095

Rv3099

ss

Rv3098

Rv3097 (lpy)

H2

B

E

A

H1

Δ-F

Δ-R

Probe (804 bp)

5'-flank

deleted part

3'-flank

Mutants

WT

1 2 3 4 5 6 7 8 9

10 11 12 13 14 15 16 17 18

C

1330 bp

1007 bp

804 bp

D

Mutants

WT

deleted part

804 bp
FIGURE 5

A

Fatty acid released (µmol/mg)

Time (min)

B

Fatty acid released (pmol/min)

LipY protein (µg)

C

Fatty acid released (nmol/mg/min)

Triolein Concentration (mM)

D

Fatty acid released (nmol/mg/min)

pH
FIGURE 6

A

Relative Activity (%)

0 100 200 300 400 500

E-600 (µM)

0 0.1 0.2 0.3 0.4 0.5

B

Relative Activity (%)

0 10 20 30 40 50 60 70 80 90 100

PMSF (mM)

0 1 2 3 4 5

C

Relative Activity (%)

0 20 40 60 80 100

Temperature (°C)

0 20 40 60 80 100
**FIGURE 8**

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TARGETING OF LONG CHAIN TRIACYLGLYCEROL HYDROLASE GENE FOR TUBERCULOSIS TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Ser. No. 11/561,460 filed Nov. 20, 2006, now U.S. Pat. No. 7,601,357, which claims priority to U.S. Provisional Patent No. 60/748,284, filed Dec. 7, 2005, which are incorporated herein by reference. Priority is claimed under 35 USC §120.

GOVERNMENT SUPPORT

This invention was made through support from the NIH, Grant Nos. A146582 and A135272. The government may have certain rights in this invention.

BACKGROUND

Tuberculosis (TB) has been a major health problem for most of recorded history and Mycobacterium tuberculosis remains one of the world’s most significant pathogens. Responsible for millions of new cases of tuberculosis annually (see e.g. Pablo-Mendez et al., (1998) New Engl. J. Med. 338, 1641-1649), it is the leading cause of death from a single infectious agent. While the incidence of the disease declined in parallel with advancing standards of living since at least the mid-nineteenth century, in spite of the efforts of numerous health organizations worldwide, the eradication of tuberculosis has never been achieved, and is imminent.

TB is acquired by the respiratory route; actively infected individuals spread this infection efficiently by coughing or sneezing “droplet nuclei” which contain viable bacilli. Overcrowded living conditions and shared air spaces are especially conducive to the spread of TB, underlying the increase in instances that have been observed in the U.S. in prison inmates and among the homeless in larger cities.

Medical experts estimate that about 10 million Americans are infected with TB bacteria, and about 10 percent of these people will develop active TB in their lifetime. However, TB is an increasing worldwide problem, especially in Africa. It is estimated that, worldwide, about one billion people will become newly infected, over 150 million people will contract active TB, and 36 million people will die between now and 2020 unless TB control is improved.

The emergence of multi-drug resistant strains of Mycobacterium tuberculosis poses serious threats to the control of this disease due to the complex nature of second-line drug treatment (WHO Report. (2004) WHO/HLM/TB/2004.343). Upon infection the bacterium goes through an initial replicative phase inside the alveolar macrophages after which it enters a non-replicative, drug-resistant state of dormancy. This state of dormancy is probably induced by the environmental stress exerted upon the pathogen by the host’s immune response. The bacterium is able to survive in this dormant state for decades until the host’s immune system is weakened when it reactivates and causes the infectious disease (Donnenberg, Jr., A. M., and Rook G. A. W. (1994) In Tuberculosis: Pathogenesis, Protection and Control, Bloom, B. R., (Ed.) American Society of Microbiology, Washington D.C.). The current anti-mycobacterial drugs are able to kill only the actively replicating mycobacteria and do not clear the latent bacteria from the host (Homer zu Bentrop, K., and Russell D. G. (2001) Trends Microbiol. 9, 597-605). Thus latency is a major problem in TB control. One-third of the world population is infected with the latent microorganism and nearly two million deaths occur annually (Dye, C., Scheele, S., Dolin, P., Pathania, V., and Raviglione M. C. (1999) JAMA. 282, 677-686, WHO Report. (2005) WHO/HLM/TB/2005). Individuals carrying a latent infection are estimated to harbor a 2-23% lifetime risk of reactivation (Zahr, T. C. (2003). Microbes Infect. 5, 159-167).

If an individual has TB disease, i.e., has active TB, the individual typically is administered a combination of several drugs. It is very important, however, that the individual continue a correct treatment regimen for the full length of the treatment. If the drugs are taken incorrectly, or stopped, the individual can suffer a relapse and will be able to infect others with TB.

When an individual becomes sick with TB a second time, the TB infection may be more difficult to treat because the TB bacteria have become drug resistant, i.e., TB bacteria in the body are unaffected by some drugs used to treat TB. Multidrug-resistant tuberculosis (MDR TB) is a very dangerous form of tuberculosis. In particular, some TB bacteria become resistant to the effects of various anti-TB drugs, and these resistant TB bacteria then can cause TB disease. Like regular TB, MDR TB can be spread to others.

To avoid drug resistance in the treatment of TB, a four-drug regimen, i.e., isoniazid, rifampin, pyrazinamide, and streptomycin, is administered to TB patients. Aminoglycosides, such as streptomycin, are important anti-TB agents, but their utility is restricted by the requirement of parenteral administration, which is inconvenient and leads to poor patient compliance. It is theorized that poor patient compliance also can lead to the development of drug resistance, and it appears that the frequency of streptomycin resistance among anti-TB drugs is surpassed only by isoniazid.

In view of the above, an urgent need exists for new anti-TB agents useful in an effective treatment regimen for both the active and latent TB, and that effectively treat TB caused by multidrug resistant (MDR) strains of bacteria. Therefore, it would be advantageous to provide compounds and compositions for administration to an individual in the treatment of tuberculosis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: TG utilization by M. tuberculosis wild type and lipY mutant cells under nutrient starvation condition. (A) Autoradiogram and (B) dichromate/sulfuric acid charring of lipids showing utilization of TG by wild type M. tuberculosis in PBS medium. After 12 days of hyopxic growth, cells were incubated with 1 µCi of 14C-oleic acid for 1 hr and the cells were washed with PBS prior to 6 hr incubation in same media. (C) Dichromate/sulfuric acid charring of lipids showing utilization of triacylglycerol (TG)-accumulated during 12 days of hypoxic growth of M. tuberculosis (wild type) and lipY mutant cells at 0 h and by 6 h incubation in PBS and nutrient rich (7H9) media. Lipids were separated on Silica gel TLC using n-hexane:diethyl ether (9:1) as developing solvent. In panel A, autoradiogram is shown from a typical experiment and the incorporation values of 14C into TG is shown as a percentage of the total 14C administered. In panel B and C, charred TLC chromatograms are shown from a typical experiment and the intensity of the TG band was determined in arbitrary units by the Alphalmager 2200 Gel Doc system. On top of each panel, the values are given as ±SEM of three independent measurements.

FIG. 2: RT-PCR assessment of the induction of lip genes upon starvation (A) and estimation of the potential contribution to TG hydolysis (B). M. tuberculosis H37Rv was incu-
bated in PBS or in 7H9 medium for 6 h after TG accumulation by hypoxic growth for 12 days. Transcript levels of lips are shown as a fraction of 23S rRNA transcripts (mean±SD from three independent experiments). The methods used for quantitation and experimental details are given in Experimental Procedures. In B, the potential relative contribution of each lip gene product to the total lipase activity was estimated by multiplying transcript level with the lipase activity of each gene product expressed in E. coli.

FIG. 3: Generation of a lipY deficient mutant of M. tuberculosis. (A) Genomic organization of lipY (Rv3097c). (B) Schematic representation of the disruption construct for lipY. A hatched region with grey flanks depicts the genome part of the deleted sequence of lipY. WT, wild type. Text. (C) PCR analysis of 5'-flank (lanes: 1-5, disruption construct. The part of the lipY gene (upward diagonal hatch) replaced with res-lipY-res gene cassette is shown. Primer pairs E/H1, H2/F, and D-F/D-R (solid square check box) were used for PCR analysis of recombinant as described in the text. (C) PCR analysis of 5'-flank (lanes: 1-5, Δ-lipY mutants; 6, wild type), deleted part of the gene (lanes: 7-11, Δ-lipY mutants; 12, wild type) and 3'-flank (lanes: 13-17, Δ-lipY mutants; 18, wild type). (D) Southern blot hybridization of five Δ-lipY mutant clones and wild type probed with the part of the deleted sequence of lipY WT, wild type.

FIG. 4: SDS-PAGE analysis of the expression and purification of LIPY. His-tagged LIPY was expressed in E. coli BL21 cells, solubilized from the 16,000 x g pellet from cell lysate and purified by cobalt affinity chromatography (TALON) as described in Experimental Procedures. Protein samples were loaded on a 12% SDS-polyacrylamide gel under reducing conditions. Lane 1, Benchmark prestained molecular weight markers (Invitrogen); lane 2, 16,000xg supernatant from solubilized inclusion bodies; lane 3, flow-through fraction from TALON resin; lanes 4-5, 10 mM imidazole wash; lanes 6-9, 150 mM imidazole eluted fractions. LIPY WT and LIPY total cell lysate are shown.

FIG. 5: Characterization of triolein hydrolase activity of purified LIPY. A) Time-course of [14C]tri olein hydrolysis at pH 8.0 was determined using 8 μg of purified LIPY protein per assay. B) Protein concentration dependence of TG hydrolysis (incubation time, 2 h) C) substrate concentration dependence of enzyme activity (incubation time, 2 h). D) Effect of pH on lipase activity. LIPY was incubated for 15 min at room temperature in 0.1 M citrate-phosphate buffer pH 6.0 or 0.1 M potassium phosphate buffer pH 7.0 or 0.1 M Tris-HCl pH 7.5 or pH 8.0 or 0.1 M glycine-NaOH buffer pH 10.0 prior to the assay. Values are mean±SD for three replicates.

FIG. 6: Effect of E-600 (A), and PMSF (B), and temperature on TG hydrolysis by LIPY. LIPY was pre-incubated with indicated concentration of inhibitor for 15 min at room temperature prior to assay at 37°C. C, LIPY was incubated at indicated temperature for 15 min prior to assay. Values are mean±SD from three separate experiments.

FIG. 7: Effect of detergents on LIPY activity. Purified LIPY was pre-incubated with the indicated concentrations of SDS (A), Triton X-100 (B) or Tween-20 (C) for 15 min at room temperature prior to assay. Activity relative to control is given as mean±SD from three replicates.

FIG. 8: Structure-based alignment of the conserved domains of LIPY with lipases from the HSL family. The multiple sequence alignment produced by ClustalW was used to align other members of the HSL family with LIPY using ClustalW and the output was manually adjusted for optimal alignment. The sequences selected for alignment with LIPY (SEQ ID NO: 69) are A. fulgidus carboxylesterase (GI 17943077; SEQ ID NO: 70), Acinetobacter esterase (GI 34559428; SEQ ID NO: 71), Human HSL (GI 21328445; SEQ ID NO: 72), Pseudomonas B11-1 lipase (GI 2853612; SEQ ID NO: 73), B. subtilis brefedlin A esterase (GI 414772; SEQ ID NO: 74), M. tuberculosis CDC1551 esterase (GI 15840511; SEQ ID NO: 75), M. tuberculosis CDC1551 PE family protein (GI 15842668; SEQ ID NO: 76), R. solanacearum putative esterase/lipase (GI 17545158; SEQ ID NO: 77), and S. aureus hypothetical protein (GI 15928114; SEQ ID NO: 78). Black shading indicates residues conserved in seven or more aligned sequences and shades of grey are used to indicate residues conserved in a few sequences. ▲, amino acids belonging to the catalytic triad. The ClustalW program was accessed from the European Bioinformatics Institute website at ebi.ac.uk/cluster.

DETAILED DESCRIPTION

The ability of Mycobacterium tuberculosis to go into a latent/dormant state and survive under such conditions for decades make TB control extremely difficult. Developing drugs targeted at the ability of the pathogen to survive under such latent conditions for long periods is one way to fight against latent TB. The invention is based, in part, on the inventors discovery of a novel TB gene (Rv3097c) encoding an enzyme required for Mycobacterium tuberculosis to process energy in order to enter, survive, or exit the dormancy (or latent) period. The gene sequence is provided as SEQ ID NO: 1. The inventors believe that tricaprylglycerol (TG) could be used as an energy source by M. tuberculosis during the dormancy period, thus its synthesis or its metabolism could be an ideal drug target against latent TB. Accordingly, the inventors have screened putative lipase/esterase gene products from M. tuberculosis for long-chain TG hydrolase activity and have discovered that the Rv3097c gene product, is active as a true lipase. LIPY (PE-PGRS63), which is a member of PE-PGRS protein family, is a lipase belonging to the hormone-sensitive lipase (HSL) family and hydrolyzes long-chain TG with high specific activity. The inventors have also found that lipY is upregulated to a much higher level than the transcripts of other lipase/esterase-like genes when mycobacteria that accumulated TG during an in vitro dormancy-like state were subjected to TG utilizing conditions indicating that this lipase plays a role in the utilization of TG for survival through dormancy. The inventors disclose herein that the ability to hydrolyze stored TG was drastically diminished in a lipY disrupted mutant (Δ-lipY).

Thus, one embodiment of the subject invention pertains to a method of screening for therapeutic agents useful in the treatment of Mycobacterium tuberculosis infection in a mammal comprising the steps of i) contacting a test compound with a M. tuberculosis TG hydrolase (MTTGH) polypeptide, ii) detecting binding of said test compound to said MTTGH polypeptide.

Another embodiment the subject invention is directed to a method of screening for therapeutic agents useful in the treatment of Mycobacterium tuberculosis infection in a mammal comprising the steps of i) determining the activity of a MTTGH polypeptide at a certain concentration of a test compound or in the absence of said test compound, ii) determining the activity of said polypeptide at a different concentration of said test compound. These and other embodiments will be described in further detail herein.

1. Screening Methods

The invention provides assays for screening test compounds which bind to or modulate the activity of an MTTGH polypeptide or bind to and inhibit or affect expression of an
MTTGH polynucleotide. A test compound preferably binds to an MTTGH polypeptide. More preferably, a test compound decreases or increases MTTGH activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

1.1. Test Compounds

Test compounds relate to agents that potentially have therapeutic activity, i.e., bind to or modulate the activity of an MTTGH polypeptide or bind to or affect expression of an MTTGH polynucleotide. Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. Examples of such compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the “one-bead one-compound” library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomers, or small molecule libraries of compounds. See Lam, Anticancer Drug Des. 12, 145, 1997.


1.2. High Throughput Screening

Test compounds can be screened for the ability to bind to and inhibit MTTGH polypeptides or polynucleotides or to affect MTTGH activity or MTTGH gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 µl. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format. Alternatively, “free format assays,” or assays that have no physical barrier between samples, can be used.

1.3. Binding Assays

For binding assays, the test compound is preferably, but not necessarily, a small molecule which binds to and occupies, for example, the active site of the MTTGH polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

In binding assays, either the test compound or the MTTGH polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to the MTTGH polypeptide can then be accomplished, for example, by direct counting of radioemission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Those skilled in the art equipped with teachings herein will appreciate that there are multiple conventional methods of detecting binding of a test compound. For example, binding of a test compound to an MTTGH polypeptide can be determined without labeling either of the interactants. A microphysiometer can be used to detect binding of a test compound with an MTTGH polypeptide. A microphysiometer (e.g., CYTOSENSOR™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and an MTTGH polypeptide (McConnell et al., Science 257, 19061912, 1992).

In another alternative example, determining the ability of a test compound to bind to an MTTGH polypeptide can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, Anal Chem. 63, 23382345, 1991, and Szabo et al., Curr. Opin. Struct. Biol. 5, 697905, 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIACore™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, an MTTGH polypeptide can be used as a “bait protein” in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Pat. No. 5,283,317; Zervos et al., Cell 72, 223232, 1993; Madura et al., J. Biol. Chem. 268, 1204612054, 1993; Bartel et al., BioTechniques 14, 920924, 1993; Iwabuchi et al., Oncogene 8, 16931696, 1993; and Brent WO94/10300), to identify other proteins which bind to or interact with the MTTGH polypeptide and modulate its activity.

In many screening embodiments, it may be desirable to immobilize either the MTTGH polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the MTTGH polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the MTTGH polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a MTTGH polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In a specific embodiment, the MTTGH polypeptide may be a fusion protein comprising a domain that allows the MTTGH polypeptide to be bound to a solid support. For example, glutathione S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the nonadsorbed MTTGH polypeptide; the mixture is then incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells
are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a MTG polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated MTG polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS (N-hydroxysuccinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a MTG polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the MTG polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the MTG polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the MTG polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a MTG polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a MTG polypeptide or polynucleotide can be used in a cell-based assay system. A MTG polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a MTG polypeptide or polynucleotide is determined as described above.

1.4. Enzyme Assays

Test compounds can be tested for the ability to increase or decrease the triacylglycerol storage (TGS) activity of a MTG polypeptide. TGS activity can be measured, for example, by adapting techniques such as that described in U.S. Pat. No. 4,529,693 (see Example 2). Enzyme assays can be carried out after contacting either a purified MTG polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound which decreases TGS activity of a MTG polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing MTG activity. A test compound which increases TGS MTG polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing TGS activity.

1.5. Gene Expression

In another embodiment, test compounds which increase or decrease MTG gene expression are identified. An MTG polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the MTG polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of MTG mRNA or polypeptide expression in the cells can be determined by methods well known in the art. For detecting mRNA or polypeptide, either qualitative or quantitative methods can be used. The presence of polypeptide products of an MTG polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunological methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined in vivo, in a cell culture, or in an in vitro translation system by detecting incorporation of labeled amino acids into a MTG polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell which expresses a MTG polynucleotide can be used in a cell-based assay system. The MTG polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

2. Pharmaceutical Compositions

The invention also pertains to pharmaceutical compositions comprising one or more therapeutic agents that are identified by screening methods that utilize MTG polypeptides and/or polynucleotides. Therapeutic agent(s) can be administered to a patient to achieve a therapeutic effect, i.e. useful in treatment of TB. Pharmaceutical compositions of the invention can comprise, for example, therapeutic agents identified by a screening method embodiment described herein, which are identified by their ability to bind to or affect activity of MTG polypeptides, or bind to and/or affect expression MTG polynucleotides. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intrapertitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa., which is incorporated herein by reference). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.
This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a therapeutic agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (for example, but not limited to, a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a MTTGH polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above described screening assays for treatments as described herein.

Those skilled in the art will appreciate that numerous delivery mechanisms are available for delivering a therapeutic agent to an area of need. By way of example, the agent may be delivered using a liposome as the delivery vehicle. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 µg of DNA per 16 nmole of liposome delivered to about 106 cells, more preferably about 1.0 µg of DNA per 16 nmole of liposome delivered to about 106 cells, and even more preferably about 2.0 µg of DNA per 16 nmol of liposome delivered to about 106 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods which are standard in the art (see, for example, U.S. Pat. No. 5,705,151). Preferably, from about 0.1 µg to about 10 µg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 µg to about 5 µg of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 µg of polynucleotides is combined with about 8 nmol liposomes.


2.1 Determination of a Therapeutically Effective Dose

The determination of a therapeutically effective dose of therapeutic agents identified by a screening method herein is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which attenuates or eliminates TB infection contrasted to TB infection or attenuation that occurs in the absence of the therapeutically effective dose.

Therapeutic efficacy and toxicity, e.g., ED₅₀ (the dose therapeutically effective in 50% of the population) and LD₅₀ (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD₅₀/ED₅₀.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending on the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

Preferably, an therapeutic agent reduces expression of an MTTGH gene or the activity of an MTTGH polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of an MTTGH gene or the activity of an MTTGH polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to MTTGH-specific mRNA, quantitative RT-PCR, immunologic detection of an MTTGH polypeptide, or measurement of MTTGH activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects. Any of the therapeutic methods described above can be applied to any subject in need of such therapy.

3. Polypeptides

M. tuberculosis TG storage (MTTGH) polypeptides according to the invention comprise at least 12, 15, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 or 265 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2, or a biologically active variant thereof, as defined below. A MTTGH polypeptide of the invention therefore can be a portion of an MTTGH protein, a full-length MTTGH protein, or a fusion protein comprising all or a portion of MTTGH protein.
3.1 Biologically Active Variants

MTTGH polypeptide variants which are biologically active, i.e., confer an ability by *Mycobacterium tuberculosis* to store and/or process TCG, are also considered MTTGH polypeptides for purposes of this application. Preferably, naturally or non-naturally occurring MTTGH polypeptide variants have amino acid sequences which are at least about 55, 60, 65, or 70, preferably about 75, 80, 85, 90, 96, or 98% identical to the amino acid sequence shown in SEQ ID NO: 2 or a fragment thereof. Percent identity between a putative MTTGH polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is determined using the Blast2 alignment program (Bloom62, Expect 10, standard genetic codes).

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valin, an aspartate with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of an MTTGH polypeptide can be found using computer programs well known in the art, such as DNASTAR software. Whether an amino acid change results in a biologically active MTTGH polypeptide can readily be determined by assaying for MTTGH activity, as described for example, in the specific Examples, below.

3.2 Fusion Proteins

In some embodiments of the invention, it is useful to create fusion proteins. By way of example, fusion proteins are useful for generating antibodies against MTTGH polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions of an MTTGH polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A MTTGH polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. For example, the first polypeptide segment can comprise at least 12, 15, 25, 50, 75, 100, 125, 150, 175, 200, 225, or 250 contiguous amino acids of SEQ ID NO: 2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise a full-length MTTGH polypeptide.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include galactosidase, glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BFP protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the MTTGH polypeptide-encoding sequence and the heterologous protein sequence, so that the MTTGH polypeptide can be cleaved and purified away from the heterologous moiety.

Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, Wis.), Stratagene (La Jolla, Calif.), CLONTECH (Mountain View, Calif.), Santa Cruz Biotechnology (Santa Cruz, Calif.), MBL International Corporation (MIC; Watertown, Mass.), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

3.3 Obtaining Polypeptides

MTTGH polypeptides can be obtained, for example, by purification of polypeptides from *M. tuberculosis*, expressed by of MTTGH polynucleotide(s) and other appropriate methods as will be appreciated by those skilled in the art in view of the teachings herein. In a specific example, the inventors have expressed poly His-tagged enzyme in *E. coli* and have purified it.

3.4 Protein Purification

MTTGH polypeptides can be purified from any cell which expresses the enzyme, including host cells which have been transfected with MTTGH enzyme expression constructs. A purified MTTGH enzyme polypeptide is separated from other compounds which normally associate with the MTTGH enzyme polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis. A preparation of purified MTTGH polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

4. Polynucleotides

An MTTGH polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for an MTTGH polypeptide. A coding sequence for MTTGH polypeptide of SEQ ID NO: 2 is shown discarding one.

Degenerate nucleotide sequences encoding MTTGH polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 60, preferably about 75, 90, 96, or 98% identical to the nucleotide sequence shown in SEQ ID NO: 1 also are triacylglycerol synthase-like enzyme polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALN GN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of MTTGH polynucleotides which encode biologically active MTTGH polypeptides also are MTTGH polynucleotides.

4.1 Identification of Polynucleotide Variants and Homologs

Variants and homologs of the MTTGH polynucleotides described above are also MTTGH polynucleotides. Typically, homologous MTTGH polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known MTTGH polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions: 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 60 minutes each; then 2xSSC, 0.1% SDS, 50°C once, 50 minutes; then 2xSSC, room temperature twice, 10 minutes each homologous sequences can be identified which contain at least about
20

25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the MTTGH polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries. It is well known that the $T_m$ of a double-stranded DNA decreases by 1-1.5°C for every 1% decrease in homology (Bonner et al., J. Mol. Biol. 81, 123 (1973)). Variants of MTTGH polynucleotides or polynucleotides of other species can therefore be identified by hybridizing a putative homologous MTTGH polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO:1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to MTTGH polynucleotides or their complements are necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding an MTTGH polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (e.g., baculovirus), plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those nontranslated regions of the vector enhancers, promoters, 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Strategene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding an MTTGH polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

4. Host Cells

According to certain embodiments of the subject invention, an MTTGH polynucleotide will need to be inserted into a host cell, for expression, processing and/or screening. A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed MTTGH polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Posttranslational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activi-

Alternatively, synthetic chemistry techniques can be used to synthesize MTTGH polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a MTTGH polypeptide having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

4.3 Expression of Polynucleotides

To express a MTTGH polynucleotide, the polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding MTTGH polypeptides and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described, for example, in Sambrook et al. (1989) and in Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a MTTGH enzyme polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (e.g., baculovirus), plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids), or animal cell systems. The control elements or regulatory sequences are those nontranslated regions of the vector enhancers, promoters, 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Strategene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding an MTTGH polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

5. Host Cells

According to certain embodiments of the subject invention, an MTTGH polynucleotide will need to be inserted into a host cell, for expression, processing and/or screening. A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed MTTGH polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Posttranslational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activi-
ties (e.g., CHO, HeLa, MDCK, HEK293, and W138), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, Va. 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high yield production of recombinant proteins. For example, cell lines which stably express MTTGH polypeptides can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 12 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced MTTGH sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. See, for example, ANIMAL CELL CULTURE, R. I. Freshney, ed., 1986.

5.1 Detecting Expression

A variety of protocols for detecting and measuring the expression of a MTTGH polypeptide, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a MTTGH polypeptide can be used, or a competitive binding assay can be employed. These and other assays are described in Hampton et al., SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., (1990) and Maddox et al., J. Exp. Med. 158, 1211,1216, 1983.

5.2 Expression and Purification of Polypeptides

Host cells transformed with nucleotide sequences encoding MTTGH polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode MTTGH polypeptides can be designed to contain signal sequences which direct secretion of soluble MTTGH polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound MTTGH polypeptide.

6. Antibodies

Antibodies are referenced herein and various aspects of the subject invention utilize antibodies specific to MTTGH polypeptide(s). As described above, one example of a therapeutic agent may pertain to an antibody. Any type of antibody known in the art can be generated to bind specifically to an epitope of an MTTGH polypeptide. “Antibody” as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab)2, and Fv, which are capable of binding an epitope of an MTTGH polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, e.g., at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of an MTTGH polypeptide can be used therapeutically, as mentioned, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen. Antibodies useful for embodiments of the subject invention may be polyclonal, but are preferably monoclonal antibodies.

7. Ribozymes

Ribozymes may be one category of test compounds potentially useful as therapeutic agents for treatment of TB infection. Ribozymes are RNA molecules with catalytic activity. See, e.g., Cech, Science 236, 1532-1539; 1987; Cech, Ann. Rev. Biochem. 59, 5435-68; 1990; Cech, Curr. Opin. Struct. Biol. 2, 60509; 1992; Couture & Stinchcomb, Trends Genet. 12, 510515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (e.g., Haseloff et al., U.S. Pat. No. 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

Accordingly, another aspect of the invention pertains to using the coding sequence of a MTTGH polynucleotide to generate ribozymes which will specifically bind to mRNA transcribed from the MTTGH polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff et al. Nature 334, 585591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete “hybridization” region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach et al., EP 321,201).

Specific ribozyme cleavage sites within a MTTGH RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate MTTGH RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease MTTGH expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter ele-
ment, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff et al., U.S. Pat. No. 5,641,673, ribozymes can be engineered so that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

8. Long Chain TG Hydrolase Activity in Mycobacterium tuberculosis

8.1 Materials and Methods

Bacterial Strains and Culture Conditions—M. tuberculosis H37Rv (ATCC 25618) and Δ-lipY mutant were grown in Middlebrook 7H10 (supplemented with 0.05% Tween 80, 10% oleic acid-albumin-dextrose-catalase enrichment, and 0.2% glycerol) and in Dubos-Tween 80-albumin medium (prepared from Dubos broth base and Dubos medium albumin as per the manufacturer’s instructions). All media were purchased from Difco. E. coli DH5α and BL21 Star (DE3) (Invitrogen) used as host strains for cloning and expression experiments were grown on Luria-Bertani broth or agar and, when required, antibiotics were added to the culture media at the following concentrations: carbenicillin or kanamycin, 50 µg/ml; hygromycin B, 150 µg/ml for E. coli or 75 µg/ml for M. tuberculosis. Other chemicals and antibiotics were from Sigma Chemical Co., Fisher Scientific and Calbiochem.

DNA restriction and modifying enzymes were procured from New England Biolabs (Beverly, Mass.).

Induction of lipase genes and TG utilization in M. tuberculosis strains under nutrient starvation after hypoxic stress—M. tuberculosis H37Rv and Δ-lipY mutant cells were grown under a hypoxic condition essentially as previously described to induce the accumulation of TG inside the mycobacterial cell (7). M. tuberculosis cells were inoculated into Middlebrook 7H10, grown aerobically at 37°C. C. and was used as control. 100 µl of cell suspension were transferred to an OD800 of 0.8 and used to inoculate Dubos-Tween-albumin medium to an OD800 of 0.015. grown up to an OD800 of 0.06 and distributed in tubes or in single neck small bottles (Chemglass) with a 0.5 head space to culture volume ratio, sealed with rubber sleeve caps and slowly stirred on a magnetic stirrer for hypoxic growth up to 12 days. Harvested cells were divided into three sets. The first set was preserved in −80°C and was used as control (0 h). The harvested cells of the second set were washed and resuspended in phosphate-buffered saline (PBS) and incubated at 37°C for 6 h. The cells of the third set were washed, resuspended in normal 7H9-OADC-Tween (7H9) medium instead of PBS and served as a nutrient supplemented control. These differentially treated cells were used to measure induction of lipase gene transcripts by semi-quantitative RT-PCR and to measure the utilization of stored TG. RNA isolation and semi-quantitative RT-PCR were performed as described before (7), using the primers shown in Table 1.

Cells from the three sets were autoclaved and total lipids were extracted with chloroform/methanol (2:1; v/v) as previously described (7). The 12 days old wild type M. tuberculosis hypoxic culture (10 ml) was also used for labeling with 1 µCi of [14C]oleic acid (specific activity, 55 Ci/mole; American Radiolabeled Chemicals) for 1 h and then the cells were washed with PBS before incubating in PBS for the next 6 h. Lipids were analyzed by silica gel thin-layer chromatography (TLC) using n-hexane/diethyl ether (9:1; v/v), and the radioactivity in the silica gel corresponding to the TG band was measured using a liquid scintillation counter (Packard). An autoradiogram of the TLC was prepared. The amount of TG was visualized by dichromate/sulfuric acid charring of the TLC plates as described before (15). The charred TLC plate was also scanned for quantification of TG accumulation by using the Alphalmerger 2200 Gel Doc system (Alphalnnootech).

Generation of lipY disrupted mutant of M. tuberculosis H37Rv—The lipY gene was disrupted by allelic exchange using specialized transducing phage as described (16). The disruption construct of lipY was made by sequential cloning of a 953-bp 5’-flank (consisting of the first 38 bp of lipY ORF and 915 bp sequence upstream of lipY ORF) and a 789-bp 3’-flank (consisting of last 4 bp of lipY ORF and 785 bp sequence downstream of lipY) of lipY, on either side of res-hyg-res gene cassette in the cosmid pYUB854. The two flanks were generated by PCR amplification using M. tuberculosis H37Rv genomic DNA as template by introducing AflII and XbaI and HindIII and SpeI sites on the ends of the 5-flanks and 3-flanks respectively for directional cloning into pYUB854 (Table-2), The PacI digested recombinant pYUB854 containing lipY disruption construct was introduced in plasmid pHAE159 and the recombinant transducing phage obtained after packaging was used to transduce M. tuberculosis. LipY disruption by allelic exchange was confirmed by PCR analyses using specific sets of primers and Southern hybridization (Table-2). Ten hygromycin resistant clones were screened by PCR using a set of primers (Δ-F and Δ-R) designed from the deleted part of the lipY gene. The allelic exchange by double cross-over was confirmed in two sets of primers, each representing a outwardly directed hyg primer (H1 and H2) and a primer (primers E and F) in the mycobacterial genome beyond the flanking gene sequences used for making the homologous arms of the disruption construct.

DNA isolation and Southern blotting—Mycobacterial genomic DNA was isolated by the guanidine thiocyanate (GTC) method as described (17). DNA samples were digested with suitable restriction enzyme, separated by electrophoresis in 1% agarose gel, transferred to Nitro membranes (Nytran Plus, Schleicher and Schuell, Keen, N.H.) and hybridized with [α-32P]dCTP labeled probes. Probe labeling and preparation were performed using recliwv[α-32P]dCTP and rediprim II random priming labeling kit (Amersham Biosciences) as per manufacturer’s instructions.

Cloning and Expression of M. tuberculosis lipase/esterase genes in E. coli—The 24 open-reading frames (ORFs) were amplified from the genomic DNA of M. tuberculosis H37Rv by PCR using Pfu Turbo Hotstart DNA polymerase (Stratagene) and cloned into pT2000 D-TOPO expression vector (Invitrogen). The directionally cloned fragments were completely sequenced to confirm the sequence integrity of each expression construct. The constructs were used to transform competent cells of E. coli BL21 Star (DE3) (Invitrogen) and the proteins were expressed as N-terminal 6x His-tagged (SEQ ID NO: 79) fusion proteins after induction with IPTG according to the manufacturer’s protocols. Total cell lysates from induced cultures were prepared in 0.1 M Tris.HCl pH 8.0 containing 1 mM DTE and used for screening for TG hydrolase activity of all lip gene products. Untransformed host cell lysate was used as control.

Solubilization and purification of LIPY—In our attempts to produce soluble LIPY protein, we performed IPTG induction in E. coli BL21 Star (DE3) cells at 16°C, 24°C and 37°C for 4 h and 12 h. Inductions were also carried out without using IPTG in Dual Media (Zymo Research). In all cases virtually all the expressed protein was insoluble. To solubilize the insoluble protein, his-tagged LIPY was expressed in 500 ml cultures by induction with 1 mM IPTG at 37°C for 12 h
in LB broth. Total cell lysates were centrifuged at 10,000×g and the pellet was solubilized in a buffer containing 1% (w/v) sodium lauryl sarcosine, 2 mM sodium dodecyl sulfate (SDS), 25 mM triethanolamine, 1.5 mM CaCl₂, 50 mM sodium phosphate buffer pH 7.0, 300 mM NaCl and 50 µg/ml aprotonin with shaking at 350 rpm at 10°C for 1 h. The solution was clarified by centrifugation at 16,000×g for 20 min at 4°C and the supernatant was loaded onto a 5 ml bed-volume cobalt-affinity resin (TALON, BD Biosciences). Unbound proteins and detergent were removed by washing the resin with 10 bed-volumes of bind/wash buffer (50 mM sodium phosphate buffer pH 7.0, 300 mM NaCl) followed by 10 bed-volumes of bind/wash buffer containing 10 mM imidazole. The bound protein was eluted in 5 bed-volumes of bind/wash buffer containing 150 mM imidazole followed by 5 bed-volumes of bind/wash buffer containing 250 mM imidazole and 5 ml fractions were collected. Aliquots of fractions collected at each step of the solubilization and purification process were analyzed on 12% SDS-PAGE followed by coomasie staining.

Lipase Assays—Lipase activity was measured by the release of [14C]oleic acid from [14C]triolein (55 mCi/mmol, American Radiolabeled Chemicals) using a modified method of Belfrage and Vaughan (18). The reaction mixture contained 50-100 µg protein from total cell lysates, 0.2 µCi [14C]triolein, 20 mM triolein, 2% gum arabic, 1 µg bovine serum albumin, 100 mM NaCl and 0.1 M Tris.HCl pH 8.0 in a total volume of 100 µl. Triolein was emulsified with gum arabic and aliquoted into the reaction mixture at the indicated concentrations prior to the addition of enzyme. After incubation at 37°C for 2 h, the reaction mixture was extracted with 1 ml chloroform:methanol:hexane (1:2.5:1:4:1, v/v/v) following the addition of 200 µl 0.1 M NaHCO₃, pH 10.5. The radioactivity released into the upper aqueous phase was measured by liquid scintillation counting. Alternatively, the reaction mixture was acidified with 50 µl 6 N HCl and extracted with 1 ml chloroform:methanol (2:1, v/v). The lipids in the lower organic phase were resolved on silica TLC plates developed in hexane:diethyl ether (65:35:2, v/v/v) and the radioactive spots were identified by autoradiography.

To investigate substrate specificity, sonicated emulsions of 20 mM [14C]dieleoin or [14C]dioleoyl phosphatidylcholine or [14C]dioleoyl phosphatidylethanolamine or [14C]cholate were used. To measure pH stability, the purified enzyme was pre-incubated in the appropriate buffer for 15 min at 24°C prior to the assay at 37°C for 2 h. Temperature stability was measured by pre-incubation of purified LIPY at the indicated temperature for 15 min prior to assay at 37°C. The effects of serine-directed reagents like diethyl-p-nitrophenyl phosphate (E-600), phenylmethanesulfonyl fluoride (PMSF), detersive like SDS, polyethylene glycol tert-octylphenyl ether (Triton X-100), polyethylene glycol sorbitan monolaurate (Tween-20) and various salts were determined by pre-incubating the purified LIPY for 15 min at 24°C with the indicated concentration of effector prior to the assay at 37°C for 2 h.

8.2 Results
Identification and cloning of M. tuberculosis lipase/esterase genes—M. tuberculosis probably uses fatty acids as a carbon source during its dormancy inside the host (8, 10). Previously, we identified 15 ts genes in the genome of the pathogen several of which are induced under culture conditions that lead to a dormancy-like state resulting in TG accumulation within the bacterial cell (7). Utilization of stored TG would require a true lipase. However, no mycobacterial gene that encodes long-chain TG hydrolase has been identified and characterized. We screened the M. tuberculosis genome for the presence of genes encoding such enzymes. The M. tuberculosis genome contains 21 ORFs annotated as putative lipase/esterase genes. We used the sequence of a putative TG lipase from M. tuberculosis strain W17 in the NCBI database (U76006.1, Acc. No. NCBI ABJ18414, Bifani et al.) to screen the M. tuberculosis H37Rv genome for homologous genes. This approach identified three additional ORFs—Rv1169c, Rv3097c and Rv1834, that we designate as lipX, lipY and lipZ, respectively, to be consistent with the present nomenclature of all the other lip genes in the M. tuberculosis genome. We included these three genes along with the 21 previously annotated ‘lip’ genes in our screen for genes encoding true lipases.

Screening of M. tuberculosis lipase/esterase gene products for long-chain TG hydrolase activity. The 24 selected M. tuberculosis ORFs encoding putative lipase/esterase proteins were expressed in E. coli and total cell lysates were assayed for triolein hydrolase activity. Lipase activity above untransformed control was normalized for the expression level of each recombinant protein in the total cell lysate. Triolein hydrolase activity in our standard radiometric assay at pH 8.0 was by far the highest in the lysates of recombinant E. coli expressing LIPY followed by LIPK, LIPL and LIPC that showed much lower activities followed by lysates expressing LIPX and LIPG that had even lower levels of activity. All other lip gene products showed little or no triolein hydrolase activity (Table 3).

Utilization of stored TG and induction of lipase genes by starvation in M. tuberculosis cells grown under hypoxic conditions. In order to test the hypothesis that lipase(s) release fatty acids from TG stores under nutrient-deprived conditions that might be encountered during dormancy, we cultured M. tuberculosis under hypoxic conditions for 12 days, a condition which was previously shown to cause TG accumulation (7), and subsequently incubated these cells in a starvation medium (PBS) to test whether the stored TG was utilized under such conditions. To label the stored TG, we incubated M. tuberculosis cells held under hypoxia for 12 days with [14C]-oleic acid for 1 h and then the labeled cells were incubated in PBS for 6 h. After incubation, about 50% of the labeled TG was utilized under nutrient deprived condition (FIG. 1A). Accumulation of TG during hypoxia and utilization during starvation was also clearly seen by dichromate/sulfuric acid charring of the silica gel-TLCs of the total lipids (FIG. 1B). The intensity of the TG band showed about 50% decrease after 6 h of incubation in PBS. Cells incubated in nutrient rich medium, used as a control, did not utilize significant amounts of the stored TG (FIG. 1C).

During the TG utilization conditions, the lipase genes involved in the release of fatty acids from TG would be expected to be induced. To test for this possibility, the transcript levels of all the 24 selected ORFs were measured by semi-quantitative RT-PCR analyses. The transcript level of each lip gene before starvation (0 h), after starvation (6 h-PBS) and after incubation in nutrient rich medium (6 h-7H9) are expressed as a fraction of the 23S rRNA transcript level of the same sample (FIG. 2A). Most of the lipase genes showed induction of transcript level during incubation for 6 h in PBS. The highest level of induction after 6 h starvation was seen in the transcripts of lipY, lipE, lipC, lipZ, lipL and lipT. Since the lip gene products manifested very different degrees of TG hydrolase activity, the possible relative contributions of the lipase genes to the hydrolysis of stored TG within the mycobacterial cell were assessed by multiplying the transcript level after 6 h starvation with the triolein hydrolase activity of each expressed gene product. Such an analysis
showed that LIPY had by far the greatest potential for hydrolyzing in vivo stores of TG in the mycobacterium under such conditions (FIG. 2B).

Disruption of lip Y in *M. tuberculosis*—LIPY showed the highest capacity to hydrolyze long chain TG among all the probable lipase gene products of *M. tuberculosis* cloned and expressed in *E. coli*. Moreover, when the organism was subjected to a nutrient deprived state after the cells had accumulated TG under hypoxia, lip Y was found to be the most highly induced gene. These results suggested that LIPY is most likely to be the major lipase involved in the hydrolysis of the stored TG. To test for this possibility, we generated a lipY knock-out mutant of *M. tuberculosis* (FIG. 3). Lip Y was disrupted by allelic exchange using specialized transducing recombinant mycobacteriophage phAE159 (16). In the constructed lipY deletion allele, 1258 bp out of 1314 bp of total lipY gene sequence was replaced with a hygromycin resistance gene cassette (res-hyg-res) flanked by res (resolvase recognition) sequences. Several mutants were identified as tentative lip Y disrupted mutant (Δ lipY) as an 804-bp sequence in the deleted lipY-segment could not be amplified by PCR (FIG. 3C). Further PCR analysis of the flanking regions of the deleted part of the gene confirmed the deletion at the correct orientation by homologous recombination (FIG. 3C). A 1330-bp 5'-flank (primer pair—E+H1) and a 1007-bp 3'-flank (primer pair—H2+F) could be amplified from the selected disruptants, but, no product could be amplified from the wild type genomic DNA (FIG. 3B, C). Southern blot of EcoRI restricted genomic DNA of five putative lipY mutants when hybridized with a 804-bp probe generated from the deleted sequence of the gene didn’t show any hybridization where as the wild type control showed the hybridized band (FIG. 3D). Lip Y transcript was absent in Δ lipY mutant and the level of induction of the transcripts of the other lip genes was similar in the Δ lipY and the wild type, when both were incubated in PBS or in 7H9 medium (data not shown) was confirmed by RT-PCR (data not shown).

TG utilization by lipY deficient mutant of *M. tuberculosis*—*M. tuberculosis* wild type and Δ lipY mutant cells were subjected to TG utilization as described above. When subjected to starvation by incubating in PBS, TG utilization in lipY mutant was drastically decreased, compared to that in the wild type (FIG. 1C). Also no significant TG hydrolysis could be detected when the cells were incubated in a nutrient rich medium (7H9) as a control.

Purification of LIPY—Since LIPY showed the highest potential for hydrolyzing the TG stored inside the *M. tuberculosis* cell, we purified LIPY and characterized its activity. LIPY was expressed as a 6x his-tagged (SEQ ID NO: 79) fusion protein in *E. coli* at 16°C, 24°C, and 37°C. under various conditions of induction and was found to partition into the 16,000g pellet after cell lysis in all cases. Therefore, we solubilized LIPY from this pellet using a low concentration of detergents that did not inhibit the activity of LIPY as determined by preliminary assays with total cell lysates containing recombinant LIPY (data not shown). The clarified supernatant from the solubilized 16,000g pellet contained a large quantity of recombinant LIPY (FIG. 4, lane 2) and was used to purify LIPY by cobalt-affinity chromatography. The 6x his-tagged (SEQ ID NO: 79) LIPY protein was bound to the TALON resin and was eluted in the 150 mM imidazole elution step (FIG. 4). The recombinant LIPY migrates on SDS-PAGE with an apparent molecular weight that was slightly lower than the theoretically predicted value of 45 KDa. The purified enzyme eluted from a pre-calibrated Superose-6 gel-filtration column just after the void volume suggesting that the purified, recombinant LIPY exists as aggregates (data not shown).

Biochemical characterization of the TG hydrolyase activity of LIPY—The purified recombinant LIPY protein showed very high activity in our standard radiometric triolein hydrolysis assay. Lipase activity increased linearly with time and protein concentration (FIGS. 5A, B). LIPY displayed typical Michaelis-Menten kinetics (FIG. 5C) and the apparent *Kₘ* and *Vₚₙ₅* values were calculated to be 7.57 mM and 653.3 nmol/mg/min respectively from the rectilinear double-reciprocal plot. LIPY hydrolyzed [14C]diolein at a lower rate (316.8±9.0 nmol/mg/min). LIPY did not show any fatty acid release when incubated with phosphatidylcholine, phosphatidyl ethanolamine or hexadecyl palmitate (data not shown). LIPY displayed optimal activity between pH 8.0 and pH 9.0 (FIG. 5D).

The effect of inhibitors like E-600 which is an organophosphorus compound that irreversibly inhibits various esterases and is known to target serine esterases/lipases was tested on LIPY. E-600 inhibited LIPY by 99.5% at 0.5 µM (FIG. 6A) and PMSF at 5 mM inhibited LIPY activity by 75% (FIG. 6B). The temperature stability of LIPY was investigated by pre-incubation of the purified protein at the indicated temperature for 15 min. The lipase activity of LIPY dropped off sharply when the enzyme was held at 50°C or higher (FIG. 6C). LIPY was very stable in storage and retained nearly all of its original activity even after 60 days at 4°C and after 4 cycles of freezing at −20°C followed by thawing. The effect of various detergents on LIPY was investigated. Lipase activity was stimulated slightly by SDS at concentrations up to 2 mM above which the activity was severely inhibited (FIG. 7A). Triton X-100 at 0.1% stimulated the activity but higher concentrations were inhibitory (FIG. 7B). The inhibition by SDS was partially reversed by Triton X-100 and 20% of the original activity was recovered (data not shown). Tween-20 inhibited LIPY at all concentrations from 0.1-2.0% (FIG. 7C). Many lipases require calcium for activity but LIPY was inhibited by CaCl₂, CoCl₂, MnCl₂, ZnCl₂, and MgCl₂ also inhibited the activity. However NaCl, KCl, sodium acetate and potassium acetate enhanced the activity (Table 4).

LIPY, a member of the HSL family—Thirteen of the 24 putative lipase/esterases can be classified as lipases belonging to the HSL family (12). LIPY was the only protein out of the twenty-four with a putative TG hydrolyase activity as annotated in the database (19). The product of lip Y would encode a protein with a predicted molecular weight of 45 KDa and a pI of 4.5. However, it showed only 9.21% global amino acid identity with the other lipase/esterase-like proteins in the mycobacterial genome (Table 5). Pair-wise alignment of the amino acid sequence of LIPY with 35 representative lipases from all the eight reported families of bacterial lipases (20) indicated that LIPY shared only 12-23% global amino acid identity with known bacterial lipases. However, LIPY possesses the conserved active-site motif GDSAG (SEQ ID NO: 61) characteristic of the HSL family. Since the crystal structures of the *Bacillus subtilis* brefeldin A esterase and the *Archeoglobus fulgidus* carboxylesterase which belong to the HSL family have been elucidated (21, 22), we used the Cn3D Version 4.1 software from the Entrez System at NCBI to produce a structure-based sequence alignment of LIPY with the members of the HSL family. The alignment of conserved domains produced by Cn3D was then used to align other selected members of the HSL family by ClustalW multiple sequence alignment program. The multiple sequence alignment output from ClustalW was then adjusted manually to achieve maximum similarity between the amino acid
sequences. As shown in Fig. 8, this alignment revealed a high degree of similarity between the C-terminal half of LIPY and other members of the HSL family. This region of high similarity includes the catalytic domain with the consensus pentapeptide GDAG (SEQ ID NO: 61) containing the active serine residue and the strictly conserved HGGG (SEQ ID NO: 68) motif of unknown function (23) located immediately upstream of the active site consensus motif. The aspartate and histidine residues of the active-site are also conserved with the other members of the HSL family.

REFERENCES

19. au.exp.asy.org/cgi-bin/niceprot.pl?P77909

TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs (5’-3’)</th>
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<td>F: GGTAGACACCTCAAGCCGCTCGGC R: GCGCTGAACCACTACCCGCTCCAG</td>
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<td>l1pD (Rv1923)</td>
<td>F: GGTGTTTCAGCGGGCGAGCGAGTTC R: GCGCTGAACCACTACCCGCTCCAG</td>
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<td>Gene</td>
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<td>--------------------------------------------------</td>
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<td>lipE</td>
<td>(Rv3775) F: TCTAGGCGGCGTACGCGACCGACGC R: ACTGGGCGGGACGACGCACGCATC</td>
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**Gene Product**

**TG hydrolase activity (nmol/mg/min)**

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**TABLE 4**

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**Primer pairs to amplify 5'- and 3'-flanks of lipY**

5'-flank A: 5'-GCTAGGCGGCGTACGCGACCGACGC-3'
B: 5'-GCTAGGCGGCGTACGCGACCGACGC-3'
3'-flank C: 5'-GGATCGACGCGGCGGCAATGACG-3'
D: 5'-GCTAGGCGGCGGCAAGACGG-3'

**Primer pairs from the deleted segment**

Δ-P: 5'-GCTAGGCGGCGGCAAGACGG-3'
Δ-R: 5'-GCTAGGCGGCGGCAAGACGG-3'

**Primer pairs to amplify genomic flanks in the mutants**

5'-flank E: 5'-GCTAGGCGGCGGCAAGACGG-3'
H: 5'-GCTAGGCGGCGGCAAGACGG-3'
3'-flank F: 5'-GCTAGGCGGCGGCAAGACGG-3'

**Long-Chain TG Hydrolase activity of expressed lip genes**

**TABLE 3**

<table>
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<th>Gene</th>
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<th>TG hydrolase activity (nmol/mg/min)</th>
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<td>LIPY</td>
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<td>LIPX</td>
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<td>LIPI</td>
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<td>LIPI</td>
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Lipase genes were expressed in E. coli BL21 cells and lysates were assayed for TG hydrolase activity with 14C-labeled trinolein as the substrate.
TABLE 4-continued

Effect of salts on LIPY activity

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration</th>
<th>Activity (nmol/mg/min)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂</td>
<td>10 mM</td>
<td>424.8 ± 21.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>22.9 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10 mM</td>
<td>465.1 ± —</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>433.6 ± —</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>103.7 ± 29.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 mM</td>
<td>692.6 ± 57.0</td>
<td></td>
</tr>
<tr>
<td>CH₃COO⁻K</td>
<td>50 mM</td>
<td>749.0 ± 73.3</td>
<td></td>
</tr>
<tr>
<td>CH₃COONa</td>
<td>50 mM</td>
<td>681.7 ± 80.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>729.3 ± 96.5</td>
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Purified LIPY protein was incubated for 15 min at room temperature with indicated concentrations of salts and then assayed radiometrically for triolein hydrolase activity as described in Experimental Procedures.

TABLE 5-continued

Lipase/Esterase-like Proteins in M. tuberculosis

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Identity (%)</th>
<th>MW kDa</th>
<th>pl</th>
<th>Active-site Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIPY (Rv3097c)</td>
<td>100.0</td>
<td>45.0</td>
<td>4.5</td>
<td>GDSAG</td>
</tr>
<tr>
<td>LIPP (Rv2643)</td>
<td>21.4</td>
<td>42.8</td>
<td>6.0</td>
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<tr>
<td>LIPM (Rv2284)</td>
<td>21.0</td>
<td>46.7</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>

Lipase/Esterase-like Proteins in M. tuberculosis

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Identity (%)</th>
<th>MW kDa</th>
<th>pl</th>
<th>Active-site Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIPY (Rv3097c)</td>
<td>100.0</td>
<td>45.0</td>
<td>4.5</td>
<td>GDSAG</td>
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<tr>
<td>LIPP (Rv2643)</td>
<td>21.4</td>
<td>42.8</td>
<td>6.0</td>
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<tr>
<td>LIPM (Rv2284)</td>
<td>21.0</td>
<td>46.7</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>

Theoretical Conserved Residues

Amino acid identity of lipase genes were compared by pairwise alignment with LIPY, which had the highest TG hydrolase activity, using the ALIGN software program at Genestream (igh.cnrs.fr/bin/align-guess.cgi).

*We identified these genes as putative lipases as described in Experimental Procedures.
tacatggtga gcccagggca ccoagtacgc tgctcacttg tggctgctgc ccotgtggctc 1020
gatggggga cctggcagat cagcaggcc tgtggcggt gaaccagccg 1080
tggctgacgc cctggtgact gcgcttgagc ggcttggctc gtagctatgt ctatctgggc 1140
tgcggggct gcctgctact aacagcctg gctgcagcag cagacagcag 1200
gccctgatc gotctgtact ggcccctgg cccttccacc ctgtgctact ctctaatgt tcagcccctc 1260
tgctgcggcc gcagagaact ccagcaactcg gtatcgccgt gctgagtc 1315

<210> SEQ ID NO 2
<211> LENGTH: 437
<212> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 2

Met Val Ser Tyr Val Val Ala Leu Pro Glu Val Val Met Ser Ala Ala Ala
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Thr Asp Val Ala Ser Ile Gly Ser Val Val Ala Thr Ala Ser Gln Gly
15 20 25 30
Val Ala Gly Ala Thr Thr Val Leu Ala Ala Ala Glu Asp Glu Val
35 40 45
Ser Ala Ala Ile Ala Ala Leu Phe Ser Gly His Gln Gln Asp Tyr Gln
50 55 60
Ala Leu Ser Ala Gln Leu Ala Val Phe His Gln Arg Phe Val Gln Aln
65 70 75 80
Leu Thr Gly Ala Ala Lys Gly Tyr Ala Ala Ala Glu Leu Ala Asn Ala
85 90 95
Ser Leu Leu Gln Ser Glu Phe Ala Ser Gly Ile Gly Asn Gly Phe Ala
100 105 110
Thr Ile His Gln Glu Ile Gln Arg Ala Pro Thr Ala Leu Ala Ala Gly
115 120 125
Phe Thr Gln Val Pro Pro Phe Ala Ala Ala Gln Ile Phe Thr
130 135 140
Gly Thr Pro Ser Gly Ala Ala Gly Phe Asp Ile Ala Ser Leu Trp Pro
145 150 155 160
Val Lys Pro Leu Leu Ser Leu Ser Ala Leu Glu Thr His Phe Ala Ile
165 170 175
Pro Asn Pro Leu Leu Ala Leu Ile Ala Ser Asp Ile Pro Pro Leu
180 185 190
Ser Trp Phe Leu Gly Asn Ser Pro Pro Pro Leu Leu Asn Ser Leu Leu
195 200 205
Gly Gln Thr Val Gln Tyr Thr Thr Tyr Asp Gly Met Ser Val Val Gln
210 215 220
Ile Thr Pro Ala His Pro Thr Glu Tyr Val Val Ala Ile His Gly
225 230 235 240
Gly Ala Phe Ile Leu Pro Pro Ser Ile Phe His Trp Leu Asn Tyr Ser
245 250 255
Val Thr Ala Tyr Gln Thr Gly Ala Thr Val Gln Val Pro Ile Tyr Pro
260 265 270
Leu Val Gln Glu Gly Gly Thr Ala Gly Thr Val Val Pro Ala Met Ala
275 280 285
Gly Leu Ile Ser Thr Gln Ile Ala Gln His Gln Val Ser Asn Val Ser
290 295 300
Val Val Gly Asp Ser Ala Gly Gln Leu Ala Leu Ala Ala Ala Gln
305 310 315 320
Tyr Met Val Ser Gln Gly Asn Pro Val Pro Ser Ser Met Val Leu Leu

325 330 335

Ser Pro Trp Leu Asp Val Gly Thr Trp Glu Ile Ser Gln Ala Trp Ala

340 345 350

Gly Asn Leu Ala Val Asp Pro Leu Val Ser Pro Leu Tyr Gly Ser

355 365 365

Leu Asn Gly Leu Pro Pro Thr Tyr Val Tyr Ser Gly Ser Leu Asp Pro

370 375 385 390 395 400

Leu Ala Gln Gln Ala Val Val Leu Glu His Thr Ala Val Gln Gly

405 410 415

Leu Ala Gln Gln Ala Val Val Leu Glu His Thr Ala Val Gln Gly

420 425 430

Leu Leu Thr Pro Trp Gly Leu Leu Ser Trp Pro Gln Ile Asn Gln Gln

435

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actgggcgcg accagcggca catc
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cgacggcgct gggcgggtgg tgct
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```
cgacgcgcct ggccgggttg ttgct
```

```
cgcgccacgg cttgcggcg tcaggt
```

```
cgcgccacgg cttgcggcg tcaggt
```

```
ctgatcatgg gcctgggcgc ccag
```

```
ctgatcatgg gcctgggcgc ccag
```

```
autcccctgta gcagcgccag cage
```

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autcccctgta gcagcgccag cage
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ggacccgtga gcagcgccag cage
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223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence, Synthetic primer

<400> SEQUENCE: 59

tggaggcgtg gtgggtcga tgcg

<210> SEQ ID NO 59
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence, Synthetic primer

<400> SEQUENCE: 59

ggaactggcg cagttcctct gggg

<210> SEQ ID NO 60
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence, Synthetic primer

<400> SEQUENCE: 60

ccaagggctg gggtgcacaa ctcc

<210> SEQ ID NO 61
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence, Synthetic peptide

<400> SEQUENCE: 61

Gly Asp Ser Ala Gly

1 5
peptide

<400> SEQUENCE: 62
Gly Trp Ser Leu Gly
1  5

<210> SEQ ID NO: 62
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 63
Gly Ala Ser Ala Gly
1  5

<210> SEQ ID NO: 64
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 65
Gly Ala Ser Met Gly
1  5

<210> SEQ ID NO: 65
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 66
Gly Glu Ser Ala Gly
1  5

<210> SEQ ID NO: 66
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 67
Gly Cys Ser Ala Gly
1  5

<210> SEQ ID NO: 67
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 68
Gly His Ser Phe Gly
1  5
His Gly Gly Gly

<210> SEQ ID NO 69
<211> LENGTH: 178
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 69
Glu Tyr Val Val Ala Ile His Gly Gly Ala Phe Ile Leu Pro Pro Ser 5 10 15
Ile Phe His Trp Leu Arg Tyr Ser Val Thr Ala Tyr Gin Thr Gly Ala 20 25 30
Thr Val Gin Val Pro Ile Tyr Pro Leu Val Gin Glu Gly Gly Thr Ala 35 40 45
Gly Thr Val Val Pro Ala Met Ala Gly Leu Ser Arg Val Ser Val Val 50 55 60
Gly Asp Ser Ala Gly Asn Leu Ala Ala Ala Gly Tyr Met 65 70 75 80
Val Ser Gin Gly Arg Pro Val Pro Ser Ser Met Val Leu Leu Ser Pro 85 90 95
Trp Leu Asp Val Gly Thr Trp Gin Ile Ser Gin Ala Thr Gly Asn 100 105 110
Leu Ala Val Asn Leu Pro Pro Thr Tyr Val Tyr Ser Gly Ser Leu Asp 115 120 125
Pro Leu Ala Gin Gin Ala Val Leu Glu His Thr Ala Val Val Gin 130 135 140
Gly Ala Pro Phe Ser Phe Val Leu Ala Pro Trp Gin Ile His Asp Trp 145 150 155 160
Ile Leu Leu Thr Pro Trp Gly Leu Leu Ser Trp Pro Gin Ile Asn Gin 165 170 175
Gln Leu

<210> SEQ ID NO 70
<211> LENGTH: 181
<212> TYPE: PRT
<213> ORGANISM: Archaeoglobus fulgidus

<400> SEQUENCE: 70
Pro Val Leu Val Tyr Tyr His Gly Gly Phe Val Ile Cys Ser Ile 1 5 10 15
Glu Ser His Asp Ala Leu Cys Arg Arg Ile Ala Arg Leu Ser Asn Ser 20 25 30
Thr Val Val Ser Val Asp Tyr Arg Leu Ala Pro Glu His Lys Phe Pro 35 40 45
Ala Ala Val Tyr Asp Cys Tyr Asp Ala Thr Ser Lys Ile Phe Val Gly 50 55 60
Gly Asp Ser Ala Gly Asn Leu Ala Ala Val Ser Ile Met Ala 65 70 75 80
Arg Asp Ser Gly Glu Asp Phe Ile Lys His Gin Ile Leu Ile Tyr Pro 85 90 95
Val Val Asn Phe Val Ala Pro Thr Pro Ser Leu Leu Glu Phe Gly Glu
100 105 110

Gly Leu Trp Ile Leu Pro Pro Ala Leu Ile Ile Thr Ala Glu Tyr Asp
115 120 125

Pro Leu Arg Asp Glu Gly Glu Val Phe Gly Gln Met Leu Arg Arg Ala
130 135 140

Gly Val Glu Ala Ser Ile Val Arg Tyr Arg Val Leu His Gly Phe
145 150 155 160

Ile Asn Tyr Tyr Pro Val Leu Lys Ala Ala Arg Asp Ala Ile Asn Gln
165 170 175

Ile Ala Ala Leu Leu
180

<210> SEQ ID NO 71
<211> LENGTH: 180
<212> TYPE: PRT
<213> ORGANISM: Acinetobacter esterase

<400> SEQUENCE: 71

Pro Leu Ile Val Phe Tyr His Gly Gly Gly Phe Val Val Gly Gly Leu
1 5 10 15

Asp Ser His Asp Glu Phe Cys Arg Leu Met Ala Val His Ala His Ala
20 25 30

Gln Val Leu Ser Val Glu Tyr Pro Leu Ala Pro Glu Ala Ser Pro Gln
35 40 45

Gln Ile Ile Gln Val Cys Glu Asp Ala Leu Asn Arg Ile Ala Val Ala
56 60

Gly Asp Ser Ala Gly Asp Ile Ala Ala Val Ala Gln Arg Ser
65 70 75 80

Ala Gln Ser Ala Tyr Ala Pro Asn Ala Gln Phe Leu Ile Tyr Pro Ala
85 90 95

Leu Asp Phe Lys Ser Arg His Pro Ser Phe Phe Ala Tyr Lys Asp Gly
100 105 110

Leu Val Leu Ala Pro Ala Phe Val Val Thr Ala Gly His Asp Val
115 120 125

Leu His Asp Glu Ala Glu Ile Tyr Ala His Lys Leu Arg Glu Asn Gln
130 135 140

Val Thr Val Lys Tyr Val Glu Tyr Leu Asp Gln Thr His Gly Phe Ile
145 150 155 160

Ser Met Thr Phe Val Ser Arg Arg Ala Lys Lys Ile Ser Ile Glu Leu
165 170 175

Cys Lys Asn Phe
180

<210> SEQ ID NO 72
<211> LENGTH: 183
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 72

Ser Leu Ile Val His Phe His Gly Gly Phe Val Ala Gln Thr Ser
1 5 10 15

Arg Ser His Glu Pro Tyr Leu Lys Ser Trp Ala Gln Glu Leu Gly Ala
20 25 30

Pro Ile Ile Ser Ile Asp Tyr Ser Leu Ala Pro Glu Ala Pro Phe Pro
35 40 45

Arg Ala Leu Glu Glu Cys Phe Phe Ala Tyr Glu Arg Ile Cys Leu Ala
Gly Asp Ser Ala Gly Gly Asn Leu Cys Phe Thr Val Ala Leu Arg Ala
65 70 75 80
Ala Ala Tyr Gly Val Arg Val Pro Asp Gly Ile Met Ala Ala Tyr Pro
85 90 95
Ala Thr Met Leu Gln Pro Ala Ala Ser Pro Ser Arg Leu Leu Ser Leu
100 105 110
Met Asp Pro Leu Leu Pro Pro Val His Ile Val Ala Cys Ala Leu Asp
115 120 125
Pro Met Leu Asp Asp Ser Val Met Leu Ala Arg Arg Leu Arg Aen Leu
130 135 140
Gly Gln Pro Val Thr Leu Arg Val Val Glu Asp Leu Pro His Gly Phe
145 150 155 160
Leu Thr Leu Ala Ala Leu Cys Arg Glu Thr Arg Gln Ala Ala Glu Leu
165 170 175
Cys Val Glu Arg Ile Arg Leu
180

<210> SEQ ID NO 73
<211> LENGTH: 183
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas sp.

<400> SEQUENCE: 73
Pro Leu Leu Val Phe Phe His Gly Gly Gly Phe Val Met Gly Asn Leu
1 5 10 15
Asp Thr His Asp Asn Leu Cys Arg Ser Leu Ala Ser Gln Thr Glu Ala
20 25 30
Val Val Val Ala Tyr Arg Leu Ala Ala Ser Gln His Phe Pro
35 40 45
Ala Ala Pro Leu Asp Cys Tyr Ala Thr Arg Arg Leu Ala Leu Ala
50 55 60
Gly Asp Ser Ala Gly Gly Asn Leu Ala Ala Ser Arg Leu Ala
65 70 75 80
Ala Gln Arg Gln Gly Pro Lys Ile Ser Tyr Glu Cys Leu Phe Tyr Pro
85 90 95
Val Thr Asp Ala Arg Cys Asp Ser Glu Ser Tyr Glu Glu Phe Ala Glu
100 105 110
Gly Tyr Phe Leu Leu Val Pro THR Leu Ile Thr Ala Glu Phe Asp
115 120 125
Pro Leu Arg Asp Glu Gly Ala Phe Ala Leu Arg Leu Gln Glu Ala
130 135 140
Gly Val Ser Val Arg Val Gln Arg Cys Glu Gly Met Ile His Gly Phe
145 150 155 160
Ile Ser Met Ala Pro Phe Val Glu Arg Ala Ala His Ala Leu Ser Asp
165 170 175
Ala Ala Ala Asp Leu Arg Arg
180

<210> SEQ ID NO 74
<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Bacillus subtilis

<400> SEQUENCE: 74
Pro Gly Leu Val Tyr Thr His Gly Gly Gly Met Thr Ile Leu Thr Thr
1 5 10 15
Asp Asn Arg Val His Arg Arg Trp Cys Thr Asp Leu Ala Ala Ala Gly 20 25 30
Ser Val Val Met Val Asp Phe Arg Asn Ala Trp Thr Ala Glu Gly 35 40 45
His His Pro Phe Pro Ser Gly Val Glu Asp Cys Leu Ser Gly Val Val 50 55 60
Val Gln Gly Glu Ser Gly Gly Gly Asn Leu Ala Ile Ala Thr Thr Leu 65 70 75 80
Leu Ala Lys Arg Arg Gly Arg Leu Asp Ala Ile Asp Gly Val Tyr Ala 85 90 95
Ser Ile Pro Tyr Ile Ser Gly Tyr Ala Trp Asp His Glu Arg Arg 100 105 110
Leu Thr Glu Leu Pro Ser Leu Pro Pro Phe Val Val Ala Val Asn Glu 115 120 125
Leu Asp Pro Leu Arg Asp Gly Ile Ala Phe Ala Arg Arg Asp Ala Ala 130 135 140
Arg Ala Gly Val Asp Val Ala Ala Arg Val Asn Ile Gly Leu Val His 145 150 155 160
Gly Ala Asp Val Ile Phe Arg His Trp Leu Pro Ala Ala Leu Gly Ser 165 170 175
Thr Val Arg Asp Val Ala Gly Phe Ala 180 185

<210> SEQ ID NO 75
<211> LENGTH: 181
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis
<400> SEQUENCE: 75
Arg Ala Val Leu Tyr Leu His Gly Gly Ala Phe Leu Thr Cys Gly Ala 1 5 10 15
Asn Ser His Gly Arg Leu Val Glu Leu Ser Lys Phe Ala Asp Ser 20 25 30
Pro Val Leu Val Asp Tyr Arg Leu Ile Pro Lys His Ser Ile Gly 35 40 45
Met Ala Leu Asp Asp Cys His Asp Gly Tyr Glu Gin Ile Val Leu Ala 50 55 60
Gly Asp Ser Ala Gly Gly Tyr Leu Ala Leu Ala Leu Ala Gin Arg Leu 65 70 75 80
Gln Glu Val Gly Glu Glu Pro Ala Ala Leu Val Ala Ile Ser Pro Leu 85 90 95
Leu Gln Leu Ala Lys Glu His Lys Gin Ala His Pro Asn Ile Lys Thr 100 105 110
Asp Ala Met Phe Leu Pro Arg Thr Leu Ile His Val Ser Gly Ser Glu 115 120 125
Val Leu Leu His Asp Ala Gin Leu Ala Ala Lys Leu Ala Ala Ala 130 135 140
Gly Val Pro Ala Glu Val Arg Val Trp Pro Gly Gin Val His Asp Phe 145 150 155 160
Gln Val Ala Ala Ser Met Leu Pro Glu Ala Ile Arg Ser Leu Arg Gin 165 170 175
Ile Gly Glu Tyr Ile 180

<210> SEQ ID NO 76
<211> LENGTH: 178
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 76
Glu Tyr Val Val Ala Ile His Gly Ala Phe Ile Leu Pro Pro Ser
1  5 10 15
Ile Phe His Trp Leu Asn Tyr Ser Val Thr Ala Tyr Gln Thr Gly Ala
20 25 30
Thr Val Gln Val Pro Ile Tyr Pro Leu Val Gln Glu Gly Gly Thr Ala
35 40 45
Gly Thr Val Val Pro Ala Met Ala Gly Leu Ser Asn Val Ser Val Val
50 55 60
Gly Asp Ser Ala Gly Asn Leu Ala Ala Ala Ala Gln Tyr Met
65 70 75 80
Val Ser Gln Gly Asn Pro Val Pro Ser Ser Met Val Leu Leu Ser Pro
85 90 95
Trp Leu Asp Val Gly Thr Trp Gln Ile Ser Gln Ala Trp Ala Gln Asn
100 105 110
Leu Ala Val Asn Leu Pro Pro Thr Tyr Val Tyr Ser Gln Ser Leu Asp
115 120 125
Pro Leu Ala Gln Gln Ala Val Val Leu Gln His Thr Ala Val Val Gln
130 135 140
Gly Ala Pro Phe Ser Phe Val Leu Ala Pro Trp Gln Ile His Asp Trp
145 150 155 160
Ile Leu Leu Thr Pro Trp Gly Leu Leu Ser Trp Leu Gln Ile Asn Gln
165 170 175

Gln Leu

<210> SEQ ID NO 77
<211> LENGTH: 181
<212> TYPE: PRT
<213> ORGANISM: Ralstonia solanacearum

<400> SEQUENCE: 77
Pro Val Val Phe Tyr Leu His Gly Gly Tyr Phe Phe Cys Ser Pro
1  5 10 15
Arg Thr His Arg Ser Ile Thr Ile Gly Leu Ala Val His Ala Arg Ala
20 25 30
Arg Val Phe Ala Leu Asp Tyr Arg Leu Ala Pro Glu His Pro Phe Pro
35 40 45
Ala Ala Val Leu Asp Ala Leu Ala Gly Tyr Ser Arg Ile Val Val Ala
50 55 60
Gly Asp Ser Ala Gly Gly Gly Leu Ser Leu Ala Leu Leu Val Ala Leu
65 70 75 80
Arg Asp Ala Gly Asp Pro Leu Pro Ala Gly Ala Ile Leu Tyr Ser Pro
85 90 95
Trp Thr Asp Leu Ala Ala Thr Gln Ala Ser Leu Val Glu Asn Asp Ala
100 105 110
Ser Asp Val Met Leu Pro Pro Leu His Cys Tyr Val Ser Thr Ser Glu
115 120 125
Val Leu Arg Asp Thr Leu Arg Met Ala Glu Arg Ala Arg Ala Ala
130 135 140
Gly Val Ser Val Ser Val Glu Leu Gly Arg Arg Leu Pro His Val Trp
145 150 155 160
Pro Ile Phe Tyr Pro Phe Leu Pro Glu Ala Arg Lys Ala Leu Arg Gln

<210> SEQ ID NO 77
<210> SEQ ID NO 78
<211> LENGTH: 179
<212> TYPE: PRT
<213> ORGANISM: Staphylococcus aureus

<400> SEQUENCE: 79
Arg Val Val Leu Tyr Ala His Gly Gly Ala Trp Phe Gln Asp Pro Leu 1 5 10 15
Lys Ile His Phe Glu Phe Ile Asp Glu Leu Ala Glu Thr Leu Asn Ala 20 25 30
Lys Val Ile Met Pro Val Tyr Pro Lys Ile Pro His Gln Asp Tyr Gin 35 40 45
Ala Thr Tyr Val Leu Phe Glu Lys Leu Tyr Lys Gin Ile Val Ile Met 50 55 60
Gly Asp Ser Ala Gly Gly Gin Ile Ala Leu Ser Phe Ala Gin Leu Leu 65 70 75 80
Lys Glu Lys His Ile Val Gin Pro Gly His Ile Val Leu Ile Ser Pro 95 90 95
Val Leu Asp Ala Thr Met Gin His Pro Glu Ile Pro Asp Tyr Leu Lys 100 105 110
Lys Asp Pro Met Leu Gly Arg Ile Thr Leu Thr Val Gly Thr Lys Glu 115 120 125
Val Leu Tyr Pro Asp Ala Leu Asn Leu Ser Gin Leu Leu Ser Ala Lys 130 135 140
Gly Ile Glu His Asp Phe Ile Pro Gly Tyr Gin Phe His Ile Tyr 145 150 155 160
Pro Val Phe Pro Ile Pro Glu Arg Arg Phe Leu Tyr Gin Val Lys 165 170 175

<210> SEQ ID NO 79
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic 6x His tag

<400> SEQUENCE: 79
His His His His His His 1 5

<210> SEQ ID NO 80
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Portion of TG hydrolase

<400> SEQUENCE: 80
Gly Asp Ser Asp Gly 1 5

<210> SEQ ID NO 81
<211> LENGTH: 4
<212> TYPE: PRT
What is claimed is:

1. A polynucleotide comprising a nucleic acid sequence encoding SEQ ID NO. 2, or a polypeptide having at least 90 percent identity to SEQ ID NO. 2, wherein said polynucleotide is in an expression vector.

2. The polynucleotide sequence of claim 1, wherein said nucleic acid sequence comprises a sequence that encodes GDSAG (SEQ ID NO. 61).

3. The polynucleotide sequence of claim 1, wherein said nucleic acid sequence comprises a sequence that encodes HGGG (SEQ ID NO. 68).

4. The polynucleotide sequence of claim 1, wherein said nucleic acid sequence comprises a sequence that encodes GDSAG (SEQ ID NO. 61) and a sequence that encodes HGGG (SEQ ID NO. 68).

5. An agent which regulates the activity of a M. tuberculosis TG hydrolase (MTTGH) polypeptide, wherein said agent is an RNA molecule, an antisense oligonucleotide, or a ribozyme that targets a nucleic acid sequence that encodes SEQ ID NO. 2.


7. A recombinant cell host containing a purified MTTGH polynucleotide or a recombinant vector comprising a MTTGH polynucleotide, wherein said MTTGH polynucleotide comprises a nucleic acid sequence that encodes SEQ ID NO. 2, or a polypeptide having at least 90 percent identity therewith.