Characterization of a Novel Cell Wall-anchored Protein with Carboxylesterase Activity Required for Virulence in Mycobacterium tuberculosis*

Shichun Luen and William R. Bishai

From the Department of Medicine, Center for Tuberculosis Research, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231

Pooled mutant competition assays have shown that the Mycobacterium tuberculosis MT2282 gene (Rv2224c, annotated as encoding a proteinase) is required for bacterial survival in mice. To understand the mechanism of this requirement, we conducted a genetic and biochemical study of the MT2282 gene and its product. MT2282 encodes a member of the microbial esterase/lipase family with active site consensus sequences of G-X-S-X-G, and we have concluded that the MT2282 protein is, in fact, a cell wall-associated carboxylesterase rather than a proteinase, as initially annotated. The MT2282 gene product preferentially hydrolyzes ester bonds of substrates with intermediate carbon chain length. Purified MT2282 is a monomer with enzymatic catalysis properties that fit in the Michaelis-Menten kinetic model. Esterase activity was inhibited by paraoxon and dichlorvos. Replacement of Ser215, Asp450, and His477 by Ala in the consensus motifs completely abolishes esterase activity, suggesting that Ser215-Asp450-His477 forms a catalytic triad with Ser215 as an active site residue. To evaluate the role of the MT2282 in pathogenesis, the gene was deleted from the M. tuberculosis genome. BALB/c mouse aerosol infections showed reduced colony-forming unit loads in lungs and spleens and less lung pathology for the ΔMT2282 mutant. High dose intravenous infection of mice with the mutant resulted in a significantly delayed time to death compared with the wild type or complemented mutant. These results indicate that MT2282 encodes a cell wall-associated carboxylesterase, which is required for full virulence of M. tuberculosis. We propose that MT2282 (Rv2224c) and its adjacent paralogous gene MT2281 (Rv2223c) be named caeA and caeB respectively, for carboxylesterase A and B.

Tuberculosis, one of the leading human infectious diseases claiming about 1.7 million deaths annually, is difficult to control because of human immunodeficiency virus/tuberculosis co-infection and emergence of virtually untreatable extensively drug-resistant Mycobacterium tuberculosis strains. Clearly, new drugs and effective vaccines are urgently needed, which creates a strategic demand for better understanding of the genetic basis for the virulence and pathogenesis of M. tuberculosis.

The M. tuberculosis genome sequence and annotation has provided an invaluable blueprint of the genomic organization and gene functionality for this slowly growing pathogen (1). However, annotation is unable to predict gene function with complete accuracy due to divergent evolution of protein motifs. Recent advances in genetic approaches such as transposon mutagenesis and DNA microarray techniques have facilitated initial functional screening of essential genes for in vitro and in vivo growth in a high throughput manner (2–6). Mycobacterial genes that are involved in lipid metabolism, carbohydrate transport and metabolism, cell division chromosomal partitioning, and secretion are more likely to be required for survival in the mouse (5, 6). For example, mutation of six of the mycobacterial membrane protein mmpL family genes severely compromises the ability of the respective mutants to multiply in mouse lungs (6).

M. tuberculosis escapes host defense mechanisms by inhibiting and evading the host immune system (7, 8), by arresting phagolysosome maturation (9, 10), and by establishing a state of persistence (11, 12). However, the underlying genetic expression and molecular mechanisms are incompletely understood. Virulence genes refer to those genes that are required for disease in the host, but not necessarily essential for in vitro growth. The MT2282 gene of M. tuberculosis CDC1551 (or Rv2224c of H37Rv) was identified as a putative virulence gene by high throughput techniques (5, 6). It is annotated as a putative proteinase (TIGR-CMR) or a probable exported protease (Tubercullist). Interestingly, isogenic M. tuberculosis mutants with an insertional inactivated MT2282 gene were attenuated in pooled mutant competition studies in mouse lungs and spleens (5, 6). This gene was also preferentially expressed in human macrophages (13). Under nutrient starvation, MT2282 was significantly up-regulated during 4–96 h after the onset of starvation (14).

In this study, we further characterized the virulence role of MT2282 in the mouse infection model. We demonstrated that the MT2282 protein is membrane-associated and possesses

* This work was supported by National Institutes of Health Grants AI36973, -37856, and -43846 (to W. R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

□ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

1 A recipient of a postdoctoral fellowship from the Heiser Program of the New York Community Trust.

2 To whom correspondence should be addressed: Dept. of Medicine, Center for Tuberculosis Research, Johns Hopkins University School of Medicine, 1550 Orleans St., Baltimore, MD 21231. Tel.: 410-955-3507; Fax: 410-614-8173; E-mail: wbishai@jhmi.edu.
carboxylesterase activity. The functional annotation of proteinase appears incorrect because no proteinase activity was found.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Media, and Growth Conditions—**M. tuberculosis CDC1551 (15) and its mutants were cultured as described previously (6). Mycobacterium smegmatis mc²155 was used for mycobacterial phage propagation was cultured in 7H9 or 7H10 medium, and incubated at 30 °C when temperature-sensitive phage was recovered. *Escherichia coli* strains (DH5α for cloning, BL21 for expression, and HB101 for *in vitro* packaging) used in this study were cultured in Luria broth (LB; Difco) at 37 °C with shaking. Hygromycin B was added to the media at a final concentration of 50 μg/ml for *M. tuberculosis* deletion mutants or 200 μg/ml for *E. coli*. Kanamycin was added to the media with a final concentration of 20 μg/ml for *M. tuberculosis*-complemented mutants or 50 μg/ml for *E. coli*.

**Gene Deletion, Complementation, and Characterization—**A specialized phage transduction approach was used to generate the MT2282 deletion mutant (16). The complementation vector was constructed by PCR amplification of a 2-kb DNA fragment containing MT2282 and its native promoter into the EcoRI site of the mycobacterial integrating vector pMH94 (17, 18) to yield a single-copy plasmid pCOM that confers kanamycin resistance. pCOM was introduced into ΔMT2282 by electroporation and kanamycin selection.

To characterize the deletion and complementation, genomic DNA from wild type (WT), ΔMT2282, and COM was isolated, restricted with XhoI, separated by 1% agarose gel electrophoresis, and Southern blotted onto nylon membranes by standard capillary transfer. The DNA templates were hybridized with PCR-generated, digoxigenin-11-DUTP-labeled gene probes, detected with an anti-digoxigenin alkaline phosphatase conjugate, and developed with CSPD³ substrate (Roche Diagnostics). Primers used for generating probes included MT2282-specific gya-F and moi-R, hygromycin-specific hyg4-F and hyg4-R, and kanamycin-specific kanf-1 and kanr-1 (see Table 1).

**MT2282 Expression, Purification, and Localization—**The MT2282 gene, without its signal sequence, was PCR-cloned into the EcoRI and XhoI sites of the glutathione S-transferase-tagged expression vector pGEX-6P-1 (Amersham Biosciences) using primers EX-2 and EXP-3 (see Table 1) to yield the expression vector pSL115. To solubilize the recombinant fusion proteins that accumulate as inclusion bodies, a mildly denaturing/refolding kit was used according to the manufacturer’s protocol (Novagen). The fusion proteins were affinity-purified using glutathione-Sepharose 4B gel chromatography, and the glutathione S-transferase tag was cleaved off with PreScission™ protease following the on-column cleavage protocol (Amersham Biosciences). Protein levels were quantified using the BCA protein assay reagent kit (Pierce). Rabbit anti-MT2282 polyclonal antibodies were generated using purified MT2282 as an immunogen according to a standard protocol (Spring Valley Laboratories, Inc.).

To detect MT2282 in the culture medium, 7H9 culture supernatants were concentrated 20-fold with an Amicon ultracentrifugal device (Millipore) before immunoblotting. *M. tuberculosis* cells were fractionated into cell envelope and cytosol by bead beating and centrifugation. Briefly, *M. tuberculosis* cell pellets were washed once and resuspended in 1 ml of phosphate-buffered saline in an O-ring tube with glass beads. After bead beating two times for 30 s each with maximum revolutions/min then centrifuging for 5 min at 13,000 revolutions/min, the upper cytosol phase was removed and passed through a 0.22-micron filter. The pellet containing the cell envelope and beads was washed once with phosphate-buffered saline and centrifuged at 2,500 revolutions/min for 2 min. The upper aqueous phase was discarded, and the middle phase containing the cell envelope fraction was collected. Standard protocols were followed for protein SDS-PAGE and semidry transfer of proteins onto nitrocellulose membranes (19). Immunohybridization and detection was carried out according to a standard ECL protocol (Amersham Biosciences) using purified polyclonal rabbit anti-MT2282 IgG as the primary antibody and goat anti-rabbit IgG horseradish peroxidase conjugate as the secondary antibody.

**Carboxylesterase Activity of MT2282—**Carboxylesterase activity of MT2282 was determined by observing the conversion of α-naphthyl acetate, α-naphthyl propionate, and α-naphthyl butyrate to α-naphthol according to Lanz and Williams (20). Briefly, each reaction mixture contained 0.1 ml of protein (0.5 mg/ml) and 1.5 ml of substrate (0.42 mM), 0.5 ml of Na₂HPO₄ buffer, pH 7.0 (0.2 M), and 0.4 ml of distilled water. The mixture was incubated for 10 min at 39 °C (20). At the end of the reaction, 0.5 ml of 10% lauryl sulfate (Sigma) solution containing 2.5 mg of fast garnet GBC (Sigma) was added. The mixture was then incubated at room temperature for 15 min for color development. Absorbance at 560 nm was measured and the yield of α-naphthol extrapolated from a standard curve (linear from 0 to 50 μM) constructed with α-naphthol (Sigma). Catalytic activity of MT2282 was also tested in the fluorogenic substrates 4-methylumbelliferyl butyrate (5 μM, 0.1 μM Tris, pH 8.0), 4-methylumbelliferyl heptanoate (5 μM, 0.1 μM Tris, pH 8.0), and 4-methylumbelliferyl oleate (50 μM, 0.1 μM phosphate, pH 7.0). 145 μl of substrate was mixed with 4.5 μl of MT2282 (2 mg/ml) in an Eppendorf tube, and 100 μl was then pipetted into the assay wells of a 96-well plate (Costar 3694). The plate was read by the fluorometer (ICN Titertek Fluoroskan II) at room temperature with an excitation of 355 nm and an emission of 460 nm. Readings were recorded for 2 h at 3-min intervals. Initial rates were calculated and reported.

To test carboxylesterase inhibitors, paraoxon, dichlorvos, phenylmethylsulfonyl fluoride, and sodium fluoride stock solutions were prepared using the manufacturer-recommended diluents (Sigma-Aldrich). 4-Methylumbelliferyl butyrate was used as substrate at 250 μM in 0.1 M Tris pH 8.0. Each inhibitor solution was added to the substrate to make final concentrations of 1, 10, 100, and 1000 μM. 100 μl of these mixtures were used as negative controls. The percentage of inhibition was determined as the ratio of the initial rate of inhibition divided by the normal rate.

---

³ The abbreviations used are: CSPD, disodium 3-[4-methoxySpiro (1,2-dioxetane-3,2’-5’-chloro)(tricyclo(3,3.1^3^)-decan)-4-Y]phenyl phosphate; (Fast Garnet) GBC, (4’-amino-2,3-dimethylbenzenene-O-aminoazotoluene); CFU, colony-forming unit; WT, wild type.
Novel Mycobacterial Protein Important for Virulence

TABLE 1

| Primer  | Sequence                      |
|---------|-------------------------------|
| gya-F   | 5'-GGACCGCTTTCAACACCTAG-3'    |
| moi-R   | 5'-ACGCTCGAGGCGACGACGCGAC-3' |
| hyg4-F  | 5'-AGGGTCATTCGCCGAACTGCTG-3' |
| hyg4-R  | 5'-TCTCGAGGACTCTCCGCACTCG-3' |
| kanF    | 5'-GCGGCGCATTTAGGTCCCAAC-3'  |
| kanR    | 5'-GCGGCGCATTTAGGTCCCAAC-3'  |
| EX-2    | 5'-GGATCCATGACGCGAACTGCTG-3' |
| EXP-3   | 5'-GCGGCGCATTTAGGTCCCAAC-3'  |
| SER1    | 5'-CCTCGAGGACTCTCCGCACTCG-3' |
| SER2    | 5'-CCTCGAGGACTCTCCGCACTCG-3' |
| Asp-F   | 5'-CCTCGAGGACTCTCCGCACTCG-3' |
| Asp-R   | 5'-CCTCGAGGACTCTCCGCACTCG-3' |
| H477-1  | 5'-GCGGCGCATTTAGGTCCCAAC-3'  |
| H477-2  | 5'-GCGGCGCATTTAGGTCCCAAC-3'  |

To characterize the active site serine and the catalytic triad, PCR-based site-directed mutagenesis was conducted to mutate Ser215, Asp450, and His477 to Ala215, Ala450, and Ala477, respectively, using primer pairs SER1 and SER2, Asp-F and Asp-R, and H477-1 and H477-2 (Table 1) and the plasmid pSL115. Mutagenesis was confirmed by DNA sequencing and restriction mapping.

Aerosol Infection—Female, 6-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) were aerosol-infected as described previously (21). Colony-forming units (CFUs) present in lungs and spleens were counted at the indicated time points (see Fig. 8). At days 14 and 42, the lungs were fixed for histological examination with hematoxylin and eosin and acid-fast bacilli staining. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Time to Death—Groups of BALB/c mice (4–6 weeks old, female; Charles River Laboratories, Wilmington, MA) were infected with 0.2 ml of M. tuberculosis by intravenous injection via the lateral tail vein. Fresh cultures grown to early log phase were used. The inoculum was 1.5 × 10⁶ CFUs for WT (n = 13), 1.76 × 10⁶ CFUs for AMT2282 (n = 13), and 1.66 × 10⁶ CFUs for COM (n = 12). Five mice of each group were sacrificed at day 1 for CFU load determination in the lungs. The death of the mice was recorded, and the mice were weighed weekly. A log-rank test was applied to compare the survival curves.

RESULTS

Generation and Characterization of an MT2282 Deletion Strain and Complemented Mutant—The MT2282 gene was deleted from the genome by the phage transduction and single step selection approach, yielding the deletion mutant (ΔMT2282). Fifteen base pairs at the 5’ and 64 base pairs at the 3’ of the gene remained (Fig. 1A). A single copy of MT2282 with its promoter was introduced into ΔMT2282 using the integrating vector pMH94 and kanamycin selection to give the complemented deletion mutant (Fig. 1A, COM). Phenotypically, ΔMT2282 was resistant to hygromycin but susceptible to kanamycin. COM was resistant to both hygromycin and kanamycin, whereas WT M. tuberculosis CDC1551 was susceptible to both antibiotics. Genotypically, Southern blotting demonstrated deletion of MT2282 in ΔMT2282 and confirmed its presence in COM and WT strains (Fig. 1B). Although ΔMT2282 and COM contained the hygromycin resistance gene, only COM retained the kanamycin resistance marker (Fig. 1B).

Localization of the MT2282 Protein—Cloning of MT2282 into the expression vector pGEX-6P-1 was validated by DNA sequencing. A glutathione S-transferase-MT2282 fusion protein was expressed and purified from E. coli BL21(DE3). Approximately 99% of the protein remained as an insoluble inclusion body; therefore, a mild denaturation/refolding procedure was applied to achieve a maximum yield. Affinity purification of recombinant MT2282 yielded a protein that was soluble and 96% pure based upon Coomassie-stained PAGE analysis. The affinity-purified MT2282 protein was shown to be a monomer and had an apparent molecular mass of 54 kDa as extrapolated from migration on 5, 7.5, 10, and 12.5% native PAGE (Fig. 2). A polyclonal rabbit MT2282 antiserum was generated using native MT2282 cell lysate (Fig. 3A), confirming the deletion. Because
SignalP 3.0 predicted a signal peptide sequence at the N terminus of MT2282. A WT culture supernatant was concentrated and tested for secreted MT2282. MT2282 immunoreactivity was also absent from the culture supernatant, indicating that the protein was not secreted (Fig. 3A). When whole cells were disrupted by bead beating and fractionated into cytosol and the envelope, MT2282 immunoreactivity was found only in the envelope of the WT and COM strains with \(1\%\) in the cytosol (Fig. 3B). A motif scan predicts that, at the N terminus of MT2282, an undecapeptide (amino acids 8–18, LAAVALVLVGC) may serve as a prokaryotic membrane lipoprotein lipid attachment site. The prediction of a lipoprotein that is associated with the membrane is in agreement with our subcellular localization data (Fig. 3B).

**MT2282 Does Not Possess Proteinase Activity as Annotated**—Sequence analysis by Pfam suggests MT2282 is a member of the \(\alpha/\beta\) hydrolase fold superfamily (\(E = 4.2 \times 10^{-70}\)). This superfamily contains a typical tertiary fold, but family members lack obvious primary sequence similarity or substrate specificity (22, 23). The MT2282 protein is annotated as a putative proteinase or a probable exported protease. Using soluble, affinity-purified recombinant MT2282, we tested the protein for a variety of proteolytic activities. Purified MT2282 lacked activity when tested with either the proteinase substrate Hide Powder Azure (Calbiochem) or with the serine proteinase substrate bis-(N-CBZ-L-arginine amide)-rhodamine 110, dihydrochloride (BZAR, Biotium) (Fig. 4). Three independent tests yielded the same results, and one set of data is shown.
again negative (data not shown). In each substrate, we observed strongly positive proteolytic activity with the positive control enzymes (which was trypsin for Hide Powder Azure and BZAR and proline iminopeptidase for PPNA; the generous gift of Tadashi Yoshimoto, Nagasaki University, Japan).

**Carboxylesterase Activity of MT2282**—In contrast to previous predictions, a sequence motif scan from the PROSITE database predicted an esterase motif in MT2282 positioned between residues 89 and 230 with an $E$ value of 0.00045 (24). An active site serine (Ser$^{215}$) was predicted within residues 213–217 (G-Y-S-Y-G), consistent with the G-X-S-X-G consensus motif for members of the esterase/lipase superfamily (25). In view of their bioinformatics findings, we tested affinity-purified MT2282 for esterase activity in vitro and found that MT2282 cleaved ester bonds and released $\text{-naphthol}$ from esterase substrates, such as 1-$\text{naphthyl acetate}$, 1-$\text{naphthyl propionate}$, and 1-$\text{naphthyl butyrate}$. Interestingly, the esterase activity of MT2282 increased with the increasing carbon chain length of the substrate ($p < 0.001$ for 1-NA versus 1-NP and $p < 0.05$ for 1-NP versus 1-NB). However, the positive control for this system, porcine liver esterase, did not show this pattern (Fig. 5A).

Using fluorogenic substrates, we further observed that MT2282 was most active on 4-methylumbelliferyl butyrate followed by 4-methylumbelliferyl heptanoate, with weakest activity using 4-methylumbelliferyl oleate (Fig. 5B). In 0.2M Na$_2$HPO$_4$ buffer, the optimum pH for MT2282 activity on 1-NP was 7.0 within the range of pH 5.0–8.5 (Fig. 5C). Enzyme activity was routinely assayed at 39 °C because of the fact that 37 °C assays were 10% lower in activity. Enzymatic kinetics study yielded a curve that fit the classical Michaelis-Menten model, indicating a single active site of MT2282.

With 4-methylumbelliferyl butyrate as the substrate, the $V_{max}$ = 128.1 relative fluorescence units/min and $K_m = 738 \mu$M (Fig. 6). Next, we conducted inhibition studies with the organophosphates paraoxon and dichlorvos that phosphorylate the active site serine of known esterases (26). These inhibitors showed concentration-dependent inhibition in the 0.1–1.0 mM range. Dichlorvos stimulated activity some 10–12% at subinhibitory concentrations. Both sodium fluoride and phenylmethylsulfonyl fluoride, which are inhibitory for serine proteases and some esterases, were non-inhibitory at low concentrations but stimulatory for enzyme activity at high concentrations. The stimulation of ester bond hydrolysis was of interest, because it would indicate that sites other than the active site serine of MT2282 were involved. Such cooperativity, in some cases, can lead to activation of enzymes (26). Plus, it has already been shown that
phenylmethylsulfonyl fluoride does not inhibit all esterases (27).

*Site-directed Mutagenesis Confirmed a Catalytic Triad*—Microbial carboxylesterases share structural similarities (an open twisted β-sheet surrounded on both sides by α-helices) and the arrangement of three catalytic residues (the catalytic triad, Ser-Asp-His) with the serine located in a sharp turn between a β-strand and α-helix that is known as the nucleophile elbow, and the His side chain hydrogen-bonded to the carboxylic group of the Asp side chain to form a charge relay system (28). ClustalW sequence alignment indicates that the G-Y-S-Y-G motif is highly conserved not only in the genus of *Mycobacterium*, but also in the genera *Arthrobacter*, *Rhodococcus*, and *Nocardia*. The same is true for the Asp450 and His477 residues (supplemental Fig. S1). To test the Ser215, Asp450, His477 catalytic triad, site-directed mutagenesis was conducted, and replacement of Ser215, Asp450, and His477 with Ala completely abolished esterase activity as assessed on 1-naphthyl propionate (Fig. 7). The same results were seen in an assay with the fluorogenic 4-methylumbelliferyl butyrate as substrate (data not shown). These observations confirmed that the primary enzymatic function of MT2282 is esterase activity, that Ser215, Asp450, His477 is the catalytic triad, and that the 213G-Y-S-Y-G217 motif is the active site for that activity.

*In Vitro Growth of the ΔMT2282 Mutant*—Previous work had shown that a transposon insertion in MT2282 yielded an insertion mutant JHU2224c-197, but the mutant failed to survive in mouse lung competition assays (4, 6). To determine whether this phenotype could be seen in a single mutant rather than in pooled mutants, we prepared a full deletion of MT2282 (as described above) and studied its growth and virulence properties alongside that of the wild type and complemented mutant strains.

In Middlebrook 7H9 medium, the growth rates by *A*$_{600}$ and CFU counting of ΔMT2282 and COM were the same as that of the WT before day 7, or *A*$_{600}$ of ~1.2 (supplemental Fig. S2, B and C). Between days 7 and 10, the ΔMT2282 CFUs plateaued, whereas the WT and COM grew further. MT2282 was not essential for *in vitro* growth, as shown by insertional inactivation using transposon mutagenesis (3, 4). Although the optical density did not show a difference, the CFUs in this study indicated that some of the bacilli in the ΔMT2282 culture were not viable toward the end of growth. Introduction of the MT2282 gene with its native promoter into ΔMT2282, as in the COM strain, completely restored its viability phenotype. In addition to increased expression of MT2282 under nutrient starvation (14), the lower viability of ΔMT2282 in late log and early stationary phases implied that MT2282 was required to maintain viability under nutritional stress and that expression of MT2282 was regulated by a stress signal through an unknown pathway.

*Deletion of the MT2282 Gene Resulted in Attenuation and Reduced Pathology in the Mouse Lung*—To assess virulence in the mouse model, we used an aerosol infection route in BALB/c mice. An inoculum of 10 ml of *A*$_{600}$ = 0.2 culture, which was subsequently found to have a closely matched titer of 1.46 × 10$^7$, 1.55 × 10$^7$, and 1.39 × 10$^7$ CFU/ml for WT, ΔMT2282, and COM, respectively, was used in the Glas-Col nebulizer for each of the three strains. We moni-
Novel Mycobacterial Protein Important for Virulence

tored CFU counts in the lungs and spleens of mice for 56 days. ΔMT2282 grew rapidly in the lungs for the first 14 days (Fig. 8A). From day 14 to day 28, the ΔMT2282 mutant replicated slowly and peaked on day 28; thereafter the CFU counts slowly decreased between days 42 and 56. Although the CFU curve of ΔMT2282 followed a similar pattern to that of the WT and COM, the CFUs were significantly lower for ΔMT2282, which indicated an attenuated mouse phenotype for ΔMT2282. We also noticed that on day 1, the CFU loads in the lungs of ΔMT2282 (2.35 log units) were lower than that of WT (3.10 log units) and COM (3.07 log units). Two possibilities could be accountable for the lower CFU loads in ΔMT2282 lungs. ΔMT2282 may be more susceptible than WT and COM to the mechanical stress of nebulization (29), or ΔMT2282 may be more susceptible than WT and COM to alveolar macrophages, because pulmonary alveolar macrophages are highly activated (30). To further characterize the attenuation of ΔMT2282 in mice, we conducted a time-to-death study using high dose intravenous mouse infection (see below). Spleen CFUs showed initial variation on day 14, but by days 28–56 a more uniform count was obtained (Fig. 8B). The 2-log difference in spleen CFUs on day 14 suggested that the MT2282 mutant may be attenuated for dissemination from the lung.

The gross pathology in the mouse lungs infected by ΔMT2282 was markedly less severe compared with lungs infected with WT and COM (Fig. 9A). Beginning at 4 weeks, the lungs infected with ΔMT2282 were smaller in size and had fewer apparent granulomas, whereas the lungs infected with WT and COM were larger and had more granulomas (Fig. 9A, upper panel). Similarly, the spleens infected with ΔMT2282 were also smaller in size (Fig. 9A, lower panel) and weighed less (Fig. 9B). Histopathology using hematoxylin and eosin staining indicated that, at day 14, there were more localized mononuclear cell aggregates in the lungs infected by WT and COM than in lungs infected by ΔMT2282 (supplemental Fig. S3A). Acid-fast-stained bacilli were identified in the infected lungs for all three groups on day 14. It was obvious that there were more bacilli in WT and COM-infected lungs than those infected by ΔMT2282 (supplemental Fig. S3B). By day 42, consolidation of lung tissue was apparent in the WT and COM, whereas in ΔMT2282-infected lungs, much more functional alveolar air space was seen (supplemental Fig. S3A). The differences in histopathology between WT and COM versus ΔMT2282 reflect functional changes to the lung that were confirmed by time-to-death studies in mice.

Deletion of the MT2282 Gene Conferred Prolonged Time to Death in Mice—To confirm the role of MT2282 in virulence, a high dose intravenous mouse infection experiment was carried out focusing on time-to-death analysis (18). At day 1, there were 5.62 ± 0.68 × 10⁶, 6.03 ± 0.54 × 10⁶, and 6.10 ± 0.29 × 10⁴ CFUs deposited in mouse lungs infected with knock-out, WT, and COM, respectively. BALB/c mice infected with WT and COM showed significantly different median survival times as compared with ΔMT2282-infected mice. As seen in Fig. 10A, mice infected with WT died within 56 days (median = 47), mice infected with COM died within 64 days (median = 52.5), whereas mice infected with ΔMT2282 survived up to 191 days (median = 154, p < 0.0001 by Logrank test for comparison to both WT and COM). It was noteworthy that mice infected with WT and COM showed significant weight loss compared with mice infected with ΔMT2282 (Fig. 10B). The fact that median survival times were ~3.0–3.3× longer in ΔMT2282-infected mice indicates a virulence role for this carboxylesterase gene.

DISCUSSION

Our results revealed that deletion of the MT2282 gene in M. tuberculosis resulted in attenuation in the mouse CFU organ burden and time-to-death models of virulence. Introduction of a single copy MT2282 gene with its native promoter completely restored the wild type phenotype both in vitro and in vivo. Attenuation is unlikely due to polar effect, because expression of the downstream genes MT2281 and MT2280 (glnA2) was detected at the mRNA level, as shown by reverse transcription PCR (supplemental Fig. S4). Although insertional inactivation of MT2281 did not have a phenotype either in vitro or in vivo in a pooled mutant study (3, 5), the function of MT2281 is worthy...
of further study, because its predicted amino acid sequence shares homology with MT2282.

Bioinformatic analysis predicted a signal peptide sequence at the N terminus of MT2282 (SigP3.0). Further in silico analysis by LipoP1.0 and PSORT algorithms predicted a lipid attachment site at residue 18 for a membrane-anchored lipoprotein (31). By Western blotting, we showed association of MT2282 with the cell envelope without evidence of secretion into the culture medium, suggesting that MT2282 is not cleaved by signal peptidase I. Therefore, it is likely that the MT2282 immature protein is cleaved by mycobacterial signal peptidase II; a potential site exists just upstream of the Cys18 residue to which a glyceride fatty acid lipid is attached (32–34). However, further studies are necessary to demonstrate MT2282 to be a lipoprotein. Our data indicate that the MT2282 protein is anchored to the surface membrane where it may function as a catalytic protein, as suggested by this study. Furthermore, it remains possible that MT2282 may also be implicated in structural and/or nutrient transport functions.

A high degree of Mycobacterium-specific gene duplication has been observed within mycobacterial genomes (35, 36). MT2282 and its adjacent downstream parologue MT2281 appear to be duplicated genes, because they share significant sequence similarities (51.2% identity, 64.6% similarity at the protein level) and are located adjacently in the same orientation with a gap of 62 base pairs (Fig. 1A). They also share the same structural fold and functional predictions, with the active site residues G-Y-S-Y-G being completely conserved. Although MT2282 is anchored to the membrane, MT2281 is predicted to be cleaved by signal peptidase I and secreted into the environment. Because MT2282 was characterized to be a carboxylesterase in this study, MT2281 likely has the same activity. Hence MT2282 and MT2281 appear to be proteins with similar function but possible divergent localization, one anchored to the membrane and the other secreted. A synergistic action between the two proteins may exist. We propose that the MT2282 gene be named as caeA and the MT2281 gene as caeB for carboxylesterases A and B.

At least two possibilities exist regarding the molecular mechanism of attenuation of the M. tuberculosis caeA mutant. Surface lipids are important for the virulence of M. tuberculosis (37, 38). For example, the peptidoglycan-linked mycolic acids that are esterified to arabinogalactan are key virulence determinants (39), and lipoproteins of M. tuberculosis have been shown to be capable of triggering activation of humoral and cellular immune responses to mycobacteria (40, 41). As a membrane-anchored protein, CaeA may play a structural role in modifying envelope composition through its esterase activity (42). Alternatively, it is also possible that CaeA is primarily the hydrolase of free esters in the local environment. Certain fatty acyl esters, for example, may possess mycobactericidal activity (43), and hence CaeA may play a protective role in degrading potential toxic lipids. Additionally, esterase action on free esters in the microenvironment could be a source of fatty acids to be used nutritionally. Further assessment of the in vivo substrates of CaeA and CaeB will be useful in distinguishing among these hypotheses.

Acknowledgments—The assistance of Naomi Gauchet is gratefully acknowledged. We thank Norman Morrison for critical reading of this manuscript. We also thank Sandeep Tyagi for technical assistance.

REFERENCES

1. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglemeier, K., Gas, S., Barry, C. E., III, Tekaiia, F., Bada, K., Basham, D., Brown, D., Chillingworth, T., Connor, N., Davies, R., Devlin, K., Feltwell, T., Gentle, S., Hamlin, N., Holroyd, S., Hornsby, T., Jingles, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, S., Squares, S., Sulkon, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) Nature 393, 537–544

2. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12712–12717

3. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) Mol. Microbiol. 48, 77–84

4. Lamichhane, G., Zignol, M., Blades, N. J., Geiman, D. E., Dougherty, A., Gross, J., Bromman, K. W., and Bishai, W. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7213–7218

5. Sassetti, C. M., and Rubin, E. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12989–12994

6. Lamichhane, G., Tyagi, S., and Bishai, W. R. (2005) Infect. Immun. 73, 2533–2540

7. Pai, R. K., Convery, M., Hamilton, T. A., Boom, W. H., and Harding, C. V. (2003) J. Immunol. 171, 175–184
Novel Mycobacterial Protein Important for Virulence

8. Post, F. A., Manca, C., Neyrolles, O., Ryffel, B., Young, D. B., and Kaplan, G. (2001) Infect. Immun. 69, 1433–1439
9. Pethe, K., Swenson, D. L., Alonso, S., Anderson, J., Wang, C., and Russell, D. G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13642–13647
10. Vergne, I., Chua, J., Lee, H. H., Lucas, M., Belisle, J., and Deretic, V. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4033–4038
11. Wayne, L. G. (1994) J. Bacteriol. 176, 732–737
12. Parrish, N. M., Dick, J. D., and Bishai, W. R. (1998) Trends Microbiol. 6, 107–112
13. Dubnau, E., Fontan, P., Manganelli, R., Soares-Appel, S., and Smith, I. (2002) Infect. Immun. 70, 2787–2795
14. Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002) Mol. Microbiol. 43, 717–731
15. Valway, S. E., Sanchez, M. P., Shinnick, T. F., Orme, I., Agerton, T., Hoy, D., Jones, J. S., Westmoreland, H., and Onorato, I. M. (1998) N. Engl. J. Med. 338, 633–639
16. Bardarov, S., Bardarov, S., Jr., Pavelka, M. S., Jr., Sambandamurthy, V., Larsen, M., Tufariello, J., Chen, J., Hatfull, G., and Jacobs, W. R., Jr. (2002) Microbiology (Read.) 148, 3007–3017
17. Lee, M. H., Pascoella, L., Jacobs, W. R., Jr., and Hatfull, G. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3111–3115
18. Chen, P., Ruiz, R. E., Li, Q., Silver, R. F., and Bishai, W. R. (2000) Infect. Immun. 68, 5575–5580
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (2006) Current Protocols in Molecular Biology, Vol. 2, pp. 10.8.4–10.8.7, John Wiley & Sons, Inc., New York
20. Lanz, W. W., and Williams, P. P. (1973) J. Bacteriol. 113, 1170–1176
21. Nuerenberger, E. L., Yoshimatsu, T., Tyagi, S., Williams, K., Rosenthal, L., O'Brien, R. J., Vernon, A. A., Chaisson, R. E., Bishai, W. R., and Grosset, J. H. (2004) Am. J. Respir. Crit. Care Med. 170, 1131–1134
22. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Vereschueren, K. H. G., and Goldman, A. (1992) Protein Eng. 5, 197–211
23. Nardini, M., and Dijkstra, B. W. (1999) Curr. Opin. Struct. Biol. 9, 732–737
24. Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C. J., Hofmann, K., and Bairoch, A. (2002) Nucleic Acids Res. 30, 235–238
25. Fojan, P., Jonson, P. H., Petersen, M. T., and Petersen, S. B. (2000) Biochimie (Paris) 82, 1033–1041
26. Rosenfeld, C. A., and Sultatos, L. G. (2006) Toxilog. Sci. 90, 460–469
27. Patel, D., and Scott, C. S. (1991) Biochem. Pharmacol. 42, 1577–1585
28. Liu, P., Wang, Y. F., Ewis, H. E., Abdelal, A. T., Lu, C. D., Harrison, R. W., and Weber, I. T. (2004) J. Mol. Biol. 342, 551–561
29. Sun, R., Converse, P. J., Ko, C., Tyagi, S., Morrison, N. E., and Bishai, W. R. (2004) Mol. Microbiol. 52, 25–38
30. Dannenberg, A. M. (2006) Pathogenesis of Human Pulmonary Tuberculosis. pp. 120–152, ASM Press, Washington D.C.
31. Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H., and Krogh, A. (2003) Protein Sci. 12, 1652–1662
32. Hayashi, S., and Wu, H. C. (1990) J. Bioenerg. Biomembr. 22, 451–471
33. Mattar, S., Scharf, B., Kent, S. B., Rodewald, K., Oesterhelt, D., and Engelhard, M. (1994) J. Biol. Chem. 269, 14939–14945
34. Klein, P., Somorjai, R. L., and Lai, P. C. (1988) Protein Eng. 2, 15–20
35. Kinsella, R. J., Fitzpatrick, D. A., Creevey, C. J., and McInerney, J. O. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10320–10325
36. Qamra, R., Prakash, P., Aruna, B., Hasnain, S. E., and Mande, S. C. (2006) Biochemistry 45, 6997–7005
37. Khuller, G. K., Taneja, R., Kaur, S., and Verma, J. N. (1982) Aust. J. Exp. Biol. Med. Sci. 60, 541–547
38. Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., Kaplan, G., and Barry, C. E., III (2004) Nature 431, 84–87
39. Draper, P., and Daffe, M. (2005) in Tuberculosis and the Tubercle Bacillus (Cole, S. T., Eisenach, K. D., McMurray, D. N., and Jacobs, W. R., Jr., eds) pp. 261–274, ASM Press, Washington, D.C.
40. Young, D. B., and Garbe, T. R. (1991) Res. Microbiol. 142, 55–65
41. Sinha, S., Kosalai, K., Arora, S., Namane, A., Sharma, P., Gaikwad, A. N., Brodin, P., and Cole, S. T. (2005) Microbiology (Read.) 151, 2411–2419
42. Trivedi, O. A., Arora, P., Vats, A., Ansari, M. Z., Tickoo, R., Sridharan, V., Mohanty, D., and Gokhale, R. S. (2005) Mol. Cell 17, 631–643
43. Kondo, E., and Kanai, K. (1977) Jpn. J. Med. Sci. Biol. 30, 171–178