Inhibition of the Sole Type I Signal Peptidase of *Mycobacterium tuberculosis* Is Bactericidal under Replicating and Nonreplicating Conditions

Infectious Disease Research Institute, Seattle, Washington, USA

Proteins secreted by bacteria perform functions vital for cell survival and play a role in virulence in *Mycobacterium tuberculosis*. *M. tuberculosis* lepB (Rv2903c) encodes the sole homolog of the type I signal peptidase (SPase). The lepB gene is essential in *M. tuberculosis*, since we could delete the chromosomal copy only when a second functional copy was provided elsewhere. By placing expression under the control of an anhydrotetracycline-inducible promoter, we confirmed that reduced lepB expression was detrimental to growth. Furthermore, we demonstrated that a serine-lysine catalytic dyad, characteristic for SPase function, is required for LepB function. We confirmed the involvement of LepB in the secretion of a reporter protein fused to an *M. tuberculosis* signal peptide. An inhibitor of LepB (MD3; a beta-aminoketone) was active against *M. tuberculosis*, exhibiting growth inhibition and bactericidal activity. Overexpression of lepB reduced the susceptibility of *M. tuberculosis* to MD3, and downregulation resulted in increased susceptibility, suggesting that LepB is the true target of MD3. MD3 lead to a rapid loss of viability and cell lysis. Interestingly, the compound had increased potency in nonreplicating cells, causing a reduction in viable cell numbers below the detection limit after 24 h. These data suggest that protein secretion is required to maintain viability under starvation conditions and that secreted proteins play a critical role in generating and surviving the persistent state. We conclude that LepB is a promising novel target for drug discovery in *M. tuberculosis*, since its inhibition results in rapid killing of persistent and replicating organisms.

One-third of the human population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Tuberculosis, an ancient disease, continues to claim over 1 million human lives every year, making it the leading cause of death from a bacterial infection (37). There is a need to understand the complex biology and pathogenic potential of *M. tuberculosis* in order to identify key pathways against which novel therapeutics can be developed (21). Protein secretion is a key cellular process; approximately one-fifth of all bacterial proteins are exported into the extracellular environment, promoting motility, cell wall biosynthesis, chemotaxis, adhesion to host cells, and nutrient uptake (32). In *M. tuberculosis*, over 250 proteins are exported across the cytoplasmic membrane through either type I or type II signal peptidase-mediated mechanisms, and many of those proteins are important in bacterial pathogenesis (2, 35). Exported proteins can remain in the cell wall or be completely released from the cell as true secreted proteins; in *M. tuberculosis*, at least 127 proteins are exported or secreted by the SecA pathway. These include the mycolyl transferases, which play a key role in cell wall biogenesis and are the most abundant proteins found in *M. tuberculosis* culture filtrate (23). A recent comprehensive review summarizes protein secretion pathways in *M. tuberculosis* and their potential as targets for drug development (16).

Type I signal peptidase (SPase I) enzymes are membrane-bound endopeptidases that are responsible for the cleavage of the signal peptide of secreted proteins during membrane translocation via the general secretion (Sec) pathway. Unfolded preproteins with an N-terminal signal sequence are recognized by the SecA ATPase and transported via the SecYE translocation pore (7). Stepwise translocation of the preprotein across the membrane is driven by SecA-mediated ATP hydrolysis (14). After translocation, LepB cleaves the signal peptide from the preprotein, releasing the mature protein into the periplasm (27). The genome of *M. tuberculosis* encodes one protein with sequence similarity to SPase I (Rv2903c/LepB). Transposon site hybridization suggests that lepB is essential for mycobacterial growth (34).

SPase I is an attractive target for the development of novel antituberculosis treatments because (i) it is essential for survival in all bacterial species examined to date, (ii) the active site of the enzyme is located on the extracellular surface of the cytoplasmic membrane, suggesting increased accessibility, (iii) the SPase I utilizes a unique Ser/Lys catalytic dyad active site, allowing the development of highly specific inhibitors, and (iv) bacterial SPases I are distinctly different from eukaryotic SPases, suggesting a promising therapeutic window in humans (27, 36). The most effective class of SPase I inhibitors belong to the group of beta lactam compounds or penem-type inhibitors. Furthermore, SPase inhibition by arylomycin has been studied extensively, and a crystal structure of arylomycin A2 bound to the *Escherichia coli* SPase I protein is available (8, 20, 26). However, the number of SPase I-specific inhibitors remains small.

**MATERIALS AND METHODS**

**Bacterial strains.** *M. tuberculosis* was grown in Middlebrook 7H9 medium containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) supplement (Beckton Dickinson) and 0.05% (wt/vol) Tween 80.

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Address correspondence to T. Parish, tanya.parish@idri.org.

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deletion allele was performed using the primer pair (29, 30). PCR screening to determine the presence of the wild-type or in antisenses followed by selection on sucrose and X-Gal as described previously. DCO strains were isolated by streaking cells onto plates lacking antibiotic. The resulting vector, pNIPPY25G, which carries both resistance markers, was verified by PCR and restriction digestion.

**Construction of in-frame deletion vector and an attempt to construct lepB deletion mutants.** An lepB deletion delivery vector was constructed as follows: primers lepB-upFl (GGG TGC AGG GCA GGT GCC GTG GCC TCC GTG TGT T-3") and lepB-upR1 (5'-GGG CAT GGC GGA GGT GCC TCC GTG TGT T-3") were used to amplify the lepB upstream flanking region (PtrsII and Sphl restriction sites are underlined). The primers lepB-downFl (5'-GGG CAT GGC GGA CGG ATG TTC CGT TCG TG-3") and lepB-downR2 (5'-CGG GTA CGG CGG AGG ATG TCC CGG TAT TGT C-3") were used to amplify the lepB downstream flanking region and were designed to carry Sphl and PstI restriction sites. Fragments were cloned into p2NIL (PtrsII-PstI) using the underlined restriction sites and the gene cassette (lacZa sacB hyg) from pGOAL19 (30) introduced as a PacFl fragment. The resulting vector, pNIPPY25G, which carries both hygromycin and kan resistance markers, was verified by PCR and restriction digestion. A total of 5 µg of UV-treated plasmid DNA (19) was electroporated into M. tuberculosis H37Rv, and single crossover (SCO) transformants were selected with kanamycin, hygromycin, and X-Gal. Double-crossover (DCO) strains were isolated by streaking cells onto plates lacking antibiotics followed by selection on sucrose and X-Gal as described previously (29, 30). PCR screening to determine the presence of the wild-type or deletion allele was performed using the primer pair lepB-C1-fwd (5'-GGG TGC AGT TAA TTA AGC GTG CCT GAC ATG TTC T-3") and lepB-C2-rev (5'-GGA AGC TTA ATT AAG TGG CCA GGT CTT GAT CCT GAT A-3").

**Construction of merodiploid strain and confirmation of lepB essentiality.** An integrating lepB complementation vector pDIAL6 (Plmyc-tetO-upR1 upR2 downR1 downR2 lepB) was constructed by amplifying the Pmyc-tetO promoter (containing a hsp60 promoter) and the lepB gene. The Plmyc-tetO promoter was cloned into p2NIL (PstI-KpnI) using the underlined restriction sites and the gene cassette. The resulting vector, pNIPPY25G, which carries both resistance markers, was verified by PCR and restriction digestion. A total of 5 µg of UV-treated plasmid DNA (19) was electroporated into M. tuberculosis H37Rv, and single crossover (SCO) transformants were selected with kanamycin, hygromycin, and X-Gal. Double-crossover (DCO) strains were isolated by streaking cells onto plates lacking antibiotics followed by selection on sucrose and X-Gal as described previously (29, 30). PCR screening to determine the presence of the wild-type or deletion allele was performed using the primer pair lepB-C1-fwd (5'-GGG TGC AGT TAA TTA AGC GTG CCT GAC ATG TTC T-3") and lepB-C2-rev (5'-GGA AGC TTA ATT AAG TGG CCA GGT CTT GAT CCT GAT A-3").

**Growth curve in the presence of ATc.** Strains were grown to mid-logarithmic phase in 10 ml 7H9-Tw-OADC supplemented with 50 ng/ml PhoA secretion assay. An lepB expression plasmid was constructed by cloning lepB into pSMG3 under the control of the lepB promoter (24) to generate plasmid pOPPY4. The plasmid was electroporated into M. smegmatis mc2719 and mc2757 (6, 28). Alkaline phosphatase activity was measured as previously described (6) with some minor modifications. Briefly, cells were diluted to a theoretical OD590 of 0.005 in 7H9-Tw-OADC. Cells were incubated at 37°C with 250 rpm shaking, harvested, and resuspended in 1× phosphate-buffered saline (PBS), and cells were incubated as standing cultures for 2 weeks at 37°C prior to the addition of 250 µM MD3 (10× MIC), 4 µM rifampin, or 2% DMSO control. For starvation, 10 ml of culture was grown to mid-logarithmic phase, harvested by centrifugation at 10 min at 2,500 × g, and resuspended in 1× phosphate-buffered saline (PBS). For subsequent quantification, 1 ml of cell suspension was plated onto 10 ml of 7H9-Tw-OADC or on Middlebrook 7H10 agar containing 10% (vol/vol) OADC. Mycobacterium smegmatis was grown in LB liquid medium containing 0.05% (wt/vol) Tween 80 or on LB agar. Where appropriate, 50 µg/ml hygromycin, 10 µg/ml gentamicin, 20 µg/ml streptomycin, 20 µg/ml kanamycin, 50 µg/ml 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside (X-Gal), and 5% (wt/vol) sucrose were used.

**RESULTS**

LepB is essential for the growth of *M. tuberculosis* in vitro. We are interested in the role of the type I signal peptide protein in the Sec secretion pathway in *M. tuberculosis*. Type I signal peptides have been the focus of drug discovery efforts for *Staphylococcus epidermidis* and *Staphylococcus aureus* infections since they are essential for bacterial viability in both Gram-positive and Gram-negative bacteria (11, 27, 38). Because of its crucial role in bacterial survival, many bacterial species contain multiple proteins with Spase I function that can complement one another (36). However, annotation of the complete genome indicates that *M. tuberculosis* has only one gene encoding Spase I function (Rv2903c: LepB) (10), suggesting that it should be essential. We wanted to determine if LepB was the sole functional homolog and if it was indeed essential for viability.

We attempted to construct an lepB deletion strain of *M. tuberculosis*.
containing no ATc (triangles), 10 ng/ml ATc (squares), and 100 ng/ml ATc (circles). Open triangles and dotted line represent the Ptet- 

Aerobic growth of M. tuberculosis. Strains of M. tuberculosis were cultured aerobically; cells were inoculated to a starting OD of 0.01 into growth medium containing no ATc (triangles), 10 ng/ml ATc (squares), and 100 ng/ml ATc (circles). Open triangles and dotted line represent the P_{tet}lepB strain without TetR tetracycline repressor (no ATc). Solid symbols represent the P_{tet}lepB strain carrying TetR.

FIG 2

FIG 1 Determination of the essentiality of lepB. (A) Schematic representation showing the chromosomal location of lepB and the region deleted in the suicide delivery vector (pNIPPY25G). (B) DCO strains were generated in the wild-type and merodiploid backgrounds. Southern analysis of DCOs isolated in the merodiploid background is shown. Genomic DNA was digested with Sacl, blotted, and hybridized to an lepB probe. Lanes: 1, size markers; 2, wild type; 3, WT-int strain (lepB_{wt} [lepB_{wt}, gm]); 4, del-int strain (lepB_{Δ} [lepB_{wt}, gm]). The bands for the wild-type, deletion, and complementing (Int) copies of lepB are indicated.
The alkaline phosphatase activity of an fbpA-PhoA fusion protein and a PhoA protein lacking a signal sequence was measured in M. smegmatis wild type and M. smegmatis expressing LepBMTB (POP4). The experiment was carried out in triplicate, and results are given as the mean values and standard deviations.

FIG 3 Increased secretion of FbpA-PhoA fusion by LepBMTB in M. smegmatis. The alkaline phosphatase activity of an fbpA-PhoA fusion protein and a PhoA protein lacking a signal sequence was measured in M. smegmatis wild type and M. smegmatis expressing LepBMTB (POP4). The experiment was carried out in triplicate, and results are given as the mean values and standard deviations.

The resident plasmid was replaced, demonstrating that none of the mutant LepB proteins were functional. We were able to replace the wild-type copy of the gene with the lepB gene carrying a point mutation in the Lys143, an amino acid residue that we predicted was not required for LepB function. These results indicate that the Ser94, Ser96, and Lys174 residues are essential for LepB function.

The SPase I inhibitor MD3 is active against replicating and nonreplicating M. tuberculosis. Since we demonstrated that LepB-mediated protein secretion is essential in M. tuberculosis, this pathway might be a promising target for the development of new antitubercular drugs. To further establish LepB as a suitable target for drug development, we evaluated the effect of a previously described SPase I inhibitor (MD3) (1) on the viability of M. tuberculosis.

We synthesized MD3 and tested it for activity; the compound was active against M. tuberculosis with an MIC99 of 17.7 μM (Table 1). The LepB-overexpressing strain was more resistant to MD3 (MIC99 = 35.4 μM), while the LepB-underexpressing strain was more sensitive to MD3 (MIC = 8.8 μM), confirming on-target activity. Interestingly, MD3 was much less active against M. smegmatis (MIC > 354 μM). As previously reported, E. coli and S. aureus were resistant to at least 35.4 μM (1).

To investigate further the effect of MD3, we determined the kill kinetics of the compound against M. tuberculosis (Fig. 5). In the first 3 days, MD3 had similar kill kinetics to rifampin, one of the frontline antitubercular drugs, resulting in approximately a 2-log kill. However, treatment with MD3 resulted in a more effective kill than rifampin in the later stages (days 3 to 7) with no detectable viable cells after 7 days of treatment. Thus, MD3 has a bactericidal effect on M. tuberculosis under aerobic, replicating conditions. There was no difference in the kill kinetics between the wild-type and lepB-overexpressing strains.

To test the potency of MD3 against nonreplicating bacilli, we looked at kill kinetics against cells placed under complete nutrient starvation (4). Under these conditions, viable cell counts decreased slightly over the course of the experiment in the untreated cells. MD3 caused a rapid reduction in cell numbers under these nonreplicating conditions, with total kill (>4 logs) in 1 day in both wild-type and overexpressing strains. In contrast, rifampin had a much slower kill rate, taking 7 days to reach total kill.

DISCUSSION

SPase I enzymes have a classic catalytic serine-lysine dyad (Fig. 4) (5); bioinformatics analysis of LepB suggested that the catalytic dyad was formed by Lys174 and either Ser94 or Ser96. We expected that this lysine and one of the serine residues would be

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**TABLE 1 MIC99 of MD3 in different bacterial species on solid medium**

**FIG 4 M. tuberculosis lepB protein sequence.** Protein topology was determined using the TOPCONS online resource (3). The conserved regions boxes B to E are indicated in boxes. Lysine residues that were modified by site-directed mutagenesis are marked in bold; serine residues that were modified are marked in bold with a gray background.
essential for functionality but found that all three residues were essential for LepB function in vivo. Based on studies in E. coli, we hypothesize that Ser94 and Lys174 form the catalytic center of the protein, while Ser96 likely stabilizes the interaction with the pre-protein and the catalytic serine residue (25). Further biochemical evaluation and crystallization of the M. tuberculosis LepB are necessary to confirm this hypothesis.

PhoA fusions are favored when investigating protein secretion since PhoA activity is dependent on extracellular localization. The secretion of PhoA fusion proteins that contain signal sequences from M. tuberculosis in M. smegmatis has also been shown (6). We chose the FbpA-PhoA fusion for analysis since signal sequences of all three mycolyl transferases (FbpA, FbpB, FbpC) contain the classic AXA motif, are recognized and cleaved by the SPase I, and are abundantly secreted (23). We observed increased activity of the FbpA signal peptide-PhoA reporter fusion in an M. smegmatis strain expressing M. tuberculosis lepB, demonstrating that it does encode a protein with a role in secretion. PhoA can function as a membrane protein when fused to a noncleavable signal sequence, or in the cell wall or culture filtrate when fused to a cleavable signal peptide which directs release from the membrane via LepB. Although we were unable to distinguish between increased secretion and/or cleavage in our system, it is likely that increased PhoA activity was due to signal peptidase activity, resulting in more efficient release and subsequent folding of PhoA into an active form outside the membrane. The level of increased secretion (2-fold) was significant but low. However, SPase I works in conjunction with the Sec translocation machinery, and it may be that other members of the Sec translocation machinery become limiting in this background when LepB is overexpressed.

To date, only a few compounds are known to inhibit SPase I activity (8, 20, 26), which is in part due to its unique catalytic dyad mechanism making standard serine protease inhibitors ineffective. MD3 is a beta-aminoketone identified as an SPase I inhibitor (1); MD3 was less effective against an LepB-overexpressing strain and more effective against an LepB-underexpressing strain, suggesting that MD3 specifically targets the SPase I in M. tuberculosis. The observed two-fold increase in resistance to MD3 in the overexpressing strains was consistent with our finding of a 2-fold increase in secretion of the FbpA-PhoA reporter fusion in M. smegmatis.

The specificity of MD3 for M. tuberculosis is interesting since M. smegmatis also appears to have a single type I signal peptidase, MSMEG_2441 (http://mycobrowser.epfl.ch/smegmalist.html). The M. smegmatis LepB is 77% identical to the M. tuberculosis LepB, making it unlikely that resistance is due to the lack of a sensitive target or the presence of an alternative functional signal peptidase. There are several possibilities to account for the difference in sensitivity; for example, MD3 may not penetrate through the cell wall as easily, the compound may be inactivated, or the target may be expressed to a much higher level in the nonpathogenic species.

Many compounds which kill M. tuberculosis in aerobic culture are ineffective or have reduced potency against nonreplicating or persistent cells. In contrast, we demonstrated that MD3 has bactericidal activity against both actively growing M. tuberculosis as well as nongrowing cells and in fact was more potent against nonreplicating cells. We did not observe any change in the kill kinetics of MD3 in the LepB-overexpressing strain, although it was 2-fold more resistant to the compound. This is likely due to the excess of compound used in these experiments, which is 10× the MIC. The mode of kill of MD3 is unknown. One possibility is that disruption of LepB activity results in accumulation of nonfunctional proteins (with signal peptides still present) in the cell membrane, as has been shown in other organisms (12). Alternatively cell death could result from the loss of specific secreted proteins, for example, the mycolyl transferases which are required for anchoring of the mycolic acids in the cell wall. There are many proteins in M. tuberculosis with predicted signal peptides which could be the substrate of LepB; of these, approximately 100 are predicted to be both essential and secreted solely through SPase I-dependent processes (23, 34).

The increasing incidence and spread of drug-resistant M. tuberculosis has led to a need for additional drugs affecting new targets and pathways. Current therapeutics target cell wall synthesis, DNA transcription, and protein translation. We are focused on identifying novel mechanisms and pathways essential for bacterial growth and pathogenesis that can be exploited to develop new agents that will be effective against drug-resistant tuberculosis (TB). The best new targets are those that are essential, novel, and distinct from or lack a human homologue. The type I signal peptidase fulfills these criteria for several reasons. The catalytic dyad mechanism of LepB is distinctively different from that of eukaryotes, so it should be possible to generate specific inhibitors. Furthermore, the active site is located on the outside of the cyto-
plasmic membrane, making it relatively accessible for small molecules. To validate LepB as a drug target for the development of new antituberculosis treatment, we confirmed its essentiality in vitro and showed that reduced levels of SPase I in M. tuberculosis result in decreased viability. These data indicate that M. tuberculosis does not have another functional homolog that can substitute for LepB activity, unlike other bacteria with multiple homologues of this key enzyme. Our data provide genetic and chemical validation of LepB as a novel drug target for M. tuberculosis and suggest that compounds active against LepB could help to shorten therapy by targeting replicating and nonreplicating bacteria.

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