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

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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 SecYEG 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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(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.

Construction of in-frame gene deletion vectors and an attempt to construct *lepB* deletion mutants. An *lepB* deletion delivery vector was constructed as follows: primers *lepB*-upF1 (GGC TGC AGG TAG GCA GTT CGC CAA A-3′) and *lepB*-upR1 (5′-CCG CAT GCC GAT GGG GAG TCC GTG GTT T-3′) were used to amplify the *lepB* upstream flanking region (PstI and SphI restriction sites are underlined). The primers *lepB*-downF2 (5′-CCG CAT GCG GTA AGG CCA GGT TGA TCG TGT-3′) and *lepB* downR2 (5′-CCG GTA CCG CCG ACG ATG TCC TTG TAT T-3′) were used to amplify the *lepB* downstream flanking region and were designed to carry SphI and KpnI restriction sites. Fragments were cloned into p2NIL (PstI-KpnI) using the underlined restriction sites and the gene cassette (*lacZ sacB hyg*) from pGOAL19 (30) introduced as a PacI fragment. The resulting vector, pNIPPY25G, which carries both *hyg* and *kan* resistance markers, was verified by PCR and restriction digestion. A total of 5 μg of UV-treated pNIPPY25G 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′-GGC TGC AGT TAA TTA ACG CTG GCT ACG CTG ATC T-3′) and *lepB*C2-rev (5′-GGA AGC TTA ATT AAG TGG CCA GGT CTT GGT CAT A-3′).

Construction of merodiploid strain and confirmation of *lepB* essentiality. An integrating *lepB* complementation vector pDIAL6 ($P_{smyc\text{-}tetO}$-*lepB*) was constructed by amplifying the $P_{smyc\text{-}tetO}$ promoter (containing a 3′ PacI site) from pSE100 (18) and cloning into pGEM-T (Promega). The L5 integration cassette (22) carrying integrase, *attP*, and gentamicin resistance was inserted as an HindIII fragment. The *lepB* coding region was amplified using the C1-fwd/C2-rev primer pair incorporating 5′PstI/PacI and 3′HindIII/PacI sites and cloned as a PacI fragment downstream of $P_{myc1\text{-}tetO}$ in the proper orientation for expression (pDIAL6). pDIAL6 (1 μg) was electroporated into an SCO strain to generate an *lepB* merodiploid. DCOs were selected/screened in the merodiploid background as described before but gentamicin was included to select for the integrated, complementing plasmid.

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 anhydrotetracycline (ATc). Cultures were harvested by centrifugation, washed twice with 7H9-Tw-OADC to remove remaining ATc, and inoculated to a theoretical optical density at 590 nm (OD_{590}) of 0.02 in 12-mm glass tubes containing 5 ml 7H9-Tw-OADC supplemented with 0, 10, or 100 ng/ml ATc. Cultures were incubated at 37°C with stirring at 250 rpm using 2-mm magnetic stir bars.

Site-directed mutagenesis of *lepB*. Site-directed mutagenesis on pDIAL6 was carried out using primer pairs S94A-1 (5′-CCT TAT CTG ATT CCG GCG GAA TCG ATG GAA CCC-3′) and S94A-2 (5′-GGG TTC CAT CGA TTC CGC CGG AAT CAG ATA AGG-3′), S96A-1 (5′-CTG ATT CCG TCG GAA GCG ATG GAA CCC ACG TTG-3′) and S96A-2 (5′-CAA CGT GGG TTC CAT CGC TTC CGA CGG AAT CAG-3′), K143A-1 (5′-TGG AAC GTT GGT TAC GCG TCG ATC CGT TCG CAC-3′) and K143A-2 (5′-GTG CGA ACG GAT CGA CGC GTA ACC AAC GTT CCA-3′), and K174A-1 (5′-GAG AAC GAC CTG GTC GCG CGT GTC ATC GCG GTC-3′) and K174A-2 (5′-GAC CGC GAT GAC ACG CGC GAC CAG GTC GTT CTC-3′). PCR amplification was performed with *Pfu*, 1× *Pfu* buffer, 100 ng/ml primers, 0.5 mM dNTPs, and 10% dimethyl sulfoxide (DMSO). Thermocycling conditions were 95°C for 5 min, followed by 16 cycles of 95°C for 1 min, 55°C for 30 s, 68°C for 9 min, and a final extension at 68°C for 10 min. Template DNA was degraded with DpnI (10U) for 1 h at 37°C. Gene switching was performed as previously described (31); 1 μg of each plasmid was transformed into the del-int strain (chromosomal deletion, integrating wild-type copy of *lepB*). Transformants were selected on hygromycin and subsequently patched onto gentamicin plates to test for sensitivity to confirm that the *gm*-containing plasmid pDIAL6 was switched with the hygromycin resistance-containing plasmid. The positive control for the switch was pDIAL6H, which is pDIAL6 containing a hygromycin resistance gene (*hyg*).

MD3 synthesis. The reference compound 1-(2,5-dichlorophenyl)-3-(dimethylamino)propan-1-one (MD3) was synthesized in 65% yield by the dimethylaminomethylation of (2,5-dichlorophenyl)methylketone with Eschenmoser's salt in hot ethanol (13, 17). Recrystallization of the resulting crude product from acetone gave the title compound as white crystals in 65% overall yield. The product was characterized by liquid chromatography mass spectrometry (LCMS) and proton nuclear magnetic resonance (NMR) analysis.

MIC and kill kinetics. MICs were determined using the agar proportion method (33). Briefly, *M. tuberculosis* strains were grown in 10 ml to mid-logarithmic phase. A total of 10^4 CFU was inoculated onto plates containing 2-fold serial dilutions of MD3 and onto antibiotic-free 7H10 plates to confirm inoculum concentration. Plates were incubated at 37°C, and CFU were scored after 4 weeks. The MIC was defined as the lowest concentration at which fewer than 1% of cells formed colonies (MIC99). Kill curves were carried out under aerobic growth conditions and under nutrient starvation conditions (4). For aerobic kill curves, cells were inoculated to a theoretical OD_{590} of 0.01 into 10 ml of medium containing either 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 for 10 min at 2,500 × *g*, 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.

PhoA secretion assay. An *lepB* expression plasmid was constructed by cloning *lepB* into pSMT3 under the control of the *hsp60* promoter (24) to generate plasmid pOPPY4. The plasmid was electroporated into *M. smegmatis* strains $mc^2$2719 and $mc^2$2757 (6, 28). Alkaline phosphatase activity was measured as previously described (6) with some minor modifications. Briefly, cells were diluted to a theoretical OD_{590} of 0.005 in 7H9-Tw-OADC. Cells were incubated at 37°C with 250-rpm shaking, harvested, and resuspended in 1 M Tris (pH 8.0). The alkaline phosphatase reaction was started by the addition of 0.1 ml of cells to 1 ml of 2 mM *p*-nitrophenyl phosphate in 1 M Tris (pH 8.0) and incubated at 37°C until the development of a yellow color. Reactions were stopped by the addition of 0.1 ml of 1 M K_2HPO_4. Cells were pelleted and OD_{420} and OD_{550} were measured. Alkaline phosphatase units (AP) were calculated using the following formula: $1{,}000 \times (OD_{420} - [1.75 \times OD_{550}])/(OD_{600} \times 0.1)$.

RESULTS

LepB is essential for the growth of *M. tuberculosis in vitro*. We are interested in the role of the type I signal peptidase protein in the Sec secretion pathway in *M. tuberculosis*. Type 1 signal peptidases 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. tuber-*

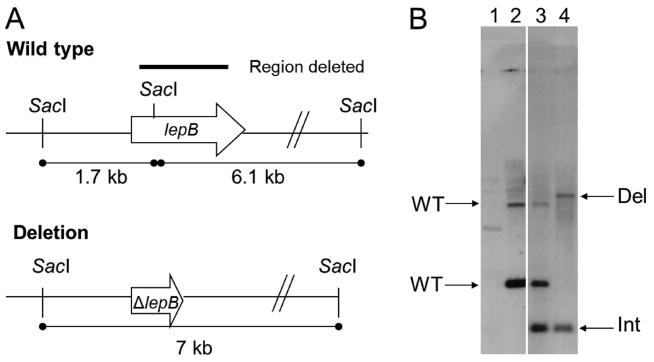

FIG 1 Determination of the essentiality of *lepB*. (A) Schematic representation showing the chromosomal location of *lepB* and the region deleted in the suicide delivery vector (pNIPPY25G). (B) DCO strains were generated in the wild-type and merodiploid backgrounds. Southern analysis of DCOs isolated in the merodiploid background is shown. Genomic DNA was digested with SacI, 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.

culosis by replacing the chromosomal copy with a truncated nonfunctional copy (in-frame, unmarked deletion) using a two-step recombination method (Fig. 1A) (30). The *lepB* deletion delivery vector was transformed into *M. tuberculosis*, and single-crossover strains were generated by homologous recombination; generation of DCOs from this strain could result in either the replacement of the wild-type gene with the deletion copy or a reversion to wild type. We analyzed 40 DCOs; all isolates carried the wild-type allele, suggesting that *lepB* is essential for growth. To confirm essentiality, we isolated DCOs in a merodiploid background, where a functional copy of *lepB* was provided on an integrating vector in the chromosome. In this genetic background, 13/40 DCOs had the deletion allele (Fig. 1B). These data strongly suggest that *lepB* is an essential gene *in vitro* (Fisher's exact test, $P < 0.0001$).

As confirmation that *lepB* is indispensable for *M. tuberculosis* survival, we attempted to replace the integrating vector carrying the functional copy of *lepB* with an empty vector as previously described (31). No isolates in which the resident vector was replaced by the empty vector could be recovered; replacement of the resident vector with a vector carrying a different selection marker (*hyg*) with a functional copy of *lepB* was successful with a frequency of 1.1×10^6 transformants per μg plasmid DNA. Thus, we were unable to recover isolates that had lost the complementing copy of the gene. Taken together, our results demonstrate that *lepB* is essential for growth of *M. tuberculosis* under these conditions.

Since *lepB* was essential in culture, we could not construct a deletion strain for phenotypic studies. Reducing the expression of essential genes often results in *M. tuberculosis* cells that have a reduced viability (23). We generated a strain of *M. tuberculosis* in which the only functional copy of *lepB* was under the control of a tetracycline-responsive promoter ($P_{smyc\text{-}tetO}$-*lepB*) (15); in this strain, the normal chromosomal copy was deleted and the integrated vector carried $P_{smyc\text{-}tetO}$-*lepB*. The TetR repressor was introduced into this strain on a multicopy plasmid to allow for anhydrotetracycline (ATc)-dependent *lepB* expression. Growth of three independent transformants was measured in various concentrations of ATc (Fig. 2); in the presence of 100 ng/ml ATc, growth of all transformants was comparable to that of the wild-type strain. However, in reduced-ATc (10 ng/ml) or no-ATc conditions, growth of two of the three recombinant strains was severely restricted (Fig. 2). One transformant (transformant 2) showed slightly reduced growth at low ATc concentrations, suggesting that the ATc-dependent regulation of *lepB* expression was lost in this strain. Escape mutants are often seen with all of the available tetracycline-regulated promoters used in *M. tuberculosis*, where accumulation of mutation(s) in the promoter/operator region leads to loss of ATc-dependent growth phenotypes (9). However, the clear ATc dependence of two of the three transformants confirms that downregulation of *lepB* expression does reduce the growth of *M. tuberculosis*.

***M. tuberculosis* LepB mediates protein secretion utilizing a Ser-Lys catalytic dyad.** We hypothesize that *lepB* is essential for *M. tuberculosis* growth due to its essential role in protein secretion, but there is no direct biochemical evidence that LepB is a functional signal peptidase, since the protein has not been expressed or purified.

In order to determine if LepB plays a role in protein secretion *in vivo*, we tested whether it could direct the secretion of a PhoA reporter protein. We used a strain of *M. smegmatis* carrying an alkaline phosphatase reporter fusion; this has an *M. tuberculosis* signal peptide fused to the PhoA reporter (6). *M. tuberculosis* LepB was expressed in *M. smegmatis* using the constitutively highly active *hsp60* promoter (24). Secretion of the *fbpA*-PhoA fusion protein was determined by measuring alkaline phosphatase activity

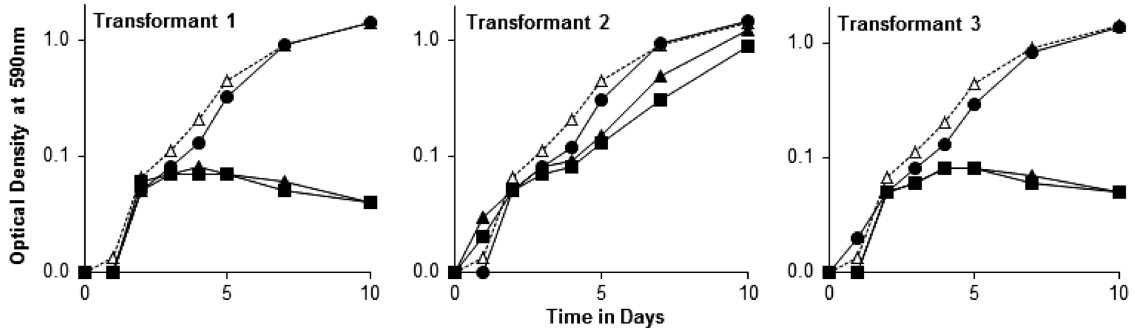

FIG 2 Aerobic growth *of M. tuberculosis.* Strains of *M. tuberculosis* were cultured aerobically; cells were inoculated to a starting OD of 0.01 into growth medium containing no ATc (triangles), 10 ng/ml ATc (squares), and 100 ng/ml ATc (circles). Open triangles and dotted line represent the P_{tet}-*lepB* strain without TetR tetracycline repressor (no ATc). Solid symbols represent the P_{tet}-*lepB* strain carrying TetR.

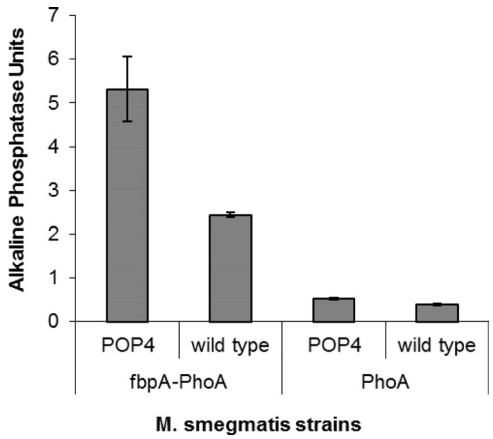

FIG 3 Increased secretion of FbpA-PhoA fusion by $LepB_{MTB}$ in *M. smegmatis*. The alkaline phosphatase activity of an *fbpA*-PhoA fusion protein and a PhoA protein lacking a signal sequence was measured in *M. smegmatis* wild type and *M. smegmatis* expressing $LepB_{MTB}$ (POP4). The experiment was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

(Fig. 3); in the presence of $LepB_{MTB}$ expression, PhoA secretion was 2-fold higher than in the control strain, confirming that there was an increased signal peptidase activity. Secretion of PhoA lacking a signal sequence was very low and was not increased in the presence of $LepB_{MTB}$ expression, confirming that the increased secretion of the PhoA fusion was due to specific activity.

Type I signal peptidases are unusual in that they normally possess a catalytic dyad, as opposed to the catalytic triad of other protease families (5). Amino acid alignments of LepB with SPase I of other bacterial species identified a short intracellular domain and a large extracellular domain containing the conserved regions boxes B, C, D, and E. The predicted catalytically active residues of the characteristic serine-lysine dyad are located in box B and box D (27) (Fig. 4). We carried out site-directed mutagenesis on Ser94, Ser96, and Lys174 and determined which mutant alleles retained functionality by gene switching; we attempted to replace the wild-type *lepB* allele with each of the mutant alleles. Vectors carrying *hyg* and mutant alleles were transformed into the del-int strain carrying the only functional copy of *lepB* on a plasmid conferring gentamicin resistance. We were unable to isolate strains in which

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  1 - VTETTDSPSE RQPGPAEPEL SSRDPDIAGQ VFDAAPFDAA
 41 - PDADSEGDSK AAKTDEPRPA KRSTLREFAV LAVIAVVLYY
 81 - VMLTFVARPY LIPSESMEPT LHGCSTCVGD RIMVDKLSYR
121 - FGSPQPGDVI VFRGPPSWNV GYKSIRSHNV AVRWVQNALS
161 - FIGFVPPDEN DLVKRVIAVG GQTVQCRSDT GLTVNGRPLK
201 - EPYLDPATMM ADPSIYPCLG SEFGPVTVPP GRVWVMGDNR
241 - THSADSRAHC PLLCTDDPLP GTVPVANVIG KARLIVWPPS
281 - RWGVVRSVNP QQGR
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Box B, Box C, Box D, Box E

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.

TABLE 1 MIC_{99} of MD3 in different bacterial species on solid medium

Strain[a]	MIC (μM)
M. tuberculosis H37Rv	17.7
M. tuberculosis LepB-OE	35.4
M. tuberculosis LepB-UE	8.8
M. smegmatis	>354
E. coli	>35.4
S. aureus	>35.4

[a] *M. tuberculosis* LepB-OE is the LepB overexpression strain carrying plasmid pOPPY4 expressing LepB from P_{hsp60}. *M. tuberculosis* LepB-UE is the LepB underexpression strain in which the sole copy of LepB is expressed from $P_{smyc\text{-}tetO}$ and cultured in the absence of ATc (uninduced promoter state).

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 MIC_{99} of 17.7 μM (Table 1). The LepB-overexpressing strain was more resistant to MD3 (MIC_{99} = 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 enyzmes 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

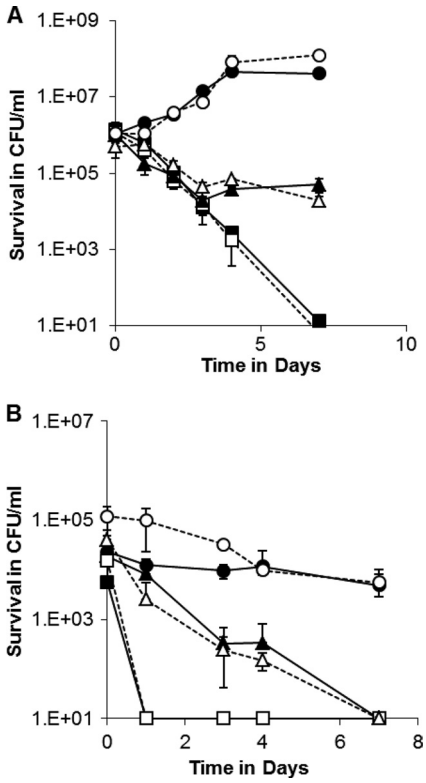

FIG 5 Kill kinetics of MD3 against *M. tuberculosis*. *M. tuberculosis* wild-type (solid line and solid symbols) and LepB-overexpressing strains (dotted line and open symbols) were inoculated from actively growing cultures into 10 ml medium (A) or PBS (B). Compounds were added at 10× MIC: 4 μM rifampin (triangles), 250 μM MD3 (squares), plus a no-compound control with 0.65% DMSO (circles). Cultures were incubated at 37°C standing, and CFU were determined. Results are the means and standard deviations from three independent biological replicates. The limit of detection was 10 CFU/ml.

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 Lys 174 form the catalytic center of the protein, while Ser96 likely stabilizes the interaction with the preprotein 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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