

Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase

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ABSTRACT

Using a component of the *Escherichia coli* protein degradation machinery, we have established a system to regulate protein stability in mycobacteria. A protein tag derived from the *E. coli* SsrA degradation signal did not affect several reporter proteins in wild-type *Mycobacterium smegmatis* or *Mycobacterium tuberculosis*. Expression of the adaptor protein SspB, which recognizes this modified tag and helps deliver tagged proteins to the protease ClpXP, strongly decreased the activities and protein levels of different reporters. This inactivation did not occur when the function of ClpX was inhibited. Using this system, we constructed a conditional *M. smegmatis* knockdown mutant in which addition of anhydrotetracycline (atc) caused depletion of the beta subunit of RNA polymerase, RpoB. The impact of atc on this mutant was dose-dependent. Very low amounts of atc did not prevent growth but increased sensitivity to an antibiotic that inactivates RpoB. Intermediate amounts of RpoB knockdown resulted in bacteriostasis and a more substantial depletion led to a decrease in viability by up to 99%. These studies identify SspB-mediated proteolysis as an efficient approach to conditionally inactivate essential proteins in mycobacteria. They further demonstrate that depletion of RpoB by ~93% is sufficient to cause death of *M. smegmatis*.

INTRODUCTION

Tuberculosis (TB) remains a global health threat and new therapies that can shorten treatment time and cure drug-resistant TB are urgently needed. Drug development

is stalled by the lack of good targets, including proteins required during both active and latent disease and those whose partial inactivation results in death of *Mycobacterium tuberculosis*, the causative agent of TB. Current approaches to the identification and validation of such targets include transcriptional silencing of the gene encoding a potential drug target to determine the vulnerability of the pathogen to target inactivation (1–8). Although it has been successfully applied to *M. tuberculosis*, transcriptional gene silencing does have limitations. The manifestation of phenotypic consequences of silencing can be slow, and stable low-abundance proteins are difficult to inactivate by this approach. Therefore, we set out to establish a system that directly regulates protein stability in mycobacteria.

Targeting bacterial proteins for degradation is achieved by multiple mechanisms, including specific recognition of N- or C-terminal degradation tags (9). One such degradation tag is encoded by *ssrA*, a small stable RNA that can mediate the addition of the amino acids it encodes to the C-terminus of a nascent polypeptide in a process called *trans*-translation (10). In *E. coli*, SsrA-tagged proteins can be degraded by several proteases, including Tsp, HflB, ClpAP, Lon and ClpXP (10). Degradation by ClpXP is specifically enhanced by the adaptor protein SspB, which tethers SsrA-tagged proteins to ClpX (11,12). ClpX and SspB recognize different amino acids of the SsrA tag: ClpX interacts with the three C-terminal residues, whereas SspB binds to the N-terminus (13).

SspB is not essential for degradation of SsrA-tagged proteins by ClpXP in *E. coli*. However, mutating the ClpX-binding region of SsrA weakened the interaction between the tagged protein and ClpX. Degradation of proteins containing such mutated tags, e.g. the so-called DAS+4 tag, was SspB-dependent in *E. coli* (14,15). We expected that this controlled proteolysis system could also function in mycobacteria because (i) ClpX from *E. coli* and mycobacteria are similar, (ii) mycobacterial

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†D.S. dedicates this work to the memory of Prof. Dr Wolfgang Hillen.

genomes do not encode SspB homologs, (iii) the DAS+4 tag differs from mycobacterial SsrA tags and thus is unlikely to be recognized by mycobacterial proteases and (iv) many residues of *E. coli* ClpX required for the interaction with SspB are conserved in mycobacterial ClpX proteins. In accordance with these expectations, we found that *E. coli* SspB can be used to control the stability of DAS+4-tagged proteins in *Mycobacterium smegmatis* and in *M. tuberculosis*. We applied controlled proteolytic inactivation to *M. smegmatis* RpoB, the β subunit of the RNA polymerase (RNAP), and demonstrated that decreasing the steady-state level of this protein mimics the mycobactericidal activities of rifamycins.

MATERIALS AND METHODS

Plasmids, bacterial strains, media and reagents

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Plasmids were constructed using standard procedures (details available upon request). *M. smegmatis* *rpoB-FLAG-DAS+4* was constructed by single-crossover homologous recombination. Candidate clones were confirmed by immunoblots and PCR followed by sequencing. *M. smegmatis* mc²155 and its derivatives were grown in Middlebrook 7H9 medium with 0.5% glycerol and 0.05% Tween 80, or on Middlebrook 7H11 agar with 0.5% glycerol. For *M. tuberculosis*, 7H9 medium was supplemented with 0.5% BSA, 0.2% dextrose and 0.085% NaCl; 7H11 agar was supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (BD). Antibiotics were added, where appropriate, at 50 μ g/ml hygromycin (Calbiochem), 20 μ g/ml kanamycin (IBI Scientific) and 25 μ g/ml nourseothricin (HKI, Jena). Anhydrotetracycline (atc) (Riedel-de Haen) was used at 100 ng/ml, unless noted otherwise. Preparation and electroporation of competent cells were performed as described previously (16).

Fluorescence and luminescence assays

M. smegmatis and *M. tuberculosis* were transformed with the appropriate plasmids and, from each transformation, four to six single colonies were inoculated separately into 1 ml 7H9 medium. After incubating at 37°C for 48–72 h (*M. smegmatis*) or 7–10 days (*M. tuberculosis*), bacteria were diluted 10- to 30-fold in 1 ml fresh medium. Cultures were incubated for another 16–20 h (*M. smegmatis*) or 3–4 days (*M. tuberculosis*). For fluorescence (GFP and RFP) measurements, cultures were concentrated 10-fold in phosphate-buffered saline (128 mM NaCl, 8.5 mM Na₂HPO₄, 3.5 mM KCl, 1.5 mM KH₂PO₄, pH 7.4). 100 μ l of cultures were used for each measurement. Optical densities were measured at 580 nm using a SpectraMax M2 or M5 plate reader (Molecular Devices). Fluorescence was measured after excitation at 400 nm and emission at 490 nm (GFP) or after excitation at 587 nm and emission at 630 nm (mCherry RFP). Luminescence was measured with a SpectraMax L plate reader (Molecular Devices) by injecting 10 μ l of decanal

(1% in ethanol, Sigma). Fluorescence and luminescence were normalized to cell density and reported as relative fluorescence units (RFU) and relative luminescence units (RLU). All measurements were performed at least twice.

Preparation of cell lysates

Bacteria were harvested by centrifugation and washed with PBS with 0.1% Tween 80. Cells were resuspended in 0.5–1 ml of PBS and transferred into a screw-cap vial containing ~250 μ l of 0.1 mm Zirconia/Silica beads (BioSpec). Cells were broken by shaking in a Precellys 24 homogenizer (Bertin Technologies) or in a Mini Bead Beater (BioSpec). Total lysates were obtained after removing beads and unbroken cells by centrifugation at 14 000 rpm twice for 10 min at 4°C.

Immunoblot analysis

Cell lysates were mixed with sodium dodecyl sulfate (SDS) loading buffer and incubated at 100°C for 3 min. Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Whatman) in an ice-cooled container with a constant voltage of 100 V in transfer buffer (192 mM glycine, 25 mM Tris, 1% w/v SDS, 20% v/v methanol) for 1 h. Polyclonal rabbit antibodies against GFP and FLAG were used at 1:5000–7500 and 1:400–1000 dilutions, respectively, in Odyssey[®] Blocking Buffer with 0.1% Tween 80. Polyclonal anti-SspB, anti-DlaT and anti-PrCB rabbit sera were used at 1:5000–10 000 dilutions. Odyssey[®] Infrared Imaging System (LI-COR Biosciences) was used for detection.

CFU analysis of *M. smegmatis* MR-*sspB*

A culture of MR-*sspB* (OD₅₈₀ ~1.0) was diluted into 1 ml fresh 7H9 with 0.5% glycerol, 0.05% Tween 80 and antibiotics in 96-deep-well blocks (Beckman) to an OD₅₈₀ of 0.0005 with atc at concentrations ranging from 0 to 100 ng/ml in quadruplicates. Serial dilutions were spotted on 7H11 agar with or without 100 ng/ml atc prior to incubation (input) and after 1 and 3 days of incubation with shaking. Colonies were counted 4–5 days later. Suppressor mutants were determined from colonies recovered on plates with 100 ng/ml atc and were subtracted from the total number of colonies recovered on plates without atc.

RESULTS

Effects of SsrA tags on the activities of GFP, RFP and LuxAB in *M. smegmatis*

We cloned *gfp-ssrAec* and *gfp-DAS+4*, which differ from *gfp* only in their 3'-ends such that the C-terminal amino acids of GFP-*ssrAec* are identical to those encoded by the *E. coli* *ssrA* RNA (AANDENYALAA) and the C-terminal amino acids of GFP-DAS+4 are those of the DAS+4 tag (AANDENYSENYADAS) (Supplementary Table S2). Episomes that constitutively transcribed these genes were transformed into *M. smegmatis* mc²155 to generate *M. smegmatis* *gfp*, *M. smegmatis* *gfp-ssrAec* and

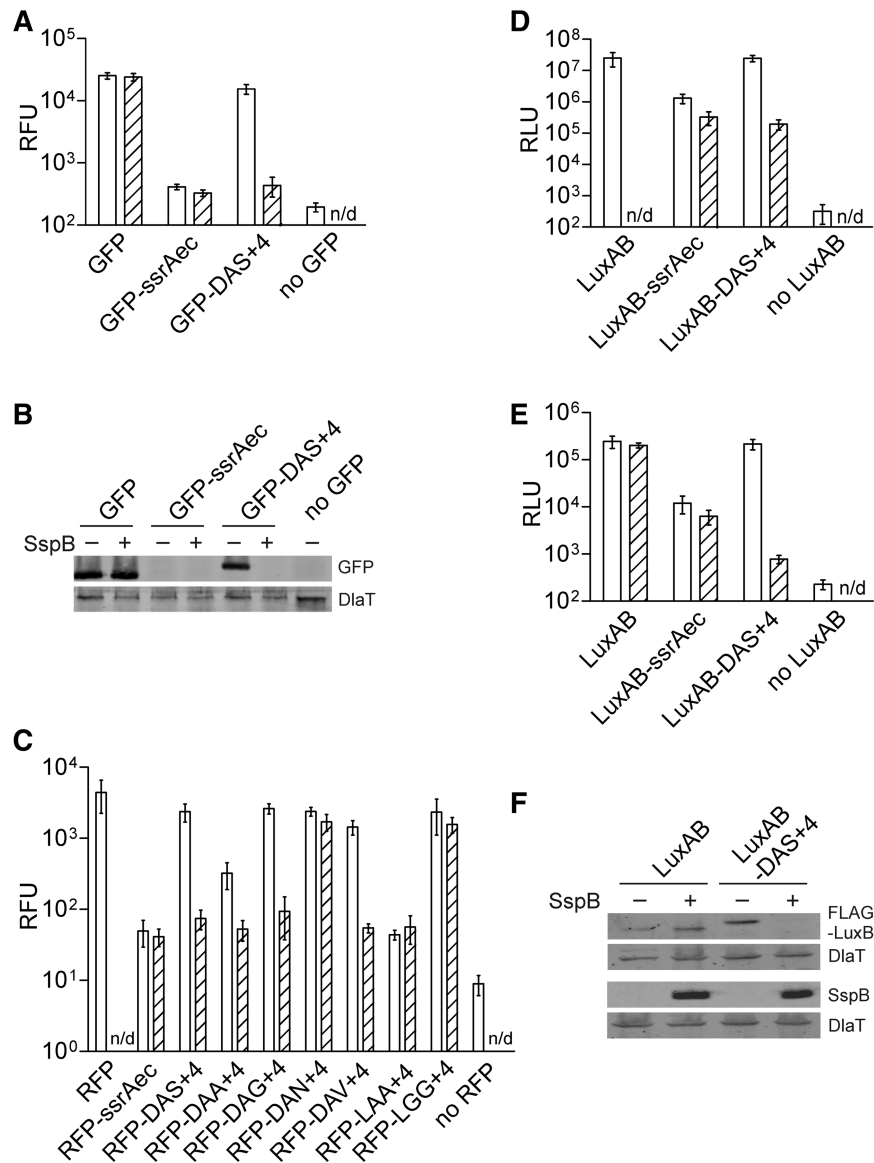


Figure 1. Effect of SsrA tags and *E. coli* SspB on GFP, RFP and luciferase (LuxAB) in *M. smegmatis*. (A) Relative fluorescence units of *M. smegmatis* *gfp*, *M. smegmatis* *gfp-ssrAec* and *M. smegmatis* *gfp-DAS+4*. White bars indicate strains without SspB; hatched bars indicate strains with a constitutively expressed SspB. n/d: not determined. (B) GFP levels detected by immunoblotting in the same strains as in (A). Dihydrolypoamide acyltransferase (DlaT) is used as a loading control. (C) Relative fluorescence units of *M. smegmatis* *rfp*, *M. smegmatis* *rfp-ssrAec*, *M. smegmatis* *rfp-DAS+4*, and six other *M. smegmatis* strains expressing variously tagged RFP. White bars indicate strains without SspB; hatched bars indicate strains with a constitutively expressed SspB. n/d: not determined. (D) Relative luminescence units of *M. smegmatis* *luxAB*, *M. smegmatis* *luxAB-ssrAec* and *M. smegmatis* *luxAB-DAS+4*. White bars indicate strains without SspB; hatched bars indicate strains with a constitutively expressed SspB. n/d: not determined. (E) Relative luminescence units of *M. smegmatis* that chromosomally express *luxAB*, *luxAB-ssrAec* and *luxAB-DAS+4* with or without constitutively expressed SspB. Open bars indicate strains without SspB; hatched bars indicate strains with a constitutively expressed SspB. n/d: not determined. (F) LuxB and SspB levels detected by immunoblotting in the same strains as in (D). DlaT is used as a loading control for each immunoblot. Data in (A,C-E) are means \pm SD of 10–20 replicates from two or three independent experiments.

M. smegmatis *gfp-DAS+4*. *M. smegmatis* *gfp* and *M. smegmatis* *gfp-DAS+4* exhibited strong green fluorescence (white bars, Figure 1A). In contrast, *M. smegmatis* *gfp-ssrAec* did not display any GFP-dependent fluorescence, consistent with previous reports demonstrating destabilization of GFP by the SsrA tag in mycobacteria (17,18). We next cloned *sspB_{myc}* that encodes the *E. coli* SspB protein but whose codon-usage was adapted to facilitate translation in mycobacteria, into an expression

vector that integrates into the attachment site of the mycobacteriophage L5. Constitutive expression of SspB did not affect growth of the bacteria (data not shown), and did not alter green fluorescence of *M. smegmatis* *gfp* but reduced green fluorescence of *M. smegmatis* *gfp-DAS+4* by 97.2%, so that it was indistinguishable from that of *M. smegmatis* *gfp-ssrAec* (hatched bars, Figure 1A). Immunoblot analyses demonstrated that the lack of green fluorescence measured in *M. smegmatis*

gfp-ssrA, with and without SspB, and *M. smegmatis gfp-DAS+4* with SspB were caused by low levels of the respective fluorescent protein (Figure 1B). For mCherry-encoded red fluorescent protein (RFP) (19), we also evaluated additional tags, some of which had improved SspB-dependent regulation of protein degradation in *Bacillus subtilis* (15). However, none of these tags yielded better regulation of RFP activity in *M. smegmatis* than the DAS+4 tag (Figure 1C).

GFP and RFP share little amino acid sequence identity and differ in their oligomeric states but their monomers fold into similar structures (20). To analyze the impact of SspB on a protein functionally and structurally unrelated to GFP and RFP, we used the heterodimeric luciferase encoded by *luxA* and *luxB*. We mutated the 3'-end of *luxB* to encode the SsrA tag or the DAS+4 tag and expressed the different *luxB* genes in operons with *luxA* using episomally replicating plasmids. Without SspB, luminescence of *M. smegmatis luxAB-ssrAec* and *M. smegmatis luxAB-DAS+4* was 5.2 and 98.2%, respectively, of that of *M. smegmatis luxAB* (white bars, Figure 1D). Expression of SspB had little effect on luminescence of *M. smegmatis luxAB-ssrAec*, but decreased luminescence of *M. smegmatis luxAB-DAS+4* by 99.2% (hatched bars, Figure 1D). In a strain that contained *luxAB-DAS+4* integrated into the *M. smegmatis* chromosome, expression of SspB reduced luminescence by 99.6% (Figure 1E). As predicted by luminescence, no LuxB protein was detected in *M. smegmatis* that expressed LuxAB-DAS+4 and SspB by immunoblot (Figure 1F). In summary, these data demonstrate that the DAS+4 tag is not recognized by a native *M. smegmatis* protease and that structurally and functionally unrelated DAS+4-tagged proteins can be inactivated by expression of SspB.

Controlled inactivation of DAS+4-tagged proteins in *M. smegmatis*

We next asked whether inactivation of a DAS+4-tagged protein can be controlled by regulating expression of SspB. For this, we cloned *sspB_{myc}* downstream of the TetR-regulated, anhydrotetracycline (atc)-inducible promoter *P_{myc1}tetO* (21) in a plasmid that also contains *tetR*. Green fluorescence of *M. smegmatis gfp-DAS+4* containing *tetR* and the inducible *sspB* was atc-dependent (Figure 2A). Without atc, green fluorescence was indistinguishable from that of *M. smegmatis gfp-DAS+4* lacking SspB; with atc, it was comparable to that of the strain in which GFP-DAS+4 was inactivated by constitutive expression of SspB (Figure 2A). Similarly complete regulation of SspB-dependent inactivation was measured for RFP-DAS+4 (Figure 2B) and LuxAB-DAS+4 (Figure 2C). We also measured luminescence with different atc concentrations for *luxAB-DAS+4* (Figure 2D), which demonstrated that increasing doses of atc caused decreasing luminescence. Repression of luminescence was maximal with as little as ~1 ng/ml atc. Taken together, these findings demonstrate that transcriptional regulation of *sspB_{myc}* provides an efficient, atc

dose-dependent approach to depleting DAS+4-tagged proteins in *M. smegmatis*.

Inactivation kinetics of GFP-DAS+4 and LuxAB-DAS+4 in *M. smegmatis*

To ascertain the time required for TetR-controlled SspB to be induced and degrade DAS+4-tagged substrates, we measured green fluorescence of *M. smegmatis* containing *gfp-DAS+4*, *tetR* and *P_{myc1}tetO-sspB_{myc}* at different times after addition of atc. Atc caused a time-dependent decrease in green fluorescence that reached half-maximal repression after ~8 h (Figure 3A). Fluorescence remained unchanged without atc. Correspondingly, GFP-DAS+4 protein levels decreased in a time-dependent manner with atc and were stable without atc (Figure 3B). In *M. smegmatis* containing *luxAB-DAS+4*, *tetR* and *P_{myc1}tetO-sspB_{myc}*, atc-induced repression of luminescence reached half-maximal levels after ~1 h (Figure 3C). Immunoblots confirmed that the decrease in luminescence corresponded with a decrease in LuxAB-DAS+4 and an increase in SspB (Figure 3D). This analysis also revealed that inactivation of LuxAB-DAS+4 reached maximal levels at a time (2 h post addition of atc) when the level of SspB was still increasing (data not shown). As in *M. smegmatis gfp-DAS+4* (not shown), expression of SspB did not affect growth of *M. smegmatis luxAB-DAS+4* (Figure 3E). These experiments demonstrate that SspB-dependent inactivation of DAS+4-tagged proteins can be rapid, that it can occur at low SspB concentrations, that the kinetics of inactivation are influenced by the tagged protein, and that expression of SspB and degradation of a dispensable DAS+4-tagged protein do not affect growth of *M. smegmatis*.

Inactivation of LuxAB-DAS+4 by SspB is inhibited by a dominant negative ClpX mutant

ClpX is predicted to be essential for normal growth of *M. tuberculosis* (22) and we expected ClpX also to be essential in *M. smegmatis*. To test this we generated a ClpX derivative, ClpX-K127R, in which the Walker A motif of the ClpX ATP binding domain was mutated. This type of ClpX mutant was dominant negative and allowed to conditionally inactivate ClpX in *Caulobacter crescentus*, in which ClpX is also required for normal growth (23,24). The inactivation of WT ClpX by ClpX derivatives with defective Walker motifs is most likely due to titration of WT ClpX into inactive heterooligomers consisting of WT ClpX and the mutated ClpX (24). We cloned *clpX-K127R* into an atc-inducible expression plasmid that replicates episomally in mycobacteria. *M. smegmatis* transformed with this plasmid was grown in liquid media without atc and then spread on agar plates with and without atc. Atc was applied on a paper disc in the center of the agar plate, which resulted in a zone of growth inhibition surrounding the disc (Figure 4A). Growth of *M. smegmatis clpX-K127R* was normal on plates that contained an atc-free paper disc. Next, we transformed *clpX-K127R* into *M. smegmatis* that constitutively expressed LuxAB-DAS+4 and SspB. Without atc this strain grew normally and showed little luminescence. However,

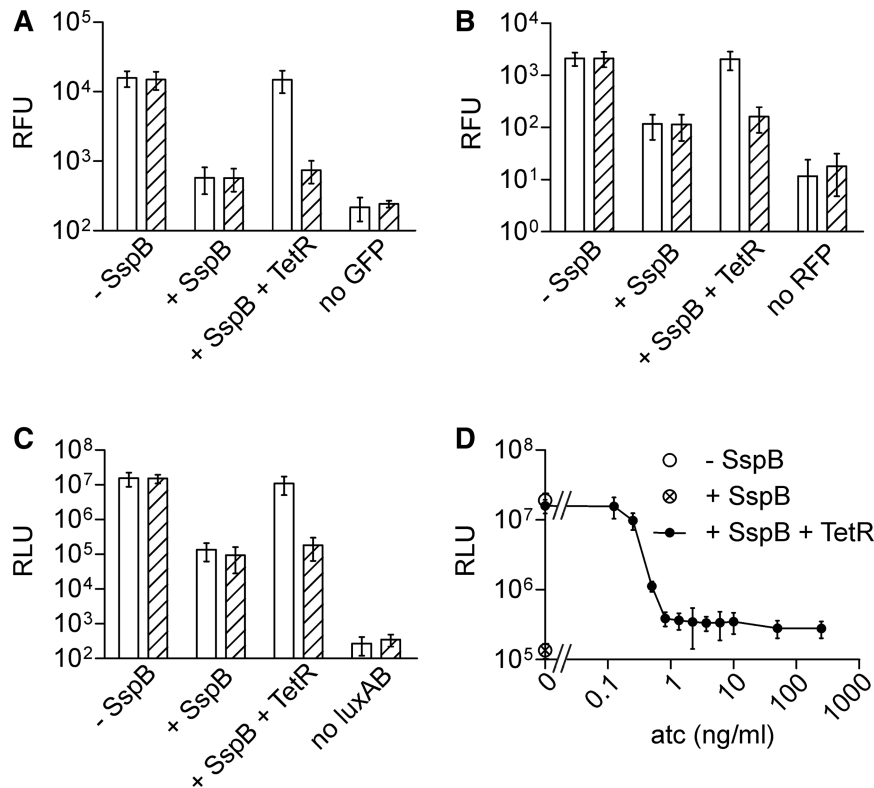


Figure 2. Inducible degradation of DAS+4-tagged proteins by SspB in *M. smegmatis*. (A) Relative fluorescence units of *M. smegmatis gfp-DAS+4* without *sspB*, with constitutively expressed *sspB*, or with *tetR* and TetR-regulated *sspB*. White bars indicate strains cultured in the absence of the inducer anhydrotetracycline (atc); hatched bars indicate those cultured in the presence of atc. (B) Relative fluorescence units of *M. smegmatis rfp-DAS+4* without *sspB*, with constitutively expressed *sspB*, or with *tetR* and TetR-regulated *sspB*. Open bars indicate strains cultured in the absence of anhydrotetracycline (atc); hatched bars indicate those cultured in the presence of atc. (C) Relative luminescence units of *M. smegmatis luxAB-DAS+4* without *sspB*, with constitutively expressed *sspB*, or with *tetR* and TetR-regulated *sspB*. White bars indicate strains cultured in the absence of atc; hatched bars indicate those cultured in the presence of atc. (D) Relative luminescence units of *M. smegmatis luxAB-DAS+4* with *tetR* and TetR-regulated *sspB* cultured with different concentrations of atc. *M. smegmatis luxAB-DAS+4* without *sspB* and *M. smegmatis luxAB-DAS+4* with constitutively expressed *sspB* cultured in the absence of atc serve as controls. Data are means \pm SD of 10–20 replicates from two or three independent experiments.

luciferase activity was restored in a dose-dependent manner when the bacteria were cultivated with increasing concentrations of atc (Figure 4B). At a concentration of 12 ng/ml atc, luminescence values approached that of *M. smegmatis* expressing LuxAB-DAS+4 without SspB. We could not test the impact of higher atc concentrations on the luminescence of strains expressing ClpX-K127R due to the atc-induced growth defect. Over-expression of WT ClpX did not affect growth (not shown) or luminescence of any of the *M. smegmatis* strains analyzed (Figure 4B). These data demonstrate that high level expression of a dominant negative ClpX mutant prevents growth of *M. smegmatis* and that lower expression interferes with the SspB-mediated inactivation of a DAS+4-tagged protein. ClpX is thus likely essential for growth of *M. smegmatis* and the activity of SspB in *M. smegmatis* is, as in *E. coli*, ClpX-dependent.

Controlled inactivation of DAS+4-tagged proteins in *M. tuberculosis*

We used GFP and RFP with the DAS+4 tag and SspB to determine if we could extend this system to

M. tuberculosis. Compared to *M. tuberculosis gfp*, green fluorescence of *M. tuberculosis gfp-ssrAec* was reduced by 98.8%, with and without SspB, and indistinguishable from that of *M. tuberculosis* without GFP (Figure 5A). In accordance with this lack of fluorescence, no GFP-ssrAec was detected by immunoblotting (Figure 5B, lanes 3 and 4). Green fluorescence of *M. tuberculosis gfp-DAS+4* was slightly reduced compared to that of *M. tuberculosis gfp* without *sspB*, but reduced by 94.2% with a constitutively transcribed *sspB* (Figure 5A). Expression of SspB also decreased the level of GFP-DAS+4 below that detectable in immunoblots (Figure 5B, lanes 5 and 6). We next analyzed *M. tuberculosis* containing *gfp-DAS+4*, *tetR* and the inducible *sspB*. Green fluorescence of this strain with atc varied more than that of the corresponding *M. smegmatis* strain, but decreased by an average of 86.3% in response to atc (Figure 5C). We furthermore measured red fluorescence of *M. tuberculosis* strains expressing the different RFPs analyzed in *M. smegmatis* (Figure 5D), and found that the activity of each RFP without SspB and the extent of inactivation achieved by coexpression of SspB were similar in *M. smegmatis* and *M. tuberculosis* (Figure 5E).

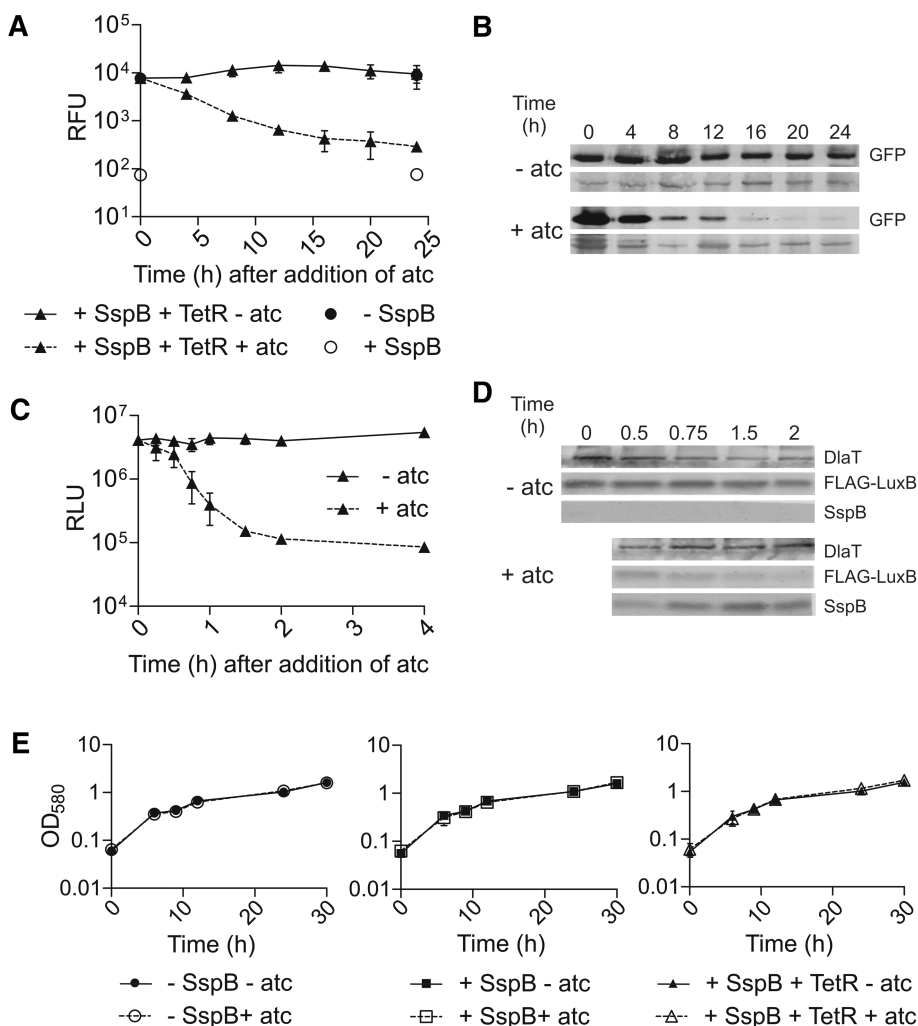


Figure 3. Kinetics of GFP-DAS+4 and LuxAB-DAS+4 degradation in *M. smegmatis*. (A) *M. smegmatis gfp-DAS+4*, *tetR* and TetR-regulated *sspB* were grown to an optical density of 0.2. Each half of the volume was then cultured without (solid line) or with (dashed line) 100 ng/ml atc. Relative fluorescence units were determined at the indicated time points. *M. smegmatis gfp-DAS+4* without *sspB* (closed circles) and with constitutively expressed *sspB* (open circles) cultured in the absence of atc and measured at 0 and 24 h serve as controls. (B) GFP levels detected by immunoblotting from cultures in (A). A non-specific band recognized by the anti-GFP antibody serves as a loading control for each immunoblot. (C) Relative luminescence units of *M. smegmatis luxAB-DAS+4*, *tetR* and TetR-regulated *sspB* in the absence (solid line) and presence (dashed line) of 100 ng/ml atc. LuxB used in these experiments contained an N-terminal FLAG tag for immunoblotting. (D) LuxB and SspB levels detected by immunoblotting from cultures in (C). DlaT serves as a loading control for each immunoblot. (E) *M. smegmatis luxAB-DAS+4* without *sspB* (left panel), with constitutively expressed *sspB* (middle panel), and with *tetR* and TetR-regulated *sspB* (right panel) were inoculated to an optical density of 0.02 without (closed figures) or with 100 ng/ml atc (open figures). Optical density was measured at the indicated time points. Data in (A), (C) and (E) are means \pm SD of four replicate cultures. The four replicate cultures in (A) and (C) were pooled to prepare lysates for immunoblotting.

In summary, these analyses indicate that the regulated expression of SspB allows controlling stability of DAS+4-tagged proteins also in *M. tuberculosis*.

Controlled inactivation of RpoB in *M. smegmatis*

An important application of transcriptional gene silencing systems is the construction of conditional knockdown mutants (1–8). We selected *rpoB*, which encodes the β subunit of RNAP, as the target to evaluate SspB-mediated proteolysis for this application. We altered the 3'-end of *rpoB* in the chromosome to encode the FLAG epitope (DYKDDDDK) followed by the

DAS+4 tag. This modification did not affect growth, compared to wild-type *M. smegmatis* (data not shown). Several transformations of *M. smegmatis rpoB-FLAG-DAS+4* with a constitutive SspB expression plasmid did not result in colonies even though expression of SspB did not affect growth of *M. smegmatis mc*²155. We then constructed two derivatives of *M. smegmatis rpoB-FLAG-DAS+4*, one that contained an atc-inducible SspB expression plasmid (*MR-sspB*) and one that contained a control vector without SspB (*MR-control*). We first checked the atc-induced growth defect on solid media. For this we inoculated the left half of agar plates with *MR-control* and the right half with *MR-sspB* and

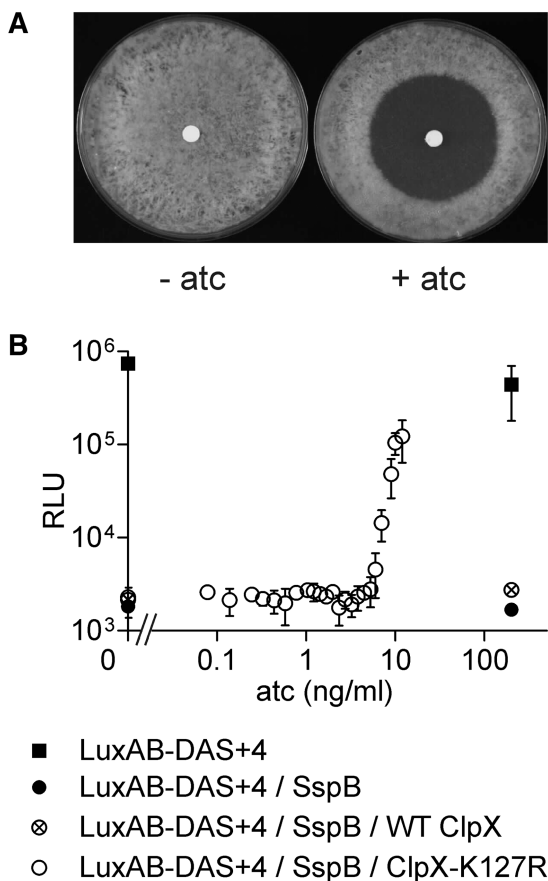


Figure 4. Involvement of ClpX in SspB-mediated degradation of LuxAB-DAS+4. (A) *M. smegmatis luxAB-DAS+4* was transformed with an episomally replicating plasmid that expresses ClpX-K127R under the control of a TetR-regulated promoter. Bacteria in logarithmic growth were incubated at 37°C for 3 days on 7H11 agar with an atc-free (left) or an atc-containing paper disc (right). (B) Relative luminescence units of *M. smegmatis luxAB-DAS+4* with constitutively expressed *sspB* and either a control plasmid (closed circles), TetR-regulated WT *clpX* (crossed circles) or TetR-regulated dominant-negative *clpX* (open circles) were cultured with different concentrations of atc. *M. smegmatis luxAB-DAS+4* without *sspB* (squares) served as control for constitutive luciferase activity. Data are means \pm SD of four or eight replicate cultures.

placed paper discs containing different amounts of atc in the center. An atc concentration-dependent zone of inhibition was visible only for *MR-sspB* and not for *MR-control* (Figure 6A). As on solid media, growth of *MR-control* in liquid media was not influenced by atc (Figure 6B). Growth of *MR-sspB* in liquid media without atc was similar to that of *MR-control*, but growth was strongly suppressed with atc. This growth defect correlated with a reduction in the protein level of the tagged RpoB. After incubating *MR-sspB* with atc for 24 h, the level of RpoB-FLAG-DAS+4 decreased to \sim 7% of the level found without atc (Figure 6C). We next asked if the growth defect of *MR-sspB* could be complemented with WT RpoB. We cloned *rpoB* including its native promoter region into a plasmid that integrates in the attachment site of the phage L5 and transformed it into *MR-sspB* to generate *MR-sspB-comp*. Growth of *MR-sspB-comp* was

not affected by atc on agar plates (not shown) or in liquid culture (Figure 6B). A derivative of *MR-control* that also expressed a second copy of *rpoB* grew normally under all conditions tested. In summary, these experiments demonstrated that combining DAS+4-tagging and regulated expression of SspB provides a straightforward approach to constructing a conditional *M. smegmatis* knockdown mutant in which addition of atc inactivates an essential protein. That the growth defect of *MR-sspB* was fully complemented by a WT copy of *rpoB* demonstrated that the cause of the growth defect was depletion of RpoB and not, for example, the generation of inhibitory degradation products.

Proteolytic inactivation of RpoB is bactericidal for *M. smegmatis*

RpoB is the target of rifamycins, an important class of antibiotics that includes the first-line tuberculosis drug rifampin (25). To determine if sensitivity of *M. smegmatis* to rifampin increased in response to partial inactivation of RpoB, we measured the growth of *MR-sspB* with different rifampin and atc concentrations. We observed an increase in activity of rifampin against *MR-sspB* at low atc concentrations on plates and in liquid culture (Figure 7A and B). In contrast, low concentrations of atc did not change the activity of other antibiotics, e.g. isoniazid (data not shown). Inhibition of RNAP inhibitors by different antibiotics can be bacteriostatic or bactericidal (26). We therefore asked if depletion of RpoB is sufficient to reduce viability of *M. smegmatis*. For this, *MR-sspB* was cultured in media containing 24 atc concentrations, and CFUs of quadruplicates were determined after different periods of incubation. Input was determined for each atc concentration \sim 15 min after inoculation. Similar CFUs were recovered from each atc concentration at this point (Figure 7C). At day 1 and more so on day 3 post inoculation, CFUs increased in cultures containing \leq 0.18 ng/ml atc. In contrast, atc concentrations between 0.22 and 1.31 ng/ml caused apparent bacteriostasis and \geq 1.64 ng/ml atc reduced CFUs recovered from both time points. Whereas the decrease in CFUs was minor after 1 day, CFUs were reduced to below the limit of detection (\sim 1% of input) after 3 days. Thus, the impact of proteolytically silencing RpoB on growth and survival of *M. smegmatis* was atc dose-dependent and mimicked the bactericidal activity of rifampin at high atc concentrations.

DISCUSSION

The *E. coli* SsrA tag caused efficient inactivation of GFP, RFP and luciferase in *M. smegmatis*; and GFP and RFP in *M. tuberculosis*. This is consistent with findings in *E. coli* where the direct interaction of ClpX with the three C-terminal amino acids of the SsrA tag (LAA) is sufficient to target a protein for degradation (13). DAS+4-tagging did not alter, or only marginally altered, the activities and steady-state levels of GFP, RFP and LuxAB in mycobacteria in the absence of SspB. Induction of SspB with atc rapidly decreased activities

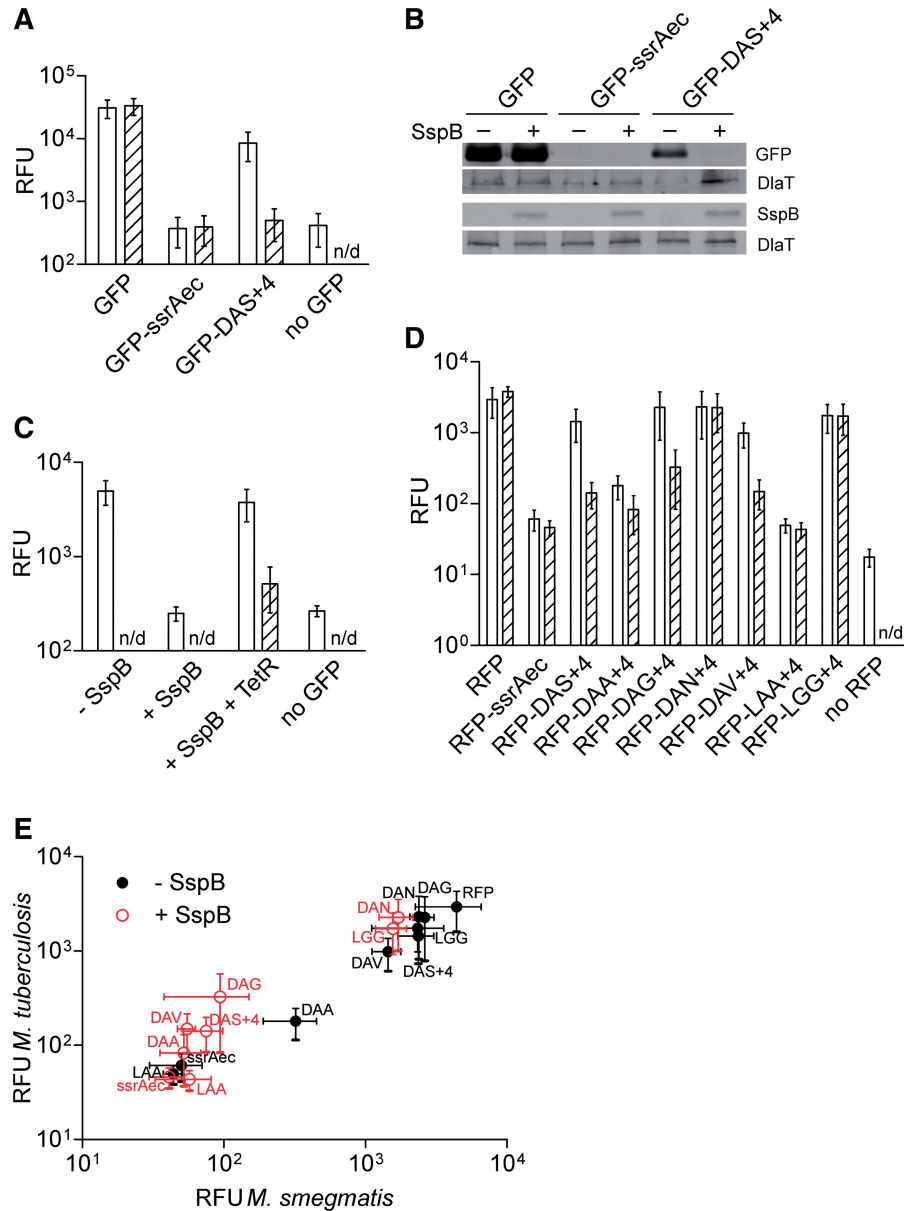


Figure 5. Effect of constitutive and TetR-regulated SspB on GFP-DAS+4 in *M. tuberculosis*. (A) Relative fluorescence units of *M. tuberculosis gfp*, *M. tuberculosis gfp-ssrAec* and *M. tuberculosis gfp-DAS+4*. White bars indicate strains without SspB; hatched bars indicate strains with a constitutively expressed SspB. n/d: not determined. (B) GFP and SspB levels detected by immunoblotting in the same strains as (A). Dlat is used as a loading control for each immunoblot. (C) Relative fluorescence units of *M. smegmatis gfp-DAS+4* without *sspB*, with constitutively expressed *sspB*, or with *tetR* and TetR-regulated *sspB*. White bars indicate strains cultured in the absence of atc; hatched bars indicate those cultured in the presence of atc. (D) Relative fluorescence units of *M. tuberculosis* expressing untagged or variously tagged RFP, as in Figure 1C. (E) Comparison of relative fluorescence units of *M. smegmatis* and *M. tuberculosis* expressing RFP with different tags as in Figures 1C and 4D. Closed black circles indicate strains without SspB; open red circles indicate those with constitutively expressed SspB. A linear relationship is observed between RFU of *M. smegmatis* expressing a tagged RFP (x-axis) and RFU of *M. tuberculosis* expressing the same tagged RFP (y-axis). Data in (A), (C) and (D) are means \pm SD of 12–20 replicates from two or three independent experiments.

and steady-state levels of the DAS+4-tagged reporters to levels observed by constitutively expressing SspB. Little, if any, degradation occurred without atc. Low concentrations of atc (~1 ng/ml) were adequate for maximal levels of proteolysis when analyzed with tagged luciferase, suggesting that a low concentration of SspB was sufficient for inactivation.

In *E. coli* and *B. subtilis* *clpX* is not essential, which allowed using *clpX* deletion mutants to demonstrate that

SspB-mediated degradation of tagged proteins is dependent on ClpX (14,15). In contrast, *clpX* is predicted to be essential in *M. tuberculosis* (22) and we expected it to also be essential in *M. smegmatis*. A ClpX variant with a mutated ATP binding site was dominant negative over WT ClpX of *C. crescentus* and was applied to study the consequences of inactivating ClpX in this organism (23,24). We therefore constructed ClpX-K127R, in which the essential lysine of the Walker A motif was

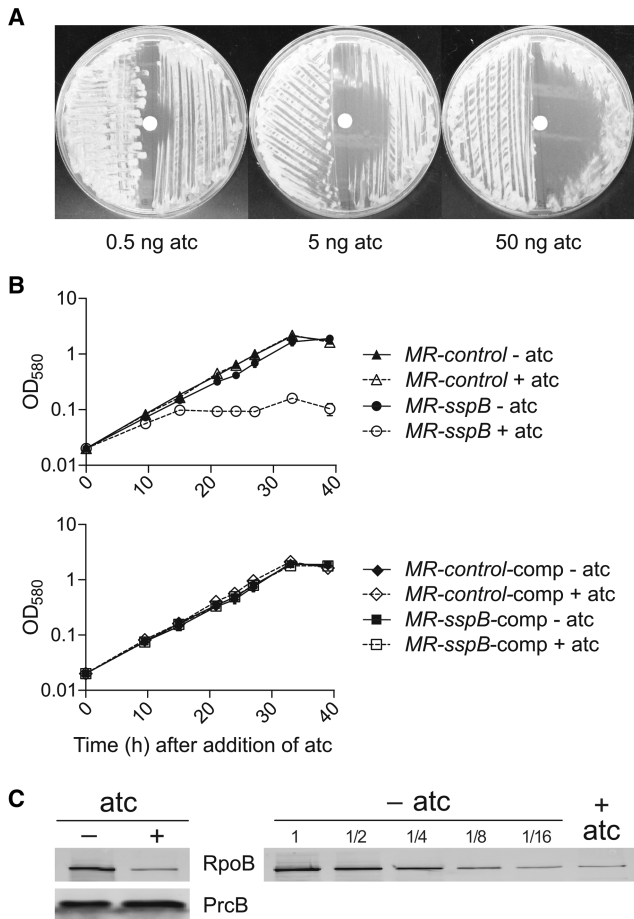


Figure 6. Controlled inactivation of RpoB in *M. smegmatis*. (A) *M. smegmatis* in which the 3'-end of the chromosomal *rpoB* gene encodes a FLAG epitope and the DAS+4 tag was transformed with a plasmid that integrates *tetR* and TetR-regulated *sspB* into the chromosome (*MR-sspB*) or a control plasmid that does not contain TetR or SspB (*MR-control*). *MR-control* (left half) and *MR-sspB* (right half) in logarithmic growth were plated on 7H11 agar with antibiotics. Atc (0.5, 5 or 50 ng) was added to a sterile disc in the center of the plate. Plates were incubated at 37°C for 2 days. (B) Top: *MR-control* (triangles) and *MR-sspB* (circles) were inoculated to an optical density of 0.02 in media without (closed symbols) or with (open symbols) 100 ng/ml atc. Bottom: *MR-control* complemented with untagged *rpoB* (diamonds) and *MR-sspB* complemented with untagged *rpoB* (squares) were inoculated to an optical density of 0.02 in media without (closed symbols) or with (open symbols) 100 ng/ml atc. Optical density was measured at the indicated time points. (C) Left: RpoB levels detected by immunoblotting with anti-FLAG antibody from *MR-sspB* cultured with or without 100 ng/ml atc for 24 h. Proteasome β subunit (PrcB) serves as a loading control. Right: two-fold dilutions of the protein lysate from *MR-sspB* - atc cultures were loaded in comparison to that from *MR-sspB* + atc for semi-quantification. Data in (B) are means \pm SD of four replicate cultures.

replaced by arginine, to determine the importance of ClpX for SspB-mediated inactivation of DAS+4-tagged proteins in *M. smegmatis*. As expected, over-expression of ClpX-K127R inhibited growth of *M. smegmatis*. Induction of ClpX-K127R to levels that did not prevent growth restored luminescence in an *M. smegmatis* strain that constitutively expressed LuxAB-DAS+4 and SspB. This strain had little LuxAB activity in the absence of

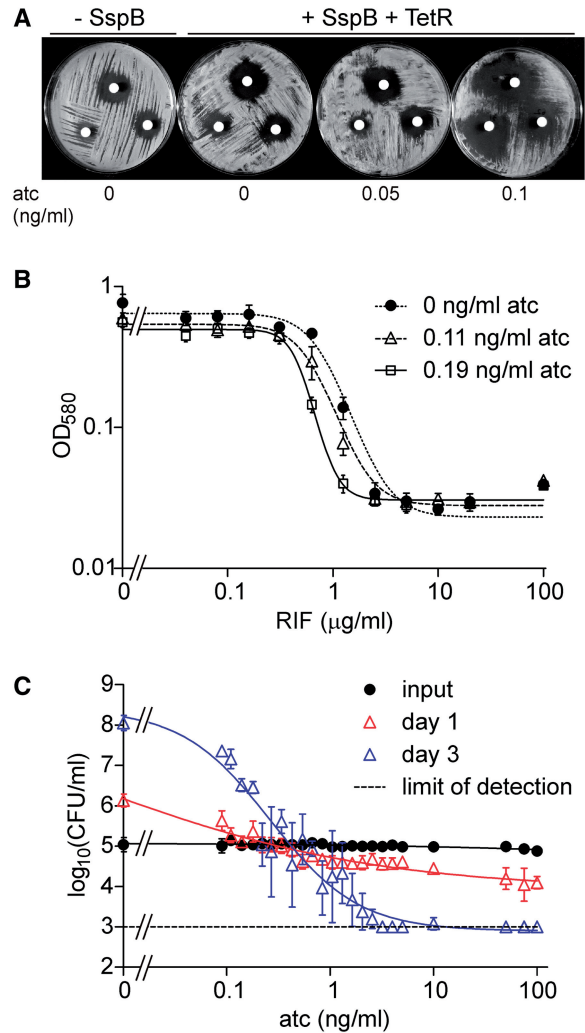


Figure 7. Susceptibility of *M. smegmatis* *MR-sspB* to rifampin and atc. (A) *MR-sspB* in logarithmic growth was plated on 7H11 agar containing 0, 0.05 and 0.1 ng/ml atc (second to fourth plates). Clockwise from the top, 125, 62.5 and 31.25 μ g of rifampin were placed on three sterile discs on the plate. Plates were incubated at 37°C for 2–3 days. *MR-control* on 7H11 agar containing no atc (first plate) was also incubated with rifampin as a control. (B) *MR-sspB* in logarithmic growth was diluted to an optical density of 0.05 in 7H9 containing 0–0.19 ng/ml atc and incubated at 37°C with shaking for 2–3 days. After the pre-incubation with atc only, cultures were diluted to an optical density of 0.05 in 7H9 with the same atc concentrations but also containing 0–100 μ g/ml rifampin. Optical density was measured after 4 days of incubation. Data are means \pm SD of four replicates. (C) CFU recovered from *MR-sspB* after 0, 1 and 3 days in 0–100 ng/ml atc. Data are means \pm SD of four replicate cultures.

ClpX-K127R. The inactivation of DAS+4-tagged proteins is thus ClpX-dependent and, as in *E. coli*, likely mediated by the delivery of tagged proteins to ClpXP by SspB. This interpretation is also supported by sequence similarities between the ClpX proteins of *E. coli*, *M. smegmatis* and *M. tuberculosis*. This includes 84% identical amino acids in the 26 amino acid zinc-binding motif of *E. coli* ClpX ZBD, which is required for the

interaction with SspB (27,28). Furthermore, the *E. coli* ClpX residues F¹⁶, A²⁹ and Y³⁴, which are important for binding SspB (28), are conserved in ClpX from *M. smegmatis* and *M. tuberculosis*.

We applied SspB-mediated proteolysis to study RpoB for two reasons. First, RNAP is required for bacterial growth and targeting *rpoB* allowed us to test the feasibility of using *in situ* DAS+4-tagging and SspB-mediated proteolysis to study essential genes in mycobacteria. Second, RpoB is the target of several antibiotics, which differ in their mechanism of RNAP inhibition and their impact on bacterial viability. For example, the rifamycins, which are often bactericidal (25,26,29–31), have no effect on RNAP once it has elongated past the promoter. Instead, rifamycins specifically interfere with an early step during transcription initiation and prevent synthesis and retention of RNAs that are longer than 2 or 3 nts (25,26,32,33). Other RNAP inhibitors, like streptolydigin, inhibit initiation, elongation and pyrophosphorolysis by bacterial RNAP but are thought to only have bacteriostatic effects (26,34–36). These data suggest that a specific mechanism of RNAP inhibition, which in the case of rifamycins includes repeated cycles of abortive transcription initiation, might be required to achieve bactericidal activity. We were therefore interested in determining if inactivation of RNAP by RpoB depletion was sufficient to cause death of *M. smegmatis*.

Addition of the DAS+4 tag to RpoB did not impair growth of *M. smegmatis* without SspB. Induction of SspB with atc decreased RpoB-DAS+4 protein levels by ~93% and prevented growth of *M. smegmatis* in an atc dose-dependent manner. However, when an additional, untagged copy of *rpoB* was expressed, the atc-dependent growth inhibition no longer occurred, indicating that the growth defect was a result of RpoB depletion. Very low concentrations of atc (0.19 ng/ml or less) did not impair growth of the RpoB knockdown strain, but increased its susceptibility to rifampin. This increase in rifampin activity was moderate, because only small decreases in the steady-state level of RpoB were tolerated before degradation of RpoB by itself prevented growth. Atc concentrations of 10 ng/ml or higher not only prevented growth but consistently reduced CFUs demonstrating that depletion of RpoB was sufficient to cause death of *M. smegmatis*. This strongly suggests that the specific mechanism by which rifamycins inhibit RNAP activity is not required to achieve killing of *M. smegmatis*. Instead, any potent inhibitor of mycobacterial RNAP will likely be mycobactericidal. The extent to which CFUs decreased after addition of atc increased over time and reached maximal levels days after growth had stopped. This indicates that inactivation of RpoB was not immediately bactericidal and suggests that secondary events induced by the inhibition of transcription were required to cause death. Some mechanisms by which drug-induced secondary effects can kill *E. coli* have recently been identified (37–40). Whether these mechanisms are relevant to killing of *M. smegmatis* after RpoB inactivation remains to be determined.

In contrast to the controlled proteolytic inactivation strategy established here, transcriptional silencing has

been used to study the function of mycobacterial genes for some time (1–8). The main advantage of transcriptional silencing is that it does not require mutating the targeted protein, whereas addition of a peptide tag is required to control protein stability. Peptide tags are, however, routinely used to facilitate purification and/or detection of proteins, which suggests that many proteins will tolerate the DAS+4-tag. An advantage of controlled proteolysis is that it leaves the native transcriptional regulation of a target unperturbed. Controlled proteolysis also actively reduces the concentration of a target protein and does not depend on cell division for target depletion, which is required in the case of stable proteins after transcriptional repression of the encoding gene. Neither transcriptional silencing nor controlled proteolysis will be universally successful. However, they might provide complementary approaches best suited for different targets. Transcriptional silencing generally inactivates highly expressed genes efficiently because, for such genes, the leakiness intrinsic to regulated promoters does not prevent a large reduction in expression of the encoded protein. In contrast, proteolysis might be most efficient for proteins whose native steady state levels are low and thus can be efficiently eliminated by proteases. Thus, regulated proteolytic degradation represents a novel approach that should facilitate the identification and validation of targets for tuberculosis drug development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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