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Phosphorylation of KasB Regulates Virulence and Acid-Fastness in *Mycobacterium tuberculosis*

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Abstract

*Mycobacterium tuberculosis* bacilli display two signature features: acid-fast staining and the capacity to induce long-term latent infections in humans. However, the mechanisms governing these two important processes remain largely unknown. Ser/Thr phosphorylation has recently emerged as an important regulatory mechanism allowing mycobacteria to adapt to their cell wall structure/composition in response to their environment. Herein, we evaluated whether phosphorylation of KasB, a crucial mycolic acid biosynthetic enzyme, could modulate acid-fast staining and virulence. Tandem mass spectrometry and site-directed mutagenesis revealed that phosphorylation of KasB occurred at Thr334 and Thr336 both in *in vitro* and in mycobacteria. Isogenic strains of *M. tuberculosis* with either a deletion of the kasB gene or a *kasB*₃₃₄₃₃₆₃₃₄₃₃₆ allele, mimicking constitutive phosphorylation of KasB, were constructed by specialized linkage transduction. Biochemical and structural analyses comparing these mutants to the parental strain revealed that both mutant strains had mycolic acids that were shortened by 4–6 carbon atoms and lacked *trans*-cyclopropanation. Together, these results suggested that in *M. tuberculosis*, phosphorylation profoundly decreases the condensing activity of KasB. Structural/modeling analyses reveal that Thr334 and Thr336 are located in the vicinity of the catalytic triad, which indicates that phosphorylation of these amino acids would result in loss of enzyme activity. Importantly, the *kasB*₃₃₄₃₃₆ allele, *kasB*₃₃₄₃₃₆ phosphomimetic and deletion alleles, in contrast to the *kasB*₃₃₄₃₃₆ phosphoablative allele, completely lost acid-fast staining. Moreover, assessing the virulence of these strains indicated that the KasB phosphomimetic mutant was attenuated in both immunodeficient and immunocompetent mice following aerosol infection. This attenuation was characterized by the absence of lung pathology. Overall, these results highlight for the first time the role of Ser/Thr kinase-dependent KasB phosphorylation in regulating the later stages of mycolic acid elongation, with important consequences in terms of acid-fast staining and pathogenicity.

Introduction

*Mycobacterium tuberculosis* (*Mtb*) is an extraordinarily versatile pathogen that can exist in two distinct states in the host, leading to asymptomatic latent infection in which bacilli are present in a non-replicating dormant form, or to active tuberculosis (TB), characterized by actively replicating organisms. Establishment of these different (patho)physiological states requires mechanisms to sense a wide range of environmental signals and to coordinately regulate multiple metabolic and cellular processes. Many of the stimuli encountered by *Mtb* are transduced via transmembrane sensor kinases, allowing the pathogen to adapt to survive in hostile environments. In addition to the 12 classical two-component systems [1], *Mtb* contains 11 eukaryotic-like Ser/Thr protein kinases (STPK) [2,3], suggesting that these two phospho-based signaling systems are of comparable importance in this microorganism. Knowledge of the substrates of each of the *Mtb* STPK is essential for understanding their function. Several kinase-substrate pairs have been identified and characterized during the last decade. In addition, a recent comprehensive understanding of in
**Author Summary**

Acid-fast staining has been used since 1882 as the hallmark diagnostic test for detecting *Mycobacterium tuberculosis*, the causative agent of tuberculosis. It has been attributed to the presence of a waxy cell envelope, and primarily to its key components, mycolic acids. Here, we report a new mechanism of regulation in which phosphorylation of KasB, involved in the completion of full-length mycolic acids, leads to shortened mycolic acids and loss of acid-fast staining. Moreover, a *M. tuberculosis* mutant strain mimicking constitutive phosphorylation of KasB is severely attenuated for growth in both immunocompetent and immunosuppressed mice and fails to cause mortality and pathophysiological symptoms. These results emphasize the critical role of kinase-dependent phosphorylation in the pathogenesis of *M. tuberculosis* by controlling the mycolic acid chain length. Our study demonstrates the importance of a regulatory mechanism governing acid-fastness and virulence of *M. tuberculosis*.

In vitro phosphorylation event in *Mtb* was gained using a mass spectrometry-based approach to identify phosphorylation sites in *Mtb* proteins [4]. This provided insights into the range of functions regulated by Ser/Thr phosphorylation, underpinning the involvement of many STPK in regulating metabolic processes, transport of metabolites, cell division or virulence [5,6,7].

Recent studies focusing on mycolic acid biosynthesis regulation have shown that most essential enzymes forming the central core of type II fatty acid synthase (FASII) are phosphorylated by STPK [6] and that, at least in *in vitro*, post-translational phosphorylation inhibits the activity of these enzymes. These include the beta-ketoacyl ACP synthase KasA, the beta-ketoacyl-ACP reductase MabA, the hydroxyacyl-ACP dehydratases HadAB and HadBC and the enoyl-ACP reductase InhA [8,9,10,11,12]. These studies culminated with the demonstration that InhA, also known as the primary target of the first-line anti-TB drug isoniazid (INH), is controlled by phosphorylation by STPK on Thr266 both *in vitro* and *in vivo* [10,11]. The physiological relevance of Thr266 phosphorylation was demonstrated using inhA-phosphoablative (T266A) or phosphomimetic (T266D/E) mutant strains. Not only was the enoylreductase activity severely impaired in the mimetic mutants *in vitro*, but introduction of *inhA* T266D/E failed to complement an *inhA*-thermosensitive *M. smegmatis* strain, in agreement with mycolic acid inhibition, in a manner similar to that by isoniazid, and growth inhibition [10]. Altogether these results strongly suggest that *Mtb* may control in a very subtle manner its FASII system by regulating each step of the elongation cycle. Since phosphorylation of HadAB and HadBC enzymes was found to be increased during stationary growth phase, it was proposed that mycobacteria shut down meromycolic acid chain production under non-replicating conditions, a view which is supported by the fact that the mycolic acid biosynthesis is growth phase-dependent and is not functional during the stationary phase [13]. However, whether phosphorylation of FASII components may also directly participate in *Mtb* virulence, through the control of the meromycolic acid chain length has not been reported yet.

It was previously demonstrated that targeted deletion of *kasB*, one of two *Mtb* genes encoding distinct beta-ketoacyl-ACP synthases, results in loss of acid-fast staining and synthesis of shorter mycolates [14]. Perhaps the most striking effect of *kasB* deletion was the ability of the mutant strain to persist in infected immunocompetent mice without causing disease or mortality. This indicated that KasB participates in the latest elongation steps by adding the last few carbon atoms to the growing acyl-ACP chains and plays a critical role in controlling *Mtb* physiopathology. From these data, it could be inferred that KasB activity may be tightly regulated in order to control the mycolic acid chain length during the infection process. We have previously reported that, like KasA, KasB was a substrate of STPK [8], suggesting that phosphorylation may represent a mechanism governing KasB activity and, as a consequence, mycolic acid chain length and *Mtb* virulence.

This prompted us to decipher an original mechanism linking post-translational phosphorylation of KasB with *Mtb* virulence in a mouse infection model. Herein, we demonstrate that phosphorylation of KasB on Thr334 and Thr336 dramatically alters the mycolic acid chain length and acid-fast staining. Importantly, a KasB phosphomimetic mutant of *Mtb* was found to be extremely attenuated in mice infection models. These results provide, for the first time, insights into the contribution and importance of FASII phosphorylation in vivo in the control of i) the clinically important feature of acid-fast staining in *Mtb* and ii) the physiopathology of TB.

**Results**

KasB is phosphorylated *in vivo* and *in vitro* on Thr334 and Thr336

Previous work demonstrated that KasB is a substrate for several Ser/Thr protein kinases with PknF being one of the most efficient kinase [8]. However, the role and contribution of KasB phosphorylation with respect to the *Mtb* physiology and pathogenicity remains unknown, mainly because of the lack of information regarding the identity of the phosphoacceptors. Therefore, recombinant wild-type KasB (unphosphorylated form) was expressed and purified from *E. coli* harboring pETPhos_kasB and used in an *in vitro* kinase assay in the presence of PknF and [γ-32P]ATP. The reaction mixture was then separated by SDS-PAGE and analyzed by autoradiography, revealing a specific band corresponding to the phosphorylated form of KasB (Fig. 1A), as reported previously [8]. To identify the number and nature of the phosphosites, the protein was phosphorylated *in vitro* with PknF and cold ATP and subjected to mass spectrometry analysis after tryptic and chymotryptic digestions, a method successfully used to elucidate the phosphoacceptors in a sequence-specific fashion for several other *Mtb* STPK substrates [7,9,10,15,16,17]. Spectral identification and phosphorylation determination were achieved with the paragon algorithm from the 2.0 database-searching software (Applied Biosystems) using the phosphorylation emphasis criterion against a homemade database that included the sequences of KasB and derivatives. The sequence coverage was 92% with the non-covered sequence free of serine or threonine residues. Phosphorylation was detected only on a single peptide 315AIQLAGLAPGDIDHVNAHATGTQVDLGAEGR345. The MS/MS spectra unambiguously confirmed the presence of two phosphate groups on this peptide (data not shown). The 315–345 peptide possesses only two Thr residues representing the potential phosphoacceptors, Thr334 and Thr336. Definitive identification of the phosphosites was achieved by site-directed mutagenesis, by replacing Thr with Ala, preventing subsequent phosphorylation. The single (T334A, T336A) and double (T334A/T336A) mutants were expressed *via* the pETPhos, purified as His-tagged proteins and individually subjected to the kinase assay. As shown in Fig. 1A, decrease of the phosphorylation signal was only partially limited in the single mutants with respect to the wild-type protein. However, phosphorylation was abrogated in the double mutant as evidenced by the absence of a specific radioactive band, confirming that, in...
Figure 1. *M. tuberculosis* KasB is phosphorylated on Thr334 and Thr336. (A) *In vitro* phosphorylation of KasB with PknF. The PknF kinase encoded by the *Mtb* genome was expressed and purified as a GST fusion and incubated with purified His-tagged KasB_WT, KasB_T334A, KasB_T336A and KasB_T334A/T336A in the presence of radiolabeled [γ-32P]ATP. Samples were separated by SDS-PAGE, stained with Coomassie Blue and visualized by autoradiography after overnight exposure to a film as indicated. Upper bands reflect the autophosphorylation activity of PknF whereas the lower bands correspond to the phosphorylation signal of KasB. (B) *In vivo* phosphorylation of KasB. A *kasB* deletion mutant of *M. bovis* BCG was transformed with either pVV16_kasB_WT or pVV16_kasB_T334A/T336A and grown in Sauton medium. Exponential (expo) or stationary (stat) phase cells were harvested, lysed and processed for KasB purification by affinity chromatography on Ni2+-containing beads. The BCG-derived KasB_WT and KasB_T334A/T336A proteins were separated by SDS-PAGE, either stained with Coomassie Blue (upper panel) or subjected to Western blot analysis after probing the membrane with anti-phosphoThreonine antibodies (lower panel). Specificity of phosphothreonine recognition was checked by probing the antibodies against recombinant KasB produced in *E. coli* strains carrying either pETPhos_kasB (non phosphorylated KasB) or pETDuet_kasB that co-expresses KasB with PknF (phosphorylated KasB). (C) Localization of Thr334 and Thr336 phospho-sites in the three-dimensional structure of KasB. Overall view (left panel) showing the KasB dimeric structure ([19]; PDB entry 2GP6) in ribbon representation with the core domain in *marine* and the cap domain in *orange*. The second chain of the dimer is in *light gray*. The Cys-His-His catalytic triad and the two phospho-sites are displayed as ball-and-stick with carbon atoms in *magenta* and *green*, respectively. Also shown with carbon atoms in *yellow* are the TLM inhibitor and the PEG molecule as observed in the structure of the KasA C171Q acyl enzyme mimic ([20]; PDB entry 2WGG). The PEG molecule is thought to delineate the acyl-binding channel [20]. Nitrogens are in *blue*, oxygens in *red*, and sulfur in *gold*. Close-up view (right panel) after a 45° rotation of the left panel along a vertical axis. Side-chains of residues delineating the active site hydrophobic tunnel and of aspartic residues at positions 334 and 336 were also represented (carbons in *cyan* and *white*, respectively).

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**KasB Phosphorylation Impairs Mtb Virulence**

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phosphorylation of KasB occurs at Thr334 and Thr336. Similar results were obtained when KasB_T334A/T336A was incubated in the presence of either PknA, PknB, PknD, PknH or PknL, indicating that these two residues are the phosphoacceptor for all six kinases (Figure S1 in Text S1).

In vitro phosphorylation of KasB was next investigated in recombinant M. bovis BCG by Western blotting using antiphosphothreonine antibodies. Specificity of the antibodies was first assessed against KasB purified from either E. coli (pETPhos_kasB) or E. coli co-expressing PknF (pETDuet_kasd), thanks to a recently developed dual strategy [18]. Phosphorylated KasB derived from pETDuet_kasd was specifically revealed with antiphosphothreonine antibodies, while the unphosphorylated isoform from pETPhos_kasd failed to react (Fig. 1B), confirming the specificity of Thr phosphorylation. To confirm the phosphorylation status of KasB in mycobacteria, and in order to exclude eventual interference/association between the endogenous and the recombinant His-tagged KasB monomers (KasB being a dimer, see below), a ΔkasB BCG mutant was transformed with pVV1::kasB_WT or pVV16::kasB_T334A/T336A in which the wild-type or phosphoablative kasB genes were placed under the control of the hsp60 promoter. BCG carrying either pVV16::kasB_WT or pVV16::kasB_T334A/T336A was harvested from exponential or stationary cultures prior to protein purification by affinity chromatography on Ni²⁺-containing agarose beads and Western blotting using phosphothreonine antibodies. Specific phosphorylation was only detected for the wild-type but not the T334A/T336A protein (Fig. 1B). The lack of reactivity with the double Ala mutant excludes the possibility of additional phosphorylation sites. Interestingly, phosphorylation of KasB was more pronounced during stationary phase than exponentially-growing conditions, indicating that KasB phosphorylation is growth phase-dependent. Taken collectively, these data suggest that phosphorylation occurs at Thr334 and Thr336, in vitro and in vivo.

Thr334 and Thr336 are in the vicinity of the catalytic triad

The crystal structure of KasB (438 residues, MW 46.4 kDa) in its apo-form has been determined to 2.4 Å resolution [19]. It consists of a dimer with each protomer adopting the typical thiolase fold decorated with specific structural features in the form of a cap (Fig. 1C). The structures of wild-type KasA (416 residues, MW 43.3 kDa), the other fatty acyl elongation kasA genes were placed under the control of the hsp60 promoter. BCG carrying either pVV16::kasB_WT or pVV16::kasB_T334A/T336A was harvested from exponential or stationary cultures prior to protein purification by affinity chromatography on Ni²⁺-containing agarose beads and Western blotting using phosphothreonine antibodies. Specific phosphorylation was only detected for the wild-type but not the T334A/T336A protein (Fig. 1B). The lack of reactivity with the double Ala mutant excludes the possibility of additional phosphorylation sites. Interestingly, phosphorylation of KasB was more pronounced during stationary phase than exponentially-growing conditions, indicating that KasB phosphorylation is growth phase-dependent. Taken collectively, these data suggest that phosphorylation occurs at Thr334 and Thr336, in vitro and in vivo.

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Loss of acid-fast staining in a M. tuberculosis phosphomimetic mutant

To study the effect of the two KasB phosphorylation sites in Mtb, the Thr334 and Thr336 amino acids were replaced either with phosphomimetic (aspartate) or phosphoablative (alanine) amino acid. Previous studies have shown that acidic residues such as aspartate qualitatively recapitulate the effect of phosphorylation with regard to functional activity [15,17,21,22]. Specialized linkage transduction [23] was used to transfer single point mutant alleles, respectively kasB T334D/T336D and kasB T334A/T336A in Mtb CDC1551 (Table S1 in Text S1, Fig. 2A). These strains contained a sacB and hgy cassettes inserted between kasB and accD6. The introduction of the sacB and hgy cassettes was confirmed by Southern blot (Fig. 2B) and presence of the point mutation(s) was verified by sequencing kasB. An additional kasB deletion strain in Mtb CDC1551 was constructed using the same plasmid (pYUB1471) as the one used for the allelic exchanges.

Quantitative reverse transcription real-time PCR analyses were conducted to measure the kasB expression level in the different mutant strains. Expression levels were standardized using the sigA internal standard (Fig. 2C). As expected, no specific kasB mRNA was produced in the ΔkasB mutant. In contrast, kasB expression levels were found to be comparable in the parental strain, the double Ala and the double Asp mutants. Similar results were obtained when using either 16S rRNA or mnap1 as alternative internal control (data not shown). These results were confirmed by Western blot analysis using antibodies raised against KasA, which cross-react with KasB, and revealing comparable levels of KasB expression in the parental strain, the double Ala and the double Asp mutants (Fig. 2D). Moreover, since kasB belong to the fasII operon and is located upstream of accD6, we also checked whether the introduction of the T/A or T/D replacements may affect expression of the downstream accD6 gene. Immunoblotting using rabbit anti-AccD6 antibodies clearly revealed the presence of similar AccD6 levels in all strains (Fig. 2E). From these data it can be inferred that mutations in kasB did not exert a polar effect on AccD6 expression.

Together, these results indicate that introduction of the phosphoablative or phosphomimetic mutations does not affect kasB gene expression in Mtb, thus allowing analysis and comparison of the phenotypes associated to these mutations.

The first phenotype tested in the KasB mutants was acid-fastness using both the carbol-fuchsin and auramine stainings (Fig. 3A). Like the parental strain, the Ala mutant remained acid-fast positive. In sharp contrast, the phosphomimetic T334D/T336D mutant strain behaved like the kasB deletion mutant, losing its ability to retain the primary stain following washing with the acid-alcohol decolorizer. The phosphomimetic and deletion strains regained acid-fast staining when a wild-type copy of kasB was introduced into these strains on a multicopy plasmid (Fig. 3B).
Figure 2. Construction of isogenic *M. tuberculosis* CDC1551 strains bearing the phosphoablative or phosphomimetic *kasB* alleles. (A) Schematic representation of the specialized transduction phage. A replicating shuttle phasmid derivative of phAE159 containing *kasB* carrying the mutations T336A/T336A or T334D/T336D, *sacB*, *hyg* resistance cassette, and the first 959 bp of *accD6* was used to transduce *Mtb* CDC1551.
This indicates that phosphorylation on Thr334/Thr336 abrogated the acid-fast property, presumably by negatively regulating the activity of KasB. The results support, for the first time, a control of acid-fastness by STPK-dependent signaling in Mtb.

Phosphorylation of KasB results in shorter mycolic acids

The lack of acid-fastness prompted us to investigate the mycolic acid content in the various strains. Since kasB deletion results in a strain producing shorter mycolic acids [14], we compared the mycolic acid profiles of the double Ala and double Asp mutants to the parental and ΔkasB strains. Mycolic acids were extracted after saponification from the strains grown to stationary phase and analyzed by thin-layer chromatography (TLC) (Fig. 4A). When separated, the mycolates of the parental and phosphoablative strains presented similar mobility shifts, whereas those from the phosphomimetic and the ΔkasB strains were found to be slightly retarded. This lower mobility shift has been earlier reported to occur in M. marinum and Mtb kasB mutants and correlated to reduced carbon chain lengths [14,24]. The normal mobility shift was restored in the corresponding complemented strains (Fig. 4A).

Individual mycolic acid species from all four strains were then purified from preparative TLC plates, re-extracted and analyzed by proton nuclear magnetic resonance (1H NMR) spectroscopy (Fig. 4B and Figure S3 in Text S1) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Fig. 4C). 1H NMR analyses revealed that mycolates from the ΔkasB and KasB T334D/T336D strains significantly differed from those of the parental strain. In particular, the relative quantification of signals at −0.33 and 0.55 ppm and signal at 0.45 ppm established that the ratio of cis-trans-cyclopropanation changed in oxygenated mycolic acids, as reported for the ΔkasB mutant [14]. For methoxy-mycolic acids, it decreased from 20% of trans-cyclopropanation in parental strain to 0% in the ΔkasB and KasB T334D/T336D strains (Figure S3B in Text S1) and for keto-mycolic acids with 50% to 0% (Figure S3C in Text S1 and Fig. 4B). In contrast, mycolates from the phosphoablative mutant exhibited spectra similar to those of the parental strain (data not shown).

Next, the mycolic acid size distribution was assessed by MALDI-TOF MS, and similarly to the 1H NMR analysis, the double Ala mutant was found to produce mycolates identical to those from the parental strain. In contrast, the double Asp mutant, like ΔkasB, synthesized shorter δ- and methoxy-, and keto-mycolic acids (up to C80:2, up to C84:1, and up to C85:1) than the parental strain (up to C84:2, up to C89:1, and up to C89:1), and accumulated a trans-unsaturated precursor of both methoxy- and keto-mycolic acids, thus confirming the slightly reduced TLC mobility shift. The average size reduction of oxygenated mycolates was higher than that of δ-mycolates because it resulted from both size reduction of aliphatic chain and loss of the -CH3 group of trans-cyclopropane groups (Fig. 4C).

Lack of trans-cyclopropanation does not result from altered cmaA2 expression

NMR analysis indicates the lack of trans-cyclopropane rings in oxygenated mycolic acids in the phosphomimetic mutant, which accumulates the corresponding trans-double bonds precursors, as found in the ΔkasB strain and in agreement with previous work [14]. Trans-double bonds have been reported to be the substrates of the trans-cyclopropane synthase CmaA2 [25]. We reasoned that lack of trans-cyclopropanation in the phosphomimetic strain may be due to the shortened oxygenated meromycolates that are poor substrates for CmaA2. Alternatively, alteration/lack of cmaA2 expression may occur in this particular strain. Indeed, multiple direct interactions have been reported to happen between various FASII enzymes in specialized multifunctional complexes [26,27]. Consequently, phosphorylation of KasB may hinder/prevent association of KasB with other partners, including CmaA2. cmaA2 expression levels were therefore measured and found to be comparable in all four strains (Fig. 2C), indicating that neither phosphomimetic mutations within KasB nor deletion of kasB altered cmaA2 expression. Similar results were obtained using sigH, 16S rRNA or mtpA1 as internal standards (data not shown). This implies that absence of trans-cyclopropane rings is likely due to the shortened mycolates that are poor substrate for CmaA2, rather than to a defect in cmaA2 expression.

Phosphorylation of KasB leads to increased cell wall permeability

Given the effect of KasB phosphorylation on the meromycolate chain length, we inquired whether shorter mycolic acids would alter drug susceptibility. The KasB strains were tested for growth in the presence of INH, ethionamide (ETH), and rifampicin (RIF) (Table 1). The KasB T334D/T336D mutant was found to be more susceptible to INH, ETH, and RIF, suggesting that this strain exhibits a cell wall permeability defect.

To confirm the permeability defect of this mutant in logarithmic phase, we compared how the phosphomimetic, phosphoablative, deletion and parental strains incorporated two different types of dye; the cyanine dye 3,3′-diethyloxacarbocyanine iodide DiOC2, which penetrates all cells, and the SYTOX Red dead cell stain, which is excluded from cells with intact membranes [28,29]. The highest amount of Sytox Red was found in the KasB phosphomimetic strain (Figure S4 in Text S1), suggesting that this strain had the most permeable membrane. This set of data support the view of a permeability defect in the KasB phosphomimetic strain.

Reduced pathogenicity and mortality of the KasB phosphomimetic strain

Growth curves of Mtb bearing either the kasB T334A/T336A or the kasB T334D/T336D allele were also similar, indicating that the phosphomimetic or phosphoablative mutations did not impact
Figure 3. Acid-fast staining of *M. tuberculosis* kasB isogenic mutant strains. (A) Cultures were fixed on glass slides and acid-fast staining was performed on the fixed smears using either the BD TB Auramine Kit or the BD Carbolfuchsin kit. The left panels show phase contrast microscopy images of *Mtb* CDC1551 parental strain and the phosphomimetic and phosphoablative *Mtb* isogenic strains. (B) Restoration of the acid-fast staining phenotype in the ΔkasB and phosphomimetic KasB mutant strains complemented with pMV261::kasB. Magnification = 100×. doi:10.1371/journal.ppat.1004115.g003
Figure 4. Structural analysis of mycolic acids in the phosphomimetic and phosphoablative kasB mutants. (A) Mycolic acid profile of the various KasB mutants and complemented strains. Culture were grown at 37°C, harvested, and FAMEs and MAMEs were extracted and analyzed by one-dimensional TLC using hexane/ethyl acetate (19:1, v/v; 3 runs). α-, methoxy- and keto-mycolic acids were revealed by spraying the plate with molybdophosphoric acid followed by charring. Mycolates migrating slightly faster in the Asp mutants than in the parental control strain can be observed. “C” indicates complemented strain (with pMV261::kasB). (B) Relative proportions of cis- and trans-cyclopropanes in mycolates established from 1H NMR spectra. The relative quantification of specific signals associated to trans- and cis-cyclopropanes revealed that oxygenated mycolates synthesized by the KasB T334D/T336D and ΔkasB strains exclusively contain cis-cyclopropane rings. The % of trans-cyclopropanes for each mycolic acid sub-species and for each strain is indicated. (C) MALDI-MS analysis. Mass spectrometry analysis revealed that all three families of mycolates isolated from the KasB T334D/T336D and ΔkasB strains display reduced sizes compared to the parental or phosphoablative strains.

Table 1. MICs of various antitubercular drugs.

| MIC (µg/l) | Parental | T334A/T336A | T334D/T336D | ΔkasB |
|-----------|----------|-------------|-------------|-------|
| INH       | 0.04–0.06| 0.04–0.06   | 0.015–0.03  | 0.02–0.03 |
| ETH       | 1–2.5    | 1–2.5       | 0.25–0.3    | 1.25–2.5 |
| RIF       | 0.03–0.06| 0.016–0.06  | 0.008–0.015 | 0.002–0.007 |

Range of MICs (µg/ml) obtained in two or three independent experiments is shown.

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Consistent with previous observations, the growth for the first three weeks following a similar kinetic (Fig. 6A). Infection indicated that both the parental and double Ala mutant aerosol and assessed for both survival and bacterial replication. 

Attenuated for growth in mice [14]. In contrast to the KasB_T334A/T336A, mice revealed multifocal, moderate infiltration after 21 days of infection. Histological examination of stained lung sections from infected C57BL/6 mice in two independent aerosol infection experiments. 

Eight weeks after infection, whereas replication of the KasB_T334D/T336D mutant was also severely attenuated for growth in mice infected with the parental strain, and to a lesser extent in the liver and spleen at different time points after infection indicated that the KasB phosphomimetic mutant was extremely attenuated, as expected, was attenuated for growth in macrophages, with day 6 CFU counts similar to day 0 numbers, versus an approximately one log increase for the parental strain. In contrast, the KasB phosphomimetic mutant strain exhibited a growth defect, albeit less pronounced than the KasB_T334D/T336D strain (Fig. 5B). 

Extensive granulomatous inflammation was visible in the lungs of SCID mice infected with Mtb KasB_T334D/T336D about 30-fold lower than the parental strain-infected mice. The bacterial burden of the KasB KasB_T334A/T336A strain was comparable to that of the parental strain. Consistent with previously published data [14], the KasB strain exhibited a growth defect, albeit less pronounced than the KasB KasB_T334D/T336D strain (Fig. 5B). 

Increased polyacylated trehalose levels in the KasB phosphomimetic mutant and the KasB_T334D/T336D mutant strain was also severely attenuated for growth in these organs (Fig. 5D). The restricted bacterial loads in both organs increased by three orders of magnitude after eight weeks of infection. In contrast, replication of the KasB KasB_T334A/ T336A strain was comparable to that of the parental strain in both organs, although a 1-log growth defect was observed in the liver eight weeks after infection, whereas replication of the KasB mutant was also severely impeded in these two organs.

Next, immunocompetent C57BL/6 mice were infected via aerosol and assessed for both survival and bacterial replication. Monitoring of CFU in the lungs at different time points after infection indicated that both the parental and double Ala mutant grew for the first three weeks following a similar kinetic (Fig. 6A). Consistent with previous observations, the KasB KasB was severely attenuated for growth in mice [14]. In contrast to the KasB mutant, the KasB phosphomimetic mutant failed to replicate, even after the first three weeks of infection and was never found in the lungs of C57BL/6 in two independent aerosol infection experiments. 

Histological examination of stained lung sections from infected mice revealed multifocal, moderate infiltration after 21 days of infection with the parental strain (Fig. 6B) but not with the KasB KasB_T334A/T336A, KasB KasB_T334D/T336D mutant strains, which may be attributed to the lower bacterial loads of these strains at this particular time point. Bacterial loads in the liver and spleen at different time points after infection indicated that the KasB phosphomimetic mutant was extremely attenuated, since CFU could not be obtained at either 3 or 8 weeks post-infection (Fig. 6C). Replication of the KasB mutant was also severely impeded in these two organs.

Together, this suggests that KasB phosphorylation regulates the growth of Mtb in both immunocompromised and immunocompetent mice. In the presence of phosphorylated KasB, the tubercle bacillus fails to establish a chronic persistent infection, and exhibits a severely attenuated phenotype.

KasB phosphorylation reduces the uptake of M. tuberculosis by macrophages

The difference in virulence between the KasB KasB and the phosphomimetic strains led us to investigate how these two strains infect and survive in macrophages, the primary cellular targets of Mtb. C57BL/6 bone marrow-derived macrophages (BMDM) were infected with the various Mtb KasB variants, lysed by 0, 1, 3 and 6 days post-infection, and the numbers of viable bacteria were counted (Fig. 7A). The parental KasB KasB_T334A/ T336A mutant grew similarly whereas the KasB KasB mutant, as expected, was attenuated for growth in macrophages, with day 6 CFU counts similar to day 0 numbers, versus an approximately one log increase for the parental strain. In contrast, the KasB phosphomimetic strain had no significant growth defect in macrophages but exhibited a marked impairment in macrophage uptake (Fig. 7A, day 0 time point). This effect was further investigated by measuring internalization of the various strains in BMDM. In three independent experiments, the uptake of the KasB phosphomimetic strain was significantly lower than the one of the parental strain by around 50% (Fig. 7B, left panel), a defect that was reversed when the phosphomimetic strain was complemented with a wild-type kasB on a multicopy plasmid (Fig. 7B, right panel). To confirm that these macrophages phenotypes were not specific to C57BL/6 BMDM, the growth of the KasB strains was also tested in the human acute monocytic leukemia cell line THP-1. In THP-1 macrophages, the phosphomimetic KasB mutant had also no growth failure and did present an uptake defect similar to the one observed in BMDM (Figure S5 in Text S1).

Taken together, this set of data suggests that phosphorylation of KasB regulates the early interaction event between Mtb and macrophages.

Increased polyacylated trehalose levels in the KasB phosphomimetic strain

The distinct in vivo phenotypes between the KasB phosphomimetic mutant and the KasB KasB mutant may reflect different physiological/metabolic status. To identify transcriptional differences that may be relevant to the differential phenotypes of these strains, a whole-genome transcriptional analysis was performed (Figure S6 in Text S1). Several genes were commonly up- or down-regulated two-fold or more in the KasB mutant strains. Most up-regulated genes were genes participating in cell wall, cell processes and lipid metabolism, whilst the majority of the down-regulated genes were associated with intermediary metabolism and respiration. Interestingly, a few genes were uniquely up-regulated in the KasB phosphomimetic strain such as the esterase/lipase lppE, proposed to play an important role in Mtb pathogenesis [30], and five genes involved in lipid or drug transport (MmpL4, MmpL5, MmpL10, MmpS4, and Rv1258c) [31,32], as well as five other genes involved in lipid metabolism (RvK3, RvK6, RvPapA1, RvPapA3, and FadD21). The genes specifically down-regulated in the KasB phosphomimetic mutant encoded: NrdF1, an enzyme involved in DNA replication; two oxidoreductases (Rv3741c, Rv3742c) which are in an operon with a triacylglycerol synthase;
two enzymes (Rv3084, Rv3085) part of the mymA operon which might be implicated in the modification, activation and transfer of fatty acids to the cell envelope [33]. Strikingly, most of the biosynthetic gene cluster (pks3/4, papA3, mmpL10, fadD21) required for the synthesis of polyacylated trehalose (PAT) [34] was specifically up-regulated in the KasB phosphomimetic mutant suggesting that PAT biosynthesis in this mutant might be altered.

Metabolic labeling with 14C-propionate and subsequent lipid analysis of parental, phosphoablative, phosphomimetic and deletion strains showed that the phosphomimetic KasB mutant produced more PAT than the three other strains (Fig. 8). Quantification of the TLC spots revealed a reproducible two-fold increase in PAT production in the KasB_334D/336D mutant compared to the three other strains and to its complemented strain, suggesting that phosphorylation of KasB positively affects PAT synthesis.

**Discussion**

Recent studies have provided clear insights into the vast range of pathways regulated by STPK in Mtb. These include multiple metabolic processes, transport of cell wall components as well as cell division or virulence functions [5,6,7]. Several STPK, such as PknH or PknG, have been reported to play a crucial role in modulating Mtb virulence [6,35,36,37,38]. However, little is known regarding the specific substrates contributing to mycobacterial virulence and regulated by these kinases. Here, we report the critical role of STPK-dependent phosphorylation of KasB, which is directly linked to Mtb virulence. Through the design of a KasB phosphomimetic Mtb mutant, we demonstrate that, in vivo, the replacement of the two Thr by Asp residues was characterized by highly pronounced phenotypes corresponding to i) loss of acid-fastness, ii) production of shorter mycolic acids with defects in trans-cyclopropanation, iii) defect in macrophage invasion, iv) incapacity to grow and establish a persistent infection in both immune-compromised and immune-deficient mice, and v) absence of pathology in infected animals (Fig. 9). The long-term persistence of the KasB phosphomimetic strain without causing disease or mortality makes it an attractive model for studying latent Mtb infections and suggests that this attenuated strain may represent a valuable vaccine candidate against TB.
Figure 6. Infection of immunocompetent C57Bl/6 mice with *M. tuberculosis* kasB isogenic strains. (A) Growth of *Mtb* kasB strains in the lungs. Low-dose aerosol infection was performed with the following *Mtb* strains: parental, KasB_T334A/T336A, KasB_T334D/T336D, and ΔkasB. Lungs from infected mice were harvested at 1, 7, 21 and 57 days post-infection, homogenized and serial dilutions were plated onto Middlebrook 7H10 plates supplemented with 10% OADC and 0.2% glycerol. (B) Pathology slides of lungs from infected mice at 21 days post-infection. Lung tissue sections from mice infected with the *Mtb* CDC1551 parental, KasB_T334A/T336A, KasB_T334D/T336D, and ΔkasB were stained with hematoxylin/eosin and observed. Magnification = ×20. (C) CFU plots in the liver and spleen three weeks (black bars) and eight weeks (grey bars) post-infection.

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By analogy with a ΔkasB mutant, these phenotypes strongly support the view that phosphorylation has a detrimental effect on KasB activity. This is also emphasized by the modelling studies highlighting the strategic localization of the two phosphosites in the vicinity of the catalytic triad. That phosphorylation of these residues is likely to perturb the condensing activity of KasB contrasts with an earlier study proposing that phosphorylation of KasA and KasB reduces and increases their condensing activity in vitro, respectively [8]. The discrepancy between these two studies could be explained by several reasons. First, in the in vitro study, KasB activity was assessed using C16-AcpM, which represents a preferred substrate for KasA, but not for KasB [39] which catalyzes the last step in the elongation cycle [14], therefore acting on very long fatty acyl chains, such as C48-C52-AcpM substrate. Second, to conduct these studies, preparation of purified KasA or KasB from recombinant BCG were used, which contain large proportions of non-phosphorylated proteins, as usually within bacteria only a small proportion of proteins gets phosphorylated. A heterogeneous preparation containing a mixture of phosphorylated and non-phosphorylated isoforms may affect the overall activity of KasB. Finally, several studies have shown that most FASII components interact together [26,27,40] and a “mycolome” concept has recently emerged from work demonstrating that FASII system of Mtb is organized in specialized interconnected complexes composed of the condensing enzymes, dehydratase heterodimers and the methyltransferases [26,27,40]. In this model of interactome, three types of FASII specialized complexes are interconnected together: i) the initiation FASII is formed by a core consisting of the reductases, FabD and FabH, linking FASI and FASII together; ii) two elongation FASII complexes consisting of a core and either KasA (E1-FASII) or KasB (E2-FASII) which are capable of elongating acyl-AcpM to produce full-length meromycolyl-AcpM (Fig. 9); and iii) the termination FASII involving Pks13 which condenses the α branch with the meromycolic branch. One may therefore hypothesize that phosphorylation of KasB may also alter/disrupt heterotypic interactions with other FASII partners of

Figure 7. Infection of C57BL/6 bone marrow-derived macrophages with M. tuberculosis kasB variants. (A) Growth of the KasB mutants and parental strain in BMDM. An MOI of 1 was used to infect the BMDM with the strains. Macrophages were lysed after 0, 1, 3 and 6 days post-infection and viable bacteria were counted by plating dilutions of the lysates on agar plates. Three independent experiments were performed and each experiment was done in triplicate. (B) Uptake of KasB strains by BMDM. The ratio between the bacterial titer after a 4 h infection and the inoculum titer is shown. A T-test (two-tailed distribution, unequal variance) was performed and showed a statistical difference between the uptake of the kasB phosphomimetic strain compared to deletion strain (left panel). Complementation with a functional KasB restores the uptake of the phosphomimetic mutant (right panel). Blue bar, parental strain; green bar, KasB_T334A/T336A; black bar ΔkasB, black bar with white stripes, ΔkasB pMV261-kasB; red bar, KasB_T334D/T336D; and red bar with white stripes, KasB_T334D/T336D pMV261-kasB.

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To address the possibility that phosphorylation of KasB could also affect the localization of the protein, recombinant strains of *M. smegmatis* and *M. bovis* BCG expressing green fluorescent protein (GFP)-tagged KasB variants were produced, comprising wild-type KasB, a phosphoablative (T334A/T336A) and a phosphomimetic (T334D/T336D) KasB isoform. We found that all KasB/eGFP fusion proteins localized in the mycobacterial cell wall, mostly on the bacterial poles (Figure S7 in Text S1). These findings strongly suggest that mycolic acid biosynthesis takes place during mycobacterial division at the bacterial poles and indicate that phosphorylation of KasB does not affect its localization in mycobacteria. Therefore, one can hypothesize that the primary consequence of KasB phosphorylation *in vivo*, is very likely to result in alteration of its enzymatic activity.

A recent study revealed that there were no detectable differences in the thickness of the cell envelope among the wild-type *Mtb* and ΔkasB mutant as determined by conventional transmission electron microscopy [41]. However, cryo-electron microscopy demonstrated that the region between the inner and outer membranes of the mutant strain, mainly composed of mycolic acids, showed a significant decrease in electron density as compared to the wild-type strain, suggesting that the altered mycolic acid pattern in the ΔkasB mutant may have affected the packing of the lipid-rich layer of the envelope [41]. One may hypothesize that the attenuated pathogenicity of the KasB phosphomimetic strain results in the increased permeability of its cell envelope due to the reduction in the number of tight bundles of mycolic acids, which facilitates the direct attack of effector molecules from the host cells. This altered mycolic acid packing and/or reduced chain length may also be responsible for the reduced capture of the dye, leading to loss of acid-fastness.

Previously, we have demonstrated that the mycolic acid cyclopropane synthase PcaA, which introduces cis-cyclopropane rings at the proximal position in ω-mycolic acids, was phosphorylated by STPK [17]. As for KasB, phosphorylation of PcaA was associated with a significant decrease in the enzymatic activity. A PcaA phosphomimetic (T168D/T183D) strain, like the ΔpcaA mutant, exhibited reduced survival in human macrophages and was unable to prevent phagosome maturation, compared to the wild-type strain. This added new insights into the importance of mycolic acid cyclopropane rings in the phagosome maturation block and provided the first evidence of a Ser/Thr kinase-dependent mechanism for modulating mycolic acid composition and phagosome maturation block [17]. The present study extends these results by adding KasB in the growing list of virulence factors associated with mycolic acid metabolism, whose activities are directly regulated by phosphorylation. However, in contrast to PcaA, phosphorylation of KasB significantly altered colonization of macrophages and, unexpectedly, this phenotype was not shared by the ΔkasB strain. These differences were reflected in the different transcriptional profiles between the two strains. Among these transcriptional differences, specific up-regulation of the PAT biosynthetic gene cluster occurred in the phosphomimetic strain and correlated with increased production of PAT. Earlier studies demonstrated that PAT deficiency affects the surface global composition of the mycobacterial cell envelope, improving the efficiency with which *Mtb* binds to and enters phagocytic host cells [42], implying that PAT production affects early interaction between *Mtb* and macrophages. From these results, one can propose that the increased PAT production in the KasB phosphomimetic strain could be, at least partially, responsible for the decreased uptake by macrophages, which in turn may also explain the attenuation of the phosphomimetic mutant in mice. As shown in Figure S8 in Text S1, the phosphomimetic strain also

Figure 8. Expression of PAT in the *M. tuberculosis* kasB variants.
Left panel: autoradiographs of thin layer chromatograms of apolar lipids derived from [1-14C]-propionate labeling in the various *Mtb* KasB strains and complemented strains carrying pMV261-kasB. Total lipids (6,000 counts) were loaded on TLC plates and developed thrice in petroleum ether/acetone (92:8, v/v) in the first direction and once in toluene/ether/acetone (95:5, v/v) in the second direction. Right panel: quantification of PAT production (corresponding to the circled spots in left panel). Results are expressed in fold increase relative to the parental strain. Results are representative of two independent experiments.

E2-FASII which may in turn affect the activation of E2-FASII, leading to shortened mycolates. This would be missed in *in vitro* assays using single purified proteins.
produced PDIM, thus excluding the possibility that the attenuated phenotype of the phosphomimetic strain could be a consequence of the loss of PDIM. However, as suggested by the microarray data, multiple genes belonging to different classes such as adaptation, cell wall processes, intermediary metabolism, and respiration or regulatory proteins were found to be differentially regulated between the KasB phosphomimetic and knock-out strains. They may also, in addition to the PAT biosynthetic genes, participate in the phenotypic differences between the two strains.

Analysis of the phosphorylation status of KasB in BCG using anti-phosphothreonine antibodies indicated that phosphorylation was more prominent in stationary cultures than in replicating cultures. These finding are reminiscent of those reporting that phosphorylation of the FASII HadAB and HadBC complexes is growth phase-dependent and that phosphorylation occurred at higher levels in non-replicating bacteria [12], suggesting that phosphorylation is a mechanism by which mycobacteria might tightly control mycolic acid biosynthesis under non-replicating conditions. Mtb bacilli have two signature characteristics: acid-fast staining and the ability to cause long-term latent infections in humans. Acid-fast staining, such as Ziehl-Neelsen (ZN) staining, remains the cornerstone of diagnosis of TB, particularly in poor countries where the infection is highly prevalent [43]. Dormant bacilli have distinct structural alterations in the cell wall and are ZN-negative [44]. It is noteworthy that in a high percentage of patients exhibiting TB symptoms, analysis of patient’s tissue samples may be positive for culture of mycobacteria and PCR analysis but negative for ZN staining. However, the reason(s) for the loss of acid-fastness during dormancy remain(s) unknown. A connection between loss of acid-fast staining and latent infection had been reported for a kasB deletion mutant [14] suggesting that regulation of KasB activity may cause these linked phenotypes. The present work provides the first evidence that phosphorylation of KasB correlates the loss of acid-fast staining and a loss of virulence allowing us to hypothesize that Mtb regulates these two related phenotypes through a signal transduction pathway. Further work will need to be done to elucidate these signals and determine which specific kinases regulate them in vivo. This knowledge might lead to better understanding of the molecular signals that trigger reactivation and TB disease. Although we show here that phosphorylation of KasB was more pronounced in stationary phase, additional studies are required to demonstrate whether this also happens in persistent mycobacteria and if it would result in the loss of acid-fast staining. This would open the way to improved methods for the diagnosis of latent TB infections.

Materials and Methods

Ethics statement

All animal experiments and protocols described in the present study were reviewed and approved by the Animal Use and Care Committee of the Albert Einstein College of Medicine (Bronx, NY) complying with NIH guidelines under the Animal Study Protocol 20120114.

Bacterial strains, plasmids, phase and growth conditions

Strains used for cloning and expression (Table S2 in Text S1) of recombinant proteins were E. coli TOP10 (Invitrogen) and
BL21(DE3)pLysS (Novagen) or BL21(DE3)Star (Novagen) grown in LB medium at 37°C. Media were supplemented with ampicillin (100 μg ml⁻¹) or kanamycin (25 μg ml⁻¹), as required. Mycobacterial strains used (Tables S1 and S2) were usually grown on Middlebrook 7H10 agar plates with OADC (oleic acid, albumin, dextrose, catalase) enrichment (Difco). Liquid cultures were obtained by growing mycobacteria either in Sauton’s medium or in Middlebrook 7H9 (Difco) supplemented with 10% OADC enrichment, 0.2% (v/v) glycerol, and 0.05% (v/v) tyloxapol (Sigma) supplemented with either kanamycin (25 μg ml⁻¹) or hygromycin (75 μg ml⁻¹) when required. The multiplicity of expression plasmid pVV16 was reported earlier [45]. All plasmids used in this study are listed in Table S2 in Text S1 and the shuttle plasmid pAE159 was described previously [18,46,47].

Cloning, expression and purification of recombinant KasB-derived mutant proteins

The kasB gene was cloned into pCR-bluntII-TOPO using primers NiermannKasB and CernmKasB (Table S3 in Text S1) to yield pCR-bluntII-TOPO:kasB. Site-directed mutagenesis was performed directly on pCR-bluntII-TOPO:kasB using inverse-PCR amplification with self-complementary primers (Table S3 in Text S1) carrying the desired mutation. The modified genes were subcloned in pETPhos using NdeI and NheI restriction sites, generating pETPhos-kasB_WT, pETPhos-kasB_T334A/T336A and pETPhos-kasB_T334D/T336D (Table S2 in Text S1). All constructs were verified by DNA sequencing. Recombinant KasB proteins were overexpressed in E. coli BL21(DE3)Star (Novagen) and purified as described previously [9]. Fractions containing pure KasB proteins were pooled, dialyzed when required and stored at −20°C until further use. The kasB gene was further subcloned from pETPhos-kasB_WT by Ncol/HindIII digest and ligated into pCDFDuet_1 vector already containing the PknF kinase domain [10], thus yielding pETDuet_kasB which allows co-expression of both PknF and KasB (Table S2 in Text S1).

A ΔkasB BCG mutant was transformed with either pVV16_kasB_WT [8] or pVV16_kasB_T334A/T336A which was derived from pVV16_kasB_WT by site-directed mutagenesis using inverse-PCR amplification with self-complementary primers carrying the desired mutation (Table S3 in Text S1). Transformants were selected on Middlebrook 7H10 supplemented with OADC enrichment, 50 μg ml⁻¹ hygromycin and 25 μg ml⁻¹ kanamycin and grown in Sauton’s broth containing the same antibiotics. Exponential and stationary phase cultures were harvested, lyzed using a French Pressure Cell and purification of soluble KasB and KasB_T334A/T336A proteins was performed on Ni-NTA agarose beads as described earlier [8].

Construction of transducing mycobacteriophages carrying kasB mutations

Mycobacteriophages used for transduction were prepared as described previously [47]. Briefly, kasB was PCR-amplified from the plasmids pETPhos_kasB carrying either the T334A/T336A or the T334D/T336D double mutations using the primers LL2 and LR1 (Table S3 in Text S1). accD6 was PCR-amplified using the primers RL and RR (Table S3 in Text S1). The kasB and accD6 PCR fragments were digested with AlwN1 and Van9I1, respectively and ligated with the 1.6 kb and 3.6 kb fragments of Van9I1-digested pYUB1471 (Table S2 in Text S1). The resulting plasmids were digested with PacI and ligated with the Pac1-digested shuttle plasmid pAE159. After packaging in vitro, the resulting plasmids were electroporated into M. smegmatis and the phages were amplified to obtain high-titer phage lysates.

Specialized transduction experiment

Mtb (50 ml) was grown to log phase, washed twice with mycobacteriophage (MP) buffer (50 mM Tris, 130 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 50 ml) and resuspended in 5 ml of MP buffer. For each transduction experiment, 0.5 ml of cell suspension was mixed with 0.5 ml of high-titer phage lysate. The suspension was incubated at 37°C for 4 h without shaking, spun down, resuspended in 0.2 ml of 7H9 media (see above) and plated on 7H10 plates supplemented with hygromycin. Plates were incubated at 37°C for 4–5 weeks and transductants were picked and patched onto two hygromycin-containing plates. Colonies from one Hyg plate were used for DNA isolation using InstaGene Matrix (BioRad). PCR was performed using 5 μl of DNA for a 50 μl PCR reaction with the primers kasB_F and kasB_R (Table S3 in Text S1) and the resulting products were sequenced to check for the presence of the desired mutations. Southern blotting was done on genomic DNA isolated from Hyg-resistant transductants digested with BglII and probed with the kasB or accD gene to confirm the allelic exchange and kasB deletion, respectively.

Acid-fast staining

The kasB mutants were grown to log phase and 10 μl of culture were spread onto a glass slide. The slides were heated at 100°C for 2 min, dipped into 10% formalin for 30 min, dried and stained using the TB Fluorescent Stain Kit M (BD, Auramine staining) or the TB Stain Kit K (BD, Carbolchulsin staining).

Mouse experiments

SCID mice and C57BL/6 mice (Jackson Laboratories) were infected via the aerosol route using a 2×10⁶ CFU/ml mycobacterial suspension in PBS containing 0.05% tyloxapol and 0.04% antifoam. Five mice from each group were sacrificed at day 1, 7, 21 and 56 to determine the bacterial burden in the lung, spleen, and liver (one aerosol experiment was carried on for 119 days). Six mice per group were kept for survival experiments. All mice infected with Mtb were maintained under appropriate conditions in an animal biosafety level 3 laboratory.

Growth in primary macrophages

C57BL/6 mice were used to obtain bone marrow-derived macrophages (BMDM). Isolated femurs were flushed with Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1x non-essential amino acids (complete DMEM). The cells were cultured for 7 days in complete DMEM containing M-CSF (biotrich) at 30–50 ng/ml and then seeded into 24-well plates (~4×10⁵ cells/well) or 48-well plates (~2×10⁵ cells/well) as described previously [48]. The cells were allowed to adhere overnight prior to infection. The strains were grown as described above, washed and resuspended in DMEM supplemented with 10% FBS and diluted in this medium to achieve the appropriate titer. The bacteria were added to the wells at an approximate multiplicity of infection (MOI) of 1. Following 4 hrs incubation at 37°C to permit bacterial uptake, macrophage monolayers were washed twice with PBS to remove extracellular bacteria, following which wells were replenished with complete DMEM containing M-CSF at 10–50 ng/ml. At various times after infection, the medium in each well was removed to a tube containing sufficient SDS to give a final concentration of 0.025%; the cell monolayers were lysed with 0.025% SDS and combined with the medium. Lysates were diluted in PBS and plated onto Middlebrook 7H10 (see above) for determination of bacterial numbers.
RNA extraction for qRT-PCR

*Mtb* strains were cultured to an OD_{600} of 0.5 in 7H9 broth supplemented with OADC, 0.2% glycerol and 2.5 ml 20% Tween 80. Cells were pelleted at 4500 rpm in 15 ml falcon tubes for 15 min, decanted, resuspended in 1 ml Trizol reagent (Invitrogen), and then transferred into new 2 ml screw cap microcentrifuge tubes containing 0.5 ml of zirconia-silica beads (diameter, 0.1 mm). Bacteria were disrupted in a Bead Beater (FastPrep Cell Disrupter, FP120) using two 45 second pulses at maximum speed of 6.5 m/sec, incubated on ice for 5 min, then 250 µl of chloroform was added, following by vigorous mixing for 15 s and then a 2–3 min room temperature incubation. Samples were then centrifuged at 12, 000 g for 5 min and the upper clear phase transferred carefully to a new tube and mixed with an equal volume of 70% ethanol, applied to an RNasy mini column (QIAGEN) and processed according to the manufacturer’s recommendations.

Reverse transcription real time PCR

Five mRNA targets (*kasB, cmaA2, sigK, 16S*, and *mdaP1*) were reverse-transcribed, using a Quantitect Multiplex RT-PCR kit (QIAGEN) according to the manufacturer’s recommendations using a primer specific for each target gene. The conditions for Reverse Transcription PCR were 50°C for 5 min, 95°C 2 min. The amount of cDNA produced was quantified by real time PCR with the corresponding molecular beacon. All primer and molecular beacon sequences are listed in Table S4 in Text S1. 

The 10 µl PCR reaction mixture consisted of 1 × PCR buffer, 250 nM dNTPs, 4 mM MgCl₂, 0.5 µM each primer, 5 ng/µl molecular beacon and 0.03 U/µl Jumpstart Taq polymerase (Sigma-Aldrich). PCRs were performed in 384-well microtiter plates using a primer specific for each target gene. The conditions for the reverse transcriptase (RT) reactions were performed in triplicate. The PCR conditions were identical for all assays. The quantity of specific target DNA was determined from the threshold cycle (CT) value with reference to a standard curve and then a 2–3 min room temperature incubation. Samples were then centrifuged at 12, 000 g for 5 min and the upper clear phase transferred carefully to a new tube and mixed with an equal volume of 70% ethanol, applied to an RNasy mini column (QIAGEN) and processed according to the manufacturer’s recommendations.

Mycolic acid extraction and purification

50–100 µl cultures of Mtb strains were grown to mid-log phase in Middlebrook 7H9 medium at 37°C. Cells were harvested and FAMEs and MAMEs were extracted as reported [56], subjected to one-dimensional thin layer chromatography (TLC) with silica gel plates (silica gel 60F₂₅₄; Merck, Germany) and developed in hexane/ethyl acetate (19:1, v/v; 3 runs). Lipids were revealed by spraying the plates with molybdophosphoric acid followed by charring, 250 nmol of a tracer (methyl-3H) in 50 µl of 7H9 medium, incubated for 15 min, and washed three times with methanol. Areas corresponding to the different mycolic acid subspecies were excised, extracted with ethyl acetate, and subjected to TLC and FAME analyses.

Microarrays

Triplicate samples were prepared by harvesting growing cultures of Mtb CDC1551 KasB strains (OD_{600 nm} = 1). Extraction of RNA, preparation of cDNA, and microarray analysis were performed as described previously [52]. The array data have been deposited in the Gene Expression Omnibus at NCBI with accession number GSE74640.

In vitro kinase assay

In vitro phosphorylation was performed as described [53] with 4 µg of KasB in 20 µl of buffer P (25 mM Tris-HCl, pH 7.0; 1 mM DTT; 5 mM MgCl₂; 1 mM EDTA) with 200 µCi [γ-³²P]ATP corresponding to 65 nM (PerkinElmer, 3000 Ci/mmol⁻¹), and 0.2 to 1.0 µg of PknF kinase in order to obtain its optimal autophosphorylation activity for 30 min at 37°C. Cloning, expression and purification of the PknFGST-tagged kinase from *Mtb* were described previously [8].

Immunoblotting

Bacteria were disrupted by bead beating with 1-mm-diameter glass beads and the total protein concentration in each cell lysate was determined using a bicinchoninic acid (BCA) protein assay. Samples were separated on 12% SDS-PAGE gels and were transferred to a nitrocellulose membrane. The membrane was saturated with 1% BSA in PBS/Tween 0.1% and either probed with rat anti-KasA antibodies (dilution:1:500) [54], rabbit anti-AC2D6 antibodies (dilution, 1:1,000) [55] or anti-phosphothreonine antibodies (dilution, 1:1,000) (Cell Signaling). After washing, the membrane was incubated with either secondary antibodies labeled with IRDye infrared dyes (Odyssey Classic) or alkaline-conjugated anti-rabbit secondary antibodies (dilution, 1:7,000) and revealed with BCIP/NBT, according to the manufacturer’s instructions.

Extraction and analysis of PAT

*Mtb* cultures (10 ml), grown in Middlebrook 7H9-OADC-glycerol-tyloxapol to an OD_{600 nm} of 0.2, were treated with either [³⁵S]C-labelled acetate (10 µCi) or [¹⁴C]C-labelled propionate (20 µCi) for 20 h or 2 days, respectively. Apolar lipids were extracted by mixing the cell pellets with methanol (2 ml), 0.3% aqueous NaCl solution (0.2 ml) and petroleum ether (1 ml) for 15 min. The suspensions were centrifuged and the upper petroleum ether phases were removed.

NMR analyses

For NMR analyses, MAMEs were dissolved into deuterated chloroform containing 0.01% of TMS and transferred into Shigemi tubes matched for D₂O. Then 0.1 ml of deuterium oxide was added to avoid solvent evaporation during long acquisition. 1D proton NMR spectra were recorded at 300K on a 400 MHz Avance II Bruker spectrometer equipped with a 5 mm broadband inverse probe.
Mass spectrometric analyses

Mass spectrometric analyses of MAMEs were performed on a Voyager Elite reflector MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA), equipped with a 357-nm UV laser. Samples were solubilized in 1 μl chloroform/methanol (2:1, v/v) and mixed on target with 1 μl of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml dissolved in chloroform/methanol 2:1, v/v).

Supporting Information

Text S1 Contains supplementary materials and Methods, supplementary Tables 1-4, supplementary Figures 1-8 and references.

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Author Contributions

Conceived and designed the experiments: CV VM DA WRJ LK. Performed the experiments: CV VM SCK JL SS LS BB JT RVC XT ST BW YG LK. Analyzed the data: CV VM YG DA WRJ LK. Wrote the paper: CV VM YG WRJ LK.
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