Mycolic acids are vital components of the *Mycobacterium tuberculosis* cell wall, and enzymes involved in their formation represent attractive targets for the discovery of novel anti-tuberculosis agents. Biosynthesis of the fatty acyl chains of mycolic acids involves two fatty acid synthetic systems, the multifunctional polyolypeptide fatty acid synthase I (FASI), which performs de novo fatty acid synthesis, and the dissociated FASII system, which consists of monofunctional enzymes, and acyl carrier protein (ACP) and elongates FASI products to long chain mycolic acid precursors. In this study, we present the initial characterization of purified KasA and KasB, two β-ketoacyl-ACP synthase (KAS) enzymes of the *M. tuberculosis* FASII system. KasA and KasB were expressed in *E. coli* and purified by affinity chromatography. Both enzymes showed activity typical of bacterial KASs, condensing an acyl-ACP with malonyl-ACP. Consistent with the proposed role of FASII in mycolic acid synthesis, analysis of various acyl-ACP substrates indicated KasA and KasB had higher specificity for long chain acyl-ACP's containing at least 16 carbons. Activity of KasA and KasB increased with use of *M. tuberculosis* AcpM, suggesting that structural differences between AcpM and *E. coli* ACP may affect their recognition by the enzymes. Both enzymes were sensitive to KAS inhibitors cerulenin and thiolactomycin. These results represent important steps in characterizing KasA and KasB as targets for antimycobacterial drug discovery.

The cell wall of *Mycobacterium tuberculosis* is a complex structure containing many components that contribute to the communication between the bacterial cell and its host (1) as well as its structural integrity and characteristic impermeability (2–4). In fact, the innate impermeability displayed by *M. tuberculosis* to many common broad spectrum antibiotics and other small hydrophilic molecules can be directly attributed to the hydrophobic nature of the cell envelope (4). The unique core structure of the cell wall consists of a covalently linked complex of peptidoglycan, arabinogalactan, and mycolic acids (5–7). The latter, which are high molecular weight, α-alkyl, β-hydroxy fatty acids, are the largest fatty acids in nature, ranging from 70 to 90 carbons (for reviews, see Refs. 5 and 8). In addition to their characteristically long acyl chains, *M. tuberculosis* mycolic acids also contain a variety of functionalities, including desaturations and cyclopropyl rings, α-methyl-branched methyl ethers, and α-methyl-branched ketones which define the α-, methoxy-, and ketomycolates, respectively (8, 9). The low permeability of the cell can be directly attributed to the nature of functional groups in mycolic acids and their effect on the fluidity of the cell wall (2, 5, 10). Variations in functional group structure can also have effects on the ability of *M. tuberculosis* to grow in macrophages (11). In addition to the influence of mycolic acids on cell wall structure and function, cell wall components such as trehalose dimycolate (cord factor) have been shown to exhibit immunomodulatory activities that can enhance the pathogenicity of *M. tuberculosis* (12).

The importance of mycolic acids to bacterial survival and pathogenesis has generated much interest in the enzymes responsible for their biosynthesis, based on the hypothesis that inhibitors of these proteins will be potential antimycobacterial agents. Indeed, the cessation of mycolic acid synthesis is one of the primary effects of isoniazid (INH),1 a front-line anti-tuberculosis drug (13). Various other compounds including ethionamide (14, 15), isoxyl (15, 16), and thiolactomycin (TLM) (17), have also been shown to inhibit mycolic acid synthesis.

Biosynthesis of mycolic acid precursors requires the interaction of two fatty acid synthase (FAS) systems, the multifunctional polyolypeptide, FASI, and the dissociated FASII system, the latter composed of monofunctional enzymes and a discrete acyl carrier protein (ACP)2 (8) (Fig. 1). Although the specific details of mycolic acid synthesis are not completely understood, the mycobacterial FASI system appears to be responsible for the de novo synthesis of C16–26 fatty acyl primers (18), which are then passed to the FASII system and elongated to produce intermediates of the long meromycolate chain (19). Such intermediates can be modified and condensed with α-branch fatty acids to form mature mycolic acids (8). The availability of the *M. tuberculosis* genome sequence has allowed the identification of putative genes encoding proteins homologous to other bac...
tional FASII enzymes (8, 20). In turn, this has facilitated the characterization of several components of the *M. tuberculosis* FASII system, including FabH (21, 22), FabD (23), and AcpM (24).

Of particular interest in the FASII system are the roles played by three condensing enzymes, FabH, KasA, and KasB. Recent reports describe the isolation of the FASII initiation enzyme FabH (21, 22) and show that this enzyme is inhibited by TLM but not by cerulenin (CER) (21). TLM also inhibits KasA and KasB, both putatively involved in fatty acid elongation, based on overexpression of the *kasA* and *kasB* genes in *Mycobacterium bovis* BCG, which resulted in increased resistance to TLM in vitro and in vivo (25). Distinct roles for KasA and KasB in mycolic acid synthesis were postulated based upon increases in the incorporation of radioactivity into fatty acids in various FAS assays after overexpression of either *kasA* or *kasB* (25). The anticytomicrobial activity of TLM suggests the essentiality of at least one of the three condensing enzymes, as is the case for orthologs in other bacterial species such as *E. coli* FabB (26) and *Streptococcus pneumoniae* FabH (27) and FabF, thus making them attractive targets for the development of screens to identify new lead compounds. Therefore, we purified and characterized KasA and KasB, and despite many similarities, these enzymes display differing enzymatic properties and may indeed play distinct roles in mycolic acid biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials and Instrumentation**—Chemicals and reagents were purchased from Sigma unless otherwise noted. All cloning steps were performed in *E. coli* DH5a cells purchased from Life Technologies, Inc. *E. coli* BL21(DE3) cells and expression vector pET28a(His6)/NdeI were purchased from Novagen. *M. tuberculosis* H37Rv genomic DNA was obtained from Dr. John Belisle at Colorado State University. Multi-domain fatty acid synthase proteins, were removed from the structural inequivalence among the aligned sequences in these regions. Thus, these regions, including the non-homologous domains of the multi-domain fatty acid synthase proteins, were removed from the alignment before phylogenetic analyses. In the final edited alignment, 395 of the 414 amino acids of *M. tuberculosis* KasA were maintained.

Phylogenetic analyses were based on the total number of sites in edited multiple-sequence alignments. Trees were constructed by neighbor-joining and maximum-parsimony methods. Neighbor-joining trees were built from pairwise distances between amino acid sequences based on the Dayhoff PAM120 substitution matrix (31) using the programs CLUSTAL (32). The programs SEQBOOT and CONSENS were used to estimate the confidence limits of branch points from 1000 bootstrap replicates. Maximum parsimony construction was accomplished with the software package PAUP*, version 4.0 (33). The number and length of minimal trees were estimated with 100 replicate random heuristic searches. Confidence limits for branch points were estimated from 1000 bootstrap replicates.

**PCR and Cloning Strategies**—To generate clones for expressing N-terminal hexahistidine-tagged KasA and KasB, the *M. tuberculosis* *kasA* and *kasB* genes (Rv2245 and Rv2246, respectively, GenBankTM accession number Z70692) were PCR-amplified from *M. tuberculosis* genomic DNA using standard PCR strategies (34) with *Pfu* DNA polymerase (Stratagene) and primers designed for directional cloning into NheI/HindIII (kasA)- or NdeI/SacI (kasB)-digested pET28a(+) (5′-CGAGGCTTGAGGCCGAGCTAGCGTGAGTCAGCCTTC-3′, forward kasA primer with NheI site underlined, and 5′-CCCGCATGCTCAAG-3′, reverse kasB primer with SacI site underlined).
CTTCGTAAGC-3', reverse kasA primer with HidIII site underlined, and 5'-GACATCGCGGCGGATCCGATCTAGGCTGTAAG-3', forward kasB primer with NdeI site underlined. PCR programs consisted of an initial 5-min denaturation step (94 °C) followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and then a final elongation step at 72 °C for 7 min. PCR products were digested with Nhe/HindIII (kasA) or NdeI/SacI (kasB) and ligated with vector using T4 DNA ligase (Life Technologies, Inc.) to produce pETkasA and pETkasB. Positive clones were identified by PCR screening using the above primers, and the plasmid insert was sequenced to assure authenticity of the construct.

**Protein Expression and Purification**—The pETkasA and pETkasB plasmids were electroporated into E. coli BL21(DE3) cells, and single colony transformants were grown at 37 °C to mid-log phase (A_{600} = 0.7) in LB broth containing kanamycin (50 μg/ml) and glucose (1%). For KasA, the cells were induced with 0.3 mM IPTG at 37 °C for 2.5 h. KasB cultures were equilibrated to 18 °C and induced with 0.3 mM IPTG for ~24 h at 18 °C. Cells were harvested by centrifugation, resuspended in Buffer A (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol (5 ml/g)), and lysed by the addition of 2 mg of lysozyme followed by 3 cycles of freezing and thawing. The KasA lysate was centrifuged at 20,000 × g for 20 min, the supernatant was discarded, and the pellet was dissolved in 20 ml of Buffer A containing 8 M urea (Buffer A'). The urea solution was centrifuged at 27,000 × g, and the supernatant containing denatured KasA was loaded onto a 5-ml nickel-chelating column. The column was washed with 5 column volumes of Buffer A' and eluted using a 100-ml linear gradient of 10–500 mM imidazole in Buffer A'. Fractions containing KasA were identified by SDS-PAGE, pooled, and diluted to an approximate concentration of 0.2 mg/ml in Buffer A'. KasA was refolded during stepwise dialyses (6–8 h/step) against 2 liters of 50 mM sodium phosphate, pH 7.5, 0.5 mM NaCl, 2 mM DTT containing 4, 2, and 1 M urea and then dialyzed against 4 liters of 50 mM sodium phosphate, pH 7.5, 0.5 mM NaCl, 2 mM DTT. Finally, the sample was dialyzed overnight against 2 liters of 50 mM Tris-HCl, pH 9.5, 0.3 mM NaCl, 2 mM DTT, and 50% glycerol and filtered through 0.45 μm filter. Aliquots of KasA were snap-frozen in liquid nitrogen and stored at −80 °C.

The KasB lysate was centrifuged at 27,000 × g, and the pellet was discarded. The soluble lysate was loaded onto a 5-ml nickel-chelating column, washed with 5 column volumes of Buffer A' and eluted with a 100-ml linear gradient of 10–500 mM imidazole in Buffer A'. Fractions containing KasB were identified by SDS-PAGE, pooled, and dialyzed against 4 liters of 50 mM sodium phosphate, 0.3 mM NaCl, 2 mM DTT, 5% glycerol, after which KasB was divided into 100-μl aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C.

**Acyl-AcpM and Acyl-AcpM Substrates**—Lauroyl (C_{12})-ACP, palmitoyl (C_{16})-ACP, and arachidoyl (C_{20})-ACP were generated using holo-AcpM (or holo-AcpM), lauric, palmitic, or arachidic acid and the E. coli Aas enzyme as previously described (24, 35, 36). Newly synthesized substrates were purified by anion-exchange chromatography on Q-Sepharose columns and characterized by SELDI-TOF mass spectrometry (SELDI-MS, and the acyl-ACPs were quantitated by amino acid analysis and enzymatically with KasA. Butyryl (C_{4})-ACP and malonyl-ACP were provided by Martin Brandt (GlaxoSmithKline). Malonyl-AcpM was synthesized using apo-AcpM, malonyl-CoA, and E. coli AcpB and purified by anion-exchange chromatography.

**Enzyme Assays**—A continuous assay format was used to monitor KasA and KasB activity by coupling the condensing activity of the Kas enzymes to a β-ketoacyl-ACP reductase, either M. tuberculosis MalA or S. pneumoniae FabF (obtained from Joshua West, GlaxoSmithKline). Both MalA and FabF reduce β-ketoacyl-ACP intermediates to the corresponding β-hydroxyacyl-ACPs, enabling the reaction course to be monitored spectrophotometrically by following the oxidation of NADPH to NADP⁺ at 340 nm. MalA was purified by expressing the malA gene as an N-terminal hexahistidine-tagged protein in E. coli followed by affinity chromatography of the soluble fraction of the whole-cell lysate. Although various concentrations of enzyme and ACP substrates were utilized in the experiments described, the details of which are included in the figure legends and text, typical KasA assays (100-μl total volume) contained 2 μM KasA, 3 μM MalA, and 36–54 μM malonyl-ACP (AcpM), 6–10 μM acyl-AcpM (AcpM), 50 μM NADPH, and 0.01% CHAPS in 50 mM HEPES buffer, pH 6.8. KasB reactions (100 μl total volume) contained 460–920 nm KasB, 3 μM MalA or FabG, 26–54 μM malonyl-AcpM, 6–10 μM acyl-AcpM, 50 μM NADPH, and 0.01% CHAPS. Fractions contain-
that KasA and KasB are more identical to each other than to homologous bacterial enzymes suggests that either kasA or kasB arose by gene duplication as opposed to horizontal transfer from another species. It was therefore of considerable interest to attempt to biochemically differentiate the two enzymes by comparing their enzymatic characteristics.

Over the course of these studies, numerous approaches to express KasA as a soluble protein in E. coli were attempted, but none were successful. Varying the level and rate of expression produced no soluble KasA (data not shown). Several E. coli strains including BL21(DE3), NovaBlue(DE3), C41(DE3), and C43(DE3) (37) were utilized to express KasA under varying conditions, but the product was always insoluble (data not shown). Additionally, expression of KasA in its native form or refolded during the slow removal of the denaturant (8 M urea) for hexahistidine-tagged KasA with the initiation methionine cleaved off. Total amino acid analysis of refolded KasA indicated no other detectable protein (data not shown). Approximately 20 mg of >95% pure refolded KasA was obtained from 0.5 liters of culture.

**TABLE I**

| Protein | % identity to KasA | % identity to KasB | % similarity to KasA | % similarity to KasB |
|---------|--------------------|--------------------|----------------------|----------------------|
| Kas proteins |                    |                    |                      |                      |
| M. tuberculosis KasA | 100 | 67 | 100 | 86 |
| M. tuberculosis KasB | 67 | 100 | 86 | 100 |
| M. leprae KasA | 93 | 67 | 99 | 86 |
| M. leprae KasB | 67 | 92 | 86 | 98 |
| FabF proteins |                    |                    |                      |                      |
| E. coli | 41 | 38 | 36 | 66 |
| M. catarrhalis | 37 | 38 | 36 | 66 |
| P. aeruginosa 1 | 42 | 40 | 40 | 69 |
| P. aeruginosa 2 | 39 | 36 | 36 | 69 |
| B. subtilis | 37 | 36 | 36 | 66 |
| E. faecalis 1 | 36 | 37 | 37 | 66 |
| E. faecalis 2 | 38 | 38 | 38 | 68 |
| S. aureus | 36 | 34 | 34 | 64 |
| S. pneumoniae | 35 | 35 | 35 | 62 |
| H. sapiens | 35 | 35 | 35 | 63 |
| FabB proteins |                    |                    |                      |                      |
| E. coli | 33 | 34 | 34 | 57 |
| H. influenzae | 35 | 35 | 35 | 61 |
| M. catarrhalis | 34 | 35 | 35 | 59 |
| P. aeruginosa | 31 | 32 | 32 | 58 |
| FAS1 proteins |                    |                    |                      |                      |
| M. tuberculosis | 23 | 24 | 24 | 51 |
| M. leprae | 24 | 23 | 23 | 51 |
| S. cerevisiae | 20 | 21 | 21 | 46 |
| H. sapiens | 22 | 24 | 24 | 50 |

**FIG. 3.** SDS-PAGE analyses of KasA and KasB purification steps. Samples were run on 10% Bis-Tris gels in either MES or MOPS SDS-PAGE buffer. A, \( \lambda_{280} \) peak fractions of KasA eluted between 65 and 210 mM imidazole during the denaturing purification. Three \( \mu \)l of each 5-ml peak fraction were loaded per lane. B, lane 1 contains refolded KasA plus colloidal precipitate after dialysis against 50 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 2 mM DTT. Lane 2 contains refolded KasA recovered after dialysis into 50 mM Tris-HCl, pH 9.5, 0.3 M NaCl, 2 mM DTT, 50% glycerol followed by filtration through a 0.45 \( \mu m \) filter. C, KasB-soluble lysate (lane 1), nickel column flow-through (lane 2), \( \lambda_{280} \) peak fraction from KasB purification (lane 3), peak fractions containing KasB which eluted between 235 and 280 mM imidazole (lanes 4–10).
Unlike KasA, ~10% of the KasB expressed in _E. coli_ BL21(DE3) cells at 18 °C was soluble in Buffer A. However, attempts to improve solubility using many of the techniques described above had no effect (data not shown). KasB was purified under native conditions from the soluble lysate of IPTG-induced cells (Fig. 3C). Combined fractions of KasB were >95% pure as judged by SDS-PAGE analysis, and the N-terminal sequence, determined to be GSSHHHHHHSSGLVPRGSHMVGVPPLAGAS, was consistent with hexahistidine-tagged KasB lacking the initiation methionine. As with KasA, total amino acid analysis showed the presence only of KasB (data not shown). 15 mg of KasB was purified from 2 liters of culture.

**Effects of Acyl-ACP and Acyl-CoA Substrates on KasA and KasB Specific Activity**—The _Kas_ activities of KasA and KasB were confirmed using a continuous assay format coupled to a β-ketoacyl-ACP reductase. The mycobacterial FASII system is incapable of _de novo_ fatty acid synthesis but instead elongates acyl primers (C₁₆₋₂₀) to larger mycolic acid intermediates (38).

For this reason, purified KasA and KasB activities were initially tested using C₁₆₀ acyl substrates. Specific activities of KasA and KasB were determined using a variety of buffers ranging from pH 6.5 to 8, and KasA was found to be most active in HEPES pH 6.8 buffer, whereas KasB had highest activity in phosphate pH 7 buffer. Both enzymes displayed ~2–3-fold higher specific activities at 37 °C compared with 30 or 42 °C. Therefore, KasA and KasB were characterized enzymatically using these optimized conditions.

To differentiate KasA and KasB from the mycobacterial FabH enzyme, which condenses acyl-CoA substrates with malonyl-ACP, KasA and KasB were assayed using C₁₆₀-ACPₖₑ or C₁₆₀-CoA and malonyl-ACPₖₑ as substrates. KasA had a specific activity of 87.8 ± 8.9 pmol/min/μg for C₁₆₀-ACPₖₑ and 5.7 ± 0.9 pmol/min/μg for C₁₆₀-CoA (Fig. 4A). Similarly, the specific activity for KasB was 31.8 ± 3.8 pmol/min/μg for C₁₆₀-ACPₖₑ and 3.4 ± 1.6 pmol/min/μg for C₁₆₀-CoA (Fig. 4B). These results indicate that both enzymes are specific for ACP-bound acyl substrates as opposed to their CoA counterparts and, therefore, function as elongation enzymes.

The linearity of the assays was determined by varying the amounts of KasA and KasB in the reaction. The activity of KasA increased linearly with increasing enzyme concentration, indicating that KasA was the limiting reagent in the assay (Fig. 4C). KasB activity also increased in a linear fashion with enzyme concentration (Fig. 4D).

**Kinetic Parameters for _Kas_ A and _Kas_ B Substrates**—Kinetic parameters for two long chain acyl-ACPₖₑ substrates (C₁₆₀- and C₂₀₀-ACPₖₑ) and malonyl-ACPₖₑ were determined for KasA and KasB (Table II). The apparent _Kₐₙ_ values for C₁₆₀- and C₂₀₀-ACPₖₑ were similar for KasA and KasB, ranging from 1.4 to 3.2 μM. _Vₐₙₐₚ_ values were also comparable with C₁₆₀-ACPₖₑ whereas the _Vₐₚₛₐₚ_ for KasB with C₂₀₀-ACPₖₑ was nearly 2-fold lower than that for KasA, suggesting that C₂₀₀-ACPₖₑ was a relatively inefficient substrate for KasB. KasB assays generally required higher enzyme concentrations to obtain observable rates as compared with KasA, which is reflected in the ~3-fold higher _kₖₐₚₐₘₚ_ value for KasA as compared with that of KasB. The apparent _Kₐₙₕ_ and _Vₐₚₛₐₚ_ values for malonyl-ACPₖₑ were nearly identical for both; however, the catalytic efficiency (ₚₐₚₛₐₚ/Kₐₚₚ) for malonyl-ACPₖₑ was ~1 log lower than that for C₁₆₀-ACPₖₑ, presumably a consequence of the higher _Kₐₙₜ_ for malonyl-ACPₖₑ. These results suggest that KasA and KasB exhibit similar binding affinities for each of the substrates, with the exception of KasB and C₂₀₀-ACPₖₑ, but that the rate of turnover (ₚₐₚₙₜ/Kₐₚₚ) is higher for KasA.

**KasA and KasB Are Specific for Long Chain Acyl-ACP Substrates**—Although the use of C₁₆₀- and C₂₀₀-ACPₖₑ for determination of kinetic parameters confirmed that KasA and KasB utilize long chain acyl-ACP derivatives, these experiments did not rule out their ability to utilize short chain acyl-ACPs as primers for elongation. Therefore, several substrates of varying acyl chain lengths were tested under saturating conditions, and specific activities were determined. As shown in Fig. 5A, KasA specific activity increased with acyl chain length. The specific activity with C₁₂₀-ACPₖₑ was ~2.4-fold higher than that with C₂₀₀-ACPₖₑ, whereas C₁₆₀- and C₂₀₀-ACPₖₑ represented a ~2.5-fold increase over that with C₁₂₀-ACPₖₑ. It is clear from these data that KasA has higher activity with long chain acyl-ACP substrates than those with short or medium length acyl groups. In contrast to KasA, KasB activity peaked with C₁₆₀-ACPₖₑ, which had a specific activity ~5.7-fold higher than that for C₁₂₀- or C₂₀₀-ACPₖₑ (Fig. 5B).

KasA and KasB are more active with acyl-ACP substrates in...
**M. tuberculosis β-Ketoacyl-ACP Synthases KasA and KasB**

**TABLE II**

*Kinetic parameters for the KAS activities of M. tuberculosis KasA and KasB*

| Substrate                  | $K_m$ (μM) | $V_{max}$ (pmol/min) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$min$^{-1}$) |
|----------------------------|------------|----------------------|----------------------|----------------------------------|
| C$_{16:0}$-ACPE$_{Ec}$     | 3.2 ± 0.3  | 63.5 ± 1.5           | 5.3                  | 1.7 × 10$^4$                     |
| C$_{20:0}$-ACPE$_{Ec}$     | 2.5 ± 0.3  | 53.2 ± 1.7           | 4.5                  | 1.8 × 10$^5$                     |
| malonyl-ACPE$_{Ec}$        | 13.5 ± 2.6 | 57.3 ± 2.6           | 4.8                  | 3.5 × 10$^4$                     |
| C$_{16:0}$-ACP$_{Ec}$      | 1.4 ± 0.2  | 64.7 ± 1.6           | 1.6                  | 1.1 × 10$^6$                     |
| C$_{20:0}$-ACP$_{Ec}$      | 1.9 ± 0.2  | 28.0 ± 0.6           | 0.6                  | 3.2 × 10$^2$                     |
| malonyl-ACP$_{Ec}$         | 13.5 ± 2.3 | 57.9 ± 2.2           | 1.4                  | 1.0 × 10$^7$                     |

As shown in Fig. 6, the specific activities of both KasA and KasB were higher with AcpM substrates as compared with ACPE$_{Ec}$. The activity of KasA increased nearly 2-fold using AcpM (161 ± 11 pmol/min/μg) over that observed for ACPE$_{Ec}$ (93 ± 8 pmol/min/μg). KasB activity was ~3.5-fold greater with AcpM (82 ± 3 pmol/min/μg) than with ACPE$_{Ec}$ (23 ± 5 pmol/ min/μg). Whether there are changes in substrate binding affinity with respect to a ACP source cannot be inferred from these data, but it is clear that the use of AcpM enhances the rate at which substrate is turned over.

**FIG. 5. Effect of acyl chain length on KasA and KasB activity.** Various acyl-ACP$_{Ec}$ substrates were tested against KasA and KasB, and the specific activities for each substrate were determined. Results are the mean of duplicate reactions with error bars depicting 1 S.D. A, specific activities of KasA with acyl-ACP$_{Ec}$ substrates. Reactions contained 240 nM KasA, 10 μM acyl-ACP$_{Ec}$, 54 μM malonyl-ACP$_{Ec}$. B, KasB activities with various acyl-ACPs. Reaction conditions were 920 nM KasB, 10 μM acyl-ACP$_{Ec}$, 54 μM malonyl-ACP$_{Ec}$.

For both KasA and KasB, the specific activities with AcpM increased nearly 2-fold compared to ACPE$_{Ec}$. The specific activities for KasA and KasB were higher with AcpM substrates as compared with ACPE$_{Ec}$. The activity of KasA increased nearly 2-fold using AcpM (161 ± 11 pmol/min/μg) over that observed for ACPE$_{Ec}$ (93 ± 8 pmol/min/μg). KasB activity was ~3.5-fold greater with AcpM (82 ± 3 pmol/min/μg) than with ACPE$_{Ec}$ (23 ± 5 pmol/min/μg). Whether there are changes in substrate binding affinity with respect to a ACP source cannot be inferred from these data, but it is clear that the use of AcpM enhances the rate at which substrate is turned over.

**Fig. 6.** The specific activities of both KasA and KasB with AcpM substrates were higher than those with ACPE$_{Ec}$ substrates. The activity of KasA increased nearly 2-fold using AcpM (161 ± 11 pmol/min/μg) over that observed for ACPE$_{Ec}$ (93 ± 8 pmol/min/μg). KasB activity was ~3.5-fold greater with AcpM (82 ± 3 pmol/min/μg) than with ACPE$_{Ec}$ (23 ± 5 pmol/min/μg). Whether there are changes in substrate binding affinity with respect to a ACP source cannot be inferred from these data, but it is clear that the use of AcpM enhances the rate at which substrate is turned over.

**Effects of TLM and CER on KasA and KasB**—Inhibitors of bacterial FASII-condensing enzymes include TLM (40, 41), a natural product inhibitor of FabF, FabB, and FabF, and CER, a fungal epoxide that irreversibly inhibits members of the FabF-FabB class (42, 43). To evaluate the sensitivity of KasA and KasB to these inhibitors, the effects of single concentrations (100 μM) of TLM and CER were tested with both ACPE$_{Ec}$- and AcpM-based substrates. Both TLM and CER inhibited KasA (Fig. 7A) and KasB (Fig. 7B) activity. Inactivation of KasA and KasB by CER was dependent on the length of pre-incubation time before initiation of the reaction (Fig. 7C). The effects of TLM on KasA and KasB differed; greater than 90% of KasA activity was inhibited compared with only ~65% inhibition of KasB. There was no statistically significant difference in the sensitivity of KasA and KasB to TLM and CER with regard to ACPE$_{Ec}$ or AcpM, suggesting similar mechanisms of inhibition for each enzyme irrespective of the ACP source.

The divergent effects of TLM on KasA and KasB in the single concentration experiment were verified by obtaining IC$_{50}$ values for TLM using C$_{16:0}$-AcP and malonyl-Acp (Fig. 8). For KasA, TLM had an IC$_{50}$ of 20 μM (Fig. 8A) whereas that for KasB was 90 μM (Fig. 8B). The 20 μM IC$_{50}$ for TLM against KasA was slightly less than that reported for M. tuberculosis FabH (24 μM) (21).

**DISCUSSION**

Bioinformatics provides powerful tools to analyze genes and their encoded proteins to glean clues about function and physiological role. Phylogenetic analyses are particularly useful in visualizing the relatedness of an uncharacterized target sequence to those from other organisms, especially if there are numerous homologous proteins spanning several enzymes classes. For example, the FASII KAS family includes FabF, FabB, and FabH, comprising the KASI, KASII, and KASIII classes of condensing enzymes, respectively. Members of these classes share varying degrees of homology both to each other and to domains of multi-functional FAS and polyketide syntheses. Our analyses confirm that KasA and KasB are indeed more closely related to FabF and FabB, thereby placing them into the KASI/II class of FASII proteins. The biochemical activity of KasA and KasB corroborates these data, exemplified by their specificity for acyl-ACP substrates as opposed to acyl-CoAs.
However, KasA and KasB can be clearly differentiated from most KASI/II enzymes by their increased activity with long chain acyl-ACP substrates than with shorter acyl primers. Edwards et al. (36) report that recombinant E. coli FabF and FabB displayed the highest activity using C_{6:0} to C_{12:0} acyl-ACP_{E. coli} substrates with minimal utilization of C_{16:0}-ACP_{E. coli}. This is in direct contrast to the specificity of KasA and KasB, both of which demonstrated higher activity for acyl-ACP_{E. coli} substrates than the C_{16:0} and C_{20:0}-ACPs. These findings are consistent with the putative role of KasA and KasB in mycolic acid biosynthesis. Both enzymes are proposed to elongate long chain acyl primers generated by FASI with subsequent conversion to acyl-ACP by FabH. The specific roles of KasA and KasB in fatty acid elongation are still unclear; however, the data suggest that both enzymes do indeed have distinctive characteristics and, therefore, may play unique roles in mycolic acid synthesis. KasA was suggested to be the condensing enzyme responsible for elongation of the initial acyl primers passed to the FASII system from FASI via FabH, and KasB was proposed to be involved later in the pathway (25). Data obtained with the purified enzymes do not contradict this scenario and support the hypothesis that these enzymes make separate contributions to fatty acid elongation. KasA and KasB share similar characteristics with respect to use of long chain acyl-ACPs, effects of temperature on activity, and sensitivity to CER, but there are also significant differences including contrasting enzymatic properties, sensitivity to TLM, activity and stability in various buffers, and solubility when expressed in E. coli.

Since the K_m values of C_{16:0}- and C_{20:0}-ACPs suggest similar binding affinities for KasA and KasB, the higher relative specific activity of KasA reflects higher catalytic constants (k_cat) for KasA. Given the prediction that KasB may be important in the later stages of fatty acid elongation (25), it is plausible that the C_{16:0} and C_{20:0} acyl primers are significantly shorter than those encountered by KasB in vivo, accounting for the lower specific activity exhibited by KasB with those substrates. It is unclear why KasB was able to utilize C_{16:0}-ACP_{E. coli} much more efficiently than C_{20:0}-ACP_{E. coli}, and further studies with long chain (> C_{20:0}) acyl-ACP substrates may help to delineate the full range of chain-length specificity for KasA and KasB. Both enzymes demonstrated higher specific activities with AcpM-based substrates than those incorporating ACP_{E. coli} and this increase was more evident for KasB than KasA. Intuitively, it might be expected that the physiologically relevant AcpM is a better substrate, but the molecular reason for this is unclear. There are many differences between AcpM and ACP_{E. coli} including size and the substitution of various conserved residues (in AcpM), which have been suggested to play key roles in KAS recognition of ACP_{E. coli} (24). It is plausible that some of these differences may affect the binding of ACP_{E. coli} to KasA and KasB; however, kinetic studies with AcpM-based substrates will be necessary to determine whether AcpM binds more tightly to the enzyme or if the increase in rate with AcpM is due to greater catalytic efficiency.

The sensitivity of KasA and KasB to TLM was significantly different, the former being more sensitive. Overexpression of M. tuberculosis FabH in M. bovis BCG did not confer TLM resistance (21), suggesting FabH is not the primary TLM target in mycobacteria, and these data correlate with the in vitro IC_{50} data. The 4.5-fold difference in TLM IC_{50} between KasA and KasB implies that overexpression of KasB in M. tuberculosis should not have any effect on the TLM MIC. However, this evidence runs contrary to a report that overexpression of KasB resulted in TLM resistance (25). It is possible that the physiological activity of KasB with its native substrates, predicted to be much longer than C_{16:0}, may be more sensitive to TLM.
CER acts as an irreversible inhibitor of KASII enzymes by forming a covalent complex with the active site cysteine (44). These enzymes are inactivated by CER in a time-dependent fashion that is governed by the rate constant of enzyme-inhibitor complex formation under varying assay conditions (28). Like their KASII counterparts, KasA and KasB are also sensitive to CER to varying extents depending on the time of preincubation. For example, KasA and KasB activity is reduced ~40% with a 5-min preincubation in 100 μM CER, but when that time is doubled, the enzymes are effectively inhibited. In the absence of the K1 and K infact values for KASII enzymes, this time dependence makes it difficult to compare CER inhibition data among them, especially without standardized assay conditions. Despite these issues, however, CER remains a useful tool for characterization of KAS enzymes.

Other compounds, which inhibit the growth of mycobacteria, including isoxy (16), ethionamide (14, 15), and the enoyl-ACP reductase inhibitor INH (45), have been shown to disrupt the synthesis of mycolic acids. INH has also been reported to affect multiple components in mycolic acid synthesis including KasA and KasB. INH has also been shown to disrupt the action of these compounds, and there is no doubt that the essentiality of INH cannot be reliably determined, and accordingly, it is not yet appropriate to say that of both of these enzymes are indeed valid targets for antimycobacterial drug discovery. Despite the differences in enzymatic properties of KasA and KasB, both are able to utilize C16:0-ACP as a substrate, and it is known that deletion of one gene will result in compensation of the lost activity by the second gene product. Despite this, the in vitro effects of TLM on KasA and KasB activity in conjunction with the overexpression data of Kremer et al. (25) provide compelling evidence that inhibition of one or both of these enzymes may lead to the discovery of lead compounds that could form the basis of an optimization program designed to deliver novel antimycobacterial drugs.

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**FIG. 8. IC50 curves for inhibition of KasA and KasB by TLM.** The IC50 values for TLM were determined by varying the TLM concentration and measuring the specific activities of KasA and KasB using AcpM-based substrates. A, KasA IC50 curve. Enzyme and substrate concentrations were 96 μM KasA, 6 μM C16:0-ACP, 25 μM malonyl-AcpM. TLM concentrations ranged from 1.6 to 200 μM. B, KasB IC50 curve. Assays contained 138 μM KasB, 6 μM C16:0-ACP, 18 μM malonyl-AcpM. The concentration of TLM varied from 12.5 μM to 800 μM.

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