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Characterization of *Mycobacterium smegmatis* Expressing the *Mycobacterium tuberculosis* Fatty Acid Synthase I (*fas1*) Gene

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Unlike most other bacteria, mycobacteria make fatty acids with the multidomain enzyme eukaryote-like fatty acid synthase I (FASI). Previous studies have demonstrated that the tuberculosis drug pyrazinamide and 5-chloro-pyrazinamide target FASI activity. Biochemical studies have revealed that in addition to $C_{16:0}$, *Mycobacterium tuberculosis* FASI synthesizes $C_{26:0}$ fatty acid, while the *Mycobacterium smegmatis* enzyme makes $C_{24:0}$ fatty acid. In order to express *M. tuberculosis* FASI in a rapidly growing *Mycobacterium* and to characterize the *M. tuberculosis* FASI in vivo, we constructed an *M. smegmatis* Δ*fas1* strain which contained the *M. tuberculosis fas1* homologue. The *M. smegmatis* Δ*fas1* (*attB*::*M. tuberculosis fas1*) strain grew more slowly than the parental *M. smegmatis* strain and was more susceptible to 5-chloro-pyrazinamide. Surprisingly, while the *M. smegmatis* Δ*fas1* (*attB*::*M. tuberculosis fas1*) strain produced $C_{26:0}$, it predominantly produced $C_{24:0}$. These results suggest that the fatty acid elongation that produces $C_{24:0}$ or $C_{26:0}$ in vivo is due to a complex interaction among FASI, FabH, and FASII and possibly other systems and is not solely due to FASI elongation, as previously suggested by in vitro studies.

Mycobacterium tuberculosis infects one-third of the world's population, and it is estimated that 1% of the world's population is newly infected with this organism each year (31). The mycobacterial cell wall is a complex structure that plays a role in both *M. tuberculosis* virulence and drug resistance (12, 16). These features of the mycobacterial cell wall are conferred by a wide variety of unique lipids that compose 60% of the cell wall. Mycolic acids (C_{74} to C_{90} α-alkyl β-hydroxyl fatty acids) are the major lipid components of the mycobacterial cell wall and the hallmark of mycobacteria and related species (16). Long-chain saturated fatty acids, which are precursors of cell membrane phospholipids, mycolic acids, and other complex lipids, are generated by the type I fatty acid synthase (FASI) in mycobacteria (4, 6, 16). Mycobacteria are unusual among prokaryotes in that they possess both FASI (typically found in parasites, fungi, and all higher eukaryotes) and the type II fatty acid synthase (FASII), which is found in most prokaryotes and plants. The multifunctional FASI enzyme is a monomer that contains seven separate domains with catalytic activities, including an active site for the prosthetic group 4′-phosphopantetheine of the acyl carrier protein (ACP) (4, 6). Mycobacterial FASI generates $C_{16:0}$ from acetyl coenzyme A (acetyl-CoA) primers and elongates the molecules to produce $C_{24:0/26:0}$ fatty acyl-CoA derivatives, which are the precursors of other fatty acid synthases and polyketide systems (8, 16). In contrast, FASII elongates the FASI products to produce meromycolate precursors, which are modified and condensed with $C_{24:0/26:0}$ to form mycolic acids (8, 16). In vitro studies have shown that mycobacterial FASI produces a unique bimodal distribution of fatty acids (15, 23). In addition to $C_{16:0}$, $C_{24:0}$ is produced by the rapidly growing organism *Mycobacterium smegmatis* (23), and $C_{26:0}$ is produced by the slow growers *M. tuberculosis* and *Mycobacterium bovis* (15).

Since fatty acid synthesis in bacteria is essential for cell survival, the enzymes involved in this pathway have emerged as promising targets for antimicrobial agents (14). The FASII enoyl-ACP reductase was identified as the target of isoniazid and ethionamide, which are first- and second-line tuberculosis drugs (1), as well as a universal bacterial target for triclosan, a consumer antimicrobial agent (13, 18–21, 28). The fungal metabolites cerulenin and thiolactomycin target the condensing enzymes of the bacterial FASII pathway (10, 17, 22, 24, 25). Two studies have shown that 5-chloro-pyrazinamide (5-Cl-PZA) (5, 32) and pyrazinamide (PZA) (32) inhibit *M. tuberculosis* FASI, indicating that FASI is also a drug target.

The use of PZA, a FASI inhibitor, in tuberculosis chemotherapy has greatly reduced the length of treatment necessary to cure a patient (3, 27). Therefore, new FASI inhibitors could be useful tools for treating tuberculosis. In order to develop a system that allows quick purification of the large quantities of *M. tuberculosis* FASI necessary for drug testing or structure-function studies, we constructed a recombinant *M. smegmatis* strain in which the native *fas1* gene was deleted and replaced with the *M. tuberculosis fas1* gene. In the course of analyzing this recombinant *M. smegmatis* Δ*fasI* (*attB*::*M. tuberculosis fas1*) strain, which was designated mc^{2}2700, we studied the in vivo elongation of $C_{16:0}$ by mc^{2}2700. The data which are presented in this paper allowed us to challenge the concept that

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TABLE 1. *M. smegmatis* strains used in this study

M. smegmatis strain	Genotype	Reference
mc^{2}155	*ept-1*	26
mc^{2}2670	*ept-1 attB*::pYUB412::*fas1*(H37Rv)	32
mc^{2}2700	*ept-1* Δ*fas1 attB*::pYUB412::*fas1*(H37Rv) *aph*	This study

$C_{16:0}$ elongation to produce $C_{24:0}$ in *M. smegmatis* or to produce $C_{26:0}$ in *M. tuberculosis* is FASI dependent, as described previously (4, 15, 23).

MATERIALS AND METHODS

Bacterial strains and media. The *M. smegmatis* strains used in this study are described in Table 1. The strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (vol/vol) ADS enrichment (50 g of albumin, 20 g of dextrose, and 8.5 g of sodium chloride in 1 liter of water), 0.2% (vol/vol) glycerol, and 0.5% (vol/vol) Tween 80 or in Mueller-Hinton broth (Difco) supplemented with 0.5% (vol/vol) Tween 80. The solid media used were the media described above with 1.5% (wt/vol) agar added.

MIC determination. The MIC was determined by a broth macrodilution test with an inoculum containing 10^5 CFU of each strain per ml in either Middlebrook 7H9 or Mueller-Hinton broth. The concentrations of 5-Cl-PZA used in these assays were 60, 40, 30, 25, 20, 10, and 5 μg/ml; a preparation containing no drug was used as a control. The assay was terminated for each strain independently when the optical density at 600 nm (OD_{600}) in the no-drug tube reached 1 to 1.2. The MIC was defined as the first concentration of drug which resulted in no visible growth (OD_{600}, <0.02).

Determination of generation time. Each strain was grown to an OD_{600} of 0.5 in Middlebrook 7H9 broth as described above and was diluted 10-fold to obtain the starting inoculum. The OD_{600} of each culture was determined every 120 min for 16 h. In addition, aliquots of each culture were taken at every other time point and plated to determine the number of CFU. The generation time was obtained by plotting the growth curves resulting from both the OD_{600} and CFU values.

Transformation experiments. *M. smegmatis* strains grown at 37°C to the mid-log phase (OD_{600}, ~0.7) were washed twice with 10% cold glycerol and resuspended in cold 10% glycerol (1 ml). A plasmid (1.5 μl) was added to each cold cell suspension (150 μl) to transform the strain, and the mixture was electroporated by using the following parameters: 2.5 V, 25 μF, and 1,000 Ω. Middlebrook 7H9 broth (1 ml) was added to the suspension, which was incubated at 37°C for 2 h before plating.

Deletion of *M. smegmatis fasI* by specialized transduction. The allelic exchange substrate was formed by amplification of the 5′ and 3′ flanking regions of *M. smegmatis fas1* from *M. smegmatis* genomic DNA. A 850-bp 5′ flanking region from nucleotide 386 of *M. smegmatis fas1* to a noncoding sequence upstream from the *fas1* start codon and a 739-bp 3′ flanking region from nucleotide 7384 of *M. smegmatis* to nucleotide 8123 of *M. smegmatis fas1* were amplified (GenBank accession number AY205337). The amplified flanking regions were cloned with a TA cloning kit into the PCR2.1 vector (Invitrogen) for sequencing and subsequent cloning. The substrate used for allelic exchange was formed by cloning the 5′ and 3′ flanking regions of *fas1* described above into pJSC285, a cloning vector containing a bacteriophage lambda *cos* site, a *Pac*I site, and a kanamycin resistance gene flanked by resolvase sites. The allelic exchange substrate on plasmid pYUB978 was packaged into phMSG104 (11) to create phAE978, as previously described (2, 11). phAE978 was used to transduce mc^{2}155 and mc^{2}2670 (32).

Transduction of *M. smegmatis* strains. *M. smegmatis* bacilli were grown to an OD_{600} of 0.8 in Luria-Bertani medium supplemented with 0.2% glycerol and 0.1% Tween 80. The cultures (10 ml) were washed twice in mycobacteriophage buffer (50 mM Tris HCl [pH 7.6], 150 mM NaCl, 10 mM, $MgCl_2$, 2 mM $CaCl_2$) to remove any traces of the Tween 80 detergent. Adsorption of phAE978 mycobacteriophage to the washed bacilli was carried out at a multiplicity of infection of 10, and the mixture was incubated at 37°C, the nonpermissive temperature, for 30 min. The bacilli were pelleted, plated on Luria-Bertani medium plates containing 25 μg of kanamycin per ml, and incubated at 37°C. Kanamycin-resistant colonies were screened for allelic exchange by Southern blotting by using PCR products from both *M. smegmatis* and *M. tuberculosis fas1* as probes.

Radiolabeling of fatty acids with [1-^{14}C]acetate and analysis by HPLC. Cultures of *M. smegmatis* strains (25 ml) were grown at 37°C to the mid-log phase (OD_{600}, ~0.8) in Middlebrook 7H9 broth and were labeled with [1-^{14}C]acetate (0.3 μCi/ml) for 1 h. Cell pellets were saponified by using a 25% methanolic KOH solution for 3 h at reflux and then acidified. The fatty acids were extracted with chloroform and derivatized to UV-absorbing *p*-bromophenacyl fatty acids (30) by using an Alltech kit (catalog no. 18036). The *p*-bromophenacyl fatty acid esters were then analyzed by high-performance liquid chromatography (HPLC) by using a reverse-phase C_{18} column, a diode array detector, and an IN/US β-RAM flowthrough beta-gamma radiation detector. The elution system was CH_3CN-H_2O (83/17, vol/vol) for 20 min, followed by a linear increase to 100% CH_3CN for 2 min and then 100% acetonitrile for another 16 min (flow rate, 2 ml/min). Use of this system resulted in separation of saturated and unsaturated fatty acids (from $C_{12:0}$ to $C_{26:0}$). The chromatogram's peaks were identified by comparison with the chromatograms of *p*-bromophenacyl fatty acid ester standards (30).

Nucleotide sequence accession number. The nucleotide sequence of the *M. smegmatis* fatty acid synthase gene (*fas1*) and the ACP synthase gene (*acpS*) have been deposited in the GenBank database under accession number AY205337.

RESULTS

Construction of mc^{2}2700, an *M. smegmatis* Δ*fas1* mutant containing the *M. tuberculosis fasI* homologue. Since fatty acid synthases are essential for bacteria, we hypothesized that *fas1* is an essential gene for mycobacteria. To delete the *M. smegmatis fas1* gene, we first generated mc^{2}2670 (Table 1), an mc^{2}155 *M. smegmatis* derivative containing the *M. tuberculosis fas1* gene integrated into the *attB* site, by using pYUB970, a site-specific integrating cosmid (from a genomic library of *M. tuberculosis* H37Rv) bearing *M. tuberculosis fas1* and its 5′ and 3′ flanking regions. The native *M. smegmatis fas1* gene was then deleted by specialized transduction (2). The allelic exchange substrate was formed by amplification of the 5′ and 3′ flanking regions of *M. smegmatis fas1* from *M. smegmatis* genomic DNA. Kanamycin-resistant (Kan^r) transductants were observed for mc^{2}2670 but not for wild-type strain mc^{2}155; these results were the results expected for an essential gene that was found to be a drug target (5, 32). Kan^r colonies were screened for allelic exchange by Southern blotting by using PCR products from both *M. smegmatis* and *M. tuberculosis fas1* as probes. The Kan^r colonies were found to contain a shifted band that was consistent with allelic exchange with a Δ*fas1* Kan^r construct, as shown in Fig. 1; this confirmed construction of mc^{2}2700, an *M. smegmatis* strain expressing *M. tuberculosis fas1* (Table 1).

The ability to exchange *M. smegmatis* FASI for the *M. tuberculosis* homologue provided an improved expression system with a rapidly growing, nonpathogenic mycobacterium strain. Moreover, it has been shown previously that *M. smegmatis*, unlike *M. tuberculosis* and *M. bovis* BCG, tolerates multiple copies of *M. tuberculosis fas1* (32). Thus, overexpression of *M. tuberculosis* FASI under different promoters is feasible in *M. smegmatis* and could be followed by allelic exchange of the *M. smegmatis fas1* gene by using the transduction system which we developed, as shown by the formation of strain mc^{2}2700.

Growth characteristics and 5-Cl-PZA susceptibility of mc^{2}2700. Strain mc^{2}2700 differs from the parental *M smegmatis* strains in several interesting properties. First, mc^{2}2700 was found to have a longer generation time (250 min in broth, compared to 210 min for wild-type strain mc^{2}155 or mc^{2}2670). The generation time was determined by measuring the OD_{600} and counting the actual CFU during exponential growth in Middlebrook 7H9 medium and then plotting the resulting growth curves. The slow-growth phenomenon was independent of the medium used. A lower growth rate was also observed in

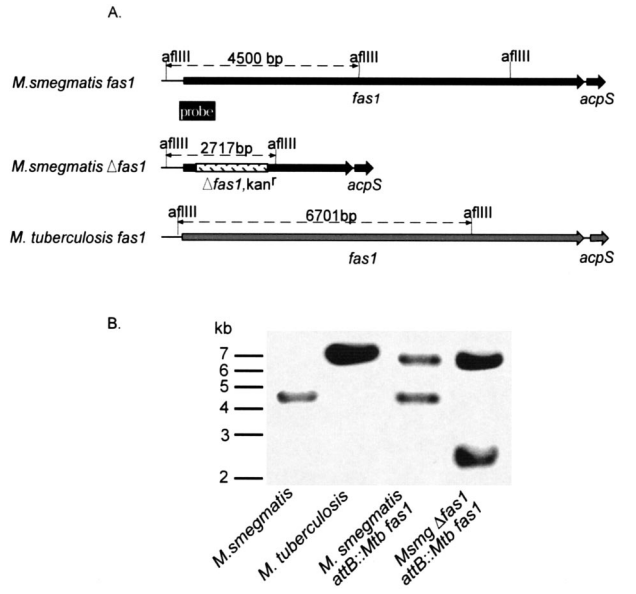

FIG. 1. Allelic exchange in mc^{2}2670, a *fas1* merodiploid strain of *M. smegmatis*, results in mc^{2}2700, an *M. smegmatis* strain bearing *M. tuberculosis fas1*. (A) Map of the *fas1* genomic region of wild-type and mutant strains of *M. smegmatis* (following allelic exchange) and wild-type *M. tuberculosis fas1*, showing AflIII sites. (B) Southern blot of AflIII-digested genomic DNA from different strains probed with the fragments shown in panel A. *Mtb*, *M. tuberculosis*; *Msmg*, *M. smegmatis*.

solid media, as single colonies of mc^{2}2700 were visualized 16 h later than single colonies of mc^{2}155 or mc^{2}2670 were visualized.

We compared the susceptibilities of wild-type strain mc^{2}155, strain mc^{2}2670, and strain mc^{2}2700 to 5-Cl-PZA. Previously, FASI was identified as a potential drug target for 5-Cl-PZA in *M. tuberculosis* (32). Another study confirmed that 5-Cl-PZA inhibits mycobacterial FASI (5). The MIC of 5-Cl-PZA were 10 μg/ml for the mc^{2}2700 strain and 25 μg/ml for mc^{2}155 and mc^{2}2670, as previously described (32). The increased susceptibility of mc^{2}2700 to 5-Cl-PZA is consistent with the previous finding that *M. tuberculosis* is more susceptible to 5-Cl-PZA than *M. smegmatis* (9, 32). This finding complements the results of a previous study in which the effect of a multicopy *fas1* gene on 5-Cl-PZA resistance was described (32). Multiple copies of *M. tuberculosis fas1* conferred only modest resistance to 5-Cl-PZA in *M. smegmatis* (there was 2.5-fold increase in the MIC), while a single copy of *M. tuberculosis fas1* (mc^{2}2670) did not confer 5-Cl-PZA resistance in *M. smegmatis*. In contrast, for a diploid strain containing one extra copy of *M. smegmatis fas1*, the MIC of this drug was fivefold greater (32). Taken together, these results show that *M. tuberculosis* is more susceptible to 5-Cl-PZA than *M. smegmatis*. Furthermore, the MICs of cerulenin, a FASI (and possibly FASII) inhibitor (22), and isoniazid, a FASII inhibitor (1, 29, 30), were the same for mc^{2}2700 and the parental *M. smegmatis* strains.

mc^{2}2700, unlike mc^{2}155, produces $C_{26:0}$ fatty acid, but $C_{24:0}$ is still the predominant end product. Previous biochemical studies showed that purified FASI of *M. smegmatis* produces $C_{16:0}/C_{24:0}$, whereas purified FASI from *M. tuberculosis* or *M. bovis* BCG produces $C_{16:0}/C_{26:0}$ (15, 23). To test the activity of

TABLE 2. Percentages of saturated fatty acids (C_{16} to C_{26}) in mc^{2}155, mc^{2}2670, and mc^{2}2700[a]

Strain	% of:					
	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$
mc^{2}155	56.9	9.3	4.4	6.7	18.3	0.0
mc^{2}2670	39.9	11.6	3.6	5.8	17.7	0.0
mc^{2}2700	31.9	29.7	3.4	6.0	11.7	4.8

[a] The cumulative percentages are not 100% because the values for other saturated and unsaturated fatty acids, such as $C_{12:0}$, $C_{14:0}$, and $C_{18:1}$, are not included.

M. tuberculosis FASI in synthesizing C_{16}-$C_{24:0}/C_{26:0}$ fatty acids in vivo, we monitored [1-^{14}C]acetate incorporation into lipids in wild-type strain mc^{2}155, strain mc^{2}2670 (*M. smegmatis* [*attB*::*M. tuberculosis fas1*]), and mc^{2}2700 (*M. smegmatis* Δ*fas1* [*attB*::*M. tuberculosis fas1*]). This experiment was repeated three times, and the results of a typical experiment are shown in Table 2 and Fig. 2.

We found that the characteristic $C_{16:0}$-$C_{24:0}$ fatty acid bimodal distribution in *M. smegmatis* was similar for wild-type strain mc^{2}155 and the merodiploid *fas1* strain mc^{2}2670 (Fig. 2). Surprisingly, the characteristic end product of *M. tuberculosis* FASI, $C_{26:0}$, was not detected in mc^{2}2670, the strain containing both *M. smegmatis fas1* and *M. tuberculosis fas1*. In contrast, mc^{2}2700 produced both $C_{24:0}$ and $C_{26:0}$. However, $C_{24:0}$ was more abundant than $C_{26:0}$ in this strain (Table 2 and Fig. 2) despite the deletion of *M. smegmatis fas1* and replacement of this gene by *M. tuberculosis fas1*. The similarity of the fatty acid profiles of the merodiploid strain of *M. smegmatis* and the wild-type *M. smegmatis* strain is compatible with the dominance of *M. smegmatis fas1* in the host strain over *M. tuberculosis fas1.* The production of larger amounts of $C_{24:0}$ than of $C_{26:0}$ in mc^{2}2700 was unexpected since in vitro studies of *M. smegmatis* and *M. bovis* BCG FASI demonstrated that *M. smegmatis* synthesizes $C_{24:0}$ and *M. bovis* BCG synthesizes $C_{26:0}$ (15, 23). Since the *M. tuberculosis fas1* gene replaced *M. smegmatis fas1* in mc^{2}2700, it was predicted that this strain would have a fatty acid profile similar to that of *M. tuberculosis* or *M. bovis* BCG strains. The presence of larger amounts of $C_{24:0}$ than of $C_{26:0}$ despite the deletion of *M. smegmatis fas1* suggests that $C_{16:0}$ elongation in vivo is not solely determined by FASI activity, as previously shown by in vitro studies in which purified FASI was used (15, 23).

Unlike the predominant synthesis of $C_{24:0}$ over $C_{26:0}$ in mc^{2}2700, the $C_{18:0}/C_{16:0}$ ratio was a direct result of the replacement of *M. smegmatis fas1* by *M. tuberculosis fas1* (Table 2). Introducing *M. tuberculosis fas1* into a mycobacterial strain increased the amount of $C_{18:0}$ synthesized by the strain. The $C_{18:0}/C_{16:0}$ ratios were found to be 0.16 for wild-type *M. smegmatis*, 0.29 for mc^{2}2670, and 0.93 for mc^{2}2700. Previous in vivo studies of *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* showed that the $C_{18:0}/C_{16:0}$ ratios were 0.17 for *M. smegmatis* (30) and 0.6 to 1 for either *M. bovis* BCG (30) or *M. tuberculosis* (32). In vitro studies of FASI activity have shown that *M. bovis* BCG FASI produces more $C_{18:0}$ than $C_{16:0}$ (15), while *M. smegmatis* FASI produces more $C_{16:0}$ than $C_{18:0}$ (23). Thus, our in vivo results for recombinant strain mc^{2}2700 confirmed

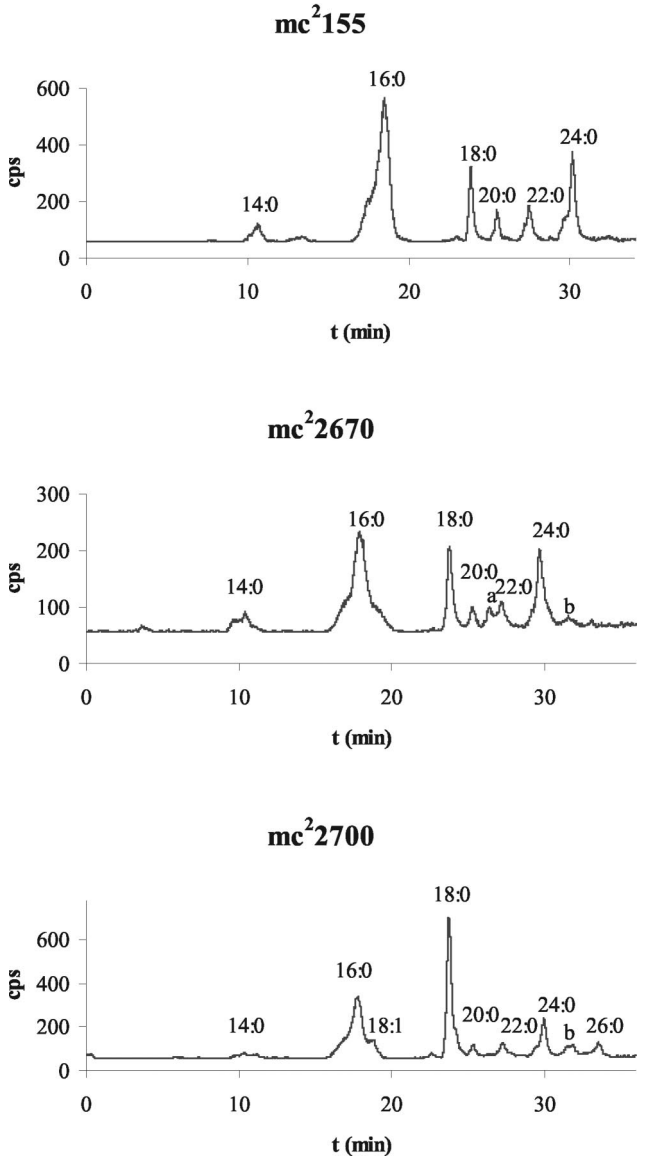

FIG. 2. HPLC analysis of 1-^{14}C-labeled fatty acids extracted from mc^{2}155 and mc^{2}2700 after labeling with [1-^{14}C]acetate for 30 min. The retention times (t) for the *p*-bromophenacyl fatty acid esters under the elution conditions described previously (30) are as follows: $C_{14:0}$, 10.5 min; $C_{16:0}$, 17.8 min; $C_{18:1}$, 18.9 min; $C_{18:0}$, 23.8 min; $C_{20:0}$, 25.3 min; $C_{22:1}$, 26.5 min (peak a); $C_{22:0}$, 27.3 min; $C_{24:0}$, 29.9 min; $C_{26:1}$,31.6 min (peak b); and $C_{26:0}$, 33.6 min.

the previous finding that *M. tuberculosis* FASI generates more $C_{18:0}$ than *M. smegmatis* FASI generates.

DISCUSSION

The profiles of $C_{16:0}$-$C_{24:0/26:0}$ synthesis are different for the wild-type strain of *M. smegmatis* and strain mc^{2}2700. In these strains, the only variable was the FASI system (*M. smegmatis fas1* in mc^{2}155 and *M. tuberculosis fas1* in mc^{2}2700). The observation that mc^{2}2700 and mc^{2}155 synthesize $C_{26:0}$ and $C_{24:0}$, respectively, and the observation that replacement of the *M. smegmatis fas1* gene by the *M. tuberculosis* homologue in *M. smegmatis* does not result in the $C_{16:0}/C_{26:0}$ bimodal fatty acid profile observed in vitro (15) suggest that this fatty acid profile is determined not only by FASI but also by the interaction of FASI with FASII. Previous studies with *M. tuberculosis* FabH, a β-ketoacyl synthase that uses acyl-CoA as its substrate, suggested that this enzymatic system acts as an interface between the type I and type II fatty acid synthase systems by funneling acyl-CoA formed by the FASI system into the FASII elongating acyl-ACP primers (7). We hypothesize that the differences in the fatty acid profiles of mycobacterial species are due to FASI interactions with FabH and FASII. Further studies of *fabH* systems in *M. smegmatis* may delineate the differences in the fatty acid profiles of *M. smegmatis* and *M. tuberculosis.* Moreover, the origin of FASI (*M. smegmatis* or *M. tuberculosis*) seems to play an important role in the amount of $C_{18:0}$ produced by a strain. The $C_{18:0}/C_{16:0}$ ratio found for mc^{2}2700 is similar to the ratio found for *M. tuberculosis* strains, while the $C_{18:0}/C_{16:0}$ ratio found for mc^{2}2670 is only reminiscent of *M. tuberculosis* FASI activity in the *fas1* merodiploid strain.

M. tuberculosis FASI is an essential enzyme and is unique among bacterial species, and therefore it is an attractive drug target. The ability to express *M. tuberculosis fas1* in *M. smegmatis* should provide a relatively rapid purification system for *M. tuberculosis* FASI, as previously described (4, 5, 15), without dependence on expression of the enzyme in *Escherichia coli* (which has been unsuccessful so far). Moreover, it has been shown previously that *M. smegmatis*, unlike *M. tuberculosis* and *M. bovis* BCG, tolerates multiple copies of *M. tuberculosis fas1* (32). Repeated attempts to transform the slowly growing mycobacteria *M. bovis* BCG and *M. tuberculosis* with multiple copies of *fas1* yielded no colonies, while *M. smegmatis* was readily transformed with multiple copies of *fas1* (32). Thus, *M. smegmatis* is an optimal bacterial system for overexpression of *M. tuberculosis* FASI. The specialized transduction system for *M. smegmatis fas1* described here should allow us to replace *M. smegmatis* FASI with overexpressed *M. tuberculosis* FASI under different promoters, which should facilitate large-scale purification. Although the FASI proteins from *M. tuberculosis* and *M. smegmatis* exhibit 90% homology, it is preferable to study the FASI protein from the pathogenic organism *M. tuberculosis*, especially if structure-function studies leading to rationale drug design for *M. tuberculosis* FASI inhibitors are planned. *M. tuberculosis* FASI studies should lead to development of new analogs of PZA and new classes of effective FASI inhibitors. In addition, the concern about emergence of drug resistance when universal bacterial sites are targeted should be eliminated as the FASI site is a unique site in bacteria. Improved agents that exhibit activity against mycobacterial FASI may provide new options for treating *M. bovis*, *Mycobacterium avium*, and drug-resistant *M. tuberculosis* infections.

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