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The Pks13/FadD32 Crosstalk for the Biosynthesis of Mycolic Acids in *Mycobacterium tuberculosis**[§]

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The last steps of the biosynthesis of mycolic acids, essential and specific lipids of *Mycobacterium tuberculosis* and related bacteria, are catalyzed by proteins encoded by the *fadD32-pks13-accD4* cluster. Here, we produced and purified an active form of the Pks13 polyketide synthase, with a phosphopantetheinyl (P-pant) arm at both positions Ser-55 and Ser-1266 of its two acyl carrier protein (ACP) domains. Combination of liquid chromatography-tandem mass spectrometry of protein tryptic digests and radiolabeling experiments showed that, *in vitro*, the enzyme specifically loads long-chain 2-carboxyacyl-CoA substrates onto the P-pant arm of its C-terminal ACP domain via the acyltransferase domain. The acyl-AMPs produced by the FadD32 enzyme are specifically transferred onto the ketosynthase domain after binding to the P-pant moiety of the N-terminal ACP domain of Pks13 (N-ACP_{Pks13}). Unexpectedly, however, the latter step requires the presence of active FadD32. Thus, the couple FadD32-(N-ACP_{Pks13}) composes the initiation module of the mycolic condensation system. Pks13 ultimately condenses the two loaded fatty acyl chains to produce α -alkyl β -ketoacids, the precursors of mycolic acids. The developed *in vitro* assay will constitute a strategic tool for antimycobacterial drug screening.

Mycolic acids, α -branched and β -hydroxylated fatty acids of unusual chain length (C30–C90), are the hallmark of the *Corynebacterineae* suborder that includes the causative agents of tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*). Members of each genus biosynthesize mycolic acids of specific chain lengths, a feature used in taxonomy. For example, *Corynebacterium* holds the simplest prototypes (C32–C36), called “corynomycolic acids,” which result from an enzymatic condensation between two regular size fatty acids (C16–C18). In contrast, the longest mycolates (C60–C90) are the products of condensation between a very long meromy-

colic chain (C40–C60) and a shorter α -chain (C22–C26) (1). These so-called “eumycolic acids” are found in mycobacteria and display various structural features present on the meromycolic chain. Eumycolic acids are major and essential components of the mycobacterial envelope where they contribute to the formation of the outer membrane (2, 3) that plays a crucial role in the permeability of the envelope. They also impact on the pathogenicity of some mycobacterial species (4).

The first *in vitro* mycolate biosynthesis assays have been developed using *Corynebacterium* cell-wall extracts in the presence of a radioactive precursor (5, 6) and have brought key information about this pathway. Yet, any attempt to fractionate these extracts to identify the proteins involved has ended in failure. Later, enzymes catalyzing the formation of the meromycolic chain and the introduction of functions have been discovered with the help of novel molecular biology tools (for review, see Ref. 1), culminating with the identification of the putative operon *fadD32-pks13-accD4* that encodes enzymes implicated in the mycolic condensation step in both corynebacteria and mycobacteria (see Fig. 1) (7–9). AccD4, a putative carboxyltransferase, associates at least with the AccA3 subunit to form an acyl-CoA carboxylase (ACC)³ complex that most likely activates, through a C₂-carboxylation step, the extender unit to be condensed with the meromycolic chain (see Fig. 1). In *Corynebacterium glutamicum*, the carboxylase would metabolize a C16 substrate (8, 10), whereas in *M. tuberculosis* the purified complex AccA3-AccD4 was shown to carboxylate C24–C26 acyl-CoAs (11). Furthermore, FadD32, predicted to belong to a new class of long-chain acyl-AMP ligases (FAAL) (12), is most likely required for the activation of the meromycolic chain prior to the condensation reaction. At last, the *cmrA* gene controls the reduction of the β -keto function to yield the final mycolic motif (13) (see Fig. 1).

Although the enzymatic properties of the ACC complex have been well characterized (9, 11), those of Pks13 and FadD32 are poorly or not described. Pks13 is a type I polyketide synthase (PKS) made of a minimal module holding ketosynthase (KS),

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³ The abbreviations used are: ACC, acyl-CoA carboxylase; ACP, acyl carrier protein; AT, acyl transferase; C-ACP, C-terminal ACP domain; KS, ketosynthase; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; N-ACP, N-terminal ACP domain; NRPS, nonribosomal peptide synthase; NRPS-PKS, hybrid NRPS-PKS system; PKS, polyketide synthase; P-pant, 4'-phosphopantetheinyl; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; WT, wild type; GC-MS, gas chromatography-mass spectrometry.

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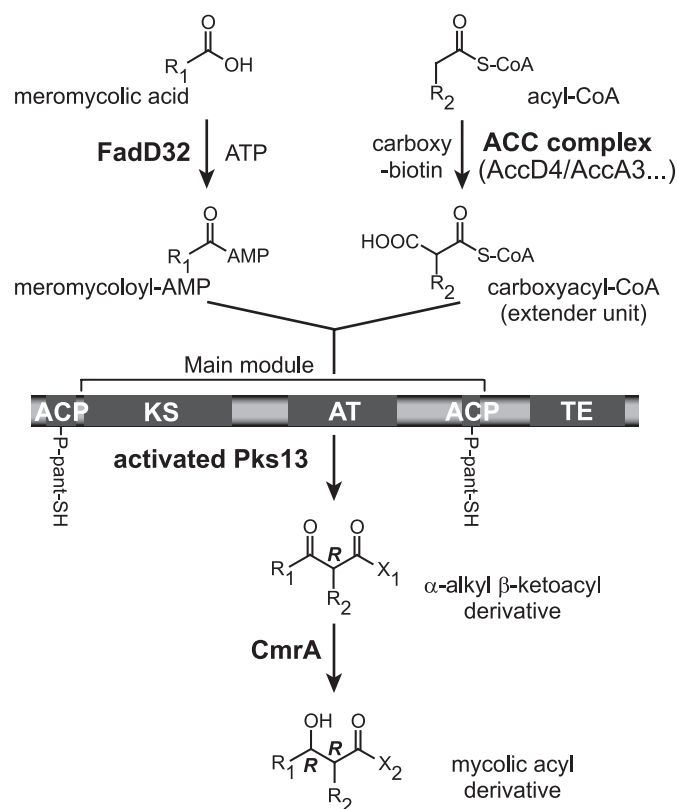


FIGURE 1. **Proposed scheme for the biosynthesis of mycolic acids.** The asymmetrical carbons of the mycolic motif have a R,R configuration. R_1 -CO, meromycolic chain; R_2 , branch chain. In mycobacteria, R_1 -CO = C40-C60 and R_2 = C20-C24; in corynebacteria, R_1 -CO = C16-C18 and R_2 = C14-C16; X_1 , unknown acceptor of the mycolic α -alkyl β -ketoacyl chains; X_2 , unknown acceptor of the mycolic acyl chains.

acyltransferase (AT), and acyl carrier protein (ACP) domains, and additional N-terminal ACP and C-terminal thioesterase domains (Fig. 1). Its ACP domains are naturally activated by the 4'-phosphopantetheinyl (P-pant) transferase PptT (14). The P-pant arm has the general function of carrying the substrate acyl chain via a thioester bond involving its terminal thiol group. In the present article we report the purification of a soluble activated form of the large Pks13 protein. For the first time, the loading mechanisms of both types of substrates on specific domains of the PKS were investigated. We describe a unique catalytic mechanism of the Pks13-FadD32 enzymatic couple and the development of an *in vitro* condensation assay that generates the formation of α -alkyl β -ketoacids, the precursors of mycolic acids.

EXPERIMENTAL PROCEDURES

Miscellaneous—The [1 - 14 C]malonyl-CoA (51.8 mCi/mmol) and [1 - 14 C]palmitic acid (57.5 mCi/mmol) were purchased from PerkinElmer Life Sciences, the [1 - 14 C]methylmalonyl-CoA (55 mCi/mmol) and C8, C12, and C14 saturated [1 - 14 C]fatty acids (55 mCi/mmol) from ARC, the [1 - 14 C]acetyl-CoA (51 mCi/mmol) from Amersham Biosciences.

Protein Production and Purification—The *pks13* (*Rv3800c*) gene of *M. tuberculosis* H37Rv was cloned into plasmid pET-26b (Novagen) upstream from a His tag coding sequence as previously described (14). The *sfp* gene was amplified by PCR

from pUC8Sfp vector (15) and inserted into pET-26b, downstream from the *T7* promoter. The resulting vector was digested with BglII and BamHI and the DNA fragment containing the *sfp* gene, plus 108 bp of sequence upstream from the start codon, a region carrying the *T7* promoter region, was inserted into the BclI site of pLysS (Novagen) to give pLSfp. *Escherichia coli* BL21(DE3) strain (Novagen) was transformed by both constructs and grown in 50-ml cultures at 37 °C in Luria Broth medium supplemented with 50 μ g/ml kanamycin and 25 μ g/ml chloramphenicol. At an A_{600} of 0.5, expression of both *pks13* and *sfp* genes was induced by adding 0.2 mM isopropyl 1-thio- β -D-galactopyranoside and a further 4-h incubation at 30 °C. Cells were collected by centrifugation, resuspended in 5 ml of lysis buffer (50 mM Tris/HCl, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) Triton X-100, pH 8.0), and stored at -80 °C. After thawing at room temperature, the suspension was sonicated using a Vibra cell apparatus (Bioblock Scientific), three times for 30 s (microtip 4, 50% duty cycle), in ice. After centrifugations at $12,100 \times g$ for 15 min and $43,700 \times g$ for 45 min, the cleared lysate was applied to a 1-ml HisTrapTM HP column (GE Healthcare) connected to an ÄKTA purifier (GE Healthcare) and equilibrated with 10 mM imidazole in 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride (buffer A). After extensive washes at 10 and 45 mM imidazole, Pks13 was eluted at 160 mM imidazole in buffer A. Fractions containing high concentrations of the protein were identified by SDS-PAGE, pooled, and applied to a HiLoad 16/60 Superdex 200 pg using 50 mM Tris/HCl, 50 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, pH 8.0, as elution buffer. After SDS-PAGE analysis, fractions corresponding to the second chromatographic peak and holding soluble activated Pks13 were pooled, concentrated by ultrafiltration, and stored at -20 °C in the presence of 50% (v/v) glycerol. FadD32 protein of *M. tuberculosis* H37Rv and *maMig* protein of *Mycobacterium avium* were produced and purified as described previously (12, 16). The *fadD26* gene was amplified by PCR from *Mycobacterium bovis* BCG genomic DNA. The PCR product was purified, digested with NdeI and HindIII endonuclease restriction enzymes, and cloned between the NdeI and HindIII sites of the pET26b vector (Novagen), upstream from a His tag coding sequence and under control of the *T7* promoter. The *E. coli* BL21(DE3) strain (Novagen) was transformed by the construct and grown in 250-ml cultures at 20 °C in autoinducing medium (17) supplemented with 50 μ g/ml kanamycin during 72 h. Cells were collected by centrifugation, washed in 50 mM Tris, pH 8, 300 mM NaCl, and 10% glycerol, resuspended in 25 ml of lysis buffer (0.75 mg/ml lysozyme, 50 mM Tris, pH 8, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride), and stored at -80 °C. The bacterial suspension was thawed at room temperature and sonicated using a Vibra cell apparatus (Bioblock Scientific) (3 pulses of 20 s, microtip 4, 50% duty cycle) in ice. After centrifugation (30 min at $20,000 \times g$), the cleared lysate was filtered on 0.2- μ m membrane (Millipore) and applied to an HisTrapTM HP (1 ml) column connected to an ÄKTA purifier (GE Healthcare) and equilibrated with 10 mM imidazole in buffer A. After washes at 50 and 100 mM imidazole, FadD26 was eluted at 250 mM imidazole in buffer A. Fractions containing high concen-

TABLE 1
Mass spectrometric analyses of Pks13 after tryptic digestion

Name (residues)	Domain		Ligand Type	Monoisotopic theoretical mass (Da)	Ligand modified tryptic fragment		Catalytic amino acid ^b
	Tryptic peptide ^a	Monoisotopic theoretical mass (Da)			Monoisotopic theoretical mass (Da)	Monoisotopic experimental mass (Da)	
N-ACP (25–91)	38–56	2000.9725	P-pant	340.0858	2341.0583	2341.0794	Ser-55
C-ACP (1236–1282)	1238–1271	3681.9038	P-pant	340.0858	4021.9896	4022.0264	Ser-1266
AT (713–1039)	794–817	2305.2066	P-pant + carboxy-C16	622.3053	4304.2091	4304.2269	Ser-801
			Carboxy-C16		2305.2066	2305.2000	
KS (117–542)	270–302	3567.7457	C12	182.1671	2587.4261	2587.4400	Cys-287
					3567.7457	3567.7600	
					3749.9128	3749.9364	

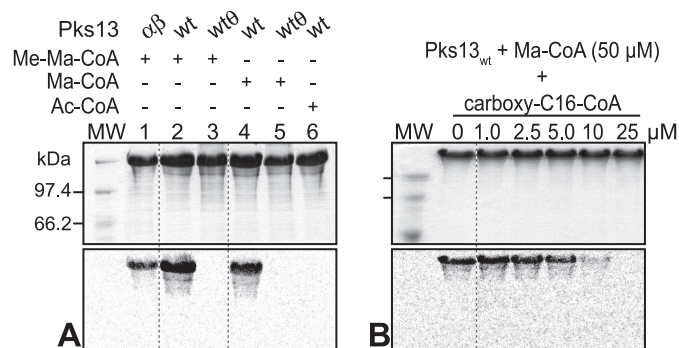
^a Tryptic peptide bearing the suspected catalytic amino acid from the domain.^b Confirmed by MS/MS fragmentation data.

FIGURE 2. Loading of carboxylated chains onto Pks13. SDS-PAGE analyses. Upper, Coomassie Blue staining; lower, phosphorimaging. MW, molecular weight standards. *A*, loading assays of radiolabeled short chain derivatives to Pks13_{wt} or Pks13_{αβ}. Lanes 1–3, [¹⁴C]methylmalonyl-CoA (Me-Ma-CoA) plus lane 1, Pks13_{αβ}; lane 2, Pks13_{wt}; and lane 3, heat-inactivated (θ) Pks13_{wt}; lanes 4 and 5, [¹⁴C]malonyl-CoA (Ma-CoA) plus lane 4, Pks13_{wt}; lane 5, heat-inactivated Pks13_{wt}; lane 6, [¹⁴C]acetyl-CoA (Ac-CoA) plus Pks13_{wt}. The radiolabeling intensity of the Pks13 band as measured by the PhosphorImager was 25,853 (lane 1), 61,118 (lane 2), and 38,491 (lane 4) (in arbitrary units). *B*, competitive loading assays between [¹⁴C]malonyl-CoA (50 μM) and cold carboxy-C16-CoA. The final concentration of the latter compound in each assay is indicated. All the assays were performed at least in triplicate. The dividing lines separate different parts of the same gel.

trations of the protein were identified by SDS-PAGE, pooled, concentrated by ultrafiltration, and stored at -20°C in the presence of 50% (v/v) glycerol.

Substrate Synthesis—The synthesis of carboxyacyl-CoAs from carboxy acids and CoASH, via the synthesis of intermediate monothiophenyl-alkylmalonates, was adapted from a previous report (18). In the case of long chain (C16) substrates, the formation of CoA derivatives required the addition of 50% (v/v) tetrahydrofuran in the reaction medium. Carboxy acids were synthesized based on a previously described procedure (19). Carboxyacyl-CoAs were purified by HPLC using a column Nucleosil 100-5-C18 (250 × 8 mm, Bischoff chromatography SNC) and the following elution program: 2 min at 60% CH₃OH, 10 min gradient up to 100% CH₃OH, 10 min at 100% CH₃OH. The HPLC grade purity of carboxyacyl-CoAs was 99%. The synthesis of acyl-AMPs was performed by reaction of AMP with a mixed anhydride formed between the fatty acid (C12 or C16) and ethylchloroformate, adapted from a previously described protocol (20) where CoASH was replaced by AMP. After adding distilled water, the acyl-AMP was precipitated at acidic pH and the precipitate was washed with CHCl₃. The intermediates and products of both syntheses were analyzed by

¹H NMR spectroscopy, GC-MS, and matrix-assisted laser desorption ionization time-of-flight MS.

Ligand Loading and Condensation Assays—The condensation assays were performed in the presence of 40 μM C12 or C16 [¹⁴C]fatty acid and 40 μM carboxy-C_x-CoA ($x = 2, 3, 8, 12, \text{ or } 16$) in the following standard medium: 50 mM Hepes, pH 7.2, 8 mM MgCl₂, 2 mM ATP, 7 mM glucose, and 7 mM trehalose. Reactions (total volume: 20 μl) were started by the addition of 1 μM FadD32 (or 2 μM FadD26) and 2.6 μM Pks13 and incubated for 6 h at 30 °C. The reaction media were treated and analyzed by TLC as described below. The ligand loading assays (total volume: 10–15 μl) were performed in the standard medium used for condensation assays, but at pH 8.0. The ligand (CoA derivative or carboxylic acid) was used at 50 μM. After the addition of either Pks13 alone (for CoA derivatives) or both FadD32 and Pks13 (for carboxylic acids) at the same concentrations as in the condensation assays, media were incubated for 1 h at 30 °C. The loading assays in the presence of different FadD enzymes were performed in the same conditions with 3 μM FadD32, 1.4 μM FadD26, or 2 μM *ma*Mig. The FAAL activities were evaluated after a 1-h incubation by TLC analysis (see below) prior to the addition of Pks13 (supplementary Fig. S4). When needed, after this 1-h incubation, FadD32 was inactivated at 100 °C for 10 min and the sample was analyzed by radio-TLC to check that acyl-AMP persisted after heating. The samples were separated by SDS-PAGE (12% polyacrylamide) and analyzed by phosphorimaging (Variable Mode Imager Typhoon TRIO, Amersham Biosciences) and Coomassie Blue staining. All the assays were performed at least in duplicate.

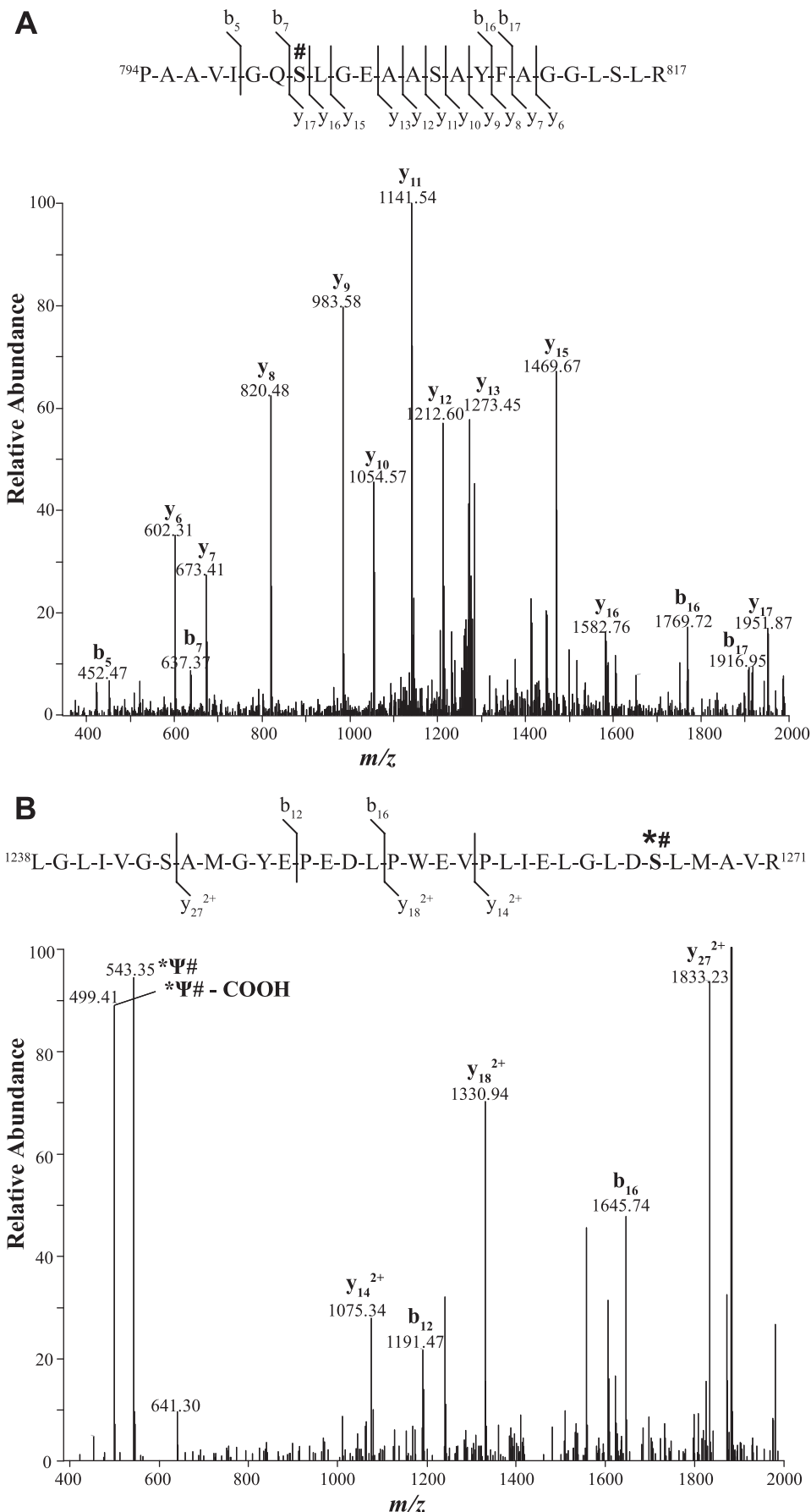
For MS analyses, condensation and loading assays were realized with cold substrates only, at 40 μM C12 fatty acid and/or 5 μM carboxy-C16-CoA, in the presence of 5.4 μM Pks13 with/without 1 μM FadD32 (stocked without glycerol) in the standard medium. After incubation at 30 °C for 6 h, samples were frozen at -20°C . In all condensation and loading assays, the solvent from the substrate solutions was evaporated before the addition of other reagents.

Treatment of Condensation Assays—For alkaline methanolysis, 500 μl of 1 M sodium methanolate was added to the assay after complete drying of the reaction medium and the mixture was incubated in a sealed tube for 30 min at 37 °C. For reduction, the dried sample was incubated at room temperature for 1 h in the presence of 100 μl of tetrahydrofuran and 500 μl of 0.5 M NaBH₄ in ethanol. Both types of reaction were stopped by the

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addition of acetic acid, and the solvent was evaporated. The residue was dissolved in 2 ml of water, and lipid compounds were extracted with diethyl ether and washed with water.

Preparation of Cell-wall Extracts of *Corynebacterium* and Mycolic Acid Biosynthesis Assays—*C. glutamicum* WT (ATCC13032 RES) and its isogenic $\Delta fadD32::km$ and $\Delta accD4::km$ (8) mutants were grown in BHI medium (Difco) supplemented with 25 $\mu\text{g/ml}$ kanamycin and the cultures were stopped at the mid-log phase of growth. Bacteria were harvested by centrifugation, washed, and suspended in 50 mM potassium phosphate buffer, pH 6.5, containing 3 mM β -mercaptoethanol (buffer B). The bacteria were disrupted by one passage through a cell disrupter (One shot cell disrupter, Constant Systems LTD) at 2.6 kbar. The suspension was centrifuged at $3,000 \times g$ for 15 min and the fluffy upper-layer material, corresponding to the cell-wall extract (21), was taken off and homogenized in buffer B. The protein concentration in the extract was determined using the Bio-Rad DC kit (Bio-Rad), after boiling samples for 10 min in 0.5 M NaOH to solubilize insoluble proteins. Mycolic acid biosynthesis assays were performed in the following standard medium: 100 mM potassium phosphate buffer, pH 7.0, 7 mM KHCO_3 , 3 mM MgCl_2 , 7 mM glucose, 3 mM ATP, 0.7 mM CoA-SH, and 0.5 mg/ml protein of cell-wall extract in a total volume of 750 μl . The medium was preincubated for 15 min at 30 $^\circ\text{C}$, and was incubated further for 90 min at 30 $^\circ\text{C}$ after adding 10 or 20 μM [$1\text{-}^{14}\text{C}$]palmitate and potentially a second substrate. The latter was either 10–44 μM carboxy-C $_x$ -CoA ($x = 12$ or 16) or 10–20 μM C $_y$ -AMP ($y = 12$ or 16). The acyl-AMP was added in tetrahydrofuran (3.3% final in the reaction). Control assays using the *C. glutamicum* WT extract showed that 3.3% tetrahydrofuran does not impair corynomycolic acid biosynthesis.



Treatment of the Mycolic Acid Biosynthesis Assays—Reactions were stopped by adding 1 volume of 10% KOH (w/v) in CH₃OH: toluene, 8:2, and incubated at 80 °C overnight. After saponification, the medium was acidified with H₂SO₄ and lipids were extracted with diethyl ether, washed with water, dried, and methylated with diazomethane.

Thin Layer Chromatography (TLC) Analyses—The whole lipid extracts from condensation or mycolic acid biosynthesis assays were dissolved in diethyl ether and separated by TLC on Silica Gel G-60 plates eluted with dichloromethane. In the case of the production of acyl-AMP by FAALs, the reactions were stopped with acetic acid (2%) and the media analyzed by TLC on Silica Gel G-60 plates eluted with butan-1-ol:acetic acid: water, 80:25:40. The radiolabeling was detected by phosphorimaging (Variable Mode Imager Typhoon TRIO, Amersham Biosciences) and quantified using the ImageQuant version 5.1 software (GE Healthcare). When indicated (see figures), highly labeled areas of the plates (e.g. origins) were masked with Scotch tape to enhance the signal in the zones of interest. The total lipids were revealed by spraying molybdophosphoric acid (10% in ethanol) and charring or with rhodamine B (0.01%, w/v, in 0.25 M NaH₂PO₄:ethanol, 9:1) and UV light (254 nm).

Nano Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) Analyses—Before MS analysis, the protein samples were precipitated in 10% trichloroacetic acid (w/v) at 0 °C and by centrifugation at 3,000 × g for 15 min at 4 °C, then washed with water. The tryptic digestion was performed by incubating 20 μl of 20 ng/μl Pks13 solution with 10 μl of 100 ng/μl modified sequencing grade trypsin (Promega) in 50 mM ammonium bicarbonate, overnight, at 37 °C. Samples were diluted 100-fold before analysis by nanoLC-MS/MS using an Ultimate3000 system (Dionex) coupled on-line to either a QStar XL (Applied Biosystems) or an LTQ-Orbitrap (Thermo Fisher Scientific) mass spectrometer. Five μl of each sample were loaded onto a C18 column (75 μm inner diameter × 15-cm PepMap, Dionex) equilibrated in 95% solvent A (95% H₂O, 5% CH₃CN, 0.2% formic acid) and 5% solvent B (20% H₂O, 80% CH₃CN, 0.2% formic acid). Peptides were eluted using a 5–50% B gradient for 55 min followed by a 50–95% B gradient for 10 min and an isocratic step at 95% for 5 min, at 300 nl/min flow rate. Data were acquired in a data dependent scan using a 60-s dynamic exclusion window. MS scans were in the 300–2000 *m/z* range. The most intense ions were selected for collision-induced dissociation fragmentation. Mascot Daemon software (version 2.2.0, Matrix Science) was used to perform data base searches in batch mode with all the raw files acquired. A peaklist was created for each sample analyzed and individual Mascot searches were performed for each fraction. Data were searched against the *M. tuberculosis* complex in the Sprot-Trembl_20070201 data base (24,522

sequences). Specificity of tryptic digestion was set for cleavage after Lys or Arg, and two missed trypsin cleavage sites were allowed. All MS/MS spectra of modified peptides of interest were manually validated.

Distance Tree of AT Domains of PKs—The radial distance tree (neighbor joining method; maximum sequence difference: 0.75) was obtained by performing a pBlast alignment (22) using as a probe the AT domain (positions 576 to 1062) of Pks13 of *M. tuberculosis*, with default parameters (100 hits maximum).

RESULTS

Production and Purification of Recombinant *M. tuberculosis* Activated Pks13 Protein—The activated (phosphopantetheinylated) Pks13 protein was produced by coexpressing the *pks13* (*Rv3800c*) gene of *M. tuberculosis* H37Rv, cloned into plasmid pET-26b, with the *sfp* gene encoding the P-pant transferase from *Bacillus subtilis*. *Sfp* was cloned in *trans* into the pLysS vector together with the T7 promoter region of pET26b. *E. coli* BL21(DE3) was co-transformed by both plasmids and the expression of *pks13* and *sfp* was induced in the presence of isopropyl 1-thio-β-D-galactopyranoside. The C-terminal His-tagged Pks13 protein was purified using a two-step chromatography procedure over His-Trap and Superdex-200 columns (supplementary Fig. S1). Only the major peak eluted from gel filtration, but not the shoulder (aggregated Pks13 protein), held the enzyme activity (data not shown). The ESI-MS spectra of the tryptic digestion products of the purified Pks13 showed two fragments of 2341 and 4022 Da, which correspond to N- and C-ACP_{Pks13} tryptic peptides carrying one P-pant arm, respectively (Table 1). ESI-MS/MS analysis of these products confirmed that these arms were covalently linked at positions Ser-55 and Ser-1266 of the ACP domains (supplementary Figs. S2 and S3).

Loading of Carboxyacyl-CoA Molecules to Pks13—To investigate the loading of the extender unit (Fig. 1), Pks13 was first incubated in the presence of commercially available radiolabeled molecules, [1-¹⁴C]malonyl-CoA (2-carboxyacetyl-CoA) and [1-¹⁴C]methylmalonyl-CoA (2-carboxypropionyl-CoA). Both molecules bound to the native enzyme, but the presence of a methyl substituent on C₂ improved the yield of loading (Fig. 2A, lanes 2 and 4), whereas no interaction between the protein and acetyl-CoA could be detected in the conditions used (Fig. 2A, lane 6). Heat inactivation of Pks13 abolished its ligand-loading capacity (Fig. 2A, lanes 3 and 5).

The predilection of Pks13 for longer chain 2-carboxyacyl-CoAs was then evaluated by competitive loading assay. We observed that a concentration of 10 μM cold synthetic carboxy-C16-CoA was sufficient to inhibit 70% of the loading of 50 μM [1-¹⁴C]malonyl-CoA (Fig. 2B). Thus, these data demonstrated (i) that Pks13 does load carboxyacyl-CoA substrates, (ii) that the presence of the carboxyl group plays a key role in this inter-

FIGURE 3. ESI-MS/MS analyses of the covalent binding of the carboxy-C16 chain to AT and phosphopantetheinylated C-ACP domains after tryptic digestion of Pks13. The MS/MS spectra were acquired using an LTQ-Orbitrap mass spectrometer. A, spectrum of the doubly charged ion at *m/z* 1294.7 corresponding to the carboxy-C16-modified AT-(794–817) tryptic peptide. B, spectrum of the triply charged ion at *m/z* 1435.7 corresponding to the carboxy-C16-modified phosphopantetheinylated C-ACP-(1238–1271) tryptic peptide. The fragmentation patterns obtained are indicated above the spectra along with the corresponding tryptic peptide amino acid sequences. In the peptide sequence, S# and S*# indicate the carboxy-C16-modified serine residue and the carboxy-C16-modified phosphopantetheinylated serine residue, respectively. The peaks labeled *Ψ# and *Ψ#-COOH on the spectrum correspond to a diagnostic fragment ion derived from the modified serine residue (P-pant arm without the phosphate moiety and with carboxy-C16 acyl chain) and to its decarboxylated form, respectively.

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action, and (iii) that, expectedly, the loading efficiency increases with the chain length of the ligand.

Binding of the Carboxyacetyl Chain to the AT Domain and Transfer onto the C-terminal ACP Domain—To examine the involvement of P-pant arms in the loading of the carboxyacetyl chain onto Pks13, a form mutated at both positions of P-pant linkage, Pks13 S55A,S1266A (Pks13_{αβ}), was produced and purified. The mutations induced a substantial decrease of labeling of Pks13_{αβ} but, remarkably, part of the [¹⁴C]methylmalonyl-CoA was still tightly associated with this isoform (Fig. 2A, lanes 1). This suggested that the carboxyacetyl chains might be loaded onto the first site before their subsequent transfer onto one of the two ACP domains. The binding sites of the carboxyacetyl-CoAs to Pks13 were sought by ESI-MS after tryptic digestion of the protein. In the absence of ligand, the predicted 794–817 tryptic peptide of 2305 Da holding the AT domain active site was observed (Table 1). In the presence of carboxy-C16-CoA in the assay, an additional peptide of 2587 Da was detected, which corresponds to the addition of a carboxy-C16 chain to the AT-(794–817) tryptic peptide (Table 1). These data demonstrated the formation of a covalent link between the AT domain of Pks13 and a carboxy-C16 chain. MS/MS experiments indicated that the covalent bond involved Ser-801 (Fig. 3A). Most importantly, in the presence of ligand, an additional peptide of 4304 Da was observed, indicating the covalent binding of the carboxy-C16 chain on the P-pant arm of the C-ACP domain (Table 1) at position Ser-1266, as demonstrated by MS/MS (Fig. 3B). Thus, the purified Pks13 protein is able to load the carboxyacetyl chain from a carboxyacetyl-CoA substrate onto its AT domain that then specifically transfers it onto the P-pant arm of the contiguous C-ACP domain.

Loading of Non-carboxylated Acyl Chains by Pks13—To investigate the loading of FadD32 reaction products onto Pks13, recombinant FadD32 protein was first produced and purified (12). Incubation of FadD32 in the presence of a radio-labeled saturated fatty acid (C12 or C16), ATP, and MgCl₂ led to the production of labeled acyl-AMP (supplementary Fig. S4A, lane 3), as expected (12). The loading of FadD32 products onto Pks13 was assayed in coupled reactions with both enzymes, in the presence of [¹⁴C]lauric acid. The labeled acyl chain of the acyl-AMPs formed in the assays tightly associated to Pks13 (Fig. 4A, lane 2). In contrast, the radioactive free acid (in the absence of FadD32) did not bind to the PKS (Fig. 4A, lane 1).

The specificity of binding of non-carboxylated acyl chains to one of the two ACP domains of Pks13 was then determined using isoforms of the enzyme mutated at the phosphopantethenyl sites, *i.e.* Pks13_α (S55A mutant of the N-ACP), Pks13_β (S1266A mutant of the C-ACP), and Pks13_{αβ}. The loading assays in the presence of labeled C16, FadD32, and Pks13 showed a clear specificity for N-ACP_{Pks13}. A significant labeling of Pks13_{wt} and Pks13_β was observed (Fig. 4B, lanes 2 and 4), whereas the weak labeling of Pks13_α and Pks13_{αβ} was at the same level as the control performed with heat-inactivated Pks13_{wt} (Fig. 4B, lanes 1, 3, and 5). Thus, the acyl chains from the acyl-AMPs are ligands of Pks13 and are specifically loaded onto the P-pant arm of its N-ACP domain.

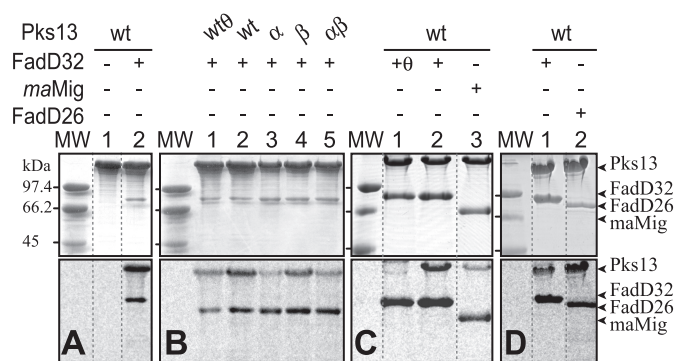


FIGURE 4. Loading of FadD32 reaction products onto Pks13 analyzed by SDS-PAGE. Upper, Coomassie Blue staining; lower, phosphorimaging. MW, molecular weight standards. A, loading of acyl-AMP onto Pks13. [¹⁴C]C12 acid was used as a precursor. Lanes 1, absence of FadD32; lane 2, presence of FadD32. B, loading assays with Pks13 mutant isoforms. [¹⁴C]C16 acid was used as a precursor. Lanes 1, heat-inactivated (θ) Pks13_{wt}; lane 2, native Pks13_{wt}; lane 3, Pks13_α; lane 4, Pks13_β; lane 5, Pks13_{αβ}. C, involvement of FadD32 in the transfer. Pks13 plus lane 1, heat-inactivated FadD32; lane 2, active FadD32; lane 3, active maMig. D, loading assays with different FAALs. [¹⁴C]C12 acid was used as a precursor. Pks13 incubated in the presence of FadD32 (lane 1) and FadD26 (lane 2). All the assays were performed at least in duplicate. The dividing lines separate different parts of the same gel.

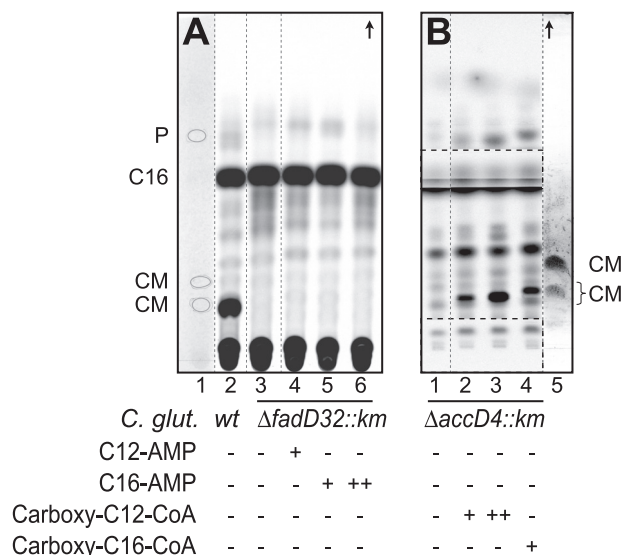


FIGURE 5. In vitro corynomycolic acid biosynthesis assays using cell-wall extracts. All assays were done in duplicate and in the presence of [¹⁴C]C16 acid. The whole lipid extract from each assay was deposited on silica gel TLC eluent, CH₂Cl₂. A, cell-wall extract from *C. glutamicum* (C. glut.) ΔfadD32::km. Lanes 1, mixture of cold palmitone (P) standard and cold racemic C32 corynomycolate (CM) standard (the lower spot is the natural 1R,2R species and the upper spot is the enantiomer 1R,2S species); lane 2, control, cell-wall extract from *C. glutamicum* WT; extract from *C. glutamicum* ΔfadD32::km with, lane 3, no addition; lane 4, addition of 10 μM C12-AMP; lane 5, addition of 10 μM C16-AMP; lane 6, addition of 20 μM C16-AMP. C16, non-incorporated [¹⁴C]C16 methyl ester. Lane 1 was sprayed with rhodamine and spots were visualized by UV detection. Lanes 2–6 were visualized by phosphorimaging. B, cell-wall extract from *C. glutamicum* ΔaccD4::km. Lane 1, no addition, or addition of: lane 2, 10 μM carboxy-C12-CoA; lane 3, 44 μM carboxy-C12-CoA; lane 4, 10 μM carboxy-C16-CoA; lane 5, cold standard racemic mixture of C32 CM. Lanes 1–4 were visualized by phosphorimaging. Lane 5 was revealed by phosphomolybdate spraying. All the assays were performed at least in duplicate. The dividing lines separate different parts of the same plate.

An Additional Function for FadD32?—To evaluate the capacity of Pks13 to metabolize the acyl-AMPs, assays were performed using cell-wall extracts of *C. glutamicum*. Although

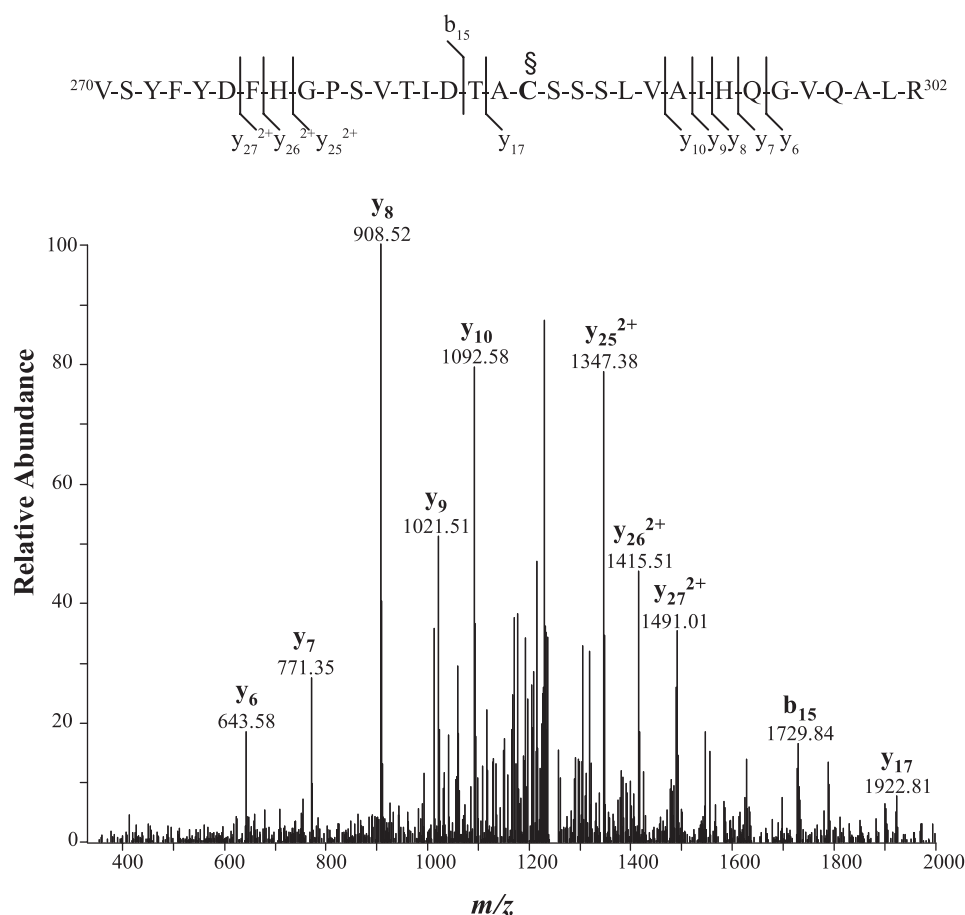


FIGURE 6. ESI-MS/MS analysis of the covalent binding of an acyl chain to the KS domain of Pks13 in the presence of FadD32, after tryptic digestion of Pks13. The spectrum was acquired using an LTQ-Orbitrap mass spectrometer. The MS/MS spectrum of the triply charged ion at m/z 1251.0 corresponds to the C12-modified KS-(270–302) tryptic peptide. The fragmentation pattern obtained is indicated above the spectrum along with the tryptic peptide amino acid sequence. In the peptide sequence, C\$ indicates the C12-modified cysteine residue.

mycolates were produced by the extract of the WT strain incubated in the presence of [$1-^{14}\text{C}$]palmitic acid alone (Fig. 5A, lane 2), no neosynthesized corynomycolic acids could be detected by TLC when the cell-wall extract of either a $\Delta\text{fadD32}::\text{km}$ or a $\Delta\text{accD4}::\text{km}$ strain was used (Fig. 5, A, lane 3, and B, lane 1), in agreement with the involvement of both FadD32 and AccD4 in the mycolic acid biosynthesis pathway (8). This feature was reversed when the extract from *C. glutamicum* $\Delta\text{accD4}::\text{km}$ was incubated in the presence of [$1-^{14}\text{C}$]C16 and cold chemically synthesized carboxy-C12-CoA or carboxy-C16-CoA (Fig. 5B, lanes 2–4). In sharp contrast, the addition of either chemically synthesized C12-AMP or C16-AMP to the extract of *C. glutamicum* $\Delta\text{fadD32}::\text{km}$ did not lead to the formation of corynomycolic acids (Fig. 5A, lanes 4–6). Thus, in the conditions used, exogenous acyl-AMPs were not able to chemically complement the lack of FadD32 protein. This suggested that FadD32 has an additional function with respect to the activation of the meromycolic chains.

FadD32 Promotes the Acyl Chain Transfer from Acyl-AMP onto ACP_{Pks13}—To examine the issue of an additional function for FadD32, [$1-^{14}\text{C}$]C12-AMP was enzymatically synthesized using FadD32. The protein was then either kept active or heat-inactivated, and Pks13 was added. On the polyacrylamide gel,

the native Pks13 protein was tightly associated with the radiolabeled C12-AMP only in the presence of active FadD32 enzyme (Fig. 4C, lane 2). When FadD32 was inactivated, no significant signal could be detected (Fig. 4C, lane 1). This showed that the presence of FadD32 was necessary for the transfer of the acyl chain from the AMP carrier onto Pks13. To determine whether this phenomenon was specific of FadD32, we performed loading assays in which FadD32 was replaced by other FadD enzymes, produced and purified from *E. coli* recombinant strains, either FadD26, another mycobacterial FAAL, or *ma*Mig, a fatty acyl-CoA ligase from *M. avium* (16) used as a FAAL *in vitro*. Indeed, *ma*Mig synthesizes acyl-AMP intermediates in the absence of CoASH (supplementary Fig. S4A, lane 4), like the proteins from the same family (12). A significant labeling of Pks13 was observed in the presence of FadD26 (Fig. 4D, lane 2) but not in that of *ma*Mig (Fig. 4C, lane 3), although the quantity of [$1-^{14}\text{C}$]C12-AMP brought by *ma*Mig in the reaction medium was the same order of magnitude as that given by FadD32 (supplementary Fig. S4A). Thus, these data showed that only certain FadD enzymes are

able *in vitro* to promote the transfer of the acyl chain of acyl-AMP onto N-ACP_{Pks13}.

Transfer of the Non-carboxylated Acyl Chains from N-ACP onto the KS Domain—To investigate the fate of the acyl chain from the acyl-AMP linked to the P-pant arm of the N-ACP domain, a MS analysis of Pks13 was performed after its incubation in the presence of FadD32, C12, and ATP, followed by tryptic digestion. A peptide of 3750 Da was observed, which corresponds to a C12 chain linked to the Cys-287 of the 270–302 tryptic peptide from the KS domain (Table 1 and Fig. 6). This data demonstrated that the non-carboxylated chain first loaded by FadD32 onto the N-ACP domain (as shown above) is subsequently transferred onto the contiguous KS domain of Pks13 (Fig. 1).

In Vitro Biosynthesis of α -Alkyl β -Ketoacids by the Purified Pks13 Enzyme—Numerous unsuccessful assays of *in vitro* biosynthesis of α -alkyl β -ketoacids were attempted prior to the discovery of the requirement of FadD32 in the condensing assays. Accordingly, the Pks13 activity was subsequently assayed by incubating the enzyme in the presence of [$1-^{14}\text{C}$]C16, ATP, MgCl_2 , FadD32, and unlabeled carboxyacyl-CoA. Control experiments lacking one of the enzymes or substrates were also performed. The reaction media were then sub-

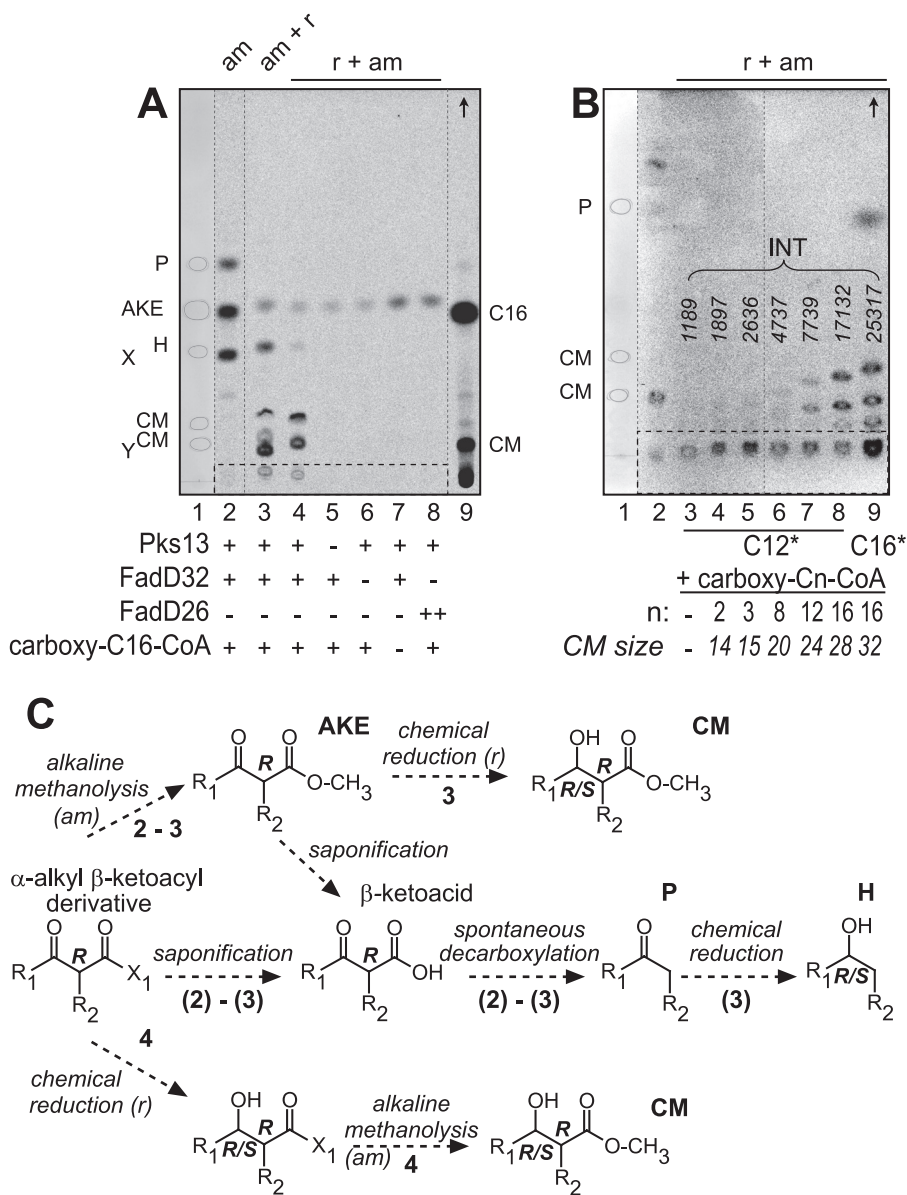


FIGURE 7. *In vitro* condensation reactions in the presence of Pks13 and FadD32. The whole reaction products were deposited on silica gel and analyzed by TLC (eluent: CH_2Cl_2). In both panels, lane 1 was sprayed with rhodamine and the spots were visualized by UV detection; lanes 2–9 were visualized by phosphorimaging. **A**, condensation assays with different treatments. All assays were done in the presence of [^{14}C]C16 and carboxy-C16-CoA. Lanes 1, mixture of cold standards: racemic C32 corynomylates (CM), hentriacontanol (H), C32 α -alkyl β -ketoester (AKE), and palmitone (P); lane 2, alkaline methanolysis (am) of the reaction mixture; lane 3, alkaline methanolysis followed by chemical reduction (am + r); lane 4, reduction followed by alkaline methanolysis (r + am); lane 5, same as lane 4 in the absence of Pks13; lane 6, same as lane 4 in the absence of FadD32; lane 7, same as lane 4 in the absence of carboxy-C16-CoA; lane 8, FadD32 was replaced by 2 eq of FadD26; lane 9, radiolabeled natural CM and C16 methyl ester standards (*C. glutamicum* whole cell extract). The light upper spots in lanes 3–8 may correspond to a fatty acid ester contaminating the [^{14}C]C16 acid stock solution. **B**, impact of the chain length of the substrates. Lane 1, mixture of cold racemic C32 CM and P standards; lane 2, radiolabeled natural CM standards (*C. glutamicum* whole cell extract); lane 3, condensation assay in the absence of carboxyacyl-CoA; lane 4, condensation assay in the presence of carboxy-C₂-CoA; lane 5, carboxy-C₃-CoA; lane 6, carboxy-C₈-CoA; lane 7, carboxy-C₁₂-CoA, lanes 8 and 9, carboxy-C₁₆-CoA. Lanes 3–8, assays in the presence of [^{14}C]C12 acid (C12*); lane 9, in the presence of [^{14}C]C16 acid (C16*). The reaction media were submitted to reduction followed by methanolysis (r + am). The expected carbon number of the CM formed is indicated in *italic*. The total intensities (INT) of the spots in the CM migration area as measured by the phosphorimager are mentioned (in arbitrary units). Zones framed with dotted lines were masked with Scotch tape before screen exposition to improve the detection of spots with weaker radiolabeling. **C**, chemical treatments used for the analysis of the condensation products. The chemical reduction (with NaBH_4) of the α -alkyl β -ketoacyl derivatives gives two mycolic acid diastereoisomers, of configurations *R,R* (the natural one) and *R,S*. *R*-CO, meromycolic chain; *R*₂, branch chain; in palmitone (C31), *R*₁ = C15 and *R*₂ = C14. *X*₁, unknown acceptor of the mycolic α -alkyl β -ketoacyl chains. Chemical reactions were labeled with the corresponding lane number in panel A. All the assays were performed at least in triplicate. The dividing lines separate different parts of the same plate.

mitted to alkaline methanolysis to cleave any potential covalent link formed between putative reaction products and Pks13 or an acceptor molecule. After extraction of the lipid compounds, TLC followed by PhosphorImager analysis revealed the presence of three main spots of radiolabeled compounds. Two spots co-migrated with the C32 α -alkyl β -ketoester (α -myristoyl β -keto stearic acid methyl ester) and palmitone standards (Fig. 7A, lane 2), suggesting that a secondary saponification reaction has occurred and has led to the formation of long chain ketones (Fig. 7C) (23). After reduction by NaBH_4 , these spots disappeared and, concomitantly, two new spots were observed at the same *R_f* as the two synthetic racemic (*1R,2R* and *1R,2S*) C32 corynomylates, and one at a *R_f* similar to that of hentriacontanol (Fig. 7, A, lane 3, and C). The unidentified spot (X) would lead to the formation of a new spot (Y) after reduction. In contrast, solely corynomylates were formed when the reduction step was performed prior to alkaline methanolysis (Fig. 7, A, lane 4, and C). The formation of the radiolabeled species was dependent on the presence of Pks13, FadD32, and carboxyacyl-CoA (Fig. 7A, lanes 5–7), showing that these compounds were the products of enzymatic reactions between the radiolabeled fatty acid and the carboxyacyl-CoA and involving both Pks13 and FadD32. Strikingly, although FadD26 is able to load the acyl moiety of the neo-synthesized acyl-AMP onto Pks13 (as shown above), the condensation did not occur when FadD32 was replaced by FadD26, even using twice the amount of the latter protein (Fig. 7A, lane 8).

Several parameters such as the nature, concentration, and pH of the buffer, and the temperature and time of incubation were varied in the condensation assays. Although the temperature change had very little impact on the reaction, if any (supplementary Fig. S5C), the yield of the condensation reaction was

greatly influenced by the pH, the nature of the buffer, and the incubation time, the optimal conditions being a 6-h incubation in Hepes, pH 7.2, at 30 °C (supplementary Fig. S5). At last, the effect of the chain length of both substrates was examined. The efficiency of the condensation reaction substantially increased with the chain length, up to the longest synthetic substrate used, *i.e.* carboxy- C_{16} -CoA (Fig. 7B, lanes 4–8), which correlated with the observed predilection of the enzyme for long chain carboxy-acyl-CoA in loading assays (see above). In the presence of carboxy- C_{16} -CoA, the $[1-^{14}C]C_{16}$ used by FadD32 gave a better yield than $[1-^{14}C]C_{12}$ (Fig. 7B, lanes 8 and 9). In conclusion, Pks13, solely in the presence of FadD32, is able to synthesize *in vitro* C14–C32 α -alkyl β -ketoesters from two substrates, a carboxylic acid and a 2-carboxyacyl-CoA. The catalytic activity of the enzymatic couple is optimal in the presence of the longest substrates available, which is in agreement with the expected function of these proteins *in vivo*.

DISCUSSION

The present work demonstrated that the activated Pks13 enzyme of *M. tuberculosis*, in adequate experimental conditions, has a condensing activity *in vitro* and is able to synthesize, in coupled reaction with FadD32, the biosynthetic precursors of mycolic acids, α -alkyl β -ketoacids, from a fatty acyl-AMP and a 2-carboxyacyl-CoA (Fig. 8). For a matter of both solubility and availability of radiolabeled molecules, the function of Pks13 was studied in the presence of substrates shorter than its presumed natural substrates (C24–C26 carboxyacyl-CoAs and C40–C60 meromycoloyl-AMP) within mycobacteria. Nevertheless, the fact that Pks13 of *M. tuberculosis* is able to condense relatively short chain substrates (C12, C16), equivalent to those used by the condensing enzyme from *Corynebacterium*, correlates with the production of C32–C34 corynomycolates upon heterologous complementation of a *C. glutamicum pks13* mutant strain by the *M. tuberculosis pks13* gene.⁴ If the Pks13 enzyme from *M. tuberculosis* presents a large specificity toward the chain length of its substrates, the length of the mero and branch chains of mycolic acids might be controlled by the enzymes that activate Pks13 substrates, *i.e.* FadD32 and the acyl-CoA carboxylase complex AccA3–AccD4 (Fig. 1). Consistently, it has been recently shown that AccA3–AccD4 from *M. tuberculosis* exhibits no activity in the presence acyl-CoA shorter than C24–C26, which perfectly matches with the required size of the extender unit during mycolic condensation in this bacterial species (Fig. 1) (11).

Investigation of the different steps involved in condensation resulted in the determination of the general catalytic scheme leading to the formation of the α -alkyl β -ketoacids (Fig. 8). The carboxyacyl-CoA unit is loaded onto Pks13 via its AT domain. The latter has a predilection for both long chain and carboxylated molecules. Pks13 is an unprecedented polyketide synthase because its extender units do not correspond to the short classical units, malonyl-CoA or methyl-malonyl-CoA, used by the other PKSs. The predilection of this enzyme for carboxyacyl-CoAs of unusual chain length is reflected in the primary structure of its AT domain.

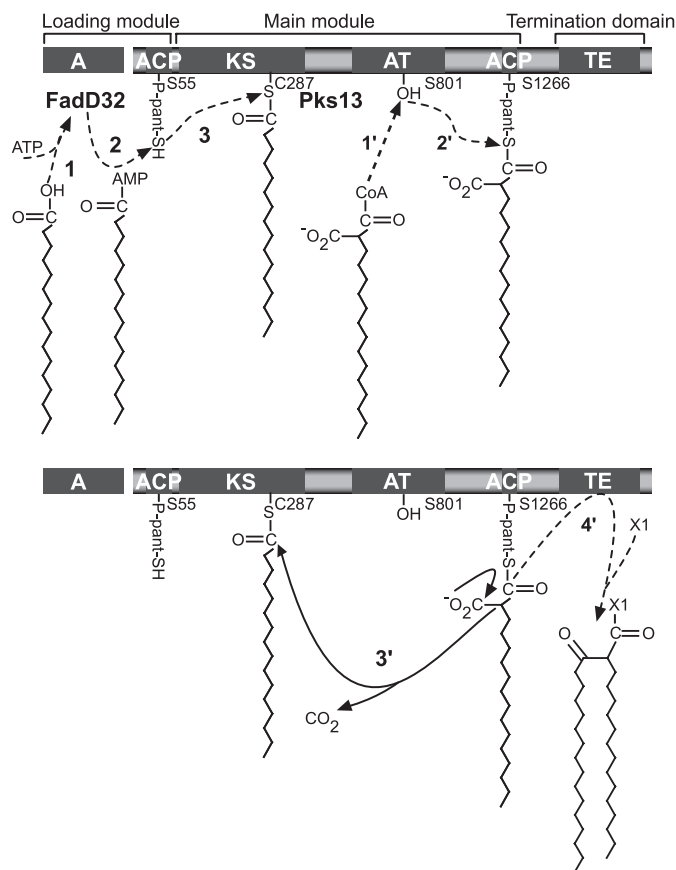


FIGURE 8. Scheme of the stepwise activity of FadD32-Pks13 PKS and its domain organization. To simplify, C16 acyl chains were drawn. FadD32 synthesizes meromycoloyl-AMPs from the meromycolic acids and ATP (1). The meromycoloyl chain of these intermediates is then specifically loaded by FadD32 onto the P-pant arm of the N-ACP domain of Pks13 (2). This is an acyl-AMP/ACP transacylation. The meromycoloyl chain is then transferred onto the KS domain (3). The extender unit carboxyacyl-CoA is specifically loaded onto the AT domain, which catalyzes the covalent attachment of the carboxyacyl chain to its active site (1') and its subsequent transfer specifically onto the C-ACP domain (2'). The KS domain catalyzes the Claisen-type condensation between the meromycoloyl and the carboxyacyl chains to produce a α -alkyl β -ketoacyl chain (3'). Then, it is likely that the thioesterase domain catalyzes the release of the α -alkyl β -ketoacyl chain and its transfer onto an unknown acceptor (X_1) (4'). Thus, FadD32 constitutes one of the two domains of the loading module of the polyketide synthase formed by both FadD32 and Pks13 polypeptides.

Indeed, the latter is relatively distant from the primary structure of the AT domain of other PKSs and fatty acid synthases, as displayed by the distance tree obtained by Blast alignment (supplementary Fig. S6).

Our data showed that the acyl chain of the acyl-AMPs produced by the FadD32 enzyme in the presence of a fatty acid and ATP are specifically transferred onto the N-ACP domain of Pks13 (Fig. 8). There are two possible mechanisms of acylation *in trans*: (i) release of the acyl-AMPs by FadD32 and adventitious acylation of the reactive –SH group of the P-pant arm of N-ACP_{Pks13} and (ii) enzyme-dependent transfer of the acyl chain from AMP to Pks13. The present work demonstrated that the transfer cannot be undertaken by the AT domain of Pks13 *in vitro*, but is dependent upon the presence of active FadD32 that catalyzes a reaction of acyl-AMP/N-ACP_{Pks13} transacylation (Fig. 8). Moreover, using Pks13 mutant proteins, we showed that FadD32 is unable to load an acyl chain onto the

⁴ C. de Sousa d'Auria and C. Houssin, personal communication.

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C-ACP_{Pks13} domain. The selectivity of FadD32 for the N-ACP domain of Pks13 might be facilitated by the substantial sequence divergence (24% identity) between the two ACP-like domains of Pks13. The specificity between an adenylation enzyme and an ACP protein has been described for PKS and NRPS (24, 25). However, to the best of our knowledge, this is the first demonstration of an enzyme-dependent loading mechanism in the case of PKSs. Interestingly, we have observed that FadD32 could not be replaced by FadD26, a mycobacterial FAAL, during the condensation reaction, although FadD26 was able to load the acyl chain of its acyl-AMP products onto Pks13 *in vitro*. A privileged interaction between FadD32 and Pks13 might be required for proper folding of the PKS necessary to the subsequent steps of transfer and catalysis. The fact that the *fadD32* gene is essential in mycobacteria (8) is consistent with our data and proves that none of the FadD enzymes display a redundant activity *in vivo*. One can reasonably propose that, *in vivo*, FadD32 activates the very long meromycolic acids into meromycoloyl-AMPs and transfers the meromycolic acyl chains onto N-ACP_{Pks13}. This is reminiscent of the double function of adenylation and transfer described for some so-called “adenylation domains” found in NRPS or NRPS-PKS enzymes (26). As in the case of these enzymes, one can propose that FadD32 together with N-ACP_{Pks13} correspond to the “loading module” of a PKS formed by both FadD32 and Pks13 (Fig. 8). The discrete FadD32 enzyme is reminiscent of the lone standing salicyl-AMP ligase domains MbtA and YbtE found in the hybrid NRPS-PKSs involved in mycobactin and yersiniabactin biosyntheses, respectively (26, 27). The knowledge of Pks13 enzymatic properties as well as of the experimental conditions for *in vitro* activity assays will now serve as fundamental tools for screening for inhibitors of this very original condensing enzyme that, because of its essentiality in mycobacteria (7) and its characteristic features, represents an excellent target for novel antimycobacterial drug design.

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