The Acyl-AMP Ligase FadD32 and AccD4-containing Acyl-CoA Carboxylase Are Required for the Synthesis of Mycolic Acids and Essential for Mycobacterial Growth

IDENTIFICATION OF THE CARBOXYLATION PRODUCT AND DETERMINATION OF THE ACYL-CoA CARBOXYLASE COMPONENTS*

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Mycolic acids are major and specific long-chain fatty acids of the cell envelope of several important human pathogens such as Mycobacterium tuberculosis, M. leprae, and Corynebacterium diphtheriae. Their biosynthesis is essential for mycobacterial growth and represents an attractive target for developing new antituberculous drugs. We have previously shown that the pks13 gene encodes condensase, the enzyme that performs the final condensation step of mycolic acid biosynthesis and is flanked by two genes, fadD32 and accD4. To determine the functions of the gene products we generated two mutants of C. glutamicum with an insertion/deletion within either fadD32 or accD4. The two mutant strains were deficient in mycolic acid production and exhibited the colony morphology that typifies the mycolate-less mutants of corynebacteria. Application of multiple analytical approaches to the analysis of the mutants demonstrated the accumulation of a tetradeccymalic acid in the ΔfadD32::km mutant and its absence from the ΔaccD4::km strain. The parental corynebacterial phenotype was restored upon the transfer of the wild-type fadD32 and accD4 genes in the mutants. These data demonstrated that both FadD32 and AccD4-containing acyl-CoA carboxylase are required for the production of mycolic acids. They also proved that the proteins catalyze, respectively, the activation of one fatty acid substrate and the carboxylation of the other substrate, solving the long-debated question of the mechanism involved in the condensation reaction. We used comparative genomics and applied a combination of molecular biology and proteomic technologies to the analysis of proteins that co-immunoprecipitated with AccD4. This resulted in the identification of AccA3 and AccD5 as subunits of the acyl-CoA carboxylase. Finally, we used conditionally replicative plasmids to show that both the fadD32 and accD4 genes are essential for the survival of M. smegmatis. Thus, in addition to Pks13, FadD32 and AccD4 are promising targets for the development of new antimicrobial drugs against pathogenic species of mycobacteria and related microorganisms.

Mycolic acids, long-chain 2-alkyl, 3-hydroxy fatty acids, are the hallmark of Corynebacteriniae, a suprageneric actinomycete group that includes corynebacteria, mycobacteria, nocardia, rhodococci, and other related microorganisms. In mycobacteria, these fatty acids, called eumycolic acids, possess a very long chain (C40–C90) and may contain various oxygen functions, cyclopropyl rings, and methyl branches, in addition to the 3-hydroxyl group (1–4). In contrast, mycolic acids found in other genera consist of homologous mixtures of saturated and unsaturated acids and contain shorter chains, e.g. C40–C50 in nocardomycolic acids and C22–C36 in corynomycolic acids (5, 6). Mycolic acids represent the major lipid constituents of the singular cell envelope of Corynebacteriniae and are found either as esters of trehalose or esterifying the terminal pentarabinofuranosyl units of arabinogalactan, a polysaccharide that forms (with peptidoglycan and mycolic acids) the cell wall skeleton of the cell envelope of the Corynebacteriniae members (1–6). Both types of mycolate-containing components have been shown to play a crucial role in the structure and functions of the cell envelope. Mycolic acids attached to the cell wall arabinogalactan are organized with other lipids to form a barrier that contributes to the very low permeability of the envelope of Corynebacteriniae and the natural resistance of these microorganisms to various antibiotics (1, 7–9). Trehalose mycolates have been implicated in numerous biological functions, notably in mycobacterial virulence, in which the structure of the mycolates has been shown to be important for the initial replication and persistence of pathogenic mycobacterial species in their hosts (10–12).

Numerous studies have been and are currently devoted to the structures and biosynthesis of mycolic acids, primarily because these substances are specific to the Mycobacterium genus, and this metabolism is also the only clearly identified

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acyl and mycoloyl residues are transferred. It has been shown to be a bimodal system capable of elongating C16, 18 fatty acids to yield C22–26 saturated fatty acids that may be either palmitoyl or CoA or palmitaldehyde to give a 2-alkyl, 3-oxo ester; and (ii) a "malonic" condensation, similar to the reaction catalyzed by β-ketoacyl synthase, in which a palmitoyl CoA is condensed with a tetradecylmalonyl-CoA, followed by decarboxylation. In both cases, an enzyme, the condensase, is required for the condensation of the two fatty acyl substrates to yield 2-alkyl, 3-keto ester that subsequently has to be reduced to produce the C32 corynomycolic acid. We have recently identified Pks13 as the condensase (18). This enzyme contains the required enzymatic domains for the condensation reaction, and the gene encoding this protein has been found in all members of the Corynebacterineae group analyzed. In Corynebacterium glutamicum, the inactivation of the pks13 gene completely abolishes the production of corynomycolates and, as a consequence, alters the structure of the cell envelope of the mutant strain (18). In mycobacteria, we demonstrated that the pks13 gene is essential for the mycobacterial growth (18). The remaining important question, addressed in the present work, is which of the two condensation mechanisms (Claissen-like or malonic reaction) is used by Corynebacterineae to synthesize the mycolic acid motif. Interestingly, two genes that may encode proteins involved in the activation of the two substrates of the condensase flank the pks13 gene in all Corynebacterineae examined (18). One of these genes, fadD32, predicted to encode an acyl-CoA synthase, was recently shown to be an acyl-AMP ligase (19). The other, accD4, is predicted to encode a subunit of an acyl-CoA carboxylase, a class of enzyme catalyzing the formation of carboxylated acyl-CoA. The present study was undertaken in order to determine the roles of the candidate enzymes in mycolic acid biosynthesis and to evaluate their importance for the physiology of corynebacteria and mycobacteria.

**FIG. 1. A proposed scheme for the terminal steps of mycolic acid biosynthesis.** R1 and R2 correspond to alkyl chains of various sizes that vary according to the Corynebacterineae species, X1 corresponds to the carrier molecule on which the newly synthesized β-ketoacyl and mycolyl residues are transferred.

Because all types of mycolic acids display a common 2-alkyl-3-hydroxy structural feature (1–6), the mycolic acid motif (Fig. 1), the enzymes involved in the formation of this motif represent good potential targets for the development of new and specific drugs against Corynebacterineae. Two mechanisms have been proposed for the synthesis of the C32 corynomycolic acid (17): (i) a "Claissen-like" condensation, in which a palmitoyl thioester is condensed with either another palmitoyl CoA or a palmitaldehyde to give a 2-alkyl, 3-oxo ester; and (ii) a "malonic" condensation, similar to the reaction catalyzed by β-ketoacyl synthase, in which a palmitoyl CoA is condensed with a tetradecylmalonyl-CoA, followed by decarboxylation. In both cases, an enzyme, the condensase, is required for the condensation of the two fatty acyl substrates to yield 2-alkyl, 3-keto ester that subsequently has to be reduced to produce the C32 corynomycolic acid. We have recently identified Pks13 as the condensase (18). This enzyme contains the required enzymatic domains for the condensation reaction, and the gene encoding this protein has been found in all members of the Corynebacterineae group analyzed. In Corynebacterium glutamicum, the inactivation of the pks13 gene completely abolishes the production of corynomycolates and, as a consequence, alters the structure of the cell envelope of the mutant strain (18). In mycobacteria, we demonstrated that the pks13 gene is essential for the mycobacterial growth (18). The remaining important question, addressed in the present work, is which of the two condensation mechanisms (Claissen-like or malonic reaction) is used by Corynebacterineae to synthesize the mycolic acid motif. Interestingly, two genes that may encode proteins involved in the activation of the two substrates of the condensase flank the pks13 gene in all Corynebacterineae examined (18). One of these genes, fadD32, predicted to encode an acyl-CoA synthase, was recently shown to be an acyl-AMP ligase (19). The other, accD4, is predicted to encode a subunit of an acyl-CoA carboxylase, a class of enzyme catalyzing the formation of carboxylated acyl-CoA. The present study was undertaken in order to determine the roles of the candidate enzymes in mycolic acid biosynthesis and to evaluate their importance for the physiology of corynebacteria and mycobacteria.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—** C. glutamicum (ATCC13032 RES167) (20) was cultured on Brain Heart Infusion medium (Difco). M. smegmatis mc²155 was grown on Middlebrook 7H9 medium (Difco) supplemented with 0.05% Tween 80 to prevent aggregation. Escherichia coli DH15a (21) or Top10 (Invitrogen) was used for the construction of plasmids and grown on Luria Bertani (LB) medium (Difco). Ampicillin, kanamycin (Km), 1 hygromycin (Hyg), chloramphenicol, and sucrose (Suc) were added when required at final concentrations of 100 μg/ml, 40 μg/ml (for M. smegmatis) or 25 μg/ml (for C. glutamicum and E. coli), 50 μg/ml, 15 μg/ml, or 30 μg/ml (for C. glutamicum and E. coli, respectively), and 5% (w/v), respectively.

**Computer Analysis—**M. tubercolosis strain H37Rv and M. leprae DNA sequences were obtained from the Pasteur Institute web site (www.pasteur.fr). Research of FadD32 and AccD4 orthologs on M. smegmatis, C. diphtheriae, C. glutamicum, and C. efficients genomes was performed at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov/) using the BLAST program. Multiple sequence alignments were performed using Clustal W (22) at the Pasteur Institute web site. The sequence of AccD4 was compared with the α and β subunits of the carboxyltransferase of E. coli (encoded by gene accA and accD) using the Needleman-Wunsch program on the Pasteur Institute web site.

The Mascot search engine (Matrix Science, London, UK) was used for protein identification with tandem mass spectrometry (MS/MS) and searching in nonredundant NCBI nr TREMBL and Swiss-Prot databases. Because most of the M. smegmatis protein sequences were not found in these databases, eight M. smegmatis proteins corresponding to the candidate subunits for being an acyl-CoA carboxylase (namely, the orthologs of AccA1, AccA2, AccA3, AccD1, AccD2, AccD3, AccD4, and AccD5 from M. tuberculosis) were added to the databases. The identification resulting from a Mascot search was confirmed by manual interpretation of corresponding MS/MS data.

The abbreviations used are: Km, kanamycin; Hyg, hygromycin; Suc, sucrose; GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; cfu, colony-forming units;
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Priming used for the construction of the C. glutamicum and M. smegmatis mutant strains and the complementation plasmids

| Primer | Sequence (5′→3′) |
|--------|-----------------|
| C. glutamicum | |
| Accel1 | ATCAAGATCTACTCCGATGGC |
| Accel2 | CTTATCGTGTTTCTACAGATCTTTCC |
| Accel3 | CGATTTCCCGGGTTGGCACTGC |
| Accel4 | TATATCCCCGCGGCCTTCAGC |
| Acc1 | GGATCCGAACAAAAGCTGATCTCGGAGGAGGATCTGCTGCA |
| Acc2 | TGCACGACTTCGAGGTGTTCG |
| AccNeo | H1 |
| Faddel1 | ATCAAGATCTACTCCGATGGC |
| Faddel2 | TATATCCCCGCGGCCTTCAGC |
| Faddel3 | CGATTTCCCGGGTTGGCACTGC |
| Faddel4 | H2 |
| Faddel5 | ACTAGTTCAGTCGGTGGCATCCGGGA |
| Fad2 | H3 |
| Fad3 | ATTGCGGGAGATCTGGCTGCTC |
| Fad4 | ATCAAGATCTACTCCGATGGC |
| Fad5 | TATATCCCCGCGGCCTTCAGC |
| Fad6 | CGATTTCCCGGGTTGGCACTGC |
| pKs2 | H4 |
| pKs6 | GGATCCGAACAAAAGCTGATCTCGGAGGAGGATCTGCTGCA |
| pKs5 | TGCACGACTTCGAGGTGTTCG |
| pKKhAm | H5 |
| pKNeo | ATCAAGATCTACTCCGATGGC |

Construction of the C. glutamicum &fadd32::km and accd4::km Mutants and Complementation Plasmids—The C. glutamicum &fadd32::km and accd4::km mutants were produced using a strategy described previously (18). Two DNA fragments (each of 0.8 kb in length) overlapping the fadd32 gene at its 5′ and 3′ extremities were amplified by PCR from C. glutamicum total DNA using primers Faddel1 + Faddel5 and Faddel3 + Faddel4 (Table I). These two fragments were inserted flanking a kanamycin resistance cassette (km) from plasmid pCGL243 (23) into the vector pMCS5 (MoBiTec, Göttingen, Germany) to give pMCSfadd. The same strategy was used to produce a plasmid in which two DNA fragments (each of 0.7 kb in length) overlapping the 5′ and 3′ extremities of the accd4 gene from C. glutamicum were cloned flanking the km resistance cassette. These two PCR fragments were obtained using primers Accel1 + Accel2 and Accel3 + Accel4 (Table I), and the plasmid was named pMCSacc. The two plasmids were transferred into C. glutamicum by electroporation, and transformants were selected on plates containing Km. Transformants in which all the deletion fragments were replaced by the mutated mycobacterial genes and the mutated plasmid-borne alleles were identified on the basis of their rough colony morphology and characterized by PCR using primers Fad2, Fad4, Fad5, pks2, and k10 for the mutant within the fadd32 gene and Acc1, AccII, AccIII, Acc5, k7, and k10 for the insertion within accd4 (Table I). Two mutant strains, C. glutamicum &fadd32::km and &accd4::km (named CGL2034 and CGL2035, respectively), were retained for further studies.

To complement the &fadd32::km mutation, a 2.5-kb DNA fragment carrying the fadd32 gene plus the 370 bp upstream of the putative start codon of this gene was amplified by PCR using primers Fad3 + Fad4 (Table I) and C. glutamicum genomic DNA. The amplified DNA fragment was inserted between the BglII and SacI sites of pCGL482, which contains a replicon functional in C. glutamicum (24), to give pCGL2319.

To complement the &fadd32::km mutation, a 2.5-kb DNA fragment carrying the fadd32 gene plus the 370 bp upstream of the putative start codon of this gene was amplified by PCR using primers AccNeo and pks6 (Table I) from the C. glutamicum genomic DNA. The primer AccNeo was designed to introduce a NcoI restriction site overlapping the putative start codon of accD4. The PCR fragment was digested with enzymes NcoI and BamHI. A second DNA fragment containing the well-characterized ptc promoter, which was previously shown to be functional in C. glutamicum (25), was generated using primers pKKBam and pKKNco (Table I) and pKK881-1 plasmid (Clontech) as template DNA. This second PCR fragment was digested with NcoI and BamHI. The two PCR fragments were then inserted simultaneously between the BamHI and pGEM-T vector sites of plasmid pCGL2316. The two plasmids, pCGL2319 and pCGL2316, were used for electroporation of the C. glutamicum &fadd32::km and &accd4::km mutants, respectively. Transformants were selected on chloramphenicol-containing plates.

Mutation in the C. glutamicum &fadd32::km and &accd4::km genes was amplified by PCR from C. glutamicum total DNA using primers Acc4a + Acc4b and F32a + F32b, respectively. The fragment overlapping the fadd32 gene was digested with SpeI and EcoRV and inserted into the corresponding sites of plasmid pJQ200 (26) to give pDP59. A hygromycin resistance cassette (hyg) was inserted between the two BamHI sites of pDP65 (located within the fadd32 gene) to yield pDP68. The fragment overlapping the accd4 gene was first inserted into the pGEM-T vector sites of the pGEM-T vector system I kit (Promega). The hyg cassette was inserted into the unique Xhol site within the cloned accd4 gene to give plasmid pDP62. A DNA fragment carrying the disrupted accd4::hyg allele was inserted between the Smal and SpeI sites of pJQ200 to yield pDP63.

Plasmids pDP60 and pDP65 were transferred into M. smegmatis by electroporation, and transformants were selected on Hyg-containing plates. Transformants in which pDP60 or pDP65 had been integrated by single crossover between the wild-type and mutated copies of fadd32 or accd4 were characterized by PCR using primers F32c, F32d, F32f, H1, and H2 for fadd32 and primers Acc4c, Acc4d, Acc4f, H1, and H2 for accd4. For each construct, one clone giving the pattern corresponding to insertion of the plasmid by single homologous recombination event was retained for further analysis. These strains, obtained by the integration of pDP60 and pDP63, were renamed PMM58 and PPM59, respectively.

To produce the complementation plasmids, the fadd32 and accd4 genes were amplified by PCR from M. smegmatis total DNA using primers F32a + F32b and Acc4d + Acc4f, respectively (Table I). The PCR fragments were inserted into the pGEM-T vector (Promega) to give pDP66 and pDP67. The PCR fragment of the fadd32 and accd4 genes, respectively. The fadd32 gene was recovered from plasmid pDP65 on a Km/SpeI restriction fragment and inserted between the corresponding sites of plasmid pMIP12. The resulting plasmid, named pDP66, contained the fadd32 gene under the control of the pBlaF promoter (27). A cassette containing the pBlaF promoter, the fadd32 and accd4 genes, and a transcriptional termination fragment were obtained in a PacI/AseI restriction fragment and inserted into thermosensitive mycobacterial plasmid pDP24 derived from pCG63 (28) by deletion of a usele AssI fragment. This new plasmid was named pDP69. A similar strategy was used with the accd4 gene. A PstI/SpeI fragment carrying the accd4 gene without promoter was inserted into the PstI and SpeI sites of pMIP12 to give pDP67. A PstI/AseI fragment containing the accd4 gene under the control of the pBlaF promoter, was then transferred into pDP62 thermosensitive vector to give pDP70.

Plasmids pDP69 and pDP70 were transferred by electroporation into PMM58 and PPM59, respectively. Transformants were selected on plates containing Km and Hyg. The second crossover events at the chromosomal fadd32 and accd4 loci were selected by plating a liquid culture of PMM58/pDP69 and PMM59/pDP70 cultured at 30 °C on plates containing Km, Hyg, and Suc, which were then incubated at 30 °C. Colonies were screened by PCR using primers F32c, F32d, F32f, H1, and H2 for fadd32 and Acc4c, Acc4d, Acc4f, H1, and H2 for accd4. Two strains, named PMM61 and PMM62, in which the wild-type chromosomal copy of fadd32 was replaced by the mutated fadd32::hyg allele, and PMM66 and PMM70, in which the wild-type chromosomal copy of accd4 was replaced by the mutated accd4::hyg allele, were selected for phenotypic analysis.

Construction of Plasmids for Immunoprecipitation—To perform the immunoprecipitation experiments in M. smegmatis, two new plasmids containing either the fadd32 gene or the accd4 gene fused to the myc tag were constructed. Briefly, two double-stranded oligonucleotides were produced by annealing either myc-PA with myc-PB or myc-KA with myc-KB in 40 μl containing 45 μl of each single-stranded oligo.
nucleotide (Table 1), 10 mM Tris, pH 7.5, and 50 mM NaCl. This mixture was incubated for 5 min at 100 °C and allowed to cool slowly to reach 4 °C. The resulting double-stranded oligonucleotides contained the coding sequence of the myc epitope. They were inserted between the BamHI and SpeI sites of the BamHI and KpnI sites of pM12 to give pMIP12mycP and pMIP12mycK, respectively. A KpnI/SpeI fragment from pDP66, containing the fadD32 open reading frame, was then inserted between the KpnI and SpeI sites of pMIP12mycK to give pDP80. Similarly, a FstI/SpeI fragment from pDP67, containing the accD4 open reading frame, was cloned into the pMIP12mycP plasmid cut with FstI and SpeI to give pDP81. The two plasmids, pDP80 and pDP81, the open reading frames corresponding to fadD32 and accD4, respectively, were fused to a sequence encoding the myc epitope. These two plasmids were transferred into the M. smegmatis strains PMM58 and PMM59. The second crossover events at the chromosomal copy of fadD32 and accD4 loci were selected by plating a liquid culture of PMM58::pDP80 and PMM59::pDP81 cultured at 37 °C on plates containing Km, Hyg, and Suc. Ten and seven colonies obtained with PMM58::pDP80 and PMM59::pDP81 cultured at 37 °C for 5 days on plates containing Km, Hyg, and Suc were labeled by incubation of exponentially growing cells with 0.5Ci/ml [14C]acetate (specific activity, 54 mCi/mmol; ICN, Orsay, France) to late growth phase (15). Fatty acids were prepared from the labeled cells and separated by analytical TLC on Durasil 25 using dichloromethane:acetone:acetic acid (1:1:1) as solvent. Fatty acid methyl esters were then prepared according to the method described previously (29). They were fractionated on a Florisil column irrigated with petroleum ether containing 0%, 5%, and 10% (v/v) methanol as eluant (27). They were quantified on a PhosphorImager (Amersham Biosciences).

Fatty acid methyl esters were obtained by saponification of cells, followed by methylation with diazomethane (28). They were fractionated on a Florisil column irrigated with petroleum ether containing 0%, 1%, 2%, 3%, and 100% (v/v) diethyl ether. The last eluted fraction, which contained polar fatty acid methyl esters, was then analyzed by gas chromatography. The polar fatty acid methyl esters were separated on a 3% (w/v) Carbowax 20M column (12 m). The temperature program was from 60 °C to 100 °C, at 20 °C/min, and then 100 °C to 310 °C at 8 °C/min.

Synthesis and Analysis of Tetrade cylmalonic Acid—The tetrade cylmalonic acid was obtained by alkaline deacylation of tetrade cylmalonic ester that was prepared according to the method described previously for the preparation of n-butylcynalon anil (30). Briefly, the malonic dimethyl ester (500 mg) was gradually added to a freshly prepared solution of 1.5 m (4 ml) sodium ethoxide, and then, after cooling to about 50 °C, 0.5 m (1 ml) 18O containing (1 g) sodium bicarbonate was added. After 5 min, the reaction mixture was acidified with concentrated hydrochloric acid, and the residue was extracted with ethyl ether. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure, and the residue was recrystallized from water.

RESULTS

Sequence Analysis of FadD32 and AccD4—We recently identified Pks13 as the condensase, the enzyme that catalyzes the condensation reaction between two activated fatty acyl substrates to yield mycolic acid precursors (16). The pks13 gene is flanked, on the genome of every Corynebacteriaceae analyzed, by two genes named fadD32 and accD4 (18). The FadD32 protein of M. tuberculosis has been recently shown to belong to a family of enzymes, the acyl-AMP ligases, involved in the activation of fatty acids as acyl-adenylates before transfer onto polyketide synthases (19). This finding suggested that FadD32 catalyzes the activation of the meromycoloyl chain before its transfer onto Pks13 in mycobacteria. Analysis of the amino acid sequence of FadD32 from C. glutamicum, C. efficiens, C. diphterieae, and Rhodococcus rhodochrous revealed that the region that typifies the acyl-AMP ligase is highly conserved (Fig. 2A). Therefore, we hypothesized that FadD32 was involved in the activation of the R1 chain (Fig. 1) in all the Corynebacte ria by a mechanism similar to that proposed for M. tuberculosis (19).

In silico analysis of the sequence of the putative AccD4 protein of M. tuberculosis shows high sequence similarities with the carboxyltransferase subunit of acyl-CoA carboxylase. In E. coli, this carboxyltransferase is itself subdivided into two subunits, the α and β subunits, encoded by the accA and accD genes, respectively. In the Corynebacteriaceae analyzed, the accD gene encodes a protein that exhibits similarities with the α subunit of the E. coli carboxyltransferase at its COOH-terminal end and with the β subunit of the E. coli carboxyltransferase at its NH2-terminal end (Fig. 2B). Therefore, in Corynebacteriaceae, the accD gene putatively encodes a protein corresponding to an entire carboxyltransferase enzyme. This enzyme may be involved in the carboxylation of acyl-CoA to form alkylmalonyl-CoA, one of the expected substrates of the condensase enzyme.

Deletion of fadD32 and accD4 in C. glutamicum and Analysis of the Phenotypical Changes of the Mutants—To investigate the roles of the acyl-AMP ligase FadD32 (19) and of the putative
carboxyltransferase subunit of acyl-CoA carboxylase AccD4 in the biosynthesis of mycolic acids, we deliberately chose to delete the wild-type copy of the corresponding genes in \textit{C. glutamicum} ATCC13032. This strategy was based on the established fact that corynebacterial species can survive without producing mycolates (18), consistent with the existence of the species \textit{C. amycolatum} (32). In contrast, the inhibition of mycolic acid production has been convincingly shown to be lethal for mycobacteria, explaining why isoniazid, an inhibitor of mycolic acid production has been convincingly shown to be lethal due to the deletion of \textit{fadD32} and \textit{accD4}. Therefore, it followed that, although a functional \textit{fadD32} or \textit{accD4} gene was not required for the viability of \textit{C. glutamicum}, as expected from our previous data on \textit{pks13} (18), the gene deletion resulted in striking phenotypes suggestive of a defect in mycolic acid production and, as a consequence, a cell envelope modification.

**Lipid Analysis of the $\Delta fadD32::km$ and $\Delta accD4::km$ Mutants of \textit{C. glutamicum}**—To determine the origin of the phenotypic changes observed for the $\Delta fadD32::km$ and $\Delta accD4::km$ mutants, the fatty acids produced by the mutant strains were isolated and compared with those produced by the parental strain. Cultures of the wild-type, the $\Delta fadD32::km$, and $\Delta accD4::km$ mutants of \textit{C. glutamicum}, as well as the mutants complemented with the wild-type copy or either $\Delta fadD32::km$ or $\Delta accD4::km$, were grown to exponential growth phase and labeled with $[^{14}\text{C}]$acetate. Pufy acids were then released from the bacterial cells by saponification. TLC and GC analyses of the fatty acid methyl esters showed a production of non-hydroxylated \textit{C}_{16}–\textit{C}_{18} fatty acid derivatives comparable for all the strains but a complete absence of corynomycolates in the $\Delta fadD32::km$ and $\Delta accD4::km$ mutants of \textit{C. glutamicum} (Fig. 4B). This observation was also supported by the lack of production by the mutant strains of palmitone, a degradation product of the 3-oxo intermediate resulting from the condensation reaction (Fig. 1) that usually appears upon alkaline hydrolysis (data not shown). As expected, complementation of the mutant strains with the wild-type copy or either $\Delta fadD32::km$ or $\Delta accD4::km$ fully restored the production of mycolic acids (Fig. 4B). These observations were confirmed by GC-MS analysis (data not shown) and

| Component | $\Delta fadD32::km$ | $\Delta accD4::km$ | WC | WC |
|-----------|------------------|------------------|---|---|
| $[^{14}\text{C}]$acetate | 100 | 100 | 100 | 100 |
| Palmiton | 0 | 0 | 100 | 100 |
| Mycolates | 0 | 0 | 0 | 0 |
| Other Fatty Acids | 0 | 0 | 0 | 0 |
clearly demonstrated that the \( \Delta \text{fadD32::km} \) and \( \Delta \text{accD4::km} \) mutants of \textit{C. glutamicum} were devoid of mycolic acids because of the deletion of either \textit{fadD32} or \textit{accD4}.

**Synthesis of Tetradecylmalonic Acid and Identification of Carboxylated Intermediates in the \( \Delta \text{fadD32::km} \) and \( \Delta \text{pks13::km} \) Mutants of \textit{C. glutamicum}**—Three successive reactions are expected to yield the 3-oxo intermediate resulting from the condensation reaction between two fatty acid molecules (Fig. 1). According to this model, the deletion of either \textit{fadD32} or \textit{pks13}, but not \textit{accD4}, may lead to the accumulation of carboxylated intermediates, provided that such an accumulation is not prevented by a possible strictly regulated enzymatic activity and/or a polar effect on the expression of the downstream gene \textit{accD4} due to the insertion of the \textit{km} cassette in the \textit{fadD32} or \textit{pks13} gene. The polar effect that may be due to the insertion of the \textit{km} cassette in \( \Delta \text{fadD32::km} \) was ruled out because the expression of the wild-type \textit{fadD32} gene in the \( \Delta \text{fadD32::km} \) mutant restored the production of mycolates (Fig. 4B). In the case of the \( \Delta \text{pks13::km} \) mutant, however, the transfer of a plasmid containing the wild-type \textit{pks13} gene did not fully complement the mycolate synthesis defect (18), suggesting that the \textit{km} cassette located within the \textit{pks13} gene has led to an \textit{accD4} expression defect in the \( \Delta \text{pks13::km} \) mutant.

To validate the proposed condensation model further, we searched for the carboxylated intermediates that may accumulate in the \( \Delta \text{fadD32::km} \) and/or \( \Delta \text{pks13::km} \) mutants. Because the putative intermediates may not be easily identifiable in fatty acid mixtures, we first synthesized the compound predicted from the expected carboxylation of the major C\(_{16}\) fatty acid by an acyl-CoA carboxylase, i.e. a tetradecylmalonic acid. The alkylmalonate was purified by recrystallization (30) and characterized. GC-MS analysis of the dimethylester of tetradecylmalonic acid (Fig. 5A) showed a pseudomolecular (M+1, 1.7%) ion peak at 329 m/z and fragments at 297 m/z (M-31, 0.6%), 145 m/z (\( \beta \) cleavage, 100%), and 132 m/z (McLafferty cleavage, 91%). The \(^1\text{H} \) NMR spectrum of the compound (Fig. 5B) showed peaks attributable to the resonances of terminal CH\(_3\) at 0.80 ppm (3H, triplet), of CH\(_2\) at 1.18 ppm (24H, multiplet), \( \beta \) CH\(_2\) at 1.81 ppm (2.4H, multiplet), CH at 3.29 ppm (1H, triplet), and OCH\(_3\) at 3.65 ppm (6.5H, singlet). Analysis of the \(^1\text{C} \) NMR spectrum (Fig. 5C) confirmed the structure of the synthesized product by the identification of most of the signals, notably those assignable to the resonances of COOH (170.28 ppm), C-2 (52.41 ppm), and OCH\(_3\) (51.76 ppm). The resonances of C-4 through C-14 were seen at 28.88–31.95 ppm.
whereas those of C-3, C-15, and C-16 were observed at 27.33, 22.69, and 14 ppm, respectively.

Based on the characteristic mass value of the McLafferty fragment of the synthetic alkylmalonate (132 m/z instead of 74 m/z for straight chain fatty acids), it was possible to focus on this mass value and thereby to identify a peak eluted at 15.43 min on GC of polar fatty acid derivatives from the \(/H9004\fadD32::km\) mutant whose mass spectrum contained an intense ion at 132 m/z (Fig. 6). A less intense signal of the 132 m/z ion peak was also detected in the mass spectra of the wild-type strain of \(C.\ glutamicum\) but was absent from that of the \(/H9004\accD4::km\) mutant (Fig. 6). Interestingly, this ion peak was observed in the mass spectrum of the polar fatty acid methyl esters of the \(/H9004\accD4::km\) mutant complemented with the wild-type \(accD4\) gene (Fig. 6). Furthermore, the entire mass spectrum of the natural compound (Fig. 6), typified by the occurrence of the 132 m/z ion fragment, was superimposable with that of the synthetic tetradecyl malonic acid methyl ester (Fig. 5A). It was thus concluded that the wild-type \(C.\ glutamicum\), but not the \(\Delta accD4::km\) mutant, contained an alkylmalonate derivative whose mass spectrum corresponded to that of the expected activated C16 fatty acid derivative destined to be condensed with the product of FadD32 to yield the 3-oxo intermediate of major C32 corynomycolic acids. This compound was accumulated in the \(\Delta fadD32::km\) mutant, and a higher homolog of the fatty acid, i.e. hexadecylmalonic acid, was also detected in this mutant as a minor peak eluted at 17.51 min on GC (Fig. 6).

Both FadD32 and AccD4 Are Essential for the Viability of Mycobacteria—Mycobacterial growth (18, 33, 34). Consequently, FadD32 and AccD4 are expected to be essential in mycobacteria if the enzymes are not redundant. To address this question, a genetic approach was used in the model strain \(M.\ smegmatis\ mc2155\). Two non-replicative vectors containing the counter-selectable marker \(sacB\) (35) and a mutated copy of either \(fadD32\) or \(accD4\) were inserted into the chromosome by single crossover between the wild-type chromosomal allele of \(fadD32\) or \(accD4\) and the mutated alleles, to yield PMM59 and PMM58, respectively (Fig. 7). Plating a culture of each strain at 30 °C on medium containing 5% Suc and Hyg generated clones with mutations in the \(sacB\) gene but failed to select the second recombination event that would have produced a strain carrying...
ing only the mutated fadD32::hyg or accD4::hyg allele (data not shown). These results suggested that both genes are essential for mycobacterial growth, consistent with the demonstrated involvement of these genes in mycocid acid production in *C. glutamicum* (see above). To firmly establish that the two genes are essential in mycobacteria, we transferred a second wild-type copy of *fadD32* or *accD4* in a thermosensitive mycobacterial vector into PMM59 and PMM55, respectively. In these constructs, named pDP69 for *fadD32* and pDP70 for *accD4*, the expression of the cloned genes was under the control of the mycobacterial promotor pBlaF5 (27). In this genetic context, the selection of clones resistant to both Suc and Hyg gave several mutants (3 of 5 Suc<sup>C</sup> and 10 of 10 Hyg<sup>R</sup> colonies tested for *fadD32* and *accD4*, respectively) in which a second recombination event had occurred in PMM58:pDP69 and PMM59:pDP70 between the two chromosomal alleles of *fadD32* or *accD4*, leaving only the non-functional copy of either *fadD32* or *accD4* on the chromosome (Fig. 7). These recombinant strains, PMM61:pDP69 and PMM60:pDP70, contained a deletion and insertion in the *fadD32* gene or an insertion in the *accD4* gene on the chromosome and a functional *fadD32* or *accD4* gene on a thermosensitive plasmid. Streaking these recombinant strains on Hyg plates at 30 °C or 42 °C revealed that they were unable to form colonies at high temperature (Fig. 8). In liquid culture, the PMM60:pDP70 strain grew as well as the wild-type strain at 30 °C, a permissive temperature for plasmid replication (Fig. 8). In the case of PMM61:pDP69, there was a weak growth defect at 30 °C illustrated by a colony size smaller for PMM61:pDP69 than for PMM60:pDP70 and the wild-type strain (Fig. 8A) and a delay in obtaining a liquid culture of the same optical density at 30 °C. When the cultures were shifted to 42 °C, a non-permissive temperature for plasmid replication, the number of viable bacteria increased during the first 12–24 h, during which time the temperature-sensitive plasmid was cured, and then remained stable during the next 24 h before declining (data not shown). After 100 h of growth at 42 °C, the number of viable bacteria was unchanged for PMM61:pDP69, compared with that inoculated, and 10 times higher for PMM60:pDP70 (Fig. 8). This higher cfu count for PMM60:pDP70 may be due to a higher stability of the pDP70 plasmid. In any case, these data demonstrated that, as expected, both fadD32 and accD4 are essential for the survival of *M. smegmatis*.

**Identification of the Other Subunits of the AccD4-containing Acyl-CoA Carboxylase**—To be functional, an acyl-CoA carboxylase requires, in addition to the carboxyltransferase subunit, a biotin carboxyl carrier protein and a biotin carboxylase (36). In *M. tuberculosis*, four genes (*accA1, accA2, accA3, and pca*) encode proteins containing the biotin carboxyl carrier and biotin carboxylase domains (37) that may thus be part of the AccD4-containing acyl-CoA carboxylase. All four genes are highly conserved in *M. smegmatis*, but only one of these genes, namely, MI0726 (the ortholog of accA3 in *M. tuberculosis*), is found in *M. leprae*, indicating that the AccA3 protein is a good candidate for being the requested portion of the acyl-CoA carboxylase in mycobacteria. In corynebacteria, two candidate genes for being the biotin carboxyl carrier protein and biotin carboxylase exist, namely, *Negl0670* and *Negl0659*. *Negl0659* is a putative pyruvate carboxylase, whereas *Negl0670*, which is homologous to both MI0726 of *M. leprae* and AccA3 of *M. tuberculosis*, is probably essential for bacterial growth and involved in the biosynthesis of fatty acids (38). To experimentally address the question of the biotin carboxyl carrier protein and biotin carboxylase subunits of the AccD4-containing acyl-CoA carboxylase involved in the formation of mycolate, we used a novel proteomic approach by looking for proteins that interact with AccD4. For this purpose, we constructed a plasmid, pDP81, carrying the accD4 gene fused with a myc tag. To demonstrate that the tagged AccD4 is functional, the pDP81 plasmid was transferred in *M. smegmatis* PMM59. The second recombination event at the accD4 chromosomal locus was selected by plating a culture of PMM59:pDP81 on Hyg<sup>R</sup>, Suc<sup>R</sup>, and Km<sup>R</sup>-containing medium. Analysis of seven Suc<sup>R</sup>, Hyg<sup>R</sup>, and Km<sup>R</sup> colonies by PCR showed that a second recombination event had occurred in five of these clones between the two chromosomal alleles of accD4 (data not shown). Because these strains are viable despite the mutated copy of accD4 on the
chromosome, it was concluded that the fused accD4-myc gene carried by the pDP81 plasmid encodes a functional carboxyltransferase. Using a similar protocol, a control strain was produced containing a mutated fadD32 gene on the chromosome and a fadD32-myc fused gene on a plasmid named pDP80. Once again, the viability of the constructed strain demonstrated that the fadD32-myc fused gene encodes a functional acyl-AMP ligase.

To identify proteins that specifically interact with the myc-tagged AccD4, we carried out immunoprecipitation of proteins extracts of M. smegmatis obtained from PMM61:pDP80 or PMM60:pDP70 using a monoclonal antibody directed against the myc epitope. The immunoprecipitated proteins were analyzed by SDS-PAGE stained with silver nitrate (Fig. 9A, lane A). A single protein band was obtained with the control strain PMM61:pDP80 (Fig. 9A, lane B). This protein had the expected molecular mass of FadD32-myc (70 kDa) and reacted with the anti-myc antibody (Fig. 9A and B). In contrast, two protein bands were detected in the protein extracts from PMM60:
Role of FadD32 and AccD4-containing Acyl-CoA Carboxylase

The observation that AccD5 is also part of the complex was more surprising but in agreement with a recent finding of Gande et al. (39), who also showed that the ortholog of AccD5 in C. glutamicum is required for the production of corynomycolate. It must be noted that no peptide sequence that may correspond to orthologs of the remaining candidate subunits for being an acyl-CoA carboxylase (namely, the orthologs of AccA1, AccA2, AccD1, AccD2, and AccD3) was found in the protein digests, showing the specificity of the immunoprecipitation reaction. Taken together, these experiments identified AccA3 and AccD5 as two other subunits of the AccD4-containing acyl-CoA carboxylase.

**DISCUSSION**

The present study was undertaken in order to identify new enzymes involved in mycolic acid biosynthesis, a key metabolism in Corynebacterineae that is a target for isoniazid, a powerful antituberculous drug. In their seminal work on the biosynthesis of C₃₂ mycolic acids, Gastambide-Odier and Lederer (17) have proposed two models for the condensation of two C₁₆ to yield the corynomycolic acid. In the malonic condensation mechanism, a carboxylated acyl-coenzyme A is condensed to a second activated acyl chain to yield a 3-oxo intermediate, which would then be reduced to form mycolic acid (Fig. 1). We have recently provided strong experimental support for this model by identifying the condensase, the enzyme responsible for the final condensation step in mycolic acid biosynthesis. This enzyme is encoded by the pks13 gene, which seems to form an operon together with two other genes, fadD32 and accD4. We postulated that both AccD4 and FadD32 would be involved in the activation of the condensase substrates because these proteins exhibit similarities with acyl-CoA synthases and subunits of acyl-CoA carboxylases, two classes of enzymes that may be involved in the substrate activation required for the final condensation reaction. Consistent with this hypothesis is the presence of the fadD32 and accD4 genes in the genomes of all the Corynebacterineae examined (18). The recent demonstration that the protein FadD32 of *M. tuberculosis* belongs to a family of enzymes, the acyl-AMP ligases, involved in the activation of fatty acids as acyl-adenylates before transfer onto polyketide synthase (19) further supported our hypothesis. To establish the functions of accD4 and fadD32, we adopted the strategy of deleting the putative genes of interest from the genomes of bacteria and measuring the consequences of the deletions both in terms of production of mycolic acids and phenotypical changes that typify cells devoid of these structurally important cell envelope constituents. Because previous works have established the essentiality of gene products involved in the biosynthesis of mycolic acids in mycobacteria and not in the phylogenetically related corynebacteria, we first deleted the accD4 and fadD32 genes in *C. glutamicum*. As expected, the two resulting mutant strains were deficient in mycolic acid production and exhibited the colony morphology that typifies the mycolate-less mutants of corynebacteria. The parental corynebacterial phenotype was restored in both mutants after expression in trans of the wild-type fadD32 and accD4 genes. Furthermore, the ∆fadD32::km mutant, but not the ∆accD4::km strain, accumulated a tetradeycymalonic acid, the expected major product of AccD4-containing acyl-CoA carboxylase in corynebacteria, a phenotype that was again fully reversed to that of the wild type by complementation of the mutant with a functional fadD32 gene. These data clearly demonstrated the following: (i) AccD4 is involved in the carboxylation of the fatty acid that will be found as the α chain of the mycolic acid, (ii) FadD32 is required for the activation of the meromycolate chain, probably through the formation of an
acyl-adenylate as shown by Trivedi et al. (19); and (iii) these two activation reactions are required for the final condensation step by Pks13 to produce mycolic acid precursors. To confirm and extend our finding, we also attempted to generate knock-out mutants in *M. smegmatis* using conditionally replicative plasmids. We showed that insertion/deletion within either *fadD32* or *accD4* was lethal for *M. smegmatis* unless functional merodiploid strains were used, thus indicating that the gene products are essential in mycolic acid biosynthesis and viability in *M. smegmatis*. As such, these enzymes represent two novel good targets for the development of new antituberculous drugs in the context of re-emergence of multiresistant strains of the tubercle bacillus. Besides, the specific implication of Fad32 in mycolic acid biosynthesis illustrates the complexity of lipid metabolism in mycobacteria that have devoted as many as 36 non-redundant FadD proteins to the activation of fatty acid substrates (37). In agreement with this observation is the result of the inactivation of *fadD26* that generates attenuated tubercule bacilli (40) that lack phthiocerol dimycolates but not other types of lipids such as mycolic acids (41).

The observation that FadD32 and AccD4, like Pks13 (18), proteins involved in the last condensation step of mycolic acids biosynthesis, are essential for the growth of *M. smegmatis* is in apparent conflict with two reports that indicate that temperature-sensitive strains isolated from *M. smegmatis* cultures after chemical mutagenesis are viable without the continued production of full-length mycolic acids and accumulate long-chain meromycolate-like fatty acids (42, 43). Biochemical analysis of one of the spontaneous mutant strains has shown that it also lacks the characteristic glycopeptidolipids and lipooligosaccharides of the parent strain (44). In addition, the latter mutant strain exhibits an abnormal ultrastructure that has not been observed in mycolate-less bacteria (45). Temperature-sensitive mutants isolated by chemical mutagenesis are known to be highly versatile, and all our attempts to maintain these strains failed and resulted in the selection of either revertants or contaminants. Thus, additional studies that should include genotypic analyses are clearly needed to clarify the status of the strains before drawing any definite conclusion. Another study that may contradict the fact that mature mycolic acids are essential for *in vitro* growth of mycobacteria is the work of Mdluli et al. (46), who have reported continued growth of *M. avium* treated with isoniazid concentrations that inhibit mycolate synthesis. Again, the resulting bacteria exhibited altered colony morphologies and an uncommon cell wall ultrastructure different from what was observed in the temperature-sensitive mycolate-less strain (44) and *C. amycolatum* (45), whose ultrastructure does not significantly differ from...
that of mycolate producer strains (1, 45). Importantly, we were unable to reproduce the data obtained on *M. avium* that also contrast strongly with those of Vilchêze et al. (33), who clearly established a correlation between the inhibition of mycolate biosynthesis and the viability of *M. smegmatis*. These latter data are supported by several recent and genetically well-controlled studies that have convincingly demonstrated the essentiality of gene products involved in the biosynthesis of the mycobacterial key cell wall components, notably mycolic acids and arabinogalactan (18, 47, 48).

As far as the mechanism of the condensation reaction that yield mycolic acids is concerned, it has been postulated from structural considerations that the C32 corynomycolic acid could result from a condensation of two molecules of C16 (17). Indeed, the intervention of two molecules of palmitic acid in this synthesis has been first proved by incubating *C. diphtheriae* cells with [1-14C]palmitic acid. In the resulting corynomycolic acid, the labeling was specifically found in carbon atoms 1 and 3 (17).

Moreover, a particular cell-free preparation of *C. diphtheriae* incubated with [1-14C]palmitate was shown to produce a β-keto ester specifically labeled on carbon atoms 1 and 3 (49). Two distinct mechanisms have been proposed for the condensation reaction: (i) a Claisen-like condensation in which a fatty acyl thioester is condensed with another CoA derivative or with palmitaldehyde to give a 2-alkyl, 3-oxo ester or a 2-alkyl, 3-hydroxy derivative; and (ii) a condensation of a palmitoyl CoA with a tetradecymalonyl-CoA followed by decarboxylation, in a manner similar to the action of the β-ketoacyl synthases during fatty acid chain elongation, to produce a 2-alkyl, 3-keto ester, which is subsequently reduced to give corynomycolic acid. The difference between the two mechanisms resides in the existence of a carboxylation step necessary to produce the malonyl CoA derivative. Experiments designed to differentiate between the two mechanisms have led to contradictory conclusions. Whereas the condensation reaction in cell-free preparations of *C. diphtheriae* was inhibited by avidin, indicating the occurrence of a carboxylation step through the intervention of a biotin enzyme in the reaction sequence (49), avidin showed no effect on the condensation reaction in cell-free extracts of *C. matruchotii* (50). More recently, labeling experiments using [2,2-3H]palmitic acid have shown that the deuterium atom (2H) was located at position C-2 in the mature corynomycolic acid. Accordingly, the authors have proposed a mechanism involving a highly activated enolate intermediate to explain the reaction (51) and concluded that palmitate condensation in whole cells of *C. matruchotii* does not involve an intermediate carboxylation. This conclusion implies, however, that the malonyl intermediate reacts further in a true malonic condensation, i.e. with the formation of a carbanion by the loss of one deuterium. Importantly, it has been convincingly shown in yeast that the malonyl intermediate does not react in this way but rather by a concerted decarboxylation with conservation of the deuterium (52). Consistent with this observation, the present work, by proving the involvement of the AccD4 carboxyltransferase and the accumulation in the ΔfadD32::km mutant of tetradeccymalonic acid, clearly discriminates between the proposed mechanisms and proves that a carboxylation step to form a malonyl derivative takes place before the condensation reaction in mycolic acid biosynthesis.

Acy-CoA carboxylases are complex enzymes composed of several catalytic domains. In *E. coli*, they contain subunits encoded by four genes: *accA*, *accB*, *accC*, and *accD* (36). Genes *accA* and *accD* encode the α and β subunits of the carboxyltransferase, respectively; *accC* encodes the biotin carboxylase; and *accB* encodes the biotin carboxyl carrier protein (36). In Corynebacterineae, our sequence analysis data revealed that AccD4 encodes a protein containing both the α and β domains of a carboxyltransferase. To identify the other subunits of the entire acyl-carboxylase enzyme, we combined comparative genomics, molecular biology approaches, and proteomics. A comparison of the genome sequences of various mycobacteria and corynebacteria suggested that the genes named *accA3* in *M. tuberculosis*, *MI0726* in *M. leprae*, and *NcgI0670* in *C. glutamicum* were likely to encode the biotin carboxyl carrier subunit and biotin carboxylase subunit of the AccD4-containing acyl-carboxylase. The identity of the protein was then clearly established in co-immunoprecipitation experiments by demonstrating that AccD4 interacts with the *M. smegmatis* ortholog of *AccA3* of *M. tuberculosis*. In these co-immunoprecipitation experiments, we also identified the ortholog of the *M. tuberculosis* carboxyltransferase AccD5 as another partner of AccD4.

This finding was surprising in view of what is known with regard to *E. coli*, but it is not unexpected because while this work was under review, Gande et al. (39) have published a series of experiments demonstrating that the ortholog of *M. tuberculosis* AccD5 in *C. glutamicum*, called AccD2 by these authors, is required for corynomycolate biosynthesis. Thus, the occurrence of two different AccD protein subunits in an acyl-CoA carboxylase appears to be a unique feature of Corynebacterineae. We now have a scenario for the final condensation reaction of the mycolic acid biosynthesis in which five proteins are involved (Fig. 1): the condensase Pks13, the acyl-AMP ligase FadD32, and the acyl-CoA carboxylase formed by AccD4, AccD5, and AccA3. In their model, Gande et al. (39) have also proposed that the carboxylation of one acyl-chain (the mero-chain) occurs after transfer of this chain onto Pks13. However, our results do not support this part of the model because an alkyl-malonyl intermediate is formed in a corynebacterial mutant deficient in the synthesis of Pks13. Rather, our findings indicate that the carboxylation of the mero-chain occurs before the transfer onto the condensase, Pks13.

To conclude, our results further extend our understanding of the biosynthesis of mycolic acids, the key lipid components of the mycobacterial cell envelope. New enzymes were identified and shown to play a role in the activation of the substrates of the condensase. These proteins, as well as the condensase, are essential for the viability of mycobacteria and specific for a restricted number of bacterial species. Therefore, they represent new and attractive targets for the development of novel drugs for the treatment of mycobacterial infections in humans.

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The Acyl-AMP Ligase FadD32 and AccD4-containing Acyl-CoA Carboxylase Are Required for the Synthesis of Mycolic Acids and Essential for Mycobacterial Growth: IDENTIFICATION OF THE CARBOXYLATION PRODUCT AND DETERMINATION OF THE ACYL-CoA CARBOXYLASE COMPONENTS

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