AccD6, a key carboxyltransferase, essential for mycolic acid synthesis in *Mycobacterium tuberculosis*, is dispensable in a non-pathogenic strain

Jakub Pawelczyk,¹ Anna Brzostek,¹ Laurent Kremer,²,³ Bozena Dziadek,⁴ Anna Rumijowska-Galewicz,¹ Marta Fiolka,⁵ and Jaroslaw Dziadek¹*

¹Institute for Medical Biology, Polish Academy of Sciences, Lodz, Poland
²Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS UMR 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France
³INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France
⁴Department of Immunoparasitology, University of Lodz, Poland
⁵Department of Immunobiology, Institute of Biology and Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland

Running title: Requirement for AccD6 in *Mycobacterium* mycolic acid biosynthesis

Key words: acetyl-CoA carboxylase, mycobacteria, mycolic acid, type II fatty acid synthase, cell wall, gene essentiality

*Corresponding author: Jaroslaw Dziadek, Institute For Medical Biology, Polish Academy of Sciences, Lodowa 106, 93-232 Lodz, Poland. Telephone: 48 42 2723610, Fax: 48 42 2723630, e-mail: jdziadek@cbm.pan.pl
Abstract

Acetyl coenzyme-A carboxylase (ACC) is a key enzyme providing substrate for mycolic acid biosynthesis. Although in vitro studies demonstrated that the protein encoded by accD6 (Rv2247) may be a functional carboxyltransferase subunit of ACC in M. tuberculosis, the in vivo function and regulation of accD6 in slow- and fast-growing mycobacteria remain elusive. Herein, directed mutagenesis demonstrated that, although accD6 is essential for M. tuberculosis, it can be deleted in M. smegmatis without affecting its cell envelope integrity. Moreover, we showed that despite being part of the type II fatty acid synthase operon, the accD6 of M. tuberculosis, but not M. smegmatis, possesses its own additional promoter (P_{acc}). The expression level of accD6_{Mtb} placed only under P_{acc} is ten-fold lower than that in wild-type M. tuberculosis, but sufficient to sustain cell viability. Importantly, this limited expression level affects growth, mycolic acid content and cell morphology. These results provide the first in vivo evidence of AccD6 as a key player in mycolate biosynthesis of M. tuberculosis implicating AccD6 as the essential ACC subunit in pathogenic mycobacteria, and an excellent target for new anti-tubercular compounds. Our findings also highlight important differences in the mechanism of acetyl carboxylation between pathogenic and non-pathogenic mycobacterial species.
One of the most interesting features of all mycobacteria is their thick and highly impermeable cell envelope (45). Besides peptidoglycan surrounding the cytoplasmic membrane, this complex lipid-rich structure consists of an outer layer of mycolic acids covalently linked to arabinogalactan. Mycolic acids are long-chain (C₆₀₋₉₀), high molecular weight β-hydroxy fatty acids with short alkyl branch (C₂₄₋₂₆) in α position (2, 9, 17, 39). The tightly packed layer of mycolate chains in the cell wall of *M. tuberculosis* is responsible for its important characteristics, including resistance to chemical injury and dehydration, low permeability to hydrophobic antibiotics or its ability to form biofilms (19, 23, 24, 52). Furthermore, mycolic acids are considered as major virulence effectors, allowing *M. tuberculosis* to persist within the host (6, 17, 24, 83).

The biosynthesis of mycolic acid is linked to the unusual presence of two fatty acid synthases in mycobacteria (Fig. S1): the mammalian-type multienzyme, fatty acid synthase-I (FAS-I); and fatty acid synthase-II (FAS-II) which is a set of autonomic enzymes, similar to those found in other bacteria (8, 59, 68, 82). FAS-I catalyzes the *de novo* synthesis of short fatty acyl primers (typically C₁₆₋₂₆), and the FAS-II system subsequently elongates these short chains into long-chain fatty acids (C₄₈₋₅₆). Mycolic acid biosynthesis is also the target of powerful antitubercular drugs such as isoniazid, ethionamide and thiolactomycin (3, 27, 31, 44, 53, 70, 72) and also emerging target for the future inhibitors. Although we know the functions of the most important enzymes involved in the *de novo* synthesis and elongation of fatty acyl chains (7, 10, 14, 22, 35, 41, 42, 60, 66, 67, 79, 84), little is known about the initial steps within this pathway, such as the synthesis of malonyl-coenzyme A (CoA).
Malonyl-CoA, the universal substrate for the synthesis of mycolic and other fatty acids, is incorporated into the growing acyl chain during the repetitive cycle of FAS-I/II reactions (Fig. S1). It is generated by the carboxylation of acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase (ACC) (80). This irreversible, biotin- and ATP-dependent reaction consists of two catalytic steps: the carboxylation of biotin to form carboxybiotin; and the transfer of a carboxyl group from the biotin to a specific substrate such as acetyl-CoA to generate malonyl-CoA. Each half-reaction is catalyzed by a specific ACC subunit: first step – biotin carboxylase (BC), second step – carboxyltransferase (CT). In mycobacteria and other bacteria, each catalytic subunit is encoded by a separate gene (16, 37).

Three genes potentially encoding biotin carboxylase (α-subunit) – accA1-3 and six genes believed to encode carboxyltransferase (β-subunit) – accD1-6 were identified in *M. tuberculosis* genome (15). Since the β-subunits confer the substrate specificity of ACC, the large number of accD genes in mycobacterial genomes may reflect the ability of mycobacteria to carboxylate not only acetyl-CoA but also several other distinct substrates, including the short acyl chains that serve as intermediates in the biosynthesis of complex mycobacterial (glyco)lipids. Transposon site hybridization (TraSH) analysis has shown that among the six carboxyltransferase genes in *M. tuberculosis*, accD4, accD5 and accD6 are essential for cell survival (63, 64, 65). To date, the roles of accD4 and accD5 in mycolic acid biosynthesis have been studied (Fig. S1) (21, 28, 38, 51, 58), and the expression profile of all accD family members in *M. tuberculosis* is known (18).

In the context of malonyl-CoA synthesis, we have focused our work on accD6, which remains the less characterized carboxyltransferase gene, despite its presumable role in the mycolic acid biosynthesis. The role of accD6 (Rv2247) in *M. tuberculosis*
mycolic acid biosynthesis has been predicted from its location in FAS-II gene cluster (15). Daniel et al. confirmed that \textit{accD6}_{\textit{Mtb}} is highly expressed during intensive mycolate biosynthesis and showed that the AccD6 and AccA3 proteins could together reconstitute an enzyme that is able to carboxylate acetyl-CoA \textit{in vitro} (Fig. S1) (18). However, due to the essential nature of \textit{accD6}, the possible involvement of AccD6 in mycolic acid biosynthesis has never been addressed \textit{in vivo}. Till now, only one study concerning the probable function of \textit{accD6} in the fast-growing non-pathogenic \textit{M. smegmatis} species has been conducted (36).

Herein, genetic studies were used to investigate and compare \textit{accD6} gene essentiality in both \textit{M. tuberculosis} and \textit{M. smegmatis}. We also demonstrated that \textit{accD6} of \textit{M. tuberculosis} is controlled by both the FAS-II promoter and also an internal promoter upstream of gene. This unexpected finding allowed to generate a \textit{M. tuberculosis} mutant strain in which \textit{accD6} expression was only driven by its own promoter regardless of the FAS-II promoter. This strain appeared to be a valuable tool to dissect the role of AccD6 expression with respect to growth, mycolic acid biosynthesis and \textit{M. tuberculosis} cell morphology.
Materials and Methods

Bacterial strains and culture conditions

*M. tuberculosis* H37Rv, *M. smegmatis* mc²155 (73), *E. coli* Top-10 (Invitrogen) and *E. coli* BL21 pLysS (Invitrogen) were used in the present study. Strains based on *M. tuberculosis* H37Rv were maintained on Middlebrook 7H10 agar or 7H9 broth (Becton Dickinson) supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) enrichment (Becton Dickinson). *M. smegmatis* derivative strains were cultured in Nutrient Broth (Becton Dickinson) supplemented with 10.0 g l⁻¹ glucose, or in Sauton medium. For selection, we used kanamycin (25 μg ml⁻¹), hygromycin (50 μg ml⁻¹), gentamycin (7.5 μg ml⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 50 μg ml⁻¹) or sucrose (2% w/v) as appropriate. *E. coli* Top-10 was used as the host for cloning whereas *E. coli* BL21 pLysS was used as the host for expressing recombinant AccD₆*₆Mb*. Both *E. coli* strains were grown in LB medium. Plasmid selection and maintenance were performed using ampicillin (10 μg ml⁻¹), chloramphenicol (34 μg ml⁻¹), hygromycin (200 μg ml⁻¹) and kanamycin (50 μg ml⁻¹).

The plasmids used in this study are listed and described in Table S1. Cell densities were determined by using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech); the presented results reflect the values remaining after subtraction of the optical density (OD)₆₀₀ of the culture medium was subtracted.

Gene cloning strategies
Standard molecular biology protocols were used for all cloning procedures (62). All PCR products were obtained using thermostable *Pfu* DNA polymerase (Fermentas). They were initially cloned into a pJET1.2/blunt vector (Fermentas), followed by sequencing and digestion with the appropriate restriction enzymes. They were then cloned into the final vectors. To facilitate subcloning, some restriction enzyme recognition sites were incorporated into the primer sequences (Table S2), while in other cases natural restriction sites were used.

Construction of accD6 gene replacement vectors

All PCR primers utilized in this study are listed in Table S2. To create unmarked deletion of the *accD6* in *M. tuberculosis* and *M. smegmatis*, a suicidal recombination delivery vector based on p2NIL was used (54). In both cases, the recombination vector carried the region upstream of *accD6* together with the 5’ end of the gene (the GR1-GR2 PCR fragment; 1595 bp for *M. tuberculosis*, 1762 bp for *M. smegmatis*) cloned next to the 3’ end of the gene together with its downstream region (the GR3-GR4 PCR fragment; 1197 bp for *M. tuberculosis*, 1237 bp for *M. smegmatis*) (Fig. S2A and Fig. S3A). The 5’ and 3’ *accD6* PCR fragments were ligated into p2NIL so that the resulting Δ*accD6* gene was devoid of an internal sequence (624 bp for *M. smegmatis*, 853 bp for *M. tuberculosis*). Since Δ*accD6* was cloned out of frame it encoded a nonfunctional protein.

Finally, the PacI screening cassette from pGOAL17 (54) was inserted into the prepared constructs, yielding suicide delivery vectors, pJPD6Ms and pJPD6Tb.

Testing the essentiality of the accD6 gene from *M. tuberculosis*
The two-step recombination protocol of Parish and Stoker (54) was used to disrupt the gene of interest at its native locus. The plasmid DNA of suicide delivery vector pJPD6Tb was treated with NaOH (0.2 mM) and electroporated into *M. tuberculosis* competent cells, where it was integrated into the chromosome by homologous recombination. The resulting single-crossover recombinant (SCO) mutant colonies were blue, Kan^R^ and sensitive to sucrose (2%). The recombination site was confirmed by PCR and Southern blot hybridization. Single SCO colony was then picked, resuspended in fresh 7H9 + OADC medium, poured onto solid 7H10 + OADC medium without any selective markers, and incubated at 37°C for 7 days to allow the second crossover to occur. Serial dilutions were plated onto media containing sucrose and Xgal to select for double crossovers (DCO). Potential double crossovers (white, sucrose-resistant colonies) carrying either wt *accD6* (wt-DCO) or the mutated *ΔaccD6* gene (mut-DCO) were screened for kanamycin sensitivity and confirmed by PCR and Southern blot hybridization. The identification of mut-DCO strains would be possible only if *accD6* is dispensable for the viability of *M. tuberculosis*.

The PCR analysis used to distinguish among SCO, wt-DCO and mut-DCO strains was performed on XhoI- and PvuII-digested chromosomal template DNA using primers TBaccD6-XbaIs and TBaccD6-HindIIIrev. The probe for Southern blot hybridization was PCR generated using the same primers, with pJPD6Tb as the template. Probe labeling, hybridization and signal detection were performed using the AlkPhos Direct Labeling and Detection System (GE Healthcare), according to the manufacturer’s instructions.

*Disruption of the M. smegmatis accD6 gene by homologous recombination*
To perform unmarked deletion of the accD6 gene from M. smegmatis, we used the above-described two-step recombination protocol. The pJPD6Ms suicide delivery vector was electroporated into M. smegmatis competent cells, and the resulting blue, Kan\textsuperscript{R} and sucrose-sensitive (2%), single-crossover recombinant (SCO) mutant colonies were streaked onto solid medium without antibiotics to allow the second crossover to occur. Potential double crossovers (white, sucrose-resistant colonies) carrying either wt accD6 (wt-DCO) or the mutated ΔaccD6 gene (mut-DCO) were screened for kanamycin sensitivity and confirmed by PCR and Southern blot hybridization. The PCR analysis used to distinguish among the SCO, wt-DCO and mut-DCO strains was performed using BamHI-digested chromosomal DNA as the template along with primers MsaccD6Xs and MsaccD6HXr. The probe for Southern blot hybridization was generated by PCR using the same primers with pJPD6Ms as the template. Probe labeling, hybridization and signal detection were performed as described above.

Construction of complementation plasmids

For complementation of the M. tuberculosis accD6 SCO strain, we constructed pFASTb2, pFD6Tb1 and pPD6Tb vectors. For pFASTb2, a PCR fragment (1028 bp after NotI/XbaI digestion), carrying the FAS-II operon promoter and the first 114 bp of the fabD gene (Fig. 2A) was amplified from M. tuberculosis chromosomal DNA using primers TbP-fas2-Not-nat and TbP-fas2-Xba-nat, and the resulting fragment was cloned into the NotI/XbaI site of the pMV306Gm integrative vector to yield pFASTb. Then another PCR fragment (2754 bp after XbaI/EcoRI digestion), carrying the
remaining 795 bp of the fabD, the full sequences of acpM and kasA and the first 259 bp of kasB, amplified using Tb-fas2-sense-Xb and Tb-fas2-reve-Ec primers, was cloned into pFASTb using the XbaI/EcoRI sites, yielding pFASTb1. Finally, a PCR fragment (2542 bp after EcoRI digestion) carrying the remaining 1058 bp of kasB and the full sequence of accD6 (amplified using primers Tb-fas2-senEcoRI and Tb-fas2-revEcoRI) was cloned into the EcoRI site of pFASTb1 to yield pFASTb2, which contained a reconstructed version of the entire FAS-II gene cluster (PfasII\(FASII_{Mtb}\)). For construction of pFD6Tb1, the entire accD6 gene (1486 bp) was amplified from \(M.\) tuberculosis chromosomal DNA using primers TBaccD6-XbaI and TBaccD6-HindIIIrev and subsequently cloned into the XbaI/HindIII site of the pMV306Hyg integrative vector to yield pFD6Tb. This latter was then used as a host for fragment carrying the FAS-II promoter (1028 bp after NotI/XbaI digestion), amplified using TbP-fas2-Not-nat and TbP-fas2-Xba-nat primers and cloned into NotI/XbaI site upstream from the accD6 gene sequence to yield final pFD6Tb1 construct \(P_{fasII\_accD6_{Mtb}}\) (see Fig. 2A).

For construction of pPD6Tb \(P_{acc\_accD6_{Mtb}}\), a PCR fragment (2542 bp after EcoRI digestion) carrying the entire accD6 (Rv2247) gene together with 1088 bp of upstream sequence (the 30 bp intergenic region and 1058 bp of kasB) (see Fig. 5B) was amplified using primers Tb-fas2-senEcoRI and Tb-fas2-revEcoRI and cloned into the EcoRI site of the pMV306Gm.

The three constructs were separately introduced by electroporation into the attB site of the \(M.\) tuberculosis accD6 SCO mutant. The obtained strains were selected for DCO mutants in which the chromosomal copy of the gene had been replaced by the plasmid-delivered version to generate strains \(\Delta accD6_{Mtb}\_P_{fasII\_FASII_{Mtb}}, \Delta accD6_{Mtb}\_P_{fasII\_accD6_{Mtb}},\) and \(\Delta accD6_{Mtb}\_P_{acc\_accD6_{Mtb}}\).
For complementation of the *M. smegmatis* ΔaccD6 DCO mutant, we constructed vectors pAceD6Ms, pAceD6Tb and pPD6Ms1. To construct pAceD6Ms (P<sub>ami</sub>accD6<sub>Msm</sub>), a PCR fragment (1425 bp) carrying the entire *M. smegmatis* accD6 (MSMEG_4329) was amplified using primers MsaccD6Xs and MsaccD6Xr, and then cloned into the XbaI site of the pJam2 shuttle vector (75), under the control of the acetamidase (P<sub>ami</sub>) promoter, induced following addition of 4 g l<sup>-1</sup> of acetamide. To construct pAceD6Tb (P<sub>ami</sub>accD6<sub>Mtb</sub>), a PCR fragment (1422 bp) carrying the entire *M. tuberculosis* accD6 (Rv2247) was amplified using primers TBaccD6B and TBaccD6X and cloned into the BamHI/XbaI site of the pJam2 shuttle vector. To construct vector pPD6Ms1 (P<sub>acc</sub>accD6<sub>Msm</sub>), a PCR fragment (1637 bp after ClaI/EcoRI digestion) carrying the entire *M. smegmatis* accD6 (MSMEG_4329) and 180 bp of sequence upstream from the accD6 start codon was amplified using primers MsD6Cns and MsD6prEr and cloned into the ClaI/EcoRI site of the pMV306Km, to generate construct pPD6Ms. Then, another PCR fragment, carrying an additional 827 bp (after ClaI digestion) upstream from the above-described 180 bp sequence was amplified using primers MsD6PCls and MsD6nCr and cloned into the ClaI site of pPD6Ms to generate pPD6Ms1, in which accD6 is placed under the control of its 1007 bp upstream sequence (see Fig. 5A).

The three constructs were separately electroporated into the *M. smegmatis* ΔaccD6 DCO mutant, to generate strains ΔaccD6<sub>Msm</sub>-P<sub>ami</sub>accD6<sub>Msm</sub>, ΔaccD6<sub>Msm</sub>-P<sub>ami</sub>accD6<sub>Mtb</sub> and ΔaccD6<sub>Msm</sub>-P<sub>acc</sub>accD6<sub>Msm</sub> strains.

Expression and purification of recombinant KasA<sub>Mtb</sub> and AccD6<sub>Mtb</sub>
The cloning and purification of recombinant KasA<sub>Mtb</sub> was previously described (32). For generation of recombinant AccD6<sub>Mtb</sub>, the accD6<sub>Mtb</sub> (Rv2247) gene was PCR amplified from <i>M. tuberculosis</i> genomic DNA using primers TBaccD6s and TBaccD6r and cloned into the BamHI/HindIII site of the pHIS.Parallel1 expression vector (69). The resulting plasmid, pHD6Tb, was verified by sequencing and introduced into <i>E. coli</i> BL21 pLysS cells. The cells were grown in 1 L LB at 37°C until the OD<sub>600</sub> reached 0.4–0.6, whereupon expression of the His-tagged AccD6<sub>Mtb</sub> fusion protein was induced with 0.4 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). After 4 h incubation at 37°C the cells were harvested by centrifugation, resuspended in Binding Buffer (Novagen) and lysed by sonication. The AccD6<sub>Mtb</sub> fusion protein was purified from cell lysates with nickel-affinity chromatography on a His-Bind column (Novagen), and subsequently was used to raise a rabbit polyclonal primary antibody (see below).

Preparation of antisera against KasA<sub>Mtb</sub> and AccD6<sub>Mtb</sub>

The preparation of rat anti-KasA<sub>Mtb</sub> antibodies was previously described (34). Antiserum against AccD6<sub>Mtb</sub> was obtained by subcutaneous immunization of New Zealand laboratory rabbit with three doses of the purified <i>M. tuberculosis</i> AccD6 antigen (150 µg, 100 µg and 100 µg), emulsified with incomplete Freund’s adjuvant (Sigma) at three-week intervals. The levels of anti-AccD6 antibodies in serum samples from immunized rabbit and preimmune serum samples (negative controls) were screened by enzyme-linked immunosorbent assay (ELISA) using purified AccD6 as the coating antigen, and rabbit serum samples diluted from 1:100 to 1:51200 as the primary antibody. The immunoenzymatic reaction was developed...
using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch), with and 2.2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) (Sigma) as the chromogen. The absorbance values were measured at \( \lambda = 405 \) nm. The optimal working dilution of polyclonal anti-AccD6 serum for Western blotting was determined in preliminary titration experiments using purified AccD6 as a standard antigen, anti-AccD6 rabbit serum in dilutions from 1:100 to 1:51200 as primary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) as the secondary antibodies, and 4-chloro-1-naphthol (Sigma) as the chromogen.

**Cell wall permeability test**

Tritiated rifampin (4-methylpiperazine-\(^3\)H; specific activity: 10 Ci mmol\(^{-1}\); Moravek Biochemicals) was used to examine the cell wall permeability of *M. smegmatis* wild-type and ΔaccD6 DCO mutant cells according to the modified protocol of Piddock et al. (57). In brief, mycobacterial cells were grown to mid-logarithmic phase (OD\(_{600}\) of 0.6) in NB medium. Fifty milliliters of the culture was centrifuged at 3 000 \( \times \) g for 20 min at 37°C, resuspended in the same medium to an optical density of 8.0, and placed in a 37°C water bath for 10 min to equilibrate. The \([\text{\textsuperscript{3}}\text{H}]\text{rifampin}\) was added at a final concentration of 0.272 \( \mu \)g ml\(^{-1}\) (3.33 \( \mu \)Ci ml\(^{-1}\)) and 500 \( \mu \)l samples were removed at various time intervals. Each sample was mixed with 1 ml of 50 mM sodium phosphate buffer (pH 7) on ice and centrifuged at 16 000 \( \times \) g for 20 min at 4°C. The resulting cell pellets were washed again in the same buffer, recentrifuged, and mixed with Ultima Gold scintillation fluid (Perkin Elmer). The cell-associated radioactivity was determined by liquid scintillation counting. Passive adsorption of
rifampin to the cell wall (background) was estimated by performing the experiments at 0°C; the results from these experiments were subtracted from the values obtained at 37°C to determine the activity of rifampin that had actively accumulated in the cells.

**RNA extraction and reverse transcription**

For quantitative, real-time PCR (qRT-PCR) and FAS-II operon analysis, RNA was extracted from wild-type *M. tuberculosis* and *M. smegmatis* strains and ΔaccD6Mtb-\(\Delta_{fasII}accD6Mtb\), ΔaccD6Mtb-\(P_{acc}accD6Mtb\) mutants using the TRIzol LS Reagent (Invitrogen). Briefly, 10 ml of aerated culture at logarithmic phase (OD\(_{600}\) of 0.6) was centrifuged at 3,000 × g for 20 min at 4°C and resuspended in 300 µl of water. The culture was transferred to screw-cap tubes containing 0.5 ml of 0.1 mm zirconia/silica beads (BioSpec Products) and 900 ml of TRIzol LS Reagent. The bacteria were lysed using a Mini-BeatBeater-8 (BioSpec Products) for 3 min, and incubated for 5 min at room temperature. Following incubation, the insoluble material was removed by centrifugation at 16,000 × g for 15 min at 4°C, and RNA was purified and treated with DNase I (Fermentas) according to the manufacturer’s instructions. Finally, the RNA samples were eluted in RNase-free water, and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Each time the RNA samples were PCR-verified in order to identify possible DNA contamination. For reverse transcription, we used a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas), and performed the reactions in total volumes of 20 µl containing 1 µg of total RNA. Subsequently, 1 µl of cDNA (equivalent to 50 ng of RNA) was used in the qRT-PCR experiments (see below).
Quantitative real-time polymerase chain reaction

qRT-PCR for the analysis of accD genes expression was performed using the Maxima SYBR Green qPCR Master Mix (Fermentas) and a 7900HT Real-Time PCR System (Applied Biosystems). Each reaction (25 µl final volume) was mixed on ice and contained 1x Maxima SYBR Green qPCR Master Mix, 50 ng of cDNA and 0.3 µM of each primer (see Table S2 for primer sequences). For expression analysis of the *M. tuberculosis* accD genes, we used a two-step cycling protocol in which the reactions were heated to 95°C for 10 min and then subjected to 40 cycles of 95°C for 20 s (denaturation) and 60°C for 60 s (annealing/extension). Data acquisition was performed during the annealing/extension step. For expression analysis of the *M. smegmatis* accD genes, we used a three-step cycling protocol in which the reactions were heated to 95°C for 10 min and then subjected to 40 cycles of 95°C for 20 s (denaturation), 63°C for 30 s (annealing) and 72°C for 30 s (extension). Data acquisition was performed during the extension step. To verify the specificity and identity of the generated PCR products, melting curve analysis was performed at the end of each PCR reaction, and the PCR products were analyzed by agarose gel electrophoresis. Each experiment was performed in triplicate, and the results are presented as means and standard errors. Our comparisons of the expression levels of various accD family members between *M. tuberculosis* and *M. smegmatis* are presented as cycle threshold (C_{T}) values, normalized with respect to the expression of *sigA* (ΔC_{T}) and converted to linear form (2^{ΔC_{T}}). For the other qRT-PCR experiments, the results reflect the fold-change in expression of a given gene in the...
mutant strain versus the wild-type strain, as calculated using the double delta method 
\(2^{-\Delta\Delta CT}\).

**Total protein isolation and Western blotting**

Ten milliliter aliquots of bacterial culture were centrifuged, the bacteria were 
resuspended in TE buffer and disrupted by bead beating with 0.1 mm zirconia/silica 
beads. The resulting cell lysates were clarified by centrifugation. The total protein 
concentration in each cell lysate was determined using a BCA Protein Assay 
Reagent Kit (Pierce). Equal amounts of proteins (20 µg) were separated on 10% 
sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred onto a 
nitrocellulose membrane (Thermo Scientific). The membrane was saturated with 5% 
skim milk in PBS, probed with rat anti-KasA antibodies (1:500 dilution) or rabbit anti- 
AccD6 antibodies (1:1000 dilution), and then washed and incubated with horseradish 
peroxidase-conjugated anti-rat or anti-rabbit secondary antibodies (1:1000 dilution). 
The protein signals were visualized directly on the membrane using 4-chloro-1- 
naphthol (Sigma) as a chromogen.

**Lipid and mycolic acid extraction and analysis**

Mycobacterial cultures at mid-logarithmic growth phase were mixed with [2-
\(^{14}\)C]acetate (specific activity: 45-60 mCi mmol\(^{-1}\); Perkin Elmer) at 1 µCi ml\(^{-1}\), and then 
further incubated at 37°C for 4 h (for *M. smegmatis*) or 1, 6, and 24 h (for *M. tuberculosis*). The \(^{14}\)C-Labeled cells were harvested by centrifugation at 2 000 × g, 
subjected to alkaline hydrolysis by incubation in 2 ml 15% tetrabutylammonium
hydroxide (TBAH) (Sigma) at 100°C overnight, and then mixed with 4 ml CH$_2$Cl$_2$, 300 µl CH$_3$I (Sigma) and 2 ml H$_2$O. After 1 h, the upper, aqueous phase was discarded and the lower, organic phase was washed twice with water and dried. The lipids were extracted using diethyl ether, dried, and then resuspended in 200 µl CH$_2$Cl$_2$. Equal counts of the fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) from wild-type and mutant strains were applied to TLC silica gel 60F$_{254}$ plates (Merck) and developed in petroleum ether/acetone (19:1, v/v).

For complex lipid analysis, the [2-14C]acetate-labeled cells were extracted with 2 ml CH$_3$OH/0.3% NaCl (10:1, v/v) and 2 ml petroleum ether. The mixture was centrifuged, the upper petroleum ether layer was removed, and an additional 2 ml petroleum ether was mixed with the lower fraction. The combined petroleum ether extracts were then evaporated under nitrogen to yield apolar lipids that were resuspended in CH$_2$Cl$_2$ prior to TLC analysis. For extraction of polar lipids, the methanolic saline extract obtained after extraction of the apolar lipids was heated at 65°C for 5 min and then mixed with 2.3 ml of CHCl$_3$/CH$_3$OH/0.3% NaCl (9:10:3, v/v). The solvent extract was then separated from the biomass by centrifugation and the supernatant was retained. The pellet was further extracted with 0.75 ml CHCl$_3$/CH$_3$OH/0.3% NaCl (5:10:4, v/v). The combined solvent extracts were mixed with 1.3 ml CHCl$_3$ and 1.3 ml 0.3% NaCl. After centrifugation the lower organic layer was collected and evaporated to dryness to yield the polar lipids which were resuspended in CHCl$_3$/CH$_3$OH/H$_2$O (10:10:3, v/v) prior to TLC analysis.

For two-dimensional TLC analysis of the complex lipids, five solvent systems were used to cover the polarity range of both polar and apolar mycobacterial lipids according to Besra et al., 1998 (4). Equal counts of the extracts were subjected to
TLC, resolved using the appropriate solvent system, dried, and exposed overnight to X-Omat film (Kodak).

Scanning electron microscopy (SEM)

The N-acetyl-L-cysteine–sodium citrate–NaOH (NALC-NaOH) procedure was used to prepare samples for SEM (49). In brief, 10 ml of 0.05 g N-acetyl-L-cysteine in 5 ml of 2.9% citric acid and 5 ml 4% NaOH was mixed with 10 ml of mycobacterial liquid culture, and the mixture was vortexed and incubated at 37°C for 20 min. Then 20 ml of water was added, and the sample was centrifuged at 3,000 × g. The supernatant was carefully discarded, and the cells were fixed with 1.5 ml of 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 6 h (76), followed by centrifugation and resuspension in phosphate buffer. The cells were then treated with OsO4, dehydrated stepwise in a graded acetone series, dried, and sputter coated with gold using a K550X Sputter Coater (Quorum Technologies). The samples were examined using a VEGA 3 scanning electron microscope (TESCAN) with 30,000 × magnification and an accelerating voltage of 30 kV.
Results

The expression profiles of the acyl-CoA carboxylase β subunits differ between pathogenic and non-pathogenic mycobacteria

The expression profile of all nine genes predicted to encode the carboxylase subunits of *M. tuberculosis* has been evaluated previously (18). Quantitative real-time PCR (qRT-PCR) analysis revealed that, only the genes encoding the AccA3, AccD4, AccD5 and AccD6 carboxylase subunits were expressed at high levels during *M. tuberculosis* exponential growth. However, the expression profile of their orthologs in fast-growing species has not been reported yet. Therefore, we compared the expression levels of various members of the *accD* family in pathogenic and non-pathogenic mycobacteria. RNA isolated from exponentially growing cells was subjected to qRT-PCR, and the relative expression level of each *accD* gene was calculated from the CT value, normalized with respect to the expression level of the endogenous control gene, *sigA*. Comparison of values obtained for *M. tuberculosis* and *M. smegmatis* indicated significant difference in the expression level limited to three specific *accD* genes that are mostly expressed and regulated during the exponential growth of pathogenic mycobacteria (Fig. 1). The expression levels of *accD1-accD3* were similar in both species. In contrast, the expression level of *accD5* in *M. smegmatis* was found to be three times lower, whereas expression levels of *accD4* and *accD6* were found to be two times lower with respect to *M. tuberculosis*. The significant difference in the expression levels of genes encoding major carboxyltransferase subunits may involve differences in the carboxylation process during mycolates biosynthesis in these species.
The accD6 (Rv2247) gene is essential for the viability of Mycobacterium tuberculosis.

The in vivo function and regulation of accD6 expression in slow- and fast-growing mycobacteria remains largely unknown. Furthermore, the belief that the accD6 gene is essential for M. tuberculosis is based solely on predictive data from high density mutagenesis studies (63, 64, 65). To carefully evaluate whether accD6 is essential for M. tuberculosis, we used a two-step homologous recombination protocol (54) to generate single crossover (SCO) strains carrying both an endogenous wild-type accD6 gene and an additional accD6 allele carrying an internal deletion (ΔaccD6).

After the second crossover PCR analysis of more than 50 individual DCO colonies generated from two independent SCO strains identified wt-DCO exclusively, thereby strongly suggesting that deletion of accD6 is lethal for M. tuberculosis (Fig. S2A). To further confirm essentiality of accD6 in M. tuberculosis and exclude potential failure in the knockout procedure, the screening was repeated with another, intact copy of the gene introduced into the attB site of the SCO strain. Two distinct constructs based on the mycobacterial integrative pMV306 vector were created and introduced into the host chromosome. In the first construct, accD6 was cloned under the control of a putative FAS-II operon promoter (P_{fasII}accD6_{Mt}). The second construct consisted of whole FAS-II operon sequence (P_{fasII}FASII_{Mt}) (Fig. 2A). The resulting strains were then subjected to second crossover to generate DCO mutants (ΔaccD6_{Mt}-P_{fasII}accD6_{Mt}; ΔaccD6_{Mt}-P_{fasII}FASII_{Mt}) that were subsequently identified by PCR (Fig. S2B) and Southern blot hybridization (Fig. 2B). The successful engineering of such strains confirmed the essentiality of accD6 in M. tuberculosis.
The accD6 (MSMEG_4329) gene is dispensable in Mycobacterium smegmatis

The observed difference in the requirement for AccD6 between pathogenic and non-pathogenic strain prompted us to revisit the question of whether the accD6 gene is essential for \textit{M. smegmatis}. SCO strains carrying both wild-type accD6 and a mutated copy of accD6 (ΔaccD6) were constructed and subjected to second crossover. This resulted in generation both wt-DCO strains and strains carrying only mutated (ΔaccD6) copy of gene (mut-DCO). The deletion of accD6 in \textit{M. smegmatis} showed that, in contrast to its ortholog in \textit{M. tuberculosis}, this gene is not essential for the viability. The genotype of three selected mutants identified within 25 analyzed DCO was PCR (Fig. S3) and Southern blot verified (Fig. 3A). The loss of AccD6 protein in \textit{M. smegmatis} ΔaccD6 mutant was confirmed by Western blotting with a polyclonal rabbit antiserum raised against the \textit{M. tuberculosis} AccD6 protein (Fig. 3B). To ensure, that deletion of the accD6 gene did not affect the expression of the neighboring kasB gene, which is also dispensable in \textit{M. smegmatis}, we Western blotted total protein extracts from the ΔaccD6 mutant with a rat antiserum raised against KasA, which can also cross-react with KasB (7). Our results revealed that deletion of accD6 did not affect the expression of either the β-ketoacyl-AcpM synthases KasA and KasB (Fig. 3C).

The permeability and lipid composition of \textit{M. smegmatis} ΔaccD6 cell envelope remains unaltered

Studies on conditional depletion of enzymes that are essential for mycolic acid synthesis in \textit{M. smegmatis} have demonstrated that loss of active protein leads to
cessation of mycolates synthesis, drop in the OD, decrease in numbers of CFU and finally to mycobacterial cell lysis (7, 79). Thus, we reasoned that inactivation of a probable acetyl-CoA carboxylase, active in FAS-II system should also introduce changes into mycolic acid biosynthetic pathway and whole envelope lipid organization that in turn should affect the cell viability. Optical density (OD) measurements of the wild-type and ΔaccD6 DCO mutant cultures growing in Sauton medium revealed only a slight decrease in mutant growth dynamic during the logarithmic phase, but in the end both mutant and wild-type M. smegmatis entered the stationary phase with the same OD value. This growth rate delay was not seen in mutant strains complemented with intact copies of accD6 from M. smegmatis or M. tuberculosis (Fig. 4A). To prepare such strains, M. smegmatis and M. tuberculosis accD6 were cloned under the control of the acetamidase (P_{ami}) promoter and the resulting constructs (P_{ami}accD6_{Msm}/P_{ami}accD6_{Mt}) where then introduced into M. smegmatis ΔaccD6 strain to generate ΔaccD6_{Msm}-P_{ami}accD6_{Msm} and ΔaccD6_{Mt}-P_{ami}accD6_{Mt}. To study the effect of accD6 deletion on M. smegmatis envelope lipids composition the M. smegmatis wild-type and ΔaccD6 DCO mutant strains were labeled with [2-^{14}C]acetate. The fatty and mycolic acids were extracted from labeled cells and methylated. Extracts of total fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were analyzed by TLC-autoradiography. As shown in Figure 4C, disruption of the accD6 gene did not cause any detectable changes in the composition or quantity of mycolic acids. We next addressed whether complementation of the ΔaccD6 mutant with an intact accD6 M. tuberculosis gene could affect the cell wall mycolic acid profile. The ΔaccD6_{Msm}-P_{ami}accD6_{Mt} mutant was cultured on rich medium with or without acetamide and labeled with [2-
As shown in Figure 4C, no significant changes in the mycolate profile were observed between the wild-type and mutant strains. To have the full picture of cell envelope lipids in the ΔaccD6 mutant strain, we investigated the extractable (polar and apolar) lipid profile by two-dimensional TLC, with the use of different solvent systems. This systematic analysis did not provide significant changes in the quantity and/or quality of complex lipids (Fig. S4A). In search for more subtle changes in the structure of cell envelope as a consequence of accD6 disruption, we determined and compared the cell wall permeability in both mutant and parental strains. This was achieved using a large, hydrophobic, tritium-labeled rifampin molecule, according to the modified protocol of Piddock et al. (57). Figure S4B clearly indicates that both the wild-type strain and ΔaccD6 mutant exhibit the same rate of radiolabeled rifampin uptake. The absence of any significant differences in permeability of the cell wall between the mutant and wild-type strains was finally confirmed by comparison of the estimated MIC99 values for rifampin and crystal violet. For both wild-type and mutant strains, the MIC values were 20 µg ml\(^{-1}\) for rifampin and 6 µg ml\(^{-1}\) for crystal violet (data not shown).

*M. tuberculosis* accD6 possesses its own promoter

Our study is the first report of a FAS-II encoded gene whose essentiality differs between pathogenic and non-pathogenic species. Interestingly, we also observed differences in the expression levels of this gene between the analyzed strains. In light of these results, we reasoned that accD6 may possibly be regulated separately from the other members of the FAS-II gene cluster. Therefore, using specific genetic
constructs for complementation of the *M. smegmatis* ΔaccD6 and *M. tuberculosis* accD6 SCO mutants, we examined whether accD6 possesses an endogenous and specific promoter (P_{acc}). In the case of *M. smegmatis*, the accD6 gene was cloned together with 1007 bp upstream sequence into the pMV306 integrative vector, and the resulting pPD6Ms1 construct was introduced into the mut-DCO (ΔaccD6) strain to generate the ΔaccD6_{Msm}-P_{acc}accD6_{Msm} mutant. Total protein extract of the ΔaccD6_{Msm}-P_{acc}accD6_{Msm} mutant was assayed by Western blot and probed with rabbit antiserum against AccD6. As shown in Figure 5A, the ΔaccD6_{Msm}-P_{acc}accD6_{Msm} mutant failed to produce AccD6, supporting the fact that the 1007 bp region upstream from accD6 in *M. smegmatis* did not confer promoter activity under our experimental conditions. Similar experiments were undertaken regarding the accD6 promoter region from *M. tuberculosis*. The accD6 gene together with its 1088 bp upstream sequence was cloned into pMV306, thus generating pPD6Tb, which was subsequently introduced into the *M. tuberculosis* SCO strain containing both the native and disrupted (ΔaccD6) form of the gene. If it would be possible for pPD6Tb construct to complement the loss of essential for *M. tuberculosis* accD6 gene, one would expect, after second crossover to obtain a DCO strain with deletion in chromosomal copy of the gene. Such strain would only contain an intact accD6 gene, expressed from its own promoter (P_{acc}accD6_{Mtb}). Following completion off all selection steps, we found that the 1088 bp DNA fragment upstream from accD6_{Mtb} carries a promoter sequence able to drive expression of accD6 on a level sufficient to ensure cell survival. This mutant, designated ΔaccD6_{Mtb}-P_{acc}accD6_{Mtb} containing accD6 under its own promoter was subsequently confirmed by Southern blot (Fig. 5B) and Western blot (Fig. 7B, Fig. 8A) analysis.

|  | 24 |
The accD6 gene belongs to the FAS-II transcriptional unit

Our data showed presence of independent promoter sequence (P_acc) that can exclusively controls accD6 expression in M. tuberculosis. Additionally, the same gene in M. smegmatis occurred non-essential for normal mycolates biosynthesis. These observations make questionable the theory that accD6 belongs to the FAS-II transcriptional unit. Although the accD6 ORF is located within a cluster of five genes that have been shown to be involved in mycolic acid biosynthesis (15), there is no any genetic evidence that they constitute a single operon. This prompted us to analyze whether expression of all five FAS-II encoded genes is of mono- or polycistronic type. If all five genes are expressed as a single multigenic transcript, we would expect that four PCR reactions performed on a total cDNA matrix with the use of four sets of primers covering four intergenic sequences between FAS-II genes should result in obtaining four distinct PCR products. Similar procedure was used to analyze the expression mode of FAS-II genes in both M. smegmatis (Fig. 6A) and M. tuberculosis (Fig. 6B). These results indicate that, in both species, all four intergenic sequences are present. This genetic evidence conclusively indicates that accD6 together with other FAS-II genes constitute the operon that can be transcribed from a single, continuous mRNA particle.

Expression of accD6_Mtb under the P_fasII promoter but not the P_acc promoter is upregulated by isoniazid treatment

The above data indicated that, despite possessing its own and independent promoter sequence, the accD6 gene of M. tuberculosis is also a member of the FAS-II
transcriptional unit. The sequence and location of the FAS-II operon promoter (P_{fasII}) is known (26). Interestingly, the first intent antitubercular drug isoniazid (INH), together with other drugs inhibiting FAS-II pathway, can stimulate expression from FAS-II operon promoter (5, 26, 71, 81). Here, we used INH to further verify that although accD6_{Mtb} can be expressed independently from P_{acc}, it can be also expressed under the control of P_{fasII} together with the rest of the FAS-II genes. One can assume that if accD6 is transcribed together with the other members of the operon, INH-mediated induction of the P_{fasII} promoter should result in increased AccD6 expression levels. Moreover, the availability of the M. tuberculosis mutant expressing accD6 from P_{acc} promoter allowed us to investigate whether the internal regulatory sequence is also sensitive to INH induction. Thus, wild-type M. tuberculosis and ΔaccD6_{Mtb}-P_{acc}accD6_{Mtb} mutant were cultured to mid-log phase and then treated with INH (2 µg ml^{-1}). After 24 hours of incubation, samples were collected from control cultures (without INH) and INH-treated cultures. The corresponding bacterial lysates were electrophoresed, blotted and probed with antibodies specific to AccD6. Figure 7A clearly show increased level of AccD6 after INH treatment in wild-type M. tuberculosis. In contrast, the ΔaccD6_{Mtb}-P_{acc}accD6_{Mtb} mutant failed to overexpress AccD6 (Fig. 7B). As a positive control for INH-mediated induction, samples were also probed with antiserum raised against KasA. The visualization of KasA-containing 80-kDa complexes formed as a result of KasA protein overexpression (33, 43) confirmed that the FAS-II genes were properly induced through INH-mediated action on the P_{fasII} promoter.

Similar experiments were performed using wild-type M. smegmatis: The M. smegmatis strain was cultured to mid-log phase and then treated with INH (15 µg ml^{-1}). Aliquots were withdrawn at several time points and subjected to disruption to
obtain total protein extracts, and the lysates were blotted with an antiserum raised against AccD6 (Fig. 7C). Visible induction of AccD6 protein expression after 4 hours of incubation with INH indicated that accD6Msm expression is strongly controlled by the PfasII promoter. The positive control for INH induction was prepared as described above for *M. tuberculosis*.

Decreased expression of accD6 in *M. tuberculosis* affects growth, cell wall lipid content and cell morphology

The generation of the ΔaccD6Mtb-PaccaccD6Mtb mutant indicates that the expression level of accD6 under Pacc promoter is sufficient to sustain the function of this essential gene in *M. tuberculosis* and allow for cell survival. In addition, accD6 placed only under the PfasII promoter successfully replaced the chromosomal copy of this gene in the ΔaccD6Mtb-PfasIIaccD6Mtb mutant. These facts led to the question of what is the accD6 expression level under the control of each promoter separately. Thus, qRT-PCR analysis was performed on total cDNA from ΔaccD6Mtb-PaccaccD6Mtb and ΔaccD6Mtb-PfasIIaccD6Mtb mutants and data were compared with those obtained for wild-type *M. tuberculosis* (Fig. 7D). Results indicated that the expression level of accD6 under the control of Pacc promoter in the ΔaccD6Mtb-PaccaccD6Mtb mutant was at least ten times lower than that in wild-type *M. tuberculosis*. This low expression of accD6 did not significantly alter the expression of the other accD genes (data not shown). Interestingly, we did not observe any significant difference in accD6 expression between wild-type and ΔaccD6Mtb-PfasIIaccD6Mtb *M. tuberculosis* strains. Our qRT-PCR data showing very low accD6 expression in ΔaccD6Mtb-PaccaccD6Mtb mutant were consistent with the results from Western blot analysis of AccD6 protein...
expression in ΔaccD6MtbpaccaccD6Mtbp mutant and wild-type M. tuberculosis (Fig. 7AB, Fig. 8A). The massive decrease in the expression of this essential gene prompted us to analyze the growth dynamics of the mutant strain. OD measurements of the three independently obtained mutants grown in rich medium revealed that the cultures terminated their dynamic growth after 96 to 120 hours of incubation, and they entered a turbidimetric plateau after 168 hours of incubation at OD600 that never exceeded value of 1.0 (Fig. 8A). It is noteworthy that at the plateau point, >60% of the cells were still viable, as confirmed by a fluorescent live/dead test (data not shown). The growth of ΔaccD6MtbpaccaccD6Mtbp was also particularly evident when plating the strain on solid 7H10/OADC medium, but the same as for liquid cultures, significant growth rate defect can be observed (Fig. 8A, inset). Given the results from the prior in vitro studies on the probable function of AccD6 in M. tuberculosis (18) and the indisputably defective growth of the ΔaccD6MtbpaccaccD6Mtbp mutants, we next analyzed their lipid content. The three independently obtained ΔaccD6MtbpaccaccD6Mtbp mutants and wild-type strain were labeled with [2-14C]acetate for 1, 6 or 24 hours, and equal volumes (10% of the total counts) of the extracted FAMEs and MAMEs were analyzed by TLC-autoradiography. As shown in Figure 8B, regardless of the labeling time, the total counts of extracted lipids were typically 3- to 4-fold lower in the ΔaccD6MtbpaccaccD6Mtbp mutant strains versus the wild-type. The difference in lipid content was the most obvious when the labeling period was short (1 hr). Additionally it is important to note that the [2-14C]acetate pulse-labeling studies as well as accD6Mtbp expression analyses were done in exponentially growing cultures, before the mutant cells terminated their dynamic growth.

Phenotypic analyses of the ΔaccD6MtbpaccaccD6Mtbp mutant offered us a unique opportunity to observe the direct in vivo effects of very low AccD6 expression. TLC
analysis showed an overall decrease in the quantity of both fatty acids (the FAS-I end-products) and mycolic acids (the FAS-II end-products), suggesting that fatty acid synthesis was inhibited at a very early stage in the mutant strain (Fig. 8B). Previous studies on genes essential for mycolic acid biosynthesis demonstrated that their inactivation or depletion in *M. smegmatis* led to the cells having an irregular surface (7, 79). Similarly, our scanning electron microscopy (SEM) analyses of the Δ*accD6*<sub>Mtb</sub>-P<sub>acc</sub>*accD6*<sub>Mtb</sub> revealed extensive changes to the surface of the mutant cell wall (Fig. 8C). 84.5 % of the bacteria observed in a total of 20 fields (586 cells analyzed in total) had a characteristic “wrinkled” appearance of grooves and dimples. This phenotype was similar to that of *M. smegmatis* cells following depletion of KasA, which is also an essential member of the FAS-II biosynthetic pathway (7). Altogether, these results are the first *in vivo* demonstration of the role of AccD6 in the mycolic acid biosynthesis pathway of *M. tuberculosis*. Early stage inhibition of fatty acid biosynthesis in the Δ*accD6*<sub>Mtb</sub>-P<sub>acc</sub>*accD6*<sub>Mtb</sub> mutant implicates AccD6 role as the essential, dedicated acetyl-CoA carboxylase subunit in pathogenic mycobacteria.
The mechanism of acetyl-CoA carboxylation – an essential reaction in fatty acid and mycolic biosynthesis - still requires elucidation in mycobacteria. Since AccD4 and AccD5 were the only carboxyltransferase subunits purified from mycobacterial cell extracts they were initially ascribed to be major constituents of ACCase complexes in tubercle bacilli (21, 51, 58). However, *in vitro* analysis showed that none of them can be considered as the subunit dedicated exclusively to acetyl-CoA carboxylation. In the context of malonyl-CoA synthesis in mycobacteria, the work of Daniel *et al.* (18) focused our attention on the third essential carboxyltransferase gene – *accD6*. *In vitro* studies showed that AccD6 protein, together with AccA3 reconstitute an enzyme that preferentially carboxylate acetyl-CoA over propionyl-CoA (18). The *accD6* is the only CT subunit- encoding gene that is a member of the FAS-II gene locus (15) and it is highly expressed during intensive mycolate biosynthesis in *M. tuberculosis* (18).

Here, we report the first detailed genetic analysis of *accD6* as the gene encoding the carboxyltransferase subunit of acetyl-CoA carboxylase in mycobacteria. The qRT-PCR analysis of all *accD* members in *M. tuberculosis* and *M. smegmatis* showed that the three *accD* genes highly expressed and regulated during mycolate biosynthesis in pathogenic mycobacteria were expressed at significantly lower levels in *M. smegmatis*. The lower expression level of *M. smegmatis accD6*, suggesting that there could be a species-specific difference in the requirement for the AccD6 protein prompted us to reconsider the essential nature of *accD6*. As the transposon mutagenesis method of Sassetti *et al.* (63, 64, 65) only predicts essentiality and may give some false results (55), we opted to use a two-step homologous recombination method (54) to examine whether we could disrupt *accD6* in the chromosomes of *M.*.
tuberculosis and M. smegmatis. This efficient method has been widely used in our laboratory for testing the essentiality of M. tuberculosis and M. smegmatis genes (11, 12, 13, 20, 29, 30). Contrary to procedures based on delivery vectors harboring thermosensitive origin of replication (25, 56), this method does not require growth temperature shifts (54). Using this technique, we provided compelling evidence that accD6 is indeed an essential gene in M. tuberculosis under described culture conditions, thereby confirming the previous predictive data.

However, in contrast to the previous report by Kurth et al. (36) we were able to remove the functional accD6 gene from the chromosome of M. smegmatis. Deletion of accD6 in M. smegmatis was performed in two independent experiments and confirmed by all typical techniques, as described in the Results. The basic difference between our findings concerning accD6 in M. smegmatis and previous work of Kurth et al. (36) may be the result of different gene knockout methodologies used in both studies. Difference in efficiency of the integration and/or allelic exchange processes as well as screening procedure that require changing the culture growth conditions may be possible reason of failure in successful isolation of ΔaccD6Msm mutant in previous studies.

This is the first report showing the difference in essentiality of particular gene that belongs to FAS-II gene locus between pathogenic and non-pathogenic mycobacteria. Similar differences between slow and fast-growing strains have been noted previously among the genes responsible for controlling cell envelope biosynthesis. Amin et al. demonstrated the essentiality of the arabinosyltransferase-encoding gene embA in M. tuberculosis but not M. smegmatis (1). accD6 is the second member of FAS-II gene locus found to be dispensable for the viability of M. smegmatis, as kasB was also shown to be non-essential for the in vitro growth of this bacteria (7).
Interestingly, we were able to simultaneously disrupt both *kasB* and *accD6* in *M. smegmatis* (our unpublished data). The double mutant was still viable, but its cell envelope was significantly more permeable which is in agreement with described previously phenotype of Δ*kasB* mutant of *M. marinum* (22). To date, only two of the five genes in the FAS-II locus have been shown to be essential for cell viability in *M. smegmatis*: *kasA* (7) and *acpM* (our unpublished data).

Phenotypic analysis of the Δ*accD6*Msm mutant showed that the absence of functional AccD6 in *M. smegmatis* is not associated with changes in the cell wall lipid content and/or permeability, indicating that the process of acetyl carboxylation was not affected in the mutant cells. This finding, in disagreement with those of Kurth *et al.* (36), suggests that there may be another AccD subunit, capable of fulfilling the function of AccD6 in *M. smegmatis*, or perhaps that AccD6 is not the functional subunit of acetyl-CoA carboxylase in this species. *In vitro* studies on *M. tuberculosis* AccD5 substrate specificity have suggested that despite its predominant activity as a propionyl-CoA carboxyltransferase, it is also able to transfer the carboxyl group on the acetyl-CoA (21, 51). A previous study showed that *accD4* was essential for *M. smegmatis* (58). We tested the essentiality of all *accD* family members in *M. smegmatis* and found that only *accD4* and *accD5* are essential for cell survival in this bacteria (data not shown). Furthermore, qRT-PCR analysis revealed that the expression levels of the *accD4* and *accD5* genes were increased in the Δ*accD6*Msm mutant (data not shown), prompting us to speculate that one or both of these genes could encode the putative subunit(s) that can compensate for the loss of AccD6 in *M. smegmatis*.

Our complementation studies on *M. smegmatis* Δ*accD6*Msm and *M. tuberculosis* *accD6* SCO mutants revealed another important between-species difference in
accD6 expression. Although accD6 in both cases is a member of FAS-II transcriptional unit and its expression is controlled by P_{fasII} promoter, we found that accD6_{Mtb} possesses its own, additional promoter (P_{acc}), placed within the first 1088 bp of its upstream sequence. Contrary to previous findings of Kurth et al. (36), such sequence is not present in case of M. smegmatis accD6 which expression is controlled exclusively by P_{fasII} promoter. Our results implicate that in case of pathogenic M. tuberculosis strain accD6 is under the influence of two (P_{fasII} and P_{acc}) regulatory sequences. qRT-PCR analysis revealed that P_{fasII} plays the dominant role in driving the expression of accD6_{Mtb} under standard in vitro growth conditions.

Although the additional P_{acc} promoter seems not to participate in supporting the physiological expression level of accD6_{Mtb} under standard growth conditions, it is able to sustain expression of this gene on the level allowing for cell survival in the absence of P_{fasII}.

Recently, Salzman et al. (61) identified transcriptional factor (MabR) regulating FAS-II operon expression through the action on P_{fasII} promoter. Also typical inhibitors of mycolic acid biosynthesis like isoniazid (INH) are able to induce transcriptional response of genes placed under the control of P_{fasII} (5, 26, 71, 81). Identification of additional P_{acc} promoter of accD6_{Mtb} is the first report showing that particular gene of FAS-II operon in M. tuberculosis can be regulated independently from the other operon members. We speculate that it might be possible under specific growth conditions through the alternative (P_{fasII}/P_{acc}) promoter usage and activity of different transcriptional regulators as was described for mammalian ACCase 1 and 2 (40, 50).

Our studies on ΔaccD6_{Mtb}-P_{acc}accD6_{Mtb} mutant treated with INH implicates that P_{acc} is regulated independently from P_{fasII} as the P_{acc} is insensitive to INH treatment. Findings by Salzman et al., (61) support this hypothesis showing that palindromic
motif recognized by MabR is localized uniquely in P\text{\textsubscript{fasII}}, thus suggesting that this transcriptional repressor is very unlikely to influence acc\textsubscript{D6\textsubscript{Mtb}} expression driven from P\textsubscript{acc} promoter. However, it does not exclude the possibility of AccD6 activity being regulated following post-translational modifications. Indeed, recent studies reported that several FAS-II components, including KasA, KasB, FabH, MabA and InhA are regulated at the level of enzyme activity through the phosphorylation (46, 47, 77, 78).

The fact that all FAS-II components investigated so far are regulated by mycobacterial Ser/Thr kinases (48) leads to the hypothesis that AccD6 may also be regulated by phosphorylation, although it requires to be experimentally demonstrated.

The expression level of acc\textsubscript{D6\textsubscript{Mtb}} placed only under P\textsubscript{acc} was more than ten times lower than that in wild-type \textit{M. tuberculosis}. Our ability to successfully obtain the Δacc\textsubscript{D6\textsubscript{Mtb}}-P\textsubscript{acc}acc\textsubscript{D6\textsubscript{Mtb}} mutant allowed us to analyze the direct phenotypic effect of low acc\textsubscript{D6} expression in tubercle bacilli. We found that this decreased expression of AccD6 protein arrested the growth of the mutant strain and inhibited proper fatty and mycolic acid biosynthesis. Inhibition of the fatty acid synthesis occurred at a very early stage, likely reflecting the impaired activity of acetyl-CoA carboxylase, which provides the essential building blocks for both FAS-I/FAS-II pathways. As shown by SEM analysis, low-level expression of AccD6 in \textit{M. tuberculosis} generated cells with an irregular “wrinkled” surface similar to that reported for INH-treated \textit{M. tuberculosis} (74). Similar changes were also observed prior to lysis of \textit{M. smegmatis} subjected to depletion of KasA or InhA (7, 79). These morphological changes, considered as the effect of reduced mycolic acid biosynthesis, support the hypothesis of a direct \textit{in vivo} involvement of Acc\textsubscript{D6\textsubscript{Mtb}} in this metabolic pathway.
Along with the *in vitro* studies on AccD6 (18) our results demonstrate and confirm the key role of AccD6 in mycolic acid biosynthesis of *M. tuberculosis*. Essentiality of AccD6 assigned only to *M. tuberculosis* may implicate its importance in pathogenesis of mycobacterial infection and makes it an excellent target for the development of new anti-mycobacterial compounds.
Acknowledgments

Research co-financed by the European Regional Development Fund under the Operational Programme Innovative Economy, grant POIG.01.01.02-10-107/09 and State Committee for Scientific Research (contract N302 035 31/3172).
References


Fig. 1  Quantitative real-time PCR analysis of the expression levels of the carboxyltransferase β subunit genes in exponentially growing cultures of *M. tuberculosis* and *M. smegmatis*. The transcript level of each subunit is indicated relative to the expression level of *sigA* (internal control). Values are given as means and standard errors.

Fig. 2  Complementation of *M. tuberculosis accD6* SCO strain. (A) Schematic explaining the construction of the complementation vectors that allowed us to replace the chromosomal copy of *M. tuberculosis accD6* with its mutated copy. The dashed lines indicate the cloning steps, and the restriction sites are shown. P1/P2 - TBaccD6-XbaI and TBaccD6-HinDIII rev primers used to amplify the *M. tuberculosis accD6* gene. (B) (top) Map showing the length of the restriction DNA fragment (1966 bp) and the internal deletion in the mutated gene (853 bp). The chromosomal localization of accD6 is represented by the gray arrow, while the internal deletion is marked by a black rectangle. (bottom) Southern blot confirming the deletion of the chromosomal copy of *accD6* from complemented *M. tuberculosis* strains. The lanes represent genomic DNA from: 1, wild-type *M. tuberculosis*; 2, SCO; 3, DCO carrying the wild-type *accD6* gene (wt-DCO); 4, Δ*accD6*Mtb-PfasII; 5, Δ*accD6*Mtb-PfasIFASII*Mtb.

Fig. 3  Confirmation of the loss of functional *accD6* gene in *M. smegmatis ΔaccD6* DCO mutant (A) (top) Schematic showing the restriction-digested DNA fragment (1421 bp) and the size of the internal deletion in the mutated gene (624 bp). The *accD6* gene is represented by the gray arrow and the internal deletion by a black rectangle. (bottom) Southern blot confirming the deletion of *accD6* in mutated *M. smegmatis*. Lanes: 1 - wild-type *M. smegmatis*; 2 - single-crossover strain; 3 - double-crossover Δ*accD6* mutant; 4 - wild-type DCO. (B) Western blot of total crude lysates from *M. smegmatis* (*Msm*) and Δ*accD6* DCO mutant (mut-DCO) strains confirming loss of AccD6 protein expression in the mutant strain, as assessed using rabbit anti-AccD6 antibodies. (C) Western blot of total crude lysates from *M. smegmatis* (*Msm*) and Δ*accD6* DCO mutant (mut-DCO) strains confirming that the protein expression levels of KasA and KasB were similar in the wild-type and mutant strains, as assessed using rat anti-KasA antibodies capable of cross-reacting with KasB.
Fig. 4 Phenotypic analysis of the *M. smegmatis* ΔaccD6 mutant (A) Growth rate analysis of wild-type *M. smegmatis* (1), the ΔaccD6 mutant (2), and strains complemented with intact copies of accD6*Msm* and accD6*Mtb* expressed under the control of acetamidase promoter - ΔaccD6*Msm*-P*ama*accD6*Msm* (3) and ΔaccD6*Msm*-P*ama*accD6*Mtb* (4). Growth rate analysis was performed on Sauton medium, and the presented OD values are given as means and standard errors from three independent experiments. (B) Western blot of total crude lysates from: 1, *M. smegmatis* wild-type strain; and 2, the ΔaccD6*Msm*-P*ama*accD6*Mtb* mutant grown in the absence (-) or presence (+) of acetamide. (C) Thin-layer chromatography of 14C-labeled FAMES and MAMES extracted from: 1, wild-type *M. smegmatis*; 2, the ΔaccD6 mutant; and 3, uninduced (-) and induced (+) ΔaccD6*Msm*-P*ama*accD6*Msm*. Equal counts (100,000 cpm) were loaded on the TLC plate and separated as described in the Materials and Methods. Symbols: α, α’, and e correspond to α-mycolates, α’-mycolates and epoxy-mycolates, respectively.

Fig. 5 *M. tuberculosis* but not *M. smegmatis* accD6 possesses its own promoter (A) (top) Schematic explaining the construction of the genetic construct for complementation of the *M. smegmatis* ΔaccD6 mutant in order to examine whether accD6 possesses its own promoter (P*acc*). The dashed lines indicate the fragment cloned into integrative vector to give pPD6Ms1 construct. (bottom) Western blot analysis of total crude lysates from the ΔaccD6*Msm*-P*ama*accD6*Msm* mutant (lane 1) and wild-type *M. smegmatis* (lane 2) strains, as assessed using rabbit anti-AccD6 antibodies. Note the lack of a detectable signal for AccD6 protein expression driven by the 1007 bp region upstream of the gene. (B) (top) Schematic explaining the construction of the genetic construct for complementation of the *M. tuberculosis* accD6 SCO mutant in order to examine whether accD6 possesses its own promoter (P*acc*). The dashed lines indicate the fragment cloned into integrative vector to give pPD6Tb construct. (middle) Southern blot confirming the deletion of the chromosomal copy of *M. tuberculosis* accD6 in the ΔaccD6*Mtb*-P*ama*accD6*Mtb* mutant, which exclusively expressed the accD6 gene from its own promoter (P*acc*). The accD6*Mtb* gene sequence was used as the probe. Lanes: 1, wild-type *M. tuberculosis*; 2, single-crossover mutant (SCO); 3, double-crossover mutant carrying the wild-type accD6 gene (wt-DCO); 4-6, three independent ΔaccD6*Mtb*-P*ama*accD6*Mtb* mutant
strains. (bottom) Map showing the restriction DNA fragment and the size of the internal deletion in the mutated gene. The chromosomal localization of accD6 is represented by the gray arrow and the internal deletion is shown with a black rectangle.

**Fig. 6** PCR analysis of the FAS-II gene cluster structure. Four PCR reactions were prepared using four sets of primers covering intergenic sequences and total cDNA matrices from *M. smegmatis* (A) and *M. tuberculosis* (B). The lane numbers (bottom) correspond to the numbered PCR products shown in the schematic (top). Expected product sizes are given. In the schematics, the genes of the FAS-II cluster are marked as thick black arrows, while the wavy line represents isolated mRNA and the gray arrows show the total cDNA obtained by reverse transcription. See Table S2 for a description of the utilized primers.

**Fig. 7** (A-C) Western blot analysis of total crude lysates of *M. tuberculosis* (A), the ΔaccD6Mtb-PaccaccD6Mtb mutant (B) grown in the presence (+) or absence (-) of INH and *M. smegmatis* (C) grown in the presence of INH during 0; 0.3; 1 and 4 hours, probed with rabbit anti-AccD6 antibodies and rat anti-KasA antibodies. The asterisk represents KasA-containing 80-kDa complex. (D) qRT-PCR analysis showing the fold-change in expression of accD6 in exponentially growing cultures of ΔaccD6Mtb-PfasiaaccD6Mtb (PfasiaaccD6Mtb) and ΔaccD6Mtb-PaccaccD6Mtb (PaccaccD6Mtb) mutants relative to the expression level in wild-type *M. tuberculosis*, with all results normalized with respect to the expression level of sigA. Values shown represent the means and standard errors.

**Fig. 8** Phenotypic analysis of the ΔaccD6Mtb-PaccaccD6Mtb mutants. (A) (left) Western blot analysis of total crude lysates from *M. tuberculosis* (Mtb) and three independently obtained ΔaccD6Mtb-PaccaccD6Mtb mutants (I, II and III), probed with rabbit anti-AccD6 antibodies. (right) Growth rate analysis of the three ΔaccD6Mtb-PaccaccD6Mtb mutants (2; 3 and 4) and wild-type *M. tuberculosis* (1) grown under aeration in 7H9 medium supplemented with OADC. All values represent the means and standard errors from three independent experiments. The inset picture represents growth of the ΔaccD6Mtb-PaccaccD6Mtb mutant (Mut) with respect to the growth of wild-type *M. tuberculosis* (Mtb) in 7H10/OADC medium (B) Thin-layer
chromatography (left) and scintillographic analysis (right) of $^{14}$C-labeled mycolic and fatty acid methyl esters extracted from exponentially growing wild-type $M$. tuberculosis ($Mtb$) and the three $Δ$accD$_6^{Mtb}$-$P_{acc^{Δ}accD6^{Mtb}}$ mutants (I, II and III). Samples were withdrawn after 1, 6 and 24 hours of labeling with $[2-{14}^C]$acetate. Symbols: $α$, M, and K correspond to $α$-mycolates, methoxy-mycolates and keto-mycolates, respectively. (C) Scanning electron micrographs of wild-type $M$. tuberculosis (left) and the $Δ$accD$_6^{Mtb}$-$P_{acc^{Δ}accD6^{Mtb}}$ mutant (right). Several fields were examined, and representative samples are shown in both panels. All scale bars represent 1.0 µm.
Fold change in accD6 expression according to wild-type M. tuberculosis