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ACCase 6 is the essential acetyl-CoA carboxylase involved in fatty acid and mycolic acid biosynthesis in mycobacteria

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Abstract

Mycolic acids are essential for the survival, virulence and antibiotic resistance of the human pathogen *Mycobacterium tuberculosis*. Inhibitors of mycolic acid biosynthesis, such as isoniazid and ethionamide, have been used as efficient drugs for the treatment of tuberculosis. However, the increase in cases of multidrug-resistant tuberculosis has prompted a search for new targets and agents that could also affect synthesis of mycolic acids. In mycobacteria, the acyl-CoA carboxylases (ACCases) provide the building blocks for *de novo* fatty acid biosynthesis by fatty acid synthase (FAS) I and for the elongation of FAS I products by the FAS II complex to produce meromycolic acids. By generating a conditional mutant in the *accD6* gene of *Mycobacterium smegmatis,* we demonstrated that AccD6 is the essential carboxyltransferase component of the ACCase 6 enzyme complex implicated in the biosynthesis of malonyl-CoA, the substrate of the two FAS enzymes of *Mycobacterium* species. Based on the conserved structure of the AccD5 and AccD6 active sites we screened several inhibitors of AccD5 as potential inhibitors of AccD6 and found that the ligand NCI-172033 was capable of inhibiting AccD6 with an IC_{50} of 8 μM. The compound showed bactericidal activity against several pathogenic *Mycobacterium* species by producing a strong inhibition of both fatty acid and mycolic acid biosynthesis at minimal inhibitory concentrations. Overexpression of *accD6* in *M. smegmatis* conferred resistance to NCI-172033, confirming AccD6 as the main target of the inhibitor. These results define the biological role of a key ACCase in the biosynthesis of membrane and cell envelope fatty acids, and provide a new target, AccD6, for rational development of novel anti-mycobacterial drugs.

INTRODUCTION

Although effective chemotherapeutic agents have been developed, *Mycobacterium tuberculosis,* the aetiological agent of tuberculosis, is still a leading cause of death worldwide, killing over two million people annually. Each year approximately nine million

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people develop active tuberculosis and this number continues to rise due to the expanding world population and the threat posed by HIV/AIDS. Moreover, the synergy between tuberculosis and the AIDS epidemic (Corbett & De Cock, 1996), coupled with the emergence of multi-drug-resistant (MDR) *M. tuberculosis* (Chopra, 1996), and more recently extensively drug-resistant (XDR) *M. tuberculosis* (Gandhi *et al.*, 2006), mainly as a result of the lack of compliance with the six-month multidrug chemotherapy regime, poses a serious threat to progress in the control of tuberculosis and could even reverse recent gains. Therefore, there is an urgent need to identify new drug targets suitable for the development of new anti-mycobacterial drugs.

The unusual lipid-rich cell wall of *M. tuberculosis* contains several components essential for both viability and pathogenicity (Brennan & Nikaido, 1995). This impermeable barrier imparts resistance against both hostile environments and therapeutic agents, and it plays an active role in modulating the host immune response (Karakousis *et al.*, 2004). The cell envelope of *M. tuberculosis* has also provided the molecular targets for several of the major anti-tubercular drugs currently in use such as isoniazid, ethambutol and pyrazinamide (Zhang, 2005). Thus, the unique structure of this cell envelope and the importance of its integrity for the viability of the organism suggest that the search for novel drug targets within the array of enzymes responsible for its construction may still prove fruitful.

Among the potentially attractive drug targets are the enzymes that provide the building blocks for lipid biosynthesis, the acyl-CoA carboxylases (ACCases) (Tong, 2005). These enzymes catalyse the biotin-dependent α-carboxylation of acetyl- and/or propionyl-CoA to generate malonyl- and methylmalonyl-CoA, respectively. In mycobacteria, these metabolites are used by the fatty acid synthase I (FAS I) for the biosynthesis of membrane fatty acids (Schweizer & Hofmann, 2004), as well as by the FAS II and the polyketide synthases for the biosynthesis of the complex lipids present in the cell wall, such as the long-chain α-alkyl,β-hydroxymycolic acids (Bhatt *et al.*, 2007), the phthiocerol dimycocerosates (Trivedi *et al.*, 2005) and sulfolipids (Jackson *et al.*, 2007). The ACCase reaction occurs in two catalytic steps (Cronan & Waldrop, 2002); in the first step, biotin carboxylase (BC) couples carbonate to a biotin residue attached to a biotin carboxyl carrier protein (BCCP) to form carboxybiotin. In the second step, carboxyltransferase (CT) transfers the carboxyl group from biotin to the acyl-CoA and generates the corresponding α-carboxylated acyl-CoA. In actinomycetes, the ACCases consist of two large polypeptides: an α-subunit that contains the BC and the BCCP domains and a β-subunit that contains the CT domain (Erfle, 1973; Henrikson & Allen, 1979; Hunaiti & Kolattukudy, 1982; Rodriguez *et al.*, 2001; Rodriguez & Gramajo, 1999). In some cases, a third subunit called ε, a unique feature of actinomycete ACCases, is essential for holo complex activity (Diacovich *et al.*, 2002; Gago *et al.*, 2006).

All the sequenced genomes from *Mycobacterium* species contain three *accA* genes (for α subunits AccA1–3) and six *accD* genes (for β subunits AccD1–6) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). So far, only two ACCase complexes from *M. tuberculosis* have been characterized at the biochemical level. ACCase 5 was reconstituted from the biotinylated α subunit AccA3, the CT β subunit AccD5 and the ε subunit AccE5 (Gago *et al.*, 2006; Oh *et al.*, 2006). The kinetics of the holo complex indicate that ACCase 5 accepts acetyl- and propionyl-CoA as substrates, with a fivefold preference for propionyl-

CoA, suggesting that its main physiological role is to provide methylmalonyl-CoA for the biosynthesis of multimethyl-branched fatty acids (Gago *et al.*, 2006). More recently, the ACCase 6 from *M. tuberculosis* was reconstituted from the AccA3 and AccD6 subunits, and the kinetic data showed that the enzyme carboxylates acetyl-CoA and propionyl-CoA with similar efficiency (Daniel *et al.*, 2007). Based on the location of *accD6* in a genetic locus that contains members of the FAS II complex, it was suggested that ACCase 6 would preferentially work as an acetyl-CoA carboxylase providing malonyl-CoA to the FAS II complex for the biosynthesis of mycolic acids. However, this hypothesis was not proved experimentally, and hence a detailed genetic and physiological characterization of this enzyme was required.

For a long time, it has been predicted that bacterial ACCases could be suitable targets for antibacterial drug discovery (Tong, 2005). However, it was not until recently that the first class of bacterial ACCase inhibitor with antibacterial activity, derived from pyrrolidine dione natural products, was characterized and proposed as a group of promising antibacterial compounds with a novel mode of action (Freiberg *et al.*, 2004; Pohlmann *et al.*, 2005). Recently, the successful determination of the crystal structure of AccD5 of *M. tuberculosis* (Lin *et al.*, 2006) allowed us to carry out an extensive *in silico* screening of several compound databases that resulted in the identification of a number of putative ACCase inhibitors.

In this paper, we present what is believed to be the first genetic and physiological characterization of an essential ACCase of mycobacteria, and propose a physiological role for it based on the analysis of an *accD6* conditional mutant generated in *Mycobacterium smegmatis*. We have also identified and characterized a novel inhibitor of AccD6, with the ability to inhibit growth of several *Mycobacterium* species, including MDR strains of *M. tuberculosis.*

METHODS

Bacterial strains, culture, and transformation conditions

Escherichia coli strain DH5α (Hanahan, 1983) was used for routine subcloning and was transformed according to Sambrook *et al.* (1989). The transformants were selected on media supplemented with the appropriate antibiotics: 20 μg chloramphenicol (Cm) ml^{-1}, 50 μg kanamycin (Km) ml^{-1}, 20 μg gentamicin (Gm) ml^{-1} and/or 100 μg streptomycin (St) ml^{-1}. *E. coli* B strain BL21 λ(DE3) and BL21 Codon Plus RIL (Stratagene) were used for the heterologous expression of *Mycobacterium* genes. *M. smegmatis* mc^2155 is an electroporation-proficient mutant of mc26 (Snapper *et al.*, 1990). Liquid cultures of *M. smegmatis* were grown at 37 °C in Sauton's medium and Middlebrook 7H9 supplemented with ADS enrichment. Antibiotics were used at the following concentrations: Km 15 μg ml^{-1}, Gm 20 μg ml^{-1} and St 20 μg ml^{-1}. *M. tuberculosis* and other *Mycobacterium* species were grown at 37 °C in Middlebrook 7H9 or 7H10 medium.

DNA manipulations and plasmid construction

Isolation of plasmid DNA, restriction enzyme digestion and agarose gel electrophoresis were carried out by conventional methods (Sambrook *et al.*, 1989). Genomic DNA of *M. smegmatis* was obtained as described by Connell (1994).

pPR27D6

The *accD6* gene from *M. smegmatis* mc^2155 was PCR amplified from genomic DNA using the oligonucleotides D6rbsNde (5′-AGACCTCATATGACAATCATGGCCCCCG-3′), to introduce an *Nde*I site (underlined) at the translational start codon of *accD6*, and D6Hind (5′-CTCGCGAAGCTTATTCTGCGTCTGCTC-3′), to introduce a *Hin*dIII site (underlined) at the end of the ORF. The PCR product was digested with *Nde*I and *Hin*dIII and cloned into *Nde*I/*Hin*dIII-cleaved pET24b, yielding pD6MS. The *M. smegmatis* mutant allele *accD6::aphA-3* was built on pUC19. For this an *Xba*I–*Hin*dIII fragment from pD6MS was cloned in pUC19 digested with the same enzymes, yielding pUCD6MS. The *aphA-3* cassette that confers Km^r was obtained from pUC4K and subcloned in *Eco*RI-digested pGEM-T Easy; from here, a 1.2 kb *Not*I fragment carrying the Km^r cassette was cloned into pUCD6MS, yielding pD6Km. Finally, pPR27D6, the construct used for allelic exchange, was obtained by transferring a 3.6 kb *Pvu*II fragment from pD6Km containing *accD6::aphA-3* into *Xba*I-cut pPR27, a temperature-sensitive ($temp^s$) mycobacterial vector carrying the counter-selectable marker *sacB* and the *xylE* reporter gene.

pCGD6

A 2.4 kb fragment including *accD6* coding sequence plus 500 bp, both upstream and downstream of this ORF, was obtained from genomic DNA of mc^2155 by PCR using oligonucleotides D6Ms2-Xba (5′-TCTAGACGGACGGCTACCACAT-3′) and D6Ms2-Hind (5′-AAGCTT-CCACCGAGGCCGAATACG-3′), sequenced to confirm fidelity and cloned in pCRBluntII-Topo (Invitrogen). From the resulting plasmids a 2.4 kb *Xba*I fragment was cloned into pCG76, to yield pCGD6.

pCGHD6

An *Nde*I–*Hin*dIII fragment from pD6MS was cloned in pVV2 digested with the same enzymes, yielding pVVD6MS. The vector pVV2 (Dhiman *et al.*, 2004) is a derivative of pMV261 that allows expression of recombinant proteins from cloned genes from the GroEL promoter as fusions with an aminoterminal hexahistidine epitope tag. The 1.73 kbp *Xba*I–*Spe*I fragment containing *M. smegmatis accD6* under control of the GroEL promoter was cloned in pCG76, yielding pCGHD6.

pD6MT

accD6 was PCR amplified from the genomic DNA of *M. tuberculosis* H37Rv by using the oligonucleotides D6UP (5′-AGACCTCATATGACAATCATGGCCCCCG-3′), to introduce an *Nde*I site (underlined) at the translational start codon of the *accD6* gene, and D6DN2 (5′-CTCGCGAAGCTTATTCTGCGTCTGCTC-3′), to introduce a *Hin*dIII site (underlined) at the end of the ORF. To generate an *accD6* His-tag fusion gene, the PCR product was

digested with *Nde*I and *Hin*dIII and cloned into *Nde*I/*Hin*dIII-cleaved pET24b, yielding pD6MT.

Southern blot analysis for the allelic replacement of *accD6*

Approximately 10 μg genomic DNA was digested overnight with an excess of *Eco*RV, and the fragments were separated by electrophoresis through 0.7 % agarose gels. Southern blotting was carried out in 10× SSC using Hybond-N^+ nylon membranes (Amersham). The probe consisted of a 666 bp fragment of the *kasB* gene amplified using the oligonucleotides 5D6EcoUP (5′-TCTGGCATTCGGGCGTTACTG AG-3′) and 5D6EcoDn (5′-CGGCACGGCTTCGATCTTGGTCT-3′). The Prime a Gene labelling system (Promega) and 5 mCi (185 MBq) [α-^{32}P]dATP were used to label the probe. Prehybridization and hybridization were carried out at 65 °C using 5×SSC, 5× Denhardt's solution and 0.5 % SDS. Serial 15 min washes were performed at 65 °C as follows: two washes with 2× SSC/0.1 % SDS and two washes with 1× SSC/1 % SDS. The filter was developed and digitalized with a Storm 840 scanner (Amersham).

Protein methods

Purified proteins were analysed by SDS-PAGE (Laemmli, 1970). Coomassie brilliant blue was used to stain protein bands. Protein contents were determined by the methods of Bradford (Bradford, 1976) or Lowry with BSA as a standard.

Heterologous protein expression and purification

For the expression and purification of *M. tuberculosis* His_6-AccD6 and AccA3 we followed the protocols described by Gago *et al.* (2006).

Enzyme assays for ACCase and CT activities

Radioactive method—ACCase activities in cell-free extracts or of *in vitro* reconstituted complexes were measured by following the incorporation of radioactive HCO_3^- into acid non-volatile material, as previously described (Bramwell *et al.*, 1996; Diacovich *et al.*, 2002; Hunaiti & Kolattukudy, 1982). One unit of enzyme activity catalysed the incorporation of 1 μmol ^{14}C into acid-stable products min^{-1}.

Pyruvate kinase-lactate dehydrogenase (PK-LDH)—The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically (Janiyani *et al.*, 2001). The production of ADP was coupled to PK and LDH, and the oxidation of NADH was monitored at 340 nm. Assays were performed in a Dynex MRX microplate reader as previously described (Diacovich *et al.*, 2002). Initial velocities were obtained from initial slopes of the recorder traces. Under the assay conditions described, the reaction was linear for at least 3 min and the initial rate of reaction was proportional to the enzyme concentration. One unit of enzyme activity catalyses the formation of 1 μmol of the respective carboxylated CoA derivative or ADP min^{-1} under the assay conditions described. Specific activity is expressed as units per mg of AccA3. Initial velocities were determined with this assay at 10–150 μM inhibitor concentrations in the presence of K_m concentrations of propionyl-CoA (100 μM) for ACCase 5 or acetyl-CoA (500 μM) for ACCase 6. The

amount of protein used for the AccD5 assay was 0.4 μM AccA3, 0.4 μM AccD5 and 4 μM AccE5, and for the AccD6 assay 0.8 μM AccA3 and 0.8 μM AccD6. IC_{50} values were obtained by fitting the data to a sigmoid dose–response equation using the GraphPad Prism 4.0 software.

Minimal inhibitory concentration (MIC) determination—MIC values for the mycobacterial species used were determined by twofold dilution in Middlebrook 7H9 broth supplemented with glycerol and ADS and colorimetric evaluation of viability as described by Palomino *et al.* (2002). *M. smegmatis* mc^2155, *M. tuberculosis* H37Rv, *M. bovis* BCG, and clinical isolates of MDR *M. tuberculosis* AI55 and AI57, *M. fortuitum*, *M. kansasii* and *M. avium* were used for this determination. Briefly, 1×10^5 c.f.u. were used to inoculate each well in a 96-well plate in which a twofold dilution (100 μl final volume) of the tested compound had been made. Rifampicin and isoniazid were used as control drugs, except for *M. fortuitum*, where imipenem was used due to the intrinsic resistance of this mycobacterium to rifampicin and isoniazid. Plates were sealed and incubated at 37 °C for 2 days (*M. smegmatis* and *M. fortuitum*), 5 days (*M. kansasii*) or 7 days (*M. tuberculosis* and *M. bovis* BCG). Viability was estimated by adding 20 μl resazurin (10 mg ml^{-1} in water) followed by further incubation for 24 h. A colour change from blue to pink indicates cell viability. The MIC was determined as the lowest concentration of drug giving no colour change.

The MIC of NCI-172033 for *M. smegmatis* overexpressing *accD6* was determined on Petri dishes containing Middlebrook 7H11 solid medium by spreading 100–200 c.f.u. of strains MS-CGD6 and MS-CGHD6 and the control strain MS-CG76 (Table 1). Four concentrations of NCI-172033 (25, 50, 100 and 200 μM) were tested. Colonies were counted after incubation at 30 °C for 6 days. Plates were also screened under an optical microscope to detect pinpoint colonies and determine colony morphology alterations. MIC_{99} was defined as the concentration of the compound under test that reduced the c.f.u. to ≤1 % of the number on control plates (no compound). The experiments were performed twice in triplicate.

Effect of NCI-172033 on mycobacterial growth—To determine whether the compound being tested was bacteriostatic or bactericidal, *M. tuberculosis* cultures containing 10^5 c.f.u. ml^{-1} were incubated with several concentrations of NCI-172033. Small aliquots (10 μl) of control (drug-free) and drug-treated (0.5–4 times the MIC value) cultures were withdrawn from a 96-well microtitre plate after 2 and 4 days incubation with the drug, diluted and plated on drug-free 7H11 Middlebrook medium. C.f.u. were counted after incubation at 37 °C for 3 weeks. A similar experiment was also carried out with *M. smegmatis*. Samples were withdrawn after 24 or 48 h incubation with the inhibitor (at 0.5–2 times the MIC), diluted and plated on drug-free 7H11 Middlebrook medium. The experiments were carried out in duplicate for two independent cultures of each of the strains under study.

Metabolite incorporation assays—Cultures of *M. smegmatis* mc^2155 and of the D6DCO2 mutant were grown in 7H9 medium supplemented with ADS at 30 or 42 °C, and at different time points aliquots of the cultures were labelled for 1 h with L-[4,5-^{3}H]leucine

(60 Ci $mmol^{-1}$), or [1-^{14}C] acetate (59 mCi $mmol^{-1}$) (New England Nuclear) at concentrations of 1 μCi ml^{-1} (37 kBq).

[^{3}H]Leucine was used to monitor metabolic activity. After labelling, cells were pelleted, washed with cold water, and resuspended in 100 μl Tris/HCl (10 mM, pH 8.0). Samples were precipitated with 6 % perchloric acid and filtered through glass fibre filters (Millipore). After washing the precipitates with 1 ml ethanol, radioactivity was determined in a Beckman liquid scintillation counter.

Fatty acid biosynthesis was analysed by incorporation of [^{14}C]acetate. Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were extracted as described by Kremer *et al.* (2000), using aliquots from cultures containing the same number of cells. The resulting solution of FAMEs and MAMEs was assayed for radioactivity in a Beckman liquid scintillation counter and then subjected to TLC using silica gel plates (5735 silica gel 60F254; Merck) and developed in hexane/ethyl acetate (9:1, v/v) or petroleum ether/diethyl ether (95:5, v/v). Autoradiograms were produced by overnight exposure to Kodak X-Omat AR film to reveal ^{14}C-labelled FAMEs and MAMEs. To analyse the effect of the inhibitors (isoniazid, cerulenin or NCI-172033) on fatty acid biosynthesis, the compounds were added at the corresponding concentrations 1 h before the radioactive label.

***In vitro* assay for FAS I activity—**The standard reaction mixture for FAS I was composed as described by Slayden *et al.* (1996). It consisted of 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM DTT, 300 μM acetyl-CoA, 100 μM NADPH, 100 μM NADH, 1 μM flavin mononucleotide, 500 μM α-cyclodextrin, 20 μM mal-onyl-CoA, 100 000 c.p.m. [2-^{14}C]malonyl-CoA and 100 μl of the cytosolic enzyme preparation (1–2 mg protein) in a total volume of 500 μl. Reactions were performed in triplicate at 37 °C for 30 min and terminated by the addition of 500 μl 20 % potassium hydroxide in 50 % methanol and incubation at 100 °C for 30 min. Following acidification with 300 ml 6 M HCl, the resultant ^{14}C-labelled fatty acids were extracted three times with petroleum ether. The organic extracts were pooled, washed once with an equal volume of water, and dried in a scintillation vial prior to measurement of radioactivity.

RESULTS

AccD6 is an essential carboxyltransferase in *M. smegmatis*

In order to demonstrate that AccD6 is the CT component of an essential ACCase involved in providing malonyl-CoA for lipid biosynthesis in mycobacteria, we reasoned that a conditional *accD6* mutant was required. For this purpose we used *M. smegmatis* as a model system, where temperature-sensitive (temp^{s}) vectors, which allow the rapid and efficient construction of conditional mutants, are available (Guilhot *et al.*, 1992).

The essentiality of *accD6* was first suggested by the impossibility of generating a knockout mutant through a two-step homologous recombination strategy (Pelicic *et al.*, 1996). For the first recombination step, the temp^{s} plasmid pPR27D6, harbouring a disrupted copy of the *accD6* gene (*accD6::aphA-3*), was introduced into *M. smegmatis* by electroporation and one of the Km^{r} transformants was plated at 42 °C to promote plasmid recombination. PCR

analysis of the chromosomal DNA from several Km^r $XylE^+$ colonies indicated that two of them resulted from a single recombination event in the 3′ end of the chromosomal copy of *accD6* (Fig. 1b); the other eight clones most probably arose from illegitimate recombination. To select for the intra-chromosomal allelic exchange, one of the *M. smegmatis* colonies, containing the correct integration of pPR27D6 and named D6SCO1, was grown in LB-Km at 30 °C and plated on LB-Km-Suc plates at 30 °C (Pelicic *et al.*, 1997). None of the thousands of colonies screened exhibited the expected phenotype (Km^r Suc^r, white after catechol spraying), strongly suggesting that *accD6* was an essential gene. Thus, to allow for the second recombination event to take place we constructed an *accD6* merodiploid strain by introducing pCGD6 into D6SCO1. In this plasmid *accD6* can only be expressed from its own promoter sequences, since pCG76 is not an expression vector. One of the Km^r St^r transformants, called D6DCO2, was grown at 30 °C in LB-Km-St and plated on LB-Km-St-Suc plates at 30 °C. At least 30 % of the colonies grown in these conditions were also white after spraying with catechol, indicating that they had undergone intra-chromosomal allelic exchange (Fig. 1c). Three of these colonies were screened by PCR (see Supplementary Fig. S1, available with the online version of this paper) and it was confirmed by Southern blot analysis that they all had the correct allelic exchange at the chromosomal *accD6* locus (Fig. 1d). These experiments support the idea that *accD6* is an essential gene in *M. smegmatis* and strongly suggest that this gene, which is considered part of the *fasII* operon (Daniel *et al.*, 2007), is most probably an independent transcription unit.

To confirm that AccD6 was essential for *M. smegmatis* growth, we investigated the ability of strain D6DCO2 to survive at 42 °C, a temperature at which the rescue plasmid pCGD6 is unable to replicate. The growth characteristics of mc^2155/pCG76 and of the *accD6* $temp^s$ mutant D6DCO2 at 30 and 42 °C are presented in Fig. 2(a, b). As expected, at 30 °C, strain D6DCO2 exhibited the same growth characteristics as the control strain mc^2155/pCG76. However, at 42 °C, the D6DCO2 mutant replicated for two to three generations and then stopped dividing after ~12 h (OD_{600} 0.7) (Fig. 2a). The growth of D6DCO2 for two to three generations after the temperature shift is consistent with the way the $temp^s$ plasmid is cured, as has been described for the pAL5000 derivative plasmids (Guilhot *et al.*, 1992). Soon after the D6DCO2 culture stops growing, a large proportion of cells can no longer replicate, consistent with the loss of the AccD6-encoding plasmid and therefore with the loss of ACCase 6 activity. This effect is reflected by a dramatic drop of c.f.u. ml^{-1} in the culture; e.g. 2 h after the culture stopped growing, fewer than 30 % of the cells are still able to yield colonies when plated on LB agar at 30 °C (Fig. 2b). All these colonies also grew on LB-St agar, indicating that all of them still contain pCGD6 (data not shown). These results unambiguously show that AccD6 plays an essential role in the physiology of this bacterium.

Characterization of the *accD6* conditional mutant of *M. smegmatis*

To study the physiological role of the ACCase 6 complex we carried out [^{14}C]acetate labelling experiments using the *accD6* conditional mutant D6DCO2 and the control strain mc^2155/pCG76 and analysed their lipid content by TLC. As shown in Fig. 2(c), when both strains were growing at the same rate at the restrictive temperature of 42 °C (labelled as −3 h in Fig. 2c), there was no difference in the content of fatty acids or mycolic acid in their membranes; however, after the conditional mutant stopped growing (OD_{600} 0.7 or ~12 h of

growth at 42 °C), *de novo* synthesis of both molecules was progressively affected until there was almost no incorporation of the labelled substrate (Fig. 2c). To confirm that this phenotype was related to the loss of the acetyl-CoA carboxylase activity of ACCase 6, cell-free extracts were prepared from the control strain and the D6DCO2 conditional mutant growing at 42 °C and assayed for this enzyme activity. In correlation with the expected loss of pCGD6, the levels of acetyl-CoA carboxylase in D6DCO2 showed a progressive and dramatic decrease after the culture stopped growing, in contrast with the steady levels of activity in the control strain (Fig. 3a). To confirm that the loss in ACCase activity in the mutant strain was not related to a pleiotropic effect caused by an immediate death of those cells that lost the plasmid, we performed [^{3}H]leucine labelling experiments at different time points in the wild-type and the mutant in order to determine the levels of metabolic activity in the cultures. As shown in Fig. 3(b), 2h after the D6DCO2 strain stopped growing, the metabolic activity of the cells was still comparable to that of strain mc^{2}155/pCG76 (Fig. 3b); however, at this point the ACCase activity had already dropped 70 % compared to the wild-type strain (Fig. 3a). At later time points the drop in ACCase activity could be correlated with a decrease in cell viability, as indicated by the reduction of [^{3}H]leucine incorporation compared to the control strain (Fig. 3b). To further support the observation that after 2 h the mutant cultures stop growing but the cells remain metabolically active, we measured the cytoplasmic levels of the NADP-dependent malic enzyme at different time points (Supplementary Fig. S2). Altogether, these results demonstrate that at least in *M. smegmatis*, AccD6 is part of an essential ACCase that provides the substrate, malonyl-CoA, for fatty acid and mycolic acid biosynthesis and that none of the other ACCases present in this bacterium can compensate for the loss of this activity.

Identification and characterization of an AccD6 inhibitor

Having demonstrated that AccD6 is the CT subunit of a key ACCase in mycobacteria, we set out to identify a ligand that could inhibit this protein and hence the synthesis of the malonyl-CoA needed for lipid biosynthesis. Based on the similarity of substrate specificities of ACCase 5 and ACCase 6 and on the crystallographic data of the active sites of AccD5 (Lin *et al.*, 2006) and AccD6 (results to be published elsewhere), implying a very similar acyl-CoA binding pocket, we reconstituted the *M. tuberculosis* ACCase 6 complex *in vitro* and tested the inhibitory effect of the hit compounds identified in the first round of *in silico* inhibitor screening carried out against the AccD5 active site (Lin *et al.*, 2006). Among all the compounds tested, only one, NCI-172033 (Fig. 4a), showed extensive enzyme inhibition when tested against the AccD6 subunit by using the malate dehydrogenase/citrate synthase coupled assay (data not shown). As the selection of this compound was originally based on the AccD5 structure, we compared its inhibition profile on both ACCase 5 and ACCase 6 complexes by the pyruvate kinase coupled method (Janiyani *et al.*, 2001). Both ACCase complexes were reconstituted from their recombinant expressed subunits and their activity was determined in the presence of different concentrations of the inhibitor. These studies showed that NCI-172033 produced a strong inhibition of the ACCase 6 activity, with IC_{50} values of approximately 8 μM. In comparison, the IC_{50} for ACCase 5 was almost 10-fold higher (70 μM) than the value obtained for ACCase 6 (Fig. 4b). The same results were obtained with the reconstituted ACCase 6 from *M. smegmatis* (data not shown).

Anti-mycobacterial activity of NCI-172033

To continue with the characterization of NCI-172033 *in vivo*, the compound was tested for its anti-mycobacterial activity against a set of fast- and slow-growing mycobacterial species. NCI-172033 was active against *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. fortuitum*, *M. kansasii* and *M. avium*, with MIC values ranging between 12.5 and 25 μM, except for *M. smegmatis*, with a MIC value of 100 μM (Table 2). It is important to point out that two clinical isolates of MDR *M. tuberculosis* were also inhibited by NCI-172033 at the same concentration as *M. tuberculosis* H37Rv (25 μM). Rifampicin and isoniazid were used as control drugs, and all the species used for this assay, except for the two MDR strains and *M. fortuitum*, were inhibited at the previously reported concentrations of these two anti-mycobacterial drugs (Palomino *et al.*, 2002). These results indicate that NCI-172033 exhibits anti-mycobacterial activity on both drug-susceptible and drug-resistant strains, and that it has no cross-resistance with the two major anti-tubercular drugs currently in use.

To determine if NCI-172033 was bacteriostatic or bactericidal, cultures of *M. tuberculosis* H37Rv were exposed for 2 or 4 days to different concentrations of the inhibitor, and the number of viable cells, from samples obtained before and after the treatment, was determined in antibiotic-free medium by c.f.u. counts. At the MIC (25 μM), the same number of c.f.u. that had been inoculated (10^4 c.f.u. ml^{-1}) was recovered after the treatment, indicating that at this concentration the compound acts as a tuberculostatic agent. However, at concentrations above the MIC (50 and 100 μM), the compound showed a clear tuberculocidal effect: the c.f.u. counts from both cultures declined 10-fold after 48 h of drug exposure and 100-fold after 96 h. Analogous results were obtained with *M. smegmatis,* where a bacteriostatic effect of the compound was observed at 100 μM (MIC), while a severe bactericidal effect was observed after the treatment of the cultures with 200 μM NCI-172033 (c.f.u. counts declined more than 100-fold after 24 or 48 h of drug exposure) (Supplementary Fig. S3). These results confirm that this antimicrobial agent behaves as a typical bactericidal compound (Walsh, 2003).

NCI-172033 affects fatty acid and mycolic acid biosynthesis at similar rates

Considering that NCI-172033 is a potent inhibitor of ACCase 6, we hypothesized that, *in vivo*, the bactericidal property of this molecule was related to the inhibition of malonyl-CoA biosynthesis, the substrate of the FAS I and II systems. To validate this hypothesis we studied the effect of increasing concentrations of NCI-172033 on the *de novo* synthesis of fatty acids in *M. tuberculosis* and *M. smegmatis*. In both cases, mid-exponential-phase cultures were grown in the presence of increasing concentrations of NCI-172033 and labelled with [^{14}C]acetate. Fatty acids and mycolic acids were extracted and analysed by TLC. As shown in Fig. 5, in both micro-organisms, *de novo* synthesis of these macromolecules was inhibited at similar rates, suggesting that inhibition of malonyl-CoA biosynthesis was the most likely reason for the absence of newly synthesized fatty acids and mycolic acids. However, inhibition of FAS I by NCI-172033 could also result in a strong inhibition of [^{14}C]acetate incorporation into both macromolecules. Therefore, in order to narrow down the *in vivo* target candidates of NCI-172033, we measured the activities of both ACCase and FAS I in cell-free extracts prepared from *M. smegmatis* cultures that had been pre-incubated in the presence of the inhibitor. As shown in Fig. 6, the acetyl-CoA-

dependent ACCase activity of the treated cell extracts showed ~50 % inhibition as compared with the non-treated extract, whereas the FAS I activity was not affected. These results strongly suggest that the inhibition of fatty acid biosynthesis in the cultures treated with NCI-172033 occurs by inhibition of an ACCase, most probably ACCase 6, that provides malonyl-CoA to FAS I and FAS II.

AccD6 as the *in vivo* target of NCI-172033

To unambiguously demonstrate that AccD6 was the main target for the growth-inhibitory effect of NCI-172033, we carried out MIC_{99} determinations on *M. smegmatis* strains containing extra copies of *accD6*. Our results showed that growth on solid medium of the control strain MS-CG76 was not altered by the presence of a 25 μM concentration of the inhibitor when compared with growth on control plates (no inhibitor). At 50 μM NCI-172033, the number of colonies was the same as on the control plate, although we did observe a reduction in colony size. As expected, at 100 and 200 μM NCI-172033 no colonies were present on the plates. Remarkably, in agreement with our hypothesis, the strains overexpressing AccD6 (MS-CGD6 and MS-CGHD6) grew normally at all the concentrations tested. Unfortunately, we could not assay higher concentrations of the inhibitor because of its low solubility above 200 μM. These results provide compelling evidence that over-expression of *accD6* protects *M. smegmatis* from the effect of NCI-172033, validating AccD6 as the *in vivo* target of this inhibitor.

DISCUSSION

In this study we demonstrated by genetic means that *accD6*, the gene encoding the CT subunit of ACCase 6, is an essential locus in *M. smegmatis* (Fig. 1). Moreover, by constructing an *accD6* conditional mutant we were able to show that the physiological role of ACCase 6 is to provide malonyl-CoA for fatty acid and mycolic acid biosynthesis (Figs 2 and 3). This is the second ACCase unambiguously characterized in actinomycetes whose physiological role is that of an acetyl-CoA carboxylase; the first one, called ACC, was studied in *Streptomyces coelicolor*, where it was demonstrated that it provides malonyl-CoA for both fatty acid and polyketide biosynthesis (Rodriguez *et al.*, 2001). Studies in *Corynebacterium glutamicum*, whose genome encodes four CT subunits, suggested that AccD1 is the CT subunit of the essential ACCase complex (Gande *et al.*, 2004). However, a mutant in this subunit is still able to grow on minimal medium, suggesting that more studies are needed to assign a clear physiological role to this subunit.

In vitro studies by Gago *et al.* (2006) showed that the ACCase 5 of *M. tuberculosis* has the ability to carboxylate propionyl-or acetyl-CoA at similar rates. *M. smegmatis* also contains orthologues of the genes encoding ACCase 5, suggesting that it also has a second ACCase complex, besides ACCase 6, with the ability to carboxylate acetyl- and propionyl-CoA. This situation raised the notion that these enzyme complexes could complement each other for their function in generating malonyl-CoA for fatty acid biosynthesis. However, our results show conclusively that this is not the case and that, *in vivo,* ACCase 6 is the dedicated acetyl-CoA carboxylase of *M. smegmatis*. Furthermore, taking into account that the genetic background of the ACCases of *M. tuberculosis* and *M. smegmatis* is highly conserved and

considering that *accD6* has also been predicted as an essential gene in *M. tuberculosis* (Sassetti *et al.*, 2003), we could predict that the CT subunit AccD6 could be considered as a new target for the development of a new class of anti-mycobacterial compounds.

By testing nine compounds that had been identified by *in silico* docking studies as putative inhibitors of the CT AccD5 (Lin *et al.*, 2006), we found that NCI-172033, a poor inhibitor for AccD5, turned out to be a potent inhibitor of AccD6 (Fig. 4b). In the literature, only two compounds with strong inhibitory activities against prokaryotic acetyl- or acyl-CoA carboxylases have been characterized in detail: CPD1 (Moraimide B), which inhibits the *E. coli* ACCase with a K_i of 0.005 μM (Freiberg *et al.*, 2004), and NCI-65828, which inhibits *M. tuberculosis* ACCase 5 with a K_i of 13.1 μM (Lin *et al.*, 2006).

NCI-172033 is also the first inhibitor of an ACCase with proven anti-mycobacterial properties. Recently, an inhibitor (NCI-65828) of AccD5, the β subunit of the ACCase 5 complex of *M. tuberculosis,* was identified; however, this ligand only inhibited *M. tuberculosis* growth at MIC values >250 μM. In contrast, the compound characterized in this work, NCI-172033, showed a more potent anti-bacterial effect (MICs between 12.5 and 25 μM) against a broad range of *Mycobacterium* strains, including two MDR strains of *M. tuberculosis* (Table 2). So far, only one group of compounds, the pyrrolidine dione antibiotics, has shown such promising antibacterial properties (Freiberg *et al.*, 2004; Pohlmann *et al.*, 2005). However, at least one of them, Moiraimide B, was not efficacious against *Mycobacterium* (unpublished results). Although the chemical nature of NCI-172033 indicates that this compound could not be considered as a new lead, it is relevant to note that the rational approach followed to identify this ligand, using the active site of AccD5 for *in silico* docking of large libraries of compounds, could also be followed with the recently solved crystal structure of AccD6 (data to be published elsewhere) to select new and more potent compounds.

Although the inhibition of acetate incorporation and the *in vitro* inhibitory activity of NCI-172033 on the AccD6 of *M. tuberculosis* and *M. smegmatis* strongly suggest that ACCase 6 is the primary target of action of this drug *in vivo*, similar results would be obtained if the compound also inhibited FAS I. By treating exponentially growing cultures with inhibitory concentrations of the drug and measuring ACCase, PCCase and FAS I activity (Fig. 6), we were able to demonstrate that ACCase, but not FAS I, is the target of NCI-172033. It is important to bear in mind that *M. tuberculosis* has six ACCases (Cole *et al.*, 1998), and that at least two of them, ACCases 5 and 6, can utilize acetyl- and propionyl-CoA as substrates (Daniel *et al.*, 2007; Gago *et al.*, 2006; Oh *et al.*, 2006). Considering that the IC_{50} of NCI-172033 for ACCase 5 is almost ten times higher than that for ACCase 6, it is not surprising that we can still detect acetyl-and propionyl-CoA carboxylase activity in cell-free extracts prepared from cultures pre-incubated with the drug.

Multiple copies of *accD6*, under the transcriptional control of its own promoter or the GroEL promoter, increased the MIC_{99} of NCI-172033 for the wild-type strain *M. smegmatis* mc^2155 at least twofold, validating AccD6 as the *in vivo* target of NCI-172033. This result, together with the *in vitro* inhibitory effect of NCI-172033 on AccD6 (Fig. 4b) and the *in vivo* inhibitory effect of this compound on the ACCase activity (Fig. 6) and on fatty acid and

mycolic acid biosynthesis (Fig. 5), strongly suggests that the main mechanism by which NCI-172033 works as a tuberculocidal agent is through the inhibition of a dedicated acetyl-CoA carboxylase activity, ACCase 6 being the best candidate.

In conclusion, we have demonstrated that AccD6 is an essential component of a dedicated acetyl-CoA carboxylase in *Mycobacterium* and therefore a good target for developing a novel class of inhibitors that affect fatty acid biosynthesis. Moreover, we identified NCI-172033 as a potent inhibitor of AccD6 and found that this compound is the first inhibitor of an ACCase with anti-mycobacterial properties, (Table 2). This work provides a solid foundation for the discovery of more potent ACCase inhibitors as novel anti-tuberculosis therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACCase	acyl-CoA carboxylase
CT	carboxyltransferase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
MAME	mycolic acid methyl ester
MDR	multi-drug resistant

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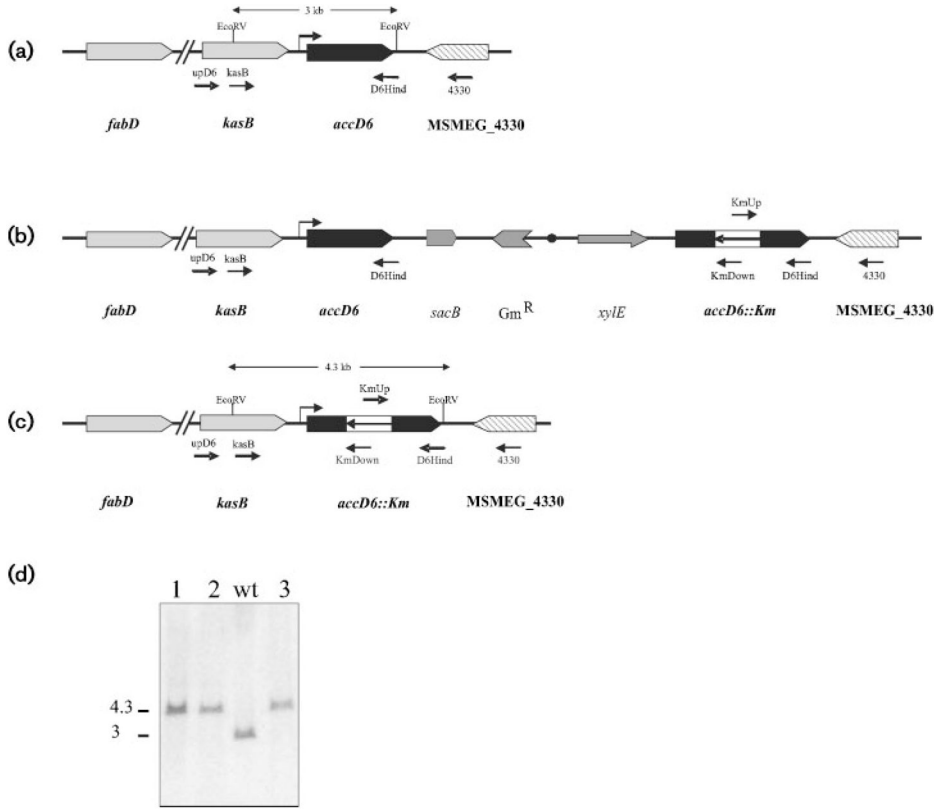

Fig. 1.

Allelic exchange of the *accD6* locus of *M. smegmatis*. (a–c) Genetic organization, partial restriction map and expected hybridization profiles of the *accD6* chromosomal region in (a) the wild-type strain mc^{2}155, (b) the single crossover strain D6SCO1 and (c) the *M. smegmatis* D6DCO2 conditional mutant. Oligonucleotides used to confirm single and double crossover events by PCR are indicated. (d) Southern blot analysis of *M. smegmatis accD6* mutants. Three mutants were picked at random (1, 2 and 3); chromosomal DNA was digested with *Eco*RV and probed for hybridization with a labelled 666 bp fragment corresponding to the 5′ region of *kasB*. *M. smegmatis* mc^{2}155 DNA was included as a control (wt). Molecular masses are indicated in kb.

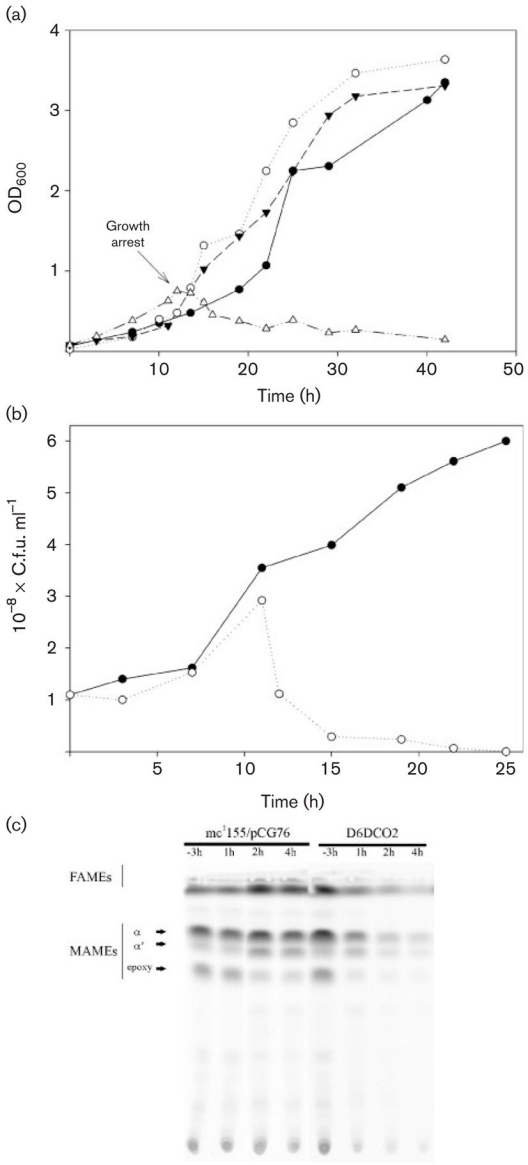

Fig. 2.

Growth characteristics and lipid composition of the *accD6* conditional mutant D6DCO2 incubated at 30 and 42 °C. (a) Growth curves of strains mc^{2}155/pCG76 (●, ○) and D6DCO2 (▼, △) incubated at 30 °C (black symbols) and 42 °C (white symbols). Saturated cultures grown at 30 °C were diluted in fresh 7H9 medium supplemented with ADS to an OD_{600} of 0.1 and further incubated at 30 or 42 °C. The arrow indicates growth arrest of D6DCO2. (b) At different time points, the number of viable cells of D6DCO2 in the cultures grown at 30 °C (●) and 42 °C (○) was evaluated by plating serial dilutions onto LB plates at 30 °C. (c) Saturated cultures of strains mc^{2}155/pCG76 and D6DCO2 were diluted in fresh 7H9 medium and incubated at 42 °C. At −3, 1, 2 and 4 h before or after D6DCO2 stopped growing (see a), aliquots from both cultures containing the same number of cells were labelled with [^{14}C]acetate for 1 h at 42 °C. One half of each sample was used to study the

fatty acid and mycolic acid compositions of the strains by TLC (solvent system: hexane/ethyl acetate, 10:1).

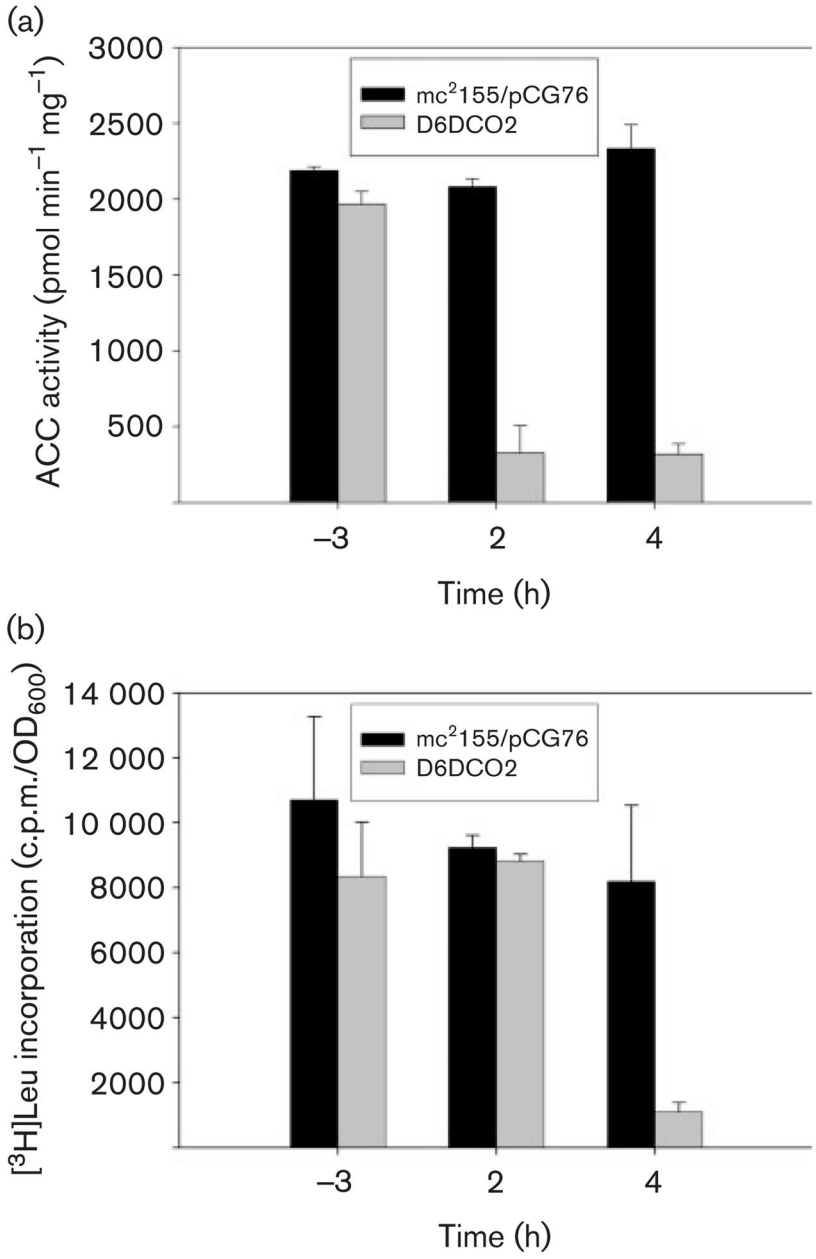

Fig. 3.

(a) Loss of acetyl-CoA carboxylase activity in the D6DCO2 mutant strain at restrictive growth temperature. Cell-free extracts were prepared from strains mc^{2}155/pCG76 and D6DCO2 growing at 42 °C (−3, 2, and 4 h before or after D6DCO2 stopped growing), and acetyl-CoA carboxylase activity was determined for each sample as indicated in Methods. Results are the means of three independent experiments. (b) Determination of metabolic activity by incorporation of [^{3}H]leucine. At different time points aliquots of mc^{2}155/pCG76 and D6DCO2 cultures growing at 42 °C were labelled for 1 h with [^{3}H]leucine and the radioactivity incorporated into the cells was measured as indicated in Methods. The results were normalized by OD_{600} and are the means of three independent experiments.

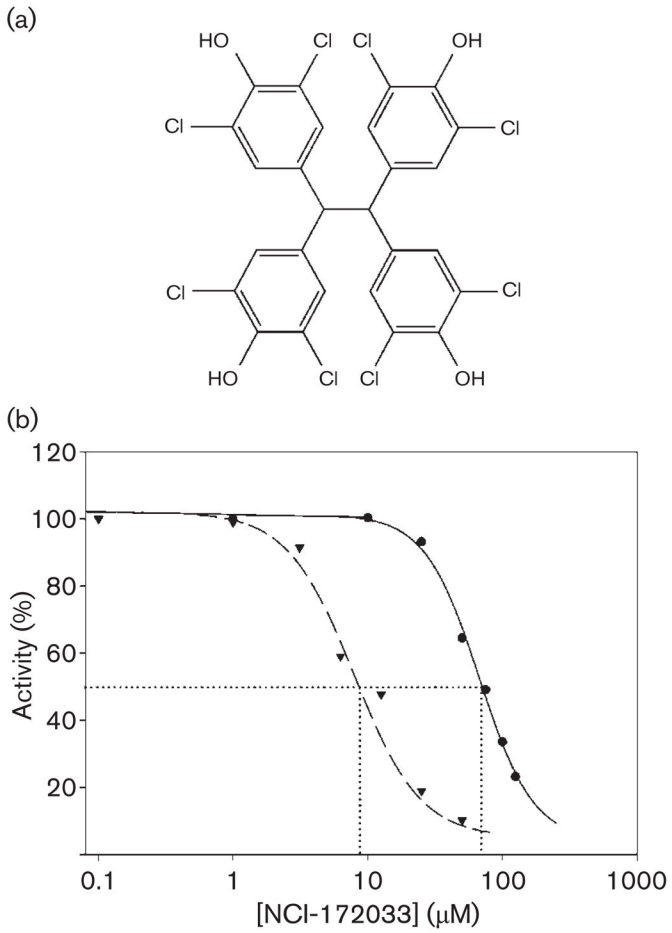

Fig. 4.

(a) Chemical structure of NCI-172033. (b) IC50 determination. ACCase activity of AccD5 (●) and AccD6 (▼) was measured using the pyruvate-kinase-coupled enzyme assay at different concentrations of NCI-172033 and K_m concentration of the acyl-CoA substrate. IC50 values of 8 μM for the ACCase 6 complex and 78 μM for the ACCase 5 complex were determined. Results are the means of at least four independent experiments with a standard error that was within ±5 % of the mean.

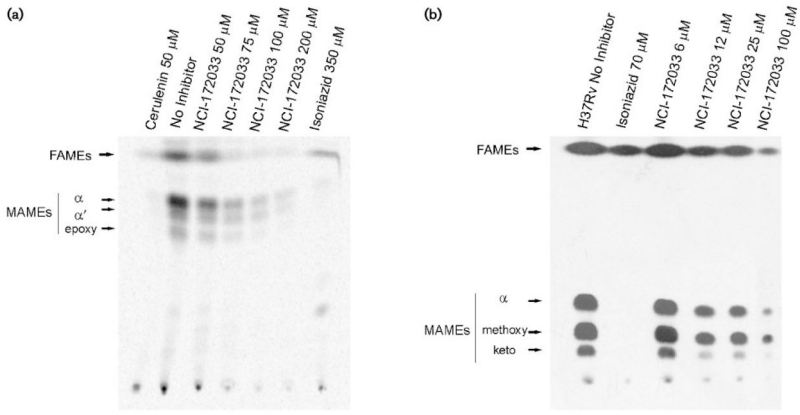

Fig. 5.

Dose–response effects of NCI-172033 on fatty acid and mycolic acid biosynthesis in *M. smegmatis* (a) and *M. tuberculosis* (b). The inhibitory effect on the incorporation of [^{14}C]acetate was assayed by labelling the individual cultures in the presence of increasing concentrations of NCI-172033 and inhibitory concentrations of isoniazid and cerulenin. The corresponding FAMEs and MAMEs were isolated and analysed by TLC.

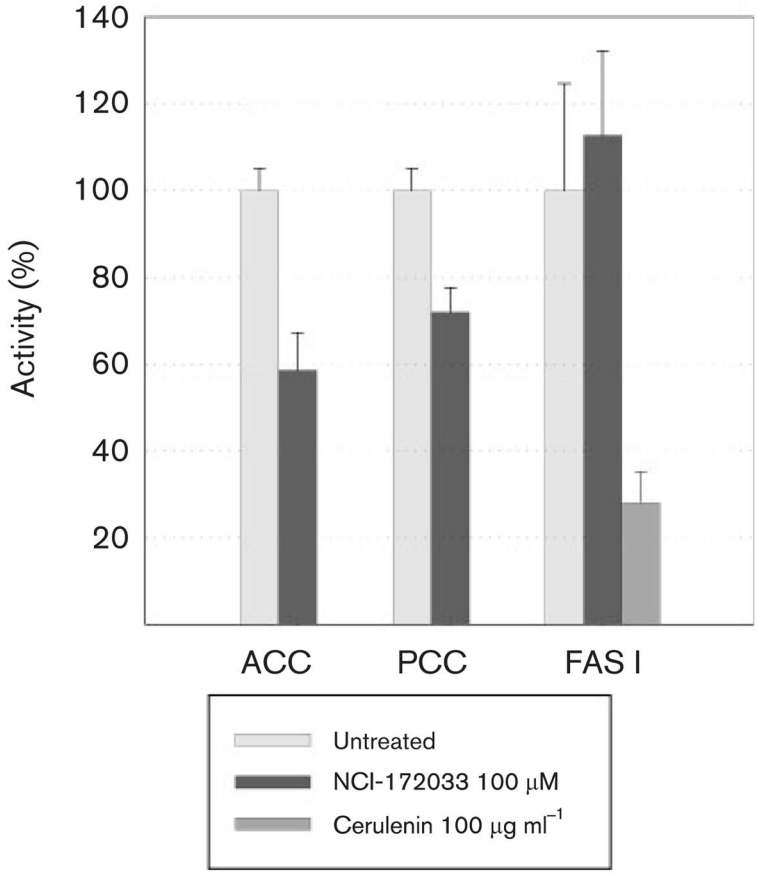

Fig. 6.

In vivo effect of NCI-172033 on the ACCase, PCCase and FAS I activities of *M. smegmatis*. Cultures of *M. smegmatis* were incubated in the presence of 100 μM NCI-172033 and the relative acetyl- and propionyl-CoA-dependent carboxylases and FAS I activities determined in the cell-free extracts. Cerulenin, a known inhibitor of the β-ketoacyl synthase domain in type I synthases, was used as a control.

Table 1

Bacterial strains and plasmids used in this work

Plasmids	Relevant genotype and/or information*	Source or reference
pET28a(+)	Phagemid vector (Km^r *lacZ′*) for expression of recombinant proteins under control of strong T7 transcription and translation signals	Novagen
pD6MT	pET28a(+) with *M. tuberculosis accD6* His-tag fusion gene, under T7 promoter control, Km^r	This study
pD6MS	pET28a(+) with *M. smegmatis accD6* His-tag fusion gene, under T7 promoter control, Km^r	This study
pD5	pET28a(+) with *M. tuberculosis accD5* His-tag fusion gene, under T7 promoter control, Km^r	Gago *et al.* (2006)
pA3	pET28a(+) with *M. tuberculosis accA3* His-tag fusion gene, under T7 promoter control, Km^r	Gago *et al.* (2006)
pUC4K	Contains *aphA-3* cassette that confers Km^r	Vieira & Messing (1982)
pPR27	*E. coli–Mycobacterium* shuttle vector, *oriM* $temp^s$, *sacB xylE* Gm^r	Pelicic *et al.* (1997)
pPR27D6	pPR27 derivative carrying *accD6::aphA-3,* Gm^r Km^r	This study
pCG76	*E. coli/Mycobacterium* shuttle vector, *oriM* $temp^s$, St^r/Sp^r	Guilhot *et al.* (1994)
pCGD6	pCG76 derivative harbouring *accD6* plus 500 bp, both upstream and downstream of this ORF from *M. smegmatis,* St^r/Sp^r	This study
pCGHD6	pCG76 derivative harbouring *M. smegmatis accD6* His-tag fusion gene, under GroEL promoter control, St^r/Sp^r	This study
E. coli		
DH5α	*E. coli* K-12 F^- Δ*lacU*169 (φ80*lacZ*ΔM15) *endA1 recA1 hsdR17 deoR supE44 thi-1* λ^- *gyrA96 relA1*	Hanahan (1983)
BL21λ(DE3)	*E. coli* B F^- *ompT* $r_B m_B$ (DE3)	Studier & Moffatt (1986)
BL21λ(DE3) Codon Plus	*E. coli* B F^- *ompT hsdS* ($r_B m_B$) *dcm*$^+$ Tet^R *gal* (DE3) *endA Hte [argU ileY leuW]*, Cm^R	Stratagene
M. smegmatis		
mc^2155	Fast-growing strain harbouring all plasmids used herein	Snapper *et al.* (1990)
D6SCO1	mc^2155 with pPR27D6 integrated into *accD6* locus, Km^r	This study
D6DCO2	Mutant containing an intrachromosomal allelic exchange at *accD6* locus in presence of pCGD6, Km^r St^r/Sp^r	This study
MS-CG76	mc^2155 harbouring pCG76, St^r/Sp^r	This study
MS-CGD6	mc^2155 harbouring pCGD6, St^r/Sp^r	This study
MS-CGHD6	mc^2155 harbouring pCGHD6, St^r/Sp^r	This study

*Gm^r, Gentamicin resistance; Km^r, kanamycin resistance; St^r, streptomycin resistance; Sp^r, spectinomycin resistance; Cm^r, cloramphenicol resistance; $temp^s$, temperature sensitivity.

Table 2

MIC determination for NCI-172033, using several mycobacterial species

1×10^5 c.f.u. were used to inoculate Middlebrook 7H9 supplemented with ADS (10 % v/v) in a 96-well plate containing twofold dilutions of the tested compound. Plates were sealed and incubated at 37 °C for 2 days (*M. smegmatis* and *M. fortuitum*), 5 days (*M. kansasii*) or 7 days (*M. tuberculosis* and *M. bovis* BCG). Viability was estimated by adding resazurin followed by further incubation for 24 h. Colour change from blue to pink indicated viability. MIC was determined as the lowest concentration of drug giving no colour change.

Strain	MIC (μM)
M. tuberculosis H37Rv	25
M. tuberculosis AI55	25
M. tuberculosis AI57	25
M. bovis BCG	25
M. kansasii	25
M. fortuitum	12.5
M. avium	12.5
M. smegmatis mc^2155	100