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Fatty acid biosynthesis in actinomycetes

Gabriela Gago[⊥], **Lautaro Diacovich**[⊥], **Ana Arabolaza**[⊥], **Shiou-Chuan Tsai**[#], and **Hugo Gramajo**[⊥,*]

[⊥]Microbiology Division, IBR (Instituto de Biología Molecular y Celular de Rosario), Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, (S2002LRK) Rosario, Argentina.

[#]Department of Molecular Biology and Biochemistry and Department of Chemistry, University of California, Irvine, CA 92612.

Abstract

All organisms that produce fatty acids do so via a repeated cycle of reactions. In mammals and other animals, these reactions are catalyzed by a type I fatty acid synthase (FAS), a large multifunctional protein to which the growing chain is covalently attached. In contrast, most bacteria (and plants) contain a type II system in which each reaction is catalyzed by a discrete protein. The pathway of fatty acid biosynthesis in *Escherichia coli* is well established and has provided a foundation for elucidating the type II FAS pathways in other bacteria (White *et al.*, 2005). However, fatty acid biosynthesis is more diverse in the phylum Actinobacteria: *Mycobacterium*, possess both FAS systems while *Streptomyces* species have only the multi-enzyme FAS II system and *Corynebacterium* species exclusively FAS I. In this review we present an overview of the genome organization, biochemical properties and physiological relevance of the two FAS systems in the three genera of actinomycetes mentioned above. We also address in detail the biochemical and structural properties of the acyl-CoA carboxylases (ACCases) that catalyzes the first committed step of fatty acid synthesis in actinomycetes, and discuss the molecular bases of their substrate specificity and the structure-based identification of new ACCase inhibitors with anti-mycobacterial properties.

Keywords

lipid biosynthesis; acyl-CoA carboxylase; *Corynebacterium*; *Mycobacterium*; *Streptomyces*; lipid homeostasis

Introduction

Within the Actinobacterial taxon (Gram-positive bacteria with high G + C content of DNA) three genera, *Mycobacterium*, *Streptomyces* and *Corynebacterium*, have been widely studied because of their remarkable impact on mankind, either as extremely dangerous pathogens, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium diphtheriae* or, at the other extreme, as very useful sources of bioactive metabolites. In this sense streptomycetes are well known for their ability to produce a vast array of secondary metabolites widely used in human health (e.g. anticancer agents, immunossupressants, and

[*]Corresponding author. gramajo@ibr.gov.ar, Phone number: 54-341-4350661, FAX number: 54-341-4390465.
Author´s contribution
G.G. and L.D. contributed equally to this article.

antibiotics)(Hopwood, 1989) and corynebacteria for their ability to produce and secrete large amounts of L-amino acids, mainly used in the food and livestock industries (Kramer, 1994).

One motivation to present a comprehensive and comparative review of fatty acid biosynthesis in these three genera of actinomycetes was to focus on the remarkable differences between them in the content of their membrane and storage lipid components and consequently on the lipid biosynthetic machinery. Even though streptomycetes undergo a complex life cycle with distinctive developmental and morphological stages (Chater and Losick, 1997), the cell wall is similar to that of other Gram-positive bacteria, composed of a simple peptidoglycan mesh surrounding the cytoplasmic membrane. By contrast, the cell walls of *Mycobacterium* spp., as well as species of related genera including *Corynebacterium*, *Gordonia*, *Nocardia*, and *Rhodococcus*, are formed by a thick *meso*-diaminopimelic acid-containing peptidoglycan covalently linked to arabinogalactan, which in turn is esterified by long-chain α-alkyl, β-hydroxy fatty acids called mycolic acids (Brennan, 2003). While in mycobacteria these fatty acids have very long carbon chains ($C_{60–90}$) and may contain various oxygen functions in addition to the β-hydroxyl group (Daffe and Draper, 1998), mycolic acids found in other actinomycetes consist of a mixture of saturated and unsaturated fatty acids with shorter carborn chains like $C_{22–36}$ in corynomycolic acids (Collins *et al.*, 1982; Minnikin and Goodfellow, 1980). Mycolic acids are believed to play a central role in the formation of a second permeability barrier functionally similar to the outer membrane in Gram-negative bacteria (Gebhardt *et al.*, 2007; Liu *et al.*, 1996). While the latter is a typical bilayer of phospholipid and lipopolysaccharide, in mycobacteria and corynebacteria it consists of a monolayer of mycoloyl residues covalently linked to the cell-wall arabinogalactan, to form mycoloyl arabinogalactan (MAG), or acylated to trehalose units fo form trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (Brennan, 2003; Daffe and Reyrat, 2008) (Fig. 1). Besides mycolic acids, the outer membrane of mycobacteria also contains an array of very complex lipids relevant in the infection process of the pathogenic mycobacteria, such as dimycocerosate esters (DIMs), sulpholipids and mycoketides (Astarie-Dequeker *et al.*, 2009; Camacho *et al.*, 1999; Cox *et al.*, 1999). In the last few years cell-free reconstitution studies demonstrated that several of these compounds are synthesized from acyl-CoA molecules, most probably derived from the fatty acid biosynthesis pathway, which are further elongated by specific FAS related enzymes known as polyketide synthases (PKS) (Onwueme *et al.*, 2005; Sirakova *et al.*, 2001; Trivedi *et al.*, 2005).

Another remarkable feature of mycobacteria and streptomycetes, but not of corynebacteria, is that *de novo* synthesized fatty acids are not only dedicated to building membrane phospholipids (PL) but are also incorporated into neutral lipid storage compounds, the triacylglycerides (TAG) (Arabolaza *et al.*, 2008; Daniel *et al.*, 2004). The synthesis of TAG and its accumulation within the cytoplasm as lipid droplets brings another layer of complexity in the lipid metabolism of these bacteria, and highliths another important difference between the three genera of actinomycetes discussed in this review.

General principles of bacterial fatty acid biosynthesis

The biosynthesis of fatty acids is the first step in the formation of membrane lipids and is essential for all cells, excepting the Archea in which the membranes are composed of glycerol-ether lipids instead of glycerol-ester lipids and are based on isoprenoid side chains (De Rosa *et al.*, 1986). The basic features of fatty acid synthesis pathways, like the constituent chemical reactions and the identity of the component enzymes, were the focus of intensive studies more than four decades ago, especially in the laboratories of Vagelos (Alberts *et al.*, 1969; Greenspan *et al.*, 1969), Bloch (Odriozola *et al.*, 1977) and Wakil

(Pugh *et al.*, 1966; Toomey and Wakil, 1966). Later on, the genes and enzymes of fatty acid and phospholipid metabolism were defined using *E. coli* as a model organism (Cronan and Rock, 1996; Rock and Cronan, 1996). However, although the *E. coli* paradigm provides a solid core of information that is shared by all bacteria, the biochemical details of the lipid metabolic pathways in other bacteria cannot be inferred only from this model. Some relevant differences between *E. coli* and other important non-actinomycetes human pathogens have been reviewed elsewhere (Rock, 2008).

The first committed step in fatty acid biosynthesis is the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC), to form the common extender unit malonyl-CoA, which is then made available to the enzymes of fatty acid biosynthesis by its conversion to malonyl-ACP by malonyl-CoA:ACP transacylase, usually called FabD. The chain elongation steps in fatty acid biosynthesis consist of an iterative series of reactions built on successive addition of a two-carbon unit to a nascent acyl group. Reaction intermediates (the growing acyl-chains) are covalently attached to the acyl carrier protein (ACP) through a thioester linkage to the terminal sulphydryl of the 4´-phosphopanteine prosthetic group. The first elongation step is initiated by the Claisen condensation of malonyl-ACP with an acyl-CoA, catalyzed by the first condensing enzyme, the β-ketoacyl-ACP synthase III or FabH, to form β-ketoacyl-ACP. In *E. coli*, two other mechanisms for the initiation of fatty acid biosynthesis have been described (Cronan and Rock, 2008). Additional cycles of elongation in the pathway are catalyzed by the FabF-FabB class of condensing enzymes. These proteins condense malonyl-ACP with acyl-ACP to extend the acyl chain by two carbons. This is the only irreversible step in the elongation cycle and so it is not surprising that the 3-ketoacyl-ACP synthases play key roles in regulating the product distribution of the pathway. Three enzymes complete the cycle by reducing the β keto group. First, the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent β-ketoacyl-ACP reductase, or FabG, reduces the β keto group to a β-hydroxyl intermediate. Second, this is dehydrated by the β-hydroxyacyl-ACP dehydratase, known as FabZ or FabA, to a *trans*-2-enoyl-ACP. The third step involves reduction of the enoyl chain by the NADPH-dependent (FabI or FabL) or NADH-dependent (FabV) enoyl-ACP reductase. On completion of each cycle of condensation and chain reduction, the growing acyl chain is transferred back to the ketosynthase to initiate the next cycle.

Based on protein architecture, FASs are classified as type I or type II. The type II FAS consists of a dissociated system formed by a discrete and highly conserved group of enzymes encoded by a series of separate genes (Fig. 2). This system has been characterized from bacteria and parasites as well as mitochondria and chloroplasts (the site of fatty acid biosynthesis in plants), where it catalyses *de novo* fatty acid synthesis (except in mycolic acid producers), usually from acetyl-CoA (Rawlings, 1998;Rock and Cronan, 1996). The classical type I FAS consists of a multifunctional protein carrying all the catalytic domains on a single polypeptide chain (Fig. 2). These enzymes are typically found in eukaryotes (Schweizer and Hofmann, 2004) (though not in plants) and as a remarkable prokaryotic exception in the mycolic acid-producing actinomycetes (Bloch and Vance, 1977). FAS I enzymes can be subdivided according to their domain arrangements and oligomerization state. Microbial type I FASs consist of a single type of polypeptide organised as a hexamer (α6) (Bloch and Vance, 1977;Wood *et al.*, 1978), with a different domain order compared to the vertebrate FAS (Schweizer and Hofmann, 2004). Mycobacteria and corynebacteria type I FASs are hexamers with a domain arrangement of: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein, 3-ketoacyl reductase and 3-ketoacyl synthase (AT-ER-DH-MPT-ACP-KR-KS), an organization that differs from that of the animal FAS (KS-AT-DH-ER-KR-ACP-TE) (Schweizer and Hofmann, 2004). Besides, animal FAS releases free fatty acids by hydrolytic cleavage catalyzed by a thioesterase (TE) domain (Schweizer and Hofmann, 2004), while the type I FASs of yeast, mycobacteria and

corynebacteria use an integral malonyl/palmitoyl transferase activity (MPT) for transacylation of palmitate (or any other end acyl-enzyme product) from the enzyme to CoA (Bloch and Vance, 1977;Lynen, 1980).

A number of functionally differentiated FAS variants, as well as a large family of FAS-related enzymes, have evolved by slight variations in the FAS pathways to produce a wide range of natural compounds in streptomycetes and complex lipids in mycobacteria (Gokhale *et al.*, 2007a; Hertweck, 2009). For instance it is well known that many polyketides (PK) natural products, which have been a rich source of therapeutic compounds, are biosynthesized with comparable logic by multimodular PKSs, which are evolutionary descendant of type I FAS, or by type II PKS complexes highly homologue to FAS II enzymes. Moreover, the two systems could also share precursors, acetyl- and malonyl-CoA, which might establish a substrate competition effect; this can be the case for the biosynthesis of mycobacteria complex lipids but probably not for PK biosynthesis that generally occurs after growth arrest. The common mechanisms and the structural similarity of type I FAS and PKSs resulted in the proliferation of hibrid PKS-FAS pathways (Smith and Sherman, 2008). As an example, the very complex lipids of mycobacteria are made via a partnership between a FAS and a modular PKS (Gokhale *et al.*, 2007b). All this functional and mechanistic overlapping brings over a very interesting question which is whether or not the fatty acid and PK synthesis share protein components.

Fatty acid synthase systems in three model actinomycetes

The complexity of lipid metabolism in actinomycetes, especially in the *Corynebacterianeae* suborder, became obvious soon after the complex lipids that form part of their cell wall started to be characterized and their biosynthetic pathways elucidated. The sequencing of the genomes of several species of *Streptomyces*, *Mycobacterium* and *Corynebacterium* soon provided very valuable information about lipid metabolism in these bacterial genera. This information, along with excellent biochemical studies, provided valuable information about the *de novo* synthesis of fatty acids as well as of complex lipids in actinomycetes.

Evolution of the fatty acid biosynthetic genes has followed a unique and very interesting path in actinomycetes. One of the most remarkable differences from the canonical *E. coli* type II fatty acid biosynthetic pathway is the type I megasynthase pathway found in the mycolic acid-producing actinomycetes. In corynebacteria, for example, none of the genes encoding FAS II components can be found, presumably because of the evolution of a FAS I system for *de novo* fatty acid biosynthesis. In mycobacteria, the FAS II system has adapted to elongate existing long-chain fatty acids to form the meromycolic chain of the mycolic acids, and this system coexists with the FAS I enzyme that is dedicated to *de novo* fatty acid biosynthesis. Finally, *Streptomyces* contains only the genes for the FAS II enzymes, as most bacteria do (Fig. 2).

Next we describe in detail the genetics and enzymology of the FAS systems used for fatty acid biosynthesis in the three model actinomycetes and their interaction with the PKS systems.

Streptomyces

Streptomyces utilizes a type II FAS to catalyze the *de novo* synthesis of fatty acids. The majority are made from branched starters such as isobutyryl, isovaleryl, and anteisovaleryl units to give odd- and even-numbered fatty acids with a methyl branch at the ω-terminus (80–90% of total fatty acid content); the rest are synthesized from unbranched starters such as acetyl and butyryl units (Kaneda, 1991; Wallace *et al.*, 1995).

Characterization of the genes/enzymes involved in fatty acid biosynthesis in *Streptomyces* has received little attention compared to other bacteria, and most of the information available comes from predictive gene functions assigned by *in silico* genome analysis. Thus, the search for fatty acid biosynthetic genes in the most relevant streptomycetes genomes revealed various putative orthologues to several of the classical FAS II components. An extended analysis of all the annotated streptomycetes genomes revealed that some of the genes encoding the core enzymes involved in saturated fatty acid biosynthesis lie in a conserved fatty acid biosynthesis (*fab*) cluster that comprises the following genes: *fabD, fabH, acpP* and *fabF*. This cluster has been partially characterized in *S. coelicolor* and its relationship with the fatty acid biosynthetic pathway confirmed through genetic and biochemical experiments (Revill *et al.*, 1995, , 1996; Revill *et al.*, 2001). Further, the alignment of several actinomycete genomic regions containing this universal set of genes highlighted a remarkable synteny (Fig. 3), indicating a high degree of conservation in the evolution of this essential biosynthetic pathway.

fabD encodes the malonyl-CoA:ACP transacylase that provides the malonyl-ACP substrate to the condensing enzymes. FabD from *S. glaucescens* (sgFabD) could functionally replace the *E. coli* FabD protein when expressed in this microorganism (Summers *et al.*, 1995). *In vitro* studies also demonstrated that sgFabD is active with several ACPs such as TcmM (the *S. glaucescens* tetracenomycin PKS ACP), AcpP (the *E. coli* FAS ACP) and FabC (the *S. glaucescens* FAS ACP) (Florova *et al.*, 2002). The relaxed specificity of sgFabD for different ACPs suggested that the functional role of sgFabD is to provide malonyl-ACP precursors for both fatty acid and type II PK biosynthetic pathways (Florova *et al.*, 2002). *S. coelicolor* has a single copy of *fabD* in the main *fab* cluster (Revill *et al.*, 1995), suggesting that, as well as its essential role in fatty acid biosynthesis, scFabD is also shared with the type II PK biosynthetic pathway (Revill *et al.*, 1995). On the other hand, *S. griseus* has three *fabD* homologues, (SGR_5108, SGR_6780, SGR_3283) suggesting the possibility of malonyl-CoA ACP transacylases dedicated to serve specific PKS ACPs.

In *S. coelicolor fabH* was proved to be essential, in accordance with its role as a gene encoding one of the FAS proteins (Revill *et al.*, 2001). The generation of an *S. coelicolor fabH* mutant in the presence of a *fabH* gene from *E. coli* (with only 35% amino acid identity to the *Streptomyces* enzyme) resulted in a mutant strain that produced predominantly straight-chain fatty acids (86%) (Li *et al.*, 2005), as expected for the presence of a FabH enzyme with a high specificity for acetyl-CoA like the ecFabH. These findings correlate with the biochemical properties of the *S. glaucescens* FabH protein, which shows broad substrate specificity for short-chain acyl-CoA substrates, consistent with its role in initiating branched- and straight-chain fatty acid biosynthesis and determining, at least in part, the fatty acid profile (Han *et al.*, 1998).

The *acpP* gene is a confirmed member of the FAS complex in *S coelicolor*, as determined by its constitutive expression, its genomic clustering and its ability to function in *E. coli* fatty acid synthesis (Revill *et al.*, 1996). The remaining two ACPs are developmentally regulated and required for the synthesis of the type II PKs synthesized by *S. coelicolor*, the blue-pigmented antibiotic actinorhodin and the grey pigment associated with the spore wall (Revill *et al.*, 1996). When *acpP* was cloned in place of SCO5089 (encoding the actinorhodin ACP), pigmented PK production was severely compromised, implying that the FAS and PKS ACPs are biochemically incompatible (Revill *et al.*, 1996).

The genes encoding the enoyl-ACP reductases, the β-ketoacyl-ACP reductase and the (3*R*)-hydroxyacyl-ACP dehydratase, have not yet been characterized in streptomycetes. However, based on the genetic and biochemical data available for these enzymes in related members of actinobacteria (see below) we can assign these functions to each of the putative genes

found in streptomycetes. For example, in *S. coelicolor*, homologues to *mabA-inhA* from *M. tuberculosis* (Cole *et al.*, 1998) (enoyl-ACP reductase and β-ketoacyl-ACP reductase, respectively) revealed a putative two-gene operon (SCO1814-SCO1815) with synteny not only in *Streptomyces* species but also among other actinomycetes (Fig. 3). SCO1814 is 47 % identical in a 263 aa overlap with InhA, and SCO1815 is 50.8% identical in a 455 aa overlap with MabA. Based on homology and synteny with the *M. tuberculosis* (3*R*)-hydroxyacyl-ACP dehydratase components (Sacco *et al.*, 2007), we propose ORFs SCO4636-SCO4637 as the putative (3*R*)-hydroxyacyl-ACP dehydratase(s) of the FAS II system of *S. coelicolor*.

Mycobacterium

Mycobacteria are unusual in possessing both type I and type II FAS systems. Fatty acid biosynthesis in mycobacteria is initiated by a unique multifunctional FAS I enzyme, catalyzing the *de novo* synthesis of long-chain acyl-CoAs ($C_{16:0}$- and $C_{18:0}$) from acetyl-CoA and using malonyl-CoA as extender unit to yield butyryl-S-Enz. The $C_{16:0}$- and $C_{18:0}$-S-Enz, could either be converted to the CoA derivatives and used primarily for the synthesis of membrane phospholipids or further elongated by FAS I to produce $C_{24:0}$-S-Enz in the fast-growing organism *M. smegmatis* (Bloch and Vance, 1977; Peterson and Bloch, 1977) and $C_{26:0}$-S-Enz products in the slow-growing *M. bovis* and *M. tuberculosis* (Kikuchi *et al.*, 1992), a characteristic behaviour of FAS I called “bimodal product distribution”. To understand further the bimodality of FAS I, Zimhony *et al* (Zimhony *et al.*, 2004) generated a recombinant *M. smegmatis* strain in which the native *fas1* gene was deleted and replaced with the *M. tuberculosis fas1* gene (Zimhony *et al.*, 2004). In the course of analyzing the recombinant *M. smegmatis ΔfasI* (*attB*::*M. tuberculosis fas1*) strain, it was found that the *in vivo* elongation of $C_{16:0}$ did not follow the *M. tuberculosis* profile; rather, the new strain contained both fatty acids $C_{24:0}$ and $C_{26:0}$, challenging the concept that $C_{16:0}$ elongation to produce $C_{24:0}$ in *M. smegmatis* or to produce $C_{26:0}$ in *M. tuberculosis* is exclusively a FAS I-dependent property, as described previously (Kikuchi *et al.*, 1992; Peterson and Bloch, 1977), but most probably the result of a more complex interaction between FAS I and FAS II components. Furthermore, the bimodal product distribution shown by FAS I of mycobacteria not only depends on the intrinsic determinants of the FAS proteins but also on external factors that interfere with the elongation process. For instance, early studies carried out with FAS I from *M. smegmatis* demonstrated that acyl-CoA-binding substances like bovine serum albumin or certain mycobacterial polysaccharides could shift the FAS product pattern of mycobacterial type I FAS toward shorter-chain acids (Peterson and Bloch, 1977). More recently, Papaioannou *et al* (Papaioannou *et al.*, 2007) studied the product-regulation mechanisms for fatty acid biosynthesis catalyzed by *M. smegmatis* FAS I. They found that the predominant formation of palmitic acid in the presence of synthetic *O*-alkyl polysaccharide is regulated by the removal of the end-product via a tight stoichiometric complex between the $C_{16:0}$ fatty acid and the *O*-alkyl polysaccharide (gate-keeper mechanism) (Papaioannou *et al.*, 2007). The bimodal pattern of product distribution has also been demonstrated with the yeast FAS, where the addition of acyl-CoA-binding protein to the assay mixture caused a dramatic decrease in the chain-length of acyl-CoA reaction products (Schjerling *et al.*, 1996). In *M. tuberculosis* the $C_{26:0}$ fatty acids synthesized by FAS I will become the substrate of a dedicated acyl-CoA carboxylase to generate the α–carboxy $C_{26:0}$ fatty acid used as one of the substrate by the PKS 13 in the biosynthesis of mycolic acids.

The FAS I and FAS II systems are connected by a key condensing enzyme: the β-ketoacyl-ACP synthase III or FabH (Fig. 2). This enzyme catalyzes a decarboxylative condensation of malonyl-ACP with the acyl-CoA ($C_{16:0} - C_{20:0}$) products of the type I FAS. The resulting 3-ketoacyl-ACP product is reduced to an acyl-ACP (extended by two carbons) and shuffled into the FAS II cycle. Enzymology studies carried out in the presence of malonyl-AcpM

have shown that mtFabH can utilize a wide range of $C_{12:0}$–$C_{20:0}$ acyl-CoA substrates, consistent with structural studies (Scarsdale *et al.*, 2001) and distinguishing it from FabH in other type II FAS systems that typically utilize C_2–C_6 acyl-CoA substrates (Qiu *et al.*, 2005;Tsay *et al.*, 1992). More recent studies have shown that in the acylation reaction performed with a range of acyl-CoAs (carried out in the absence of malonyl AcpM) mtFabH selectively forms a product with the dodecanoyl CoA (Sachdeva *et al.*, 2008). These results support a previous hypothesis about the role of AcpM in modulating the substrate specificity of mtFabH (Brown *et al.*, 2005). Structural and site-directed mutagenesis studies have helped to understand the molecular basis of the differences in substrate specificity between the *E. coli* and *Mycobacterium* FabH enzymes (Brown *et al.*, 2005;Scarsdale *et al.*, 2001). Both the *E. coli* and the *M. tuberculosis* FabH are homodimers with a CoA/malonyl-ACP binding channel leading from the enzyme surface to the buried active site cysteine residue. Unlike ecFabH, mtFabH contains a second hydrophobic channel leading from the active site. In the ecFabH structure this channel is blocked by a Phe^{87} residue, which constrains specificity to acetyl-CoA, while in mtFabH this residue is a Thr^{87}, which permits binding of longer acyl chains. This same channel in mtFabH is capped by an α-helix, which limits bound acyl chain length to 16 carbons.

In mycobacteria, the genes encoding the FAS II enzymes lie in different positions in the genome in three independent transcription units (Fig. 3). The first is formed by *fabD-acpM-kasA-kasB-accD6* (Fig. 3A), the second contains two genes, *mabA-inhA* (Fig. 3B) (Cole *et al.*, 1998), and the third consists of three genes, *hadA*, *hadB* and *hadC* (Fig. 3C). The first ORF of the five-gene operon encodes an enzyme with significant homology to FabD of other bacteria, which is a malonyl-CoA:ACP acyl transferase (MAT) (Fig. 3A). This enzyme catalyzes the formation of malonyl-ACP, which is then used by the condensing enzyme system for the extension of a growing fatty acid chain. mtFabD catalyzes *in vitro* the transacylation of malonate from malonyl-CoA to activated holo-AcpM (Kremer *et al.*, 2001). A second enzyme with MAT activity was identified in *M. tuberculosis* and named FabD2 (Huang *et al.*, 2006). This protein is 28% identical to FabD but its physiological role remains unknown. The second ORF of this operon encodes the acyl carrier protein AcpM, a C-terminally-extended homologue of bacterial ACPs, involved in carrying the growing acyl chain from one reactive centre to another until the complete cycle of fatty acid biosynthesis is finished. The elongation steps that follow the FabH-dependent condensation reaction of the FAS II system are conducted by the β-ketoacyl-AcpM enzymes encoded by *kasA* and *kasB* in the five-gene operon. KasA and KasB are 65% identical to each other and 46.8% and 45% identical to the *S. coelicolor* β-ketoacyl-ACP synthase II, FabF, respectively. Both KasA and KasB catalyze the condensation of acyl-AcpM with malonyl-AcpM, elongating the growing meromycolate chain by a further two carbons (Kremer *et al.*, 2000) (Schaeffer *et al.*, 2001). KasA and KasB have different chain-length specificities, suggesting a distinct role for each enzyme in the elongation of meromycolic acids. In fact, cell lysates of *M. smegmatis* overproducing KasA of *M. tuberculosis* could elongate fatty acids up to 40 carbons, while the overproduction of KasA and KasB resulted in the production of longer chain fatty acids up to 54 carbon atoms (Slayden and Barry, 2002). Based on these *in vitro* studies it was proposed that KasA is implicated in the initial elongation of mycolates which are extended to the full length molecules by KasB (Kremer *et al.*, 2002;Slayden and Barry, 2002). This hypothesis was confirmed *in vivo* by generating *kasB* mutants both in *Mycobacterium marinum* (Gao *et al.*, 2003) and in *M. tuberculosis* (Bhatt *et al.*, 2007). Transposon mutagenesis in *M. marinum* gave rise to a *kasB* mutant strain which produced meromycolate chains two to four carbons shorter than the wild-type mycolates, with a significant reduction of the keto-mycolates (Gao *et al.*, 2003). Similarly, a *kasB* mutant of *M. tuberculosis* also generated similar shortened mycolates (by up to six carbon atoms), but also showed the loss or reduction of the *trans*-cyclopropanated oxygenated mycolates. A KasA mutant could not be obtained (Bhatt *et al.*, 2005), indicating that *kasA* is the only

essential β-ketoacyl-ACP synthase II-encoding gene and that KasA is most probably the enzyme involved in initial extension of the mycolate chains, whereas KasB is primarily involved in the full-length extension of these molecules. Interestingly, the *M. tuberculosis kasB* mutant also lost its acid-fast staining and was unable to persist in immunocompetent infected mice, suggesting KasB as a putative new target for novel therapeutics.

The genes encoding the FAS-II reductases are found in the *M. tuberculosis* genome in a cluster containing *mabA* and *inhA* (Fig. 3B), which encode proteins that are functionally and structurally related. The *inhA* gene was identified as a putative target for INH (isoniazid) and ETH (ethionamide) in *M. tuberculosis* (Banerjee *et al.*, 1994) and the InhA protein was demonstrated to catalyze the 2-*trans*-enoyl-ACP reduction, with a preference for long-chain substrates (Quemard *et al.*, 1995). MabA encodes a β–ketoacyl-ACP reductase with preference for long-chain substrates and was shown to be involved in the elongation activity of FAS II (Marrakchi *et al.*, 2002). In comparison with other bacterial reductases, MabA has unique functional and structural properties, such as a large hydrophobic substrate-binding pocket, which correlates with its preference for long-chain substrates consistent with its role in mycolic acid biosynthesis (Cohen-Gonsaud *et al.*, 2002;Marrakchi *et al.*, 2002). So far, there have been no successful reports of the generation of any *mabA* or *inhA* gene knockout, strongly suggesting their essentiality for mycobacterial viability.

Dehydration of the β–hydroxyacyl-ACP intermediate into *trans*-2-enoyl-ACP is catalyzed by the β–hydroxyacyl-ACP dehydratases in the *M. tuberculosis* cluster *hadA-hadB-hadC* (Sacco *et al.*, 2007) (Fig. 3C). Heterologous expression of these proteins in *E. coli* led to the formation of two heterodimers, HadAB and HadBC, both with the properties expected for the mycobacterial FAS-II dehydratases: a marked specificity for both long-chain and ACP-bound substrates.

Corynebacterium

Corynebacteria also utilize the multifunctional type I FAS for *de novo* biosynthesis of fatty acids, which in these organisms are incorporated as part of the cell-membrane phospholipids or used as precursors for mycolic acid biosynthesis (Fig. 2). It is important to highlight that corynebacteria lack a type II FAS system that could elongate the $C_{16:0}$ or $C_{18:0}$ fatty acids generated by the type I FAS, so corynomycolic acids are synthesized through the direct condensation of the FAS I end products (Kalinowski *et al.*, 2003).

The catalytic domain organization of the corynebacterial type I FAS follows the same pattern as in mycobacteria: AT-ER-DH-MPT-ACP-KR-KS (Schweizer and Hofmann, 2004). This, together with the high overall identity at the amino acid level (~ 50 %), suggests a conserved physiological function.

An *in silico* analysis of all the *Corynebacterium* genome sequences highlights the presence of two putative type I FASs in most species, with some exceptions in the pathogen *Corynebacterium diphtheriae* that only contains one type I FAS and in *Corynebacterium jeikeium* or *Corynebacterium urealyticum* that contain none, reflecting their strict dependence for growth on the presence of exogenous fatty acids (Tauch *et al.*, 2005; Tauch *et al.*, 2008). The presence of two FAS I enzymes in this genus was initially described in the former *Brevibacterum ammoniagenes* (Stuible *et al.*, 1996), currently reclassified as *Corynebacterium ammoniagenes* and later in *Corynebacterium glutamicum* (Radmacher *et al.*, 2005). In both cases it was shown through gene knockout experiments that one of these enzymes is essential for cell survival, FAS A and FAS-IA of *C. ammoniagenes* and *C. glutamicum*, respectively, while the second (FAS B or FAS-IB) is supplementary; however both enzymes are necessary to produce the characteristic lipid environment of this organism. Purification and further characterization of FAS A and FAS B from *C. ammoniagenes*

showed that FAS A synthesizes principally palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids while FAS B could only synthesize saturated $C_{16:0}$ and $C_{18:0}$ (stearic acid) fatty acids. In agreement with this biochemical characterization, mutational inactivation of the gene encoding FAS-IA in *C. glutamicum* results in an unsaturated fatty acid requirement (Radmacher *et al.*, 2005). Supporting these data, both FAS A and FAS-1A contain a FabA-like domain which, in the type II FAS system, represents the enzymatic activity involved in the synthesis of unsaturated fatty acids. To date, our knowledge on the redundancy of type I FAS systems in the same organism is very limited.

Acyl-CoA carboxylases

Acetyl-CoA carboxylases

The first committed step in the biosynthesis of long-chain fatty acids in all animals, plants and bacteria is carried out by the acetyl-CoA carboxylase (ACC) (Wakil *et al.*, 1983). ACC is a key enzyme that catalyzes the production of malonyl-CoA, which is further utilized by all the FAS systems for the elongation of the growing acyl chain. Malonyl-CoA is generated by the carboxylation of acetyl-CoA in a reaction that involves two separate hemi-reactions (Wakil *et al.*, 1983) (Fig. 4A).

ACC is composed of three different components which allow it to carry out these two distinct reactions (Fig. 4B). The biotin carboxylase component (BC) catalyzes the first half-reaction, which involves the phosphorylation of bicarbonate by ATP to form a carboxyphosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin (Blanchard *et al.*, 1999a;Blanchard *et al.*, 1999b;Janiyani *et al.*, 2001;Levert *et al.*, 2000;Waldrop *et al.*, 1994). *In vivo*, the cofactor biotin is attached to the biotin carboxyl carrier protein (BCCP) via an amide bond between the valeric acid side chain of biotin and the ε-amino group of a specific lysine residue (Athappilly and Hendrickson, 1995;Cronan, 2001;Reddy *et al.*, 2000;Roberts *et al.*, 1999;Yao *et al.*, 1997). In the second reaction, catalyzed by the carboxyltransferase (CT) component, the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA (Fig. 4A and B) (Blanchard and Waldrop, 1998;Lane and Lynen, 1963;Levert and Waldrop, 2002;Lynen, 1979). The “swinging arm” of biotin, consisting of a thumb-like loop region on BCCP, the lysine on the loop and biotin itself, is capable of moving alternatively between the BC and CT domains in order to transport the CO_2 (Fig. 4B) (Perham, 2000).

Mammalian, yeast, and most other eukaryotic ACC complexes are large multifunctional enzymes containing the three components distributed in active domains on one polypeptide chain (Fig 4C) (Cronan and Waldrop, 2002). In contrast, in *E. coli* the three components are on four separate polypeptides that constitute the ACC complex (Fig 4C) (Cronan and Waldrop, 2002;Lane *et al.*, 1974). This enzyme has long served as model system for biochemical and mechanistic studies of biotin-dependent carboxylases (Alberts *et al.*, 1972;Blanchard *et al.*, 1999b;Cronan and Waldrop, 2002).

Acyl-CoA carboxylases from actinomycetes

The first attempts to purify the ACC complex from actinomycetes resulted in the characterization of several enzymes with the ability to carboxylate not only acetyl-CoA but also other short chain acyl-CoA substrates, including propionyl- and butyryl-CoA (Erfle, 1973; Henrikson and Allen, 1979; Hunaiti and Kolattukudy, 1982). Consequently, these enzymes were referred to as acyl-CoA carboxylases (ACCase), and all of them consist of two subunits, a larger one (the α-chain) with the ability to carboxylate its covalently bound biotin group, and a smaller subunit (the β-chain) bearing the CT activity. More recently a third subunit, called ε, was also identified as part of several ACCase complexes (Diacovich

et al., 2002; Gago *et al.*, 2006). So far, this subunit has been identified only in ACCases from actinomycetes (Fig. 4C).

Genetic organization, substrate specificity and physiological role of acyl-CoA carboxylases from actinomycetes

The increasing volume of sequence information revealed by the genome sequencing projects facilitated the studies of the ACCases from several actinomycetes, which rapidly revealed major aspects of their biochemical, physiological and structural properties (Daniel *et al.*, 2007; Diacovich *et al.*, 2002; Gago *et al.*, 2006; Gande *et al.*, 2004; Gande *et al.*, 2007; Oh *et al*., 2006). *In silico* analysis of the *S. coelicolor*, *M. tuberculosis* and *C. glutamicum* genomes for the presence of putative ACCase components revealed a clear correlation between the number of putative ACCase genes and the diversity of lipid metabolism present in these microorganisms (Table 1). *S. coelicolor*, for example, has four genes encoding putative α subunits (*accA1, accA2, pccA, SCO4381*), four genes encoding β subunits (*accB, pccB, SCO2776, SCO4380*) and two genes encoding ε subunits (*accE, pccE*) (Bramwell *et al.*, 1996; Diacovich *et al.*, 2004; Rodriguez and Gramajo, 1999; Rodriguez *et al.*, 2001). *M. tuberculosis*, as well as other free-living mycobacteria, also has an unusual number of genes related with putative ACCase complexes in their genomes; these genes encode three α subunits (*accA1–3*), six β subunits (*accD1–6*) (Cole *et al.*, 1998) and one ε subunit (*accE5*) (Gago *et al.*, 2006; Oh *et al.*, 2006) (Table 1 and Fig. 5). Each of the six putative ACCases presumably serves a different physiological role, providing a vast variety of extender units for the biosynthesis of the rich diversity of mycobacterial lipids. The three genes (*accA1, accA2, accA3*) encoding the ACCase α subunits are highly conserved in mycobacteria, although in *M. leprae* only the ortholog of *accA3* in *M. tuberculosis*, namely *Ml0726*, is found. This suggests that *accA3* encodes the α subunit of the essential ACCase complexes. The focus in *M. tuberculosis* has been on the three putative essential ACCases (ACCase 4, ACCase 5 and ACCase 6) (see bellow), according to the transposon site hybridization (TraSH) analysis carried out by Sassetti et al (Sassetti *et al.*, 2001; Sassetti and Rubin, 2003). The genome of *C. glutamicum* has four genes encoding the β subunits (*accD1–4*) of putative ACCases and only one gene encoding an α subunit (*accBC*) and one for an ε subunit (*accE*) (Table 1 and Fig. 5).

A phylogenetic analysis, including the information for most of the predicted β subunits (CT) associated with the carboxylase group of enzymes from several actinomycetes, defined six groups that can be clearly distinguished (I to VI) (Fig. 5). Group I includes the two CTs, AccD1 and AccD2, corresponding to the *Mycobacterium* ACCase complexes that have been implicated in the degradative metabolism of odd-numbered fatty acids (Cole *et al.*, 1998). The *accA1* and *accD1* genes encode the α and β subunits of a putative carboxylase and are organized as an operon clustered in a locus next to *fadD35* and *fadE10*, with a partial synteny with the locus containing *pccA* from *S. coelicolor*. In addition, the *accA2* (α2) and *accD2* (β2) genes are in a locus together with *fadE12, fadE13*, and *echA7*. The relationship of these two enzyme complexes with β-oxidation, as proposed by Cole *et al* (Cole *et al.*, 1998), is possible but still remains an open question. In *Streptomyces* two of the β subunits included in Group I are also organized as operons together with their putative α subunits, suggesting that they form a discrete enzyme complex. This is the case for the α and β subunits *pccA* and *SCO2776* and for *SCO4381* and *SCO4380*, respectively. An enzymatic PCC complex was partially characterized in *S. coelicolor* (Bramwell *et al.*, 1996) consisting of a biotinylated protein, PccA, of 88 kDa, and the CT of 66 kDa (product of *SCO2776*). No ε subunits have been associated to this enzyme complex. To date, the putative complex formed by the proteins encoded by *SCO4381* and *SCO4380* has not been studied. In this group, no CT orthologs were found for corynebacteria.

Group II is the only one where each organism analyzed is represented at least once, suggesting that members of this group might be involved in fundamental functions in actynobacteria. In *M. tuberculosis*, the main locus comprising *accA3* (α3) (*Rv3285*) also contains *birA* (biotin ligase) (*Rv3279*), *accD5* (β5) (*Rv3280*) and *accE5* (ε) (*Rv3281*). This locus is syntenous with orthologous gene sets in *S. coelicolor* where the genes encoding the β and ε subunits, *pccB* (*SCO4026*) and *pccE* (*SCO4025*), also organized as an operon, are found in the same locus as *accA2* (*SCO4021*) and the biotin ligase *birA* (*SCO4027*) (Fig. 5). In *C. glutamicum* the *accD2* (*CG0811*), *accE* (*CG0810*), and *accBC* genes are also clustered, showing a partial synteny with their corresponding genes from streptomycetes and mycobacteria that includes the gene *birA* (*CG0814*). In addition to *accD2* this cluster includes a second gene, named *accD1* (*CG0813*), encoding a β subunit highly homologous to AccD2 (identity of 69 %), suggesting that one of these genes originated by a gene duplication event (Table 1 and Fig. 5).

The first studies that integrated the biochemical and genetic analysis of the actinomycete ACCases were initiated with the identification of the α and β subunits of two ACCases from *S. coelicolor* named PCC (Rodriguez and Gramajo, 1999) and ACC (see below, group IV) ((Rodriguez and Gramajo, 1999; Rodriguez *et al.*, 2001). The PCC complex is composed by PccB (β subunit), PccE (ε subunit) and the α subunits AccA1 or AccA2, which are encoded by two identical genes named *accA1* (*SCO6271*) and *accA2* (*SCO4021*). Kinetic studies demonstrated that this enzyme efficiently carboxylates butyryl- and propionyl-CoA, with a clear preference for the latter (Diacovich *et al.*, 2002), but not acetyl-CoA. The ε subunit resulted in a distinctive feature of the actinomycete ACCases (Diacovich *et al.*, 2002) and its presence dramatically stimulates the specific activity of the enzyme complexes. The ε subunits are small basic proteins (pIs between 9 and 11) that, when present, seem to be essential for the formation of a stable α-β complex. *In vivo*, PCC may provide methylmalonyl- and ethylmalonyl-CoA for the biosynthesis of polyketides associated with *S. coelicolor*. In this sense the α subunit AccA1, which is identical to AccA2 but is found in a different locus and is expressed at the transition phase (Rodriguez *et al.*, 2001), could be part of a stationary-phase PCC complex together with PccB and PccE, generating the substrates for a type III PKS (*SCO7221*) that utilizes methylmalonyl- and ethylmalonyl-CoA as extender units for the biosynthesis of germicidin (Song *et al.*, 2006). Besides, the genes encoding a type I PKS (*SCO6273*, *SCO6274* and *SCO6275*) are separated from *accA1* by only 2 Kb, suggesting that this PKS could also be the receptor of the α-carboxylic acids synthesized by a stationary phase ACC or PCC.

The essential complex ACCase 5 from *M. tuberculosis* was also reconstituted from its purified components, the α biotinylated subunit AccA3, the β subunit AccD5, and the ε subunit AccE5 (*Rv3281*) (Gago *et al.*, 2006). The close phylogenetic relationship between AccD5 and PccB from *S. coelicolor* (Fig. 5) helped to predict the biochemical properties of this enzyme, which was later confirmed by *in vitro* enzyme kinetics. ACCase 5 shows a clear substrate preference for propionyl-CoA compared with acetyl-CoA (specificity constant five fold higher), indicating that the main physiological role of this enzyme is to generate methylmalonyl-CoA for the biosynthesis of branched-chain fatty acids. Also, similarly to *S. coelicolor*, the addition of the ε subunit, which in this case binds tightly to the α-β subcomplex and not to the β subunit as it happens in streptomycetes, is essential for obtaining maximal enzyme activity (Gago *et al.*, 2006). Controversial results about the size of the active ε subunit of this complex have been published (Oh *et al.*, 2006). Significantly, in the paper published by Oh *et al.* the activity of the ACCase 5 complex, containing an ε subunit with extra amino acids in its N-terminal sequence, resulted in an enzyme with a specific activity 300-fold lower than the enzyme complex reconstituted by Gago *et al.* (Gago *et al.*, 2006) with a shorter version of the ε subunit.

The putative ACC of *C. glutamicum* consists of the biotinylated α-subunit AccBC (63 kDa), the β-subunit AccD1 (58 kDa) included in group II, and the small peptide AccE of 8.9 kDa (Gande *et al.*, 2007). This enzyme complex carboxylates acetyl- and propionyl-CoA, with higher affinity for the first one. *AccD1* mutants grow very poorly; however, when the medium is supplemented with sodium oleate together with butter hydrolysate, growth of the *accD1* mutant is restored to normal levels, confirming a direct relationship of *accD1* with fatty acid biosynthesis (Gande *et al.*, 2004).

In group III, we only found enzymes that belong to the mycolic acid-producing branch of actinomycetes, suggesting that they represent CTs with more specialized functions. In agreement with this finding, it has been recently demonstrated that members of this group are directly involved in mycolic acid biosynthesis (Gande *et al.*, 2004; Gande *et al.*, 2007; Portevin *et al.*, 2005). So far, the ACCase 4 complex of *M. tuberculosis* has not been well characterized at the biochemical level. The fact that *accD4* (*Rv3799c*) is clustered with and transcribed in the same orientation as the genes *pks13* (*Rv3800c*) and *fadD32* (*Rv3801c*) (Fig. 5), a condensase and an acyl-AMP ligase involved in the condensation reaction leading to mycolic acid formation (Trivedi *et al.*, 2004), suggested that AccD4 should be the β component of a long-chain acyl-CoA carboxylase that generates the α-C_{26} carboxylic acid involved in the last condensation step of mycolic acid biosynthesis. Moreover, phylogenetic analysis shows that AccD4 ortologs are present only in the mycolic acid containing bacteria, confirming a specific role for this CT subunit in the biosynthesis of this complex lipid. Co-immunoprecipitation studies carried out in cell-free extracts of *M. smegmatis* demonstrated that AccD4 interacts with the α subunit AccA3 and the β subunit AccD5 (Portevin *et al.*, 2005), suggesting that the ACCase 4 components would be AccA3, AccD4 and AccD5. Experiments carried out in our laboratory directed to reconstitute the AccD4-AccD5 hetero-oligomers *in vitro* and *in vivo* were unsuccessful (Kurth and Gago, unpublished), while the ACCase 5 containing AccD5 as the unique β subunit was unambiguously characterized as a PCC. Consequently, more sophisticated biochemical, structural and genetic studies will be needed to characterize in detail the enzyme dedicated to provide the α chain of mycolic acids in mycobacteria. In *C. glutamicum* the putative ACCase involved in corynomycolic acid biosynthesis was studied in more detail. Similarly to *M. smegmatis*, the AccD3 and AccD2 β subunits of corynebacteria (orthologous of the AccD4 and AccD5 subunits from *M. smegmatis*) co-immunoprecipitated with the α subunit AccBC (the orthologous of AccA3) and the ε subunit AccE when anti-His antibodies where used to treat cell-free extracts of *C. glutamicum* expressing either His_6-AccD2 or His_6-AccD3 (Gande *et al.*, 2007). These experiments suggested that in this microorganism the ACCase involved in providing the substrates for corynomycolic acid synthesis is formed by all these subunits. The immunoprecipitation experiments were also supported by the construction of knockout mutants in the two β subunits. The *accD2* and *accD3* mutations resulted in the complete loss of free and extractable mycolates without affecting phospholipid synthesis and the possible accumulation of an unknown intermediate (Gande *et al.*, 2004). In addition, in the *accD2* and *accD3* mutants, the cell wall-bound mycolic acids were absent, illustrating that both genes could be involved in mycolic acid synthesis. However, the biochemical studies performed on the cell-free extracts of these mutants or on cell-free extracts of cells overexpressing either AccD2 or AccD3 were less convincing (Gande *et al.*, 2007). Furthermore, experiments carried out *in vitro* with the purified components, AccBC-AccD2-AccD3-AccE, show enzyme activities barely detectable either with acetyl-CoA or palmitoyl-CoA, and curiously the assays were not conducted using propionyl-CoA as a substrate which, based on the high identity (75%) found between AccD2 of *C. glutamicum* and AccD5 of *M. tuberculosis*, would be the preferred substrate of such complex.

Mycobacteria and corynebacteria lack group IV enzymes; this is particularly noteworthy as members of this group are probably essential ACC complexes that primarily recognize

acetyl-CoA as a substrate, as has been demonstrated for the AccB subunit of *S. coelicolor* (Diacovich *et al.*, 2002; Rodriguez *et al.*, 2001). In this bacterium the genes encoding the β and ε subunits *accB* (*SCO5535*) and *accE* (*SCO5536*) are clustered as an operon (Table 1 and Fig. 5). As occurs with the PCC complex (see group II), the α subunit that conform the ACC complex in *Streptomyces* are either AccA1 or AccA2. Consequently, the α subunits are shared by the two complexes, although the β and ε subunits of the ACC complex are AccB and AccE while for PCC the β and ε subunits are PccB and PccE, respectively. Notably, in *S. coelicolor* the ε subunits interact with the corresponding β subunit and are not functionally interchangeable (Diacovich *et al.*, 2002). ACC complex was reconstituted *in vitro* from their purified components, and their kinetic characterization was conducted with several acyl-CoA substrates (Diacovich *et al.*, 2002; Rodriguez and Gramajo, 1999; Rodriguez *et al.*, 2001). This study denoted a relaxed substrate specificity of this enzyme, with a similar specificity constant for acetyl-, propionyl-, and butyryl-CoA, to provide malonyl-, methylmalonyl-, and ethylmalonyl-CoA, respectively (Diacovich *et al.*, 2002). The primary role of the *S. coelicolor* ACC appears to be the generation of malonyl-CoA for the biosynthesis of fatty acids during exponential phase, and for the biosynthesis of the polyketide actinorhodin during the stationary phase of growth (Rodriguez *et al.*, 2001). Gene disruption experiments showed that AccB and AccA2 are essential for *S. coelicolor* viability (Rodriguez and Gramajo, 1999), which confirmed that these subunits are part of a dedicated ACC in this microorganism.

Group V contains proteins found only in mycobacteria (Fig. 5). The CT AccD6 that belongs to this group of CT subunits was recently characterized as the β component of an essential ACC from *M. smegmatis* (Kurth *et al.*, 2009), suggesting that this group of proteins could represent the CTs of the essential ACCs of mycobacteria that have diverged from other known ACCs due to the intracellular lifestyle of this group of bacteria. A complex with acetyl-CoA carboxylase activity was reconstituted *in vitro* with the α subunit AccA3 and the CT β subunit AccD6 (Daniel *et al.*, 2007); this enzyme shows similar specificity for both acetyl- or propionyl-CoA and does not require a ε subunit for full activity. The physiological role of this enzyme as the ACC in charge of providing malonyl-CoA for mycolic acid biosynthesis was first predicted based on the location of *accD6* (*Rv2247*) as part of the *fasII* operon (*fabD-acpM-kasA-kasB-accD6*) (Fig. 3A) and later confirmed in *M. smegmatis* by the construction of a conditional mutant in *accD6* (Kurth *et al.*, 2009). Studies carried out on this mutant allowed AccD6 to be assigned as the essential CT component of the ACCase 6 enzyme complex, whose biological role is to generate malonyl-CoA for the biosynthesis of membrane and cell envelope fatty acids by the type I and type II FAS systems. The essentiality of *accD6* was also demonstrated by genetic experiments (Kurth *et al.*, 2009).

Recently it has been suggested that members of the most distantly related group VI, including the gene encoding the CT *accD3* from mycobacteria, are not directly involved in lipid biosynthesis (Gande *et al.*, 2004). It is hard to predict their functional role since none of the other members of this group have a known activity or a predicted function (Table 1 and Fig 5). In *C. glutamicum*, the *accD4* mutant (the *accD3* orthologue of *M. tuberculosis*) did not exhibit a phenotype in terms of total fatty acid or mycolic acid content. However, this mutant resulted in a uniform and significant decrease in ACCase activity (^{14}C fixation) in *C. glutamicum* cell-free extracts, and overexpression of *accD4* led to a uniform increase in activity, in both cases independently of the substrate used (acetyl-CoA, palmitoyl-CoA or palmitic acid). Therefore, the physiological role of this CT subunit remains unknown.

A remarkable feature that arises from this sequence alignment analysis is that all of the β subunits present in groups II and IV have a putative ε subunit genetically associated with the corresponding β-subunit-encoding gene, suggesting that the new group of ACCases originally described in streptomycetes is ubiquitously present in all the actinomycetes

analyzed so far. This new class of ACCases could be considered as specific targets for the development of new antimycobacterial drugs based on their distinctive structure.

In summary, the ACCase complexes from actinomycetes not only catalyze the first committed step in fatty acid biosynthesis, providing malonyl-CoA for the iterative condensation steps that occurs during the synthesis of these macromolecules, but they also catalyze the carboxylation of different substrates, such as propionyl- butyryl- or long-chain acyl-CoAs, generating precursors for the synthesis of a variety of polyketides and complex lipids in these microorganisms.

Structural studies of acyl-CoA carboxylases: identification of inhibitors

The quaternary structure of ACCase complexes has been reported for *M. smegmatis* (α6β6) (Haase *et al.*, 1982), *M. tuberculosis* (α6β6) (Gago *et al.*, 2006), *Sacharopolyspora erythraea* (α4β4) (Hunaiti and Kolattukudy, 1982), *Myxococcus xanthus* (α6β6) (Kimura 1998) and *S. coelicolor* (Diacovich *et al.*, 2002), and in all cases the stoichiometry of the complex subunits was 1:1. The individual analysis of each subunit shows that in most cases the β subunit adopts a stable hexameric conformation (Diacovich *et al.*, 2004; Gago *et al.*, 2006; Lin *et al.*, 2006), while the α subunit is found both as a trimer and as a hexamer (Gago *et al.*, 2006). As we mentioned above, some of these complexes need an ε subunit for full enzyme activity (Diacovich *et al.*, 2002; Gago *et al.*, 2006; Oh *et al.*, 2006; Rodriguez *et al.*, 2001), but the exact molar ratio between this subunit and the α and β subunits has not been determined.

Due to the instability of the bacterial ACCs, the structural details of the different subunits were studied separately. First, the crystal structure of the BC component of *E. coli* ACC was solved (Waldrop *et al.*, 1994), and this was followed by the crystal structure of the biotinoyl domain of *E. coli* BCCP (Athappilly and Hendrickson, 1995) and the CT domain (Bilder *et al.*, 2006). Other CT domains from biotin-dependent carboxylases were also solved by X-ray crystallography, including the 1.3S transcarboxylase subunit (Reddy *et al.*, 2000) and the 12S carboxyltransferase (for pyruvate carboxylation) (Hall *et al.*, 2003) of *P. Shermanii*, the tetrameric pyruvate carboxylase (Xiang and Tong, 2008; Yu *et al.*, 2009), and the glutaconyl-CoA decarboxylase (for sodium ion pump) (Wendt *et al.*, 2003).

The crystal structures of *S. coelicolor* AccB and PccB

PccB is a 65 kDa protein that forms a hexamer in solution and in the crystal structure (Fig. 6A). The hexameric stoichiometry of PccB implies an overall stoichiometry of $\alpha_6\beta_6\varepsilon$? for the holo complex, with a molecular weight of > 1 Mda. Similar to the *Propionibacterium shermanii* 12S transcarboxylase (Hall *et al.*, 2003), the PccB hexamer has a 32-fold symmetry, where a 3-fold axis is perpendicular to a 2-fold axis (Fig. 6A). Specifically, A-B-C and D-E-F monomers form two tightly interacting trimer rings, while A–D, B–E, and C–F dimers interact extensively (Fig. 6A). Like 12S and yeast-CT (Hall *et al.*, 2003;Zhang *et al.*, 2003), PccB also reveals two domains (N- and C-domains) in each monomer, and both domains have a crotonase fold that consists of seven β-sheets and α-helices (Fig. 6B and C) (Holden *et al.*, 2001).

The biotin and acyl-CoA binding pockets of CTs are identified by the co-crystal structure of PccB bound with the substrate biotin and propionyl-CoA (Diacovich *et al.*, 2004) (Fig. 7A). The binding pockets of acyl-coenzyme A and biotin are perpendicular to each other and meet at the junction of the L-shaped active site, near the intersection of Nβ5-Nα6 of one monomer and Cβ5-Cα6 of its dimeric partner (Fig. 7A and B), and shows that acyl-CoA binds predominantly from the N domain of the first monomer, while biotin binds from the C domain of its dimeric partner. Such a dimeric, di-domain active site arrangement is

conserved across the biotin-dependent CTs, including PccB, 12S (a decarboxylase) (Hall *et al.*, 2003), GCD (a sodium-transport decarboxylase) (Wendt *et al.*, 2003) and yeast-CT (the β-subunit of ACC) (Zhang *et al.*, 2003). Based on the biotin-propionyl-CoA co-crystal structure of PccB and its comparison with other CT structures, the binding motif of acyl-CoA and biotin to CT should be ubiquitous among biotin-based CTs, where dimeric, di-domain interactions are crucial for substrate binding. Although the overall structures of PccB, AccB (Tsai, unpublished results) and 12S are similar, there is a large difference in surface properties among these CTs (Fig. 6D). This may reflect a major difference in protein-protein interaction and in their corresponding biochemical roles.

Structure-based mutation: molecular basis of substrate specificity of *S. coelicolor* ACCases

The molecular basis of substrate specificity, i.e. how the CTs differentiate acetyl- from propionyl-CoA, has been addressed with the *S. coelicolor* β subunits AccB and PccB. Significantly, nearly all residues in the acyl-CoA binding pocket are conserved between AccB and PccB, except residue 422 of PccB (420 in AccB), which lies at the end of the acyl-CoA binding pocket (Fig. 7C). Residue 422 is an Asp in PccB and an Ile (420) in AccB. Sequence comparison indicates that for different CTs that accept propionyl-CoA as a substrate, from *S. coelicolor* to the human PccB, this position is occupied by small residues, such as Asp or Cys (Fig. 7C). On the other hand, for CTs that accept acetyl-CoA as a substrate, such as AccB, this residue is a larger hydrophobic residue. This led to the hypothesis that the amino acid at this position may help to define the shape of the acyl-CoA binding pocket. To test this hypothesis, the D422I:PccB and I420D:AccB mutants were compared. Wild type PccB does not accept acetyl-CoA as a substrate; however the D422I mutant of PccB should make the active site into a “mock AccB” to accept acetyl-CoA. The PccB D422I mutant not only accepted acetyl-CoA as its substrate, but also exhibited similar substrate specificity as wild type AccB for propionyl- and butyryl-CoA. Further, the AccB I420D mutant (the “mock PccB”), cannot accept acetyl-CoA as its substrate (Diacovich *et al.*, 2004). In essence, by a single mutation in the active site, the substrate specificities of mutant ACC and PCC have been interchanged. Systematic mutations of PccB D422 and AccB I420 further confirmed the importance of this residue for substrate specificity (Arabolaza *et al.*, 2010b). In summary, the above results confirmed that the CT domain is the major determinant for acyl-CoA specificity.

The crystal structure of *M. tuberculosis* AccD5

As mentioned above, *M. tuberculosis* contains six ACCase β subunits (AccD1–6) (Cole *et al.*, 1998). In order to determine the molecular basis of substrate specificity for ACCase5 complex, a crystal structure of AccD5 was solved at 2.9 Å resolution (Lin *et al.*, 2006). AccD5 consists of a 360-kDa hexamer (Fig. 8A), similar to the *S. coelicolor* PccB (Diacovich *et al.*, 2004); however AccD5 differs from the dimeric yeast CT domains (Zhang *et al.*, 2003). Similarly to PccB, the dimeric interface is also conserved in AccD5 (Fig. 8B). All of the biotin-binding residues are conserved between AccD5 and PccB. Significantly, residues in the acyl-CoA binding pocket are also highly conserved surrounding the acyl, pantetheine, ribose and adenine ring, while residues that interact with the bis-phosphate group, such as Q472, R471, and F468, are partially conserved among AccD4–6 and PccB (Lin *et al.*, 2006) (Diacovich *et al.*, 2004).

Significantly, the AccD5 crystal structure shows that the active site cannot accommodate an acyl chain with more than three carbons (Fig. 8C). Contrary to the previous proposal that the AccD4–5 complexes accept long-chain acyl-CoAs as their substrates (Gande *et al.*, 2004), both crystal structure and kinetic assay consistently showed that AccD5 preferentially accepts propionyl-CoA as its substrate, producing methylmalonyl-CoA, the substrate for the

biosynthesis of multimethyl-branched fatty acids such as mycocerosic, phthioceranic, hydroxyphthioceranic, mycosanoic and mycolipenic acids (Minnikin *et al.*, 2002;Takayama *et al.*, 2005), many of which are important factors for virulence, survival, multi-drug resistence, and latency development.

Structure-based inhibitor design of *M. tuberculosis* ACCases

Acetyl-CoA carboxylases have crucial roles in fatty acid metabolism and are attractive targets for drug discovery against a variety of human diseases, including diabetes, obesity, cancer and microbial infections. ACCs have also been the targets of herbicides that are currently used as commercial products. The reader can find excellent reviews on these topics elsewhere (Gronwald, 1994; Heath *et al.*, 2001; Levert and Waldrop, 2002; Pacheco-Alvarez *et al.*, 2002; Shen *et al.*, 2004; Tong, 2005; Tong and Harwood, 2006).

The first antibacterial compounds that targeted the ACC were the pseudopeptidic pyrrolidinedione natural products moiramide B and andrimid that selectively inhibit the ACC-carboxyltransferase reaction (Freiberg *et al.*, 2004; Pohlmann *et al.*, 2005) and synthetic variations of each moiety of the modularly composed pyrrolidinediones have showed highly improved activities against gram-positive bacteria compared to those of previously reported variants. Unfortunately, no studies have been published addressing the efficacy of these compounds in actinobacteria. As an effort to find inhibitors for the ACCases of *M. tuberculosis* a structure-based *in silico* screening was conducted with UC Irvine ChemDB database (Chen *et al.*, 2005) and the crystal structure of AccD5 (Lin *et al.*, 2006). More than 100 putative AccD5 inhibitors were identified and assayed *in vitro* for inhibition of AccD5 and for the whole ACCase 5 enzyme complex. One of the inhibitors, NCI-65828, was found to be a competitive inhibitor of AccD5 with a K_I of 13.1 μM (Lin *et al.*, 2006). Based on the conserved structure of the AccD5 and AccD6 active sites, several inhibitors of AccD5 were also screened as potential inhibitors of AccD6 and it was found that the ligand NCI-172033 was capable of inhibiting AccD6 with a K_I of 8 μM (Kurth *et al.*, 2009). This compound also showed bactericidal activity against several pathogenic mycobacteria by causing 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 and as a target for the development of new antimycobacterial agents (Kurth *et al.*, 2009)

A key issue of structure-based inhibitor design is the versatility of the docking software and the lack of self-improving ability of the most commonly used docking softwares (Anderson, 2003). Building on the above promising results, the enzymatic assays of AccD5 and AccD6 were combined with virtual high-throughput screening, aiming to discover new AccD5 and AccD6 inhibitors as potential tuberculosis drugs (Swamidass *et al.*, 2009). Using a new method (Influence Relevance Voter, IRV), the *in silico* screening program was turned into an artificial intelligence that self-improves by feeding the enzyme inhibition assay data back to the program. In this way, IRV achieves significantly more accurate prediction than other traditional screening programs. Further, IRV retrieves three times as many experimentally proven “hits” in its prediction-sorted list. This successfully developed “smart” program allows feedback of actual experimental data to improve its prediction power, which leads to improved inhibitors against AccD5 and AccD6. These results pave the way towards structure-based development of anti-tuberculosis therapeutics.

Controlling lipid homeostasis in actinomycetes

The *de novo* fatty acid biosynthetic pathway is a major focal point for the regulatory events that control lipid homeostasis. Regulation at the level of fatty acid biosynthesis is crucial not only because the biophysical properties of membranes are determined in large part by the

composition of the fatty acids that are produced by *de novo* biosynthesis, but also for the metabolic energy that is expended in the formation of these molecules. Therefore, fatty acid production must be precisely controlled to support membrane biogenesis and prevent the wasteful expenditure of ATP.

Although significant progress has been made in recent years, most of the regulatory mechanisms that control fatty acid metabolic pathways have been elucidated in model bacteria, such as *E. coli* (Cronan and Subrahmanyam, 1998; DiRusso and Nystrom, 1998; Rock and Cronan, 1996; Zhang *et al.*, 2006) and *Bacillus subtilis* (Matsuoka *et al.*, 2007; Schujman *et al.*, 2003; Schujman *et al.*, 2006). These investigations revealed that this process involves both a biochemical control of key enzymes as well as a transcriptional regulation of the lipid genes. There is a rapid response of the integrated biochemical network, acting at the level of the activities of lipid biosynthetic enzymes and a central control at the level of gene expression involving specific transcription factors.

Regulation of expression of the genes for fatty acid synthesis is complex (Zhang and Rock, 2008). Currently, six transcriptional regulators have been identified, four of them - FadR (*E. coli*), DesR (*B. subtilis*), FabR (*E. coli*) and DesT (*P. aeruginosa*) - adjust the levels of unsaturated fatty acids in membrane phospholipids, while FapR (*B. subtilis*) and FabT (*S. pneumonieae*) are global transcriptional repressors that simultaneously control the expression of many genes involved in fatty acid and phospholipid metabolism (Cronan and Subrahmanyam, 1998; Lu and Rock, 2006; Schujman *et al.*, 2003; Schujman *et al.*, 2006; Schujman and de Mendoza, 2008; Zhang and Rock, 2009). An extensive bioinformatic analysis carried out in different actinomycetes genome databases demonstrated that these microorganisms lack FadR, FapR or FabR homologues. However, the existence of a transcriptional control of the genes required for fatty acid biosynthesis was recently reported both in *S. coelicolor* and in *M. tuberculosis* (Arabolaza *et al.*, 2010a; Salzman *et al.*, 2010). These studies describe the characterization of a novel type of transcription factor widely distributed among the actinobacteria (see below).

In most bacteria, the fatty acids produced by the biosynthetic pathway are incorporated exclusively into the membrane. The actinomycetes represent an exception since the product of microbial fatty acid biosynthesis, long chain acyl-ACP, can be directed to other cellular components such as complex structural lipids (e.g. mycolic acids or PDIM) or storage lipids (triacylglycerols). This feature adds a higher level of sophistication to the regulation of lipid homeostasis in these bacteria and the mechanisms involved in this global coordination are only recently coming to be understood.

Biochemical control of lipid biosynthesis in actinomycetes

While fatty acid biosynthesis has been reasonably well characterized in some genera of actinobacteria, the mechanisms that regulate the production of fatty acids and lipid molecules, either at the biochemical or at the genetic levels, are largely unknown. Only recent reports proposed a model for mycolic acid synthesis regulation based on the phosphorylation of KasA, KasB, FabH and MabA (Molle *et al.*, 2006; Veyron-Churlet *et al.*, 2009; Veyron-Churlet *et al.*, 2010). Protein phosphorylation/dephosphorylation is a central mechanism for transduction of specific signals to various cellular processes, such as regulation of growth, differentiation, mobility and survival (Stock *et al.*, 1989); in this sense these works represent the first evidence of this kind of regulation for the lipid metabolism. KasA and KasB are phosphorylated at high rates *in vivo* in *Mycobacterium bovis* BCG, mainly at two positions, and can be dephosphorylated by the mycobacterial Ser/Thr phosphatase PstP. *In vitro* experiments demonstrated that phosphorylation of KasA and KasB differentially modulates their condensing activities in the presence of either malonyl-ACP or C_{16}-ACP (Molle *et al.*, 2006). MtFabH is efficiently phosphorylated both *in vitro*

and *in vivo* by several mycobacterial STPKs (serine, threonine and tyrosine kinases), particularly by PknF and PknA (Veyron-Churlet *et al.*, 2009). A mutant variant of mtFabH, constructed to mimic constitutive phosphorylation, exhibited a marked decrease in transacylation, malonyl-ACP decarboxylation and condensing activities compared with the wild-type protein. MabA is also phosphorylated *in vitro*, as well as *in vivo*, by several *M. tuberculosis* STPKs (Veyron-Churlet *et al.*, 2010). In this work, the authors provide a fundamental insight into the molecular mechanism(s) controlling mycolic acid biosynthesis through STPK-dependent phosphorylation of a FAS II enzyme. Thus the mycobacterial STPKs appear to play an important role in regulating the metabolism of mycolic acids, although whether phosphorylation of these proteins regulates the mycolic acid profile *in vivo* in response to environmental changes, and/or affects *M. tuberculosis* virulence in infected mice, has yet to be demonstrated.

On other hand, the Ser/Thr/Tyr phosphorylation phosphoproteome in *C. glutamicum* revealed that most of the phosphorylated proteins identified are enzymes acting on central metabolic pathways such as glycolysis, tricarboxylic cycle, fatty-acid metabolism and components of the protein synthesis machinery (Bendt *et al.*, 2003). Particularly, an acyl-CoA synthetase (NgCl2774) and the acyl-CoA carboxylase α subunit (*accBC*) were found to be phosphorylated; however how this post-translational modification affects their enzyme activities and how this influences lipid metabolism is not known.

Transcriptional control of the fatty acid biosynthetic genes in actinomycetes

A major advance in the recognition of a transcriptional control of actinobacterial lipid synthesis occurred by the identification of FasR and MabR; two transcription factors that regulate the expression of the main *fab* operon in *S. coelicolor* and *M. tuberculosis*, respectively (Arabolaza *et al.*, 2010a; Salzman *et al.*, 2010). These novel regulators are highly conserved in most of the actinomycete genomes sequenced to date, including *Arthrobacter, Frankia, Rhodococcus, Nocardia, Acidothermus, Bifidobacterium, Streptomyces* and *Mycobacterium*, and the coding genes lie immediately upstream of their corresponding *fab* operons (Fig. 3A). An exception to this situation occurs in *Corynebacterium*, where no type II FAS exists and therefore no homologues to these transcription factors. The 21-bp imperfect palindromic motif (5´-TTTTGT-N9-ACAAAA-3´) identified as the regulator binding site was found to be highly conserved within actinomycetes and always associated with *fab* operons. Remarkably, although the binding motifs of *S. coelicolor* and *M. tuberculosis* lie at a comparable distance from the 5´ end of the first gene of the *fab* operon, at approximately −65 to −80 bp from the TSS (Fig. 3A), the binding of FasR to its operator sequence results in an activation of the *fab* genes in *S. coelicolor* (Arabolaza *et al.*, 2010a) while the binding of MabR to its operator sequence results in a repression of these genes in *M. tuberculosis* (Salzman *et al.*, 2010). Furthermore, these orthologues show another important difference. TraSH (transposon site hybridization) experiments carried out in *M. tuberculosis* suggested that *mabR* is an essential gene (Sassetti *et al.*, 2001), and Salzman *et al.* (Salzman *et al.*, 2010) demonstrated that the chromosomal copy of *M. smegmatis mabR* could only be disrupted in the presence of a wild-type copy of the gene provided in *trans*, giving a rigorous proof of the essentiality of this gene in mycobacteria. In sharp contrast, FasR has been shown to be non-essential for the survival of *S. coelicolor* (Arabolaza *et al.*, 2010a). Thus, the evolutionary divergence found for this regulatory protein in *S. coelicolor* and in *M. tuberculosis* could be correlated with the different physiological roles of the FAS systems that each of them regulates.

Concluding Remarks

Actinomycetes include a very interesting group of bacteria that are ubiquitous in nature and well known for their distinct impacts on mankind. In actinomycetes, lipid metabolism is

relevant not only for the generation of cell membrane phospholipids but also for the production of PK compounds with useful pharmaceutical properties, for the generation of storage compounds (TAG) with possible technological applications and for the synthesis of complex lipids, crucial for the permeability and pathogenicity of some pathogenic species. Therefore, the identification and further biochemical and structural characterization of each of the components of the fatty acid biosynthetic pathways, as well as understanding the regulation of lipid homeostasis, should have a major impact on the biology, biotechnology and disease control related with each of the organisms discussed in this review.

The genomic information available for several species of corynebacteria, mycobacteria and streptomycetes provides excellent opportunities for comparative genome analysis and for the extrapolation of gene functions between these genera. We have shown that several genes involved in fatty acid metabolism can be identified and analyzed in this way. This information when complemented with transcriptomic, proteomic and metabolomic data should result in a major step toward the understanding of this essential but poorly understood metabolic pathway in this group of organisms.

One of the novel aspects highlighted by this review in relation to lipid metabolism is a comparative analysis of the enzymes (ACCases) that catalyze the carboxylation of different acyl-CoAs and whose products are utilized not only in fatty acid biosynthesis but also in the synthesis of complex lipids and in the production of PK compounds. ACCases from actinomycetes present unique characteristics within this group of enzymes at both the biochemical and structural levels. The wide diversity of substrates recognized by the ACCase β subunits is an example of a remarkable divergent evolution of this class of enzymes, and provides great opportunities not only for understanding the molecular bases that dictate substrate specificity of the CTs but also for the use of these enzymes in protein engineering for the generation of new substrates for polyketide biosynthesis as well as the use of some of them as attractive target for the design of novel antibacterial drugs (Cronan and Waldrop, 2002; Heath *et al.*, 2001). For instance, studies combining the structural data of some of the essential CTs subunits of *M. tuberculosis* with *in silico* screening of compounds available at the NCI database allowed the identification and characterization of two structurally different CT inhibitors, one of them with interesting bactericidal activity against MDR *M. tuberculosis*. In the future, investigation of fatty acid biosynthesis and its regulation in actinomycetes could open new avenues for therapeutics.

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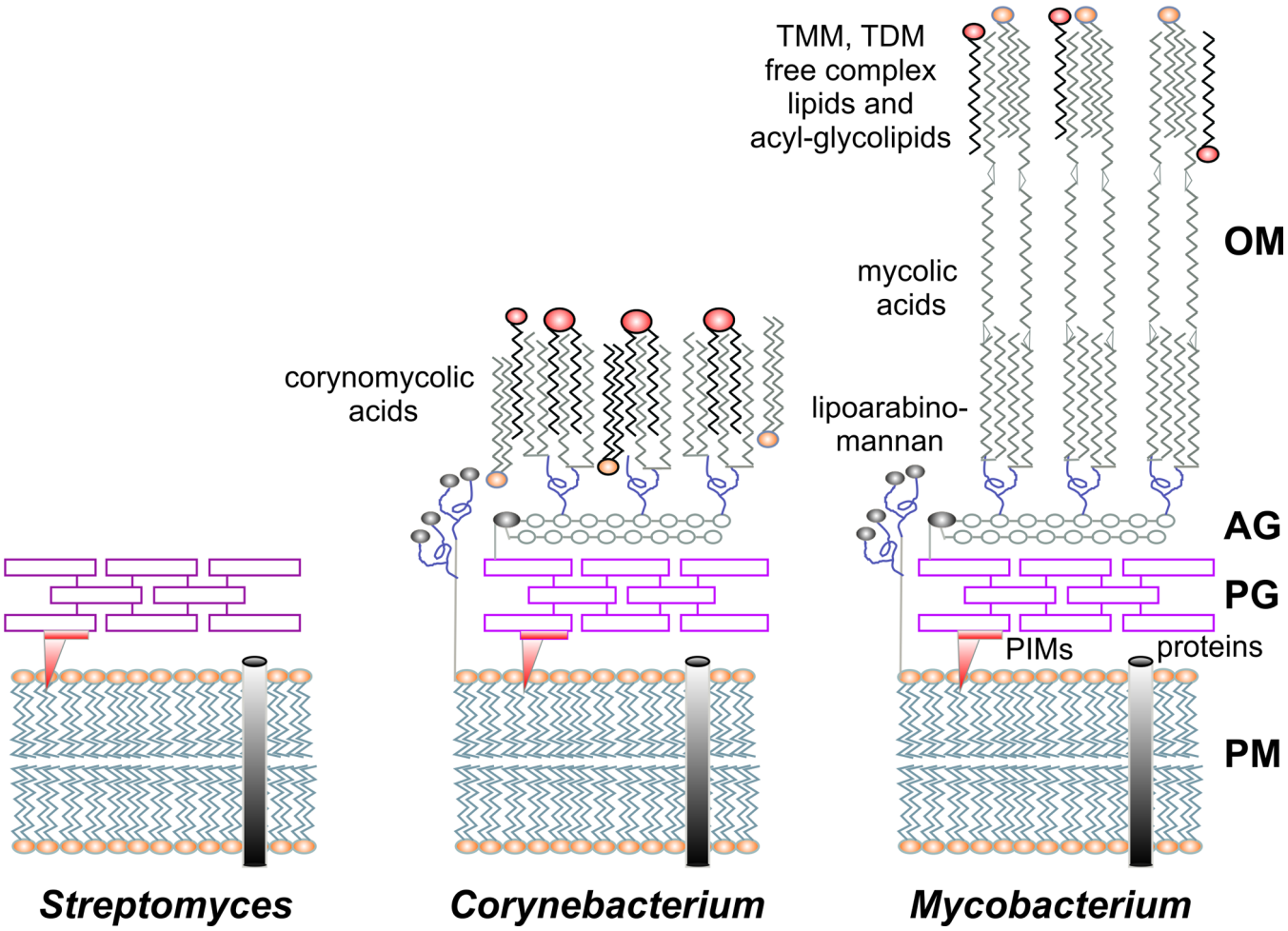

Fig. 1. Schematic representation of the cell envelope of actinomycetes

Comparison of the cell wall structures from *Streptomyces*, *Corynebacterium* and *Mycobacterium*. In streptomycetes, as in other Gram-positive bacteria, the cell wall consists of a peptidoglycan layer (PG) that covers the cytoplasmic membrane (PM). Corynebacteria and mycobacteria contain a more complex cell wall including an additional layer of arabinogalactan (AG) and an outer membrane (OM) composed of mycolic acids, trehalose monomycolate (TMM), trehalose dimycolate (TDM) and free lipids. The mycobacterial cell wall has a high proportion of covalently attached mycolic acid residues, which constitute a permeability barrier contributing to antibiotic resistance and pathogenicity. PIMs: phosphatidylinositol-mannosides.

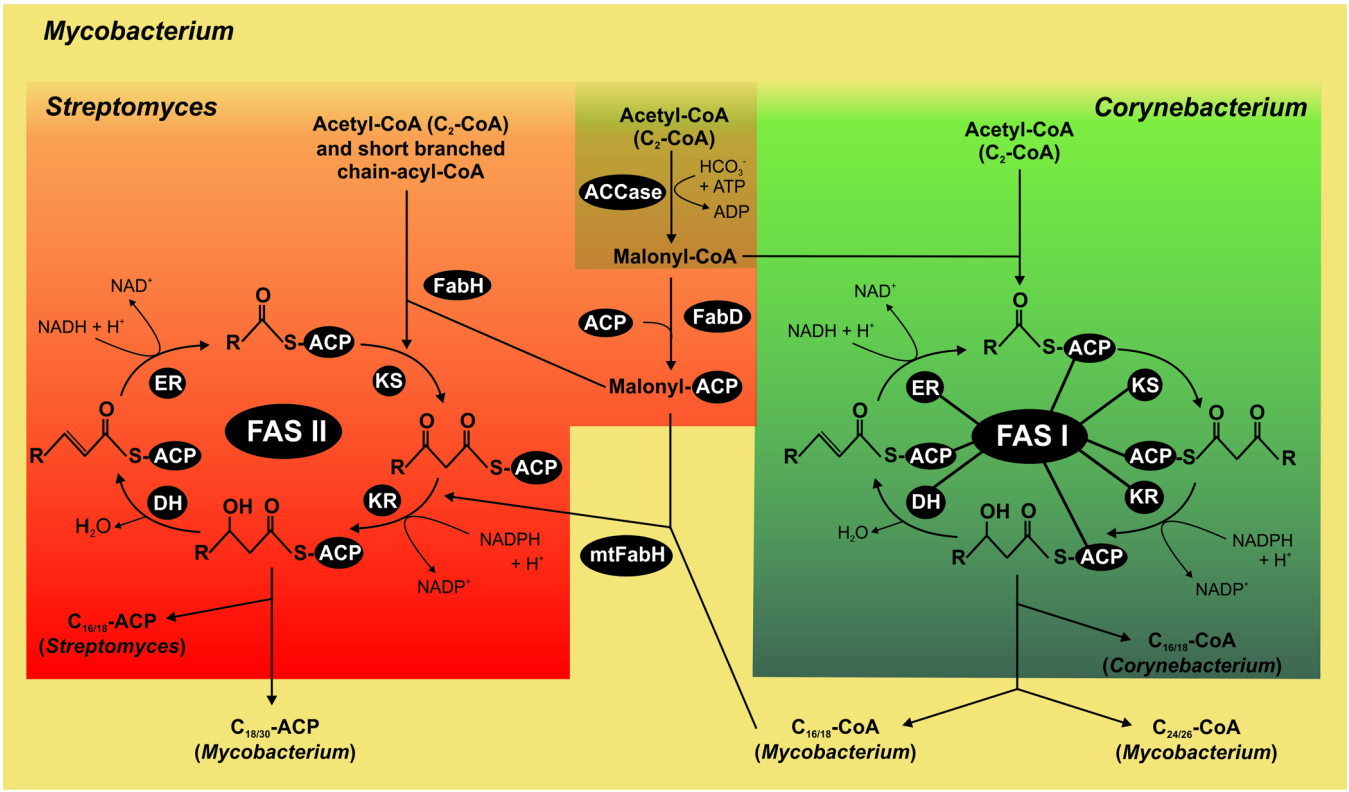

Fig. 2. Comparative model of fatty acid biosynthesis in actinomycetes

Streptomyces Corynebacterium and *Mycobacterium* share the initiation step for fatty acid biosynthesis which is the carboxylation of acetyl-CoA to produce malonyl-CoA, catalyzed by the ACCase complex (brown). Both in *Mycobacterium* and *Corynebacterium* spp., malonyl-CoA and acetyl-CoA are condensed to be utilized by the multifunctional type I FAS for *de novo* biosynthesis of fatty acids (green). In *Streptomyces*, malonyl-CoA is converted to malonyl-ACP by FabD and then condensed with acetyl-CoA by FabH to form β-ketoacyl-ACP, which is used by the type II FAS dissociated system for *de novo* biosynthesis of fatty acids (red). Both in FAS I and FAS II systems, the chain elongation steps consist of an iterative series of reactions built on successive addition of a two-carbon unit to a nascent acyl group, and reaction intermediates are covalently attached to the acyl carrier protein (ACP). In *Mycobacterium* both systems are present, FAS I for *de novo* fatty acid biosynthesis and FAS II for the elongation of fatty acids for mycolic acid production. The differential features of *Mycobacterium* are shown in yellow.

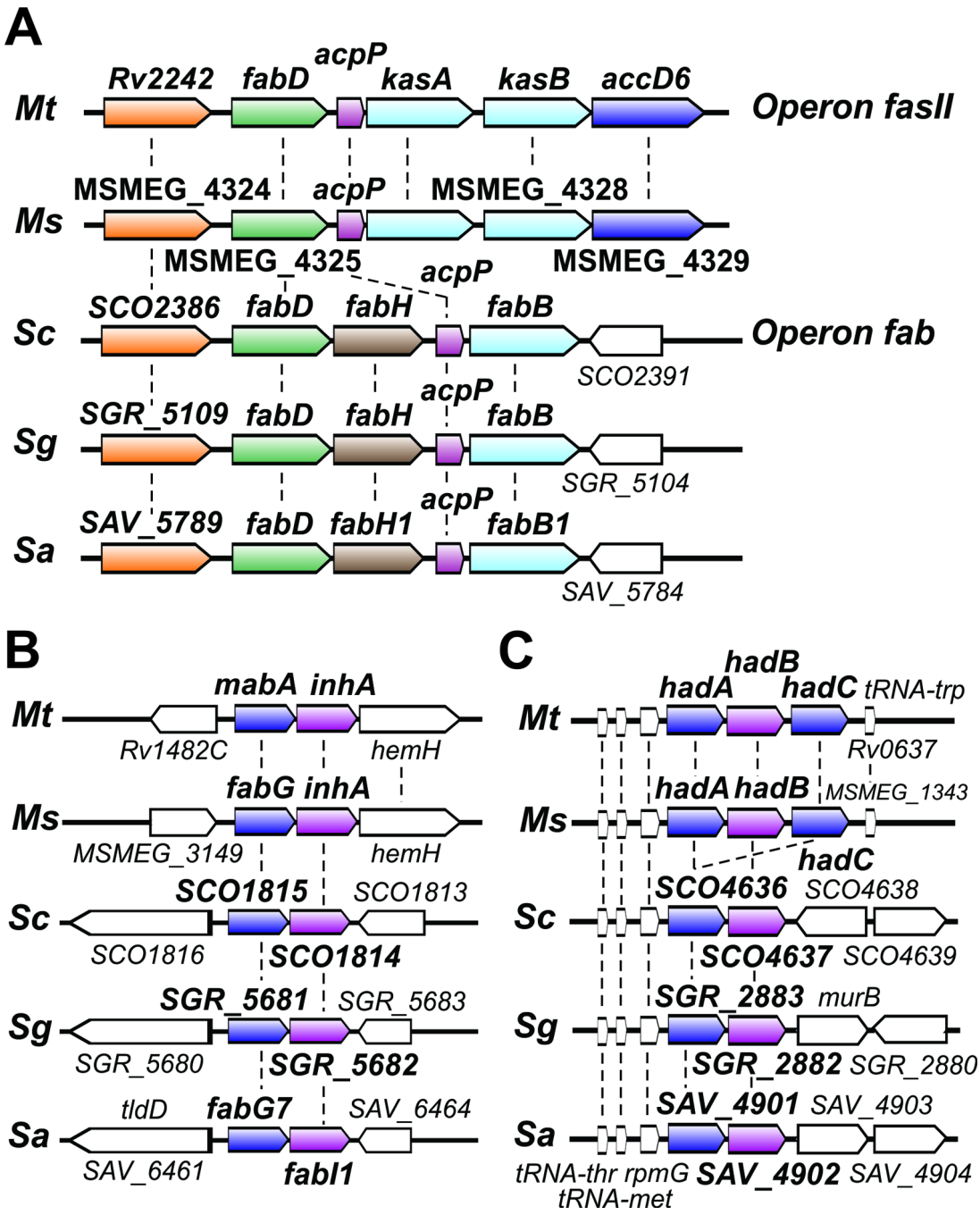

Fig. 3. Genetic organization of FAS II genes

Synteny present in the *fas* II (*fab*) operons **(A)**, the cluster of genes encoding for the FAS-II reductases **(B)** and the FAS II dehydratases **(C)** in some of the most relevant species of mycobacteria and streptomycetes: *M. tuberculosis* (Mt), *M. smegmatis* (Ms), *S. coelicolor* (Sc), *S. griseous* (Sg) and *S. avermitilis* (Sa).

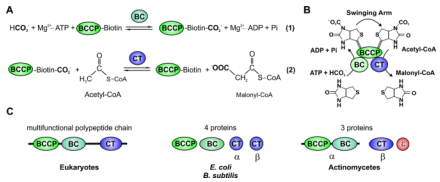

Fig. 4. Schematic diagram of ACC reaction and of domains and/or subunits of the biotin-dependent ACC

(A) Stepwise enzymatic reactions of ACC. **(B)** ACC is composed of three different components, the biotin carboxylase component (BC), the biotin carboxyl carrier protein (BCCP) and carboxyltransferase (CT). The "Swinging Arm" of biotin-BCCP transports CO_2 between the components BC and CT. Then the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA. **(C)** The eukaryotic ACC is a multifunctional polypeptide chain that contains the three components (BC, BCCP and CT). Bacterial ACC is composed of multiple subunits. The *E. coli* and *B. subtilis* ACCs are formed by four proteins: the subunit BC, the subunit BCCP, and two proteins (α and β) that form the CT subunit. In actinomycetes the ACCase complex is made up of two main subunits: the α subunit, which contains the BC and BCCP components and the β subunit, which has the CT activity. In addition, actinomycete ACCases may include a third subunit, ε, the presence of which dramatically stimulates the specific activity of the enzyme complexes.

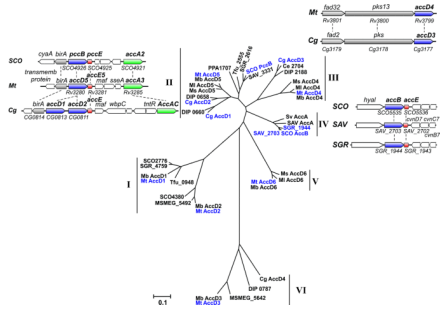

Fig. 5. Phylogenetic analysis and genetic organization of CTs β subunits of ACCase complexes found in actinomycetes

The unrooted phylogenetic tree was generated using the neighbor-joining method of the freely available Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Bootstrap values for the different families described in the text are all over 80. The tree is drawn to scale and the lengths of the branches are proportional to the inferred evolutionary distances. The bar indicates the relative number of substitutions per site. The organism key is as follows: Ce, *Corynebacterium efficiens*, Cg, *Corynebacterium glutamicum*; DIP, *Corynebacterium diphtheriae*; Mb, *Mycobacterium bovis*; Ml, *Mycobacterium leprae*; Mt, *Mycobacterium tuberculosis*; Ms, *Mycobacterium smegmatis*; PPA, *Propionibacterium acnes*; Sa, *Streptomyces antibioticus*; SAV, *Streptomyces avermitilis*; SCO, *Streptomyces coelicolor*; SGR, *Streptomyces griseus*; Sv, *Streptomyces venezuelae*; Tfu, *Thermobifida fusca*. The genetic organization and synteny are shown for CTs that have been characterized (Mt AccD4, Mt AccD5, Cg accD1, Cg AccD2, Cg AccD3, SCO PccB, and SCO AccB).

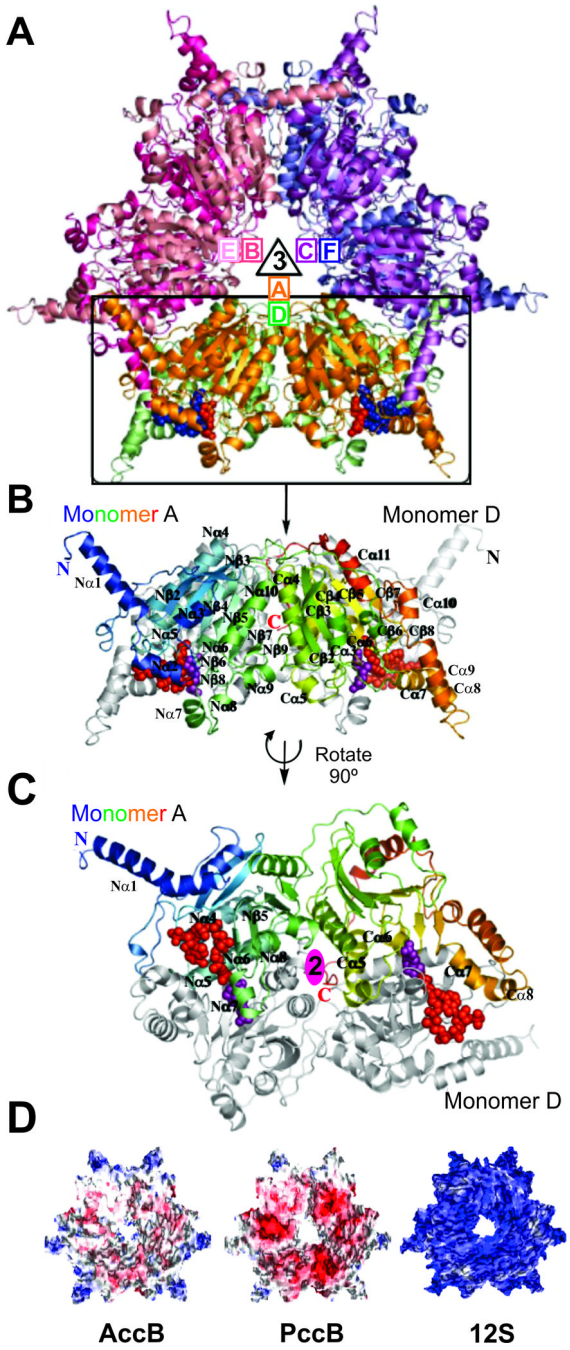

Fig. 6. Crystal structure of PccB from *S. coelicolor*

(A) The overall hexameric structure of PccB formed by two stacks of three monomers (A-B-C and D-E-F) related by the 3-fold axis. **(B)** Monomer A of PccB, colored from the N-terminus (blue) to the C-terminus (red), with two structurally similar domains (N and C). Monomer D is shown in black and white under monomer A. **(C)** The dimeric, di-domain interaction between monomer A and monomer D (after a 90° rotation) of PccB, related by the 2-fold axis. (D) Electronegative surfaces of AccB, PccB, and 12S (Diacovich *et al.*, 2004; Hall *et al.*, 2003) reveal key differences between these very similar CTs. Surfaces are colored according to their charges from red (−) to white to blue (+).

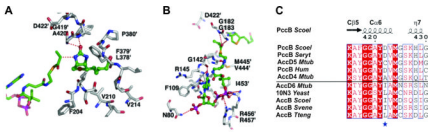

Fig. 7. PccB active site

(A) The binding pockets of acyl-CoA and biotin in PccB are perpendicular to each other and meet at the junction of the L-shaped active site. **(B)** The binding pocket of propionyl-CoA reveals that residue 422, at the end of the pocket, is the only residue different in AccB and PccB. **(C)** Sequence comparison of different CTs that accept propionyl-CoA as a substrate, like PccB, where position 422 (in PccB) is occupied by small residues (blue star), such as Asp or Cys, and CTs that accept acetyl-CoA as a substrate, such as AccB, where this residue is a larger, hydrophobic residue. *Scoel* (*S. coelicolor*), *Seryt* (*S. erythraea*), *Mtub* (*M. tuberculosis*), *Hum* (Human), *Yeast*, *Svene* (*S. venezuelae*), *Tteng* (*T. tengcongensis*).

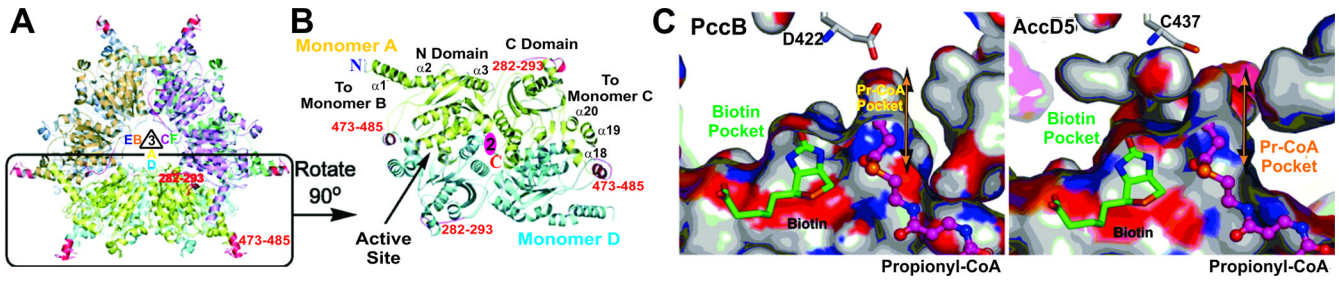

Fig. 8. Crystal structure of AccD5 from *M. tuberculosis*

(A) The overall hexameric fold of AccD5 is similar to that of PccB. **(B)** The active site lies at the dimeric, di-domain interface. **(C)** A comparison of the active sites between PccB and AccD5 shows that AccD5 cannot accommodate a substrate larger than propionyl-CoA (Lin *et al*., 2006)

Table 1

Composition of acyl-CoA carboxylase complexes in actinomycetes.

Complex/ Organism	ACCase (ACC)	ACCase (PCC)	ACCase (long substrate)	ACCase?	ACCase?	ACCase?
Streptomyces						
	α: *accA2* (*accA1*)	α: *accA2* (*accA1*)		α: *pccA*	α: *SCO4381*	
	β: *accB*	β: *pccB*	-	β: *SCO2776*	β: *SCO4380*	-
	ε: *accE*	ε: *pccE*				
Mycobacterium						
	α: *accA3*	α: *accA3*	α: *accA3*	α: *accA1*	α: *accA2*	
	β: *accD6*	β: *accD5*	β: *accD4*	β: *accD1*	β: *accD2*	β: *accD3*
		ε: *accE5*	β: *accD5*			
Corynebacterium						
	α: *accBC*	α: *accBC*	α: *accBC*			
	β: *accD1*	β: *accD2*	β: *accD3*			
		ε: *accE*	β: *accD2*	-	-	β: *accD4*
			ε: *accE*			

ACCase: acyl-CoA carboxylase, ACC: acetyl-CoA carboxylase, PCC: propionyl-CoA carboxylase, α: subunit containing BC and BCCP domains, β: subunit containing CT domain, ε: small subunit necessary for maximal ACCase activity.

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