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## Functional reconstitution of the *Mycobacterium tuberculosis* long-chain acyl-CoA carboxylase from multiple acyl-CoA subunits

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### Abstract

*Mycobacterium tuberculosis* produces a large number of structurally diverse lipids that have been implicated in the pathogenicity, persistence and antibiotic resistance of this organism. Most building blocks involved in the biosynthesis of all these lipids are generated by acyl-CoA carboxylases (ACCase) whose subunit composition and physiological roles have not yet been clearly established. A rather controversial data in the literature refers to the exact protein composition and substrate specificity of the enzyme complex that produces the long-chain  $\alpha$ -carboxy-acyl-CoAs; one of the substrates involved in the last step of condensation mediated by the polyketide synthase Pks13 to synthesize mature mycolic acids. Here we have successfully reconstituted the so called long-chain acyl-CoA carboxylase complex (LCC) from its purified components: the  $\alpha$ -subunit AccA3, the  $\epsilon$ -subunit AccE5 and the two  $\beta$ -subunits AccD4 and AccD5, and demonstrated that the four subunits are essential for its LCC activity. Furthermore, we also showed by substrate competition experiments and the use of a specific inhibitor of the AccD5 subunit, that its role in the carboxylation of the long acyl-CoAs, as part of the LCC complex, was structural rather than catalytic. Moreover, AccD5 was also able to carboxylate its natural substrates, acetyl-CoA and propionyl-CoA, in the context of the LCC enzyme complex. Thus, the supercomplex formed by these four subunits has the potential to generate the main substrates, malonyl-CoA, methylmalonyl-CoA and  $\alpha$ -carboxy-C<sub>24–26</sub>-CoA, used as condensing units for the biosynthesis of all the lipids present in this pathogen.

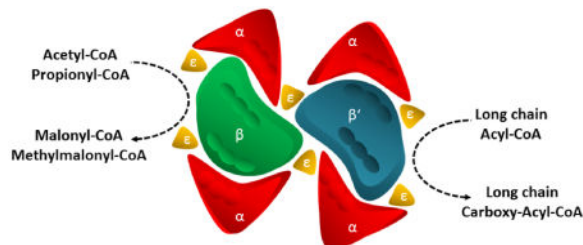
### Graphical abstract

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#### AUTHOR CONTRIBUTIONS

BBL, LD, GG, AQ, FB and HG designed and analyzed the experimental data. BBL, LD, GG performed all the enzyme related experiments. BBL, FB, LS and AL performed the meromycolic acids analysis. BBL, LD, GG and HG wrote the paper. All the authors read and approved the final manuscript.

A new long-chain acyl-CoA carboxylase complex (LCC) formed by a biotinylated  $\alpha$  subunit (AccA3), two different carboxyltransferase subunits  $\beta$  (AccD4) and  $\beta'$  (AccD5) and an  $\epsilon$  subunit (AccE5), was characterized and showed to be able to carboxylate short- as well as long-chain acyl-CoAs. This supercomplex could provide the substrates needed for fatty acids and mycolic acid biosynthesis, as well as for methyl-branched lipid biosynthesis of *M. tuberculosis*.



## Keywords

Acyl-CoA carboxylase; Mycolic acids; *M. tuberculosis*; lipid biosynthesis

## Introduction

The hallmark of *Mycobacterium tuberculosis* is its unusual lipid-rich cell envelope that contains several components essential for its viability and pathogenicity. This highly impermeable barrier imparts resistance against both hostile environments and therapeutic agents, and it plays an active role in modulating the host immune response [1,2]. Thus, the unique structure of this cell envelope and the importance of its integrity for the viability of the organism, imply that enzymes involved in its biosynthesis could be good targets for the development of novel therapeutic agents. For instance, the frontline antitubercular drug isoniazid (INH) has as a target InhA, the 2-trans-enoyl-acyl carrier protein reductase of the type II fatty acid synthase (FAS-II) involved in the biosynthesis of the major lipid components of the mycobacteria outer membrane, the mycolic acids [3]. Following this rationale, the acyl-CoA carboxylases (ACCases), enzymes that provide the building blocks for fatty acids, mycolic acids and complex lipid biosynthesis in mycobacteria, could also be considered as potential targets for the development of new antituberculosis drugs [4,5].

ACCases are ubiquitous multifunctional enzymes that catalyze the biotin-dependent carboxylation of coenzyme A esters, in which the site for carboxylation is either the  $\alpha$ -carbon position of saturated acid esters, such as acetyl-CoA, propionyl-CoA or long-chain acyl-CoAs, or the  $\gamma$ -carbon position of  $\alpha$ - $\beta$  unsaturated acid esters [6]. The carboxylation reaction occurs in two catalytic steps [7]: first, the biotin carboxylase (BC) couples carbonate to a biotin residue attached to a biotin carboxyl carrier protein (BCCP) to form carboxybiotin; second, the carboxyltransferase (CT) transfers the carboxyl group from biotin to the acyl-CoA and generates the corresponding carboxylated acyl-CoA.

In actinomycetes, the ACCases are generally organized into oligomeric assemblies composed of two different subunits: an  $\alpha$ -subunit that contains the BC and the BCCP domains, and a  $\beta$ -subunit that contains the CT domain [8–12]. A third subunit, called  $\epsilon$ , a

unique feature of actinomycetes, has been found to be essential for holo complex activity of only some of the ACCase complexes characterized so far [13,14]. In *M. tuberculosis*, three  $\alpha$  subunits (*accA1-3*), six  $\beta$  subunits (*accD1-6*) and one  $\epsilon$  subunit (*accE5*) encoding genes have been identified [14]. However, despite this high number of putative ACCase subunits present in this microorganism, to date, only one  $\gamma$ -acyl-CoA carboxylase, formed by the AccA1 and AccD1 subunits [15], and two  $\alpha$ -acyl-CoA carboxylase enzyme complexes have been thoroughly characterized at the biochemical and physiological levels. The two  $\alpha$ -ACCases are ACCase 6, that was successfully reconstituted *in vitro* from the biotinylated  $\alpha$  subunit AccA3 and the  $\beta$  subunit AccD6 [16–18], and ACCase 5, reconstituted from the  $\alpha$  subunit AccA3, the  $\beta$  subunit AccD5 and the  $\epsilon$  subunit AccE5 [14,19]. These two enzyme complexes are able to carboxylate both acetyl-CoA and propionyl-CoA; however, enzyme kinetic studies demonstrated that ACCase 6 has a substrate preference towards acetyl-CoA, while ACCase 5 preferentially carboxylates propionyl-CoA [14,16]. These results, together with the physiological analysis of conditional mutants in the essentials *accD6* and *accD5* genes, have determined that *in vivo* ACCase 6 functions as an acetyl-CoA carboxylase (ACC) that generates malonyl-CoA, the main building block for straight-chain fatty acids biosynthesis, including mycolic acids, while ACCase 5 would be, most probably, the propionyl-CoA carboxylase (PCC) that synthesizes methylmalonyl-CoA for the biosynthesis of methyl-branched lipids of the mycobacterial envelope [14,19].

Until now there were no concluding biochemical or physiological evidences regarding the precise protein composition and/or the specific role of each of the subunits proposed to be part of the long-chain acyl-CoA carboxylase (LCC), the enzyme complex involved in the  $\alpha$ -carboxylation of the very long-chain acyl-CoAs (C<sub>24</sub>-C<sub>26</sub>) prior to their condensation with a C<sub>50</sub>-C<sub>60</sub> meromycolic acid by Pks13, to produce mycolic acids. Genetic and biochemical studies carried out with an enzyme complex purified from *Corynebacterium glutamicum* suggested that the LCC enzyme could have a fairly complex subunit composition in this and in other actinomycetes. On this regard, an enzyme complex containing two CT subunits, AccD3 (an ortholog to AccD4 of *M. tuberculosis* and whose gene is genetically linked to *pks13*) and AccD2 (an ortholog to AccD5 of *M. tuberculosis*), the  $\alpha$  subunit AccBC (an ortholog to AccA3 of *M. tuberculosis*) and the  $\epsilon$  subunit AccE was purified from *C. glutamicum*. This complex, containing a rather unique protein composition, showed carboxylase activity against C<sub>16</sub>-CoA, to produce the  $\alpha$ -carboxylated-C<sub>16</sub>-CoA for the biosynthesis of corynomycolic acids [20,21]. However, these studies did not explore the essentiality or the specific role of each of the protein subunits in the enzyme complex activity. On the other hand, the limited studies performed on the mycobacterial LCC bring more uncertainty into this issue. For example, co-immunoprecipitation experiments carried out in cell-free extracts of *M. smegmatis* showed that using an antibody against a tagged AccD4 resulted in coprecipitation of AccD4 with the AccA3 and AccD5 subunits [22]. A second study, carried out by using a single-step avidin chromatography, led to the purification of a complex containing the biotinylated subunit AccA3, the  $\beta$  subunits AccD4 and AccD5 and the  $\epsilon$  subunit AccE5 from *M. smegmatis*. However, no activity assays were performed with the purified complexes [23]. Interestingly, the same work also reported that a LCC activity was reconstituted *in vitro* in the presence of both AccA3 and AccD4 subunits, while the  $\epsilon$  subunit AccE5 partially inhibited this enzyme activity. Surprisingly, the specific

activities were about 500 times lower compared with those obtained for other reconstituted mycobacterial ACC complexes, and 100 lower than the values obtained for the *C. glutamicum* LCC [21,23]. More recent studies carried out with cell free extracts prepared from a conditional AccD5 mutant of *M. smegmatis* showed both a reduction in the bacterial LCC activity and a decrease in the carboxy-C<sub>24</sub>-CoA content when the *accD5* expression was dropped-down, suggesting that AccD5 could also play an active role in the LCC complex [19]. Taking all these data in consideration, it is clear that there are still inconclusive evidences in terms of the subunits that form part of the LCC enzyme in mycobacteria and the role that each subunit has in this enzyme complex, essential for the synthesis of mycolic acids and for the survival of these organisms.

In the present study, we report the successful *in vitro* reconstitution of the LCC of *M. tuberculosis* from its purified subunits, and determine the specific role that each of the  $\beta$  subunits, AccD4 and AccD5, plays in this enzyme complex. We also present data about the kinetic parameters for the preferred substrates of this holocomplex and provide strong evidences that the LCC enzyme of mycobacteria is a supercomplex with the ability to carboxylate different chain-length acyl-CoAs, which could potentially provide the substrates needed for fatty acid and mycolic acid biosynthesis, as well as for methyl-branched lipid biosynthesis.

## Results

### Dissection of the subunit composition of the LCC enzyme complex of *M. tuberculosis*

The existing data related with the subunit composition, the substrate specificity and the role of each of the  $\beta$  subunits present in the so called LCC enzyme complex of mycolic acid containing actinomycetes, remains poorly characterized in mycobacteria. The main difference between the *C. glutamicum* and the *M. tuberculosis* LCC complexes is the minimal set of subunits that each of them appears to require in order to carboxylate their corresponding long-chain acyl-CoA substrate [20,22,23]. Therefore, in the first part of this work we aimed to determine the minimal set of subunits that compose the LCC enzyme complex of *M. tuberculosis*.

With these precedents and with the aim of studying the biochemistry of the enzyme complex responsible of synthesizing the  $\alpha$ -carboxy-C<sub>(24-26)</sub>-CoA in *M. tuberculosis*, the precursors of the  $\alpha$  alkyl-chain of mycolic acids, we developed an LC/MS method to efficiently and reproducibly assay LCC activity *in vitro*. For this, the four subunits proposed to be part of the LCC enzyme complex of *M. tuberculosis* were independently expressed in *E. coli* as N-terminal His-tagged proteins and purified by Ni<sup>2+</sup> affinity chromatography (Figure 1A). The oligomeric state of the purified AccD4 and AccD5 subunits was analyzed by size exclusion chromatography and found that both proteins are hexamers (Figure 1B) [14]. The *in vitro* reconstitution of the LCC enzyme complex was tested by mixing the four purified subunits in an arbitrary AccA3:AccD4:AccD5:AccE5 molar ratio of 1:1:1:4 (and the LCC activity assayed in the presence of 40  $\mu$ M of C<sub>20</sub>-CoA as the enzyme substrate. The enzymatic reaction was carried out at 30 °C for 4 h and the reaction mixes analyzed by LC/MS (Figure 2). The *m/z* of 1104.4, 1126.4 and 1142.3 corresponding to [M-H]<sup>-</sup>, [M+Na-H<sub>2</sub>]<sup>-</sup> and [M+K-H<sub>2</sub>]<sup>-</sup> adducts of the carboxy-C<sub>20</sub>-CoA, respectively, were indentified (Figure 2B). The

identity of the carboxy- $C_{20}$ -CoA was further supported by MS/MS analysis of the charged ion at  $m/z$  1104.40 (Figure 2C). The only combination of subunits that generated the carboxy- $C_{20}$ -CoA was the one containing a mix of AccA3, AccD4, AccD5 and AccE5 proteins, while the absence of any of these subunits provoked a total loss of the long-chain acyl-CoA carboxylase activity of the complex (Figure 2A). These results showed, for the first time, that the four subunits are essential, at least *in vitro*, for the reconstitution of an active LCC enzyme complex. However, the fact that AccD5 and AccE5 have been previously characterized as the  $\beta$  and  $\epsilon$  subunits of an essential ACCase involved in the carboxylation of propionyl-CoA and acetyl-CoA (ACCase 5) [14], now opens the question about a putative new role of the AccD5 and AccE5 proteins in the context of the LCC enzyme complex.

Once the minimal set of proteins necessary to generate LCC activity *in vitro* was determined, the optimal molar ratio of the four subunits was studied by combining various molar concentrations of the individual proteins and then carrying out the enzyme assay described above. As seen in Figure 3A, we achieved the highest carboxylase activities towards  $C_{20}$ -CoA when the AccA3:AccD4:AccD5:AccE5 molar ratio was 1:1:1:2, respectively. Increasing the molar ratio of AccE5 above 2 or of any of the other LCC subunits above 1 did not improve the specific enzyme activity. Based on these results, we used the molar ratio indicated above for the rest of the study.

### Substrate specificity of the LCC complex

Following the enzymatic characterization of the LCC complex, and in order to determine the tolerance of this enzyme for different chain-length acyl-CoAs, we carried out enzyme assays in the presence of  $C_8$ -,  $C_{16}$ -,  $C_{18}$ -,  $C_{20}$ - and  $C_{24}$ -CoA, each of them at 40  $\mu$ M. Carboxylase activity was detected with all the long-chain substrates assayed ( $C_{16}$  to  $C_{24}$ ), although at very different levels, revealing a high degree of substrate tolerance for this enzyme complex (Figure 3B). Unexpectedly, the highest specific activity was obtained with  $C_{20}$ -CoA, and not with the supposedly preferred substrates of this enzyme, the  $C_{24}$ -CoA and  $C_{26}$ -CoA, whose  $\alpha$ -carboxy derivatives become the major  $\alpha$ -alkyl chains of the mycolic acids present in *M. tuberculosis*, after their condensation with the meromycolyl-AMP by Pks13. This result could be explained by the detergent properties of such long-chain acyl-CoAs at the concentration tested (40  $\mu$ M), that could inhibit the enzyme activity. However, when  $C_{20}$ -CoA and  $C_{24}$ -CoA were added to the same reaction mix at 40  $\mu$ M each, carboxy- $C_{20}$ -CoA was generated at the same levels as in the absence of  $C_{24}$ -CoA, suggesting that the enzyme remains fully active in the presence of both substrates (Figure 3C). One possible explanation for this result is that acyl-CoA substrates  $>C_{20}$  may not become completely available to the enzyme in the conditions set up for the assay due to their low solubility. Furthermore, when a mix of substrates ( $C_{18}$ -CoA,  $C_{20}$ -CoA and  $C_{24}$ -CoA), each of them at a final concentration of 40  $\mu$ M, were incubated in the same reaction mix we were able to identify the corresponding carboxy-acyl-CoA for each of the substrates present in the reaction mix (Figure 4). These results not only highlight the high degree of substrate tolerance for this enzyme complex, but open the question of how the LCC can select the very long-chain acyl-CoAs ( $C_{24}$ -CoA and  $C_{26}$ -CoA) *in vivo*.



### Kinetic parameters for the preferred LCC substrates

Since the LCC complex intrinsically contains the ACCase 5 (PCC) enzyme subunits (AccA3, AccD5 and AccE5), we proceeded to determine the kinetic parameters for the substrates of the two radically different enzyme activities present in the LCC enzyme complex. Typical Michaelis-Menten profiles were obtained for C<sub>20</sub>-CoA and for acetyl- and propionyl-CoA with the reconstituted LCC. The LCC enzyme converted C<sub>20</sub>-CoA to the corresponding  $\alpha$ -carboxy- derivative with a  $K_m$  32  $\mu$ M and a  $V_{max}$  of 2.44 nmol mg<sup>-1</sup> min<sup>-1</sup> (Table 1). The kinetic values obtained for the two short-chain acyl-CoAs were very similar to those obtained for the PCC complex itself (Table 1) [14]. The  $K_{cat}/K_m$  values determine that the catalytic efficiency of the complex for the C<sub>20</sub>-CoA is 10 and 50 fold lower compared with that of acetyl-CoA and propionyl-CoA, respectively. These results suggest that the four subunits that form the LCC complex would carboxylate efficiently, and probably independently, short-chain acyl-CoAs to synthesize the substrates for straight- or branched-chain fatty acid biosynthesis, as well as the long-chain acyl-CoAs for the synthesis of the  $\alpha$ -alkyl branch of mycolic acids.

### Role of AccD5 in the LCC complex of *M. tuberculosis*

Considering that CTs are the protein subunits that determine the substrate specificity of the ACCases [5], and based on the biochemical and structural studies that show the high specificity of AccD5 for acetyl- and propionyl-CoA, at least in the context of the PCC complex [14,24], we asked ourselves if the role of AccD5 in the carboxylation of long-chain acyl-CoAs by the LCC enzyme was catalytic or structural. To solve this question, we conducted a series of experiments. Firstly, a classical substrate competition assay was performed, and for that, a reaction mix containing AccA3, AccD4, AccD5, and AccE5 was incubated in the presence of 40  $\mu$ M C<sub>20</sub>-CoA (the  $K_m$  for this substrate) and different concentrations of propionyl-CoA (0, 40  $\mu$ M and 1 mM). In this context, if the two main activities of the LCC complex function independently, AccD5 together with the other subunits of the ACCase 5 complex (AccA3 and AccE5), should carboxylate its natural substrate propionyl-CoA, while AccD4 should carry out the carboxylation of the C<sub>20</sub>-CoA. On the other hand, if AccD5 is also involved in the catalytic carboxylation of C<sub>20</sub>-CoA, this latter reaction should be competitively inhibited by the presence of up to 1 mM propionyl-CoA (a concentration 6.5 fold times its  $K_m$ ) in the reaction mix. Analysis of the chromatograms showed that the same levels of C<sub>20</sub>-CoA carboxylase activity were found for the three different conditions tested, as if C<sub>20</sub>-CoA was the only substrate present in the reaction mix (Figure 5A). Moreover, the 40  $\mu$ M of propionyl-CoA present in the assay were completely converted into methylmalonyl-CoA at the end of the reaction, while approximately half of the propionyl-CoA remained at the end of the enzyme reaction when it was present at 1 mM concentration in the enzyme assay (Figure 5A). This result indicates that there is no competition between these two substrates and that each  $\beta$  subunit that form part of the LCC complex catalyses the carboxylation of its own substrate; AccD4 carboxylates the C<sub>20</sub>-CoA while AccD5 carboxylates the propionyl-CoA yielding  $\alpha$ -carboxy C<sub>20</sub>-CoA and methylmalonyl-CoA, respectively.

A second experiment to determine the independent activity of the two CT subunits present in the LCC complex was carried out with an AccD5 inhibitor recently identified through a

high-throughput screening method. The compound, named D5-6, was found to interact with AccD5 and we first tested its inhibitory effect on the propionyl-CoA carboxylase activity of the ACCase 5 complex. In a typical ACCase coupled reaction, the D5-6 compound showed a 90 % inhibition of the PCC activity at a concentration of 10  $\mu$ M. The inhibitory effect of D5-6 on the PCC activity was then examined varying the inhibitor concentration (0.5 to 20  $\mu$ M) at a fixed concentration of propionyl-CoA. As shown in Figure 5B, the compound inhibited the activity of the enzyme complex in a concentration dependent manner with an  $IC_{50}$  of  $2.2 \pm 0.2$   $\mu$ M. Furthermore, when the inhibitor was added to the LCC complex at a concentration of 50  $\mu$ M it completely inhibited the inherent PCC activity of the supercomplex, while the carboxylase activity against  $C_{20}$ -CoA remained unmodified (Figure 5C).

Altogether, these results have the following implications: 1) AccD5 and AccD4 recognize completely different chain-length substrates and carboxylate either short-chain acyl-CoAs (acetyl-CoA and propionyl-CoA) or very long-chain acyl-CoAs ( $C_{16}$ -CoA), respectively; 2) AccD5 appears to have a structural role, rather than a catalytic one, in the long-chain carboxylase activity of the LCC complex of *M. tuberculosis*, and 3) the LCC complex, formed by the AccA3, AccD4, AccD5 and AccE5 subunits, constitute a “supercomplex” with multiple acyl-CoA carboxylase activities, and with the potentiality to produce malonyl-CoA, methylmalonyl-CoA and long-chain carboxy-acyl-CoAs, the main substrates for the biosynthesis of fatty acids, mycolic acids and other complex lipids that are relevant for the survival and/or pathogenicity of *M. tuberculosis*.

### Accumulation of mycolic acids intermediates at suboptimal *accD5-accE5* expression levels

Having established that AccD5 and AccE5 are components of an enzyme complex containing ACC, PCC and LCC activities, we investigated further the metabolic consequences that suboptimal levels of the AccD5 and AccE5 subunits have in the *accD5-accE5* conditional mutant strain D5 MUT, constructed and partially analyzed in our previous work [19]. For this, we studied in more detail the lipids synthesized *de novo* by the mutant strain growing in the absence or presence of anhydrotetracycline (ATc), which represses *accD5-accE5* expression. Lipid biosynthesis was followed by radiolabeling with  $^{14}C$ -acetate, after shutting down the expression of both *accD5* and *accE5* genes during increasing times of ATc treatment (13, 16 and 19 h) and then analyzing the labeled lipids by thin-layer chromatography (TLC). As shown in Figure 6, there was a clear drop in the overall mycolic acid biosynthesis and the content of the three types of mycolic acid,  $\alpha$ ,  $\alpha'$  and epoxy, decreased simultaneously. This effect was accompanied by the appearance of a previously undetected spot of lipidic nature (highlighted with an X in Figure 6A). This spot was not present in samples obtained from the isogenic strain ISO-D5 grown in the absence or presence of ATc, suggesting that the lower levels of AccD5-AccE5 in D5 MUT is what induces the accumulation of these lipid molecules [19]. Given the retention factor (Rf) of this new spot we could hypothesize that they were either long chain ketones or methyl esters of meromycolic acids. To differentiate between these two chemical entities we carried out a saponification reaction; the sensitivity of these molecules to this reaction suggested that they could be meromycolic esters (Figure 6B). Therefore, to assess whether the suboptimal levels



of AccD5-AccE5 provokes the accumulation of meromycolic acids *in vivo*, we grew the D5 MUT conditional mutant for nine to ten hours in 7H9 broth, divided it into two equal aliquots and one of them was treated with ATc for 19 h (T3). Lipids were extracted from both cultures, purified and analyzed by UPLC-QTOF MS. As shown in Figure 7, spot X corresponded most probably to a mixture of  $\alpha$  and  $\alpha'$  meromycolic acids, precursors of the  $\alpha$ - and  $\alpha'$ -mycolic acids, and an increase in their content was clearly detected in the presence of ATc. This increment was progressive through time, supporting the idea that these long-chain fatty acids were actively synthesized and became accumulated after the drop of the AccD5 and AccE5 subunits. The UPLC-QTOF MS analyses allowed the accurate determination of each chain length of the different types of meromycolic and mycolic acids present in the wild type and mutant strains. Data showed that a significant drop of  $\alpha$  and  $\alpha'$  mycolic acids occurred in the D5 MUT depleted in AccD5-AccE5, which was accompanied with an accumulation of their precursors, C<sub>30</sub>-C<sub>60</sub> meromycolic acids. This phenomenon also affected the elongation of intermediate molecules leading to an accumulation of shorter meromycolic acids (C<sub>37</sub>-C<sub>47</sub> for  $\alpha$ -meromycolic acids and C<sub>30</sub>-C<sub>35</sub> for  $\alpha'$ -meromycolic acids). Compared to the radiolabeling experiments, the variations are underestimated because of the important background linked to the molecules preexisting before shutting down *accD5-E5* expression. No epoxy-meromycolic acids were detected in either condition, suggesting that the epoxy-MAs are natural oxidation products of  $\alpha$ -mycolic acids as proposed previously [25]. A global analysis of these experiments indicates that soon after the addition of ATc, the lower levels of AccD5-AccE5 have a negative impact in the biosynthesis of both fatty acids and mycolic acids. In principle this result could be explained by a drop in the ACC activity of the ACCase 5, as has been reported in the primary characterization of this mutant strain [19]. However, the recovery on fatty acid biosynthesis at longer incubation times after the addition of ATc would mean that malonyl-CoA biosynthesis is reestablished by a different pathway, most probably from ACCase 6, and that mycolic acid synthesis is still impaired for the lower activity of the LCC complex, where the presence of AccD5 and AccE5 play an essential role according to our *in vitro* experiments. Therefore, these data show that AccD5 and AccE5 proteins play an important role in the maturation of meromycolic acids into mycolic acids.

## Discussion

In the present study the successful *in vitro* reconstitution of the LCC complex of *M. tuberculosis* from its purified subunits allowed us to dissect, for the first time, the minimal subunit composition of this multimeric enzyme, as well as to unravel the role of each of the two CT subunits, AccD4 and AccD5, that form part of this complex. Previous attempts to reconstitute an active LCC from the proposed *M. tuberculosis* subunits resulted in almost negligible enzyme activities, likely related to the incomplete set of subunits used for the reconstitution of the enzyme complexes. For these reasons, and in order to have reliable and reproducible enzyme activities, we first optimized a direct LC-ESI-MS methodology that let us identify the products of the reaction, and then carry out UV quantification of the products of the enzymatic reaction.

One of the first conclusions that emerged from our enzyme reconstitution experiments was that the four protein subunits that were either copurified or immunoprecipitated as part of a

proposed *C. glutamicum* or *M. tuberculosis* LCC complex in previous experiments [20–22], were in fact essentials to reconstitute an active long-chain acyl-CoA carboxylase with activity levels 500 fold higher compared with a previously reconstituted LCC complex [23]. These *in vitro* results carried out with purified proteins of the complex, came also to confirm a series of indirect evidences, biochemical and genetics, which suggested that two different CT subunits would be needed in order to have a fully active LCC enzyme complex. Thus, the putative presence of two different CT subunits as part of the LCC enzyme complex in mycobacteria was another interesting and somehow conflicting issue to solve. The main question about this result is why AccD5, the CT subunit of a carboxylase complex (together with AccA3 and AccE5) that only recognizes acetyl-CoA and propionyl-CoA as substrates *in vitro* [14], would also be necessary in a more complex enzyme structure that carboxylates much larger and hydrophobic acyl-CoAs, such as the C<sub>24–26</sub>-CoA of *M. tuberculosis*. Considering that the crystal structure of the stable oligo hexameric AccD5 reveals an acyl-CoA binding pocket with high similarity in size and shape to the PccB active site of the propionyl-CoA carboxylase of *S. coelicolor* (organism that do not synthesizes long-chain carboxy-acyl-CoAs), and with no space to accommodate a long-chain acyl group in its active site [24,26], it is difficult to imagine that AccD5 plays a catalytic role, together with AccD4, in the carboxylation of long-chain acyl-CoAs. On the other hand, the possibility of these two  $\beta$  subunits to form hetero-hexamers with a new active site on their interacting aminoacid residues is less probable considering the high stability of the homo-hexamers of both subunits in solution (Figure 1B). Therefore, we could speculate that AccD5, together with AccE5, play a structural role in relation to the long-chain carboxylase activity of the LCC complex, probably by stabilizing the interactions of AccD4 with AccA3 and/or determining a correct positioning of AccD4 to interact with its substrate and the carboxyl-biotin arm of AccA3.

The substrate competition experiments carried out in the presence of C<sub>20</sub>-CoA and propionyl-CoA, and the use of the specific AccD5 inhibitor D5-6 which only inhibited the PCC activity and not the LCC activity, strongly suggest that each CT subunit independently carboxylates its corresponding substrate within the LCC enzyme complex. However, a consideration to bear in mind in the enzyme activity experiments carried out with the four subunits that form the LCC complex, is the possibility that the LCC and the PCC complexes coexist in equilibrium as two independent functional entities. In this situation, the LCC would carboxylate both short- and long-chain acyl-CoAs while the PCC complex would exclusively react with short acyl-CoAs. However, a titration of the AccA3 subunit with different concentrations of AccD4 (with ratios from 1 to 4 AccD4 with respect to AccA3 and AccD5), which should result in a shift of the equilibrium towards the LCC complex, did not increase the specific activity in the presence of C<sub>20</sub>-CoA (Figure 3A). This result implies that 1:1:1:2 (AccA3:AccD4:AccD5:AccE5) is the minimal subunit composition that gives optimal activity against the C<sub>20</sub>-CoA and infers that all (or most of) the subunits are forming part of the LCC complex. This is also in good agreement with other *in vivo* experiments that show that AccD4 strongly interacts with AccD5 and with AccA3 [22]. More recently, protein/protein interaction experiments, carried out with purified  $\alpha$ ,  $\beta$  and  $\epsilon$  subunits from *M. tuberculosis*, confirmed the previous interaction data centered on the AccD4 subunit, and extended the interactome to the  $\epsilon$  subunit, suggesting a mycobacterial holocomplex

analogous to the *C. glutamicum* LCC complex, and formed by the mycobacterial AccD4, AccD5, AccA3 and AccE5 subunits [15].

Considering that C<sub>24</sub> and C<sub>26</sub> are the main  $\alpha$ -alkyl branch of the mature mycolic acids of *M. tuberculosis*, we expected that C<sub>24</sub>-CoA would have been the best substrate for the LCC enzyme. However, the enzymatic assays demonstrated that C<sub>20</sub>-CoA was a much better substrate. This result is difficult to be explained, particularly because we demonstrated that a larger C<sub>24</sub>-CoA does not inhibit the enzyme activity against the C<sub>20</sub>-CoA. We could speculate that *in vitro* C<sub>24</sub>-CoA is not readily available to the enzyme as a substrate because of its very low critical micellar concentration and that it might need a carrier for its transfer and presentation to the enzyme within the cell. Recently, the mycobacterial EchA6 protein was shown to be a shuttle of long-chain acyl-CoAs, possibly for the FAS-II system [27]; one can imagine that this mechanism might also serve for other metabolic complexes that also use very long-chain fatty acyl-CoAs as substrates, such as the LCC complex. The low  $K_m$  determined for the C<sub>20</sub>-CoA, 10 and 5 fold lower than the values obtained for acetyl-CoA and propionyl-CoA, respectively, is coherent with the low concentration of the very long-chain acyl-CoAs found within mycobacteria cells [28]. Yet, the catalytic efficiency of the LCC complex is between 10 and 50 fold lower, compared with the ACC and PCC activities of the same complex, respectively; probably reflecting the lower need for very long-chain carboxy-acyl-CoAs with respect to malonyl-CoA and methylmalonyl-CoA which are required for an array of fatty acid elongation pathways in mycobacteria. Recently, a novel single-chain, multi-domain biotin-dependent long-chain acyl-CoA carboxylase has been characterized in bacteria [29]. This enzyme has been named as a LCC for its preference for long-chain acyl-CoA substrates; however, it also carboxylates short-chain and medium-chain acyl-CoAs. The holoenzyme is a homo-hexamer and the crystal structure of the long-chain acyl-CoA carboxylase holoenzyme from *Mycobacterium avium* subspecies *paratuberculosis* revealed an architecture that is strikingly different from those of related biotin-dependent carboxylases [30].

Our work provides conclusive evidences regarding the essential subunits that form a key ACCase complex of *M. tuberculosis*, the so-called long-chain acyl-CoA carboxylase. The data converge towards a model where the LCC enzyme would correspond to a supercomplex that contains all the enzymatic activities needed to synthesize the main substrates, malonyl-CoA, methylmalonyl-CoA and carboxy-C<sub>24-26</sub>-CoA, used by the different fatty acid synthases or polyketide synthases to produce the lipid pathogenicity factors which compose the protective coat of *M. tuberculosis*. Further structural analyses will be required to draw a precise picture of the topology of the unique mycobacterial LCC complex.

## Material and Methods

### Bacterial strains, culture and transformation conditions

*Escherichia coli* strain DH5 $\alpha$  [31] was used for routine subcloning and was transformed according to [32]. The transformants were selected on LB media supplemented with the appropriate antibiotics: 50  $\mu$ g kanamycin (Km) ml<sup>-1</sup> and/ or 20  $\mu$ g chloramphenicol (Cm) ml<sup>-1</sup>. *M. smegmatis* mc<sup>2</sup>155 is an electroporation proficient mutant of mc<sup>2</sup>6 [33]. Liquid cultures of the *M. smegmatis* *accD5-accE5* conditional mutant D5 MUT and the isogenic

strain ISO-D5 [19] were grown at 37 °C in Middlebrook 7H9 media supplemented with glycerol and the appropriate antibiotics at the following concentrations: Kanamycin 15 µg ml<sup>-1</sup>, Apramycin 50 µg ml<sup>-1</sup> and Streptomycin 10 µg ml<sup>-1</sup>. The same antibiotic concentrations were used in solid LB media. To repress *accD5-accE5* expression, we grew the D5 MUT conditional mutant for nine to ten hours in 7H9 broth, divided it into equal aliquots and treated each of them with 200 ng ml<sup>-1</sup> anhydrotetracycline (ATc) for 13 h (T1), 16 h (T2) and 19 h (T3).

### DNA manipulations and plasmid construction

Isolation of plasmid DNA, restriction enzyme digestion and agarose gel electrophoresis were carried out by conventional methods [32].

### Expression, Purification and Protein methods

Recombinant *M. tuberculosis* His-tagged ACCase subunits AccA3, AccD5, AccD4, and AccE5, were expressed heterologously in *E. coli* and purified as described to yield >95% pure protein [14]. Proteins were analysed by SDS-PAGE [34]. Protein contents were determined by measuring its A<sub>280nm</sub> and/or Quant-iT™ Protein Assay Kits and Qubit® fluorometer (Invitrogen).

### Size Exclusion chromatography

Molecular mass was estimated by size exclusion chromatography using an ÄKTA basic high-performance liquid chromatograph (GE). Samples containing 200 µg of AccD5 or AccD4 were loaded onto a Superdex S200 column (GE). The column was equilibrated in 50 mM potassium phosphate, pH 7.6, 50 mM NaCl, and 0.5 mM DTT and eluted with the same buffer. Absorbance at 280 nm was recorded. The column was calibrated with the following molecular mass standards: blue dextran, 2,000,000; thyroglobulin, 669,000; apoferritin, 443,000; β-amylase, 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; and lysozyme, 14,300.

### ACCase enzyme assays

**Pyruvate kinase-lactate dehydrogenase (PK-LDH) assay**—The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically [35]. The production of ADP was coupled to PK and LDH, and the oxidation of NADH was monitored at 340 nm [36]. Assays were performed in a Synergy2 microplate reader as previously described [13]. Under the assay conditions described, the reaction was linear for at least three min and the initial rate of reaction was proportional to the enzyme concentration. Initial velocities were obtained from initial slopes of the recorder traces. One unit of enzyme activity catalyses the formation of 1 mmol of the respective carboxylated CoA derivative or ADP min<sup>-1</sup> under the assay conditions described. Specific activity is expressed as units per mg of AccD4. The kinetic parameters of the LCC complex for the short-chain acyl-CoAs were obtained with the method described above but varying the acetyl-CoA or propionyl-CoA concentrations between 1 and 1000 µM. The reaction was carried out in the presence of 0.4 µM of the enzyme complex reconstituted with a molar ratio of 1:1:1:2 for the AccA3, AccD4, AccD5 and AccE5 subunits, respectively.

**LCC activity assay**—LCC activities in *in vitro* reconstituted complexes, obtained by mixing purified proteins, were measured following the detection of the carboxylated acyl-CoA product by LC-ESI-MS. Reactions were performed in 20  $\mu$ l, containing 50 mM HEPES pH 7.2, 2 mM ATP, 8 mM MgCl<sub>2</sub>, 0.01% Brij, 50 mM HCO<sub>3</sub><sup>-</sup>, 1  $\mu$ M – 1 mM long-chain acyl-CoA, and proteins AccA3, AccD4, AccD5, and/or AccE5 at different concentrations (0.1 – 1  $\mu$ M for AccA3, AccD4, AccD5 and 0.1 – 5  $\mu$ M AccE5). Most experiments were carried out at 1  $\mu$ M AccA3, AccD4 and AccD5, and 2  $\mu$ M AccE5. Reactions were started by the addition of proteins, incubated at 30°C at different times (0.5 – 24 h) and stopped by transferring the tubes to –20°C. Under the assay conditions described, the reaction was linear for at least 6 h and the initial rate of reaction was proportional to the enzyme concentration. Before quantification by LC-ESI-MS, 10  $\mu$ l C<sub>16</sub>-CoA was added as standard (40  $\mu$ M). In the reactions containing C<sub>16</sub>-CoA as substrate, C<sub>24</sub>-CoA was used as standard. Separation of the different acyl-CoAs was made by ion pairing-reverse phase high performance liquid chromatography (IP-RP-HPLC) as described in Cabruja et al [28]. UV<sub>260nm</sub> chromatogram peaks were integrated using Bruker Daltonics's Compass™ DataAnalysis (Bruker Corporation, Billerica, MA, United States) software; peak areas were copied to spreadsheets and used for the quantification of the substrates and products. Kinetic parameters of the LCC complex for a long-chain acyl-CoA were obtained by the method described above but varying the C<sub>20</sub>-CoA concentration between 1 and 500  $\mu$ M. The reaction was carried out for 4 h in the presence of 1  $\mu$ M of the enzyme complex reconstituted with a molar ratio of 1:1:1:2 for the AccA3, AccD4, AccD5 and AccE5 subunits, respectively.

When indicated, AccD5 inhibitor D5-6 was added at a final concentration of 50  $\mu$ M.

#### Determination of IC<sub>50</sub> of the AccD5 inhibitor D5-6

*In vitro* reconstituted PCC activities were assayed by coupling ADP production to pyruvate kinase and lactate dehydrogenase, as described above. AccD5 inhibitor D5-6 (initial concentration 10mM dissolved in DMSO) was incubated at different final concentrations with the protein mixture for 30 min at room temperature before starting reaction. IC<sub>50</sub> value was obtained by fitting the data to a sigmoid dose–response equation using the GraphPad Prism 6.01 software.

#### Determination of acyl-CoAs by mass spectrometry

High resolution mass spectrometry was performed using a Bruker micrOTOF-QII a Q-TOF instrument (Bruker Corporation, Billerica, MA, United States) with an electrospray ionization source (ESI; Bruker Corporation, Billerica, MA, United States). ESI parameters were optimized for acyl-CoAs detection as described in Cabruja *et al* [28].

#### Analysis of fatty acids and mycolic acids biosynthesis on *M. smegmatis*

*M. smegmatis accD5-accE5* conditional mutant D5 MUT and the isogenic strain ISO-D5 were grown as described above. Nine to ten hours after inoculation, the culture was divided into two aliquots and ATc was added to one of them. *De novo* fatty acid and mycolic acid biosynthesis were followed by labeling 5 ml of D5 MUT or ISO-D5 strains culture aliquots with 1  $\mu$ Ci/ml [1-<sup>14</sup>C]-acetate (specific activity: 55.3 mCi/mmol; Perkin Elmer) for 1 h at

37 °C at 13 h (T1), 16 h (T2) and 19 h (T3) after addition of ATc. Fatty acid and mycolic acid methyl esters were extracted from samples containing equivalent amounts of bacteria as described by [37]. 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). Samples were normalized by culture OD and developed in CH<sub>2</sub>Cl<sub>2</sub>. The radiolabeling was detected by phosphorimaging (Variable Mode Imager Typhoon TRIO, Amersham Biosciences).

### Saponification of FAMES and MAMES

Radioactive FAMES and MAMES were saponified by adding one volume of KOH 10% (w:v) in methanol:toluene 8:2 and incubated at 80°C for 1 h. Then, 400 µl of H<sub>2</sub>SO<sub>4</sub> 20% was added (final pH ~ 1). Lipids were extracted three times with diethylether, washed with water and dried. Saponified lipids were subjected to TLC as described above.

### Purification and UPLC-QTOF analysis of MAMES and meromycolic acids

The different types of mycolic acid methyl esters (MAMES) and mero-MAMES (spot X) were purified after separation by preparative HPTLC. An equivalent weight of total lipids of each sample was applied on an HPTLC on Silica gel 60 plate (Merck) with ATS4 (Camag system) and developed in dichloromethane with ADC2 (Camag system). The lipids of interest were scrapped off and eluted from silica gel with diethyl ether. Mero-MAMES were further saponified in 2-methoxymethanol/KOH 5%, at 110°C for 4 h and the resulting mero-MAs were extracted with diethyl ether, washed with water and dried. The molecules of the different samples were then separated and analyzed according to their chain length by UPLC-QTOF. Aliquots of 5 µL were injected and separated over an Acquity UPLC C18 1.7µm column (Waters) using an isocratic gradient at a flow rate of 0.1 mL/min (Acquity System, Waters). Molecules were detected using a mass spectrometer QTOF Xevo G2 (Waters), in a positive mode for MAMES and in a negative mode for mero-MAs. Each molecule was quantified by integration of the peak area.

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### Abbreviations used in this work

<b>ACC</b>	Acetyl-CoA carboxylase
<b>ACC<sub>ase</sub></b>	Acyl-CoA carboxylase
<b>ATc</b>	Anhydrotetracycline



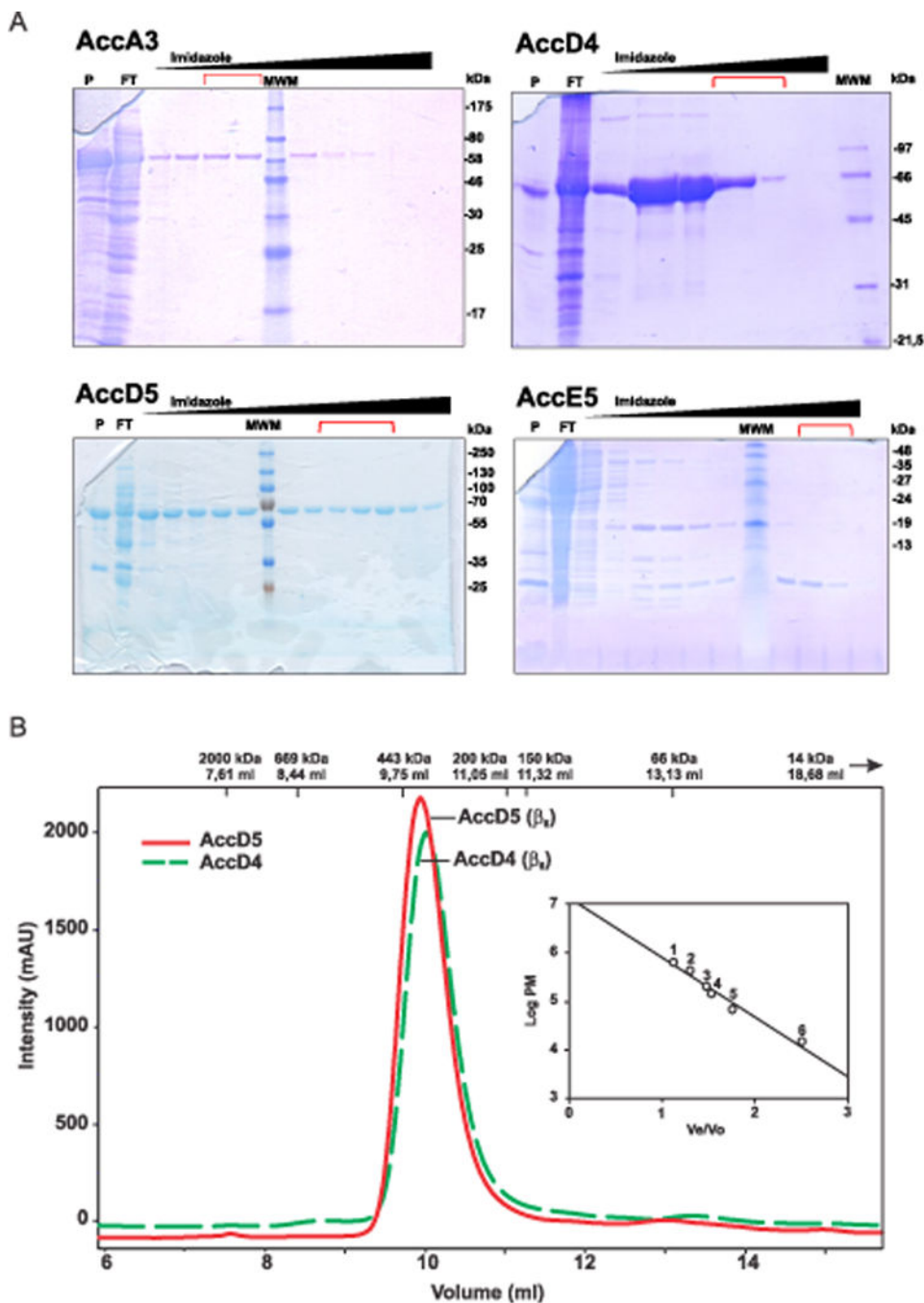
<b>BC</b>	Biotin Carboxylase
<b>BCCP</b>	Biotin Carboxyl Carrier Protein
<b>CT</b>	Carboxyl Transferase
<b>INH</b>	Isoniazid
<b>LCC</b>	Long chain acyl-CoA Carboxylase
<b>LC-MS</b>	Liquid chromatography – Mass spectrometry
<b>PCC</b>	Propionyl-CoA Carboxylase
<b>TLC</b>	Thin Layer Chromatography

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**Figure 1. Purification and analysis of the LCC subunits**

A) Purification of LCC subunits. Each His-tagged protein was purified as described in Gago *et al* [14]. Fractions highlighted in red were collected, dialyzed and used for further experiments. 12% Tris-Glycine SDS PAGE was used for AccA3, AccD4 and AccD5, and 18% Tris-Tricine SDS-PAGE for AccE5. P, pellet fraction; FT, flow through; MWM, Molecular Weight Marker. B) Determination of the oligomeric state of the carboxyltransferase  $\beta$  subunits, AccD5 and AccD4. The protein profiles ( $A_{280nm}$  in milli-

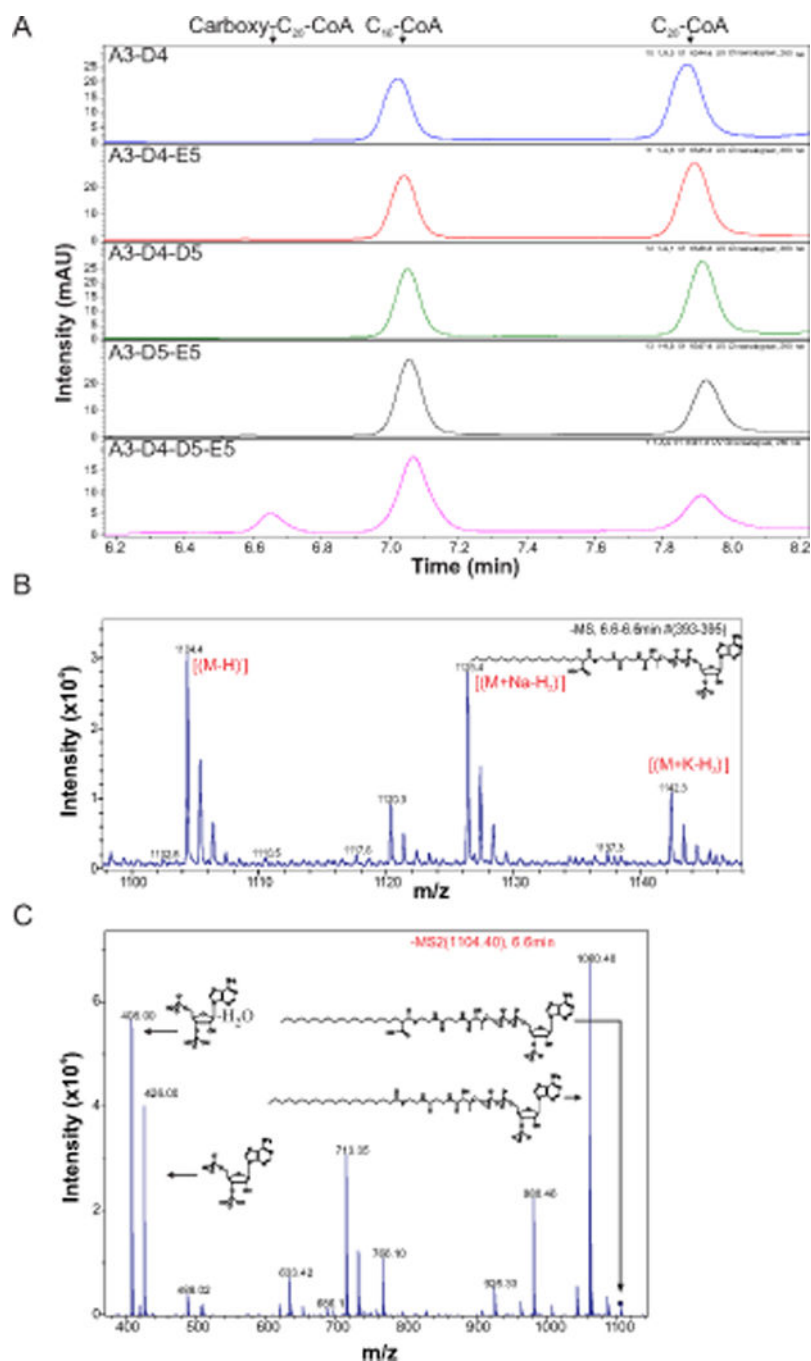
absorbance units [mAU]) and molecular masses of protein standards used are shown. The calibration curve is shown in the inset.

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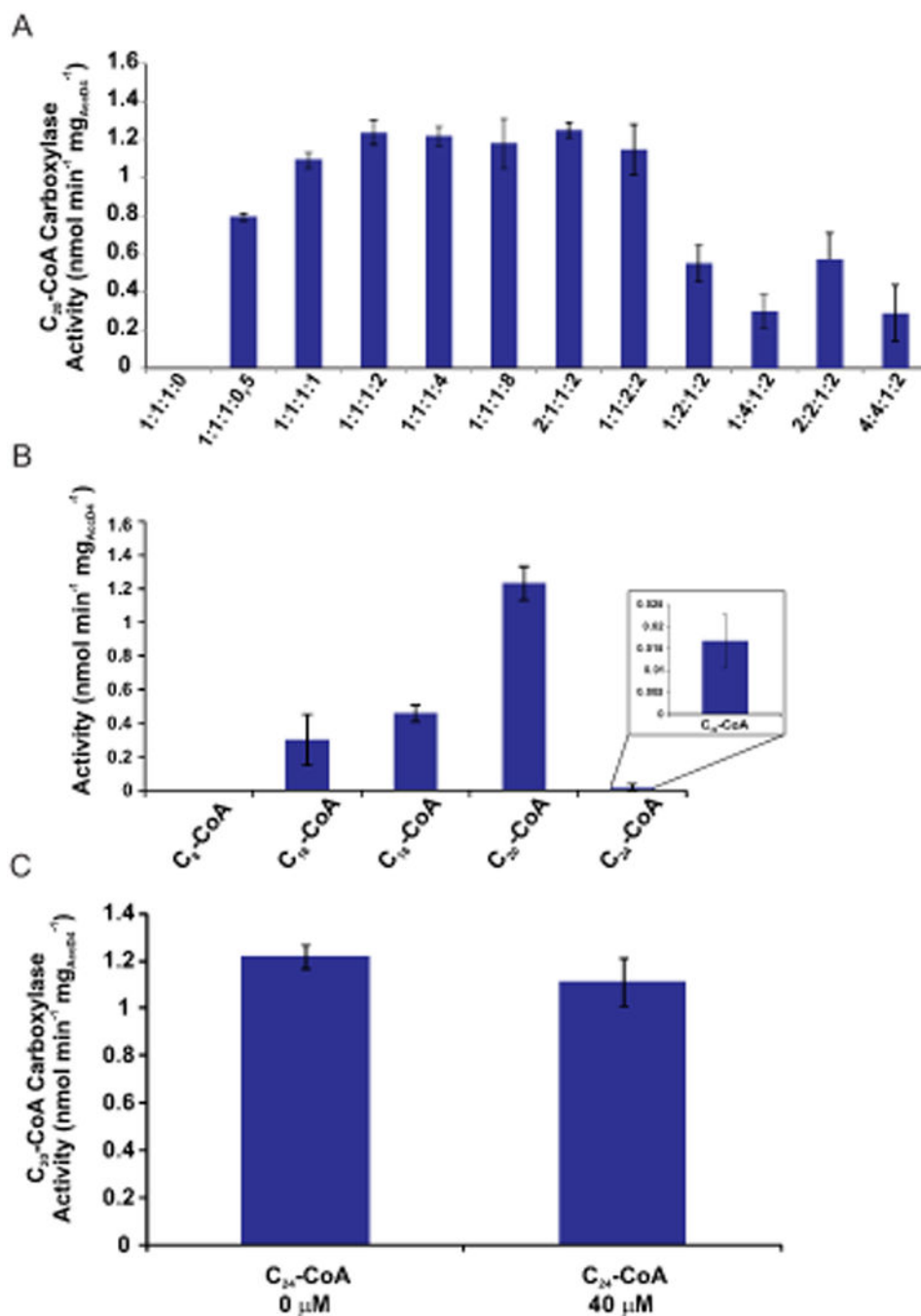
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**Figure 2. Product analysis of the reconstituted LCC enzyme complex**

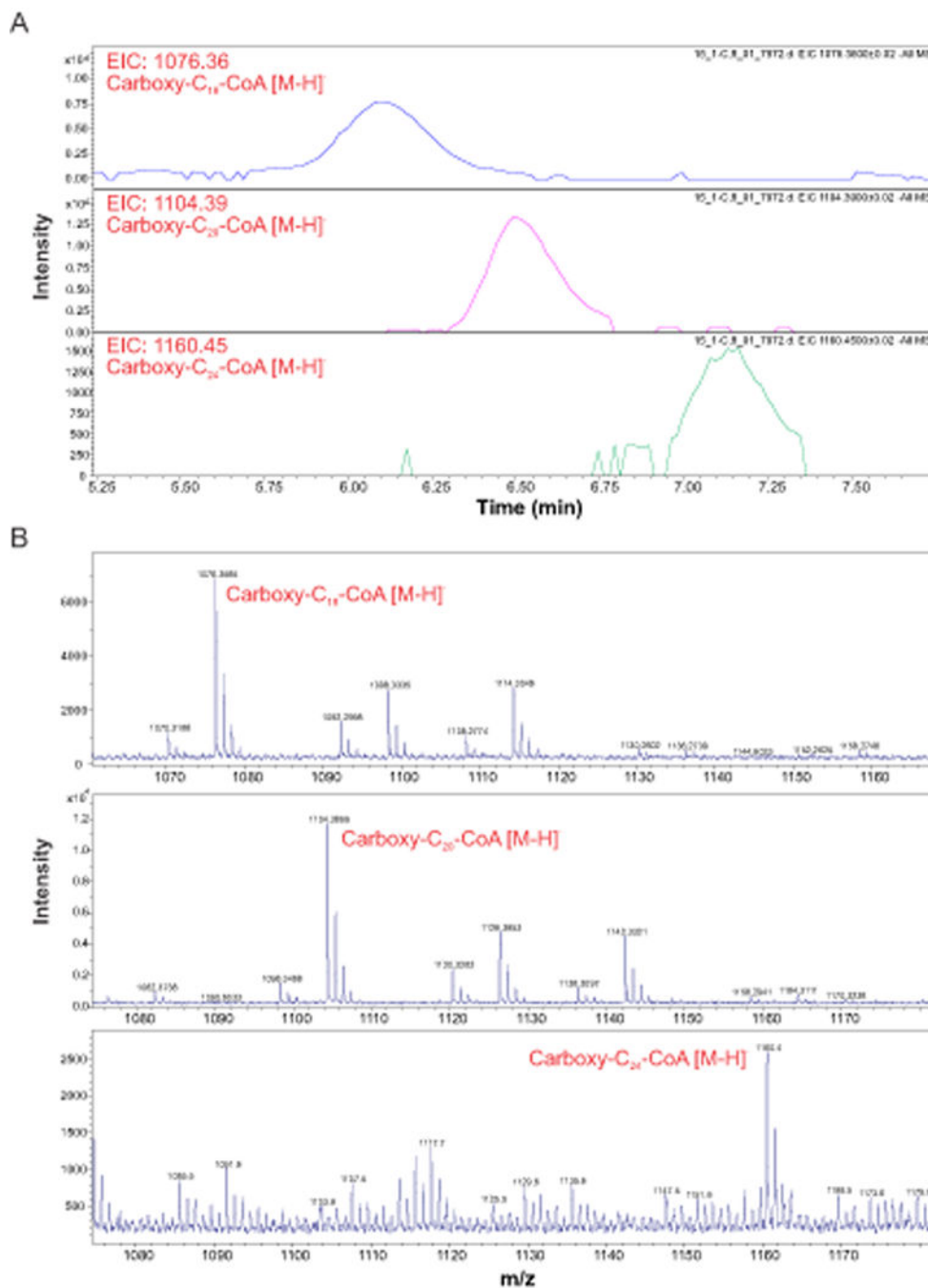
A) UV<sub>260nm</sub> chromatograms corresponding to the LCC reaction assay in the presence of different ACCase subunits.  $C_{16}$ -CoA was added as internal standard. Reaction took place at 30°C using  $C_{20}$ -CoA as enzyme substrate. B) Mass spectrum of the carboxy- $C_{20}$ -CoA.  $[M-H]^-$ ,  $[M+Na-H_2]^-$  and  $[M+K-H_2]^-$  ions are observed. C) Carboxy- $C_{20}$ -CoA fragmentation products obtained by tandem mass spectrometry analysis,  $C_{20}$ -CoA  $[M-H]^-$  (1060.4 m/z) product ion is observed.





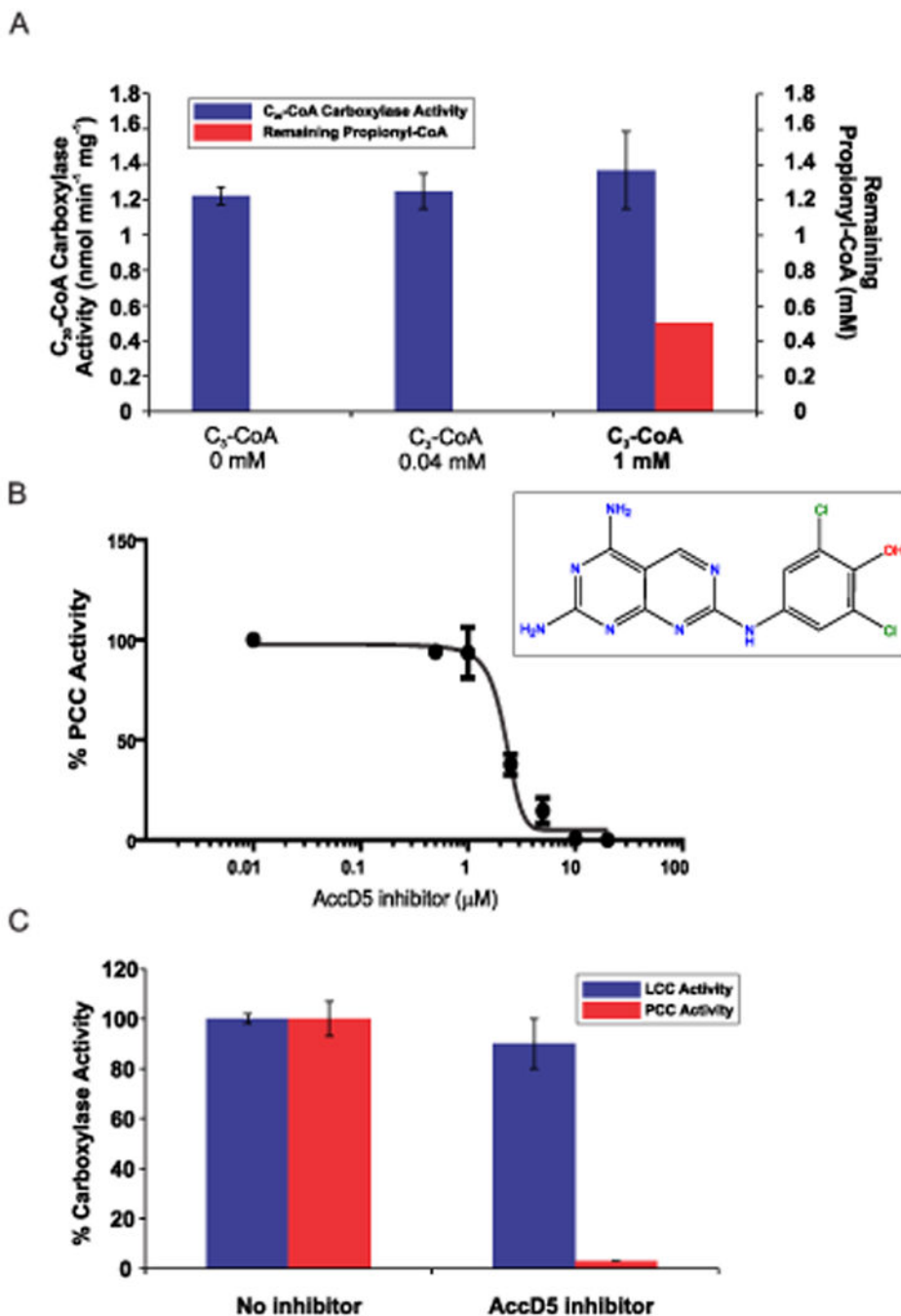
**Figure 3. Substrate tolerance of LCC enzyme complex**

A) Determination of optimal molar ratio for the reconstitution of acyl-CoA carboxylase activity. Purified subunits were mixed in the indicated ratios. The molar ratios of subunits are indicated as AccA3:AccD4:AccD5:AccE5. B) *In vitro* LCC enzyme activity in the presence of different length of long-chain acyl-CoAs as substrates (40 μM). Inset: Zoom of the LCC activity in the presence of C<sub>24</sub>-CoA. C) C<sub>20</sub>-CoA carboxylase activity in the presence or absence of C<sub>24</sub>-CoA. Results are the means of three independent experiments ± standard deviations (n = 3).



**Figure 4. Carboxy-acyl-CoAs synthesized *in vitro* by the LCC complex in the presence of a mixture of long chain acyl-CoAs**

A) Extracted Ion Chromatograms (EIC) of the respective carboxy-acyl-CoAs, corresponding to the LCC reaction assay in the presence of the LCC complex and a mix of 40  $\mu$ M of different acyl-CoAs (C<sub>18</sub>-CoA, C<sub>20</sub>-CoA and C<sub>24</sub>-CoA) as substrates. B) Mass spectra of the carboxy-C<sub>18</sub>-, carboxy-C<sub>20</sub>-, and carboxy-C<sub>24</sub>-CoA.



**Figure 5. AccD5 has a structural role into the LCC enzyme complex**

A) *In vitro* C<sub>20</sub>-CoA carboxylase activity (blue bar) of the LCC enzyme complex in the presence of propionyl-CoA (0; 0,04 and 1 mM). Remanent propionyl-CoA concentration (red bar) was estimated by the detection of the [(M-H)<sup>-</sup>] product ion at 822.13 m/z. B) IC<sub>50</sub> determination of the AccD5 inhibitor D5-6. PCC activity of the ACCase 5 complex was measured using the PK-LDH coupled enzyme assay at different concentrations of the inhibitor and a *K<sub>m</sub>* concentration of propionyl-CoA. IC<sub>50</sub> value of 2.2 ± 0.2 was determined. Results are the means of three independent experiments. Inset: chemical structure of the

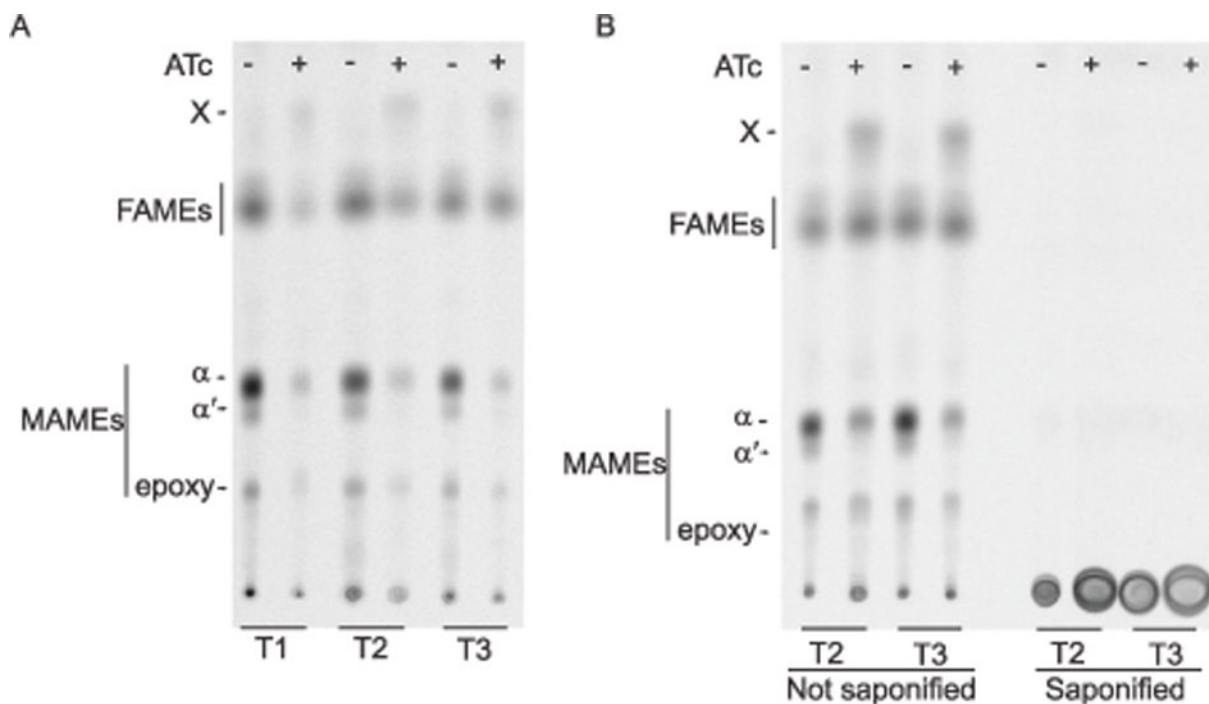
D5-6 inhibitor. C) Acyl-CoA carboxylase activity in the presence or absence of the AccD5 inhibitor D5-6 (50  $\mu$ M) using C<sub>20</sub>-CoA or propionyl-CoA as substrates. Results are the means of three independent experiments  $\pm$  standard deviations (n = 3).

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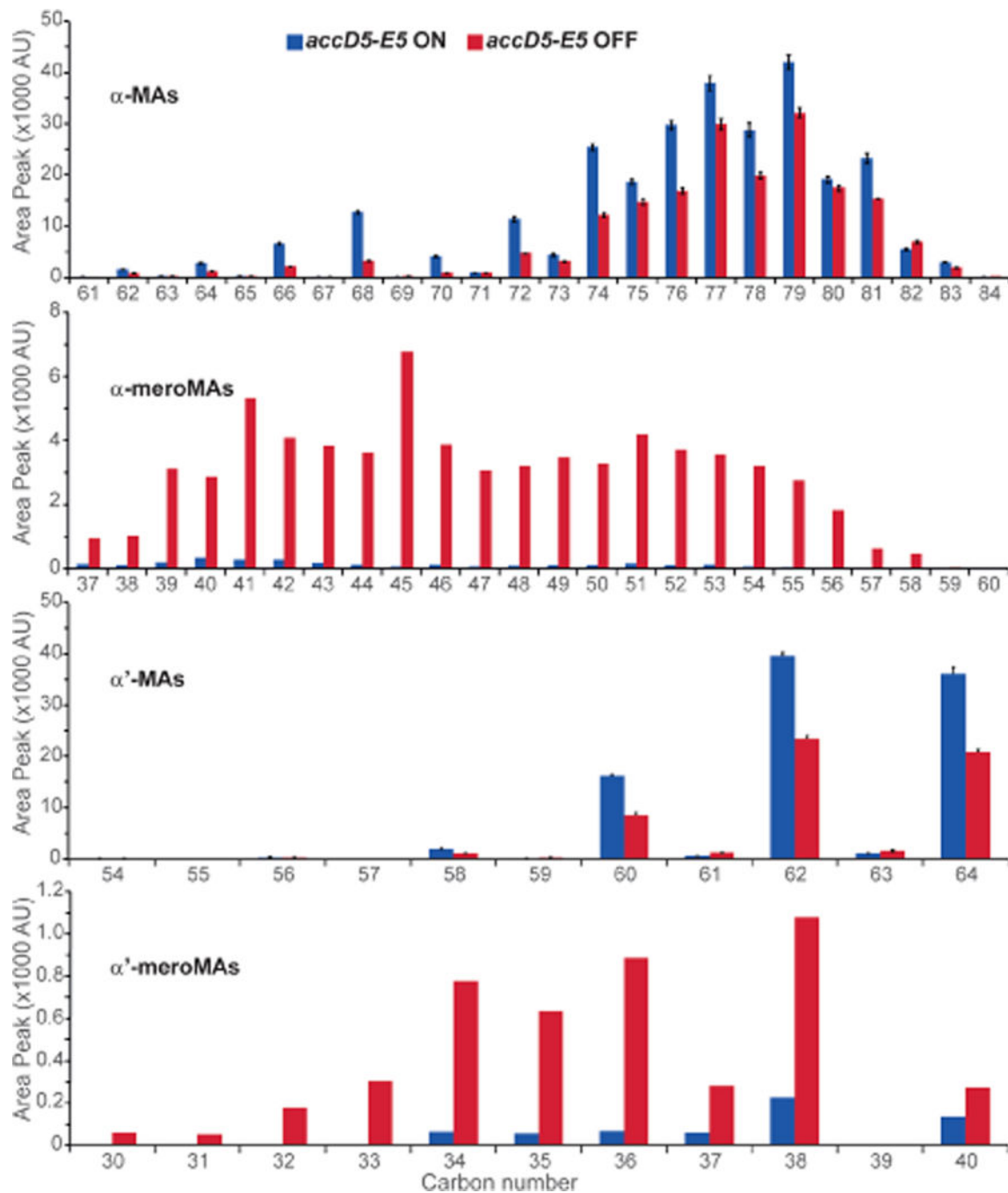
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**Figure 6. Fatty acid and mycolic acid biosynthesis in D5 MUT**

Times T1, T2 and T3 correspond to 13, 16, and 19 hours after the addition of ATc to the media (see Materials and Methods). A) At the indicated times, aliquots from D5 MUT cultures incubated in absence or presence of ATc were labelled with [ $^{14}\text{C}$ ]-acetate for 1 hour at 37°C. Fatty acids and mycolic acids methyl esters were extracted from samples containing equivalent amounts of bacteria and were analyzed by TLC developed in  $\text{CH}_2\text{Cl}_2$ . An uncharacterized spot (X) is present in samples grown in the presence of ATc. B) 40 000 cpm of FAMES and MAMES from D5 MUT were subjected to a saponification reaction giving the free acid forms of the compounds and developed in the same TLC as the non-saponified samples (methyl ester forms). Solvent:  $\text{CH}_2\text{Cl}_2$ .



**Figure 7. Quantitative analysis of mycolic and meromycolic acid production upon *AccD5-AccE5* depletion**

Mycolic acids (under methyl ester forms, i.e. MAMEs) and compound X (under free acid forms) purified from time point T3 (see Figure 6), were analyzed with respect to their chain lengths by UPLC-QTOF MS in positive and negative modes, respectively. Each mycolic acid (MA) and its corresponding meroMA are superimposed (based on the major  $\alpha$ -branch size of  $C_{24}$ ). The chain lengths indicated are those of the free MAs and mero-MAs. The same weight of total lipids was injected for samples from *accD5-E5*-expressing bacteria (*accD5-E5 ON*; without ATc) and *AccD5-E5*-depleted bacteria (*accD5-E5 OFF*; with ATc).



Note that differences between AccD5-E5-depleted samples and control samples are minimized because molecules present before stopping *accD5-E5* expression are also detected. Results are the means of three independent experiments  $\pm$  standard deviations (n = 3).

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**Table 1**

Kinetic parameters of the reconstituted LCC and ACCase 5 complexes.

	LCC			ACCcase 5		
	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (nmol/min/mg)	Catalytic Efficiency <sup>a</sup> $K_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{sec}^{-1}$ )	$K_m$ ( $\mu\text{M}$ ) <sup>b</sup>	$V_{\text{max}}$ (nmol/min/mg) <sup>b</sup>	Catalytic Efficiency $K_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{sec}^{-1}$ )
<b>C<sub>20</sub>-CoA</b>	32 ± 4	2.44 ± 0.5	63	ND	ND	ND
<b>Acetyl-CoA</b>	300 ± 29	250 ± 31	833	220 ± 55	120 ± 8	769
<b>Propionyl-CoA</b>	156 ± 22	500 ± 39	3205	240 ± 35	680 ± 43	4359

<sup>a</sup> Calculated from apparent kinetic parameters assuming the molar concentration of the AccD4 subunit.

<sup>b</sup> Values obtained from Gago *et al* [14]. ND: not determined