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ANALYSIS OF COENZYME A ACTIVATED COMPOUNDS IN ACTINOMYCETES

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Abstract

Acyl-CoAs are crucial compounds involved in essential metabolic pathways such as the Krebs cycle, lipid, carbohydrate and amino acid metabolism and they are also key signal molecules involved in the transcriptional regulation of lipid biosynthesis in many organisms. In this study we took advantage of the high selectivity of mass spectrometry and developed an ion-pairing reverse-phase high pressure liquid chromatography electrospray ionization high resolution mass spectrometry (IP-RP-HPLC/ESI-HRMS) method to carry on a comprehensive analytical determination of the wide range of fatty acyl-CoAs present in actinomycetes. The advantage of using a QTOF spectrometer resides in the excellent mass accuracy over a wide dynamic range and measurements of the true isotope pattern that can be used for molecular formula elucidation of unknown analytes. As a proof of concept we used this assay to determine the composition of the fatty acyl-CoA pools in *Mycobacterium*, *Streptomyces* and *Corynebacterium* species, revealing an extraordinary difference in fatty acyl-CoA amounts and species distribution between the three genera and between the two species of mycobacteria analyzed; including the presence of different chain-length carboxy-acyl-CoAs, key substrates of mycolic acid biosynthesis. The method was also used to analyze the impact of two fatty acid synthase inhibitors on the acyl-CoAs profile of *Mycobacterium smegmatis* which showed some unexpected low levels of C_{24} acyl-CoAs in the isoniazid treated cells. This robust, sensitive and reliable method should be broadly applicable in the studies of the wide range of bacteria metabolisms in which acyl-CoA molecules participate.

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Compliance with ethical standards

Compliance with ethical standards is not required in this study.

Conflict of interests

All the authors declare that they have no conflict of interest.

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Keywords

Mycobacterium; *Corynebacterium*; *Streptomyces*; acyl-CoA; IP-RP-HPLC-ESI-HRMS; lipid synthesis

INTRODUCTION

Coenzyme A (CoA) and CoA-thioesters are essential molecules involved in amino acid, lipid, and carbohydrate metabolisms. Apart from participating in numerous metabolic pathways as substrates and intermediates (Haynes 2011), CoA and a number of its thioester derivatives, such as acetyl-CoA, can also regulate directly the activity of proteins by allosteric mechanisms and by affecting protein acetylation reactions (Shi and Tu 2015). It has also been found that CoA-thioesters have the ability to regulate gene expression by modulating the affinity of transcription factors for their target DNA (Black et al. 2000; Mondino et al. 2013).

Our research group has an ongoing interest in studying lipid metabolism and polyketide production in different actinomycetes, with emphasis in the genera *Mycobacterium*, *Streptomyces* and *Corynebacterium.* These organisms 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, in the other extreme, as very useful sources of bioactive metabolites (Hopwood 1989; Krämer 1994). Both, fatty acid synthases and polyketide synthases use a wide range of acyl-CoA esters as substrate, or substrate precursors, for fatty acids or polyketide biosynthesis. Therefore, the development of a rapid, sensitive, and selective analytical method for detecting and quantifying CoA-thioesters in actinomycetes will have a significant impact on the biomedical and metabolic engineering research fields, by enabling improved process and strain development, rapid and accurate metabolic flux analysis, and monitoring of cellular response to changes in environment.

Early methods for measuring acyl-CoA pools include enzymatic assays, paper and thin layer chromatography and HPLC (Corkey 1988; Bieber 1992; Mandrup et al. 1993; Wadler and Cronan 2007), but all of these methods have low sensitivity. More recently, the sensitivity of the assays was improved by using gas chromatography followed by mass spectrometry (GC-MS) or fluorimetric analysis upon derivatization (Larson and Graham 2001; Shimazu et al. 2004). To exploit the selectivity of mass spectrometry on discriminating CoA-compounds, but circumventing the laborious and potentially error-prone derivatization step required for GC-MS, methods of liquid chromatography-mass spectrometry were developed, especially those using tandem mass spectrometry (LC-MS/MS) (Magnes et al. 2007; Onorato et al. 2010; Zimmermann et al. 2013). To fulfill the above mentioned requirements we developed a quantitative IP-RP-HPLC/ESI-HRMS (ion-pairing reverse-phase high pressure liquid chromatography electrospray ionization high resolution mass spectrometry) method suitable for the analysis of a wide range of fatty acyl-CoAs in actinomycetes. The strategy consisted in determine the IP-RP-HPLC/ESI-HRMS conditions to identify and quantify several commercial fatty acyl-CoAs followed by the optimization of the extraction protocol of the

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acyl-CoA pools from *Mycobacterium* cells. Finally, using the appropriate internal standards and the optimized extraction conditions we unambiguously characterized for the first time the intracellular fatty acyl-CoA pools of different actinomycetes.

MATERIALS AND METHODS

Chemicals

Methanol (HPLC grade), acetonitrile (HPLC grade) and 2-Propanol (> 99.7%, v/v) were obtained from Merck. Acyl-CoAs are from Avanti Polar Lipids. All other reagents were purchased from Sigma-Aldrich. Commercially available CoA-thioesters were dissolved in 10 mM acetic acid.

Preparation of Standard Solutions

Stock solutions of acyl-CoAs 5 mM were prepared in 10 mM acetic acid and stored at −80 °C. Internal standards were prepared by diluting stock solutions with 10 mM acetic acid. Calibration standard mixtures containing 5 acyl-CoAs of different chain length were prepared by mixing equal amounts of each compound (C_2-CoA, C_{16}-CoA, C_{17}-CoA, C_{20}-CoA and C_{26}-CoA). The exact concentration of each acyl-CoA standard solution was determined using UV-absorption at 230/260 nm (Durchschlag et al. 1981).

Bacterial experiments

Liquid cultures of *M. smegmatis* mc2 155 were grown in Middlebrook 7H9 media supplemented with 2.75 g/l glycerol and 0.03 % (w/v) tyloxapol at 37 °C and 180 rpm.

M. bovis BCG cultures were grown until OD_{600} ~1 in Middlebrook 7H9 based medium supplemented with 10 % (w/v) ADS, 2.75 g/l glycerol and 0.03% (w/v) tyloxapol at 37 °C and 180 rpm.

Corynebacterium glutamicum was cultured until OD_{600} ~6 in Luria broth (LB) supplemented with 2 % (w/v) glucose at 30 °C and 180 rpm and *Streptomyces coelicolor* M145 was cultured for 26 h in SMM at 30 °C and 180 rpm.

Cerulenin and isoniazid (INH) were added to *M. smegmatis* cultures in mid-exponential growth phase (OD_{600} ~1) at a final concentration of 7.5 μg/mL and 100 μg/mL respectively; and cells were collected after 1 h treatment. C17:0 fatty acid at a final concentration of 0.01 % (w/v) was added to *M. smegmatis* cultures at OD_{600}~1 and cells were collected after 1.5 h of incubation.

All bacterial strains used in this work are deposited in the ATCC publicly available collection: *M. smegmatis* mc2 155 (ATCC 700084), *M. bovis* BCG (ATCC 35737), *C. glutamicum* (ATCC 13032), *S. coelicolor* M145 (ATCC BAA-471).

Extraction method

Acyl-CoAs were extracted following a modified version of the protocol described by Sun *et al.* (Sun et al. 2006). All solutions and solvents used for extraction were precooled at 4 °C, except the ammonium sulfate saturated solution, which was used at room temperature.

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Bacterial cultures, corresponding to a biomass of 50 mL of OD_{600} ~1, were harvested by centrifugation at 5,800 x *g* for 15 min at 4 °C and resuspended in 500 μL of cold potassium dihydrogen buffer (100 mM; pH 4.9) freshly prepared. 500 μL of 2-propanol, 1μL of triethylamine (TEA) and 0.625 nmol of ^{13}C2:0-CoA and ^{13}C16:0-CoA, were added in each sample as internal standards. Cells were disrupted using a Bioruptor™ UCD-200 (Diagenode Inc. Sparta, NJ 07871 – USA) for 10′ (15″ on/15″ off) at 4 °C. 1 mL of acetonitrile and 60 μL of a saturated solution of ammonium sulfate were added and immediately vortexed for 2′. Finally, the samples were centrifuged at 25,500 x *g* for 20 min at 4 °C, and the supernatant collected and cold dried under gentle N_2 flow. The dry extract was resuspended in 200 μL of 4:1 (v/v) 2-propanol/1 mM acetic acid and centrifuged at 25,500 x *g* for 20 min at 4 °C. The supernatants were transferred to autosampler vials and stored at −80 °C until further analysis.

Liquid chromatography/ Mass spectrometry

Separation of the different chain-length acyl CoAs was made by ion pairing-reverse phase high performance liquid chromatography (IP-RP-HPLC) with an Agilent 1200 SL instrument (Agilent Corporation, Santa Clara, CA, United States) having a Hypersil GOLD C_{18} column (dimensions: 2.1 mm × 150 mm × 3 μm, Thermo Fisher Scientific, Waltham, MA, United States) at 30 °C. The flow rate was 0.2 ml/min in binary gradient mode with the following elution program: the column was equilibrated with mobile phase A [H_2O:CH_3CN (95:5, v/v), containing 0.05 % (v/v) TEA] 85 % and mobile phase B [H_2O:CH_3CN (10:90, v/v), containing 0.05 % (v/v) TEA] 15 %. 10 μL of each sample were injected, and the same elution conditions continued for 1 min, followed by a 9 min gradient to 100 % mobile phase B. Afterward, the column was washed for 9 min with 100 % mobile phase B, followed by a 1 min gradient to reach 85 % A and 15 % B and holding 5 more min at those conditions to equilibrate the column before injection of the next sample.

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 CoA's detection as follows: Nebulizer pressure 1.0 bar, desolvation gas (N_2) flow 4.0 L/min, dry heater 200 °C, capillary voltage 2,800 V, end plate offset −500 V. The target mass scan was set from 50 *m/z* to 3,000 *m/z*. The mass spectrometer parameters were set so the entire acyl CoA's ions were detected in the single deprotonated form $(M-H)^-$ operating in the negative mode. The collision energies for tandem mass spectrometry analysis were set at 26 eV and 40 eV for short and long chain acyl-CoA's respectively.

To determine the dynamic range of the method, the different commercial acyl-CoAs used as standards were serially diluted and analyzed as described in the results section. The linearity of the method for acyl-CoAs with acyl chains longer than 8 carbon atoms ranged between 1.8 pmol to 1.2 nmol. For short chain acyl-CoAs (2 to 4 carbon atoms) the method showed a dynamic range shifted to higher values: 3.6 pmol to 2.4 nmol (Figure S1). When an acyl-CoA was present at concentrations outside the linear range of the method, acyl-CoA concentrations were determined from diluted samples.

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Data analysis

Quantitative data analyzes was performed as follows: extracted ion chromatogram peaks for internal standards and endogenous fatty acyl-CoAs were integrated using Bruker Daltonics's Compass™ DataAnalysis (Bruker Corporation, Billerica, MA, United States) software and peak areas were copied to spreadsheets. To estimate the concentration of the endogenous analytes, a cognate internal standard was selected based upon their similarity of the acyl chain length. Calibration curves were performed by serial dilutions for different acyl CoA's standards to test correlation between chromatogram peaks areas and analyte quantities. The mass tolerance was set to +/− 0.02 *m/z*. All samples were analyzed in triplicate and results were reported as the average with standard deviation.

RESULTS

Optimization of an IP-RP-HPLC/ESI-HRMS method for the analysis of a wide range of CoA thioesters

In order to develop a reliable method for the direct determination of the broad spectrum of acyl-CoAs present in complex biological samples, we first set up the chromatographic and ionization conditions to detect a mixture of five commercially available acyl-CoA derivatives (C_2-CoA, C_{16}-CoA, C_{17}-CoA, C_{20}-CoA and C_{26}-CoA) using IP-RP-HPLC/ESI-HRMS. Figure 1-A shows an extracted ion chromatogram with the ionization pattern of the five acyl-CoAs present in the mixture using the elution system described in the experimental section. We then optimized the mass spectrometer parameters in order to get the whole spectrum of the fatty acyl-CoAs with the same settings. A characteristic mass spectrum was obtained for each component in the mixture, and the individual analysis for palmitoyl-CoA is illustrated in Figure 1-B. The spectrum shows the isotope pattern of the compound which allows the validation of the molecular formula.

To further confirm the identity of each of the acyl-CoAs present in the sample, tandem MS was performed for selected acyl-CoAs, where fragment ions *m/z*= −408 and *m/z*=−426, characteristic of CoA and CoA-thioesters were detected as shown in Figure 1-C (Haynes et al. 2008). The structure of the CoA molecule is illustrated in Figure 2, where the CoA molecule fragmentation regions and *m/z* values for the resulting ions, are indicated. This fragmentation pattern is characteristic for each CoA thioester, and allows the unambiguous identification of these compounds.

Analysis of the acyl-CoA pools in actinomycetes

In order to validate the analytical method developed we set up to analyze the complete fatty acyl-CoA profile from different actinomycetes bacteria, in particular from *Mycobacterium, Streptomyces* and *Corynebacterium* cells. To do this we developed a new extraction protocol based in a previous method developed by Sun *et al.* (Sun et al. 2006). Briefly, pellets of *M. smegmatis* cells corresponding to a biomass of 50 mL at an OD_{600} ~1 were dissolved in the extraction buffer [1:1 (v/v) potassium dihydrogen buffer: 2-propanol] supplemented with triethylamine (TEA). Complete cell disruption was achieved using a cellular disruptor (Bioruptor™). In order to normalize and quantify the acyl-CoAs of the sample, an internal reference standard was added to compensate for the variations during sample preparation

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and analysis. We used two stable isotope labeled acyl-CoAs: ^{13}C-acetyl-CoA, for the quantification of short chain acyl-CoAs (C_2 to C_4) and ^{13}C-palmitoyl-CoA for the quantification of medium to very long chain acyl-CoAs (C_8 to C_{26}). The internal standards were added at different stages during the extraction protocol to determine the stability and recovery of the acyl-CoAs throughout the whole procedure and the results showed that both standards were stable during the complete process. The recovery rates were the same for both standards ~15 % and in all the experiments performed.

Once the acyl-CoAs extraction protocol was established, we processed cells of *M. smegmatis* from exponential phase cultures and intracellular levels of short to very long chain acyl-CoAs were quantified (Table 1). The short chain acyl-CoAs detected included acetyl-CoA, propionyl-CoA and malonyl-CoA; key metabolites of lipid biosynthesis. Considering the medium, long and very long-chain CoA derivatives, the most abundant species found were palmitoyl-CoA ($C_{16:0}$), stearoyl-CoA ($C_{18:0}$), oleoyl-CoA ($C_{18:1}$) and lignoceryl-CoA ($C_{24:0}$).

We further examined if the method developed for *M. smegmatis* was suitable for the analysis of the acyl-CoA pools of other related actinomycetes: *C. glutamicum*, *S. coelicolor* and the slow growing *M. bovis* BCG. Very long chain fatty acyl-CoAs with alkyl chain lengths longer than C_{20} constitute ~ 5 % of the total fatty acyl-CoAs of *Mycobacterium* cells, while they are undetectable in *S. coelicolor* and *C. glutamicum*. This is in accordance with the bimodal behavior of *Mycobacterium* FAS I (Peterson 1977)(Banis et al. 1977). The acyl-CoA profile obtained for *S. coelicolor* and *C. glutamicum* also reflects the fatty acid composition of their membranes. Most fatty acids from *S. coelicolor* are made from branched chain starter units such as isobutyryl, isovaleryl and ante-isovaleryl to give odd- and even-numbered fatty acids with a methyl branch at the ω-terminus, the rest are synthesized from acetyl and butyryl units (Kaneda 1991; Wallace et al. 1995). Accordingly, the most abundant acyl-CoAs were myristoyl-CoA (C_{14}), pentadecanoyl-CoA (C_{15}) and palmitoyl-CoA (C_{16}). In contrast, *Corynebacterium* only employs acetyl units as starters and the eukaryotic-like FAS I enzymes present in these bacteria synthesize palmitoyl-CoA ($C_{16:0}$), stearoyl-CoA ($C_{18:0}$) and oleoyl-CoA ($C_{18:1}$), which are also the main acyl-CoA molecules found in *C. glutamicum* (Table 1).

Determination of carboxy acyl-CoAs

Our next aim was to determine if the method developed was also capable of detecting the long-chain 2-carboxy-acyl-CoA activated molecules needed for mycolic acid biosynthesis in *Mycobacterium* and *Corynebacterium*. For this, we searched in each chromatogram for the $(M\text{-}H)^-$ ion corresponding to the 2-carboxy-acyl-CoA reported for the bacteria analyzed. We found ions with *m/z* corresponding to a carboxylated C_{24}-CoA in *M. smegmatis*, a carboxylated C_{26}-CoA in *M. bovis* BCG and a carboxylated C_{16}-CoA in *C. glutamicum*, as expected (Table 1). The three ions were analyzed by tandem mass spectrometry. In all cases, the specific fragmentation of the carboxy-acyl-CoA ion gave rise to the corresponding acyl-CoA ion that result from the loss of a CO_2 molecule. Figure 3 shows the data obtained for the carboxy-C_{24}-CoA. To confirm these results we synthesized 2-carboxy-acyl-CoA from C_{20}-CoA *in vitro* using the reaction of the long chain acyl-CoA carboxylase complex of *M.*

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tuberculosis (Oh et al. 2006). The product of this reaction, the 2-carboxy-C_{20}-CoA, was analyzed by LC/MS and LC/MS/MS and the chromatographic retention time and fragmentation product where the same as those found in the biological samples, confirming our findings (Figure 4).

Effect of FAS inhibitors and fatty acid supplementation in the composition of the acyl-CoA pools in M. smegmatis

Mycobacteria have the ability to efficiently use exogenously supplied fatty acids as carbon and energy sources through the β-oxidation cycle, and this feature is particularly relevant for the pathogen *M. tuberculosis* that uses a broad range of lipids from the host (Russell et al. 2010). To gain further knowledge about the changes in acyl-CoA composition in response to different environmental stimulus, we analyzed the acyl-CoA profile of *M. smegmatis* cells grown in 7H9 media supplemented with heptadecanoic acid (C_{17}). Activation of fatty acids by thioesterification to coenzyme A is a fundamental metabolic process that can be found in all organisms from Archaea to man (Watkins 2008). Upon uptake, fatty acids are immediately converted to fatty acyl-CoA thioesters by an acyl-CoA synthetase and later used either for the biosynthesis of phospholipids and triacylglicerol or degraded through the β-oxidation cycle. The β-oxidation pathway acts in a cyclic manner, each cycle resulting in shortening of the input acyl-CoA by two carbon atoms to give acetyl-CoA. In our studies we added C_{17} fatty acid to the growth media and analyzed the effect on the intracellular acyl-CoA pool (Table 2). We observed that the levels of heptadecanoyl-CoA and pentadecanoyl-CoA (the product of the first round of β-oxidation) increased ~1.5 and 5 times, respectively, after 1 hour treatment. Moreover, CoA derivatives coming from the β-oxidation of heptadecanoyl-CoA were also detected. Table 2 shows the presence of C17-β-hydroxyl-CoA, the intermediate (C_{17}-OH) formed by the hydration of the enoyl-CoA by the enoyl-CoA hydratase, in the fatty acid treated cells but not in the non-treated cells.

Furthermore, we determined the complete acyl-CoA profile of *M. smegmatis* cells treated with different antibiotics that interfere with lipid biosynthesis. *M. smegmatis* cells were treated for one hour with cerulenin, an antibiotic that irreversibly inhibits fatty acid synthases by forming a covalent bound with the active site cysteine (Parrish et al. 1999). Analysis of these cells revealed large differences in the acyl-CoA levels compared with the control (non-treated) cells (Table 2). As expected, cerulenin treatment led to a depletion of the medium and long chain acyl-CoAs, with the concomitant increase of malonyl-CoA content. We also analyzed the acyl-CoA profile of *M. smegmatis* cells in the presence of the mycolic acid biosynthesis inhibitor isoniazid (INH), a front-line drug of choice for the treatment of tuberculosis. INH is a prodrug that requires activation once it enters the mycobacterial cell. Following activation by KatG, INH reacts with NAD(H) to form an adduct that inhibits InhA, the enoyl reductase in the mycobacterial FAS II pathway that is involved in mycolic acid biosynthesis (Rozwarski et al. 1998). The FAS II pathway is responsible for elongating fatty acyl-CoAs provided by the mycobacterial FAS I pathway, and thus impairment of mycolic acid biosynthesis by INH impact the synthesis of the long fatty acyl chain precursor required for mycolic acid biosynthesis. It has also been shown that INH does not have an effect on the FAS I system and that inactivation of InhA causes accumulation of the end products of FAS I (Vilchèze et al. 2000). As shown in Table 2, the

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levels of short and medium chain length acyl-CoAs are clearly increased after 1 hour treatment of *M. smegmatis* cells with isoniazid and unexpectedly, the levels of the very long chain acyl-CoA (C_{24}) were reduced.

DISCUSSION

In actinomycetes, lipid metabolism is not only related to the biosynthesis of cell membrane phospholipids but also to the production of polyketide compounds with useful pharmaceutical properties, to the generation of storage compounds (triacylglycerides) with promising technological applications and to the synthesis of complex lipids, crucial for the permeability of the cell wall and pathogenicity of some pathogenic species. Therefore, a detailed characterization of the key intermediate metabolites of lipid metabolism, the fatty acyl-CoAs, provides a valuable tool to start deciphering poorly understood metabolic pathways that could have a major impact on the biology, biotechnology and disease control related with this group of bacteria.

In this work we describe a comprehensive and reliable IP-RP-HPLC/ESI-HRMS method to identify and quantify a broad range of fatty acyl-CoAs derived from different actinomycetes bacteria. Using IP-RP-HPLC, the retention time of each of the acyl-CoAs is established based on the commercially available standards analyzed, while the ESI-HRMS method allows the validation of the elemental composition of each acyl-CoA through the analysis of the isotope pattern of their main ion [$(M-H)^-$]. Therefore, with the information of the retention time, the accurate mass, and the molecular formula, it is possible to determine the whole set of fatty acyl-CoAs present in a sample. The method relies on a simple sample preparation where the final CoA activated molecules remain stable for several months when stored at −80 °C. In addition, and in opposition to the methods that use MS/MS for the identification of only pre-characterized analytes only, the HRMS allows the identification of all the CoA activated compounds present in the sample based on their exact mass and isotope pattern, giving the possibility to re-analyze the raw data at any time and search for the presence of new or unexpected analytes, without the need to prepare and run the samples again as it is the case for several of the published methods.

Considering the complex cell envelope that surrounds most actinomycetes bacteria, and the variation in the polarity and solubility of fatty acyl-CoA esters with a broad range of fatty acyl chains and with different numbers of unsaturated carbon/carbon double bonds, we first had to set up a cell disruption protocol and to develop an appropriate solvent system to ensure complete extraction and avoid discrimination between the different chain lengths of the extracted molecules during the purification process.

The IP-RP-HPLC/ESI-HRMS method developed allowed the analysis, in a single analytical run, of the complete fatty acyl-CoA profile of *M. smegmatis, M. bovis* BCG, *C. glutamicum and S. coelicolor.* This is a clear improvement compared to other analytical methods (Sun et al. 2006; Zimmermann et al. 2013). The acyl-CoA profiles obtained were in complete accordance with the fatty acid composition reported for the different microorganisms analyzed in this study (Li et al. 2005; Radmacher et al. 2005), and also confirmed the bimodal activity described for *Mycobacterium* fatty acid synthase I (FAS I), which is known

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to synthesize and release long chain acyl-CoAs as well as the very long chain C_{24-26}-CoA (Peterson 1977; Zimhony et al. 2004). The results shown in Table 1 revealed large differences in fatty acyl-CoA levels and subspecies distribution between the three different genera of bacteria analyzed. Very long chain fatty acyl-CoAs were only detected in *Mycobacterium* cells. However, a key difference between the two *Mycobacterium* species analyzed was readily detected. As expected, and based on the reported properties of the *M. smegmatis* and *M. bovis* BCG FAS I enzymes (Zimhony et al. 2004), C_{26}-CoA was only found in this latter organism. In contrast, *Corynebacterium* FAS I synthesizes only long chain acyl-CoAs and accordingly, palmitoyl-CoA ($C_{16:0}$), stearoyl-CoA ($C_{18:0}$) and oleoyl-CoA ($C_{18:1}$) were the main acyl-CoA detected in this bacterium (Table 1).

Our laboratory is actively studying lipid metabolism in mycobacteria and related actinomycetes (Rodríguez et al. 2001; Gago et al. 2006; Kurth et al. 2009; Salzman et al. 2010; Mondino et al. 2013; Comba et al. 2014; Menendez-Bravo et al. 2014; Bazet Lyonnet et al. 2014). The structure and biosynthesis of the unique fatty acids found in the complex lipids that form the *M. tuberculosis* cell envelope has been the subject of intense research efforts (Daffé 2008), primarily because the enzymes involved in their metabolism offer attractive and selective targets for the development of antimycobacterial drugs or for their use in biotechnological processes (Zhang 2005; Menendez-Bravo et al. 2014). However, there are still several aspects of mycobacterial fatty acid biosynthesis that need to be addressed in order to fully understand the complex regulatory processes involved in maintaining lipid homeostasis in these microorganisms. Thus, the ability to determine the complete acyl-CoA profile (from C_2 to C_{24-26}) in mycobacteria in a single analytical run, provides a strong tool for the analysis of the effect of new antibiotics that target lipid biosynthesis or its regulation and also for the analysis of different mutant strains impaired or altered in lipid synthesis.

Mycolic acids are the major and most specific lipid components of the mycobacterial cell envelope and have been shown to be essential for the survival of *Mycobacterium* (Marrakchi et al. 2014). In the alarming context of the emergence of multidrug-resistant, extremely drug-resistant, and totally drug-resistant tuberculosis, understanding the biosynthesis of these critical molecules is an important goal to achieve, because it may open an avenue for the development of novel antimycobacterial agents. *Corynebacterium sp.* have the shortest α branch of the mycolic acid containing bacteria, 16 carbons, while *M. smegmatis* presents an α branch of 24 carbons, and the *M. tuberculosis* complex mainly have 26 carbons in the α branch (Marrakchi et al. 2014). On this regard, the method developed not only allowed the identification and quantitation of the long-chain acyl CoAs but also of their activated molecules (2-carboxy-acyl-CoAs) which are finally converted in the α branch of the different mycolic acids present in these organisms (Table 1). Finally this analytical method was also used to analyze the intracellular changes on the fatty acyl-CoAs levels expected to occur under fatty acid degradation conditions or as a result of fatty acid synthesis inhibition by the addition of the antibiotics cerulenin and INH. As mentioned above, mycobacterial FAS I has bimodal activity, releasing long chain acyl-CoAs as well as very long chain C_{24}-CoA in *M. smegmatis* (Peterson 1977; Zimhony et al. 2004). The finding of reduced levels of C_{24}-CoA in INH treated cells was unexpected. We hypothesize that inhibition of the FAS II system, causes an accumulation of the short and medium products synthesized by FAS I,

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as they cannot be used as starters by the FAS II system and that these intermediates could be modulating the activity of FAS I, thus preventing the biosynthesis of the long and toxic C_{24}-CoA.

The relevance of acyl-CoA molecules, not only as intermediate of fatty acids, complex lipids and polyketide biosynthesis, but also as signal molecules involved in transcriptional regulation, protein modification and other cell metabolisms, highlight the importance of having a simple, robust and reliable method to identify and quantitate a broad range of acyl-CoA molecules in one analytical run. The method developed in this study meets all these criteria and should become widely applicable in a diverse set of metabolic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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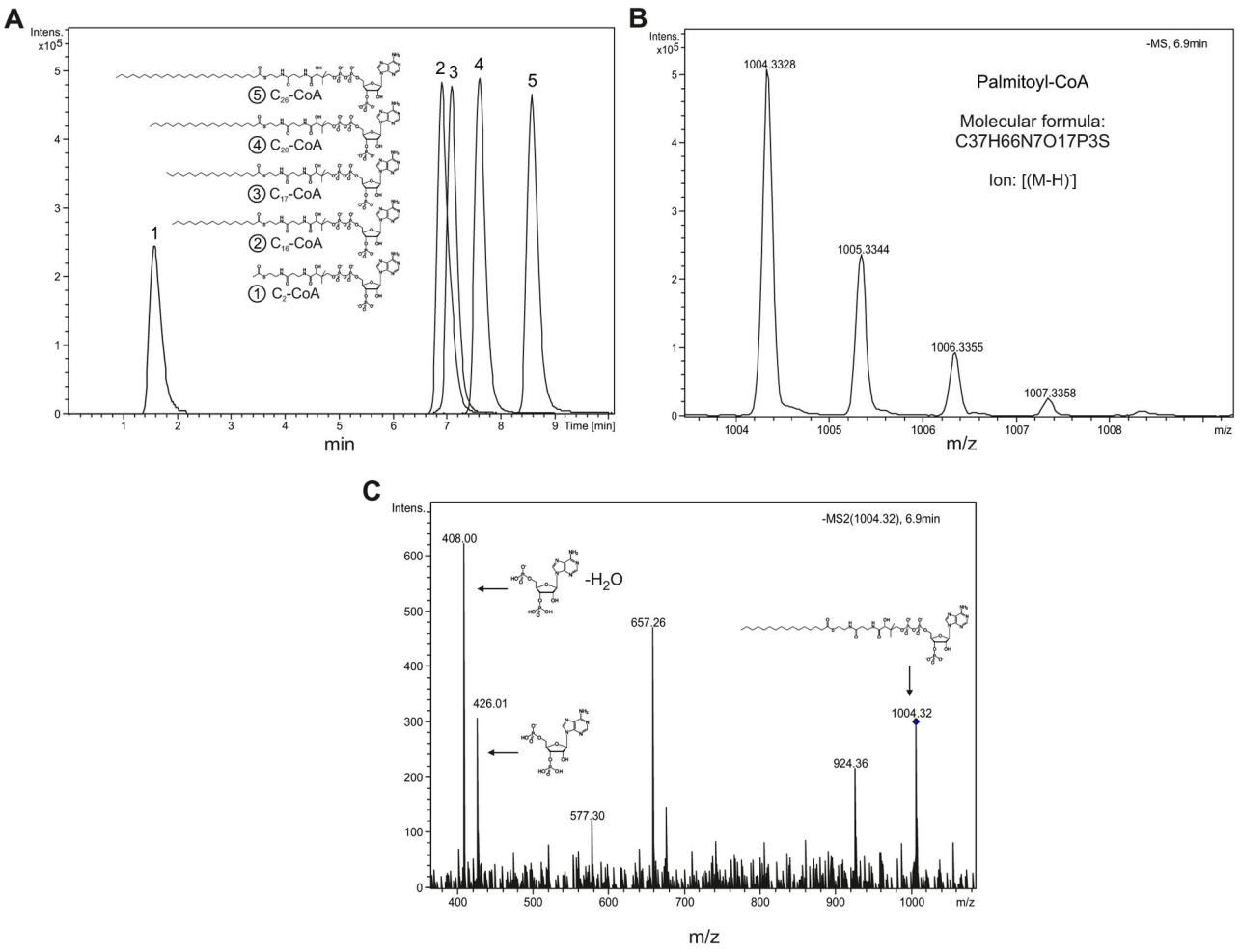

Figure 1. Analysis of a mixture of commercially available CoA thioesters

A. IP-RP-HPLC-ESI-HRMS of five acyl-CoAs standards: (1) C_2-CoA, (2) C_{16}-CoA, (3) C_{17}-CoA, (4) C_{20}-CoA and (5) C_{26}-CoA. **B.** C_{16}-CoA $[(M-H)^-]$ ion isotope pattern observed with high resolution mass spectrometry (HRMS). **C.** C_{16}-CoA fragmentation products obtained by tandem mass spectrometry (MS-MS) analysis.

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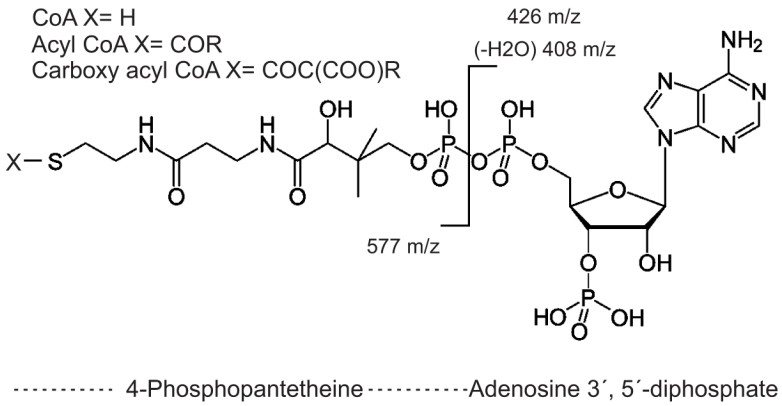

Figure 2. Structure and fragmentation pattern of coenzyme A ester derivatives

Chemical structure of coenzyme A ester derivatives and its most abundant fragment product obtained by tandem mass spectrometry using negative ionization mode.

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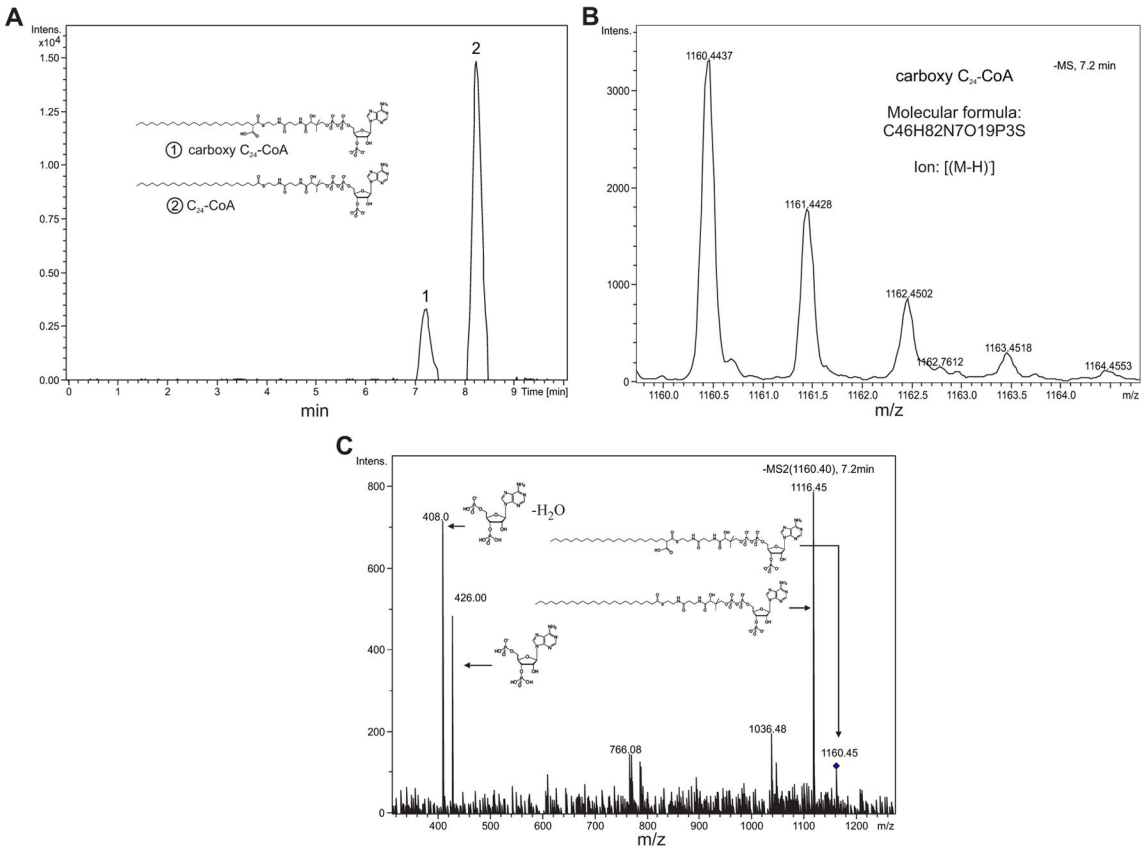

Figure 3. Analysis of carboxy-C_{24}-CoA in *M. smegmatis*

A. IP-RP-HPLC-ESI-HRMS separation of carboxy-C_{24}-CoA (1) and C_{24}-CoA (2) from *M. smegmatis*. **B.** Carboxy-C_{24}-CoA [$(M-H)^-$] ion isotope pattern observed with HRMS. **C.** Carboxy-C_{24}-CoA fragmentation products obtained by tandem mass spectrometry analysis, C_{24}-CoA (1116.45 *m/z*) product ion is observed.

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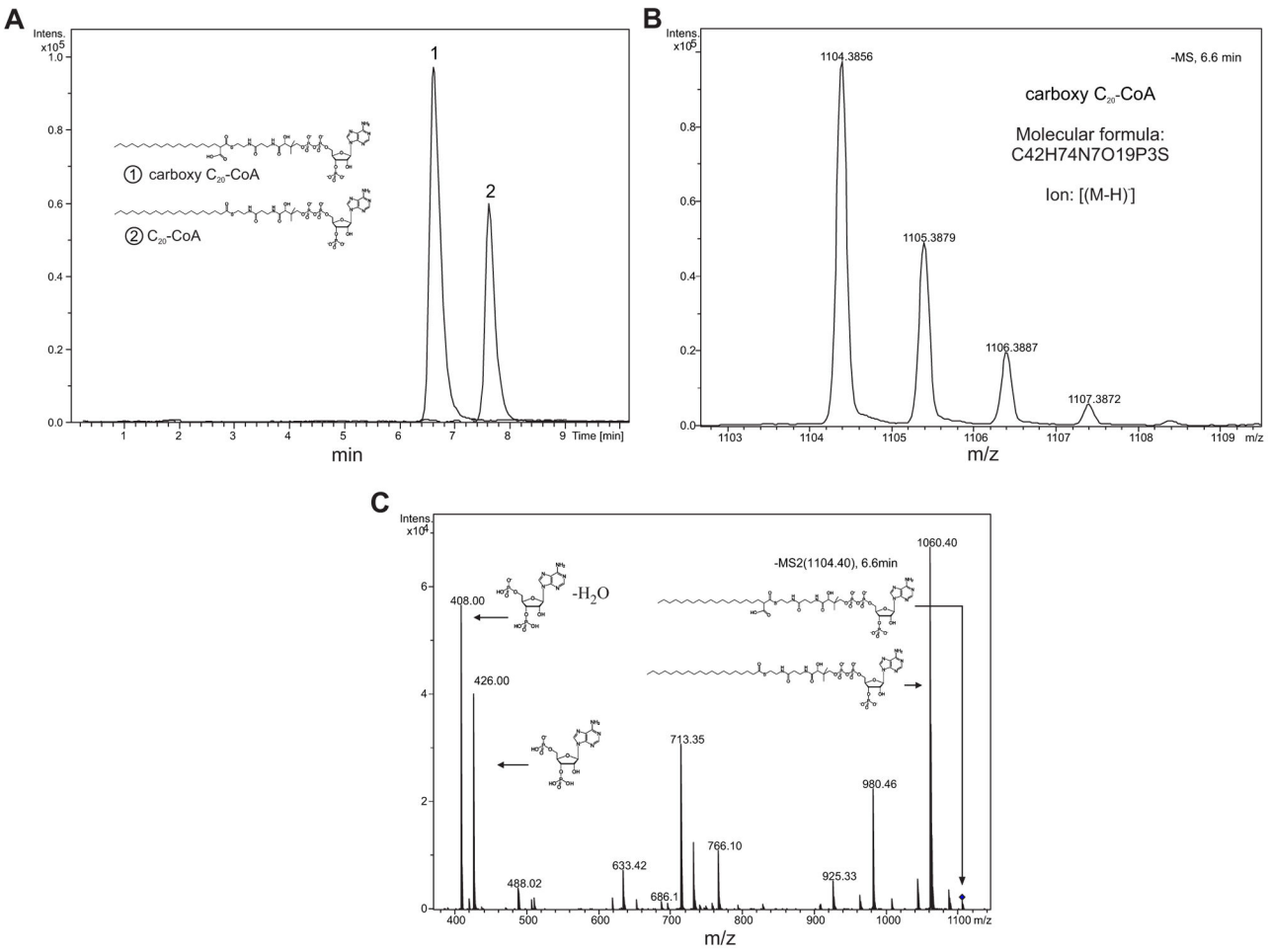

Figure 4. Analysis of carboxy-C_{20}-CoA and C_{20}-CoA standards

A. IP-RP-HPLC-ESI-HRMS separation of C_{20} derivative standards: carboxy-C_{20}-CoA (1) and C_{20}-CoA (2). **B.** Carboxy-C_{20}-CoA [$(M-H)^-$] ion isotope pattern observed with HRMS. **C.** Carboxy-C_{20}-CoA fragmentation products obtained by tandem mass spectrometry analysis, C_{20}-CoA (1060.40 *m/z*) product ion is observed.

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Table 1

Intracellular levels of acyl-CoAs in related actinomycetes

Fatty acid CoA	Acyl CoA extraction [pmol]			
	M. smegmatis	*M. bovis* BCG	*C. glutamicum*	*S. coelicolor*
C2 (Acetyl)	3,842 ± 323	1,137 ± 74	5,649 ± 800	21,418 ± 25
C3 (Propionyl)	462 ± 23	25 ± 0.2	n/d	186 ± 1
Carboxy C2 (Malonyl)	283 ± 26	27 ± 3	n/d	154 ± 31
C8:0	3.4 ± 0.6	7 ± 1	6.4 ± 0.6	n/d
C10:0	4.8 ± 1	24 ± 3	42 ± 6	n/d
C12:0	4.5 ± 0.3	67 ± 7	5 ± 0.4	9.6 ± 1
C13:0	2.75 ± 0.04	59 ± 6	n/d	13.4 ± 1
C14:0	6.8 ± 0.4	144 ± 30	11.8 ± 3.4	34.4 ± 0.6
C15:0	1.67 ± 0.02	140 ± 17	n/d	33.8 ± 0.4
C16:0	97 ± 4	330 ± 43	42 ± 1.8	23.4 ± 0.6
C16:1	34 ± 1	17 ± 1	6.6 ± 2	3 ± 0.4
C17:0	35 ± 4	92 ± 5	n/d	n/d
C18:0	224 ± 46	87 ± 19	5 ± 0.4	n/d
C18:1	88 ± 9	25 ± 0.2	28.6 ± 3.6	n/d
C20:0	66 ± 4	61 ± 2	n/d	n/d
C22:0	49 ± 0.4	34 ± 8	n/d	n/d
C24:0	95 ± 4	83 ± 9	n/d	n/d
C26:0	n/d	29.2 ± 3.6	n/d	n/d
Carboxy C16:0	n/d	n/d	10 ± 0.4	n/d
Carboxy C24:0	3.3 ± 0.5	n/d	n/d	n/d
Carboxy C26:0	n/d	2.8 ± 0.4	n/d	n/d

n/d: Not detected

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Table 2

Intracellular levels of acyl-CoAs in *M. smegmatis*

Fatty acid CoA	Acyl CoA extraction [pmol]			
	M. smegmatis	***M. smegmatis* Fatty acid C17**	***M. smegmatis* Cerulenin**	***M. smegmatis* Isoniazid**
C2 (Acetyl)	3,842 ± 323	3,025 ± 287	1,584 ± 453	8,189 ± 444
C3 (Propionyl)	462 ± 23	535 ± 4	275 ± 23	387 ± 16
Carboxy C2 (Malonyl)	283 ± 26	203 ± 36	647 ± 45	237 ± 37
C13:0	2.75 ± 0.04	3.4 ± 0.3	n/d	12 ± 1.6
C14:0	6.8 ± 0.4	9.7 ± 0.4	2 ± 0.5	38 ± 4.5
C15:0	1.67 ± 0.02	6.85 ± 0.01	n/d	7 ± 0.2
C16:0	97 ± 4	126 ± 8	7 ± 1.5	213 ± 8
C16:1	34 ± 1	31 ± 3	6 ± 1	165 ± 22
C17:0	35 ± 4	56 ± 1	5 ± 1	55 ± 4
C17-OH	n/d	3.7 ± 0.3	n/d	n/d
C18:0	224 ± 46	187 ± 23	12 ± 2	323 ± 32
C18:1	88 ± 9	74 ± 13	9 ± 1	311 ± 30
C20:0	66 ± 4	59 ± 9	2.4 ± 0.4	30 ± 5
C22:0	49 ± 0.4	38 ± 4	n/d	20 ± 6
C24:0	95 ± 4	96 ± 15	n/d	46 ± 8
Carboxy C24:0	3.3 ± 0.5	3.7 ± 0.4	n/d	2.1 ± 0.5

n/d: Not detected