A novel mycolic acid species defines two novel genera of the Actinobacteria, Hoyosella and Amycolicicoccus

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Corynebacterineae are characterized by the presence of long-chain lipids, notably mycolic acids (α-alkyl, β-hydroxy fatty acids), the structures of which are genus-specific. Mycolic acids from two environmental strains, Amycolicicoccus subflavus and Hoyosella altamirensis, were isolated and their structures were established using a combination of mass spectrometry analysis, 1H-NMR spectroscopy and chemical degradations. The C2–C3 cleavage of these C30–C36 acids led to the formation of two fragments: saturated C9–C11 acids, and saturated and unsaturated C20–C25 aldehydes. Surprisingly, the fatty acids at the origin of the two fragments making up these mycolic acids were present in only minute amounts in the fatty acid pool. Moreover, the double bond in the main C24 aldehyde fragment was located at position ω16, whereas that found in the ethylenic fatty acids of the bacteria was at ω9. These data question the biosynthesis of these new mycolic acids in terms of the nature of the precursors, chain elongation and desaturation. Nevertheless, they are consistent with the occurrence of the key genes of mycolic acid biosynthesis, including those encoding proteins of the fatty acid synthase II system, identified in the genome of A. subflavus.

Altogether, while the presence of mycolic acids and analysis of their 16S rDNA sequences would suggest that these strains belong to the Mycobacteriaceae family, the originality of their structures reinforces the recent description of the novel genera Amycolicicoccus and Hoyosella.

INTRODUCTION

The suborder Corynebacterineae forms a large group of actinomycete species characterized by the presence of specific lipids, notably mycolic acids (α-alkyl, β-hydroxy long-chain fatty acids). The variability of their chain lengths and the complexity of their structures contribute to the definition of the genera, from the simplest corynomycolic acids of Corynebacterium to the most complex and species-specific mycolic acids of Mycobacterium, with intermediate chain-lengths in Rhodococcus, Nocardia and Gordonia (Barry et al., 1998). Recently two coccolial strains have been isolated from environmental sources, Hoyosella altamirensis, from the Altamira cave in Spain, and Amycolicicoccus subflavus, from saline soil contaminated with crude oil in the Daqing Oilfield of eastern China (Jurado et al., 2009; Wang et al., 2010). By a polyphasic approach, the taxonomic position of the two species has been determined, i.e. Actinomycetales cell wall chemotype IV, menaquinones MK-8, diagnostic phosphatidylethanolamine and tuberculostearic acid. On the basis of 16S rRNA gene sequence analysis, the two species were shown to be most closely related to the genus Mycobacterium (95.6% similarity with the Mycobacterium fallax type strain for Hoyosella, and 92.7–94.9% similarity with Mycobacterium spp. for Amycolicicoccus). The two species differ from each other by their DNA G+C content, which is 49.3% for H. altamirensis, to date the lowest among all taxa included in the suborder Corynebacterineae, and 62% for A. subflavus (Cai et al., 2011). However, in these studies, no mycolic acids were found in the whole-cell fatty acids of both

Abbreviations: ESI-MS, electrospray ionization-MS; FAME, fatty acid methyl ester; GC/MS, gas chromatography/MS; MAME, mycolic acid methyl ester; TMS, trimethylsilyl.

A supplementary figure is available with the online version of this paper.

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species, indicating that they do not belong to mycolic acid-producing organisms, known as ‘mycolata’ (Jurado et al., 2009; Wang et al., 2010). According to phylogenetic tree and chemotaxonomic character analysis, the authors proposed that these species formed a distinct lineage in the suborder Corynebacterineae, and the novel genera Hoyosella and Amycolicicoccus have been defined (Jurado et al., 2009; Wang et al., 2010). In order to clarify the taxonomic status of these two strains, their lipid content was revisited, leading to the characterization of a novel class of short-chain mycolic acids displaying an asymmetrical structure.

**METHODS**

**Strains and media.** The type-strains of *H. altamirensis* DSM 45258 (Jurado et al., 2009) and *A. subflavus* DSM 45089 (Wang et al., 2010) were grown under shaking at 30°C in Trypcase-Soy broth (bioMérieux) supplemented with 2 g glycerol L⁻¹.

**Lipid extraction and purification.** Wet cell pellets were treated with mixtures of CHCl₃/CH₃OH (1 : 2; 1 : 1 then 2 : 1; v/v). The organic phases were pooled and dried under vacuum. The crude lipid extracts were analysed on TLC Silica Gel 60 plates (Macherey-Nagel) using petroleum ether/diethylether 90 : 10 (v/v) for apolar lipids, CHCl₃/CH₃OH 90 : 10 (v/v) and CHCl₃/CH₃OH/H₂O 65 : 25 : 4 (v/v) for polar lipids (phospholipids and glycolipids). Characterization of lipids was performed by using specific sprays: the Dittmer–Lester reagent (phospholipids), 0.2% anthrone in H₂SO₄ (glycolipids), 0.2% ninhydrine in acetone (lipooamino compounds).

**Mycolic acid isolation.** Whole cells or bacterial residues obtained after lipid extraction with solvents were treated with a mixture of 40% KOH and methoxyethanol (1 : 7; v/v) at 110°C for 3 h in screw-capped tubes (Daffe et al., 1983). After acidification, fatty acids were extracted with diethyl ether and methylated with diazomethane. The mycolate patterns of the strains were determined by analytical TLC on Silica Gel 60 plates (Macherey-Nagel) using either dichloromethane or petroleum ether/diethylether 90 : 10 (v/v) for apolar lipids, CHCl₃/CH₃OH 90 : 10 (v/v) and CHCl₃/CH₃OH/H₂O 65 : 25 : 4 (v/v) for polar lipids (phospholipids and glycolipids). Characterization of lipids was performed by using specific sprays: the Dittmer–Lester reagent (phospholipids), 0.2% anthrone in H₂SO₄ (glycolipids), 0.2% ninhydrine in acetone (lipooamino compounds).

**Preparation of trimethylsilyl (TMS) ether derivatives of mycolic acids.** Hydroxy-esters were treated by silanes in anhydrous conditions: fatty acid methyl esters (FAMEs) (up to 5 mg) were dissolved in pyridine (six drops); hexamethyldisilazane (4 drops) and trimethyl chlorosilane (2 drops) were added and, after shaking, the reaction was left at room temperature for 15 min. After drying under nitrogen, petroleum ether was added and the mixture was analysed by gas chromatography (GC)/MS as described below.

**Oxidative cleavage of double bonds.** Ethylenic esters were cleaved by permanganate-periodate oxidation (Von Rudloff, 1956). Briefly, a mixture of t-butanol (4 ml), aqueous 2 mM sodium carbonate (2.4 ml), distilled water (1.2 ml) and periodate-permanganate solution (2.7 ml) was added to a solution of lipids (around 5 mg) dissolved in benzene (0.6 ml). The oxidant solution contained 0.1 M sodium periodate and 2.5 mM potassium permanganate in water. The reaction was carried out in a screw-capped tube at 30°C overnight with shaking. The reaction was stopped by adding sodium metabisulphite until discoloration. After acidification with a few drops of 20% H₂SO₄ in water, the resulting oxidation products were extracted with diethyl ether. The acids obtained from the oxidative cleavage were methylated with diazomethane and purified by preparative TLC using dichloromethane as eluent. Compounds were identified by GC/MS according to their retention times and their fragmentation patterns (Oldham & Stenhalgen, 1972).

**Radiolabelling of mycolic acids.** [1-14C]Palmitic acid (0.34 μCi ml⁻¹; Perkin Elmer) was added to a growing culture of *H. altamirensis*. After a 3 h incubation with shaking, bacteria were harvested by centrifugation and the cell pellet was saponified as above. The mixture was acidified and fatty acids were extracted with diethyl ether and esterified by diazomethane. Radiolabelled methyl esters were separated by TLC using CH₃Cl as running solvent, and the detection was performed using a PhosphorImager (Typhoon, Amersham). Mycolic acid methyl esters (MAMEs) were isolated by scraping off the plate and were subsequently submitted to oxidative cleavage before being analysed by TLC and PhosphorImager detection.

For kinetic experiments, 0.05 μCi ml⁻¹ [1-14C]palmitic acid was added to growing *H. altamirensis* cells which were cultured at 30°C with shaking. Aliquots were removed after 0, 15, 30, 60, 180 and 300 min labelling. After centrifugation, cells were treated as above.

**Instrumentation.** Infrared spectra were recorded using a Perkin Elmer model FTIR 1600 apparatus. Samples were analysed as a film between two NaCl disks. The optical rotations of purified molecules were determined at 589 nm with a Perkin-Elmer model 241 polarimeter. Samples were dissolved in 5 mg CHCl₃ ml⁻¹.

1H-NMR spectra of purified MAMEs were obtained in CDCl₃ (100 % D) using a Bruker AMX-500 spectrometer at 298 K. Chemical shift values (in p.p.m.) were relative to the internal CHCl₃ resonance (at 7.27 p.p.m.).

MALDI-TOF MS analysis of purified MAMEs was performed in reflectron mode on a 4700 Proteomics Analyser (Voyager DE-STR; Applied Biosystems) equipped with an Nd:YAG laser (355 nm) operating with pulses of 500 ps with a frequency of 200 Hz. The shots (2500 total) were accumulated in positive ion mode and MS data were acquired using the instrument default calibration. Mycolate samples were dissolved in chloroform, at a concentration of 1 mM, and were directly spotted onto the target plate as 0.5 μl droplets, followed by the addition of 0.5 μl matrix solution. Samples were allowed to crystallize at room temperature. The matrix used was 2,5-dihydroxybenzoic acid (10 mg ml⁻¹) in CHCl₃/CH₃OH (1 : 1; v/v) (Laval et al., 2001).

Electrospray ionization (ESI)-MS of free mycolic acids was carried out on a QSTAR XL (AB Sciei) hybrid quadrupole (Q) TOF MS/MS system equipped with an ion-spray source, which was connected out on a QSTAR XL (AB Sciex) hybrid quadrupole (Q) TOF MS/MS system equipped with an ion-spray source, which was connected.
with the Analyst QS 1.1 software. For ESI-MS and ESI-MS/MS, all samples were scanned over a mass range of 100–2000 m/z and 80–1500 m/z, respectively, with an ion-spray voltage of 4.5 kV in negative-ion mode. The mass spectrum was accumulated for 1 min. For MS/MS analysis, the precursor ion was chosen manually and the collision energy was 30 eV.

GC/MS analysis was performed on a Hewlett Packard 5890A series II gas chromatograph, fitted with an OV1 fused-silica capillary column (12 × 0.30 mm), and connected to a Hewlett Packard 5989X mass spectrometer in EI mode with an ionization potential of 70 eV. The gas chromatograph, fitted with an OV1 fused-silica capillary column GC/MS analysis was performed on a Hewlett Packard 5890A series II collision energy was 30 eV.

For MS/MS analysis, the precursor ion was chosen manually and the negative-ion mode. The mass spectrum was accumulated for 1 min.

Sequence analysis. Analysis of genome sequences was done via the NCBI web server (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The sequenced genomes considered were: Mycobacterium tuberculosis H37Rv (NC 000962) and A. subflavus DQ53-9A1 (CP002786).

Sequence alignments were performed using the BLASTP program (NCBI, Altschul et al., 2011) with the protein table of the complete genome sequence of A. subflavus (Cai et al., 2011) with the M. tuberculosis H37Rv proteins as query.

RESULTS

A. subflavus and H. altamirensis lipids

Amycolicoccus and Hoyosella, two recently described actinomycete genera, have recently been shown to belong to the Corynebacterineae suborder with a cell wall chemotype IV and a lipid profile compatible with this group, but are surprisingly devoid of mycolic acids (Jurado et al., 2009; Wang et al., 2010). To address the taxonomic status of the strains, their lipid contents were revisited; phosphatidylethanolamine was identified in the strains, both by its positive reaction with the ninhydrin reagent (data not shown), in agreement with previous reports. This phospholipid represents a chemotaxonomic marker of Corynebacterineae as it is present in all genera of ‘mycolata’ except in some strains of Corynebacterium and Dietzia (Jurado et al., 2009). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositoldimannoside (PIM2) were the main acidic phospholipids, a composition expected for Corynebacterineae. However, the two phospholipids reported previously in A. subflavus, i.e. phosphatidylcholine and a glucosamine-containing phospholipid (Wang et al., 2010), could not be detected among the lipids of the strain analysed. Importantly, two putative trehalose-based glycolipid spots, typified by their characteristic blue-grey colour, were revealed by the anthrone reagent and exhibited migrations of dimycoloyl trehalose- and monomycoloyl trehalose-like compounds, suggesting that mycolate-containing glycolipids occur in both strains.

The fatty acids resulting from the saponification of whole cells were analysed as they represent useful taxonomic tools (Barry et al., 1998). For both strains, the main cellular volatile FAMES were identified by GC/MS as C16:0, C18:1ω9 and 10-methyl stearic acid (tuberculostearic acid); odd-chain acids with 15 and 17 carbon atoms were also characterized. Traces of short-chain esters with 9, 10, 11 and 12 carbon atoms were observed and minute amounts of long-chain esters (up to C19) were also detected, a fatty acid composition similar to that previously described for H. altamirensis (Jurado et al., 2009).

Interestingly, TLC analysis of methyl esters revealed the presence of putative hydroxy-esters in both A. subflavus and H. altamirensis. Their migration was similar to that of authentic corynomycolic acid methyl ester (C32) from Corynebacterium diphtheriae but lower than that of nocardomycolates (C30–C60) from Nocardia asteroides and γ-mycylates from M. fallax (C72–C77), the closest relative strain to Hoyosella (Fig. 1).

Structure of hydroxy-acids

A. subflavus and H. altamirensis putative hydroxylated compounds were purified by preparative TLC. The infrared spectrum of the compounds from both origins showed absorption bands corresponding to those of the free hydroxyl group (at 3650–3590 cm⁻¹), ester (at 1735 cm⁻¹) and long hydrocarbon chain (at 720 cm⁻¹). The occurrence of a hydroxyl function was confirmed by a shift in the mobility on TLC upon treatment with silanes.

The chain lengths of the compounds were evaluated by MALDI-TOF MS (Fig. 2). In both strains, the masses were similar to those of corynomycolic acids with 30–34 carbon atoms corresponding to [M + Na]⁺ ions of saturated esters at m/z 505, 519, 533, 547, 561 and 575, and unsaturated 531, 545, 559 and 573 homologues (Fig. 2). The main representative of this series was a C34 monounsaturated [M + Na]⁺ at m/z 559. Interestingly, odd and even chains of mycolic acids were present, in agreement with the presence of significant amounts of penta- and heptadecanoic acids in the bacterial fatty acid pool. The shift of 72 atomic mass units in the MALDI-TOF mass spectrum of the hydroxy-esters upon silylation indicated that only one hydroxyl per molecule was present, as expected, and the major peak at m/z 631 observed upon silylation confirmed this interpretation for the main homologue C₃₄:₁.

The ¹H-NMR spectra of MAMEs from both species were very similar and showed the characteristic signals typifying long-chain esters (Fig. 3): methylene proton resonances at 1.28 p.p.m. (broad signal), terminal methyl groups at 0.89 p.p.m. (triplet), and methyl ester at 3.71 p.p.m. (singlet). Specific resonances due to the presence of cis double bonds were also observed, notably a signal at 5.35 p.p.m. assigned to ethylenic proton resonances and those of methylene adjacent to double bonds at 2.00 p.p.m. Moreover, a characteristic broad signal at 3.58 p.p.m., assigned to the resonance of the proton on the C-3 carrying a hydroxyl group, and a multiplet at 2.43 p.p.m. due to the deshielded protons on the asymmetrical carbon at position C-2 adjacent to the carboxyl group typified the mycolic
acid structure. These attributions were confirmed by 2D $^1$H-NMR (data not shown).

The occurrence of the mycolic unit, i.e. 2-alkyl, 3-hydroxy was confirmed by the C$_2$–C$_3$ cleavage produced by pyrolysis on GC/MS, allowing detection of fatty esters with 9, 10, 11 and 12 carbon atoms on the one hand, and long chain aldehydes containing 20–26 carbon atoms on the other hand, an unusual combination for corynomycolic acids. In order to support these results, free mycolic acids were analysed by ESI-MS. This method allowed determination of the overall chain length as well as the $\alpha$-alkyl chain length and, by deduction, the aldehyde fragment (Fig. 4a). The [M-H]$^-$ ions generated by ESI-MS underwent dissociation to eliminate the mero-aldehyde residue, leading to the formation of carboxylate anions containing $\alpha$-alkyl chains (Shui et al., 2007; Hsu et al., 2011). The ESI mass spectra profiles of free mycolic acids (Fig. 4b) exhibited [M-H]$^-$ ions ranging from $m/z$ 493 to 537 corresponding to mycolic acids with 32–36 carbon atoms presenting odd and even homologues, in agreement with MALDI-TOF MS.

Fragmentation of the $m/z$ 509 homologue (mycolic acid with 33 carbon atoms) yielded ions at $m/z$ 157, 171 and 185 corresponding to saturated acids with 9, 10 and 11 carbon atoms, respectively (Fig. 4b, left-hand insert). Fragmentation of the $m/z$ 521 ions (unsaturated mycolic acid with 34 carbon atoms) yielded only a fragment ion at $m/z$ 171 (Fig. 4b, right-hand insert). Peaks corresponding to aldehydes were not observed and their masses were deduced from the calculated difference and given in Table 1. Both fragments resulting from the C$_2$–C$_3$ cleavage (Table 1) have odd and even homologues and display

![Fig. 1. Corynebacterineae mycolic acids. (a) TLC of MAMEs from C. diphtheriae (C$_{32}$) (1), H. altamirensis (2), A. subflavus (3), Rhodococcus rhodochrous (C$_{34}$–C$_{48}$) (4), Nocardia asteroides (C$_{44}$–C$_{60}$) (5), M. fallax (C$_{72}$–C$_{77}$) (6). The solvent was CH$_2$Cl$_2$. Visualization was by phosphomolybdic acid spray followed by heating. The arrow indicates the solvent front. (b) Structure of the C$_{32}$ corynomycolic acid: 2R-tetradecyl, 3R-hydroxy octadecanoic acid from C. diphtheriae, where $R$ indicates the stereochemistry of carbons 2 and 3 (I). Structure of the C$_{80}$ dicyclopentanyl mycolic acid ($\alpha$-mycolic acid) from M. tuberculosis (II).](image1)

![Fig. 2. MALDI-TOF mass spectra of MAMEs from A. subflavus (a) and H. altamirensis (b) displaying the [M+Na]$^+$ ions. The correspondence between the the total carbon number of free acids and the mass values are as follows: 29:0, 491; 30:0, 505; 31:1, 517; 31:0, 519; 32:1, 531; 32:0, 533; 33:1, 545; 33:0, 547; 34:1, 559; 34:0, 561; 35:1, 573; 36:1, 587.](image2)
differences in chain lengths: those corresponding to acids were C₉–C₁₂ and those to aldehydes were C₂₂–C₂₆. In addition, the double bond is always located on the longest chain (the aldehyde fragment). Further analysis of the TMS derivatives of methyl-mycolates by GC/MS (Table 2) confirmed these predictions and indicated an asymmetrical structure for these mycolic acids with short-chain acids from 9 to 11 carbon atoms, and long-chain aldehydes from 20 to 24 carbon atoms, an unusual combination for corynomycolic acids (Nishiuchi et al., 1999, 2000).

**Fig. 3.** ¹H-NMR spectra of MAMEs from A. subflavus (a) and H. altamirensis (b). Spectra were recorded in CDCl₃ at 298 K at 500 MHz. The chemical functions are presented and the assigned protons are in bold.

**Fig. 4.** ESI-MS of free mycolic acids of H. altamirensis. (a) Fragmentation scheme of mycolic acids. Bold type indicates the fragments characterized by MS/MS. (b) Total spectrum of mycolic acids and MS/MS fragmentation of ions with m/z 509 (left-hand insert) and m/z 521 (right-hand insert). Similar results were obtained with A. subflavus mycolic acids.
Identical results were obtained from *A. subflavus* and *H. altamirensis*. As the only asymmetrical centres present in the mycolic acid are C2 and C3, the molecular rotation ([M]D) of the molecule was determined and compared with that of *C. diphtheriae* corynomycolic acid methyl ester. The positive values of the [M]D of *H. altamirensis* (38°) and *A. subflavus* (34°) mycolic acids were very similar to that of *C. diphtheriae* (+40°) and allowed the stereochemistry 2R, 3R for the esters isolated from both strains to be deduced, in agreement with the value observed for all mycolic acids from *Corynebacterineae* studied so far (Asselineau et al., 1970).

The occurrence of a double bond(s) in the MAMEs was confirmed by AgNO3-impregnated TLC, where two bands were observed; the most retained band contained the ethylenic homologues, mainly C34 : 1, as revealed by MALDI-TOF MS. In order to localize the double bond, MAMEs from both strains were submitted to permanganate-periodate oxidation, followed by methylation and

Table 1. Structure of mycolic acids from *A. subflavus* and *H. altamirensis* deduced from ESI-MS/MS

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*Percentages indicate the relative intensity of acid from α-branch obtained after cleavage of the C2–C3 bond as shown in Fig. 4(a). (tr), trace, i.e. values less than 5 %.

Table 2. Molecular species of MAMEs from *H. altamirensis* deduced from GC/MS analyses of their TMS derivatives

Similar results were obtained with *A. subflavus* methyl mycolates. Example of mass fragmentation of TMS-ether derivatives of methyl mycolate by GC-MS. The masses of the fragments [A]+ and [B]+ allowed the number of carbon atoms of each part of the molecule to be deduced (mero-chain and pyrolysis ester). The diagram below indicates the structure of the MAMEs.
analysis by TLC and GC/MS and gave identical results (Fig. 5). Two compounds were separated by TLC, one migrating as FAMEs (compound II) and the other exhibiting a lower Rf (compound III). The GC/MS analysis (Odham & Stenhagen, 1972) allowed identification of compound II as methyl hexadecanoate (based on its intense peak at m/z 74 and the molecular ion at m/z 270) and compound III, not stable at high temperature, thus cleaved in the GC injector into two fragments, IIIa and IIIb (Fig. 5b). The esters of IIIb were characterized by the basal peak at m/z 74 for the McLafferty rearrangement and corresponded to esters from pyrolysis (the main ester being C_{10:0} with a molecular ion at m/z 186, accompanied by small amounts of C_{11} and C_{12} at m/z 200 and 214, respectively). On the other hand, a fragment with a molecular mass at m/z 172 was observed, corresponding to the mass of aldehyde-ester with eight carbon atoms (compound IIIa). This analysis allowed a structure to be attributed (I) to the main ethylenic homologue C_{34} as a 2R-octanoyl, 3R-hydroxy, 10-hexacosenoic acid (Fig. 5a).

**Biosynthesis of mycolic acids**

The unusual structures of *A. subflavus* and *H. altamirensis* mycolic acids pointed to a clear difference between their production and the synthesis of corynemycolates by corynebacteria, despite their similar overall lengths. Indeed, the asymmetry observed in both α-branched and mero-chains is reminiscent of the mycolic acids from *Nocardia* and *Mycobacterium*. The mycolic acid synthesis in these two genera proceeds through the condensation of two fatty acids, one of them (the mero-chain) being the result of the elongation of a precursor, whereas in *Corynebacterium* it results from the condensation of two short-chain fatty acids present in the bacterial pool.

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Fig. 5. (a) Structure of the C_{34} monounsaturated mycolic acid from *A. subflavus* and *H. altamirensis* as deduced from oxidative degradation. The ethylenic MAMEs (I) yield, after oxidative cleavage, a monoacid (II) and a dicarboxylic acid (III). (b) GC/MS profile of the reaction mixture after oxidative cleavage of *A. subflavus* mycolic acids: the annotated peaks correspond to the compounds depicted in (a). Compound III under these conditions is cleaved, thus liberating a C_{10} ester (compound IIIb) and a C_{8} aldehyde-ester (compound IIIa). Other peaks exhibiting higher retention times correspond to aldehydes from pyrolysis of saturated mycolic acids.
In order to investigate the construction of the meromycolic chain, growing *H. altamirensis* cells were incubated with [1-\textsuperscript{14}C]palmitic acid. Interestingly, the kinetics of labelling showed that palmitic acid is rapidly incorporated into mycolic acids in less than 5 min (data not shown). After saponification of the cells grown in these conditions (after 180 min labelling), the fatty acids were extracted, methylated and analysed by TLC (Fig. 6a). Two bands corresponding to the two isomers of labelled mycolic esters were detected, these are 2R, 3R and 2S, 3R, the latter appearing in alkaline conditions (Etemadi, 1967c). To determine which part of the molecule was labelled, the purified mycolates corresponding to the 2R, 3R isomer were oxidatively treated to induce the cleavage of the double bond. After oxidation and extraction of fatty acids, the methyl esters were analysed by radio-TLC (Fig. 6b). The labelled ester obtained in the FAME region indicated that the [1-\textsuperscript{14}C]palmitic acid was incorporated at the distal part of the unsaturated mero-chain (ω end), thus indicating the elongation of the precursor, in agreement with the proposed models for mycolic acid biosynthesis in *M.*

![Fig. 6. Radio-TLC of FAMEs of *H. altamirensis* after incubation with [1-\textsuperscript{14}C]palmitic acid. (a) TLC profile of methyl esters resulting from the saponification of labelled whole cells. C16*, residual [1-\textsuperscript{14}C]palmitate, exhibiting the FAME migration. The two MAME bands correspond, respectively, to the natural 2R, 3R (lower band) and its 2S, 3R epimer (upper band) that is an experimental artefact produced during the alkaline treatment. (b) TLC of labelled MAME before (1) and after (2) oxidative cleavage of the double bond. Lanes: 1, purified labelled MAME of *H. altamirensis*; 2, products of oxidative cleavage of mycolates. The labelled FAME obtained from the oxidation of the unsaturated mycolate homologues corresponds to the ω-part of the molecule. The unchanged mycolates correspond to the uncleaved saturated MAME. Solvent: CH\textsubscript{2}Cl\textsubscript{2}; detection was performed using PhosphorImager. The arrow indicates the solvent front. Similar results were obtained with *A. subflavus* mycolic acids.]

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<td>AS9A_1223</td>
<td>fabF</td>
<td>398</td>
<td>3-Oxoacyl-[acyl-carrier-protein] synthase</td>
</tr>
<tr>
<td></td>
<td>AS9A_1222</td>
<td>fabG1</td>
<td>256</td>
<td>3-Oxoacyl-[acyl-carrier-protein] reductase</td>
</tr>
<tr>
<td></td>
<td>AS9A_0395</td>
<td>had</td>
<td>141</td>
<td>(3R)-Hydroxyacyl-ACP dehydratase subunit</td>
</tr>
<tr>
<td></td>
<td>AS9A_0396</td>
<td>hadB</td>
<td>142</td>
<td>(3R)-Hydroxyacyl-ACP dehydratase subunit</td>
</tr>
<tr>
<td></td>
<td>AS9A_0397</td>
<td>hadD</td>
<td>263</td>
<td>Acid-CoA carboxylase subunit beta</td>
</tr>
<tr>
<td></td>
<td>AS9A_0398</td>
<td>hadA</td>
<td>85A</td>
<td>Mycoloyltransferase 85A</td>
</tr>
</tbody>
</table>

Table 3. *A. subflavus* ORFs putatively involved in mycolic acid biosynthesis.

* M. tuberculosis H37Rv protein used as Query in the blastp 2.2.35+ program (NCBI).
smegmatis and N. asteroides (Etémadi, 1967b; Etemadi & Lederer, 1965; Bordet & Michel, 1969).

**Genome sequence analysis in light of the structural data**

The genome of A. subflavus was recently released (Cai et al., 2011), which allowed the search for the presence of mycolic acid biosynthesis genes. Indeed, all the genes encoding the key enzymes of mycolic acid biosynthesis were identified: components of the type II-fatty acid synthase dissociated system (FAS-II), the mycolic acid condensing enzyme Pks13 and mycoloyltransferases (Table 3). In the A. subflavus genome, only one copy of *fas* (AS9A_3620), the gene encoding the multifunctional type I-FAS (FAS-I), was present, as is the case in *M. tuberculosis* (Cole et al., 1998) and N. farcinica (Ishikawa et al., 2004), and in contrast with *C. glutamicum* that has two *fas* copies (Ishikawa et al., 2004; Radmacher et al., 2005). The putative orthologues of the genes encoding the four catalytic steps of FAS-II (Marrakchi et al., 2008) were also present: the β-ketoacyl-ACP synthase *fabF*/*kasA* (AS9A_1223), the β-ketoacyl-ACP reductase *fabG1*/*mabA* (AS9A_1921), the β-hydroxyacyl-ACP dehydratase *hadB* (AS9A_0396) and the enoyl-ACP reductase *inhA* (AS9A_1922) (Table 4). In *A. subflavus*, only one copy of the *fabF/kasA* is found in the genome. In contrast with *M. tuberculosis*, where two heterodimers of dehydratases (HadAB and HadBC) define the chain length selectivity (Sacco et al., 2007), a single substrate-selectivity subunit (had, AS9A_0395) is present in *A. subflavus*, in addition to the catalytic subunit HadB (AS9A_0396). The three genes encoding the enzymes involved in the mycolic acid condensation (Portevin et al., 2004, 2005) were conserved, in the same order, in *M. tuberculosis* and *A. subflavus*: *fadD32* (AS9A_0107), *pks13* (AS9A_0108) and *accD4* (AS9A_0109) (Table 4). Finally, four genes encoding putative mycolyltransferases, homologous to the *M. tuberculosis* Ag85A (Cole et al., 1998), were identified in the *A. subflavus* genome, whereas six such genes were reported in *C. glutamicum* (De Sousa-D’Auria et al., 2003). No gene encoding the SAM-dependent mycolic acid methyltransferases was detected, consistent with the characterized structures of mycolic acids devoid of methyl branches.

Previous publications have shown that *Hoyosella* and *Amycolicicoccus* are most closely related to the genus *Mycobacterium* (Jurado et al., 2009; Wang et al., 2010). Analysis of 16S rDNA genes of both strains showed that *H. altamirensis* and *A. subflavus* display the pattern of 16S rRNA nucleotide signatures of the *Mycobacteriaceae* family (Supplementary Fig. S1, available with the online version of this paper), thus confirming that both genera are members of the *Mycobacteriaceae* family (Zhi et al., 2009).

**DISCUSSION**

Since 2000, a large number of novel *Corynebacterineae* strains have been discovered from various sources and novel genera have been defined on the basis of taxonomic studies. In this context, the lipid composition of the strains had been crucial for the definition of genera and species, mainly in the mycolic acid-containing strains (Barry et al., 1998). Two strains recently isolated from environmental sources, *A. subflavus* and *H. altamirensis*, display characteristics in common with *Mycobacteriaceae*, yet the search for mycolic acids was reported to be negative (Jurado et al., 2009; Wang et al., 2010). In order to define the status of these two strains, a comparative and more thorough analysis of their fatty acids was undertaken. Mycolic acids,
the hallmark of Corynebacterineae, were characterized in A. subflavus and H. altamirensis in both extractable and bound lipids. They consist of short-chain mycolic acids from 30 to 36 carbon atoms with chain lengths similar to those of Corynebacterium (22–38C), but characterized by almost equal contents of even- and odd-numbered chains. The occurrence of mycolic acids has not been reported in previous studies (Jurado et al., 2009; Wang et al., 2010), which is puzzling. The lack of mycolic acid detection by these authors could be due to the method used, i.e. HPLC conditions for long-chain mycolic acids. In the present study, TLC clearly demonstrated the presence of hydroxylated compounds in the saponification products of whole cells (Fig. 1a). Furthermore, the MALDI-TOF of the purified compounds of A. subflavus and H. altamirensis revealed the presence of MAMEs with 29–35 carbon atoms with odd- and even-chain lengths (Fig. 2). This marked a difference from the structures of mycolic acids from related genera (Corynebacterium and Rhodococcus), where even-chain lengths were preponderant (Table 4), with the exception of those found in members of the genus Dietzia (Nishiuchi et al., 2000).

Another difference between A. subflavus and H. altamirensis mycolic acids and corynomycolic acids is the fact that ‘meromycolic’ and ‘α-branch’ chains (Fig. 1b) were significantly different in length. They consisted of a short α-branched chain and a mero-chain bearing 20–24 carbon

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Fig. 7. A hypothetical scheme for the biosynthesis of the C34 mycolic acid supported by data from Etemadi & Lederer (1965), Bordet & Michel (1969) and summarized by Tarnok (1978). Putative A. subflavus genes for mycolic acid synthesis are indicated. The radiolabelled carbon atom is indicated by an asterisk (*). n=6 for this homologue.
atoms, a difference from *Corynebacterium* but comparable to *Nocardia*, *Rhodococcus* and *Mycobacterium* mycolic acids, where an asymmetry of the two chains is also observed (Barry *et al*., 1998) (Table 4). Interestingly, an asymmetry of the chains was previously reported in *Corynebacterium bovis* (Collins *et al*., 1982), where the C2–C3 cleavage of the corynomycolic acid (C22–C32) generates C6–C9 acids, a characteristic distinctive among corynebacteria species.

Mycolic acids have been shown to be the result of the condensation of two fatty acids by the enzyme Pks13 (Portevin *et al*., 2004). The acid devoted to the mero-chain is activated as an acyl-AMP (by FadD32); the other acid at the origin of the z-chain is activated as alkyl-malonate by a carboxylase (AccD4) (Portevin *et al*., 2005). The orthologues of both genes were identified in the genome of *A. subflavus* (Table 3) and it is very likely that they are involved in mycolic acid synthesis (Fig. 7). Concerning the substrates of the condensing enzyme Pks13, it is known that they are found in *Corynebacterium* in the fatty acid pool; thus, C32:0 corynomycolic acid is produced when palmitic acid is the main representative fatty acid, as in *C. diphteriae*. Similarly, C34:1 and C36:2 corynomycolic acids are produced when C18:1 is present, as in *C. glutamicum*. The data presented in the present work do not fit with this paradigm and reveal the specificity of the mycolic acid-condensing enzyme of *A. subflavus* and *H. altamirensis*. Indeed, fatty acids used for the z-chain were short-chain acids (C9, C10, C11) while the acids devoted to the ‘meromycolic’ chain were longer (C20–C24) with even and odd homologues, all of them being present only in trace amounts in the fatty acid pool (Jurado *et al*., 2009). These results suggest that specific substrates are devoted to the last condensation step. Interestingly, the presence of long meromycolic chains in mycolic acids of *A. subflavus* and *H. altamirensis* would imply an elongation process, as in *Mycobacterium*. This correlates with the identification of orthologues of genes encoding proteins involved in the FAS-II complex in the genome of *A. subflavus*; FAS-II is responsible for the elongation of fatty acids to yield the precursors of mycolic acids in mycobacteria (Marrakchi *et al*., 2008).

It is currently known that the elongation of the mycobacterial meromycolic chain proceeds by the successive addition of C2 units on a molecule of palmitic acid (Etemadi & Lederer, 1965) by β-ketoacyl-ACP synthases KasA to give meromycolic acids around C30, and KasB (meromycolic acids C48–62) (Marrakchi *et al*., 2008) yielding even chain lengths. The fact that only the orthologues of kasA are present (and not those of kasB) in the genome of *A. subflavus* is consistent with short and even meromycolic chain length: for the meromycolic chain, one may hypothesize that the addition of four C2 units to palmitic acid would lead to a C24 acid. Yet this assumption would not explain the odd-numbered meromycolic chains. For the unsaturated C24:1 homologue, at least two possibilities should be considered: either the action of a putative oxidative Δ8 desaturase operating on the saturated C24 acid or the elongation of a cis 3-enoyl intermediate (Marrakchi *et al*., 2008). Another alternative has been proposed that deserves attention: building of the meromycolic chain of mycobacteria and nocardia mycolic acids would result from a ‘head to tail’ condensation of fatty acids through ω-oxidation (Kanemasu & Goldman, 1965; Ratledge, 1976; Bordet & Michel, 1969; Asselineau *et al*., 2002).

In conclusion, based on chemical features of mycolic acids from ‘mycolata’ genera, *A. subflavus* and *H. altamirensis* were confirmed as belonging to the *Corynebacterinae* suborder. These original mycolic acid structures validate this inclusion of both strains in the novel genera, as reported previously (Jurado *et al*., 2009; Wang *et al*., 2010). A comparative study of the key enzymes of these mycolic acid-producing organisms should be of high significance for further understanding of mycolic acid biosynthesis. Although we have no direct evidence for the presence of a mycomembrane in *Amycolicoccus* and *Hoyosella*, the fact that mycomembrane has been characterized in both *Corynebacterium* and *Mycobacterium* (Hoffmann *et al*., 2008; Zuber *et al*., 2008) suggests that it should also be present in members of both genera.

**ACKNOWLEDGEMENTS**

We are grateful to Marie Ruiz and Pauline Joubel for technical assistance and to Dr Olivier Saurel for performing part of NMR analyses. This work was supported in part by a grant from the European Community (SysteMTb HEALTH-F4-2010-241587). The NMR spectrometers were financed by the CNRS, the University Paul Sabatier, the Région Midi-Pyrénées and the European Structural Funds (FEDER).

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Edited by: W. Bitter