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 (ω-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 (ω-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 coccoidal 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 92.7–93.9% 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.

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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 Trypticase-Soya 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/diethyl ether 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 acetonitrile (lipoamino compounds).

**Mycolic acid isolation.** Whole cells or bacterial residues obtained after lipid extraction with solvents were treated with a mixture of CH₂Cl₂/petroleum ether (10:1; v/v) and purification was achieved by preparative TLC using silica gel TLC plates and analysed.

**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 trimethylchlorosilane (2 drops) were added and, after shaking, the reaction was left at room temperature for 15 min. After drying under nitrogen, petrol 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 (Odham & Stenhagen, 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.

Upon labelling, MAMEs were obtained in CDC₁₂ (100% D) using a Bruker AMX-500 spectrometer at 298 K. Chemical shift values (in p.p.m.) are 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 ScieX) hybrid quadrupole (Q) TOF MS/MS system equipped with an ion-spray source, which was connected to a 100 μl syringe, which in turn was driven forward at a rate of 20 μl min⁻¹ by the integrated QSTAR Harvard 22 syringe pump. Approximately 100 μg lipids, dissolved in 100 μl chloroform/methanol (1:2 v/v), was loaded into the syringe. The m/z response of the mass spectrometer was calibrated daily with standards from the manufacturer. The QSTAR XL was operated in direct infusion mode.
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 injector temperature was at 260 °C and the temperature separation program involved an increase from 100 to 300 °C, at the rate of 5 °C min⁻¹, followed by 10 min at 300 °C. For pyrolysis conditions, the injector temperature was increased to 290 °C (Etemadi, 1967a). Alternatively, a GC Varian 3800 apparatus coupled to a mass spectrometer Varian Saturn 2000 was used.

**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* DQS3-9A1 (CP002786). Sequence alignments were performed using the BLASTP program (NCBI, Altschul et al., 1997) on 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; phosphatidyethanolamine was identified in the strains, both by its mobility on TLC and by its positive reaction with the ninhydrin reagent (data not shown), in agreement with previous reports. This phospholipid represents a chemo-taxonomic 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, C₁₈:₁₁₉₉ 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 diphteriae* but lower than that of nocardomycolates (C₃₀–C₆₀) from *Nocardia asteroides* and ω-mycolates 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.
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 $[\text{M-H}]^-$ 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

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**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$_{80}$ dicyclopropa- 
nated mycolic acid ($\alpha$-mycolic acid) from M. tuberculosis (II).

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**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.
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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<th>Carbon number</th>
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<th>Aldehyde⁺</th>
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<td>36 : 1</td>
<td>549</td>
<td>12 : 0</td>
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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.

**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 monocarboxylic (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 \[^{14}C\]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 2\(^{R}\), 3\(^{R}\) and 2\(^{S}\), 3\(^{R}\), the latter appearing in alkaline conditions (Etemadi, 1967c). To determine which part of the molecule was labelled, the purified mycolates corresponding to the 2\(^{R}\), 3\(^{R}\) 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 \[^{14}C\]palmitic acid was incorporated at the distal part of the unsaturated mero-chain (\(\omega\) end), thus indicating the elongation of the precursor, in agreement with the proposed models for mycolic acid biosynthesis in *M. tuberculosis*.

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

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<th>Function</th>
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<th>Proposed function</th>
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<th>Identity (%)</th>
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<td>Rv0503c (FabH)</td>
<td>54</td>
<td>2e-122</td>
</tr>
<tr>
<td></td>
<td>AS9A_2908</td>
<td><em>fabH3</em></td>
<td>335</td>
<td>3-Oxoacyl-ACP synthase</td>
<td>Rv0503c (FabH)</td>
<td>52</td>
<td>7e-122</td>
</tr>
<tr>
<td></td>
<td>AS9A_1921</td>
<td><em>fabG1</em></td>
<td>256</td>
<td>3-Oxoacyl-[acyl-carrier-protein] reductase</td>
<td>Rv1483 (MabA)</td>
<td>64</td>
<td>3e-113</td>
</tr>
<tr>
<td></td>
<td>AS9A_0395</td>
<td><em>had</em></td>
<td>141</td>
<td>(3R)-Hydroxyacyl-ACP dehydratase subunit</td>
<td>Rv0635 (HadA)/Rv0637 (HadC)</td>
<td>43/48</td>
<td>8e-34/-3e-38</td>
</tr>
<tr>
<td></td>
<td>AS9A_0396</td>
<td><em>hadB</em></td>
<td>142</td>
<td>(3R)-Hydroxyacyl-ACP dehydratase subunit</td>
<td>Rv0636 (HadB)</td>
<td>57</td>
<td>2e-57</td>
</tr>
<tr>
<td></td>
<td>AS9A_1922</td>
<td><em>inhA</em></td>
<td>263</td>
<td>Enoyl-acyl carrier protein reductase</td>
<td>Rv1484 (InhA)</td>
<td>64</td>
<td>9e-123</td>
</tr>
<tr>
<td>Condensation complex</td>
<td>AS9A_0107</td>
<td><em>fadD32</em></td>
<td>630</td>
<td>Long-chain-fatty-acid-CoA ligase</td>
<td>Rv3801c (FadD32)</td>
<td>48</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>AS9A_0108</td>
<td><em>pks13</em></td>
<td>1619</td>
<td>Polylactide synthase</td>
<td>Rv3800c (Pks13)</td>
<td>60</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>AS9A_0109</td>
<td><em>accD4</em></td>
<td>514</td>
<td>Acyl-CoA carboxylase subunit beta</td>
<td>Rv3799c (AccD4)</td>
<td>61</td>
<td>0.0</td>
</tr>
<tr>
<td>Transfer on cell wall</td>
<td>AS9A_0103</td>
<td></td>
<td>348</td>
<td>Mycolyltransferase 85A</td>
<td>Rv3804c (fbpA, 85A)</td>
<td>41</td>
<td>3e-64</td>
</tr>
</tbody>
</table>

* M. tuberculosis H37Rv protein used as Query in the BLASTP 2.2.25+ program (NCBI).
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 (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 inbA (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,

### Table 4. Comparative data of the structures of mycolic acids from different genera based on overall chain lengths, pyrolysis acids and degree of unsaturation

<table>
<thead>
<tr>
<th>Genus</th>
<th>Overall chain length</th>
<th>Pyrolysis ester</th>
<th>Degree of unsaturation*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynebacterium</td>
<td>22–38 even; 34–38</td>
<td>8–18 even</td>
<td>0, 1, 2</td>
<td>Nishiuchi et al. (2000)</td>
</tr>
<tr>
<td>Dietzia</td>
<td>Even and odd; 15, 16, 17</td>
<td>Even and odd; 15, 16, 17</td>
<td>0, 1, 2</td>
<td>Nishiuchi et al. (2000)</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>30–54</td>
<td>12–16 even</td>
<td>0, 1, 2</td>
<td>Soddell et al. (2006)</td>
</tr>
<tr>
<td>Millisia</td>
<td>44–52</td>
<td>ND</td>
<td>ND</td>
<td>Nishiuchi et al. (1999)</td>
</tr>
<tr>
<td>Nocardia</td>
<td>48–60</td>
<td>12, 18 even</td>
<td>0–3</td>
<td>Nishiuchi et al. (2000)</td>
</tr>
<tr>
<td>Gordonia</td>
<td>40–66</td>
<td>16, 18 even</td>
<td>1–4</td>
<td>Chun et al. (1997)</td>
</tr>
<tr>
<td>Skernania</td>
<td>58–64</td>
<td>16, 20</td>
<td>2–6</td>
<td>Daffe et al. (1988)</td>
</tr>
<tr>
<td>Tsukamurella</td>
<td>64–78</td>
<td>20, 22</td>
<td>1–6</td>
<td>Yassin &amp; Hupfer (2006)</td>
</tr>
<tr>
<td>Williamsia</td>
<td>ND</td>
<td>16, 18</td>
<td>ND</td>
<td>Barry et al. (1998)</td>
</tr>
<tr>
<td>Mycobacterium</td>
<td>60–90</td>
<td>20–26</td>
<td>1–2</td>
<td>Butler et al. (2005)</td>
</tr>
<tr>
<td>Segniliparus</td>
<td>&gt;90</td>
<td>22, 24</td>
<td>ND</td>
<td>Adachi et al. (2007)</td>
</tr>
<tr>
<td>Smaragdicococcus</td>
<td>43–49</td>
<td>ND</td>
<td>ND</td>
<td>This work</td>
</tr>
<tr>
<td>Hoyosella</td>
<td>Even and odd; 30–35</td>
<td>Even and odd; 9, 10, 11, 12</td>
<td>0–1</td>
<td>This work</td>
</tr>
<tr>
<td>Amycolicicoccus</td>
<td>Even and odd; 30–36</td>
<td>Even and odd; 9, 10, 11, 12</td>
<td>0–1</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Degree of unsaturation refers to the number of double bonds and/or cyclopropane.
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=8 for this homologue.
Novel mycolic acids in Hoyosella and Amycolicicoccus

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 C$_2$–C$_4$ cleavage of the corynomycolic acid (C$_{22}$–C$_{32}$) generates C$_{20}$–C$_{30}$ 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 $\alpha$-branch is activated as alkyl-malate 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, C$_{32}$-0 corynomycolic acid is produced when palmitic acid is the main representative fatty acid, as in C. diphtheriae. Similarly, C$_{34}$-1 and C$_{36}$-2 corynomycolic acids are produced when C$_{18}$-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 $\alpha$-branch were short-chain acids (C$_9$, C$_{10}$, C$_{11}$) while the acids devoted to the ‘meromycolic’ chain were longer (C$_{30}$–C$_{34}$) 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 C$_2$ units on a molecule of palmitic acid (Etemadi & Lederer, 1965) by $\beta$-ketoacyl-ACP synthases KasA to give meromycolic acids around C$_{30}$ and KasB (meromycolic acids C$_{48}$–C$_{62}$) (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 C$_2$ units to palmitic acid would lead to a C$_{34}$ acid. Yet this assumption would not explain the odd-numbered meromycolic chains. For the unsaturated C$_{24}$:1 homologue, at least two possibilities should be considered: either the action of a putative oxidative $\Delta$8 desaturase operating on the saturated C$_{24}$ 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 $\omega$-oxidation (Kanemasa & 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 Amycolicicoccus 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.

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