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Short communication

Revisited mycolic acid pattern of *Mycobacterium confluentis* using thin-layer chromatography

Silvia Secanella-Fandos[a], Marina Luquin[a], Míriam Pérez-Trujillo[b], Esther Julián[a,*]

[a] Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[b] Servei de Ressonància Magnètica Nuclear, Universitat Autònoma de Barcelona, Bellaterra, Spain

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ABSTRACT

The profile of mycolic acids from *Mycobacterium confluentis* has not been adequately published. However, the definition of the composition of mycolic acids is a critical element for describing new mycobacterial species. Thus, an erroneously published profile can lead to confusing citations. The aim of this article is to make the protocols clear, by using thin layer chromatography as a tool, for defining the discrete pattern of mycolic acids of any newly reported mycobacterial species. By using this method, and corroborated using nuclear magnetic resonance analysis, we demonstrated that *M. confluentis* contains α-mycolates (type I) and epoxymycolates (type V mycolic acids).

1. Introduction

Mycobacterium confluentis (DSM 44017^T) is a saprophytic mycobacteria described in 1992 by Kirschner et al. [1]. Based on a unique sputum isolate, the authors described a non-pigmented rapid grower of no clinical significance. Later, it was confirmed that *M. confluentis* is a non-pathogenic mycobacteria in animal models, being cleared after intravenous infection in BALB/c and even in Interferon-gamma knockout mice [2]. From the isolate described by Kirschner et al. [1], no other isolates of *M. confluentis* have been described in the literature.

One of the important features for defining a new *Mycobacterium* species is its pattern of mycolic acids. The description of the profile of mycolic acids is a key point for identifying mycobacterial species [3,4]. These lipids are the main components of the mycobacterial cell wall. There are seven different types (representative figures of the different mycolic acid structures are shown in Fig. 1) and each mycobacterium has a determined pattern that can be shared with other mycobacteria. All of them have a common α-alkyl, β-hydroxy core structure of high molecular weight, ranging from 60 to 90 total carbons, with two long chains. Whereas the α-branch, except for length, is fundamentally constant in all types of mycolic acids, the β-chain (called meromycolate chain) may consist of a wide variety of chemical structures [7]. There are usually two positions that could be occupied by cyclopropane rings, methyl branches, combinations of cis and trans double bonds (unsaturated chains), and hydroxy, methoxy, ester or epoxy groups (Fig. 1).

With very few exceptions, each mycobacterium species has a determined composition of mycolic acids, for which reason they are considered to be taxonomically marked. In fact, mycolates are essential elements of the mycobacteria cell wall, not only for taxonomic studies but also because of their functions. Mycolic acids are critical building blocks in the mycobacteria structure conferring properties such us low permeability of the cell wall to hydrophobic compounds and resistance to dehydration, which is critical for survival inside host cells [8,9]. They are potential targets for antimycobacterial drugs. Moreover, these lipids are recognized for the host immune system being implicated in the immunopathology of mycobacterial diseases [10,11].

There are different techniques for defining the pattern of mycolic acids, all of which are based on chromatographic tools. Reverse-phase high performance liquid chromatography (HPLC) is the most employed method in medical/routine laboratories for analyzing the mycolic acid content of mycobacterial isolates. Although different species share the same mycolic acids pattern, the exact proportion of each class of mycolate and their structure vary among different species [6,7]. In HPLC analysis, the mycolic acids are separated on the basis of their polarity and the carbon chain length, with the more polar and shorter eluting first [12]. Using this technique, it is possible to obtain a chromatogram with clusters of peaks. The arrangement and height of major peaks together with their position on the basis of retention times can be compared to commercially available software libraries of reference patterns, allowing the identification of the majority of clinically interesting species

Abbreviations: HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

* Corresponding author. Tel.: +34 93 581 3096; fax: +34 93 581 2387.
E-mail address: esther.julian@uab.cat (E. Julián).

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Type	Mycolate	Structure
I	α-Mycolate	$CH_3-(CH_2)_a-A-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
II	α'-Mycolate	$CH_3-(CH_2)_a-CH=CH-(CH_2)_b-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
III	Methoxymycolate	$CH_3-(CH_2)_a-CH(CH_3)-CH(OCH_3)-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
IV	Ketomycolate	$CH_3-(CH_2)_a-CH(CH_3)-C(=O)-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
V	Epoxymycolate	$CH_3-(CH_2)_a-CH(OH)-CH(O)-CH-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
VI	Carboxymycolate	$OH-C(=O)-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$
VII	ω-1-Methoxymycolate	$CH_3-CH(OCH_3)-(CH_2)_a-A-(CH_2)_b-B-(CH_2)_c-C^{\beta}H(OH)-C^{\alpha}H(COOH)-(CH_2)_d-CH_3$

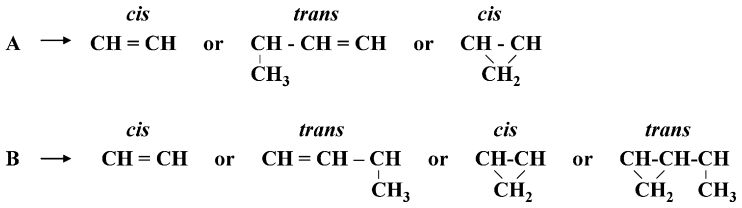

Fig. 1. Structures of the different types of mycolic acids. Modified from [5,6]. A, distal; B, proximal. I, α-mycolates; II, α′-mycolates; III, methoxymycolates; IV, ketomycolates; V, epoxymycolates; VI, carboxymycolates. The values of *a*, *b*, and *c* are ranging from 11 to 19. The value of *d* is ranging from 19 to 23. A and B symbolize a double bond or cyclopropane ring. At B, adjacent methyl branches can be present.

[12,13]. Using HPLC, it is not possible to identify the different structural types of mycolic acid; however, they can be distinguished by one- or two-dimensional thin layer chromatography (TLC) analysis. TLC allows the differentiation of the seven known mycolic acid types. The methyl esters of mycolic acids were found to migrate on TLC in accordance with the nature of their characteristic functions rather than with other differences in their molecular structures [14]. For instance, the same mycolate type with double bonds instead of cyclopropane rings maintain the same polarity co-migrating in TLC analysis [15].

On analyzing the mycolic acid content of different mycobacteria using TLC, we realized that *M. confluentis* possesses type V mycolic acid instead of type IV mycolic acid as was described before. We performed an acid methanolysis procedure to extract the lipids from the mycobacterial cell wall. Using this technique, type V mycolic acid molecule is characteristically broken down, leading to the presence of more polar long-chain components: dihydroxy derivatives and hydroxymethoxy compounds [16,17], and this may be observed by TLC analysis. The purpose of this article is to clarify which mycolic acid content has *M. confluentis* and make it clear which protocols are to be followed using TLC chromatography to describe the mycolic acid composition of a mycobacterium.

2. Materials and methods

2.1. Strains and culture conditions

M. confluentis DSM 44017^T was obtained from the German Collection of Microorganisms and Cell Cultures, and *Mycobacterium smegmatis* (ATCC 35797), *Mycobacterium phlei* (ATCC 11758) and *Mycobacterium bovis* BCG (ATCC 35737, Japan strain) were obtained from the American Type Culture Collection. All the strains were grown on Middlebrook 7H10 agar (Difco Laboratories, Surrey, United Kingdom) supplemented with 10% oleic-albumin-dextrose-catalase enrichment media at 37 °C for 7 days, with the exception of BCG which was cultured for 15 days.

2.2. Mycolic acid extraction

Cells were treated following two different protocols. Some of the cells were saponified and then the lipids extracted were methylated with diazomethane as previously described [18]. Briefly, a spadeful of bacteria (10 mg wet weight) was scraped from the surface of Middlebrook 7H10 agar plates and treated with 2 ml of a methanol-benzene solution (8:2, v/v) containing 5% (w/v) potassium hydroxide in a test tube. The mixture was heated at 110 °C for 3 h. After cooling at room temperature, the samples were acidified by the addition of 20% (v/v) sulphuric acid, and the lipids were extracted into diethyl ether. The ether extracts were washed with water until neutral, dried over anhydrous sodium sulphate, filtered and concentrated by evaporation to dryness. Lipids were then methylated with freshly prepared diazomethane. Three grams of N-Nitroso-N-methylurea (Sigma, St. Louis, MO, USA) were dissolved in a pre-cooled solution containing 45 ml of diethyl-ether, and 9 ml of 40% (w/v) KOH in distilled water. The mixture was agitated, and the supernatant was then removed and placed in a new tube cooled in ice containing potassium hydroxide pellets. A 2-ml portion of this solution was poured into each tube containing the dried lipids, and

methylation was achieved within 15 min. Diazomethane was then evaporated. Lipid extracts were resuspended in n-hexane, and then applied to silica gel plates.

From the rest of the cells, lipids were extracted and methylated by acid methanolysis as previously described [19] with slight modifications. Briefly, a spadeful of bacteria was scraped from the surfaces of agar plates. The cells were mixed with 2 ml of a reagent composed of 30 ml of methanol, 15 ml of toluene, and 1 ml of concentrated H_2SO_4 in a screw-cap test tube. The mixture was heated at 80 °C overnight. After being cooled at room temperature, the samples were extracted twice with 2 ml of n-hexane. The hexane upper layer was then removed, placed in a clean tube, and evaporated to dryness in a bath at 40 °C under a stream of nitrogen. The residue was dissolved in n-hexane to be applied to silica gel plates.

2.3. TLC analysis

Methyl mycolates were developed in one-dimensional (1D)-TLC using hexane/diethyl-ether (85:15, v/v) three times, or using dichloromethane (100%) once, and in two-dimensional (2D)-TLC using a triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction followed by a single development with toluene/acetone (97:3, v/v) in the second direction [20,21]. TLC plates were then sprayed with 10% (w/v) molybdophosphoric acid in absolute ethanol and heated at 120 °C.

For each TLC run, mycolate profiles obtained from *M. confluentis* strains were compared with those from reference strains chosen to represent the various mycolates [14,20,21].

2.4. NMR spectroscopic analysis

NMR analysis were performed on pure mycolates purified by preparative TLC. The mycolic acid methyl ester scraped from the TLC plates were recovered with diethyl ether. Etereal extracts were concentrated by evaporation and dissolved in 600 μl of $CDCl_3$ (99.80% D, Cortecnet, Voisins-le-Bretonneux, France) and transferred to NMR tubes.

NMR spectra of the resolved analytes were obtained on a Bruker Avance II 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm TBI probe with Z-gradients, operating at a ^1H and ^{13}C NMR frequencies of 600.13 and 150.90 MHz respectively and at 298.0 K of temperature. 1D ^1H NMR spectra were acquired using a standard 90° pulse sequence, with an acquisition time of 1.71 s and a relaxation delay of 2 s. The data were collected into 32 K computer data points, with a spectral width of 9590 Hz and as the sum of 128 transients. The resulting free inductions decays (FIDs) were Fourier transformed, manually phased, and baseline corrected. 2D NMR experiments such as ^1H–^1H COSY (Correlation Spectroscopy) and ^1H–^{13}C HSQC (Heteronuclear Single Quantum Correlation) were performed using standard pulse sequences (Bruker) and acquired under routine conditions. All the spectra were calibrated using the residual solvent signal ($CHCl_3$, 7.27 and 77.0 ppm for ^1H and ^{13}C respectively). Chemical shift data are expressed in ppm and coupling constant values in Hz.

3. Results and discussion

As the first four lanes of Fig. 2 show (lanes 1–4), the pattern of mycolic acids of *M. smegmatis*, *M. confluentis*, *M. phlei* and BCG seems to share type I and type IV mycolic acids. Only in the other four rows (lanes 5–8) is it possible to observe that *M. smegmatis* and *M. confluentis* have type V mycolic acid but *M. phlei* and BCG have

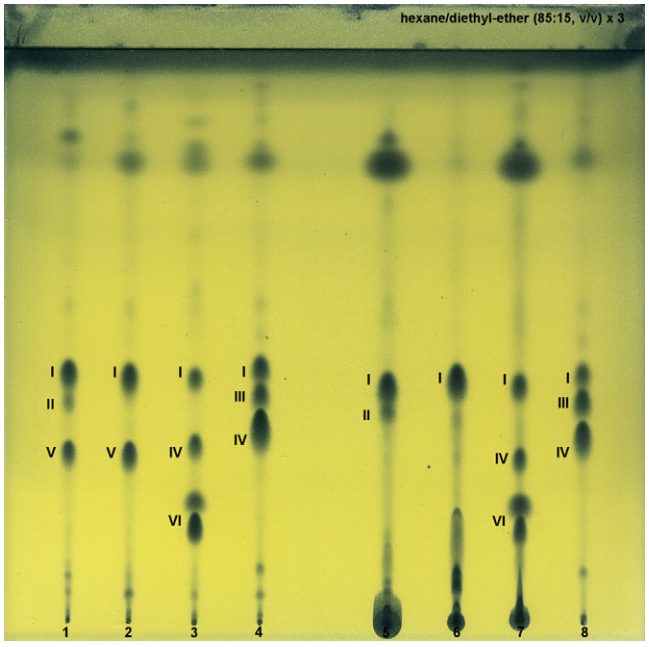

Fig. 2. One-dimensional thin-layer chromatography of methyl mycolates from *M. smegmatis* (lanes 1 and 5), *M. confluentis* (lanes 2 and 6), *M. phlei* (lanes 3 and 7) and BCG (lanes 4 and 8) obtained by saponification (lanes 1–4) or by acid methanolysis protocol (lanes 5–8). I, α-mycolates; II, α′-mycolates; III, methoxymycolates; IV, ketomycolates; V, epoxymycolates; VI, carboxymycolates. TLCs were developed three times with hexane/diethyl-ether (85:15, v/v), and revealed with molybdophosphoric acid.

type IV mycolic acid. If the samples are run in a 2D-TLC the situation is similar than in the 1D-TLC analysis. As Fig. 3(A–C) shows mycolic acid IV and V have a similar mobility pattern. Again, only in Fig. 3a–c, it is possible to differentiate the spot corresponding to mycolic acid V (Fig. 3a and b) and IV (Fig. 3c). The presence of type V mycolates in the mycobacterial cell wall is only confirmed when the result of acid methanolysis is shown. In both cases, 1D- and 2D-TLC analysis, the absence of the spot corresponding to type V mycolic acid can be appreciated together with the presence of more polar long-chain components next to the application point of the TLC. Thus, the results show that *M. confluentis* contain α-mycolates (type I) and epoxymycolates (type V) (Fig. 2, lane 2; and Fig. 3B).

In order to describe *M. confluentis*, Kirschner et al. [1] analysed the mycolic acid pattern of the isolate by TLC, comparing the profile with the known pattern of other mycobacteria run in parallel. They identify the two spots obtained as α-mycolates and keto-mycolates (type I and IV mycolic acids, respectively), when compared with *M. smegmatis* or *M. phlei* profiles among others. But there were some misinterpretations. On the one hand, *M. smegmatis* has three mycolic acids (type I, II and V) [17,22], but does not possess type IV mycolate, and the spot corresponding to type V mycolic acid appears in the same position as the type IV mycolic acid of *M. phlei*. Indeed, following the 2D-TLC protocol described by the authors, the spot corresponding to type IV could be confused with that belonging to type V mycolates (epoxymycolates) as we (Fig. 3) and others [16,23] have shown. Thus, neither in one-dimensional, using hexane/diethyl-ether (Fig. 2 and Table 1), nor in two-dimensional ([16,23], and Fig. 3) TLC elution systems it is possible to discern between type IV and V mycolic acids, since both lipids present the same mobility. On the other hand, Kirschner et al. followed an acid methanolysis procedure but there appears to be an acid-stable component both in *M. smegmatis* and *M. confluentis* analysis. As was showed before (Figs. 2 and 3) in agreement

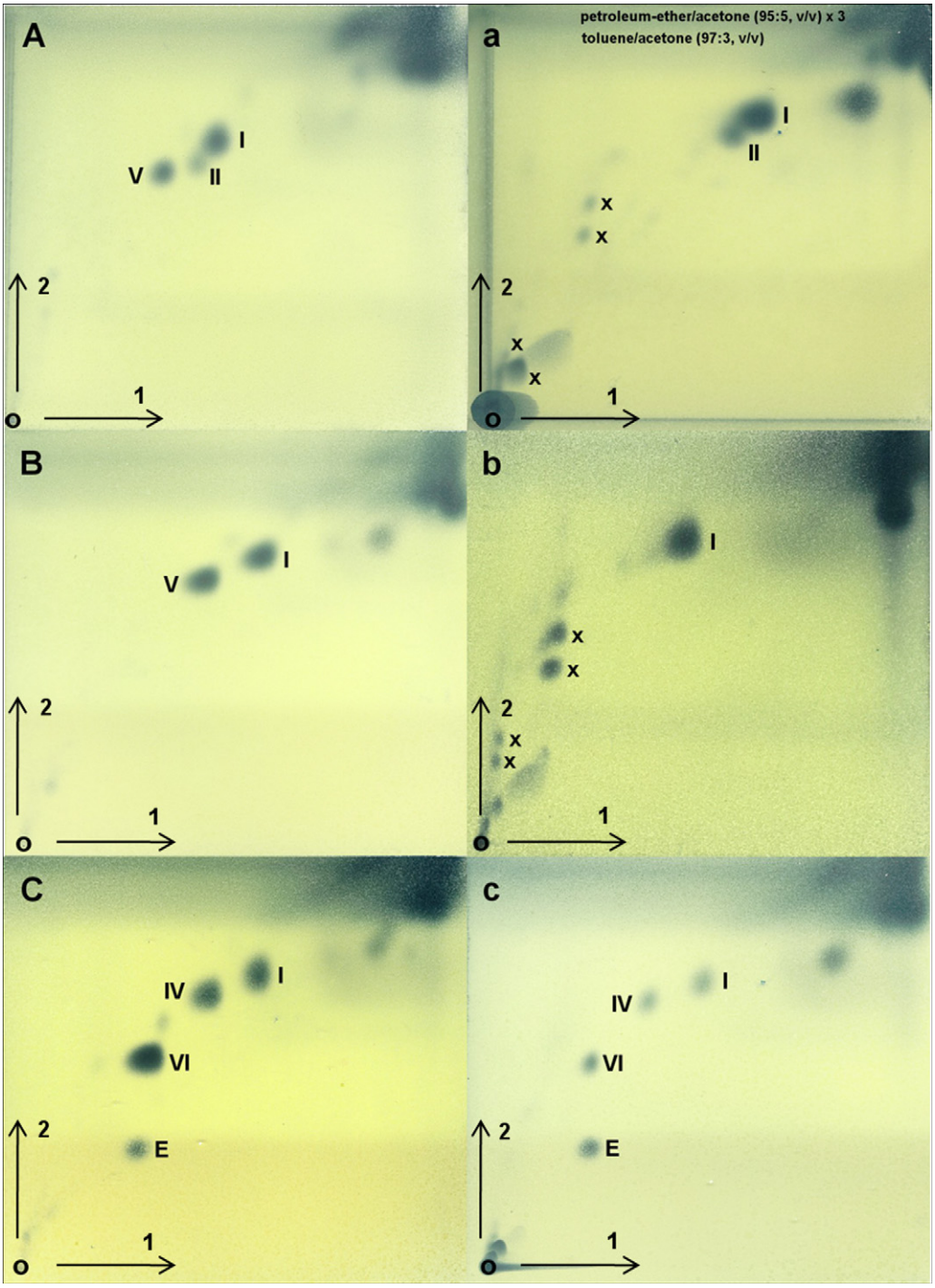

Fig. 3. Two-dimensional thin-layer chromatography of methyl mycolates from *M. smegmatis* (A and a), *M. confluentis* (B and b) and *M. phlei* (C and c) obtained by saponification (A–C) or by acid methanolysis protocol (a–c). I, α-mycolates; II, α′-mycolates; IV, ketomycolates; V, epoxymycolates; VI, carboxymycolates; E, long-chain alcohol (2-eicosanol and homologues); ×, components derived from epoxymycolates by acid methanolysis. TLCs were developed using a triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction, followed by a single development with toluene/acetone (97:3, v/v) in the second direction, and revealed with molybdophosphoric acid.

with the literature [16,17], type V mycolates are acid-sensitive and they degrade to hydroxymethoxy derivatives on release by acid methanolysis. Therefore, although it is possible that Kirschner et al., confused the pattern of mycolic acids due to the TLC elution systems used, it is not possible that the spot corresponding to type V mycolic acid appear after acid methanolysis extraction procedure.

There are two options to differentiate both mycolic acids: type IV and V. First, in order to avoid possible misidentifications and to know exactly which types of mycolic acid a mycobacterium has, the best option is to always carry out two different protocols: saponification and methanolysis of the cell wall, run both extracted lipids in parallel using TLC and compare the patterns

Table 1
Rf values of the different mycolic acid methyl esters on different 1D-TLC analysis.

Mycolic acid type	Rf values		
	Acid methanolysis[a]	Saponification[a]	
	Hexane/diethyl-ether (85:15, v/v)[b]	Hexane/diethyl-ether (85:15, v/v)[b]	Dichloromethane (100%)[b]
I	0.43–0.44	0.44–0.45	0.37–0.39
II	0.38	0.4	0.33
III	0.39	0.41	0.29
IV	0.31–0.33	0.35	0.27–0.29
V	–	0.31	0.21
VI	0.23	0.23	0.18

I, α-mycolates; II, α′-mycolates; III, methoxymycolates; IV, ketomycolates; V, epoxymycolates; VI, carboxymycolates. 1D-TLC, one-dimensional thin layer chromatography. Rf, retardation factor. Rf of each separated spot on the TLC plate was calculated using the following equation: distance traveled by the mycolic acid divided by distance traveled by the solvent front.

[a] Extraction procedure to obtain mycolic acids methyl esters as indicated in Section 2.

[b] Solvent system used to run TLC.

obtained (Fig. 2 and Table 1). A second option to differentiate type IV and V mycolates is to use dichloromethane as an elution system in TLC analysis. As Fig. 4 and Table 1 show, when TLC were run using dichloromethane and the saponification procedure was performed, type IV mycolic acid can be differentiated from type V, because type IV has the same mobility as type III mycolic acid, both (type III and IV) being combined at a unique spot (Fig. 4, lanes 1–4; Table 1).

Results described above were unambiguously confirmed by means of NMR spectroscopic analysis of the TLC spots assigned as mycolic acid methyl ester type V for *M. smegmatis* and *M. confluentis* and type IV for BCG (Fig. 5).

Fig. 5A shows the ^{1}H NMR spectrum of mycolic acid methyl esters isolated from the TLC spot assigned as type V for *M. smegmatis* (Fig. 2, lane 1). The spectrum shows a doublet of triplets at 2.72 ppm (coupling constants, J, 5.6 and 2.2 Hz) corresponding to the protons of the epoxy group [24]. An analogous peak is observed in the ^{1}H NMR spectrum of analytes isolated from the TLC spot assigned as type V for *M. confluentis* (Fig. 2, lane 2) (Fig. 5B). In the case of the BCG, shown in Fig. 5C (mycolic acid methyl esters isolated from the TLC spot assigned as type IV, Fig. 2, lane 4), no signal of protons from an epoxy group were observed. Furthermore, a multiplet at 2.51 ppm corresponding to a methynic proton adjacent to a keto group and a doublet at 1.06 ppm (J, 6.9 Hz), assigned to a methyl group bounded to the former methynic group were observed [25]. These peaks were corroborated by 2D NMR experiments such as ^{1}H–^{1}H-COSY and ^{1}H–^{13}C-HSQC.

In addition, peaks corresponding to ethylenic protons were observed in the *M. confluentis* sample (5.34 and 5.24 ppm), along with those of the methylene adjacent to double bond (2.01 ppm). Characteristic signals of cis-substituted cyclopropyl rings (0.65, 0.57 and −0.33 ppm) were observed in the BCG sample. In all cases typical signals of mycolic acid methyl esters were identified, such as the methyl ester singlet (3.72 ppm), β-carboxylic proton (3.66 ppm), α-carboxylic proton (2.44 ppm), methylene protons adjacent to the α-carboxylic group (1.71 ppm), methylene chains (1.27 ppm, broad), and terminal methyl groups (0.89 ppm, triplet) (indicated in Fig. 5C as an example) [24,25].

As far as we know, the only mention of the lipidic composition of an *M. confluentis* cell wall after Kirschner's [1] description was in the later review by Butler and Guthertz [12] who reported, using HPLC, that *M. confluentis* shares a late-emerging, complex, single-cluster peak pattern of mycolic acids with other mycobacteria. As we mentioned before, using HPLC it is not possible to determine the discrete composition of these lipids. The question remained unclear until now, the profile described initially by Kirschner et al. having been widely accepted [7,26].

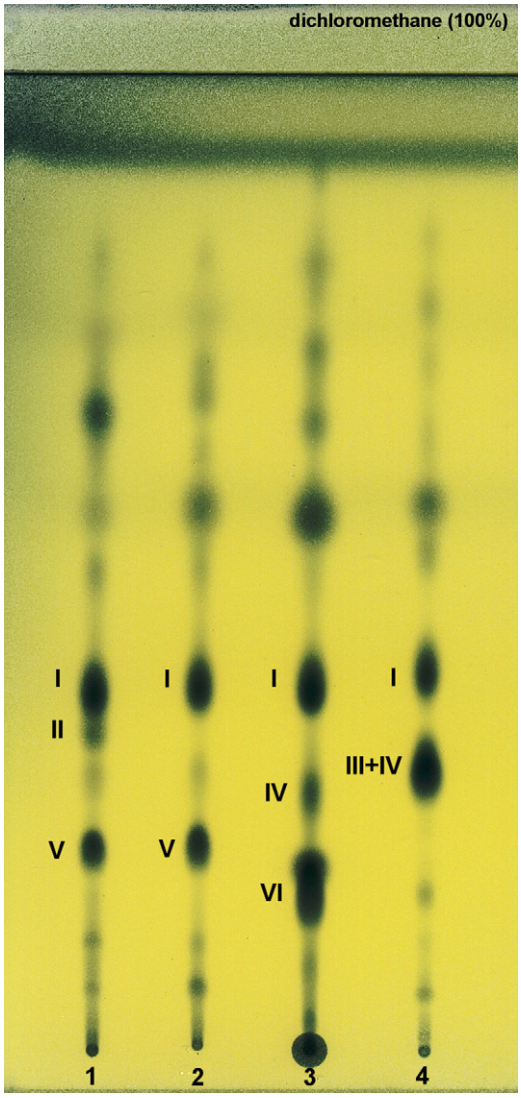

Fig. 4. One-dimensional thin-layer chromatography of methyl mycolates from *M. smegmatis* (lane 1), *M. confluentis* (lane 2), *M. phlei* (lane 3) and BCG (lane 4) obtained by saponification. I, α-mycolates; II, α′-mycolates; III + IV, methoxymycolates + ketomycolates; V, epoxymycolates; VI, carboxymycolates. TLCs were developed once with dichloromethane (100%), and revealed with molybdophosphoric acid.

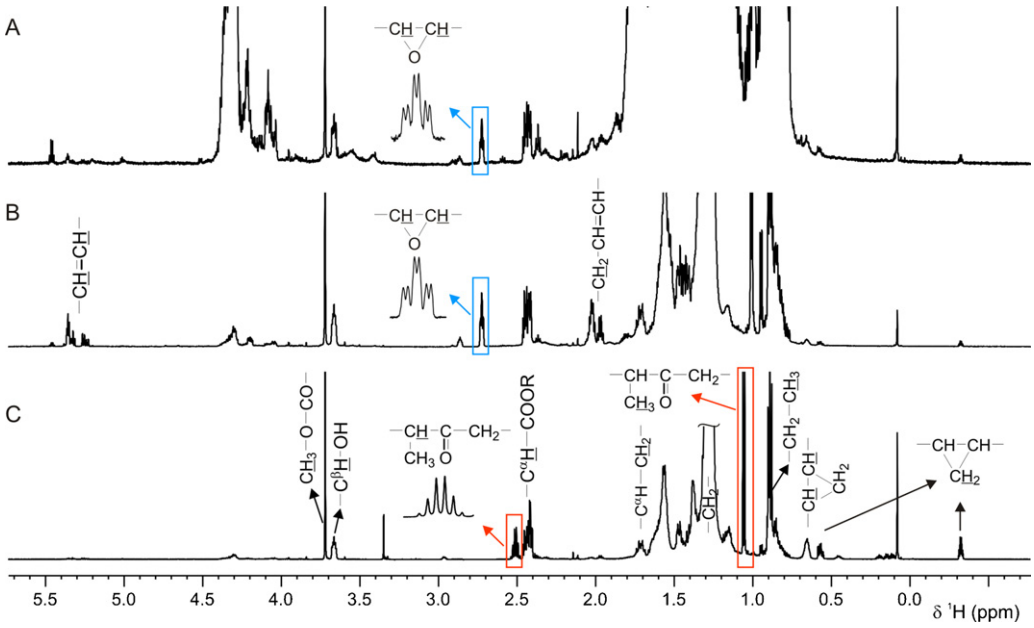

Fig. 5. ^{1}H NMR spectra of purified mycolic acid methyl esters from *M. smegmatis*, *M. confluentis* and BCG: (A) type V TLC spot from *M. smegmatis* (Fig. 2, lane 1), (B) type V TLC spot from *M. confluentis* (Fig. 2, lane 2) and (C) type IV TLC spot from BCG (Fig. 2, lane 4). Spectra were acquired at 298.0 K and at a magnetic field of 600 MHz.

4. Conclusion

In recent years, newly described mycobacteria species have been emerging incessantly. Due to the importance of the mycolic acid pattern for describing new species, it is important to make the content of each species clear, being thin-layer chromatography an advantageous tool. Accordingly, *M. confluentis* has to be considered to contain α-mycolates (type I) and epoxymycolates (type V mycolic acids).

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References

[1] P. Kirschner, A. Teske, K.H. Schröder, R.M. Kroppenstedt, J. Wolters, E.C. Böttger, Int. J. Syst. Bacteriol. 42 (1992) 257.
[2] S. Ehlers, E. Richter, Clin. Exp. Immunol. 124 (2001) 229.
[3] V.V. Lévy-Frébault, F. Portaels, Int. J. Syst. Bacteriol. 42 (1992) 315.
[4] S. Hartmans, J.A.M. de Bont, E. Stackebrandt, in: M. Dworkin, S. Falkow, E. Rosenberg, K.H. Schleifer, E. Stackebrandt (Eds.), The Prokaryotes, Springer, New York, 2006, p. 889.
[5] H.P. Hinrikson, G.E. Pfyffer, Med. Microbiol. Lett. 3 (1994) 49.
[6] F. Laval, M.A. Lanéelle, C. Déon, B. Monsarrat, M. Daffé, Anal. Chem. 73 (2001) 4537.
[7] C.E. Barry III, R.E. Lee, K. Mdluli, A.E. Sampson, B.G. Schroeder, R.A. Slayden, Y. Yuan, Prog. Lipid Res. 37 (1998) 143.
[8] L.Y. Gao, F. Laval, E.H. Lawson, R.K. Groger, A. Woodruff, J.H. Morisaki, J.S. Cox, M. Daffé, E.J. Brown, Mol. Microbiol. 49 (2003) 1547.
[9] J. Liu, C.E. Barry III, G.S. Besra, H. Nikaido, J. Biol. Chem. 271 (1996) 29545.
[10] J. Korf, A. Stoltz, J. Verschoor, P. De Baetselier, J. Grooten, Eur. J. Immunol. 35 (2005) 890.
[11] V. Rao, F. Gao, B. Chen, W.R. Jacobs Jr., M.S. Glickman, J. Clin. Invest. 116 (2006) 1660.
[12] W.R. Butler, L.S. Guthertz, Clin. Microbiol. Rev. 14 (2001) 704.
[13] N.C. Toney, S.R. Toney, W.R. Butler, Diag. Microbiol. Infect. Dis. 67 (2010) 143.
[14] A.F. Yassin, C. Binder, K.P. Schaal, Zentralbl. Bakteriol. 278 (1993) 34.
[15] D. Barkan, Z. Liu, J.C. Sacchettini, M.S. Glickman, Chem. Biol. 16 (2009) 499.
[16] M. Luquin, L. Margarit, M.J. Condom, V. Ausina, Int. J. Syst. Bacteriol. 37 (1987) 75.
[17] D.E. Minnikin, S.M. Minnikin, I.G. Hutchinson, M. Goodfellow, J.M. Grange, J. Gen. Microbiol. 130 (1984) 363.
[18] M. Daffé, M.A. Lanéelle, C. Asselineau, V. Lévy-Frebault, H. David, Ann. Microbiol. (Paris) 134B (1983) 241.
[19] D.E. Minnikin, J.H. Parlett, G. Dobson, M. Goodfellow, M. Magnusson, M. Ridell, in: M. Casal (Ed.), Mycobacteria of Clinical Interest, Elsevier Science Publishers, Amsterdam, 1985, p. 75.
[20] D.E. Minnikin, S.M. Minnikin, J.H. Parlett, M. Goodfellow, M. Magnusson, Arch. Microbiol. 139 (1984) 225.
[21] D.E. Minnikin, I.G. Hutchinson, A.B. Caldicott, M. Goodfellow, J. Chromatogr. 188 (1980) 221.
[22] M. Luquin, M.A. Lanéelle, V. Ausina, M. Garcia Barceló, F. Belda, C. Alonso, G. Prats, Int. J. Syst. Bacteriol. 41 (1991) 390.
[23] D.E. Minnikin, S.M. Minnikin, A.G. O'Donnell, M. Goodfellow, J. Microbiol. Methods 2 (1984) 243.
[24] P. Dinadayala, F. Laval, C. Raynaud, A. Lemassu, M.A. Lanéelle, G. Lanéelle, M. Daffé, J. Biol. Chem. 278 (2003) 7310.
[25] M. Watanabe, A. Ohta, S. Sasaki, D.E. Minnikin, J. Bacteriol. 181 (1999) 2293.
[26] E. Tortoli, Clin. Microbiol. Rev. 16 (2003) 319.