Analysis of the structure of mycolic acids of *Mycobacterium simiae* reveals a particular composition of α -mycolates in strain 'habana' TMC 5135, considered as immunogenic in tuberculosis and leprosy

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Structural analysis of mycolic acids from *Mycobacterium simiae* (including some 'habana' strains) was carried out using ¹H-NMR and MS. Results indicated that this species presents a general pattern of α -, α' - and keto-mycolates. α -Mycolates were composed of a complex mixture of 82 to 89 carbon atoms (C82–C89), with the predominant molecular species containing two di-substituted cyclopropane rings. Among keto-mycolates (C84–C89), those containing one *trans* di-substituted cyclopropane ring were the most abundant. The α' -mycolates were monounsaturated (C64, C66). According to MS and ¹H-NMR data, the strains studied differed in fine structural details of α -mycolates and keto-mycolates. Notably, strain 'habana' TMC 5135 (belonging to the 'habana' group, and considered as highly immunogenic in tuberculosis and leprosy) presented a particular composition of α -mycolates, with a major component (C87) containing one *cis* plus one *trans* di-substituted cyclopropane ring, unlike the type strain of *M. simiae* and other strains of the 'habana' group (IPK-220 and IPK-337R), in which the major component (C84) contained two *cis* di-substituted cyclopropane rings. In spite of this finding, the 'habana' strains were closely related to each other and mainly differed from the type strain of *M. simiae* in some details of the fine structure of keto-mycolates. The present work indicated that within an identical general pattern of mycolic acids, there is a complex composition in *M. simiae* and structural variation among different strains, as reported for pathogenic species of the genus. Noteworthy was the particular composition of α -mycolates in strain 'habana' TMC 5135.

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INTRODUCTION

The genus *Mycobacterium* includes several opportunistic and saprophytic bacteria, together with the causative agents of tuberculosis and leprosy. *Mycobacterium simiae* is considered an opportunistic pathogen (Falkinham, 1996). Included in this species is a group of strains, originally isolated in Cuba from people suffering pulmonary infections, that was named '*Mycobacterium habana*' (Valdivia, 1973). Strains of '*Mycobacterium habana*' are phenotypically identical to serotype 1 of *Mycobacterium simiae*, and show the same serological specificity (Meissner & Schröder, 1975). Although the synonymy between *M. simiae* and '*M. habana*' has been clearly demonstrated (Weiszfeiler & Karczag, 1976), the epithet '*habana*' is maintained for the

original collection of strains, in particular because a series of investigations has suggested a vaccine potential for strain '*M. habana*' TMC 5135 in tuberculosis and leprosy (reviewed by Mederos *et al.*, 2006).

Mycolic acids are long-chain α -alkyl-branched, β -hydroxylated fatty acids present in the cell envelope of mycobacteria and related micro-organisms. They are mainly linked to the arabinogalactan of the cell wall, although they also appear among the extractable lipids of the cell envelope of mycobacteria, for example esterified to trehalose, forming cord factor (dimycolates of trehalose) (Brennan & Nikaido, 1995; Daffé & Draper, 1998). In the genus *Mycobacterium*, the mycolic acids can be roughly separated into two groups: those containing additional oxygenated functions (methoxy-, keto-, epoxy-, carboxymycolates) in the so-called 'mero' chain, and those that do not (α - and α' -mycolates). Pathogenic mycobacteria contain complex

Abbreviations: MAME, mycolic acid methyl ester; EI-MS, electron-impact MS; FAB-MS, fast-atom bombardment MS.

mixtures of α -mycolates with, in general, two *cis* di-substituted cyclopropane rings, and oxygenated mycolic acids, with one di-substituted cyclopropane ring (Minnikin, 1982; Watanabe *et al.*, 2001). Molecular evidence suggests that *cis* di-substituted cyclopropane α -mycolates and *trans* di-substituted oxygenated mycolic acids are implicated in some aspects of the pathogenesis of experimental tuberculosis (Riley, 2006). On the other hand, mycolic acids are currently employed as chemotaxonomic markers, and their analysis has been applied in the identification of the most important pathogens of the genus *Mycobacterium* (Barry *et al.*, 1998; Butler & Guthertz, 2001).

The structure of polar glycopeptidolipids of '*M. habana*' TMC 5135 has been reported (Khoo *et al.*, 1996), as well as the general composition of lipids of several strains within the '*habana*' group (Mederos *et al.*, 1998), and this has taxonomic potential. However, the analysis of the fine structure of the mycolic acids of *M. simiae* is still pending. In the present work, we focused on this subject, including the type strain of the species and three '*habana*' strains. The results obtained indicated that '*M. habana*' TMC 5135, an immunogenic strain in tuberculosis and leprosy (Mederos *et al.*, 2006), presents a particular composition of α -mycolates within the general pattern of α -, α' - and keto-mycolates found in *M. simiae* (Minnikin *et al.*, 1984).

METHODS

Strains studied and culture conditions. The following strains of *M. simiae* were studied: ATCC 25275^T, '*habana*' TMC 5135, '*habana*' IPK-220 and '*habana*' IPK-337R. All of them belong to the collection of the 'Instituto de Medicina Tropical Pedro Kourí (IPK), La Habana', Cuba. They were cultivated for 3 weeks at 37 °C in liquid UIT-L medium (a modified Löwenstein-Jensen medium) (Mederos *et al.*, 1992), checked for purity, collected, washed with saline, killed at 120 °C for 20 min, and dried in a vacuum over P₂O₅.

Extraction and purification of mycolic acids. Dried cells (0.5–1.0 g) were extracted overnight at room temperature with ethanol/diethyl ether (1:1, v/v), filtered, and re-extracted with chloroform/methanol (1:1, v/v) under the same conditions (Khoo *et al.*, 1996). Extractable lipids were employed for polar glycopeptidolipid analysis (data not shown). Delipidated cells were subjected to acid methanolysis in a mixture of dried methanol/toluene/H₂SO₄ (30:15:1, v/v), overnight at 75 °C (Minnikin *et al.*, 1980). The extract was evaporated to dryness under nitrogen, and the liberated mycolic acid methyl esters (MAMEs) were purified by silica gel 60 (Merck) (particle size 0.063–0.200 μ m) column chromatography employing diethyl ether in hexane (0, 2, 5, 7, 10, 15 and 20 %) as solvent. Fractions (3–5 ml) were collected, evaporated to dryness and analysed by analytical TLC (aluminium-backed silica-gel plates, Merck) using dichloromethane as solvent. Purified fractions with the same structural type of MAMEs were combined and analysed by ¹H-NMR and MS (see below). When necessary, these compounds were repurified using preparative TLC (silica-gel plates, Merck), as previously described (Mederos *et al.*, 1998). We did not find a significant influence of acid methanolysis on cyclopropane rings or double bonds, because no artefacts were revealed by ¹H-NMR or MS. On the other hand, the recommendations of Watanabe *et al.* (2001) in the purification of mycolates were followed, to avoid mistakes with the proportion of *cis/trans* isomerism.

Structural analysis of MAMEs. ¹H-NMR was carried out in a Bruker instrument, at 400 or 600 MHz at room temperature, with the samples dissolved in deuteriochloroform (5–10 mg ml⁻¹). Electron-impact MS (EI-MS) and fast-atom bombardment MS (FAB-MS) were performed in a VG AutoSpec instrument, as previously reported (Astola *et al.*, 2002). For FAB-MS, *m*-nitrobenzyl alcohol was used as matrix and the samples were doped with NaCl. EI-MS provides information on the meroaldehyde chains, where cyclopropane rings and oxygenated functions are present (Minnikin, 1982), and on the methyl esters, which are diagnostic of the branched chains located at the α position (Minnikin, 1982). FAB-MS reveals [M+Na]⁺ ions indicative of the molecular masses of the different molecular species of mycolates (Astola *et al.*, 2002).

RESULTS

As previously reported (Mederos *et al.*, 1998; Minnikin *et al.*, 1984), α -, α' - and keto-mycolates were present in the four strains studied. All mycolates could be identified without ambiguity by comparison of the ¹H-NMR spectra obtained with those reported elsewhere (Watanabe *et al.*, 1999, 2001). Illustrating these results, the ¹H-NMR spectrum of α -mycolates from strains '*M. habana*' TMC 5135 (Fig. 1a) and '*M. habana*' IPK-220 (Fig. 1b) are given. Signals for α - and keto-mycolates centred at -0.33, 0.57 and 0.66 p.p.m. were attributed to a *cis* di-substituted

Fig. 1. ¹H-NMR spectra of α -mycolic acids (methyl esters) from *M. simiae*. (a) '*M. habana*' TMC 5135; (b) '*M. habana*' IPK-220. The different resonances are identified. *cis*-cp and *trans*-cp, *cis* and *trans* di-substituted cyclopropane rings, respectively; *trans*-db, *trans* double bond; CH₃t, terminal CH₃; CH₃b, branched CH₃; TMS, tetramethyl silane; H₂O, water as an impurity.

cyclopropane ring, and those at 0.09–0.18 and 0.45 p.p.m. to a *trans* di-substituted cyclopropane ring. An additional proton resonating in the region centred at 0.66 p.p.m. was attributed to a CH–CH₃ adjacent to a *trans* di-substituted cyclopropane ring. Chemical shifts of *trans* double bonds were located at 5.21 and 5.34 p.p.m. On the other hand, terminal CH₃ and branched CH₃ (adjacent to a *trans* di-substituted cyclopropane ring) resonances overlapped in the region 0.87–0.90 p.p.m. Branched methyl groups adjacent to a *trans* double bond and to the C=O (keto-mycolates) resonated as doublets at 0.94 and 1.05 p.p.m., respectively. Signals for the *cis* double bond in α' -mycolates resonated at 5.36 p.p.m. The ratio terminal CH₃/*cis* double bond for α' -mycolates was always ~2.0, indicating that this lipid contained one double bond only.

The area ratios for the main signals considered of α - and keto-mycolates are depicted in Table 1. It was deduced that all strains studied contained at least one *cis* di-substituted cyclopropane ring in α -mycolates, and that molecular species with two *cis* di-substituted cyclopropane rings, with one *cis* plus one *trans* di-substituted cyclopropane ring, and with one *cis* di-substituted cyclopropane ring plus one *trans* double bond were present in the complex mixture of this compound (see below, Table 2). Similarly, major keto-mycolates with one *trans* di-substituted cyclopropane ring were predicted, although homologous series with one *cis* di-substituted cyclopropane ring or with one *trans* double bond completed the mixture of this kind of mycolate (see below, Table 3). Examining the data from Table 1, it was evident that strains of the 'habana' group exhibited more internal relationship among themselves than with *M. simiae* ATCC 25275^T, although 'M. habana' TMC 5135 tended to have more *trans* cyclopropanation in α -mycolates. On the other hand, *M. simiae* ATCC 25275^T contained more *cis* cyclopropanation in α -mycolates, and notably in keto-mycolates, than the 'habana' strains (Table 1).

Meroaldehyde chains of α - and keto-mycolates are given in Tables 2 and 3, respectively. In general, all the strains studied produced the same meroaldehydes from the

mycolates detected, although their relative abundances varied among the strains (Tables 2 and 3). Thus, in strains ATCC 25275^T, IPK-220 and IPK-337R, *m/z* 824.6 (C58) predominated, whereas in strain TMC 5135, *m/z* 866.6 (C61) reached higher levels (Table 2). The major meroaldehyde from keto-mycolates was always *m/z* 882.6 (C61) (Table 3). Both α - and keto-mycolates mainly produced C26:0 methyl ester (*m/z* 410.2), and a small amount of C24:0 methyl ester (*m/z* 382.2). The major meroaldehyde from α' -mycolates was detected at *m/z* 574.5, whereas methyl esters appeared at *m/z* 410.2 and *m/z* 382.2 (the proportions of *m/z* 410.2 to *m/z* 382.2 varied from 4:1 to 2:1 depending on the strains).

The chain lengths of α - (Table 2) and keto- (Table 3) mycolates were predicted from EI-MS spectra, and confirmed by FAB-MS. FAB-MS spectra of α -mycolates are shown in Fig. 2. Notably, α -mycolates from strain TMC 5135 varied from C84 to C89 (as free acids), with a major C87 component (Table 2), unlike ATCC 25275^T, IPK-220 and IPK-337R, in which C84 appears as the major component (Table 2). Keto-mycolates ranged from C84 to C89, with a major component, in all cases, of C87 (Table 3). α' -Mycolates were mono-unsaturated derivatives of C66 and C64. Examining ¹H-NMR and MS data (Tables 1–3), and taking into account earlier work (Watanabe *et al.*, 2001, 2002), it was deduced that α -mycolates belong to the so-called α 1 (no double bonds present) and α 2 (with *trans* double bonds) series (Table 2), and keto-mycolates to the keto1 (no double bonds) and keto2 (with *trans* double bonds) series (Table 3). Based on earlier complete analyses of mycolic acids from different mycobacteria (Minnikin 1982; Watanabe *et al.*, 2001, 2002), the even series of α -mycolates should be composed by two *cis* di-substituted cyclopropane rings (α 1) or one *cis* di-substituted cyclopropane ring plus one *trans* double bond (α 2), whereas uneven chain length series of α -mycolates should mainly contain one *cis* di-substituted plus one *trans* di-substituted cyclopropane ring (α 1). On the other hand, the keto1 series with uneven chain length should mainly include one *trans* di-substituted cyclopropane ring in the structure (Minnikin, 1982; Watanabe *et al.*, 2001), whereas the keto2

Table 1. ¹H-NMR analysis of mycolic acids from several strains of *M. simiae*

*cis*Δ, *cis* di-substituted cyclopropane ring; *trans*Δ, *trans* di-substituted cyclopropane ring; *trans*-db, *trans* double bond. Total mycolates were calculated according to Watanabe *et al.* (2001).

Strain	Mycolic acid	<i>cis</i> Δ/ <i>trans</i> Δ	<i>cis</i> Δ/ <i>trans</i> -db	<i>trans</i> Δ/ <i>trans</i> -db	Total mycolates/ <i>cis</i> Δ	Total mycolates/ <i>trans</i> Δ
TMC 5135	α	1.47	2.80	1.90	1.01	1.50
	Keto	0.11	0.42	3.72	10.20	1.15
ATCC 25275 ^T	α	3.14	4.91	1.56	1.08	3.39
	Keto	0.55	1.11	2.03	2.23	1.22
IPK-220	α	2.26	3.00	1.32	1.03	2.33
	Keto	0.13	0.15	5.23	6.66	1.17
IPK-337R	α	2.42	2.43	1.00	1.04	2.52
	Keto	0.09	0.31	3.61	13.50	1.19

Table 2. MS analysis and proposed general structure of α -mycolic acids of several strains of *M. simiae*

Tentative structures for major compounds (in bold italic type) for strains TMC 5135 and ATCC 25275^T are given in Fig. 3. *cis* Δ + *trans* Δ or *cis* Δ + *cis* Δ indicates that the α -mycolate contains one *cis* plus one *trans* di-substituted cyclopropane ring, or two *cis* di-substituted cyclopropane rings, respectively (see Watanabe *et al.*, 2001, 2002). ND, Not done; *trans*-db, *trans* double bond.

Strain	Meroaldehyde chain			Chain length (free acid)	[M + Na] ⁺⁺		Type
	<i>m/z</i>	Chain length	Relative proportion		Monoisotopic mass	Relative proportion	
TMC 5135	824.6	C58	62	C84	1258.5	61	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
	838.6	C59	48	C85	1272.5	57	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
	852.6	C60	49	C86	1286.4	21	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
	866.6	C61	100	C87	1300.6	100	α1 (<i>cis</i>Δ + <i>trans</i>Δ)
	894.6	C63	42	C89	1328.7	11	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
ATCC 25275 ^T	796.6	C56	34	C82	1230.2	58	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
	810.6	C57	28	C83	1244.2	16	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
	824.6	C58	100	C84	1258.3	100	α1 (<i>cis</i>Δ + <i>cis</i>Δ) and α2 (<i>cis</i>Δ + <i>trans</i>-db)
	838.6	C59	64	C85	1272.3	36	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
	852.6	C60	21	C86	1286.3	30	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
IPK-220 and IPK-337R	866.6	C61	16	C87	1300.3	29	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
	796.6	C56	15/20	C82	ND	ND	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
	824.6	C58	100/100	C84	ND	ND	α1 (<i>cis</i>Δ + <i>cis</i>Δ) and α2 (<i>cis</i>Δ + <i>trans</i>-db)
	838.6	C59	35/45	C85	ND	ND	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)
	852.6	C60	42/73	C86	ND	ND	α 1 (<i>cis</i> Δ + <i>cis</i> Δ) and α 2 (<i>cis</i> Δ + <i>trans</i> -db)
	866.6	C61	42/70	C87	ND	ND	α 1 (<i>cis</i> Δ + <i>trans</i> Δ)

*Experimental [M + Na]⁺⁺ ions for methyl esters of α -mycolic acids are given with a maximum error of 160 p.p.m.

series with an even chain length should contain one *trans* double bond. In the specific case of strain ATCC 25275^T, keto3 mycolates (with one *cis* double bond) may also be predicted.

To arrive at a plausible tentative structure of the major mycolate types for the different strains of *M. simiae*, putative fragments of the meroaldehyde chain were checked in the EI-MS spectra (region *m/z* 250–650) (not shown). In strain '*M. habana*' TMC 5135, a meroaldehyde C61 was predicted for *m/z* 866.6 (Table 2), and a major fragment attributed to the meroaldehyde was found at *m/z* 613.3. It was considered that a further major fragment located at *m/z* 321.1 was compatible with the structure proposed for α 1-mycolates in Fig. 3(a). In a similar way, the major meroaldehyde for *M. simiae* ATCC 25275^T, '*habana*' IPK-220 and '*habana*' IPK-337R was at *m/z* 824.4 (C58) (Table 2). In the case of *M. simiae* ATCC 25275^T, the major fragments for the C58 meroaldehyde were tentatively assigned to *m/z* 571.2 and *m/z* 307.1, thus justifying the

structure proposed in Fig. 3(b). However, strains '*habana*' IPK-220 and '*habana*' IPK-337R gave similar abundances for *m/z* 613.3 and *m/z* 571.2 (even *m/z* 613.3 tended to be higher), probably indicating that the abundances of C58 and C61 meroaldehydes were actually equivalent. The major meroaldehyde from keto1-mycolates (C87) contained C61 in all strains studied (Table 3), and it was thought to fragment at *m/z* 601.3, and subsequently at *m/z* 293.2, as found in the EI-MS (not shown): a tentative structure is proposed in Fig. 3(c).

DISCUSSION

The general pattern and structure of the mycolic acids of the different strains of *M. simiae* were similar, as expected (Mederos *et al.*, 1998; Minnikin *et al.*, 1984). The major α -mycolates combined two di-substituted cyclopropane rings, and the major oxygenated mycolates (keto-mycolates) contained one *trans* di-substituted cyclopropane ring.

Table 3. MS analysis and proposed structure of keto-mycolic acids of several strains of *M. simiae*

Tentative structures for major compounds (in bold italic type) for strains TMC 5135 and ATCC 25275^T are given in the text. *cis*Δ or *trans*Δ indicates that the keto-mycolate contains one *cis* or one *trans* di-substituted cyclopropane ring in the structure, respectively. *trans*-db, *trans*-double bond. ND, Not done.

Strain	Mero chain			Chain length of the free acid	[M + Na] ⁺ *		Type
	<i>m/z</i>	Chain length	Relative proportion		Monoisotopic mass	Relative proportion	
TMC 5135	840.6	C58	25	C84	1274.5	4	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	854.6	C59	61	C85	1288.5	67	keto1 (<i>trans</i> Δ)
	868.6	C60	26	C86	1302.2	4	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	882.6	C61	100	C87	1316.6	100	keto1 (<i>trans</i>Δ)
	910.6	C63	41	C89	1344.8	10	keto1 (<i>trans</i> Δ)
ATCC 25275 ^T	840.6	C58	53	C84	Not detected	0	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	854.6	C59	69	C85	1288.3	38	keto1 (<i>trans</i> Δ)
	868.6	C60	56	C86	1302.2	4	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	882.6	C61	100	C87	1316.4	100	keto1 (<i>trans</i>Δ)
	910.6	C63	28	C89	1345.0	8	keto1 (<i>trans</i> Δ)
IPK-220 and IPK-337R	840.6	C58	28/35	C84	ND	ND	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	854.6	C59	51/49	C85	ND	ND	keto1 (<i>trans</i> Δ)
	868.6	C60	52/78	C86	ND	ND	keto2 (<i>trans</i> -db), keto1 (<i>cis</i> Δ)
	882.6	C61	100/100	C87	ND	ND	keto1 (<i>trans</i>Δ)
	910.6	C63	0/37	C89	ND	ND	keto1 (<i>trans</i> Δ)

*Experimental [M + Na]⁺ ions for methyl esters of keto-mycolic acids are given with a maximum error of 350 p.p.m.

Thus, they belonged to the α1- and keto1-mycolate types, respectively, as defined by Watanabe *et al.* (2001, 2002). As depicted in Tables 2 and 3, α2- and keto2-mycolates could

Fig. 2. FAB-MS spectra of α-mycolic acids (methyl esters) from *M. simiae*. (a) '*M. habana*' TMC 5135; (b) *M. simiae* ATCC 25275^T. The chain lengths of α-mycolates (as free acids) are indicated. The different *m/z*, [M + Na]⁺, are rounded up to the next highest unit.

Fig. 3. Tentative structures of major α1- (a, b) and keto1- (c) mycolic acids (methyl esters) of *M. simiae*. (a) '*M. habana*' TMC 5135 (the tentative mass spectral fragmentation is indicated); (b) *M. simiae* ATCC 25275^T, '*M. habana*' IPK-220, '*M. habana*' IPK-337R. The general structure shown in (a) has been detected elsewhere in α-mycolates from *M. kansasii* and the *M. avium* complex (Watanabe *et al.*, 2002).

also be detected in all strains examined. The α -mycolates appeared unexpectedly long in comparison with those found in pathogenic mycobacteria (Minnikin, 1982; Watanabe *et al.*, 2001, 2002), but it is uncertain if additional di-substituted cyclopropane rings or double bonds are also present in the mero chains of α - and keto-mycolates, as found for different strains of the *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii* and *Mycobacterium avium* (Watanabe *et al.*, 2001, 2002). The same general mycolic acid composition as that of *M. simiae* has also been found in *M. avium* (Watanabe *et al.*, 2001, 2002), although pathogenic mycobacteria mostly contain α 1-mycolates with two *cis* di-substituted cyclopropane rings (Minnikin, 1982; Watanabe *et al.*, 2001, 2002); however, this is also similar to the mycolic acid composition of the saprophytic species *M. goodii* (Astola *et al.*, 2002).

'*M. habana*' TMC 5135 tended to have longer α -mycolates, and the major ones combined one *cis* plus one *trans* di-substituted cyclopropane ring in their structure. This kind of α -mycolate has been detected elsewhere in *M. kansasii* and the *M. avium* complex (Watanabe *et al.*, 2002). Data from Table 2 strongly suggested that this kind of mycolate prevails in strain TMC 5135, in contrast to other strains, in which α -mycolates with two *cis* di-substituted cyclopropane rings could be predicted to be the most abundant. This was not reflected in the HPLC pattern previously reported for these strains (Mederos *et al.*, 1998), probably because *trans* or *cis* isomerism does not influence the results of HPLC. Also noteworthy was the fact that the major α - and keto-mycolates presented similar chain length in '*M. habana*' TMC 5135, unlike the other strains studied. This suggests that this strain has some particular variations in the general routes of biosynthesis of α -mycolates (see Takayama *et al.*, 2005), because major α -mycolates are slightly shorter than keto-mycolates (Minnikin, 1982; Watanabe *et al.*, 2001, 2002).

Although, in general, similar molecular species were predicted for the different strains, the results obtained revealed that the '*habana*' group were closely related to one another, differing from the type strain of *M. simiae*. We do not assign taxonomic implications to this finding, because DNA–DNA hybridization analyses have confirmed the synonymy between the two micro-organisms (Baess & Magnusson, 1982). On the other hand, variations in the fine structure of mycolates have been noted within strains of the same species of the genus *Mycobacterium* (Watanabe *et al.*, 2001, 2002).

A particular and distinctive composition of α -mycolates in '*M. habana*' TMC 5135 could be defined. This strain is considered highly immunogenic in experimental tuberculosis in mice, and has received interest from several investigators as a possible vaccine candidate in tuberculosis and leprosy (reviewed by Mederos *et al.*, 2006). Obviously, at present, it is not known if the fine structure of α -mycolic acids has an influence on the immunogenic properties of this strain. Recent experimental data indicate that strains '*M.*

habana' TMC 5135 and '*M. habana*' IPK-220, which differ in the fine structure of mycolates (this work), are not equally immunogenic or virulent in a BALB/c mouse model of progressive pulmonary tuberculosis (Valdés *et al.*, 2007a, b). Thus, the load of '*M. habana*' IPK-220 was higher than that of '*M. habana*' TMC 5135, and the latter strain rapidly produced small granulomas (Valdés *et al.*, 2007b). In connection with this finding, '*M. habana*' TMC 5135 induces the production of interferon (INF)- γ in vaccinated mice when different type of cells are stimulated with various antigens of *M. tuberculosis*, whereas '*M. habana*' IPK-220 fails in the induction of this molecule (Valdés *et al.*, 2007a). These results cannot be directly attributed to mycolic acid composition, because both strains also differ in the polar glycopeptidolipid TLC profile (Mederos *et al.*, 1998). It was noteworthy, however, that mutants of *M. tuberculosis* lacking a proximal *cis* di-substituted cyclopropane ring in α -mycolates (Rao *et al.*, 2005) or a *trans* di-substituted cyclopropane ring in oxygenated mycolates (Rao *et al.*, 2006) differ from wild strains in their pathogenic and immunogenic properties. Moreover, mutants of *M. tuberculosis* altered in their capacity to synthesize oxygenated mycolates present attenuated virulence in mice (Bhatt *et al.*, 2007; Dubnau *et al.*, 2000). The true significance of these data for human tuberculosis is still obscure (Riley, 2006), although they might indicate that the fine structure of mycolic acids has some influence in the pathogenesis of tuberculosis. The mechanisms involved are unknown, but it should be noted that mycolic acids (Beckman *et al.*, 1994) and other lipids (de la Salle *et al.*, 2005; Schaible & Kaufmann, 2000) are recognized by a subset of T cells in a CD1-restricted way. It has also been reported that infected macrophages release and traffic a variety of mycobacterial lipids, mostly glycolipids (Rhoades *et al.*, 2003), and that the '*in vivo*' induction of several cytokines [interleukin (IL)-1a, IL-1b, tumour necrosis factor (TNF)- α] related to granuloma formation is mainly due to trehalose di-mycolates (Geisel *et al.*, 2005). In this context, it would be interesting to know the exact structure of the trehalose di-mycolates of '*M. habana*' TMC 5135 and compare their biological activity to that of other strains of *M. simiae*.

In conclusion, the present work reveals that within a similar mycolic acid pattern, the different strains examined of *M. simiae* present variations in the fine structure of these compounds, a finding that has also been noted in several pathogenic species of the genus (Watanabe *et al.*, 2001, 2002). '*M. habana*' TMC 5135 shows a particular combination of α -mycolates that mostly combines one *cis* di-substituted plus one *trans* di-substituted cyclopropane ring. It remains open to further research if the fine structure of the mycolic acids of '*M. habana*' TMC 5135 has an influence on the immunogenic properties of the strain.

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