Analysis of lipids reveals differences between '*Mycobacterium habana*' and *Mycobacterium simiae*

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Fatty and mycolic acids and the pattern of glycolipids were studied in a collection of 34 strains of '*Mycobacterium habana*' and in two strains of *Mycobacterium simiae*. Major glycolipids of these micro-organisms were assigned to the glycopeptidolipid (GPL) structural type, but both mycobacteria differed in the patterns obtained by TLC. The strains of '*M. habana*' were separated into four groups (A–D), taking into account the presence or absence of several polar GPLs: group A contained GPL-I, GPL-II and GPL-III; group B contained GPL-I, GPL-II', GPL-II and GPL-III; group C contained GPL-II', GPL-II and GPL-III; group D did not contain any of these compounds. Fatty acids of both bacteria were similar, and ranged from 14 to 26 carbon atoms, hexadecanoic, octadecenoic and tuberculostearic acids being predominant. Mycolic acids were also similar by TLC and HPLC, and consisted of α -, α' - and ketomycolates. Partial structural analysis by MS carried out in strains '*M. habana*' TMC 5135 and *M. simiae* ATCC 25275^T revealed that α - and ketomycolates ranged, in general, from 79 to 87 carbon atoms, and α' -mycolates from 58 to 67 carbon atoms. The α - and ketomycolates belonged to several structural series, and minor variations were found between the two strains examined. The data obtained justified the synonymy between '*M. habana*' and *M. simiae* but indicated, in turn, that the former can be distinguished on the basis of GPL analysis. Most strains of '*M. habana*' can be defined by the presence of GPL-II and GPL-III, a finding that could be useful in the quality control of potential vaccine strains.

Keywords: '*Mycobacterium habana*', *Mycobacterium simiae*, lipids, glycopeptidolipid, chemotaxonomy

INTRODUCTION

The genus *Mycobacterium* includes the aetiological agents of tuberculosis and leprosy, and also some 'atypical' species that produce a variety of infections in humans. Among the latter, *Mycobacterium simiae* is an opportunistic non-tuberculous mycobacterial species mainly causing lung infection (Wayne & Sramek, 1992) and is also implicated in disseminated infections in patients with AIDS (Lévy-Frébault *et al.*, 1987; Vander-cam *et al.*, 1996).

'*Mycobacterium habana*' was first isolated in Cuba from humans suffering from lung disease and proposed as a new species within the genus *Mycobacterium* (Valdivia, 1973; Valdivia-Álvarez *et al.*, 1971). However, biochemical and serological analyses (Meissner & Schröder, 1975; Weiszfeiler *et al.*, 1981) led to it being considered synonymous with *M. simiae*, a species defined in 1965 (Karasseva *et al.*, 1965) for which two serotypes have been described.

'*M. habana*' was included in *M. simiae* serotype I, but some serological differences between both micro-organisms remained without satisfactory explanation (Meissner & Schröder, 1975; Pattyn & Dommissie, 1973) and the name '*M. habana*' has been maintained in

Abbreviations: GPL, glycopeptidolipid; HPTLC, high-performance TLC.

the literature for strains originally isolated in Cuba (Valdivia, 1973; Valdivia-Álvarez *et al.*, 1971). Moreover, a recent study (Khoo *et al.*, 1996) demonstrated that '*M. habana*' TMC 5135 and several *M. simiae* strains differ in glycopeptidolipid (GPL) composition. This fact, together with a renewed interest in the study of 'true' strains of '*M. habana*' as a candidate vaccine against tuberculosis and leprosy (Singh *et al.*, 1989, 1995, 1997), led us to carry out a comparative analysis of some representative lipids of both bacteria, work so far not undertaken. Moreover, since the synonymy was established, no other phenotypic features have been defined in '*M. habana*'. It is worth mentioning that chemical markers are of special interest in taxonomy and identification of mycobacteria, so that the definition of recognized species must include a description of their fatty and mycolic acids. These compounds are studied by chromatographic techniques. One of these, HPLC, has been defined as a powerful method for differentiating mycobacteria, even in cases in which a close phylogenetic relationship is found (Glickman *et al.*, 1994; Tortoli *et al.*, 1997).

METHODS

Strains and culture conditions. Thirty-three strains of '*M. habana*' (IPK 220, IPK 294, IPK 296, IPK 300, IPK 319, IPK 332, IPK 337, IPK 337-R, IPK 342, IPK 343, IPK 355, IPK 434, IPK 437, IPK 438, IPK 439, IPK 451, IPK 453, IPK 454, IPK 455, IPK 482, IPK 493, IPK 495, IPK 626, IPK 744, IPK 882, IPK 908, IPK 1321, IPK 1338, IPK 1339, IPK 1349, IPK 1354, IPK 1539 and IPK 2407), belonging to the collection of the Instituto Pedro Kourí (IPK), La Habana, Cuba (Valdivia-Álvarez *et al.*, 1971), were studied, together with strain TMC 5135. The last strain was previously included in several chemical (Khoo *et al.*, 1996) and immunological (Singh *et al.*, 1989, 1995, 1997) studies, and was kindly provided by Dr P. Draper, National Institute for Medical Research, London, UK. Strains of *M. simiae* were ATCC 25275^T (serotype I) and W 59 (from IPK) (serotype II).

For lipid analysis all strains were grown on Löwenstein-Jensen medium for 3 weeks at 37 °C. The cells were recovered, washed in saline, killed by autoclaving and freeze-dried.

Analysis of fatty and mycolic acids. Cells (approx. 10 mg) were subjected to acid methanolysis (Minnikin *et al.*, 1980) in 2 ml of a mixture of toluene/acetone/sulphuric acid (30:15:1, by vol.) overnight at 75 °C. Liberated fatty and mycolic acid methyl esters were extracted with hexane, and the extract was washed with distilled water until the pH was neutral, evaporated to dryness under N₂ and redissolved in hexane.

The total extract of methyl esters was studied for fatty acid content by GLC and GLC-MS (Sempere *et al.*, 1993) in a Hewlett Packard GC-MS system (GC 5890, MS 5790) fitted with a HP-5 capillary column (25 m, 0.25 µm film) that was programmed from 60 °C to 280 °C at 8 °C min⁻¹.

Overall composition of mycolic acids was determined by two-dimensional TLC on aluminium-backed silica gel plates (Merck 5554) (Valero-Guillén *et al.*, 1986), employing dichloromethane (first direction) and light petroleum (b. p. range 60–80 °C)/acetone (95:5, v/v; three times) (second direction) as solvents. Pyrolysis GLC of mycolic acid methyl esters was carried out as described by Luquin *et al.* (1991),

with the injector at 350 °C. Partial structural analysis of mycolic acids was performed for '*M. habana*' TMC 5135 and *M. simiae* ATCC 25275^T. The mycolic acids were obtained as above and purified by preparative TLC (silica gel plates; Merck 5735) using dichloromethane as solvent. The different compounds were visualized under UV light (366 nm), after spraying the plates with rhodamine 6G (Merck) (0.1%, w/v, in ethanol), scraped from the gel and eluted with diethyl ether. Rhodamine was eliminated by aluminium oxide column chromatography employing diethyl ether as eluent. MS was performed in a VG AutoSpec spectrometer operated in the electronic impact mode (70 eV; temperature range 60–400 °C).

For HPLC of mycolic acids, cells (approx. 10 mg) from '*M. habana*' strains IPK 220, IPK 319, IPK 337-R, IPK 343, IPK 908 and TMC 5135 (representing the GPL groups defined; see below) and *M. simiae* strains ATCC 25275^T and W 59 were subjected to saponification, converted to *p*-bromophenacyl derivatives (Glickman *et al.*, 1994), and separated in a HPLC system (Waters) equipped with a quaternary pump (Waters 600) and a UV/visible detector (Waters 486). A Nova-Pack C₁₈ column was used and the mycolic acids were eluted using a linear gradient of chloroform/methanol (from 98:2, v/v, to 30:70, v/v). A high molecular mass standard (Ribi, Immuno-Chem Research) was used as an internal standard to assist in the identification of the peaks. The labelling system of the peaks (S1–S9, F1–F4, A1, A4, A5, M1–M6, B1–B9, E1 and E2) is arbitrary, and they are identified by comparing their retention times with that of the internal standard within a variation range (Glickman *et al.*, 1994). For a given species the same peaks are generally found and their intensities are similar. Pattern recognition software, Pirouette (Infometrix), was employed to evaluate the similarity of the HPLC chromatograms obtained, and used in conjunction with a library containing the most relevant *Mycobacterium* species and related bacteria. This system is currently employed by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, and is described elsewhere (Glickman *et al.*, 1994; Ramos, 1994).

Glycolipid analysis. Cells (approx. 20 mg) were extracted with chloroform/methanol (1:1, v/v) (overnight, room temperature), then with chloroform/methanol (2:1, v/v) under the same conditions. The extracts were combined and evaporated to dryness under N₂, redissolved in 0.2 ml chloroform and partitioned in chloroform/methanol/water (8:4:3, by vol.). The lipids present in the lower phase were evaporated to dryness, redissolved as above and precipitated in acetone (–20 °C, overnight) (Kates, 1986). After centrifugation (1500 g, 4 °C, 15 min) the soluble acetone fraction (containing glycolipids) was subjected to further analysis.

Glycolipids were studied by single-dimensional TLC on aluminium-backed silica gel plates (Merck 5554; cut 10 × 10 cm) or high-performance TLC (HPTLC) silica gel plates (10 × 10 cm; Merck 13727), with chloroform/methanol/water (60:12:1, by vol.) or chloroform/methanol/water (65:25:4, by vol.) as solvents (Khoo *et al.*, 1996). The separated components were visualized after spraying the plates with molybdophosphoric acid (7%, w/v, in ethanol), for all lipids, or orcinol (0.1%, w/v, in 40% sulphuric acid), for glycolipids, and heating at 120–140 °C for 5–10 min. GPLs were revealed as yellow-gold or brown-yellow spots resistant to mild alkaline hydrolysis (Khoo *et al.*, 1996).

GPL-II' and GPL-II (see below) were purified by single-dimensional TLC (10 × 10 cm silica gel plates; Merck 5735)

from strains IPK 882 and TMC 5135, respectively, employing chloroform/methanol/water (65:25:4, by vol.) as solvent (Khoo *et al.*, 1996). The separated components were visualized with iodine vapour and the GPLs were scraped from the gel and eluted with chloroform/methanol (1:1, v/v) (Kates, 1986). The silica gel was removed by centrifugation (15 min, 1500 g) and the extract was evaporated to dryness. The purity of the compounds was checked by analytical TLC (Merck 5554) employing the same solvent. Sugars from GPL-II' and GPL-II were liberated by acid hydrolysis in 2 M trifluoroacetic acid, transformed to trimethylsilyl ethers (Sempere *et al.*, 1993) and studied by GLC-MS in the Hewlett Packard system described above, the HP-5 column being programmed from 60 °C to 290 °C at 6 °C min⁻¹. Identity of the peaks was evaluated by comparison with some data in the literature (DeJongh *et al.*, 1969) and with the reported composition for GPL-II of '*M. habana*' TMC 5135 (Khoo *et al.*, 1996).

RESULTS

Fatty acids

Fatty acids of '*M. habana*' and *M. simiae* strains were similar and ranged from 14 to 26 carbon atoms in composition (Table 1). Hexadecanoic (16:0), octadecenoic (18:1) and tuberculostearic (10-methyloctadecanoic; TBS) acids predominated. Hexadecenoic acid (16:1) always separated in two peaks, indicating the existence of at least two different positions for the double bond, as previously reported for several mycobacterial species, *M. simiae* included (Springer *et al.*, 1993).

Table 1. Fatty acid composition of '*M. habana*' and *M. simiae*

Range of variation (molar %) of the different fatty acids detected by GLC. ND, Not detected; T, traces (<1%); n, number of strains analysed.

Fatty acid*	' <i>M. habana</i> ' (n = 34)	<i>M. simiae</i> (n = 2)
14:0	1-14	6-13
15:0	ND-4	ND
16:1	1-5	1-2
16:0	30-46	30-40
17:1	ND-2	ND
17:0	ND-2	ND
18:2	ND-4	ND
18:1	8-34	20-32
18:0	2-10	2-3
TBS	9-27	16-18
20:0	ND-2	T
22:0	ND-1	T
24:0	ND-4	1-2
26:0	ND-9	1-7

* The fatty acids are identified by the number of carbon atoms and the number of double bonds. TBS, tuberculostearic acid (10-methyloctadecanoic acid).

Fig. 1. Two-dimensional TLC of mycolic acid methyl esters from '*M. habana*' TMC 5135. α -, α' - and ketomycolates are indicated. F, Fatty acid methyl esters; 1, first direction (dichloromethane); 2, second direction [light petroleum/acetone (95:5, v/v; three times)]. Plates were revealed at 140 °C, after spraying with molybdophosphoric acid (7%, w/v, in ethanol).

Mycolic acids

Two-dimensional TLC demonstrated the existence of α -, α' - and ketomycolates (Fig. 1) in all the strains studied. The pyrolysis products of these compounds were identified as hexacosanoic (26:0) (the major one) and tetracosanoic (24:0) acid methyl esters, as revealed by pyrolysis GC.

HPLC patterns of mycolic acids of the selected strains of '*M. habana*' (IPK 220, IPK 319, IPK 337-R, IPK 343, IPK 908 and TMC 5135) and the strains of *M. simiae* were similar (Fig. 2a, b). As mentioned above, the different peaks were named according to the nomenclature developed by the CDC, where no precise structural assignation of the mycolic acids is given. Characteristic peaks detected in all the strains examined were M4, M6, B1, B4, B5B, B6, B8, B9, E1 and E2. As shown in Table 2, peaks M6, B5B and B9 predominated. All the strains were identified as *M. simiae* by the software system incorporated into the HPLC system.

MS of mycolic acids of '*M. habana*' TMC 5135 and *M. simiae* ATCC 25275^T revealed M⁺, M⁺-18, M⁺-32 and M⁺-50 ions. An M⁺-18 peak predominated in α - and ketomycolates, and M⁺-50 predominated in α' -mycolates. A careful examination of the mass spectra indicated that α -mycolates were composed of at least two series: monocyclopropyl (or monounsaturated) (α -1) and dicyclopropyl (or diunsaturated, or mono-unsaturated + cyclopropyl) (α -2). Two series could be defined for ketomycolates: saturated (keto-1) and monocyclopropyl (or monounsaturated) (keto-2).

A significant difference between the two strains studied was found among the α -1 series. The predominant compound in '*M. habana*' contained 82 carbon atoms (M⁺-18 = 1190), whereas in *M. simiae* a component of

Fig. 2. HPLC pattern of mycolic acids (*p*-bromophenacyl derivatives) of *M. simiae* ATCC 25275^T (a) and '*M. habana*' TMC 5135 (b). Characteristic peaks are labelled following an arbitrary system (Glickman *et al.*, 1994) (see text). IS, High molecular mass internal standard.

81 carbon atoms reached the higher intensity. Both strains presented α -1 mycolates varying between 79 and 84 carbon atoms.

Among the α -2 mycolates, a compound of 81 carbon atoms ($M^+ - 18 = 1174$) predominated in both strains, and the total chain length varied from 79 to 84 carbon atoms.

The series of ketomycolates varied between 80 and 86 carbon atoms in both strains, the major ones containing 82 carbon atoms ($M^+ - 18 = 1206$) for keto-1 and 84 carbon atoms ($M^+ - 18 = 1232$) for keto-2. Notably, *M. simiae* ATCC 25275^T presented high amounts of a keto-1 of 79 carbon atoms ($M^+ - 18 = 1164$).

The α' -mycolates were exactly the same for '*M. habana*' and *M. simiae*. They varied from 58 to 67 carbon atoms, with 60:1 ($M^+ - 50 = 812$) and 62:1 ($M^+ - 50 = 840$) predominating.

Glycolipids

TLC of glycolipid extracts revealed complex mixtures of these compounds when analysed using chloroform/methanol/water (60:12:1, by vol.) as solvent and aluminium-backed silica gel plates. This system essentially separated the apolar GPLs and GPL-I (see below), but polar GPLs remained at the origin of the plates (data not shown). Better results were obtained by combining HPTLC plates and the solvent chloroform/methanol/water (65:25:4, by vol.) (Khoo *et al.*, 1996). Thus, identification of polar GPLs, GPL-I to GPL-III, previously detected in '*M. habana*' TMC 5135 (Khoo *et al.*, 1996), was easily performed. It should be mentioned that in most cases GPL-III was detected as a faint spot. When this solvent system was applied to mild-alkali-treated glycolipid extracts, GPLs presented similar mobilities to those observed for the intact compounds (not shown); however, other components on the chromatograms, designated GL (see Fig. 3), disappeared, thus being considered as glycolipids other than GPLs.

Strains of '*M. habana*' were divided into four groups (A–D) (Table 3; Fig. 3) according to the distribution of the aforementioned GPLs and a new one designated GPL-II'. Most strains of '*M. habana*' were included in group A, together with the strain TMC 5135 (Fig. 4, lane 4). Eleven strains contained GPL-II', but they were subsequently divided into two groups because some of them lacked GPL-I: group B (Fig. 3, lanes 2 and 3) and group C (Fig. 3, lane 5). The pattern of strains 220 and 908 was unique, lacking all the described GPLs and presenting two different polar components (Fig. 3, lane 1). Finally, strain 337-R did not contain polar GPLs (not shown).

Chemical identification of individual polar GPLs was done only for GPL-II', purified from strain IPK 882, and for GPL-II, obtained from strain TMC 5135. Sugar composition was similar for both compounds, as determined by GLC-MS, both in the retention times and in the mass spectra obtained (Table 4). No data are available to explain the different chromatographic mobilities of these glycolipids. The identification given was initially based on the sugar pattern of GPL-II published by Khoo *et al.* (1996). Although no data were found in the literature for trimethylsilyl derivatives of the different O-methyl sugars, the mass spectra obtained are in agreement with the identification proposed. For example, for 2,4-di-O-methylglucuronic acid (retention time 17.77 min), a characteristic fragment at m/z 101 was interpreted as a result of a breakdown between C-5 to C-6 with further rearrangement and loss of 18 mass units (H_2O): a C-5 to C-6 breakdown produces an m/z 103 in trimethylsilyl derivatives of monosaccharides (DeJongh *et al.*, 1969). For the same compound, the finding of a high intensity fragment at m/z 191, together

Table 2. Mycolic acid composition of '*M. habana*' and *M. simiae*

Mycolic acids were studied by HPLC and peaks were labelled according to the designations described in Glickman *et al.* (1994). The relative percentages of the different peaks are given.

Peak*	' <i>M. habana</i> '						<i>M. simiae</i>	
	TMC 5135	IPK 220	IPK 319	IPK 337-R	IPK 343	IPK 908	ATCC 25275 ^T	W 59
M4	6.6	12.0	6.1	8.2	7.7	10.1	7.8	7.5
M6	22.9	25.5	22.4	21.4	21.3	28.5	21.1	21.5
B1	3.9	4.8	3.9	3.7	4.4	6.3	4.4	4.3
B4	10.4	6.3	9.4	10.8	9.7	4.7	6.1	11.8
B5B	16.5	10.8	16.1	16.4	15.3	10.5	14.5	16.0
B6	6.4	6.1	7.8	6.2	8.4	7.1	12.8	5.5
B8	8.2	8.0	9.3	9.1	8.3	5.8	4.8	11.6
B9	11.8	12.9	12.7	12.2	12.7	10.9	10.0	12.6
E1	10.2	10.5	9.8	9.4	8.9	12.0	12.4	6.9
E2	3.1	3.0	3.5	2.6	3.3	4.1	6.1	2.3

* Although the HPLC system did not identify the structural types of mycolic acids, some data from this study suggest that M4, M6 and B1 could correspond to α' -mycolates, B4, B5B and B6 to ketomycolates, and B8, B9, E1 and E2 to α -mycolates.

Fig. 3. HPTLC of glycolipid extracts of '*M. habana*'. Lanes: 1, strain IPK 220, group D; 2, strain IPK 294, group B; 3, strain IPK 296, group B; 4, strain IPK 300, group A; 5, strain IPK 319, group C. GPL-I, GPL-II and GPL-III are the GPLs previously described by Khoo *et al.* (1996). GPL-II' is a new GPL detected in some strains of '*M. habana*'. aGPLs, apolar GPLs; GL, glycolipid (sugar-containing lipid labile to mild alkaline saponification); O, origin; F, front. Plates were developed with chloroform/methanol/water (65:25:4, by vol.) and revealed at 120 °C after spraying with orcinol (0.1%, w/v, in 40% sulphuric acid).

with a concomitant low intensity fragment of m/z 133, favours the presence of *O*-methyl groups at positions 2 and 4 (DeJongh *et al.*, 1969). The existence of *O*-methyl groups in the molecule is revealed by a fragment at m/z 146. Also, m/z 277 represents a breakdown of the ring conserving C-3 to C-6; a further loss of $-\text{OCH}_3$ with retention of two ring hydrogens gives m/z 247 (Table 4). Peaks with retention times at 18.63 min and 19.11 min were identified as 6-*O*-methylglucose, mainly because their mass spectra were similar to α -methylglucose

(DeJongh *et al.*, 1969), with the exception of the absence of m/z 146 (Table 4). The presence of *O*-methyl groups was deduced from fragments at m/z 435 ($M^+ - 15 - 32$) (loss of $-\text{CH}_3$ plus $-\text{OCH}_3$) and m/z 345 ($M^+ - 15 - 32 - 89$) [$89 = \text{OSi}(\text{CH}_3)_3$]. Other fragments were in accordance with the molecular mass proposed for 6-*O*-methylglucose, m/z 393 ($M^+ - 89$) and m/z 305 ($M^+ - 89 - 89$) (Table 4), all of them characteristic of this kind of derivative (DeJongh *et al.*, 1969).

The GPL profiles of the two strains of *M. simiae* included in this work (Fig. 4, lanes 1 and 2) were clearly different to those obtained for the '*M. habana*' strains. Both strains presented three major polar GPLs, with slightly different mobilities as shown by HPTLC, together with several apolar GPLs.

DISCUSSION

The data from the present study clearly establish that chemical markers of taxonomic value, such as fatty and mycolic acids, are similar in '*M. habana*' and *M. simiae*. The general patterns of both types of compound have been published previously for *M. simiae* (Daffé *et al.*, 1983; Minnikin *et al.*, 1984) and our results are in general agreement with these.

The HPLC profile of mycolic acids of *M. simiae* has been reported in several comparative studies with related species which also contained α -, α' - and ketomycolates, such as *Mycobacterium genavense* (Coyle *et al.*, 1992), *Mycobacterium malmoeense* (Tortoli *et al.*, 1995) and *Mycobacterium lentiflavum* (Springer *et al.*, 1996; Tortoli *et al.*, 1997). In all cases, the pattern obtained for *M. simiae* was specific and composed of the set of peaks depicted in Fig. 2(a, b) for *M. simiae* ATCC 25275^T and '*M. habana*' TMC 5135. This represents a definitive basis to support the synonymy between the two mycobacteria, because, as suggested elsewhere (Glickman *et*

Table 3. GPL profiles of '*M. habana*' strains

GPLs were studied by HPTLC with chloroform/methanol/water (65:25:4, by vol.) as solvent.

Strain*	Group	GPL
IPK 300, IPK 332, IPK 337, IPK 355, IPK 434, IPK 437, IPK 438, IPK 451, IPK 453, IPK 454, IPK 455, IPK 482, IPK 493, IPK 495, IPK 626, IPK 744, IPK 1321, IPK 1338, IPK 1339, TMC 5135	A	GPL-I, GPL-II, GPL-III
IPK 294, IPK 296, IPK 343	B	GPL-I, GPL-II, GPL-II', GPL-III
IPK 319, IPK 342, IPK 439, IPK 882, IPK 1349, IPK 1354, IPK 1539, IPK 2407	C	GPL-II', GPL-II, GPL-III
IPK 220, IPK 337-R, IPK 908	D	Others*

* Strains IPK 220 and IPK 908 contain similar polar GPLs, but not GPL-I, GPL-II', GPL-II or GPL-III. Strain IPK 337-R did not contain polar GPLs.

Fig. 4. HPTLC of glycolipid extracts of *M. simiae*. Lanes: 1, ATCC 25275^T (serotype I); 2, *M. simiae* W 59 (serotype II). GPL_{SIM}, polar GPLs of *M. simiae*. For comparative purposes, partially purified GPL-II' and the glycolipid extract of '*M. habana*' TMC 5135 are included in lanes 3 and 4, respectively. The positions of GPL-I to GPL-III are indicated. aGPLs, apolar GPLs. Chromatographic conditions are as in Fig. 3.

al., 1994; Tortoli *et al.*, 1997), HPLC appears to correlate very well with genotypic criteria. In this context, it is noteworthy that a strain of '*M. habana*' (337-5, not studied in the present work) was included in a DNA/DNA hybridization analysis, giving 90.2% homology with the type strain of *M. simiae* (Baess & Magnusson, 1982).

HPLC profiles of *M. simiae* ATCC 25275^T slightly differed from '*M. habana*' strains in peaks B4, B6, E1 and E2 (Table 2). This fact, however, did not affect the identification proposed by the software system. Moreover, by HPLC, *M. simiae* W 59 was more closely related to '*M. habana*' than to the type strain of the species (Table 2).

As stated above, differences in the intensities of several M⁺-18 ions of the α - and ketomycolate series were also

found in the mass spectra of mycolic acids of *M. simiae* ATCC 25275^T and '*M. habana*' TMC 5135. From these data, which are not taxonomically relevant, it can be speculated that the series of peaks labelled B4, B5B and B6 should correspond to ketomycolates, and peaks B8, B9, E1 and E2 to α -mycolates. It also seems reasonable to assign peaks M4, M6 and B1 to α' -mycolates. The series of peaks found by HPLC are related to the structural series detected by MS. Although no structural analyses have been done on peaks eluted by HPLC, it can be assumed that the α - and ketomycolate series are similar to those described in other mycobacteria, whose overall structure includes cyclopropyl rings, unsaturations and methyl branches (Minnikin & Goodfellow, 1980; Minnikin, 1982), sometimes coexisting in the same strain. Usually one, and sometimes two, unsaturations are found in α' -mycolates (Minnikin & Goodfellow, 1980; Minnikin, 1982).

Although closely related by fatty and mycolic acid composition, '*M. habana*' and *M. simiae* differed in GPL composition, as demonstrated by TLC. Our results confirm and extend a recent report on the glycolipids of these micro-organisms (Khoo *et al.*, 1996), and reveal a certain degree of heterogeneity within '*M. habana*' that supports the establishment of at least four groups of strains. However, most strains (31 out of 34) of '*M. habana*' contained GPL-II and GPL-III.

As suggested previously (Khoo *et al.*, 1996), the serological differences between '*M. habana*' and *M. simiae* (Meissner & Schröder, 1975) noted by others could be, in part, explained by a different GPL composition. These compounds are antigenic (Khoo *et al.*, 1996) and display similar structures to those of the *Mycobacterium avium* complex (Brennan, 1989).

As established by Khoo *et al.* (1996), the structural basis of the differences between the GPLs of '*M. habana*' and

Table 4. Chromatographic and mass spectrum features of sugars of GPL-II' and GPL-II from '*M. habana*'

The different sugars were studied as trimethylsilyl ethers by GLC-MS.

Sugar	Retention time (min)*	Fragment (m/z)	M ⁺ †
3,4-Di-O-methylrhamnose	12.42, 12.78	73, 133, 146, 189, 204	336
4-O-Methylrhamnose	13.67, 14.36	73, 133, 146, 191, 204, 290	394
6-Deoxy-3-O-methyltalose	14.75, 14.82	73, 133, 146, 217, 290	394
3-O-Methylfucose	14.94, 15.38	73, 133, 146, 217, 290, 305, 347	394
Rhamnose	15.49, 16.39	73, 147, 191, 204, 437	452
2,4-Di-O-methylglucuronic acid	17.77	73, 101, 133, 146, 191, 217, 247, 277, 371, 391, 423	438
6-O-Methylglucose	18.63, 19.11	73, 147, 191, 204, 305, 345, 395, 435	482

* Two peaks were, in general, detected for each compound.

† The molecular ion was not detected in any case.

M. simiae are due to the number and position of O-methyl groups and to the glycosidic linkages. Nevertheless, several derivatives of the same sugars (rhamnose, talose, fucose, glucose and glucuronic acid) are present in both micro-organisms, so that, although retaining sufficient specificity, they share common epitopes leading to serological cross-reactivities (Khoo *et al.*, 1996).

In conclusion, a chemotaxonomic analysis of a large collection of '*M. habana*' strains confirms the synonymy between this micro-organism and *M. simiae*. However, although heterogeneous in GPL composition, '*M. habana*' could be defined by the presence of GPL-II and GPL-III. This approach will be useful to assess future work with potential vaccine strains, and also to identify possible new isolates of this variant within *M. simiae*.

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