The HPLC-double-cluster pattern of some *Mycobacterium gordonae* strains is due to their dicarboxy-mycolate content

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The mycolic acids of several strains of *Mycobacterium gordonae* were examined by chromatographic and spectroscopic techniques. Both HPLC and TLC revealed two patterns of mycolates among the *M. gordonae* strains studied. As determined by TLC, one pattern was composed of α -, methoxy- and keto-mycolates; the other was composed of these mycolates plus an additional component, which was identified as dicarboxy-mycolates. The dicarboxy-mycolates were only found in those *M. gordonae* strains that displayed a so-called HPLC-double-cluster pattern. Detailed structural analyses of the dicarboxy-mycolates indicated that these compounds contained predominantly 61–65 carbon atoms (C_{63} was the major component) and a *trans*-1,2-disubstituted cyclopropane ring. Thus, the dicarboxy-mycolate content of strains of *M. gordonae* determines their HPLC pattern. In spite of the differences in their HPLC patterns, and although they belonged to different PCR-restriction length polymorphism clusters, all of the *M. gordonae* strains examined in this study were closely related on the basis of the structural features of their α -, keto- and methoxy-mycolates; the predominant α -mycolates contained two *cis*-1,2-disubstituted cyclopropane rings, the major keto-mycolates contained a *trans*-1,2-disubstituted cyclopropane ring and the methoxy-mycolates contained one *cis*- or one *trans*-1,2-disubstituted cyclopropane ring. It is noteworthy that the strains containing dicarboxy-mycolates also displayed significant amounts of α -mycolates that contained one *cis*-1,2-disubstituted cyclopropane ring and one *cis* double bond. The results obtained in this study demonstrate heterogeneity among *M. gordonae* strains.

Keywords: mycolic acids, high-performance liquid chromatography, *M. gordonae*

INTRODUCTION

Mycobacterium gordonae is a slow-growing scotochromogenic species of *Mycobacterium*, which is widely distributed in natural water sources and soils (Wayne & Kubica, 1986). It has been found in bottled table waters (Papapetropoulou *et al.*, 1997) and is the most common mycobacterial isolate from swimming-pool environments (Leoni *et al.*, 1999), probably due to its resistance to chlorine disinfection (Le Dantec *et al.*, 2002). Several

M. gordonae-associated infections have been observed in endoscopy patients; these are thought to have been due to the use of improperly cleaned or disinfected instruments (Vogiatzakis *et al.*, 1998). Although *M. gordonae* is considered to be non-pathogenic, there have been some well-documented cases of infections caused by this organism in patients with underlying conditions, e.g. AIDS or cancer (Bernard *et al.*, 1992; Clague *et al.*, 1985; London *et al.*, 1988; Rusconi *et al.*, 1997).

Molecular-based methodologies for the rapid identification of *M. gordonae* isolates have revealed genetic variability within this species. In contrast to other mycobacteria, which show conservation of rDNA sequences at the species level, *M. gordonae* strains exhibit

Abbreviations: EI-MS, electron-impact MS; FAB-MS, fast-atom bombardment MS; Gen-Probe, Gen-Probe Rapid Diagnostic System; PRA, PCR-restriction length polymorphism analysis.

variation within a region of their rDNA; this region is a common target for diagnostic species-specific probes (Kirschner & Böttger, 1992). The microheterogeneity observed among strains of *M. gordonae* could be the reason for the hybridization problems reported by Walton & Valesco (1991) when using the Gen-Probe Rapid Diagnostic System (Gen-Probe). Studies using other molecular-based identification methods, such as PCR-restriction length polymorphism analysis (PRA), have concluded that *M. gordonae* is the most heterogeneous member of the genus *Mycobacterium* studied to date, generating several closely related subclusters upon analysis of strain data (Plikaytis *et al.*, 1992; Telenti *et al.* 1993).

Mycolic acids are high-molecular-mass 3-hydroxy, 2-alkyl-branched fatty acids found in all *Mycobacterium* species. The different structural types of mycolic acids include the so-called α -mycolates and the mycolates which have other oxygen functions (i.e. the keto-, methoxy-, dicarboxy-, epoxy- and ω -1 methoxy-mycolates) in addition to the 3-hydroxy acid unit (Minnikin, 1982; Minnikin *et al.*, 1982; Luquin *et al.*, 1990). The use of TLC to analyse mycolic acid methyl esters allowed the different structural types of mycolic acids to be separated. The mycolate patterns obtained upon separation of mycolic acid methyl esters by TLC have been widely used in the classification and identification of mycobacteria (Daffé *et al.*, 1983; Minnikin *et al.*, 1984; Luquin *et al.*, 1991; Valero-Guillén *et al.*, 1985). *M. gordonae* exhibits a TLC mycolate pattern characterized by the presence of α -, methoxy- and keto-mycolates. Analysis of mycolic acids by HPLC has permitted a large proportion of mycobacterial species to be identified by their unique and reproducible chromatographic patterns (Butler & Guthertz, 2001). However, *M. gordonae* isolates have been shown to produce two different HPLC mycolic acid patterns – a single-cluster pattern, which is the most common pattern, and a double-cluster pattern (Cage, 1992; Butler *et al.*, 1991). To date, no studies have been done to correlate these patterns with differences in mycolate production between strains of *M. gordonae* or to identify the compounds responsible for the second cluster of peaks seen in the double-cluster pattern.

The aim of this work was to elucidate the lipidic compounds of *M. gordonae* that give rise to the HPLC-double-cluster pattern. We studied five clinical isolates of *M. gordonae* that produced the HPLC-double-cluster pattern; for comparative purposes, we also studied two reference strains of *M. gordonae*. The lipidic component responsible for the second peak in the double-cluster pattern was purified and identified by spectrometric methods as dicarboxy-mycolate, a mycolic acid that has not been described in *M. gordonae* until now.

METHODS

Strains, cultivation and identification. Five strains from clinical samples (CR-178, CL-333 and CL-361, from sputa; CL-416C, from an alveolar lavage; CL-554, from a broncho-

aspirate) were examined, together with *M. gordonae* strains ATCC 14470^T (HPLC-single-cluster pattern) and ATCC 35759 (HPLC-double-cluster pattern). All of the strains were grown on Sauton's medium at 32 °C for 6 weeks.

Identification of the strains was done by using standard biochemical methods (Tsukamura, 1967) and Gen-Probe for *M. gordonae* (used according to the manufacturer's instructions; Biomérieux). The strains were also typed by using PRA, as described by Telenti *et al.* (1993). Briefly, a region of DNA (439 bp) corresponding to the gene encoding Hsp65 was amplified by PCR and then digested with *Hae*III or *Bst*EIII; the fragments obtained were separated by agarose-gel electrophoresis and visualized under UV.

Extraction and analysis of fatty acids, mycolic acids and alcohols. Two to three loopfuls of bacteria (20–30 mg wet wt of each strain) were collected from the surface of the Sauton's agar plates. Fatty acids, mycolic acids and alcohols were liberated from each strain sample by saponification. These compounds were then extracted with diethyl ether. For each strain, an aliquot of the extract was treated with diazomethane to obtain the methyl esters of the fatty acids and mycolic acids (Daffé *et al.*, 1983); in another aliquot of the extract the mycolic acids were transformed to *p*-bromophenacyl derivatives (Butler *et al.*, 1991).

The *p*-bromophenacyl derivatives of the mycolic acids were separated in a HPLC system (Waters Associates) equipped with an UV/visible detector. A reverse-phase C18 column (Nova-Pack 60A, 4 μ m, 3.9 $\times$ 75 mm; Waters Associates) was used in the system; the mycolic acids were eluted using a linear gradient of methanol/chloroform [from 98:2 (v/v) to 30:70 (v/v)]. A high-molecular-mass standard (Rib; ImmunoChem Research) was used as an internal standard, to assist in the identification of the peaks. Pattern-recognition software (PIROUETTE; Infometrix) was employed to evaluate the similarity of the HPLC chromatograms obtained, and was used in conjunction with a library containing the chromatograms of the most relevant *Mycobacterium* species and related bacteria (Glickman *et al.*, 1994). Designation of HPLC peaks followed the arbitrary nomenclature used by several authors in the literature (Glickman *et al.*, 1994).

Mycolic acid methyl esters were studied by analytical one-dimensional TLC using silica gel 60 TLC plates (Merck). A triple development with a mixture of *n*-hexane/diethyl ether (85:15, v/v) was performed to separate the individual mycolates. The components separated by TLC were revealed as dark-blue spots by spraying the TLC plates with 10% (w/v) molybdophosphoric acid (Merck) in ethanol and heating them at 120 °C for 5 min. The methyl mycolates on the TLC plates were identified by comparing their positions on the plates with the mycolate patterns of reference strains (Luquin *et al.*, 1991).

Fatty acid methyl esters, alcohols and methyl mycolate cleavage products were determined by GLC and GLC-MS as described previously (Luquin *et al.*, 1991), employing a fused-silica capillary column (cross-linked methyl silicone, 15 m $\times$ 0.25 mm, HP-1; Hewlett Packard) that was programmed from 175 to 300 °C at 8 °C min⁻¹ and maintained at 300 °C for 15 min.

Purification and structural analysis of mycolates. Crude methyl mycolates from *M. gordonae* strains ATCC 14470^T, ATCC 35759 and CL-416C were obtained by precipitating their lipidic extracts with cold methanol (–20 °C, overnight). The samples were then centrifuged at 1500 *g* for 30 min at 4 °C. Unless indicated otherwise, the boiling point of the petroleum ether used in the following procedures was 60–

80 °C. For purification of the methyl mycolates, the precipitates were recovered, dissolved in the smallest possible volume of petroleum ether and applied to a silica gel 60 (Merck; particle size 0.063–0.200 µm) column equilibrated in petroleum ether. Successive elutions, 3–5 bed-volumes each, were performed with petroleum ether followed by increasing concentrations of diethyl ether (5, 10, 20 and 100%) in petroleum ether. The eluates were separated by TLC, as described above. Dicarboxy-mycolates were obtained in the 20% (v/v) diethyl ether/petroleum ether fraction.

Purified mycolic acid methyl esters were identified by MS and NMR. Electron-impact MS (EI-MS) (70 eV) and fast-atom bombardment MS (FAB-MS) (8 eV) were performed in a VG AutoSpec (Fison) mass spectrometer. FAB-MS was carried out in the positive mode, employing *m*-nitrobenzyl alcohol as the matrix. Because FAB-MS seemed to produce $(M+Na)^+/\alpha$ pseudomolecular ions, we doped the matrix with NaCl in some analyses. Dicarboxy-mycolates from strain CL-416C were also analysed by FAB-MS in the negative mode and by chemical ionization (methane; temperature of ionization, 180 °C) in the negative mode. For the latter method of analysis, a Thermoquest-Trace mass spectrometer (Thermo) with a temperature range of 60–300 °C was used.

All methyl mycolates from strains ATCC 14470^T, ATCC 35759 and CL-416C were analysed by ¹H-NMR at 300 MHz in a Varian NMR spectrometer. Dicarboxy-mycolates from strains ATCC 35759 and CL-416C were also studied by ¹³C-NMR (75 MHz) in the same spectrometer. In all cases, the spectra were recorded in deuteriochloroform [10 mg (ml sample)⁻¹] at 25 °C.

RESULTS

Biochemical and genetic identification

The five strains of clinical origin used in this study were identified as *M. gordonae* by standard biochemical tests and by Gen-Probe. Upon PRA, four of the clinical strains (CR-178, CL-361, CL-416C and CL-554) exhibited PRA pattern V, as defined by Telenti *et al.* (1993); CL-333 exhibited PRA pattern III. *M. gordonae* ATCC 35759 exhibited pattern III and *M. gordonae* ATCC 14470^T exhibited pattern I.

Mycolate chromatographic patterns

Upon HPLC analysis of their mycolates, the five clinical strains of *M. gordonae* and strain ATCC 35759 showed an HPLC-double-cluster pattern (Cage, 1992) (Fig. 1a). TLC analysis of the mycolates revealed that the six strains contained α -, methoxy-, keto-mycolates and additional components, such as secondary alcohols and dicarboxy-mycolates (Fig. 1b). *M. gordonae* ATCC 14470^T showed the most common HPLC-single-cluster pattern (Cage, 1992) (data not shown); TLC revealed this strain to contain only α -, methoxy- and keto-mycolates in its cell wall (Fig. 1b; lane 4). HPLC of the *p*-bromophenacyl derivatives of the total mycolic acids from *M. gordonae* CL-416C are shown in Fig. 1(a). As further demonstrated by HPLC (data not shown), dicarboxy-mycolates purified from strains ATCC 35759 and CL-416C and transformed to their *p*-bromophenacyl derivatives eluted as three peaks, which

Fig. 1. (a) HPLC trace of mycolates from *M. gordonae* CL-416C, showing a double-cluster pattern. Nomenclature of the peaks is arbitrary but its correspondence to the different structural types of mycolates is given. IS, internal standard. (b) TLC of mycolic acids of different strains of *M. gordonae*. Lanes: 1, CR-178; 2, CL-416C; 3, CL-554; 4, ATCC 14470^T; 5, CL-333. Petroleum ether/diethyl ether (85:15, v/v) was used as the solvent, twice. The b.p. of the petroleum ether was 60–80 °C. The different structural types of mycolates (α , methoxy and keto) are indicated. Alcohols, secondary alcohols 2-octadecanol and 2-eicosanol; FAMES, fatty acid methyl esters. Compounds migrating above the α -mycolates probably correspond to epimerization products of the different mycolates.

corresponded exactly to peaks A1, A4 and A5 in Fig. 1(a). Other components detected in the chromatogram, peaks B2–B8 (Glickman *et al.*, 1994), corresponded to a mixture of keto-, methoxy- and α -mycolates (Fig. 1a).

Fatty acids and alcohols

GLC analysis (data not shown) of the fatty acids of the clinical strains and of strains ATCC 35759 and ATCC 14470^T indicated the presence of fatty acids with 14 to 24 carbon atoms in these strains, of which hexadecanoate and octadecanoate were the most prominent. Minor amounts of 2-methyl tetradecanoic acid were detected in

Fig. 2. ^1H -NMR spectrum (300 MHz) of dicarboxy-mycolates from *M. gordonae* ATCC 35759 in deuteriochloroform. Resonances of the different protons are indicated. TMS, tetramethylsilane.

the strains, whereas tuberculostearic acid (10-methyl octadecanoic acid) was not present in any of the strains examined. Two secondary alcohols, 2-octadecanol and 2-eicosanol, were detected only in those strains showing the HPLC-double-cluster pattern. The methyl esters derived from thermal cleavage of the methyl mycolates (Luquin *et al.*, 1991) were docosanoate and tetracosanoate, which were present in varying amounts.

Structures of the dicarboxy-mycolates of *M. gordonae*

In the ^1H -NMR spectrum of strain ATCC 35759 (Fig. 2), major resonances were detected between 0.1 and 3.7 p.p.m. A multiplet centred at 0.15 p.p.m. (3H) and another one at 0.47 p.p.m. (1H) were attributed to a *trans*-1,2-disubstituted cyclopropane ring (Draper *et al.*, 1982; Watanabe *et al.*, 1999). A signal at 0.67 p.p.m. (Fig. 2) was assigned to a $-\text{CH}-\text{CH}_3$ adjacent to the cyclopropane ring (Watanabe *et al.*, 1999); the protons of the methyl branch resonated as a doublet at 0.90 p.p.m., and overlapped a signal of a terminal methyl at 0.88 p.p.m. (6H in total) (Draper *et al.*, 1982). Singlets at 3.66 and 3.70 p.p.m. corresponded to two carbomethoxy groups, and the triplet at 2.29 p.p.m. was assigned to a $-\text{CH}_2-$ attached to a carbonyl group. The protons of other $-\text{CH}_2-$ groups resonated between 1.2 and 1.6 p.p.m. Finally, the signal at 2.42 p.p.m. was due to the proton of C-2 of the molecule. Minor resonances situated at -0.3 and 0.73 p.p.m. (Fig. 2) were due to the presence of low amounts of a compound with a *cis*-1,2-disubstituted cyclopropane ring (Watanabe *et al.*, 1999).

In the ^{13}C -NMR spectrum of strain ATCC 35759 (data not shown), two signals at 174.30 and 176.19 p.p.m. indicated the existence of two carbonyl groups in the molecule, which were complemented to the resonances

of two carbomethoxy groups at 50.98 and 51.46 p.p.m. Resonances at 10.50, 18.62 and 26.14 p.p.m. were assigned to a *trans*-1,2-disubstituted cyclopropane ring; the resonances situated at 19.70 and 38.13 p.p.m. were assigned to a methyl branch and to a methyne, respectively, which were both adjacent to the aforementioned ring (Watanabe *et al.*, 1999). The presence of a hydroxyl group at C-3 was justified by the resonance at 72.29 p.p.m.

These data indicate that the additional component detected by TLC and HPLC in the *M. gordonae* clinical strains and in strain ATCC 35759 can be identified as a dicarboxy-mycolate (dimethyl ester form) that contains predominantly a *trans*-1,2-disubstituted cyclopropane ring with a methyl branch adjacent to it (Fig. 3; Table 1).

EI-MS (Fig. 3) revealed fragments related to the general breakdown (between C-2 and C-3) pattern of mycolates, which produce meroaldehydes and methyl esters (Draper *et al.*, 1982). Methyl esters appeared at m/z 382 (tetracosanoate, $\text{C}_{24}:\text{O}$) and 354 (docosanoate, $\text{C}_{22}:\text{O}$). Fragments due to $M^+ - 50/z$ (water and methanol loss) were detected at m/z 908, 936 (the most predominant) and 964, which predicted molecular ions (M^+/z) at 958, 986 and 1014; these were further confirmed by positive-mode FAB-MS, because pseudomolecular ions, $(M + \text{Na})^+/z$, appeared at 981, 1009 and 1037 (data not shown).

In the region of the EI-MS trace attributed to meroaldehydes (Fig. 3), a major fragment at m/z 601 was accompanied by another one at m/z 632; other fragments were present at lower intensities. A formula for dicarboxy-mycolates was not satisfactory when m/z 601 was considered. However, negative-mode FAB-MS (data not shown) revealed very low intensities for pseudomolecular ions, $(M - \text{H})^-/z$, but important fragments at $(M - 15)/z$, which were tentatively assigned to the loss of $-\text{CH}_3$ from a carbomethoxy group. The elimination of a methyl group to give a negatively charged molecule (a carboxylate ion) could apparently take place in the meroaldehyde part of the structure, because intense fragments at m/z 381 and 353 (similar intensities) were clearly related to methyl esters derived from a breakdown between C-2 and C-3 of the molecule. Thus, an intense fragment at m/z 617 was detected by negative-mode FAB-MS and was considered to be related to a meroaldehyde at m/z 632 seen in the EI-MS analysis. Finally, by chemical ionization an important fragment (relative abundance 90%) was detected at m/z 631 (data not shown). Thus, it was concluded that the major meroaldehyde produced by the compound under study was actually m/z 632. Moreover, this fragment satisfied the formula given in Fig. 3, when an $n2 + n3$ value equal to 34 was considered. Then, the meroaldehyde at m/z 632 fragments to m/z 601 due to loss of $-\text{OCH}_3$ (Fig. 3a), because of the existence of the second ester group at the ω end. Similarly, an m/z 573 (Fig. 3a) was derived from a meroaldehyde at m/z 604 (very low intensity) ($n2 + n3$ equal to 32).

The value of $n3$ was deduced by taking into account the

Fig. 3. (a) Partial EI-MS trace of dicarboxy-mycolates from *M. gordonae* ATCC 35759. (b) The structure and the general fragmentation pattern of the major component are included, showing the m/z ions corresponding to meroaldehydes (m/z 632, 601 and 292) methyl esters (m/z 354 and 382) and $M^+ - 50/z$ (m/z 936; loss of water and methanol).

presence of a very intense fragment at m/z 292 in the EI-MS analysis (Fig. 3a), which was derived from a breakdown between the cyclopropane ring and the carbon attached to the methyl branch and a further rearrangement. This fragment should indicate that for m/z 632, n_3 is equal to 16 and n_2 is equal to 18, and suggested that the major dicarboxy-mycolate from *M. gordonae* contains 63 carbon atoms, with an alkyl chain at C-2 of 20 carbon atoms (producing a $C_{22:0}$ methyl ester). For a meroaldehyde at m/z 604 ($n_2 = 16$, $n_3 = 16$), a compound with a molecular mass of 986 is also obtained assuming an alkyl chain of 22 carbon atoms at C-2 (producing a $C_{24:0}$ methyl ester), thus justifying a more intense peak at m/z 936 ($M^+ - 50$) (see above and Fig. 3) and at $(M + Na)^+ / z$ 1009 (data not shown).

The two remaining homologous compounds seen in the EI-MS analysis can also be formulated in a similar way. Thus, a C_{65} dicarboxy-mycolate with a molecular mass of 1014 is composed combining the m/z 632 meroaldehyde and the m/z 382 methyl ester; and a C_{61} dicarboxy-mycolate (molecular mass 958) is composed by combining m/z 604 (a meroaldehyde) and m/z 354 (a methyl ester).

Both $^1\text{H-NMR}$ (Fig. 2) and MS [EI-MS (Fig. 3) and FAB-MS (data not shown)] predicted the presence of minor amounts of dicarboxy-mycolates with a *cis*-1,2-disubstituted-cyclopropane ring in the strains producing a HPLC-double-cluster pattern. The major meroaldehyde for this series was detected at m/z 646 (fragment at m/z 615; Fig. 3a), and the chain length varied between C_{60} and C_{64} according to the MS: pseudomolecular ions, $(M + Na)^+ / z$, were situated at 967 (C_{60}), 995 (C_{62} , probably the predominant pseudomolecular ion) and 1023 (C_{64}) (data not shown).

Structures of the other mycolates of *M. gordonae*

The structures of the remaining mycolates (α -, methoxy- and keto-mycolates) of strains ATCC 35759, CL-416C and ATCC 14470^T were established by $^1\text{H-NMR}$ and MS (EI-MS and positive-mode FAB-MS); the results of these analyses are presented in Table 1.

α -Mycolates from *M. gordonae* strains ATCC 35759 and CL-416C contained mainly two *cis*-1,2-disubstituted cyclopropane rings (their protons were located at -0.32 , 0.59 and 0.64 p.p.m.), and even chain

Table 1. Structural characteristics of the major mycolates of strains of *M. gordonae*

Strain	Mycolate type	Chain length	Cyclopropane ring*		<i>cis</i> Double bond*	Major component		
			<i>cis</i>	<i>trans</i>		Chain length	<i>n</i> 1/ <i>n</i> 2/ <i>n</i> 3	
ATCC 35759	Dicarboxy	C ₆₁ –C ₆₅	—	+	—	C ₆₃	–/18/16	
	α	C ₇₂ –C ₈₄	+	—	—	C ₇₈ , C ₈₀	17/12/17†	
	α	C ₇₃ –C ₇₇	+	—	+	C ₇₃		
	Methoxy	C ₇₃ –C ₈₅	+	—	—	C ₈₁	15/16/17†	
	Methoxy	C ₇₄ –C ₈₄	—	+	—	C ₈₂	15/16/18†	
CL-416C	Keto	C ₇₉ –C ₈₅	—	+	—	C ₈₁ , C ₈₃	17/16/16†	
	Dicarboxy	C ₆₁ –C ₆₅	—	+	—	C ₆₃	–/18/16	
	α	C ₇₂ –C ₈₄	+	—	—	C ₇₆ , C ₇₈	17/12/17†	
	α	C ₇₃ –C ₇₇	+	—	+	C ₇₃		
	Methoxy	C ₇₃ –C ₈₇	+	—	—	C ₇₉ , C ₈₁	15/16/17†	
	Methoxy	C ₇₄ –C ₈₄	—	+	—	C ₈₂	15/16/18†	
	Keto	C ₇₉ –C ₈₅	—	+	—	C ₇₉ , C ₈₁	17/16/16†	
	ATCC 14470 ^T	α	C ₇₄ –C ₈₀	+	—	—	C ₇₆ , C ₇₈	17/12/17†
		Methoxy	C ₇₃ –C ₈₇	+	—	—	C ₈₁	15/16/17†
		Methoxy	C ₇₄ –C ₈₄	—	+	—	C ₈₂	15/16/18†
Keto		C ₇₉ –C ₈₅	—	+	—	C ₈₁ , C ₈₃	17/16/16†	
Dicarboxy-mycolates (<i>trans</i> -1,2-disubstituted cyclopropane)					α-Mycolates (di- <i>cis</i> -1,2-disubstituted cyclopropane)			
Methoxy-mycolates (<i>trans</i> -1,2-disubstituted cyclopropane)					α-Mycolates (<i>cis</i> double bond + <i>cis</i> -1,2-disubstituted cyclopropane)			
Methoxy-mycolates (<i>cis</i> -1,2-disubstituted cyclopropane)					Keto-mycolates (<i>trans</i> -1,2-disubstituted cyclopropane)			
$\text{R}=\text{--CHOH--CH--(CH}_2\text{)}_{19,21}\text{--CH}_3$ <div style="text-align: center;">COOCH_3</div>								

* –, Absent; +, present.

† The given values are only tentative.

lengths of C₇₂–C₈₄ (major series at C₇₆ and C₇₈ for CL-416C, and at C₇₈ and C₈₀ for ATCC 35759). Major meroaldehyde ions (*m/z* 740, 768 and 796) and their fragments (*m/z* 279, 307, 487, 515 and 543), together with methyl ester fragments at *m/z* 354 and 382, permitted the tentative structures in Table 1 to be proposed (Draper *et al.*, 1982). Moreover, other compounds probably containing one *cis* double bond (resonance at 5.34 p.p.m.) and one *cis*-1,2-disubstituted

cyclopropane ring were also predicted in the two aforementioned strains; both EI-MS and FAB-MS signalled chain lengths of C₇₃–C₇₇. The ratio of –CH=CH– protons to 1,2-disubstituted cyclopropane ring protons was 21 and 22.7 for strains ATCC 35759 and CL-416C, respectively, implying that these compounds could represent approximately 20% of the total amount of α-mycolates present in these strains. Finally, *M. gordonae* ATCC 14470^T contained α-mycolates with only two *cis*-

1,2-disubstituted cyclopropane rings, which ranged from C_{74} to C_{80} (major compounds C_{76} and C_{78}).

The methoxy-mycolates of strains ATCC 35759, CL-416C and ATCC 14470^T were identical (Table 1). They were clearly identified by ¹H-NMR due to characteristic overlapping signals at 0.85 (a methyl branch adjacent to the methoxy group of the meroaldehyde chain), 0.88 (a terminal methyl group) and 0.90 p.p.m. (a methyl branch adjacent to a 1,2-disubstituted cyclopropane ring), and also by signals at 2.95 (–CH–OCH₃) and 3.33 p.p.m. (–OCH₃). Methoxy-mycolates containing *cis*- (–0.32, 0.59 and 0.64 p.p.m.) or *trans*- (0.15 and 0.47 p.p.m.) 1,2-disubstituted cyclopropane series of C_{73} – C_{87} were detected, with series of C_{81} and C_{82} predominant for strains ATCC 35759 and ATCC 14470^T, and series of C_{81} and C_{79} predominant for strain CL-416C. The ratio of *cis* to *trans* was approximately 1:1, and given the structure of methoxy-mycolates of other mycobacteria (Minnikin, 1982; Watanabe *et al.*, 2001), it seems that the odd series can be attributed to *cis* compounds and the even series can be attributed to *trans* compounds. Meroaldehydes from methoxy-mycolates were not found in EI-MS due, probably, to the loss of 31 mass units (–OCH₃), which resulted in a series of *m/z* ions ranging from 769 to 867 (separated by intervals of 14 mass units) that predicted meroaldehydes at *m/z* 800–898. Other fragments characteristic of meroaldehydes were located at *m/z* 297 (intensity 72%), 307 (intensity 19%) and 321 (intensity 16%); considering the *m/z* 354 and 382 for methyl esters, the tentative structure presented in Table 1 can be postulated for the major compounds found in the strains studied.

Keto-mycolates (Table 1) were characterized by resonances at 0.90 (a methyl branch adjacent to a 1,2-disubstituted cyclopropane ring), 1.05 (a methyl branch adjacent to the keto group in the meroaldehyde chain) and 2.32 p.p.m. (–CH₂–CO–). The major series varied from C_{79} to C_{85} and had one *trans*-1,2-disubstituted cyclopropane ring in the structure (resonances at 0.15 and 0.47 p.p.m.). C_{81} and C_{83} were the predominant series for strains ATCC 14470^T and ATCC 35759; C_{81} and C_{79} were the predominant series for strain CL-416C. FAB-MS also predicted very minor series of mycolates with a chain length of C_{78} – C_{80} in the three aforementioned strains, probably containing a *cis*-1,2-disubstituted cyclopropane ring, as suggested by ¹H-NMR (data not shown). The tentative structure of the major keto-mycolate series is given in Table 1 and was based on the presence of important fragments at *m/z* 293, 491, 547 and 601 (derived from meroaldehydes), and at *m/z* 354 and 382 (methyl esters).

DISCUSSION

Analyses of mycolic acids are well established in mycobacterial taxonomy. Extensive studies have described mycolic acids as phenotypically stable chemical compounds, although the discontinuous distribution of the different structural types of mycolic acids described has important chemotaxonomic implications. Since the

1950s, GLC and TLC have been the chromatographic techniques most widely used to analyse the composition of mycobacterial mycolic acids as methyl esters (Daffé *et al.*, 1983; Minnikin *et al.*, 1984; Luquin *et al.*, 1991; Valero-Guillén *et al.*, 1985). In the 1990s, phenacyl esters of mycolic acids were separated by HPLC and detected under UV light (Butler & Guthertz, 2001). The strain-identification data generated by HPLC agree with 97% of the results from standard biochemical tests and 98% of the results from DNA probes; hence, HPLC can be used to correctly identify the majority of mycobacterial species of clinical interest (Butler & Guthertz, 2001; Thibert & Lapierre, 1993).

The elucidation of the structures of mycolic acids uses a variety of techniques (Minnikin, 1982), the most widely used of which are EI-MS and NMR. Recently, new techniques, such as matrix-assisted laser desorption ionization/time-of-flight MS (MALDI/TOF-MS) (Laval *et al.*, 2001; Watanabe *et al.*, 2001) and FAB-MS (Barry *et al.*, 1998), have been applied to the elucidation of these structures. In this study, FAB-MS data complemented the data obtained by EI-MS, giving more precise information on the molecular mass of the different structural types of mycolic acids present in *M. gordonae*. This information is not always evident when using EI-MS, as there is a tendency for several meroaldehydes (e.g. methoxy and dicarboxy) to break down further due to loss of –OCH₃.

Using HPLC, Cage (1992) demonstrated two different mycolic acid patterns for *M. gordonae* – a single-cluster pattern and a double-cluster pattern. In the present study, we have demonstrated that the second cluster of peaks present in the double-cluster pattern actually corresponds to dicarboxy-mycolates, and that these compounds essentially contain a *trans*-1,2-disubstituted cyclopropane ring, although a minor series with a *cis*-1,2-disubstituted cyclopropane has also been detected. As reported by Lanéeelle & Lanéeelle (1970), dicarboxy-mycolates and secondary alcohols (2-eicosanol and 2-octadecanol) are naturally combined in the cell wall to form wax-ester mycolates. These compounds are widely distributed in mycobacteria (Barry *et al.*, 1998; Luquin *et al.*, 1991; Minnikin *et al.*, 1984, 1985a), but only a limited number of studies have dealt with their structural elucidation. Thus, the dicarboxy-mycolates of *Mycobacterium phlei* (see Minnikin, 1982) are mixtures of unsaturated and cyclopropyl homologues, and differ from those found in *M. gordonae*, in which only cyclopropyl derivatives were detected.

Taking into account the presence of 2-octadecanol and 2-eicosanol in the *M. gordonae* strains containing dicarboxy-mycolates, the chain length of wax-ester mycolates from *M. gordonae* should range from C_{81} to C_{85} for the principal components, which is similar to the chain lengths detected for the principal keto-mycolates. Like the keto-mycolates, the major series of the wax-ester mycolates contain a *trans*-1,2-disubstituted cyclopropane ring, thus supporting the hypothesis that keto- and wax-ester mycolates are biosynthetically related (Minnikin, 1982; Barry *et al.*, 1998).

Partial structural analyses of α -, methoxy- and keto-mycolates from *M. gordonae* have been reported previously (Daffé *et al.*, 1981; Minnikin *et al.*, 1985b). However, for the first time, our work has established the nature of the double bonds and the cyclopropyl rings in this species. Thus, α -mycolates from *M. gordonae* are mostly of type-1, as defined by Watanabe *et al.* (2001) – i.e. they contain two cyclopropyl rings with no double bonds. Most slow-growing mycobacterial species examined to date (Minnikin, 1982; Watanabe *et al.*, 2001) contain predominantly this type of α -mycolate, with a *cis* to *trans* ratio of 1:0 (Watanabe *et al.*, 2001), as in the case of *M. gordonae*, although minor series of *trans*-cyclopropyl α -mycolates have been found in *Mycobacterium kansasii* and in the *Mycobacterium avium* complex (Watanabe *et al.*, 2001). However, other types of α -mycolates have been defined in several mycobacterial species (Watanabe *et al.*, 2001), but only the so-called type-3 α -mycolate (one *cis*-cyclopropyl ring plus one *cis* double bond) seems to be present in the *M. gordonae* strains containing dicarboxy-mycolates (this study). This type of α -mycolate has also been characterized in several strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium microti* and in strains of the *M. avium* complex (Watanabe *et al.*, 2001). The aforementioned species also contain a variety of methoxy-mycolates, the major ones being of the type methoxy-mycolate-1 (Watanabe *et al.*, 2001) (one series with a *cis*-cyclopropyl ring and one series with a *trans*-cyclopropyl ring), a characteristic shared by *Mycobacterium leprae* (Draper *et al.*, 1982) and, according to our results, by *M. gordonae*. As for its α -mycolates, *M. gordonae* seems to lack *trans* double bonds or additional cyclopropyl rings in the mero-aldehyde chain and, contrary to other mycobacteria (Watanabe *et al.*, 2001), it lacks methoxy-mycolates with *cis* double bonds. Among the keto-mycolates of *M. gordonae*, we only detected the keto-mycolate-1 (Watanabe *et al.*, 2001), generally with a *trans*-cyclopropyl ring. Hence, the keto-mycolates of *M. gordonae* resemble those of species such as *M. kansasii* and *M. avium*, and differ from those of the *M. tuberculosis* complex, where, in general, the *cis* to *trans* ratio is more balanced (Watanabe *et al.*, 2001).

According to the results cited above, it seems that a high proportion of the mycolic acids of *M. gordonae* have *trans* cyclopropanation, but like other mycobacteria (Barry *et al.*, 1998; Minnikin, 1982; Watanabe *et al.*, 2001) *M. gordonae* still maintains a *cis* configuration in the cyclopropyls of its α -mycolates. An appropriate ratio in the *cis* to *trans* geometry of both double bonds and cyclopropyls seems to have physiological significance for the bacterial cell wall, but its true relevance remains to be elucidated (Barry *et al.*, 1998).

The *M. gordonae* clinical strains studied here exhibit PRA patterns III and V, which are only present in 12% of *M. gordonae* isolates (Telenti *et al.*, 1993). The mycolic acid pattern exhibited by these clinical isolates is also uncommon in members of the genus *Mycobacterium*, as the presence of α -, methoxy-, keto- and

dicarboxy-mycolates has only been reported in two additional species of the genus, *Mycobacterium komosense* and *Mycobacterium bohemicum* (Minnikin *et al.*, 1985a; Torkko *et al.*, 2001).

We conclude that the variability in the mycolic acid patterns of strains of *M. gordonae*, as detected by HPLC and TLC, exists because of the presence of additional components (dicarboxy-mycolates) in some strains of this species. These additional components give rise to the HPLC-double-cluster pattern. Hence, our findings reiterate the heterogeneity of *M. gordonae* reported previously by several authors (Kirschner & Böttger, 1992; Plikaytis *et al.*, 1992; Telenti *et al.*, 1993; Walton & Valesco, 1991).

ACKNOWLEDGEMENTS

The authors are grateful to Dr Gary D. Cage (Department of Health Services, Arizona, USA) for his kind collaboration in this work. The technical assistance of Lidia Hernández is gratefully acknowledged. We are also indebted to Dr J. Rodríguez (SACE, University of Murcia, Spain) for mass spectrometry analyses. This work was supported by grants PM98-0180, FIS 97/0584 and AYA 9800/01.

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Received 16 April 2002; revised 11 July 2002; accepted 12 July 2002.