Structure and Molecular Species Composition of Three Homologous Series of α -Mycolic Acids from *Mycobacterium* spp.

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Three homologous series of α -mycolic acids (dicyclopropanoyl acids, monocyclopropanoyl monoenoic acids and dienoic acids) from 16 rapidly growing and 14 slowly growing mycobacteria were separated by argentation thin-layer chromatography and analysed by gas chromatography/mass spectrometry of their trimethylsilyl ether derivatives. Mycobacterial species were separated into five groups. Strains of group A contained similar amounts of even and odd carbon-numbered dienoic acids, with a methyl branch on the odd acids and a C₂₄- α -unit, as typified by *Mycobacterium fortuitum* and *M. chitae*. Group B strains possessed similar amounts of even carbon-numbered dicyclopropanoyl α -mycolic acids and odd carbon-numbered unsaturated acids with C₂₂- and C₂₄- α -units, as found in *M. phlei* and *M. diernhoferi*. Group C strains contained mainly even carbon-numbered dicyclopropanoyl acids with C₂₂- and C₂₄- α -units, as shown by *M. vaccae* and *M. aurum*. Group D strains possessed mainly odd carbon-numbered dienoic acids with a methyl branch and a C₂₄- α -unit, as seen in *M. triviale* and *M. nonchromogenicum*. Group E strains had mainly even carbon-numbered dicyclopropanoyl acids with C₂₄- or C₂₆- α -units, as found in *M. avium* and *M. tuberculosis*. Many rapidly growing mycobacteria also produced α' -mycolic acids which were shorter in the length of the main carbon chain but whose α -units were the same as those in α -mycolic acids from the same species. These α' -mycolic acids had either one or two double bonds and showed variations in both their unsaturation and overall size, which may be useful in taxonomic studies.

INTRODUCTION

The analysis of subclasses of mycolic acids, the most characteristic cell wall component of the acid-fast bacteria, by thin-layer chromatography (TLC) (Daffé *et al.*, 1983) or by two-dimensional TLC (Minnikin, 1982; Minnikin *et al.*, 1980, 1984, 1985) has shown that several characteristic patterns are useful for the classification of mycobacteria. As for the structural determination of mycobacterial α -mycolic acids, several species such as *Mycobacterium phlei* (Kusamuran *et al.*, 1972), *M. smegmatis* (Etémadi *et al.*, 1967), *M. chelonae* (Minnikin *et al.*, 1982), *M. fortuitum* (Lacave *et al.*, 1987), *M. tuberculosis* (Minnikin & Polgar, 1967),

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Abbreviations: AgNO₃-TLC, argentation thin-layer chromatography; TMS, trimethylsilyl; mono Δ , monocyclopropane; di Δ , dicyclopropane.

M. parafortuitum (Lanéelle & Lanéelle, 1970), *M. avium* (Lamonica & Etémadi, 1967), *M. kansasii* (Etémadi, 1967) and *M. marianum* (*scrofulaceum*) (Bruneteau & Michel, 1968) (for a review, see Minnikin, 1982) have been examined by mass spectrometry (MS) of their degradative products after pyrolysis or ozonolysis and by nuclear magnetic resonance spectroscopy. By isolating each molecular species of mycolic acid using high-performance liquid chromatography, α -mycolic acids of *M. smegmatis* (Danielson & Gray, 1982) and *M. tuberculosis* (Qureshi *et al.*, 1978) were successfully analysed for each molecular species using MS. We have clarified the structure and the molecular species composition of α -mycolic acids in *Nocardia* spp., *Rhodococcus* spp. and *Mycobacterium* spp., using gas chromatography (GC), GC/MS and mass chromatography. The molecular species composition is characteristic for each species, and GC/MS and mass chromatographic analysis of mycolic acids is very precise and informative (Yano *et al.*, 1978; Toriyama *et al.*, 1978; Kaneda *et al.*, 1986*a,b*). Three different homologous series of mycobacterial α -mycolic acids have been reported, dicyclopropanoyl acids, monocyclopropanoyl monoenoic acids and dienoic acids (Minnikin, 1982; Daffé *et al.*, 1983). In a previous paper on the molecular species composition of 25 mycobacterial species (Kaneda *et al.*, 1986*b*), we did not distinguish cyclopropane rings from double bonds in the main chain portion of α -mycolic acids, because the mass spectra of α -mycolic acids did not permit this information. In this study, we have differentiated these three homologous series of α -mycolic acids in 30 mycobacterial species. Their distribution and molecular species characteristics have been studied using argentation thin-layer chromatography (AgNO₃-TLC), GC/MS combined with a hydrogenation technique and mass chromatography.

METHODS

Strains and culture conditions. The following 16 rapidly growing species were studied. Photochromogenic species: *M. vaccae* VA-1 and *M. parafortuitum* PA-1, PA-4, 19686. Scottochromogenic species: *M. phlei* 14002; *M. rhodesiae* RHO-1; *M. gilvum* GI-1; *M. duvalii* DU-2, 29505; *M. aurum* AU-1; and *M. thermoresistibile* 01028, laboratory strain. Nonchromogenic species: *M. chelonae* R-1, R-2, 22011 (subsp. *abscessus*), 19009 (subsp. *chelonae*); *M. smegmatis* Takeo, Rabinobitz; *M. chitae* CH-2, CH-3; *M. fortuitum* F-6, 18001, 18112, E-11592 and E-11620 (subsp. *acetamidolyticum*); *M. peregrinum* PE-4; *M. porcinum* 19506; *M. diernhoferi* 41002, 41004; *M. pulveris* 33505; and *M. agri* 90002, 90012. Cultures of the above species were incubated on a shaker at 30 °C until mid-exponential phase (about 3–5 d) in medium (pH 7.0) containing 1% (w/v) glucose, 0.5% peptone and 0.2% yeast extract, except that *M. fortuitum* subsp. *acetamidolyticum* was cultured for 7 d and *M. thermoresistibile* was grown at 37 °C for 7 d.

The following 14 slow growing species were studied. Photochromogenic species: *M. kansasii* laboratory strain; *M. marinum* 08010; and *M. simiae* 93001. Scottochromogenic species: *M. gordonae* T-12109. Nonchromogenic species: *M. terrae* 38016; *M. nonchromogenicum* 09003; *M. novum* 24018; *M. triviale* 37014; *M. avium* 11016; *M. intracellulare* 13023; *M. ulcerans* 28504; *M. shimoidei* 43501; *M. bovis* BCG, Ravenel; and *M. tuberculosis* H₃₇Rv. These cultures were grown for 3–5 weeks at 37 °C in Sauton medium, pH 7.2 or on slants containing Ogawa egg medium, which contained 100 ml of a basal solution (1%, w/v, sodium glutamate and 1%, w/v, KH₂PO₄), 200 ml whole egg, 6 ml glycerol and 6 ml 2% (w/v) malachite green solution.

Preparation of fatty acid methyl esters. Whole cells, harvested by centrifugation, were hydrolysed with 15% (w/v) KOH at 90 °C for 3–4 h. After acidification with HCl, the fatty acids liberated were extracted with n-hexane and subsequently methylated in 5–10 ml benzene/methanol/concentrated H₂SO₄ (10:20:1, by vol.) at 90 °C for 1.5 h.

TLC and AgNO₃-TLC. The fatty acid methyl esters were developed on a TLC plate of Silica gel G (Analtech) with n-hexane/diethyl ether (4:1, v/v). After locating with iodine vapour each mycolic acid subclass was recovered with chloroform. Isolated α -mycolic acid methyl esters were further separated on a 10% (w/v) AgNO₃-TLC plate of silica gel 60G (Merck) with n-hexane/diethyl ether (94:6, v/v); this developing procedure was repeated four times. The spots were visualized with charring after being sprayed with 50% (v/v) H₂SO₄. For recovery, TLC samples were located with iodine vapour and AgNO₃-TLC samples were located with 2',7'-dichlorofluorescein.

Hydrogenation experiments. α -, α' - or $\alpha + \alpha'$ -mycolic acids separated on TLC, or one class of α -mycolic acids separated on AgNO₃-TLC, were recovered with chloroform and hydrogenated to distinguish double bonds from cyclopropane rings in the molecules; double bonds can be hydrogenated in neutral solvents, whereas cyclopropane rings can be hydrogenated only in acidic solvent systems. The sample was hydrogenated in chloroform/methanol (2:1, v/v) with a few milligrams of platinum oxide (PtO₂: Adams catalyst) for 1 h at room temperature or in glacial acetic acid for 4 h at 40 °C to cleave the cyclopropane rings (Kaneshiro & Marr, 1961). After removal of the catalyst by filtration, the solvent was evaporated off.

Preparation of trimethylsilyl (TMS)-ether derivatives of mycolic acid methyl esters. For GC and GC/MS analysis, α - and α' -mycolic acid methyl esters before or after the hydrogenation were converted to the TMS derivatives, which are stable at high temperatures (Yano *et al.*, 1978; Toriyama *et al.*, 1978). Pyridine (0.1 ml) and bistrimethylsilyl-trifluoroacetamide (0.2 ml) were added to the dried methyl mycolate (1–5 mg) and incubated at 70 °C for 20 min. The solvent and by-products were evaporated off.

GC, GC/MS and mass chromatographic analysis of the TMS-ether derivatives of mycolic acid methyl esters. The procedure has been described in detail previously (Kaneda *et al.*, 1986b). TMS derivatives of methyl mycolate were analysed by GC (Hitachi 063) with a glass column (0.4 m $\times$ 3 mm, 1% OV-101 on Gaschrom Q, column temperature 330 °C isothermally) and subsequently by a GC/MS system (Hitachi M-80B) with the same column (the ionization voltage was 20 eV and the accelerating voltage, 3 kV). The mass spectra were represented as the average value of several scans around the peak top on the chromatograms of the total ion current. Mass chromatograms were obtained by monitoring $[M - 15]^+$ ions of the TMS derivatives of each carbon-number class of mycolic acid methyl esters. On mass chromatograms each molecular species could be shown as an independent peak, and by measuring its peak area the molecular species composition and the average carbon number $[\Sigma(\text{carbon number} \times \text{percentage}/100)]$ could be determined. As well as determining the overall size of mycolic acids, the size of the so-called C_{22} -, C_{24} - and C_{26} - α -units was determined by mass chromatogram monitoring of ions resulting from cleavage between the second and third carbon in the α -mycolic acid derivatives.

RESULTS

Subclass composition of mycolic acids

On TLC plates spots corresponding to non-polar fatty acid methyl esters were observed close to the solvent front and below them several spots were seen corresponding to each subclass of mycolic acid methyl ester. α -Mycolic acids, the most hydrophobic among all the mycolic acid subclasses, were usually a major component in every mycobacterial species except for *M. duvalii* and *M. shimoidei*, which contained only a trace amount. α' -Mycolic acids, whose main carbon chains are shorter than those of α -mycolic acids, were present in many rapidly growing species (all except *M. diernhoferi*, *M. gilvum*, *M. phlei* and *M. rhodesiae*) and in two slow growers, *M. simiae* and *M. shimoidei*.

Analysis of α -mycolic acids

AgNO₃-TLC. On AgNO₃-TLC, purified α -mycolic acid methyl esters both from rapidly and from slowly growing mycobacteria were further separated (Fig. 1). Each consisted of one or two components (upper and lower spots). In the rapid growers *M. chitae*, *M. fortuitum*, *M. smegmatis* and *M. chelonae*, a lower spot predominated but, except for *M. chitae*, they also had a small amount of an upper spot. *M. rhodesiae*, *M. diernhoferi*, *M. phlei* and *M. parafortuitum* contained both upper and lower spots, and *M. pulveris*, *M. thermoresistibile*, *M. aurum*, *M. vaccae*, *M. gilvum* and *M. agri* possessed primarily an upper spot. The slow growers *M. nonchromogenicum*, *M. terrae*, *M. triviale* and '*M. novum*' possessed mainly a lower spot with a very minor upper component. With the exception of *M. bovis* BCG, which also contained a smaller amount of a lower spot, *M. tuberculosis*, *M. kansasii*, *M. simiae*, *M. marinum*, *M. gordonae*, *M. avium*, *M. intracellulare* and *M. ulcerans* contained only an upper spot.

GC. The gas chromatograms of α -mycolic acid derivatives gave generally well-separated peaks ranging from C_{70} to C_{82} in rapid growers (Fig. 2) and from C_{72} to C_{86} in slow growers (Kaneda *et al.*, 1986b). The carbon number of the α -mycolic acids in each peak was determined from its mass spectrum as described later. The α -mycolates from *M. parafortuitum*, *M. rhodesiae*, *M. phlei* and *M. diernhoferi*, however, showed gas chromatographic profiles with poorly separated peaks, probably due to a higher content of odd carbon-numbered acids.

GC/MS. The structure of the molecular species of α -mycolic acids separated by GC was determined from the mass spectra. From the mass number of the characteristic fragment ions of the TMS methyl α -mycolate, i.e. $[M]^+$ (molecular ion), $[M - 15]^+$ (loss of $-\text{CH}_3$), $[M - 90]^+$ (loss of trimethylsilanol), $[A]^+$ (TMS-alkoxy ion resulting from C_2 – C_3 cleavage), $[A - 90]^+$ (loss of trimethylsilanol from $[A]^+$), $[B]^+$ (TMS-oxymethylene carboxylic acid ester resulting from C_3 – C_4 cleavage) and $[B - 29]^+$ (loss of CHO from $[B]^+$), the numbers of carbons and double bonds (or cyclopropane rings) of the whole molecule $[\text{RCH}(\text{OH})\text{CH}(\text{R}')\text{COOH}]$, the β -unit

Fig. 1. AgNO_3 (10% v/v)-TLC of α -mycolic acid methyl esters from rapidly growing mycobacteria (a) and slowly growing mycobacteria (b). Chromatograms were developed four times with n-hexane/diethyl ether (94:6, v/v). (a) Lanes: 1, *M. chitae* CH-2; 2, *M. fortuitum* 18112; 3, *M. smegmatis* Takeo; 4, *M. chelonae* R-1; 5, *M. rhodesiae* RHO-1; 6, *M. diernhoferi* 41002; 7, *M. phlei* 14002; 8, *M. para-fortuitum* PA-1; 9, *M. thermoresistibile* Lab.; 10, *M. pulveris* 33505; 11, *M. vaccae* VA-1; 12, *M. aurum* AU-1; 13, *M. gilvum* GI-1; 14, *M. agri* 90012. (b) Lanes: 1, '*M. novum*' 24020; 2, *M. triviale* 37014; 3, *M. terrae* 38021; 4, *M. tuberculosis* H₃₇Rv; 5, *M. bovis* Ravenel; 6, *M. bovis* BCG; 7, *M. kansasii* Lab.; 8, *M. simiae* 93001; 9, *M. marinum* 08010; 10, *M. gordonae* T-12109; 11, *M. intracellulare* 13542; 12, *M. avium* 11016; 13, *M. ulcerans* 28504.

[RCH(OH)] and the α -unit [CH(R')COOH] could be determined. For example, the mass spectrum of one major GC peak of α -mycolic acids in *M. vaccae* (Fig. 3) showed $[\text{M}]^+ = 1194$, $[\text{M} - 15]^+ = 1179$ and $[\text{M} - 90]^+ = 1104$, which corresponded to C_{76} - α -mycolic acids with two double bonds (or cyclopropane rings); $[\text{A}]^+ = 841$, 813 and $[\text{B}]^+ = 455$, 483, indicating that these C_{76} - α -mycolic acids consisted of two different molecules, i.e., $\text{C}_{54:2(\text{or } 54\text{-di}\Delta)}(\beta\text{-unit}) + \text{C}_{24:0}(\alpha\text{-unit})$ and $\text{C}_{52:2(\text{or } 52\text{-di}\Delta)}(\beta\text{-unit}) + \text{C}_{24:0}(\alpha\text{-unit})$. In every mycobacterial species, α -mycolic acids possessed two double bonds (or the equivalent in cyclopropane rings). A double bond and a cyclopropane ring in α -mycolic acids could not be differentiated from each other by the mass spectrum only, because dicyclopropanoyl α -mycolic acid derivatives showed mass numbers equivalent to the dienolic species.

GC or GC/MS analysis combined with the hydrogenation technique. To clarify whether the β -unit of the α -mycolic acids possessed a cyclopropane ring or a double bond, we combined GC or GC/MS analysis with the hydrogenation technique. When the α -mycolic acid derivatives of *M. chitae*, which showed only a lower spot on AgNO_3 -TLC (Fig. 1), were analysed after hydrogenation in neutral solvents the retention time of the GC peak became longer by about 0.7 carbon numbers and the mass spectral $[\text{M} - 15]^+$ and $[\text{A}]^+$ ions increased by four mass units, showing that these α -mycolic acids were dienolic and that they received four hydrogens to become saturated acids (Fig. 3). On the other hand, when α -mycolic acid derivatives of *M. vaccae*, which showed only an upper spot on AgNO -TLC (Fig. 1), were hydrogenated in the neutral solvent, no change was observed. After hydrogenation in the acidic solvent, however,

Fig. 2. Gas chromatograms of TMS-ether derivatives of α' - and α -mycolic acid methyl esters from rapidly growing mycobacteria, showing characteristic profiles. The carbon numbers of the esters in each peak were determined from the mass spectra. (a), *M. chitae* CH-2; (b), *M. smegmatis* Rabinovitz; (c), *M. chelonae* R-2; (d), *M. fortuitum* F-6; (e), *M. fortuitum* subsp. *acetamidolyticum* E-11620; (f), *M. parafortuitum* PA-4; (g), *M. rhodesiae* RHO-1; (h), *M. vaccae* VA-1; (i), *M. aurum* AU-1; (j), *M. agri* 90002; (k), *M. thermoresistibile* Lab.

the retention time of the GC peak became shorter by about 1.5 carbon numbers and its mass number of $[M - 15]^+$ and $[A]^+$ increased by four mass units with no change in the mass number of $[B]^+$, showing that the α -mycolic acids were dicyclopropanoyl and that they received four hydrogens in the acidic solvent to cleave cyclopropane rings and to become dimethyl branched saturated acids (Fig. 3). For *M. phlei*, which showed two spots, upper and lower, on AgNO_3 -TLC (Fig. 1), we examined the α -mycolic acids of each spot by mass spectrometry after hydrogenation in the neutral solvent. Derivatives of the α -mycolic acids in the upper spot showed no change, indicating that they were dicyclopropanoyl acids, but the mass spectral $[M - 15]^+$ and $[A]^+$ ions from the lower spot increased by two mass units, indicating that they were monocyclopropanoyl monoenoic acids (the lower spot also contained a small amount of dienoic acids as indicated by coexistence of a slightly higher intensity mass spectral $[A]^+$ ion increased by four mass units) (Fig. 4). In *M. diernhoferi*, *M. rhodesiae* and *M. parafortuitum*, the

Fig. 3. Gas chromatograms and mass spectra of the main GC peak with the corresponding carbon skeleton structure of α -mycolic acid derivatives from *M. chitae* CH-2 (a, b) and *M. vaccae* VA-1 (c-e) before hydrogenation (a, c) after hydrogenation in chloroform/methanol (b, d) or after hydrogenation in glacial acetic acid (e).

upper spots on $AgNO_3$ -TLC were also shown to be dicyclopropanoyl α -mycolates and the lower spots to be composed of similar amounts of dienoic and monocyclopropanoyl monoenoic acid derivatives from the two strong $[M - 15]^+$ ions with increases of two and four mass units after hydrogenation in the neutral solvent (data not shown). The upper spots in most slow growers

Fig. 4. Mass spectra of *M. phlei* 14002 C_{78} - α -mycolic acid derivatives from an upper (a, b) and a lower (c, d) spot obtained on $AgNO_3$ -TLC, before (a, c) and after (b, d) hydrogenation in chloroform/methanol.

(Fig. 1) were shown to be dicyclopropanoyl acid derivatives and the lower spots in the *M. nonchromogenicum* complex which includes '*M. novum*', *M. terrae* and *M. triviale* (Fig. 1) to be dienoates. *M. bovis* BCG, exceptionally, contained a small amount of dienoic α -mycolates (lower spot) (Fig. 1) in addition to the major dicyclopropanoyl species (upper spot).

Mass chromatography. Since the gas chromatographic peaks of mycobacterial α -mycolic acid derivatives tended to overlap with their neighbours due to their relative broadness and the relatively high contents of odd carbon-numbered acids (as described later), the molecular species composition of α -mycolic acids was determined by measuring the area of each peak shown independently on a mass chromatogram which monitored the mass number of $[M - 15]^+$ ions of each carbon-number class of diunsaturated α -mycolic acids (Fig. 5). Data on the molecular species composition of α -mycolic acids obtained by this method and the average carbon number in representative mycobacterial species were reported previously (Kaneda *et al.*, 1986b). There was a marked specificity in the distribution of α -mycolic acid molecular species among the mycobacteria. For example, the main peak of α -mycolic acids was C_{74} in *M. vaccae*, *M. parafortuitum*, etc., C_{76} in *M. fortuitum*, *M. aurum*, etc., C_{77} in *M. chitae*, *M. nonchromogenicum*,

Fig. 5. Mass chromatograms of total (upper + lower spots) α -mycolic acid derivatives from *M. vaccae* VA-1 (a), *M. chitae* CH-2 (b) and *M. phlei* 14002 (c), the components of the upper (d) and lower (e) spots on AgNO_3 -TLC from the mycolates of the latter strain are also shown. The mass numbers shown on the right correspond to the $[M - 15]^+$ ions of TMS-ether derivatives of the dienolic or dicyclopropanoyl α -mycolic acid methyl esters whose carbon numbers are given in parentheses. The thick solid line is the gas chromatogram. Thin solid lines and dotted lines are mass chromatograms of even and odd carbon-numbered acid derivatives, respectively. The carbon numbers C_{72} - C_{80} , shown at the bottom of the figure indicate the relative retention of each unbranched dienolic or dicyclopropanoyl α -mycolic acid.

etc., C_{78} in *M. gilvum*, *M. bovis*, etc., C_{79} in *M. terrae*, *M. triviale*, etc. and C_{80} in *M. agri*, *M. tuberculosis*, etc. The molecular species composition of a given mycobacterial species did not vary significantly as long as the growth conditions did not change. The proportion of even carbon-numbered acids was characteristic to each species and was conveniently divided into three groups. (1) Even acids up to 30%: *M. terrae* 38016* (25.2%); *M. nonchromogenicum* 09003* (26.0%); '*M. novum*' 24018* (27.0%). (2) Even acids from 30 to 70%: *M. smegmatis* Jucho.* (32.4%); *M. chitae* CH-2 (37.3%); *M. chelonae* 19009* (48.9%); *M. rhodesiae* RHO-1* (50.1%);

M. phlei 14002 (57.8%); *M. fortuitum* 18001* (58.1%); *M. parafortuitum* PA-1 (61.1%); *M. diernhoferi* 41002 (67.1%). (3) Even acids more than 70%: *M. bovis* BCG (72.7%); *M. marinum* 08010* (73.0%); *M. pulveris* 33505 (73.6%); *M. vaccae* VA-1 (76.4%); *M. tuberculosis* H₃₇Rv* (77.4%); *M. agri* 90012* (81.3%); *M. avium* 11016* (82.0%); *M. intracellulare* 13023* (82.0%); *M. bovis* Ravenel* (82.0%); *M. thermoresistibile* Lab.* (82.3%); *M. aurum* AU-1* (86.0%); *M. gordonae* T-12109* (87.5%); *M. gilvum* GI-1 (89.0%); *M. ulcerans* 28504 (89.5%); and *M. kansasii* Lab.* (94.3%). [The values for the species with an asterisk were calculated from the data in our previous paper (Kaneda *et al.*, 1986*b*); the remaining species were newly examined or re-examined in this study.]

Mass chromatograms, monitoring [M - 15]⁺ ions of α -mycolic acids of *M. vaccae* and *M. chitae*, showed that the retention time of the odd carbon-numbered acids differed between these two species; in *M. vaccae*, odd acids came essentially between the even acids, whereas in *M. chitae*, odd acids appeared close to the retention time of an even acid derivative with one carbon less (Fig. 5). Considering the data on the α -mycolic acids of *M. smegmatis* (Danielson & Gray, 1982), which shows a similar pattern to *M. chitae*, and the fact that the methyl-branched-chain fatty acids appear just after the non-branched-chain parent fatty acids on the gas chromatograms, we believe that the latter type of odd carbon-numbered α -mycolic acids possesses one methyl branch on the straight alkyl portion (β -unit).

We further examined the mass chromatograms of α -mycolic acid derivatives from an upper spot (dicyclopropanoyl acids) and a lower spot (unsaturated acids) when there were two spots on AgNO₃-TLC (Fig. 1), as in *M. phlei* (Fig. 5). There was a significant difference between these two spots not only in the chemical structure but also in the molecular species composition. In *M. phlei*, dicyclopropanoyl α -mycolic acids were composed mainly of even carbon-numbered acids and the odd acids appeared between the even acids on the mass chromatograms, indicating that there was no methyl branch on the β -unit. The unsaturated acids (mainly monocyclopropanoyl monoenoic acids as revealed by the hydrogenation experiment) were composed of similar amounts of even and odd acids, and, from the similar retention time of the even acid derivatives to those of the odd acid derivatives with one carbon less, the even acids possessed one methyl branch on the β -unit. The dicyclopropanoyl and unsaturated acids were distributed within a similar carbon number range, and their average carbon numbers were also similar (Table 1). The molecular species composition and the existence of a methyl branch on the β -unit was also determined for other mycobacterial species which showed two spots for their mycolate derivatives on AgNO₃-TLC (Table 1). Mycobacterial dicyclopropanoyl α -mycolic acids were generally non-methyl-branched and consisted mainly of even acids; the proportion of even acids was 76.4% in *M. vaccae* VA-1, 76.5% in *M. phlei* and 82.2% in *M. bovis* BCG. On the other hand, the unsaturated acids (dienoic acids and/or monocyclopropanoyl monoenoic acids) consisted mainly of odd acids (the proportion of even acids was 21.1% in *M. parafortuitum* PA-1, 20.1% in *M. diernhoferi* 41002, and less than 30% in the *M. nonchromogenicum* complex) or of a similar amount of even and odd acids (the proportion of even acids was 46.7% in *M. phlei* 14002 and 37.3% in *M. chitae* CH-2), often possessing one methyl branch on the β -unit of the even acids (*M. parafortuitum* and *M. phlei*) or on that of odd acids (*M. chitae*, *M. smegmatis*, *M. fortuitum* and the *M. nonchromogenicum* complex) (Table 1). In the case of mycobacterial species which possessed a similar amount of both even acid-dominated dicyclopropanoyl acids and odd acid-dominated unsaturated acids, as in *M. phlei*, *M. parafortuitum*, *M. diernhoferi* and *M. rhodesiae*, the proportion of even carbon-numbered α -mycolic acids amounted to 40–60%.

From the mass spectra of each molecular species (Fig. 3) or from mass chromatogram monitoring of [B - 29]⁺ ions (426, 454 and 482) the sizes of the chains in the α -position of the α -mycolic acids could be determined. Mycobacterial species were grouped according to whether the α -unit was C₂₂, C₂₄ or C₂₆. (1) Mycolic acids with essentially a C₂₂- α -unit: *M. parafortuitum* and *M. diernhoferi*. (2) Mycolic acids with C₂₂- and C₂₄- α -units: *M. phlei*, *M. rhodesiae*, *M. gilvum*, *M. vaccae*, *M. aurum*, *M. pulveris* and *M. duvalii*. (3) Mycolic acids with essentially a C₂₄- α -unit: *M. chelonae*, *M. smegmatis*, *M. chitae*, *M. fortuitum*, '*M. peregrinum*', *M. porcinum*, *M. thermoresistibile*, *M. agri*, *M. terrae*, *M. nonchromogenicum*, '*M. novum*', *M. triviale*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. gordonae*, *M. ulcerans* and *M. shimoidi*. (4) Mycolic acids with a C₂₆- α -unit: *M. simiae*, *M. bovis* and *M. tuberculosis*.

Table 1. Structure and molecular species composition of the α -mycolic acid homologous series from each spot separated on AgNO_3 -TLC

Species	Spot on AgNO ₃ -TLC	Structure		Molecular species composition (%)																Average carbon no.	Percentage proportion of even carbon- numbered acids			
		Methyl branch†		Carbon no.:																				
		Even	Odd	71	72	73	74	75	76	77	78	79	80	81	82									
<i>M. chitae</i> CH-3	Total (= lower)	—	+					7.3	9.0	17.9	34.2	12.1	19.5											37.3
<i>M. vaccae</i> VA-1	Total (= upper)	—	—			4.2	37.4	9.0	34.0	4.7	5.6													76.4
<i>M. diernhoferi</i> 41002	Total*				5.9	18.1	11.9	25.0	11.1	21.1	4.0	2.9												67.3
	Upper	—	—		2.3	18.4	3.8	35.6	5.0	29.1	2.6	3.3												86.3
	Lower	—	—		14.3	6.4	34.0	7.2	26.0	6.5	5.6													20.1
<i>M. parafortuitum</i> PA-1	Total*					8.3	16.0	26.1	18.8	24.6	4.1	2.1												61.1
	Upper	—	—			6.3	8.0	29.3	12.3	35.7	8.4													71.3
	Lower	+	—		4.2	3.4	35.7	9.0	32.2	8.8	6.8													21.1
<i>M. phlei</i> 14002	Total*								9.2	22.8	20.5	31.0	12.5	4.0										57.8
	Upper	—	—						1.8	27.7	11.5	43.9	10.2	4.9										76.5
	Lower	+	—						15.9	14.8	34.0	28.8	3.4	3.0										46.7
<i>M. bovis</i> BCG	Total*							1.8	1.9	13.1	10.0	38.7	12.8	19.1	2.6									72.7
	Upper	—	—					2.2	3.1	12.3	6.6	39.5	5.9	21.4	2.2	6.7								82.2
	Lower	—	—							10.4	21.2	15.7	30.6	8.2	13.8									34.4

* Total = upper + lower spot.

† A methyl branch on even or odd carbon-numbered acids.

Fig. 6. Mass spectra of the main peaks of α' -mycolic acid derivatives from *M. vaccae* VA-1 (a, b) and *M. fortuitum* 18001 (c, d) before (a, c) and after (b, d) hydrogenation in chloroform/methanol.

Analysis of α' -mycolic acids

Several peaks of α' -mycolic acid derivatives with sizes ranging from C₅₈ to C₇₀ were clearly observed on gas chromatograms (Fig. 2). In *M. vaccae*, the mass spectrum of the main peak showed: $[M]^+ = 972$, $[M - 15]^+ = 957$ and $[M - 90]^+ = 882$, which corresponded to C₆₂- α' -mycolic acids with one double bond (or a cyclopropane ring); $[A]^+ = 619$, 591 and $[B]^+ = 455$, 483, indicating that these C₆₂- α' -mycolic acids contained C_{40:1}(or 40-mono Δ)(β -unit) + C_{22:0}(α -unit) and C_{38:1}(or 38-mono Δ)(β -unit) + C_{24:0}(α -unit) (Fig. 6). In *M. fortuitum* the main component was determined as C_{68:2}(or 68-di Δ)- α' -mycolic acids with a C_{24:0}- α -unit. After hydrogenation in the neutral solvent, the α' -mycolic acids both of *M. vaccae* and of *M. fortuitum* showed mass numbers equivalent to those of the saturated acids, indicating that these two α' -mycolic acids were monoenoic and dienoic, respectively (Fig. 6). In this study, α' -mycolic acids of every mycobacterial species were shown to have one double bond except in *M. fortuitum* and related taxa (*M. fortuitum* subsp. *acetamidolyticum*, '*M. peregrinum*' and *M. porcinum*), which possessed two double bonds. Although the C_{66,68}- α' -mycolic acids of *M. smegmatis*, as reported by Etémadi *et al.* (1967), and the C₆₆- α' -mycolic acids of *M. chelonae* consisted of a significant amount of dienoic acids as revealed by mass chromatography monitoring the $[M - 15]^+$ ions of both dienoic and monoenoic α' -mycolic acids (data not shown), these dienoic acids only made up a small proportion of the total α' -mycolic acids. Furthermore, these monoenoic or dienoic α' -mycolic acids did not have a methyl branch on their β -unit (deduced from the fact that the odd carbon-numbered acids were always situated between even acids on the mass chromatograms produced by monitoring $[M - 15]^+$ ions). From the mass chromatograms of α' -mycolic acids monitoring $[B - 29]^+$ ions of C₂₂-, C₂₄- and C₂₆- α -unit-containing mycolic acids, the proportions of these three homologous series of α' -mycolic acids were shown to be similar to those of the α -mycolic acids from the same bacterial species.

The molecular species composition of α' -mycolic acids was determined by measuring each peak area on gas chromatograms or mass chromatograms, and the average carbon number was calculated (Table 2). The average carbon number of α' -mycolic acids varied from species to species; it was about 60 in *M. parafortuitum* and *M. vaccae*, about 62 in *M. chitae*, *M. duvalii*, *M. aurum* and *M. pulveris*, about 64 in *M. smegmatis*, *M. chelonae*, *M. shimoidei* and *M. thermoresistibile*, about 66 in *M. simiae* and about 68 in *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, '*M. peregrinum*' and *M. agri*. Mass chromatographic analysis revealed that α' -mycolic acids from every mycobacterial species were composed mainly of even carbon-

Table 2. *Molecular species composition of α' -mycolic acids in mycobacteria*

With the exception of *M. pulveris*, *M. shimoidei* and *M. agri*, species containing less than 15% of odd carbon-numbered α' -mycolic acids are not listed.

Species	Molecular species composition (%)*														α -Unit	β -Unit	Average carbon no.	Double bond no.	
	56	57	58	59	60	61	62	63	64	65	66	67	68	69					70
<i>M. parafortuitum</i> PA-1	1.0				59.8		14.6		1.0							22	34-42	59.7	1
<i>M. vaccae</i> VA-1			23.4		49.3		19.8		1.4							22, 24	36-44	59.9	1
<i>M. chitae</i> CH-2			1.3		23.2		56.1		18.4		1.0					24 (22)†	36-42	61.9	1
<i>M. duvalii</i> DU-2			3.4		16.1		42.0		31.1		6.3		1.1			22, 24	36-44	62.5	1
<i>M. pulveris</i> 33505				1.1	15.1	4.7	36.8	7.2	3.2	4.6						22, 24	37-42	62.5	1
<i>M. aurum</i> AU-1					10.8		43.8	34.7	9.6				1.1			22, 24	38-44	62.9	1
<i>M. smegmatis</i> Rabino.					4.3		39.5	41.8	11.7				2.7			24	36-44	63.4	1 (2)†
<i>M. chelonae</i> R-1							11.1	83.9	5.0							24	38-42	63.9	1
<i>M. thermoresistibile</i> Lab.					3.7		15.2		44.2	32.8			4.1			24	36-44	64.4	1
<i>M. shimoidei</i> 43501							10.4	5.0	43.2	8.0	25.7	2.5	5.1			24	38-44	64.6	1
<i>M. simiae</i> 93001									18.5		72.5		9.0			26 (24)†	38-44	65.8	1
<i>M. fortuitum</i> 18001									3.4		25.2		63.5			24	40-46	67.5	2
<i>M. peregriinum</i> PE-1									1.7		29.9		63.8			24	40-46	67.4	2
<i>M. agri</i> 90002									3.0	4.4	21.0	13.6	50.6	5.3		24	42-46	67.3	1

* Values shown in bold are the main components of a given species.

† A small but a significant proportion of mycolic acids with the structure shown in parentheses coexist.

numbered acids. The proportion of odd carbon-numbered α' -mycolic acids was generally up to about 20% except in *M. agri*: *M. vaccae* VA-1 and *M. fortuitum* 18001 (trace amount); *M. simiae* 93001 (6.5%); *M. chelonae* 22011 (7.8%); *M. chitae* CH-3 (8.2%); *M. duvalii* 29506 (9.7%); *M. parafortuitum* PA-1 (9.8%); *M. smegmatis* Rabinovitz (11.3%); *M. thermoresistibile* Lab. (11.3%); *M. shimoidei* 43501 (15.5%); *M. pulveris* 33505 (16.2%) and *M. agri* 90002 (23.3%).

Variation in the structure and the molecular species composition of α -mycolic acids from 16 rapid growers and 14 slow growers allows mycobacterial species to be divided into five groups, A–E, as indicated in Table 3.

DISCUSSION

Mycobacterial α -mycolic acids possess two dicyclopropanoyl rings, one cyclopropane ring plus one double bond or two double bonds on the β -unit (Minnikin, 1982; Daffé *et al.*, 1983). These three different homologous series of α -mycolic acids were identified from the mass ion shift after hydrogenation in neutral or acidic solvents. The upper spot on AgNO₃-TLC was shown to be dicyclopropane and the lower one was olefinic acid derivatives – monocyclopropanoyl monoenoic acids and/or dienoic acids. It is uncertain why the latter two homologous series of α -mycolic acids could not be clearly separated on AgNO₃-TLC. However, Minnikin (1982) reported that one of the two double bonds on the odd carbon-numbered, mono-methyl branched (or monocyclopropanoyl) α -mycolic acids were not *cis* monoenoic but *trans* monoenoic. Since it is well known that *trans* monoenoic fatty acids migrate between the saturated and the *cis* monounsaturated acids on AgNO₃-TLC, α -mycolic acids may also behave similarly. In this study, by examining the α -mycolic acids of each upper and lower spot on AgNO₃-TLC, it was clearly shown that a significant difference existed in the molecular species composition between the dicyclopropanoyl acids and the olefinic acids. Dicyclopropanoyl α -mycolic acids were composed mainly of even carbon-numbered acids and possessed no methyl branch on the β -unit, whereas olefinic acids were composed either of predominantly odd carbon-numbered acids or of a similar amount of odd and even carbon-numbered acids, often possessing one methyl branch on the β -unit. The result obtained here (that a methyl branch often existed in the α -mycolic acids, possessing one or two double bonds) is in accordance with the structural formula of α -mycolic acids presented by Minnikin *et al.* (1984). This is reasonable because a methyl branch is considered to be introduced on the adjacent carbon of a double bond during its *cis* to *trans* conversion (Minnikin, 1982).

Many rapid growers produce α' -mycolic acids which have shorter main chain β -units than those in the α -mycolic acids. In the slow growers, only two species (*M. shimoidei* and *M. simiae*) contained this type of mycolic acid subclass. However, there was no difference in the structure or the composition of the α' -mycolic acids from rapid growers and those from slow growers. The α' -mycolic acids of *M. fortuitum* and related taxa were characteristically dienoic, whereas in many other species they were monoenoic. These α' -mycolic acids, both monoenoic and dienoic, possessed no methyl branch on the β -unit and contained only a small amount of odd carbon-numbered acids (*M. agri*, *M. shimoidei* and *M. pulveris* possessed relatively more odd carbon-numbered acids, but always less than 30% of the total α' -mycolic acids). The carbon chain length of α' -mycolic acids in groups B and C was generally shorter (up to C₆₂) than in group A (more than C₆₄).

It has been shown that α' -mycolic acids are not merely small forms of α -mycolic acids, but have rather independent structures, e.g. (1) in groups B and C, the α' -mycolic acids were monoenoic, whereas the α -mycolic acids were mainly dicyclopropanoyl derivatives; (2) in group A, the odd carbon-numbered α -mycolic acids were methyl-branched, but the α' -mycolic acids were not; (3) the proportions of odd carbon-numbered acids were very low in α' -mycolic acids; and (4) there were no obvious correlations in the carbon chain length between α - and α' -mycolic acids within the same bacterial species – *M. chitae*, *M. smegmatis* and *M. thermoresistibile*, which contained longer α -mycolic acids (C_{77–80}), possessed shorter α' -mycolic acids (C_{62–64}) and, conversely, *M. fortuitum* and '*M. peregrinum*', which possessed intermediate sized α -mycolic

Table 3. Chemical structure and molecular species composition of α - and α' -mycolic acids in mycobacteria

Species	α -Mycolic acids				α' -Mycolic acid				Chromogenesis§
	Structure		Composition		Structure		Composition		
	β -Unit* (main component)	α -Unit†	Main peak	Proportion of even acid‡	β -Unit* α -Unit†	Main peak	Proportion of even acid‡		
Rapid growers									
Group A									
<i>M. chelonae</i>	III, III'	24	77	x	IV	64	0	N	
<i>M. smegmatis</i>	III, III'	24	77	x	IV	64	0	N	
<i>M. chitae</i>	III, III'	24 (22)	77	x	IV	62	0	N	
<i>M. fortuitum</i>	III, III'	24	76	x	III	68	0	N	
' <i>M. peregrinum</i> '	III, III'	24	76	x	III	68	0	N	
<i>M. porcinum</i>	III, III'	24	77	x	III	68	0	N	
<i>M. fortuitum</i> subsp. <i>acetamidolyticum</i>	III, III'	24	78	x	III	68	0	N	
Group B									
<i>M. diernhoferi</i>	I, II, III	22	74	x	—	—	—	N	
<i>M. phlei</i>	I, II, IV'	22, 24	78	x	—	—	—	S	
<i>M. rhodesiae</i>	I, II, IV', III, III'	22, 24	74	x	—	—	—	S	
<i>M. parafortuitum</i>	I, II, IV', III, III'	22	74	x	IV	60	0	P	
Group C-1									
<i>M. gilvum</i>	I	22, 24	78	0	—	—	—	S	
<i>M. vaccae</i>	I	22, 24	74	0	IV	60	0	P	
<i>M. aurum</i>	I	22, 24	76	0	IV	62	0	S	
<i>M. pulveris</i>	I	22, 24	78	0	IV	62	0	N	
<i>M. dualii</i>	I	22, 24	76	ND	IV	62	0	S	
Group C-2									
<i>M. thermoresistibile</i>	I	24	80	0	IV	64	0	S	
<i>M. agri</i>	I	24	80	0	IV	68	0	N	

Slow growers									
Group D									
<i>M. terrae</i>	III, III'	24	79	xx	—	—	—	—	N
<i>M. nonchromogenicum</i>	III, III'	24	77	xx	—	—	—	—	N
' <i>M. novum</i> '	III, III'	24	79	xx	—	—	—	—	N
<i>M. triviale</i>	III, III'	24	79	xx	—	—	—	—	N
Group E-1									
<i>M. avium</i>	I	24	80	o	—	—	—	—	N
<i>M. intracellulare</i>	I	24	80	o	—	—	—	—	N
<i>M. kansasii</i>	I	24	80	o	—	—	—	—	P
<i>M. goodii</i>	I	24	78	o	—	—	—	—	S
<i>M. marinum</i>	I	24	76	o	—	—	—	—	P
<i>M. ulcerans</i>	I	24	76	o	—	—	—	—	N
<i>M. shimoidei</i>	ND	ND	ND	ND	IV	24	64	o	N
Group E-2									
<i>M. simiae</i>	I	26 (24)	82	o	IV	26 (24)	66	o	P
<i>M. bovis</i>	I	26	78	o	—	—	—	—	N
<i>M. tuberculosis</i>	I	26	80	o	—	—	—	—	N

ND; Not determined.

• The structural formulae are cited from Minnikin *et al.* (1984) as shown below.

† Carbon number.

‡ o, > 70%; x, 30–70%; xx, < 30%.

§ N, Nonchromogenic; S, scotochromogenic; P, photochromogenic.

|| A small but a significant proportion of mycolic acids with the structure shown in parentheses coexist.

acids (C_{76}), contained longer α' -mycolic acids (C_{68}). The proportions of the C_{22} - and C_{24} - α -units were, however, similar between the α - and α' -mycolic acids from the same bacterial species. The compositions of the α -units in the mycolic acids analysed here (Table 3) are in good agreement with the results obtained by pyrolysis gas chromatography (Kusaka & Mori, 1986).

Chromogenicity can be used to divide rapid growers into two groups. The chromogenic species include many saprophytic mycobacteria isolated from soil or water and the non-chromogens may include several opportunistic pathogens such as *M. fortuitum* and *M. chelonae* besides other non-pathogenic species (Goodfellow & Wayne, 1982). The grouping of rapid growers based on mycolic acid patterns was concordant with chromogenicity. Groups B and C included many chromogenic species but the mycobacteria in group A were all nonchromogenic. There is, however, no obvious correlation between the biosynthesis of carotenoid pigments and mycolic acids. Furthermore, Groups B and C included several nonchromogenic species, e.g. *M. diernhoferi*, *M. pulveris* and *M. agri*, and there was no correlation between chromogenicity and mycolic acid patterns in the slow growers. Although chromogenicity is often used as a convenient criterion for identifying or classifying mycobacterial species, the significance of pigment production in the physiology of mycobacterial species and its taxonomic value are not yet clear (Tsukamura, 1963; Minnikin, 1982). Furthermore, during repeated culture, nonchromogenic species often acquire a slight chromogenicity which sometimes causes confusion in identification. The grouping of mycobacteria by mycolic acid patterns is more reliable because mycolic acids are always major structural components of acid-fast bacteria and many characteristic enzymes must be involved in their biosynthesis (Minnikin, 1982; Takayama & Qureshi, 1984; Lacave *et al.*, 1987). Furthermore mycolic acid patterns are relatively constant if the cultural conditions do not vary.

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