Separation and characterization of individual mycolic acids in representative mycobacteria

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Total mycolic acid methyl ester fractions were isolated from 24 representatives of *Mycobacterium tuberculosis*, *Mycobacterium bovis* (including BCG), *Mycobacterium microti*, *Mycobacterium kansasii* and *Mycobacterium avium*. The total mycolate functional group composition was estimated from ¹H-NMR spectra. Mycolates were separated into α -mycolates, methoxymycolates and ketomycolates and each class was further separated by argentation chromatography into mycolates with no double bonds, with one *trans*-double bond and with one *cis*-double bond. Mass spectrometry revealed the mycolate chain lengths and ¹H-NMR the *cis*- and *trans*-double bond and cyclopropane ring content. The same species had similar mycolate profiles; the major type of each class had *cis*- or *trans*-cyclopropane rings and lacked double bonds. Minor proportions of possible unsaturated precursors of the cyclopropane mycolates were commonly encountered. Among unusual α -mycolates, many strains had tricyclopropyl components with chains extended by 6 to 8 carbons. Significantly, *M. tuberculosis* (Canetti) and *M. avium* had α -mycolates with a *trans*-double bond and cyclopropane ring, whose chain lengths suggested a relationship to possible precursors of oxygenated mycolates. The methoxy- and ketomycolates from a majority of *M. tuberculosis* strains had minor amounts of components with additional cyclopropane rings, some of whose chains were also extended by 6 to 8 carbons. These latter mycolates were major components in the attenuated *M. tuberculosis* H37Ra strain, whose mycolate profile was distinct from those of other strains of *M. tuberculosis*.

Keywords: mycobacterial mycolates, cyclopropane rings, double bonds, stereochemistry, tuberculosis

INTRODUCTION

Mycobacterial mycolic acids, whose general structures are indicated in Fig. 1, are high-molecular-mass 2-alkyl branched, 3-hydroxy fatty acids, which are characteristic components of the cell envelope of mycobacteria. Although they are present in the extractable lipids, mostly as trehalose dimycolates, the major portion is covalently bound in the cell envelope esterified to the 5-hydroxy groups of arabinofuranosyl residues to form the terminal [5-mycoloyl- β -Araf-(1 $\rightarrow$ 2)-5-mycoloyl- α -Araf-(1 $\rightarrow$)] units of a major arabinogalactan polysaccharide (McNeil *et al.*, 1991). The mycoloyl arabino-

galactan is, in turn, attached to the shape-forming peptidoglycan.

Schematic models of mycobacterial envelope structure have been proposed to show possible arrangements for the mycolic acids and other lipid components (McNeil & Brennan, 1991; Minnikin, 1991; Paul & Beveridge, 1994). According to the currently accepted structural model (Brennan & Nikaido, 1995; Daffé & Draper, 1998; Draper, 1998), the long hydrocarbon chains of the mycolic acids are arranged in an orderly fashion, in parallel, with the methyl ends towards the outside surface. This arrangement results in the electron-transparent layer of the cell envelope, the presence of which is revealed by electron micrographs of ultra-thin sections of mycobacterial cells (Paul & Beveridge, 1992, 1994).

The mycolic acids usually have two intra-chain groups

Abbreviations: MAC, *M. avium*–intracellular complex; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry.

Fig. 1. Generalized structures of major mycobacterial mycolic acids, indicating possible combinations of intra-chain functional groups, X and Y. Type-1 mycolates have no double bond, type-2 mycolates contain a *trans*-double bond and type-3 mycolates have a *cis*-double bond. The precise orientations of some of the X and Y groups are unknown, and in type-2 and type-3 α -mycolates X and Y may be reversed.

in the major 'meromycolate' chain (Fig. 1), which vary in the type, stereochemistry and spacing. These functional groups will affect the packing of the long hydrocarbon mycolate chains, thereby influencing the intrinsic physiological functions of the cell envelope. Mycolic acids are major components of the mycobacterial cell envelope, amounting to as much as 34% by weight of the cell wall skeleton in *Mycobacterium microti* (Davidson *et al.*, 1982), for example.

A true understanding of the importance of mycolic acids in cell envelope structure cannot be achieved until complete knowledge is established regarding the composition and the nature of the individual components of mycolic acids. Most previous studies have simply characterized the major mycolic acids in certain species (Davidson *et al.*, 1982; Qureshi *et al.*, 1978) or recorded the general distribution of mycolate types, the essential functional groups and the range of chain lengths of mycolic acids in representative mycobacteria (Minnikin *et al.*, 1984a).

In the present study, detailed assays of the composition and structure of mycolic acids from representative mycobacteria were performed, following normal-phase silica gel and argentation chromatographic separation into individual components. Each of the separated component mycolates was assayed by ^1H -NMR spectrometry and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) to reveal the stereochemistry of the double bonds and cyclopropane rings and their chain lengths. The ratios between the α -, methoxy- and ketomycolates in those mycobacteria were also estimated on the basis of the ^1H -NMR spectra.

METHODS

^1H -NMR and mass spectra. ^1H -NMR spectra (CDCl_3) were recorded on a Bruker AM400 (400 MHz) and MALDI-TOF/MS (matrix: 2,5-dihydroxybenzoic acid) on a PerSeptive Biosystems Voyager Rs, in reflector mode. EI/MS was used to identify the size of the alkyl chain in the 2-position.

Fig. 2. ^1H -NMR spectrum of the total mycolic acid methyl esters from *M. bovis* BCG Tokyo, in CDCl_3 . Complex signals are magnified: **a**, *cis*-olefinic protons, **a'**, *trans*-olefinic protons, **b**, protons for methylene and methine groups adjacent to keto carbonyls, **b'**, protons on C2 and C3-OH, **c**, protons for methylene groups adjacent to *cis*-double bonds, **c'**, protons for methylene and methine groups adjacent to *trans*-double bonds.

Silica gel column chromatography and TLC. The silica gel used for column chromatography was 40–50 μm spherical beads (Kanto Kagaku, Japan). For preparative TLC, Merck 5744 plates were used, with a 2% ethanolic spray solution of Rhodamine 6G for detection under UV light.

Argentation TLC. AgNO_3 -treated silica gel plates were prepared by treatment of precoated Merck 5744 silica gel plates with a 15% solution of AgNO_3 in acetonitrile, followed by drying.

Cell materials. The mycobacteria used were 19 strains of the *M. tuberculosis* complex [*M. tuberculosis* including clinical isolates (10), *M. bovis* (2), *M. bovis* BCG (6), *M. microti* (1)], 3 strains of *M. kansasii* and 2 strains of the *M. avium-intracellulare* complex (MAC), as listed in Table 1. Of the *M. tuberculosis* strains, O and K were newly separated clinical isolates, subcultured on egg slants only three times before the present culture in Kirchner's medium (Watanabe *et al.*, 1994). Aoyama B is a Japanese standard strain used for the preparation of PPD. Strains 9829/87, 4610/91 and 2668/92 were isolated from patients in Sweden, subcultured twice on Löwenstein–Jensen slants and once on solid Sauton's medium before mass cultivation on the latter.

Preparation of total mycolate fraction. The mycolic acid fractions were prepared from hexane-washed or chloroform-washed defatted dry cell mass by a modification of a previous

method (Hamid *et al.*, 1993). Briefly, the cell mass was heated in aqueous 15% tetrabutylammonium hydroxide at 70 °C overnight followed by centrifugation and extraction of the acidified hydrolysate with diethyl ether. Further alkaline hydrolysis of the washed residue with solutions of either tetrabutylammonium hydroxide or NaOH produced no additional mycolic acid, as shown by TLC. The diethyl ether extract was placed on a silica gel column, eluted first with hexane and then with a series of diethyl ether/hexane (6:100, 1:9 and 3:7, v/v) mixtures; the latter eluent gave mycolic acids. The crude mycolic acid fraction was treated with trimethylsilyldiazomethane (Aldrich) (Hashimoto *et al.*, 1981) and the methyl mycolate fraction was subjected to silica gel column chromatography, eluting first with hexane and then with diethyl ether/hexane (3:7, v/v) to give a purified total methyl mycolate fraction. For mycolate functional group assays, by ^1H -NMR, a part of the total methyl mycolate fraction was further purified by preparative silica gel TLC with the same solvent system.

Separation of component mycolates. The total methyl mycolate fraction was subjected to silica gel column chromatography eluting with diethyl ether/hexane (6:100, v/v) to separate the mixture into α -mycolate, methoxymycolate and ketomycolate. Care was taken to collect the whole of each class of mycolate, as the stereochemistry of double bonds and cyclopropane rings in mycolates tended to affect the elution

Table 1. Distribution of functional groups in total mycolate fraction from representative mycobacteria on the basis of $^1\text{H-NMR}$ spectra

Strain*	Double bonds†		Cyclopropyls	Mycolate ratios‡	
	Content (%)	Nature		α :methoxy:keto	α :(methoxy + keto)
<i>M. tb</i> Aoyama B ^a	6.0	c/t	1:0.18	1:0.74:0.25	1:0.99
<i>M. tb</i> MNC1397 ^b	4.0	c/t	1:0.11	1:0.78:0.28	1:1.06
<i>M. tb</i> O ^a	5.0	c/t	1:0.13	1:0.49:0.10	1:0.59
<i>M. tb</i> K ^a	5.1	c/t	1:0.13	1:0.50:0.13	1:0.63
<i>M. tb</i> 9829/87 ^c	9.5	$\ll$ t	1:0.15	1:0.63:0.34	1:0.97
<i>M. tb</i> 4610/91 ^c	6.4	c/t	1:0.14	1:0.72:0.29	1:1.01
<i>M. tb</i> 2668/92 ^c	9.6	c > t	1:0.14	1:0.57:0.40	1:0.97
<i>M. tb</i> Canetti MNC1485 ^c	18.0	$\ll$ t	1:0.19	1:0.64:0.80	1:1.24
<i>M. tb</i> H37Rv ^c	10.7	c > t	1:0.14	1:0.54:0.49	1:1.03
<i>M. tb</i> H37Ra ^c	22.2	cis	1: < 0.01	1:0.57:0.45 ^a	1:1.02
<i>M. bovis</i> MNC8 ^b	5.9	c $\ll$ t	1:0.17	1:0.72:0.35	1:1.07
<i>M. bovis</i> MNC27 ^b	—	—	—:—	1:0.65:0.30	1:0.95
<i>M. bovis</i> BCG Danish 1331 ^d	12.9	c/t	1:0.12	1: < 0.01:1.80	1:1.81
<i>M. bovis</i> BCG Danish GB425 ^c	15.3	c > t	1:0.09	1:0.05:1.76	1:1.81
<i>M. bovis</i> BCG Danish MNC5 ^b	8.4	c/t	1:0.24	1: < 0.01:1.73	1:1.74
<i>M. bovis</i> BCG Glaxo 10-F ^d	8.5	c > t	1:0.04	1: < 0.01:1.80	1:1.81
<i>M. bovis</i> BCG Pasteur 1173-p2 ^d	9.0	c > t	1:0.06	1:0.04:1.46	1:1.50
<i>M. bovis</i> BCG Tokyo 172 [9 days] ^d	—	—	—:—	1:0.60:2.96 ^b	1:3.56
<i>M. bovis</i> BCG Tokyo 172 [2 weeks] ^d	—	—	—:—	1:0.94:2.59 ^b	1:3.53
<i>M. bovis</i> BCG Tokyo 172 [3 weeks] ^d	21.0	c > t	1:0.17	1:0.85:2.67	1:3.52
<i>M. bovis</i> BCG Tokyo 172 [4 weeks] ^d	—	—	—:—	2.38 ^b	1:3.58
<i>M. bovis</i> BCG Tokyo 172-V ^e	18.1	c $\gg$	1:0.18	1:0.97:2.57	1:3.54
<i>M. microti</i> OV248 ^f	17.2	c > t	1:0.12	1:0.50:1.16	1:1.66
<i>M. kansasii</i> 20-01 ^d	10.3	c/t	1:0.37	1:0.49:0.68	1:1.17
<i>M. kansasii</i> 304 ^d	8.7	c/t	1:0.53	1:0.50:1.00	1:1.50
<i>M. kansasii</i> 429 ^d	7.2	c/t	1:0.38	1:0.46:0.78	1:1.24
<i>M. avium</i> complex KK41-24 ^d	30.4	$\ll$ t	1:0.61	1:—:1.71 ^c	—:—
<i>M. avium</i> complex MNC10 ^b	25.0	c < t	1:0.75	—:—:—	—:—

* *M. tb*, *M. tuberculosis*. *a*, Strains obtained from Research Institute of Tuberculosis, Kiyose, Tokyo; surface cultured in Kirchner's medium at 37 °C for 4 weeks. *b*, Cells surface cultured in Sauton's medium in Statens Seruminstitut, Copenhagen, Denmark; see Minnikin *et al.* (1984a). *c*, Cells surface cultured in Sauton's medium in the Department of Medical Microbiology, University of Gothenburg, Sweden; see also Minnikin *et al.* (1984a). *d*, Strains from Research Institute of Tuberculosis, Kiyose, Tokyo; shake cultured in Sauton's medium at 37 °C for 3 weeks unless specified [figures in brackets represent cultivation period]. *e*, BCG Tokyo 172 cells which had grown at 42 °C were subcultured at 42 °C, and then shake cultured in Sauton's medium at 37 °C for 3 weeks. *f*, For culture conditions, see Davidson *et al.* (1982).

† 'Content' is percentage content per whole mycolate. *cis* means *trans* not positively detected; c $\gg$, mostly *cis*; c > t, more *cis* than *trans*; c/t, *cis/trans* ratio about 1:1; $\ll$ t, mostly *trans*. Estimation on the basis of $^1\text{H-NMR}$ signal shapes. —, Either the signal was not detected or measurement was not performed.

‡ *a*, Ratio of the weights recovered. The calculated ratio was 1:0.44:0.23. See text. *b*, Estimation on the basis of HPLC. *c*, Mixture of ketomycolate and unhydrolysed wax-ester mycolate, in approximate ratio 0.6:1.

rates in silica gel chromatography with diethyl ether/hexane (see below). For the MAC mycolates, tetrabutylammonium hydroxide hydrolysis gave an inseparable mixture of keto- and wax-ester mycolates, so the mixture was further hydrolysed according to Jenkins & Goren (1986). Briefly, a keto- and

wax-ester mycolate mixture (100 mg) was heated at 80 °C with 2-propanol (2 ml) and crushed KOH (200 mg) in a sealed tube for 2 h and treated in the usual manner to obtain an acid fraction, which was methylated with trimethylsilyl diazomethane. The methyl ester fraction was then subjected to

Fig. 3. MALDI-TOF/MS charts, in which all peaks are $[M+Na]^+$ ions. (a) α -Mycolate-3 of *M. tuberculosis* H37Ra (Table 2); one series (m/z 1161, 1189 1133, 1217) is for α -mycolate-3, having one *cis*-double bond and one *cis*-cyclopropane ring, and the other series (m/z 1201, 1229 > 1173, 1257) is for mycolates with one *cis*-double bond and two *cis*-cyclopropane rings. (b)

silica gel column chromatography, eluting with hexane/ethyl acetate (10:1, v/v) to separate the resulting keto-, ω -carboxy-mycolate and 2-alkanols. No further assays were conducted on the wax-ester mycolates.

Each of the α -, methoxy- and ketomycolates was applied to $AgNO_3$ -treated silica gel TLC plates, respectively developed three to five times with 6:100, 8:100 and 1:10 (v/v) diethyl ether/hexane mixtures. Each mycolate class was separated into type-1 mycolate (mycolate-1, no double bonds), type-2 mycolate (mycolate-2, one *trans*-double bond), and type-3 mycolate (mycolate-3, one *cis*-double bond) (Fig. 1), as the top, middle and lowest bands, respectively. When the double bond content of the original material was very low or the amount of the mycolates available was very small, only the major type-1 mycolates were separated and characterized.

Effect of *cis/trans* stereochemistry on mycolate chromatography. The methyl mycolate fraction (about 300 mg) of *M. kansasii* 304 was placed on a silica gel column (22 $\times$ 180 mm) and diethyl ether/hexane (6:100, v/v) eluates were collected in 20 ml fractions. Each fraction was assayed for *cis/trans*-double bond and *cis/trans*-cyclopropane ring content by 1H -NMR.

Estimation of mycolate compositions for each strain. The ratios between the α -, methoxy- and ketomycolates in the total mycolate fractions were calculated by using the following equations, (1) and (2) or (2'). These equations assumed that the α -mycolate contained two cyclopropane rings or one cyclopropane ring and one double bond, the methoxymycolate one methoxy group and one cyclopropane ring or one double bond, and the ketomycolate one keto group and one cyclopropane ring or one double bond. The bracketed abbreviations α , MeO and Keto represent the content of α -, methoxy- and ketomycolates, respectively, estimated from the area of each characteristic 1H -NMR signal.

(Double bond) + (cyclopropane ring)

$$= 2(\alpha) + (\text{MeO}) + (\text{Keto}) \quad (1)$$

$[(\text{Methyl ester methyl protons}) + (C3-H)]/4$

$$= (\alpha) + (\text{MeO}) + (\text{Keto}) \quad (2)$$

$(C2-H) + (C3-OH) + (CH \text{ and } CH_2 \text{ next to keto})$

$$= 2(\alpha) + 2(\text{MeO}) + 5(\text{Keto}) \quad (2')$$

RESULTS

Characteristic mycolate 1H -NMR signals

The 1H -NMR spectrum of the methyl mycolate fraction from *M. bovis* BCG (Tokyo strain) is shown in Fig. 2 for reference; component signal profiles for *cis*- and *trans*-

Methoxymycolate-1 of *M. tuberculosis* H37Ra (Table 3); one series (m/z 1263 > 1235, 1291, 1319) is for mycolates with one methoxy and one *cis*-cyclopropane ring and another series (m/z 1303, 1331 > 1275, 1359) is for those with one methoxy and two *cis*-cyclopropane rings. (c) α -Mycolate-1 of *M. avium* complex KK 41-24 (Table 2); a major series of peaks (m/z 1203 > 1231 1175, 1259) is for the components having two *cis*-cyclopropane rings and a minor series (m/z 1245 > 1217, 1273) is for mycolates with one *cis*- and one *trans*-cyclopropane ring. In addition, there is another minor series of peaks (m/z 1335 > 1383) with three *cis*-cyclopropane rings and an additional 152 atomic mass units, when compared to the major series (m/z 1175, 1203, 1231, 1259).

Table 2. Distribution of *cis*- and *trans*-double bonds and cyclopropane rings in α -mycolates

Abbreviations: *M. tb*, *M. tuberculosis*; p, l, m, n and n' refer to Fig. 1, db, di-substituted double bond; cy.prop, di-substituted cyclopropane ring; c, *cis*; t, *trans*. –, Sample not available, signals not detected or measurement not performed.

Strain	α -Mycolate-mix				α -Mycolate-1†		α -Mycolate-2‡		α -Mycolate-3‡	
	db (%) [*]	cy.prop <i>cis:trans</i>	p	cy.prop <i>cis:trans</i>	2 cy.prop l+m+n	3 c-cy.prop l+m+n+n' (%)‡	1 c-cy.prop+1 t-db l+m+n	2 c-cy.prop+1 t-db l+m+n+n' (%)‡	1 c-cy.prop+1 c-db l+m+n	2 c-cy.prop+1 c-db l+m+n+n' (%)‡
<i>M. tb</i> Aoyama B	5.0	1:0	23	1:0	<u>44,46 > 42,48</u>	52,54(<)	–	–	–	–
<i>M. tb</i> MNC1397	5.0	1:0	23	1:0	<u>44,46 > 42,48</u>	52,54 > 56(<)	–	–	–	–
<i>M. tb</i> O	1.6	1:0	23	1:0	<u>44,46 > 42,48</u>	52,54(<)	–	–	–	–
<i>M. tb</i> K	3.0	1:0	23	1:0	<u>44,46 > 42,48</u>	52,54 > 56(<)	–	–	–	–
<i>M. tb</i> 9829/87	4.0	1:0	23	1:0	<u>44,46 > 42,48</u>	–	–	–	–	–
<i>M. tb</i> 4610/91	5.3	1:0	23	1:0	<u>46 > 44,48</u>	54 > 52,56(<)	–	–	–	–
<i>M. tb</i> 2668/92	6.0	1:0	23	1:0	<u>44,46 > 48</u>	52 > 54 > 56(<)	–	–	–	–
<i>M. tb</i> Canetti MNC1485	c < t 6.7	1:0	23	1:0	<u>44,46 > 42,48</u>	52,54(<)	<u>49 > 51 > 47</u>	–	<u>46 > 44,48</u>	46 > 44,48(18)
<i>M. tb</i> H37Rv	7.6	1:0	23	1:0	<u>44,46 > 48,50</u>	52,54(<)	–	–	<u>44 > 42,46</u>	–
<i>M. tb</i> H37Ra	21.2	1:0	23	1:0	<u>46 > 44 > 48</u>	–	–	–	<u>44,46 > 42,48</u>	44,46 > 42,48(43)
<i>M. bovis</i> MNC8	2.8	1:0	23	1:0	<u>44 > 46,48 > 50</u>	–	–	–	–	–
<i>M. bovis</i> MNC27	4.4	1:0	23	1:0	<u>46 > 44,48 > 50</u>	52 > 54(<)	–	–	–	–
<i>M. bovis</i> BCG Danish 1331	2.4	1:0	23	1:0	<u>44 > 46 > 48 > 42</u>	–	–	–	–	–
<i>M. bovis</i> BCG Danish MNC5	5.2	1:0	23	1:0	<u>44,46 > 48 > 42</u>	–	–	–	–	–
<i>M. bovis</i> BCG Glaxo 10-F	3.0	1:0	23	1:0	<u>44 > 46 > 48 > 42</u>	–	–	–	–	–
<i>M. bovis</i> BCG Pasteur 1173 P2	2.0	1:0	23	1:0	<u>44 > 46 > 48 > 42</u>	52 > 54(<)	–	–	<u>46 > 44 > 48</u>	–
<i>M. bovis</i> BCG Tokyo 172	15.3	1:0	23	1:0	<u>44 > 46 > 48 > 42</u>	52 > 54 > 56 > 50(<)	–	–	<u>44,46 > 48</u>	–
<i>M. bovis</i> BCG Tokyo 172-V	28.0	1:0	23	1:0	<u>44 > 46 > 48 > 42</u>	52 > 54(<)	–	–	<u>44,46 > 48</u>	–
<i>M. microti</i> OV248	24.0	1:0	23	1:0	<u>44 > 46 > 42,48</u>	52,54 > 56(<)	–	–	<u>46 > 44 > 48</u>	44 > 42 > 40,46(12)
<i>M. kansasii</i> 20-01	4.4	1:0.02	21	1:0.01	<u>48 > 46,50</u>	56 > 58(<)	–	–	–	–
<i>M. kansasii</i> 304	5.0	1:0.04	21	1:0.01	<u>48 > 46,50</u>	–	–	–	–	–
<i>M. kansasii</i> 429	5.3	1:0.03	21	1:0.01	<u>48 > 46 > 50</u>	–	–	–	–	–
<i>M. avium</i> complex KK41-24	c < t 30.0	1:0.09	21	1:0.10	<u>48 > 50 > 46,52</u>	49 > 47,51§	<u>49 > 47,51</u>	–	<u>48,50 > 46,52</u>	46,48 > 50(16)
<i>M. avium</i> complex MNC10	c < t 23.0	1:0.03	21	1:0.05	<u>48 > 46,50</u>	47 > 49§	<u>49 > 47 > 45,51</u>	57 > 55(<)	<u>48 > 46,50 > 44</u>	48 > 46 > 44,50(16)

* Percentage in whole mycolate. c < t means more *trans*-double bond than *cis*-double bond. Unless specified, the double bond is mostly *cis*-disubstituted.

† Each column gives chain lengths of the mycolates with the groups specified. In each series, the components are grouped in order of decreasing abundance. Major series are italicized and underlined.

‡ Figures in parentheses are the percentage of three intra-chain group-containing species in each type of mycolate. < denotes that the content was less than 10%.

§ Series containing one *cis*- and one *trans*-disubstituted cyclopropane ring.

olefinic protons are also shown. The signals were assigned according to a previous study on the mycolic acids of MAC (Watanabe *et al.*, 1999). The characteristic signals and coupling constants (J , in Hz) for each mycolate or functional group were as follows. The signals for *cis*-double bond protons were at 5.36 p.p.m. (2H, t, J 4.7) and those for the protons of two methylenes adjacent to *cis*-double bonds were at 2.02 p.p.m. (4H, dt, J 4.7, 6.8). The signals for *trans*-double bonds were at 5.22 p.p.m. (1H, dd, J 15.3, 7.4) and 5.35 p.p.m. (1H, dt, J 15.3, 6.4); the protons for the methylene and the methine groups, adjacent to *trans*-double bonds, were at 1.99 p.p.m. (2H, dt, J 6.5, 6.7) and 2.03 p.p.m. (1H, m), respectively. The *cis/trans* ratios of double bonds given in Tables 1 to 5 were estimations from the shape of the signal profiles. The signals used for di-substituted *cis*- and *trans*-cyclopropane rings were those for the highly shielded *cis*-cyclopropane ring proton at -0.34 p.p.m. (1H, ddd, J 4.2, 5.3, 5.3) and those for the three *trans*-cyclopropane ring protons at 0.09–0.21 p.p.m. (3H, m), respectively. The signal of the methoxy-methyl protons was at 3.34 p.p.m. (3H, s) and that of the proton on the methoxy-bearing carbon was at 2.97 p.p.m. (1H, m). The signals used for the keto group were those of the protons of methylene and methine groups adjacent to the keto carbonyl at 2.42 p.p.m. (2H, dt, J 7.4, 1.5) and at 2.50 p.p.m. (1H, m), respectively. The signals for representing the whole amount of mycolate were the complex at 3.6–3.8 [methyl ester methyl protons (3.78 p.p.m., 3H, s) + C3-H (3.66 p.p.m., 1H, m)], and the signal at 2.35–2.55 p.p.m. [C2-H (2.43 p.p.m., 1H, dt, J 4.4, 5.3) + C3-OH (2.38 p.p.m., 1H, d, J 8.8)].

Mycolate composition

Calculations were performed from the above equations using the areas of the characteristic signals for each group. Thus, the signal area at 5.18–5.4 p.p.m. ($\times 1/2$) gave the double bond content, that at 3.34 p.p.m. ($\times 1/3$) or at 2.97 p.p.m. gave the methoxymycolate content and those at -0.34 p.p.m. and at 0.19–0.25 p.p.m. ($\times 1/3$) gave the *cis*- and *trans*-cyclopropane ring content, respectively. Since the comparison is more accurate between signals of similar sizes, in a narrower magnetic field range, equation (2') was used rather than (2). Thus, the signal area in the range of 2.3–2.55 p.p.m. represented the sum of the three protons adjacent to the ketomycolate keto group and the C2-H and of C3-OH protons represented the whole mycolate.

The calculated mycolate ratios are given in Table 1, along with the number and type of double bonds and the *cis/trans* ratios of cyclopropane rings. The calculated ratios generally agreed well with the ratios of the weights of mycolates recovered from chromatography. For the mycolates of *M. tuberculosis* H37Ra, the weight ratio is given in Table 1, as it was different from the calculated ratio. Table 1 shows that there was some systematic variation in the ratios of α -mycolate to total oxygenated mycolates (methoxymycolate + ketomycolate).

MALDI-TOF/MS proved to be a reliable procedure for determining the chain lengths of mycolic acid methyl esters and recognizing the presence of particular types of unsaturation and additional homologous series, as illustrated in Fig. 3 for representative mycolates. In general, homologous series, with 28 atomic mass unit differences, could be clearly distinguished for all classes of mycolates (Tables 2–4).

Characteristics of α -mycolates

Table 2 shows the number and stereochemistry of the double bonds and cyclopropane rings in α -mycolates, determined by $^1\text{H-NMR}$. It also shows the chain lengths of the three components, α -mycolate-1 with no double bonds, α -mycolate-2 with a *trans*-double bond and α -mycolate-3 with a *cis*-double bond. There was a clear difference between the chain lengths of α -meromycolates of the *M. tuberculosis* complex and those of *M. kansasii* and MAC (Table 2).

In all strains tested, the major component was α -mycolate-1 containing two cyclopropane rings. These rings were *cis* in the *M. tuberculosis* complex strains and *cis*, with a small amount of *trans*, in the α -mycolates from MAC and *M. kansasii*. These major α -mycolates from the *M. tuberculosis* complex strains had $l+m+n$ values in the range 42–50 (Table 2). In α -mycolates-1, MALDI-TOF/MS often detected signals for a series of components, with an additional cyclopropane ring, which were larger than the major mycolates by 152 atomic mass units (Fig. 3c, Table 2).

Of the *M. tuberculosis* complex, only the Canetti strain gave substantial amounts of an α -mycolate-2 with a *trans*-double bond and these mycolates were slightly larger ($l+m+n = 47$ –51). However, careful chromatographic separation and $^1\text{H-NMR}$ analysis demonstrated that earlier chromatographic eluates of α -mycolates from all *M. tuberculosis* strains, except H37Ra, contained a small amount of mycolates with *trans*-double bonds whose sizes were not determined (data not shown). The type-2 α -mycolate is, however, a substantial characteristic component in the MAC strains (Table 2).

In the type-3 α -mycolates, $^1\text{H-NMR}$ showed that the *cis*-cyclopropane ring content per mycolate molecule was considerably higher than 1. This implies the presence of species containing one *cis*-double bond and two *cis*-cyclopropane rings. These species were confirmed by MALDI-TOF/MS, as shown (Fig. 3a) for the type-3 α -mycolates of *M. tuberculosis* H37Ra. The spectrum shows two series of peaks $(M+Na)^+$ for the components containing one *cis*-double bond and one cyclopropane ring (major peak, m/z 1161) and the other for the components containing one *cis*-double bond and two *cis*-cyclopropane rings (major peak, m/z 1201). Type-3 α -mycolates, with one *cis*-double bond and two *cis*-cyclopropane rings were limited to *M. tuberculosis* H37Ra and Canetti, *M. microti* and MAC; these strains,

Table 3. Distribution of *cis*- and *trans*-double bonds and cyclopropane rings in methoxymycolates

Abbreviations: *M. tb*, *M. tuberculosis*; p, l, m, n and n' refer to Fig. 1; db, di-substituted double bond; cy.prop, di-substituted cyclopropyl; c, *cis*; t, *trans*; c ≫, mostly *cis*; c > t, more *cis* than *trans*; c/t, *cis/trans* ratio about 1/1; ≪ t, mostly *trans*. –, Sample not available, signals not detected or measurement not performed.

Strain	Methoxymycolate mix			Methoxymycolate-1†			Methoxymycolate-2†			Methoxymycolate-3†	
	db (%) ^a	cy.prop <i>cis:trans</i>	p	cy.prop <i>cis:trans</i>	1 cy.prop		2 cy.prop l+m+n+n' (%)§	1 t-db l+m+n	1 t-db+1 cy.prop l+m+n+n' (%)§	1 c-db l+m+n	1 c-db+1 cy.prop l+m+n+n' (%)§
					l+m+n(c)‡	l+m+n(t)‡					
<i>M. tb</i> Aoyama B	≪ t 11.3	1:0.37	23	1:0.48	<u>50 > 48,52</u>	49,51 > 47,53	58 > 56,60(<)	<u>49 > 51 > 47</u>	–	<u>50 > 48 > 52</u>	50 > 48 > 52 (20)
<i>M. tb</i> MNC1397	c/t 8.6	1:0.27	23	1:0.29	<u>50 > 48,52</u>	49,51 > 53	58 > 56,60(<)	<u>49 > 51 > 47</u>	57 > 59 > 55(<)	<u>50 > 48,52</u>	48,50 > 46 > 52(23)
<i>M. tb</i> O	c/t 12.8	1:0.45	23	1:0.50	<u>50 > 48,52</u>	49,51 > 47,53	58 > 60(<)	<u>49 > 47,51</u>	–	<u>50 > 48 > 46,52</u>	48,50 > 46 (<)
<i>M. tb</i> K	c/t 15.0	1:0.43	23	1:0.48	<u>50 > 48,52</u>	49,51 > 47	58(<)	<u>49 > 47,51</u>	57 > 55,59(<)	<u>50 > 48 > 46,52</u>	48 > 46,50 (17)
<i>M. tb</i> 9829/87	c < t 13.8	1:0.38	23	1:0.41	<u>50 > 52 > 48</u>	49 > 51 > 47	–	<u>49,51 > 47,53</u>	–	–	–
<i>M. tb</i> 4610/91	c < t 8.7	1:0.37	23	1:0.40	<u>52 > 50 > 48,54</u>	51 > 49,53	–	<u>49,51 > 53</u>	–	<u>50 > 48,52</u>	50 > 48 > 46 > 52(21)
<i>M. tb</i> 2668/92	c < t 8.5	1:0.26	23	1:0.30	<u>50,52 > 48 > 54</u>	51 > 49,53	58 > 60(<)	–	–	–	–
<i>M. tb</i> Canetti MNC1485	≪ t 26.0	1:0.34	23	1:0.36	<u>52 > 50 > 48,54</u>	51 > 49 > 53	60 > (<)	<u>51 > 49,53</u>	59 > 57,61(<)	–	–
<i>M. tb</i> H37Rv	≪ t 12.5	1:0.32	23	1:0.31	<u>50,52 > 48,54</u>	51 > 49,53	58 > 60(<)	<u>49 > 51 > 47,53</u>	57 > 55,59(<)	<u>50 > 48,52 > 54</u>	50 > 48,52(<)
<i>M. tb</i> H37Ra	c ≫ 22.4	1:trace	23	1:0.01	<u>48 > 46,50,52</u>	–	48,50 > 46,52(33)	–	–	–	48,50 > 46,52(100)
<i>M. bovis</i> MNC8	c < t 10.9	1:0.39	23	1:0.39	<u>48,50 > 52</u>	49,51 > 53	–	<u>49 > 47,51</u>	–	<u>50 > 48 > 52</u>	50 > 46,48,52(44)
<i>M. bovis</i> MNC27	c < t 5.0	1:0.46	23	1:0.48	<u>50 > 52 > 48,54</u>	51 > 49,53	–	–	–	–	–
<i>M. bovis</i> BCG Danish 1331	–	–	–	–	–	–	–	–	–	–	–
<i>M. bovis</i> BCG Danish MNC5	–	–	–	–	–	–	–	–	–	–	–
<i>M. bovis</i> BCG Glaxo 10-F	–	–	–	–	–	–	–	–	–	–	–
<i>M. bovis</i> BCG Pasteur 1173-P2	–	–	–	–	–	–	–	–	–	–	–
<i>M. bovis</i> BCG Tokyo 172	c > t 9.2	1:0.07	23	1:0.07	<u>50 > 48,52</u>	49 > 47,51	58 > 60 > 56(14)	<u>49 > 47,51</u>	–	<u>50 > 48,52</u>	48,50 > 46,52(30)
<i>M. bovis</i> BCG Tokyo 172-V	c > t 9.0	1:0.05	23	1:0.06	<u>50 > 48,52</u>	49 >	58 > 56,60(13)	<u>49 > 47,51</u>	–	<u>50 > 48,52</u>	50 > 48 > 52(42)
<i>M. microti</i> OV248	c > t 8.6	1:0.21	23	1:0.21	<u>50,52 > 48,54</u>	49,51 > 47,53	58,60(<)	–	–	<u>50 > 48,52</u>	50 > 48 > 52(45)
<i>M. kansasii</i> 20-01	c > t 3.6	1:0.59	21	1:0.60	<u>50,52 > 48,54</u>	51,53 > 49,55	–	<u>49 > 51 > 47</u>	–	–	–
<i>M. kansasii</i> 304	c/t 5.9	1:0.58	21	1:0.58	<u>52 > 50 > 48,54</u>	51 > 49,53	58,60 > 62(<)	–	–	–	–
<i>M. kansasii</i> 429	c/t 4.4	1:0.64	21	1:0.65	<u>50,52 > 48,54</u>	51 > 49,53	58 > 60(<)	–	–	–	–

^a Percentage in whole mycolate.

† In each series, the components are grouped in order of decreasing abundance. Major series are italicized and underlined. The cyclopropane rings in methoxymycolates-2 and -3 appear to be predominantly *cis*. It was not possible to determine the stereochemistry of the additional cyclopropane ring in methoxymycolates-1.

‡ (c) denotes *cis*-cyclopropane ring-containing series and (t) denotes *trans*-cyclopropane ring-containing series.

§ Figures in parentheses are percentages of three intra-chain group-containing species in each type of mycolate. < denotes that the content is less than 10 %.

H37Rv and some BCG strains also had type-3 mycolates with one *cis*-double bond and one *cis*-cyclopropane ring (Table 2).

Characteristics of methoxy- and ketomycolates

In the methoxy- and ketomycolates, differences in the meromycolate chain lengths, as seen in the α -mycolates, were not noted between the *M. tuberculosis* complex strains and the *M. kansasii* and MAC strains (Tables 3 and 4). The double bond content of the methoxy- and ketomycolates suggested that the major mycolates of these classes were also of type 1, containing one oxygen function (methoxy or keto) and one *cis*- or *trans*-cyclopropane ring. The proportion of *trans*-cyclopropane rings was much higher in the ketomycolate-1 than in the methoxymycolate-1 in all the strains tested, including *M. tuberculosis* H37Ra. However, the *trans*-cyclopropane ring content in H37Ra was quite low, with

less than 1 % in the methoxymycolate-1 and less than 3 % in the ketomycolate-1. As for α -mycolates, cyclopropyl methoxy- and ketomycolates-1 were the major components; smaller amounts of type-2 and -3 homologues containing one *trans*-double bond and one *cis*-double bond, respectively, were also detected.

As in the case of α -mycolate-1, in type-1 and -2 methoxy- and ketomycolates, MALDI-TOF/MS detected components which were heavier than the respective major mycolates by 152 atomic mass units and apparently contained an additional cyclopropane ring (Tables 3 and 4). The longer type-1 and -2 methoxy- and ketomycolates had values (l+m+n+n') around 57–60 in comparison with the 48–52 values (l+m+n) for the major type-1 and -2 mycolates. However, the type-3 methoxy- and ketomycolates, with an additional cyclopropane unit, were not elongated (Tables 3 and 4). ¹H-NMR showed that, in the type-3 mycolates of these classes, there was often a substantial proportion of *cis*-

Table 4. Distribution of *cis*- and *trans*-double bonds and cyclopropane rings in ketomycolates

Abbreviations: *M. tb*, *M. tuberculosis*; p, l, m, n and n' refer to Fig. 1. db, di-substituted double bond; cy.prop, di-substituted cyclopropyl; c, *cis*; t, *trans*; c ≫, mostly *cis*; c > t, more *cis* than *trans*; c/t, *cis/trans* ratio about 1/1; ≪ t, mostly *trans*. –, Sample not available, signals not detected or measurement not performed.

Strain	Ketomycolate mix			Ketomycolate-1†		Ketomycolate-2†		Ketomycolate-3†	
	db (%)*	cy.prop <i>cis:trans</i>	p	cy.prop <i>cis:trans</i>	1 cy.prop		1 t-db l+m+n	1 t-db+1 cy.prop l+m+n+n' (%)§	1 c-db l+m+n
					l+m+n(c)‡	l+m+n(t)‡			
<i>M. tb</i> Aoyama B	c < t 18.6	1:3.3	23	1:3.3	52 > 50 > 48	<u>51 > 49 > 53</u>	–	<u>49 > 51 > 47</u>	<u>50 > 48,52</u>
<i>M. tb</i> MNC1397	c < t 12.7	1:2.0	23	1:2.1	52 > 50 > 48,54	<u>51 > 49 > 53</u>	59 > 57(<)	<u>49,51 > 47,53</u>	–
<i>M. tb</i> O	c < t 20.0	1:2.8	23	1:2.8	50,52 > 48,54	<u>49,51 > 47,53</u>	59 > 57(<)	<u>49 > 47,51</u>	48,50 > 46 > 52(27)
<i>M. tb</i> K	c < t 16.3	1:3.3	23	1:3.3	52 > 50 > 48,54	<u>51 > 49 > 47,53</u>	59 > 57(<)	<u>49 > 47,51</u>	<u>50 > 48,52</u>
<i>M. tb</i> 9829/87	≪ t 25.0	1:3.2	23	1:3.5	52 > 50,54	<u>51 > 49,53</u>	59 > 57(<)	<u>49,51 > 47,53</u>	–
<i>M. tb</i> 4610/91	c < t 13.8	1:2.1	23	1:2.1	52 > 50,54	<u>51 > 49 > 47,53</u>	59 > 57(<)	<u>49,51 > 53</u>	–
<i>M. tb</i> 2668/92	c < t 16.7	1:1.7	23	1:1.9	52 > 50,54	<u>51 > 49,53</u>	–	–	–
<i>M. tb</i> Canetti MNC1485	≪ t 35.0	1:3.8	23	1:4.0	52 > 50 > 54	<u>51,53 > 49,55</u>	59,61 > 57(<)	<u>51 > 49,53</u>	–
<i>M. tb</i> H37Rv	c < t 17.5	1:1.4	23	1:1.4	52 > 50,54	<u>51 > 49,53</u>	–	<u>51 > 49 > 47,53</u>	<u>52 > 50,54</u>
<i>M. tb</i> H37Ra	c ≫ 25.0	1:trace	23	1:0.03	<u>50 > 48,52 > 46</u>	–	50 > 48,52(45)	–	48,50 > 46,52(100)
<i>M. bovis</i> MNC8	≪ t 16.2	1:3.2	23	1:3.3	50,52 > 48	<u>49,51 > 47,53</u>	–	<u>49 > 47,51</u>	–
<i>M. bovis</i> MNC27	≪ t 10.0	1:3.1	23	1:3.2	<u>52 > 50 > 54</u>	<u>51 > 49,53</u>	–	–	–
<i>M. bovis</i> BCG Danish 1331	c/t 8.3	1:0.42	23	1:0.42	<u>50 > 52 > 54</u>	<u>51 ></u>	–	–	–
<i>M. bovis</i> BCG Danish MNC5	c/t 14.5	1:0.75	23	1:0.74	<u>52 > 50 > 48,54</u>	<u>51 > 49,53</u>	–	<u>49 > 51 > 47</u>	57 > 59(<)
<i>M. bovis</i> BCG Glaxo 10-F	c/t 10.4	1:0.25	23	1:0.25	<u>50 > 52 > 54</u>	49 >	–	–	–
<i>M. bovis</i> BCG Pasteur 1173 P2	c/t 10.0	1:0.25	23	1:0.29	<u>52 > 48,54</u>	51 > 49,53	–	–	–
<i>M. bovis</i> BCG Tokyo 172	c/t 16.5	1:0.25	23	1:0.38	<u>52 > 50 > 46</u>	51 > 49,53	60 > 58 > 56(<)	<u>49 > 47,51</u>	<u>50 > 52 > 48</u>
<i>M. bovis</i> BCG Tokyo 172-V	c/t 17.0	1:0.44	23	1:0.46	<u>50,52 > 48,54</u>	51 > 49 > 47,53	60 > 62(<)	<u>49,51 > 47,53</u>	<u>50 > 48,52</u>
<i>M. microti</i> OV248	c > t 25.0	1:0.2	23	1:0.25	<u>50 > 48,52 > 46</u>	49 > 47,51	–	–	<u>46,48 > 44,50</u>
<i>M. kansasii</i> 20-01	c/t 7.6	1:5.2	21	1:5.2	52 > 50,54	<u>51,53 > 49,55</u>	59,61(<)	–	–
<i>M. kansasii</i> 304	< t 7.0	1:6.3	21	1:6.3	52 > 50,54	<u>51,53 > 49,55</u>	59 > 61(<)	–	–
<i>M. kansasii</i> 429	< t 8.0	1:5.8	21	1:5.6	50,52 > 48	<u>51 > 49,53</u>	59 > 57,61(<)	<u>49 > 47 > 45,51</u>	–
<i>M. avium</i> complex KK41-24	< t 23.0	1:6.3	21	1:8.0	52 > 50,54	<u>51 > 53 > 49</u>	59 > 61(<)	<u>51 > 49,53</u>	<u>52 > 50,54</u>
<i>M. avium</i> complex MNC10	–	–	21	–	–	<u>51 > 49 > 47,53</u>	–	–	51 > 49 > 47,51(16)

* Percentage in whole mycolate.

† In each series, the components are grouped in order of decreasing abundance. Major series are italicized and underlined. The cyclopropane rings in ketomycolates-2 and -3 appear to be predominantly *cis*. It was not possible to determine the stereochemistry of the additional cyclopropane ring in ketomycolates-1.

‡ (c) denotes *cis*-cyclopropyl group-containing series and (t) denotes *trans*-cyclopropyl group-containing series.

§ Figures in parentheses are percentages of three intra-chain group-containing species in each type of mycolates. < denotes that the content is less than 10%.

cyclopropane rings. MALDI-TOF/MS demonstrated that the type-3 methoxy- and ketomycolates each consisted of two series, one containing one oxygen function and one *cis*-double bond and the other with one oxygen function, one *cis*-double bond and one normally *cis*-cyclopropane ring. In the ketomycolate-3 of the MAC strain, however, the cyclopropane ring was *trans*.

The exceptional methoxy- and ketomycolates-1 of *M. tuberculosis* H37Ra included 33% and 45%, respectively, of homologues containing two *cis*-cyclopropane rings (Tables 3 and 4). MALDI-TOF/MS confirmed this estimation by giving two series of peaks (Fig. 3b), but these type-1 dicyclopropyl methoxy- ($l+m+n+n' = 46-52$) and ketomycolates ($l+m+n+n' = 48-50$) were similar in size. As noted above, the type-1 and type-2 methoxy- and ketomycolates from the H37Rv and other

M. tuberculosis strains were all elongated ($l+m+n+n' = 55-60$), when an additional double bond or cyclopropane group was present. The methoxy- and ketomycolates-3 of H37Ra apparently consisted exclusively of mycolates ($l+m+n+n' = 46-52$) containing one oxygen function, one *cis*-double bond and one *cis*-cyclopropane ring. There was a clear distinction between the 15 varieties of mycolates produced by *M. tuberculosis* H37Rv and the 9 varieties detected in the attenuated variant H37Ra (Tables 2–4).

Effect of *cis/trans* stereochemistry on mycolate chromatography

The results for the silica gel chromatography of total mycolates from *M. kansasii* 304 are summarized in Table 5. Mycolates having *trans*-double bonds or *trans*-

Table 5. Stereochemistry of double bonds and cyclopropane rings in different chromatographic fractions of *M. kansasii* mycolates

Silica gel column chromatography of total methyl mycolate fraction, eluting with diethyl ether/hexane (6:100, v/v). Each fraction was examined by ¹H-NMR spectrometry.

Mycolate fraction	Fraction no.	Yield (mg)	Double bond		Cyclopropane ring	
			Content (%)	Nature*	<i>cis</i>	<i>trans</i>
α -Mycolate	a-1	18	4.5	<i>cis/trans</i>	1	0.06
	a-2,3,4	85	5.0	<i>cis</i> $\gg$ <i>trans</i>	1	0.04
Methoxymycolate	m-1	4	4.4	<i>cis</i> < <i>trans</i>	1	1.6
	m-2	25	5.0	<i>cis/trans</i>	1	1.0
	m-3	20	5.8	<i>cis/trans</i>	1	0.5
	m-4	8	8.4	<i>cis</i> $\gg$ <i>trans</i>	1	0.2
Ketomycolate	k-1,2	70	6.8	<i>cis</i> $\ll$ <i>trans</i>	1	7.1
	k-3,4	30	6.4	<i>cis/trans</i>	1	5.0
	k-5	10	8.0	<i>cis</i> > <i>trans</i>	1	4.0
	k-6	2.5	20.0	<i>cis</i> $\gggg$	1	1.8

* Estimation of *cis*- and *trans*-double bond contents from ¹H-NMR signal profiles. *cis* > *trans*, more *cis* than *trans*; *cis/trans*, *cis/trans* ratio about 1/1.

cyclopropane rings tended to elute faster than the corresponding mycolates containing *cis* unsaturations.

DISCUSSION

Overall mycolate composition

As shown in Table 1, the overall *cis/trans* ratios for mycolate cyclopropane rings were similar for strains of the same species. The ratios between the α -, methoxy- and ketomycolates, calculated from ¹H-NMR signal areas, generally agreed well with the ratios between the weights recovered from chromatography, with exception of the *M. tuberculosis* H37Ra mycolates. This disagreement resulted from the assumption that each mycolate contained two functional groups, whereas a large proportion of the mycolates of this strain contained three functional groups, as seen in Tables 2, 3 and 4. Therefore, for this strain, the weight ratio given represents its actual mycolate ratio.

The mycolic acids from *M. tuberculosis* and *M. bovis* were distinguished from those of *M. bovis* BCG, *M. microti*, *M. kansasii* and MAC by details of chain length and other structural features (Tables 1–4).

Distribution of major mycolates

Small differences were noted in the mycolate ratios among the *M. tuberculosis* complex strains. Davidson *et al.* (1982) observed changes in the proportions of methoxy- and ketomycolates during cultivation of *M. microti*. An analogous phenomenon was noted in *M. bovis* BCG Tokyo; the ketomycolate content decreased and the methoxymycolate content increased as the cultivation was continued (Table 1), while the α -

mycolate content remained almost constant. When the α -mycolate content was compared with the oxymycolate (methoxy- + ketomycolate) content (Table 1), however, these *M. tuberculosis* strains gave values of about 1:1 (for *M. tuberculosis* H37Ra, on a weight basis). The Canetti strain, however, gave a slightly higher value and the two newly isolated Japanese clinical strains, O and K, gave lower values. The lower α -mycolate to oxymycolate ratios ($\sim$ 1:0.6) of these two recent clinical isolates were due to their low ketomycolate content. For the recent Swedish patient isolates (9829/87, 4610/91, 2668/92), the ratios of α -mycolates to oxymycolates were more in line ($\sim$ 1:1) with other strains of *M. tuberculosis*. A previous report (Yuan *et al.*, 1997), on 10 newly isolated USA strains, gave a mean α -mycolate to oxymycolate ratio of 1:0.77, which is intermediate between the present results for the Japanese and Swedish strains. Mycolate ratios varied with cultivation time for *M. bovis* BCG Tokyo but the overall α -mycolate to oxymycolate ratio was relatively constant ($\sim$ 1:3.5) (Table 1). The remaining BCG preparations differed from BCG Tokyo, in that the same ratios were in the range 1:1.5 to 1.8; a similar result (1:1.66) was found for *M. microti*.

The mycolate compositions and/or stereochemistry of cyclopropane rings and double bonds reported by different authors do not always agree with each other and our present results. For example, it is reported that the proportion of *cis/trans*-groups for the mycolates from *M. tuberculosis* H37Rv is 1:0.02 (Yuan *et al.*, 1997), whereas the corresponding value for our culture was 1:0.14 (Table 1). The *trans*-cyclopropane ring and *trans*-double bond contents of the methoxy- and ketomycolate of the H37Rv strain were reported (Yuan *et*

al., 1997) to be less than 1:0.05 and 1:0.2, respectively, whereas the corresponding data for our culture were 1:0.32 and 1:1.4, respectively. Small differences in the *cis/trans* ratios of cyclopropane rings might be attributable to different chromatographic procedures. Mycolates with *trans*-double bonds and cyclopropane rings tended to move faster than the corresponding ones with *cis*-groups on silica gel chromatography (Table 5), so that the earlier chromatographic eluates might be richer in *trans*-groups. However, such a wide discrepancy may be due to laboratory selection or phenotypic changes. Thus, cultures bearing the same strain name should not always be considered to be identical, as illustrated by *M. bovis* BCG strains. Of the BCG strains, only the Tokyo strain produced a substantial amount of methoxymycolate, as reported previously (Minnikin *et al.*, 1984b), whereas the others contained only a trace amount, detectable in ¹H-NMR spectra (Table 1).

Our present results support the view that methoxy- and ketomycolates are biosynthetically related to each other (Dubnau *et al.*, 1997; Yuan & Barry, 1996; Yuan *et al.*, 1997). Both the methoxy- and ketomycolates contained significant amounts of *trans*-double bonds and *trans*-cyclopropane rings, and the total chain lengths of the major mycolates in each of these two classes of the same strains were apparently the same. Although the methoxy- and ketomycolate content ratios varied with the cultivation period and in different preparations, the sum of the methoxymycolate content and the ketomycolate content versus the α -mycolate content was almost always constant (Table 1), implying a close relationship between the two oxygenated mycolates. However, the *trans*-cyclopropane ring content was much higher in the ketomycolates than in the corresponding methoxymycolates and the same tendency was noted in the *trans*-double bond content, as shown in Tables 3 and 4. This does not suggest an immediate common precursor for the two oxygenated mycolates (Dubnau *et al.*, 1997).

Distribution of minor mycolates

In general, molecular mass comparisons of the methoxy- (Table 3) and ketomycolates (Table 4) indicated that the minor *trans*-mono-olefinic type-2 mycolates were directly related to the major *trans*-mono-cyclopropyl type-1 mycolates, as were the corresponding minor *cis*-mono-olefinic type-3 and major *cis*-mono-cyclopropyl type-1. However, in the unusual type-3 methoxy- and ketomycolates, the additional cyclopropane ring was normally *cis* even when the major type-1 mycolates had *trans*-cyclopropane rings, as in the *M. tuberculosis* ketomycolates. The additional cyclopropane ring, however, was *trans* in the ketomycolates-3 of the MAC strains. Further studies will be needed to clarify the relationships of these type-2 and -3 oxygenated mycolates, having an additional cyclopropane ring, to the major established mycolate types.

It is interesting that the type-3 methoxy- and ketomycolates, with an additional cyclopropane ring, have

$l+m+n+n'$ values (Tables 3 and 4) around 48, 50 but the corresponding type-2 mycolates have values of 57, 59. These two classes are not interrelated, therefore, by a simple methylation step, converting a *cis*-double bond to a *trans*-double bond, with an adjacent methyl branch. It would be necessary to incorporate an elongation step at a key stage. These type-2 mycolates perhaps share a common oxygenated di-*cis*-olefinic unsaturated precursor ($l+m+n+n' = 48, 50$) with the dicyclopropyl type-1 oxygenated mycolates. In the case of the H37Ra strain, this postulated precursor may be simply methylated to give, successively, mycolates-3 (1 *cis*-double bond + 1 cyclopropane ring) ($l+m+n+n' = 48, 50$), mycolates-1 (2 *cis*-cyclopropanes) ($l+m+n+n' = 48, 50$). In the others, it appears that oxygenated mycolates-3 (1 *cis*-double bond + 1 cyclopropane ring) ($l+m+n+n' = 48, 50$), or a related component, are elongated before their *cis*-double bonds are converted into either *cis*-cyclopropanes (type-1) or *trans*-double bonds with an adjacent methyl branch (type-2).

Related transformations are apparent in the α -mycolate series (Table 2). A di-*cis*-olefinic precursor in H37Ra may be converted to type-3 (1 *cis*-double bond + 1 cyclopropane ring) and type-1 (di-cyclopropyl) mycolates. The H37Ra strain may also introduce another unsaturation in the type-3 mycolates, with 1 *cis*-double bond + 2 cyclopropane rings, but it cannot elongate or convert its *cis*-double bonds to *trans*-double bonds with adjacent methyl branches. The attenuated H37Ra strain appears to be deficient in mycolate elongation and *trans*-double bond formation. Traces of both series of type-3 α -mycolates were indicated in most strains of *M. tuberculosis* but the amounts were insufficient to allow full characterization.

The chain length of the α -mycolates from the *M. tuberculosis* complex was shorter than that of the corresponding methoxy- and ketomycolates by 6–8 methylene units. This is in accordance with previous views (Brennan & Nikaido, 1995; Kremer *et al.*, 2000) that distinct biosynthetic pathways operate for the α -mycolate and for the oxygenated methoxy- and ketomycolates. In *M. kansasii* and the MAC, however, all α -, methoxy- and ketomycolates had similar chain lengths. It was previously indicated (Brennan & Nikaido, 1995) that the α -mycolates did not have components with *trans*-cyclopropane rings and adjacent methyl branches. The present results showed that *trans*-cyclopropane rings were present in the α -mycolate from *M. kansasii* and the MAC complex, although the proportions were extremely low compared with those in the two oxygenated mycolates. Small proportions of *cis*- and *trans*-double bonds were detected in all α -mycolates investigated. Although the double bonds of α -mycolate had mostly *cis*-stereochemistry, *trans*-isomers were substantial in the α -mycolates from the Canetti and MAC strains (Table 2). An early ¹H-NMR study (Minnikin & Polgar, 1967) noted the presence of doublet at τ 9.01 (δ 0.99), corresponding to a methyl branch adjacent to a *trans*-double bond, in the spectrum of the α -mycolates from *M. avium*.

The chain lengths ($l + m + n = 47-51$) of the novel *trans*-olefinic, *cis*-cyclopropyl α -mycolate-2 (Table 2) from the *M. tuberculosis* Canetti strain are apparently comparable with those ($l + m + n = 48-54$) of the Canetti *cis*-cyclopropyl type-1 methoxy- and ketomycolates (Tables 3 and 4). It is tempting to suggest that the α -mycolates-2 of the Canetti strain may be related to possible precursors of methoxy- and ketomycolates. Oxygenated mycolates have been shown to arise by methylation of a precursor with a distal *cis*-double bond, followed by hydroxylation of the intermediate carbocation (Dubnau *et al.*, 1997; Yuan & Barry, 1996; Yuan *et al.*, 1997). This intermediate carbocation could also lose a proton to give this type-2 α -mycolate, having a *trans*-double bond and an adjacent methyl branch. It is notable that traces of similar type-2 α -mycolates were detected in other strains of *M. tuberculosis* (see Results). The type-2 *trans*-olefinic, *cis*-cyclopropyl α -mycolates from the MAC strains (Table 2) may similarly be related to possible precursors of ketomycolates-1. Further clarification of any possible relationship of these type-2 α -mycolates with oxymycolates must await a detailed structural comparison with the methoxy- and ketomycolates, particularly the location of double bonds and cyclopropane rings.

Mycolates and virulence

One of the most striking differences in the mycolate profiles is the clear distinction seen between the attenuated *M. tuberculosis* H37Ra and the other virulent strains, including the H37Rv type strain. The possible presence of unusual dicyclopropyl methoxy- and ketomycolates in *M. tuberculosis* H37Ra had been previously indicated by Takayama *et al.* (1979) and this study has substantiated this finding (Tables 3 and 4). The H37Rv strain appears capable of synthesizing a total of at least 15 distinct homologous mycolate series, but the H37Ra strain has only 7 such series (Tables 2-4). As indicated above, the H37Ra variant appears to have deficiencies in both mycolate elongation and the transformation of *cis*-double bonds to *trans*-double bonds, with an adjacent methyl branch. There is, however, a marked proficiency in H37Ra for the production of mycolates with an additional cyclopropane ring, as exemplified by the α -mycolate-3 (Table 2) and methoxy- and ketomycolates-1 and -3 (Tables 3 and 4). Detailed studies will be needed to determine the significance and interrelation of these unusual mycolates. The distinct mycolate composition of the attenuated H37Ra variant may contribute to its lack of virulence but it also has profiles of free lipids that are deficient in comparison with H37Rv (Minnikin *et al.*, 1986). The single *M. tuberculosis* Canetti variant, with smooth colony morphology, has an unusual mycolate profile but it is a virulent strain.

The attenuated strains of *M. bovis* BCG have variable mycolate composition (Tables 1-4); the clearest difference is the trace amount of methoxymycolates in the Danish, Glaxo and Pasteur strains, as compared with

the Tokyo strain (Minnikin *et al.*, 1984b). The Tokyo strains are notable in having increased proportions of oxygenated mycolates, including methoxymycolates-1 and -3 (Table 3) and ketomycolates-3 (Table 4) with an additional cyclopropane ring. The vole bacillus, *M. microti*, is not a human pathogen and its mycolates are notable only in having enhanced proportions of methoxy- and ketomycolates-3, with the additional cyclopropane ring (Tables 3 and 4). The BCG strains, in particular, have been repeatedly subcultured so that it is difficult to make meaningful comparisons with modern virulent strains. The same comment is also pertinent for *M. tuberculosis* H37Rv and H37Ra.

Conclusions

The present systematic study has clearly shown, for the first time, that there is considerable variation in the mycolate composition of members of the *M. tuberculosis* complex and related mycobacteria. For example, well-studied reference strains, such as Aoyama B and MNC1397, showed the expected patterns of dicyclopropyl α -mycolate, and two series of methoxy- and ketomycolates with *cis*- and *trans*-cyclopropane rings. However, in addition to these five expected mycolate series, these two strains produced an extra seven and nine minor homologous series, respectively (Tables 2-4).

Some of the minor mycolate series are undoubtedly biosynthetic precursors of the major types, examples being α -mycolates-3 (1 *cis*-double bond and 1 *cis*-cyclopropane) and the methoxy- and ketomycolates-2 and -3 with single *cis*- or *trans*-double bonds. Others, such as the *cis*-cyclopropyl, *trans*-olefinic α -mycolates-2 found in the Canetti and MAC strains, may be 'missing links', possibly related to precursors of the oxygenated mycolates as detailed above. It must be stressed, however, that the characterization of a mycolate series, which superficially could be transformed to another, does not confirm it as a direct precursor. The key transformation may have happened earlier, perhaps at the meromycolate stage, with both substrate and product subsequently being transformed into complete mycolates.

The clear identification of mycolate series with additional cyclopropane rings raises many intriguing questions. In some cases, these mycolates are elongated, as for the tricyclopropyl α -mycolates-1 (Table 2) and the dicyclopropyl methoxy- and ketomycolates-1 and -2 (Tables 3 and 4). In other series, such as the *cis*-unsaturated dicyclopropyl α -mycolates-3 (Table 1) and the *cis*-unsaturated monocyclopropyl methoxy- and ketomycolates-3, the chains are not extended. An understanding of the true interrelation of these novel mycolates must await further studies, including precise structural analysis. The presence of an additional point of unsaturation, and increased chain length, in these mycolates must affect how they pack together in the mycobacterial outer membrane (Minnikin, 1991; Brennan & Nikaido, 1995; Draper, 1998) and a specific role for these minor components must not be ruled out.

The overall biosynthetic pathways to mycolic acids and other key mycobacterial cell envelope lipids are starting to be intensively studied using genetic approaches (Kremer *et al.*, 2000). The novel mycolates, highlighted in this paper, will provide valuable signposts to correlate with such biosynthetic studies.

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