SEPARATION OF C_{50-60} AND C_{70-80} MYCOLIC ACID MOLECULAR SPECIES AND THEIR CHANGES BY GROWTH TEMPERATURES IN *MYCOBACTERIUM PHLEI*

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1. Introduction

Mycolic acids are unusually high molecular weight 3-hydroxy fatty acids possessing a long-chain alkyl branch at the 2-position and known to be a specific constituent of glycolipids or cell wall skeletons of Mycobacteria, Nocardia and Corynebacteria [1–4]. In the past decade, intensive structural studies have been carried out, mainly by French groups. However, owing to their instability at high temperatures, the successful gas chromatographic separation of the individual molecular species has not yet been achieved. The structures of the mycolic acids [5,6] have been correlated with the biological functions of the lipids containing them and with the physiological properties of the cell walls in Mycobacteria. To obtain more information about the functions of the individual molecular species of mycolic acids, their analysis by gas chromatography seemed to be essential. We have developed GC–MS analysis of such fatty acids ranging from C_{20} to C_{68} from Nocardia [7], Corynebacteria [8] and the '*Mycobacterium rhodochrous* complex' [9,10].

The present communication first describes the GC–MS analysis of C_{70-80} mycolic acids from a 'true' Mycobacterium, *Mycobacterium phlei*. In two subclasses of mycolic acids (M_1 and M_3) from *M. phlei*, the major species of α-mycolic acids (M_1) were found to be C_{72}, C_{74}, C_{76}, C_{77}, C_{78}, C_{79} and C_{80} dienoic (or dicyclopropanoic) monocarboxy mycolic acids, while the dicarboxy mycolic acids (M_3) were shown to be C_{56}, C_{58}, C_{59}, C_{60}, C_{61}, C_{62}, C_{63} and C_{64} monoenoic (or monocyclopropanoic) acids, both of which possessed C_{20} or C_{22} branch at 2-position. It was also demonstrated that the mycolic acid composition varied dramatically with the growth temperatures: the longer-chain molecular species increased at the higher temperatures and the shorter ones at the lower, in both subclasses (M_1 and M_3) of mycolic acids.

2. Materials and methods

Mycobacterium phlei and other acid-fast bacteria were grown in a medium containing 1% glucose, 0.5% peptone and 0.2% yeast extract, with the pH adjusted to 7.0, for 24–93 h. To compare the mycolic acid composition from cells grown at different temperatures, cells were grown at 23, 30, 40 and 51°C, and harvested at the stationary growth stage. After the cells were harvested by centrifugation, the packed pellets were hydrolyzed with 10% methanolic KOH for 3 h. After the mixture was acidified with 6 N HCl, the fatty acids were extracted and then transmethylated with benzene–methanol–H_2SO_4 (10:20:1, v/v/v). The methyl esters thus obtained were separated into non-polar species and subclasses of mycolic acid esters on a thin-layer plate of Silica gel G (Merck) with a solvent system of hexane–diethyl ether (4:1, v/v). The individual esters were recovered from the thin-layer plates with chloroform. After the solvent was evaporated to dryness, the residues were trimethylsilylated with BSTFA (*N*, *O*-bis-trimethylsilyl-trifluoroacetamide) at 50°C for 20 min. The TMS derivatives of each subclass of mycolic acid esters were injected into a combined GC–MS (Hitachi RMU 6MG, type M-60). The GC–MS was carried out

with a glass-coil column coated with 2% OV-1 on Chromosorb W at 300–340°C, and the molecular separator and the ion source were kept at 330°C and 250°C, respectively. The ionization current was 60 μA, the electron energy was 20 eV and the accelerating voltage, 3.2 kV.

3. Results and discussion

Thin-layer chromatography of the total fatty acid methyl esters from *M. phlei* gave four major spots (fig.1), the fastest moving corresponding to non-polar acid esters, the second to α-mycolic acid esters (designated for M_1), the third unknown (but probably keto mycolic acid esters (M_2)), and the fourth, the most polar subclass of mycolic acid esters (M_3).

The slowest moving spot (M_3) probably consisted of esters of dicarboxy mycolic acids, which have been reported in *M. phlei* [11–13], as judged by the chromatographic behaviour.

The $M_1 + M_3$ acids together accounted for ~50% of the total cellular fatty acid methyl esters (fig.1).

The gas chromatogram of TMS derivatives of M_1 gave at least 6 major peaks corresponding to C_{72}, C_{74}, C_{76}, C_{77}, C_{78} and C_{79} acids, judged from the relationships between log-retention times and carbon numbers (fig.2) [10].

The mass spectra were recorded at the top of each peak and the prominent ions on mass spectrum are summarized in table 1.

From the $[M]^+$, [M-15] and [M-90] ions, the total carbon and double bond numbers of each species were calculated. The ions [A] and [A-90], due to C_2–C_3 cleavage showed straight chain alkyl units (β units), while the ions [B] and [B-29], due to C_3–C_4 cleavage indicated the branched chain structure at the 2-position (α unit), as reported [7,9,10]. Thus, in the case of C_{76} α-mycolic acid, mass peaks due to $[M]^+$, [M-15] and [M-90] were present at *m/e* 1194, 1179 and 1104, showing that this species possessed dienoic (or dicyclopropanoic) structure. Since both fragment ions [A] at *m/e* 813 and 841 and the fragment ions [B] at *m/e* 483 and 455 are prominent, the C_{76} mycolic acid was indicated to be a mixture of molecular species possessing different straight chain alkyl units ($C_{52:2}$ and $C_{54:2}$) and side chains at the 2-position ($C_{22:0}$ and $C_{20:0}$).

All other components of α-mycolic acids (M_1) from *M. phlei* were identified similarly.

On the other hand, the gas chromatograms of the TMS derivatives of M_3 gave at least 7 major peaks

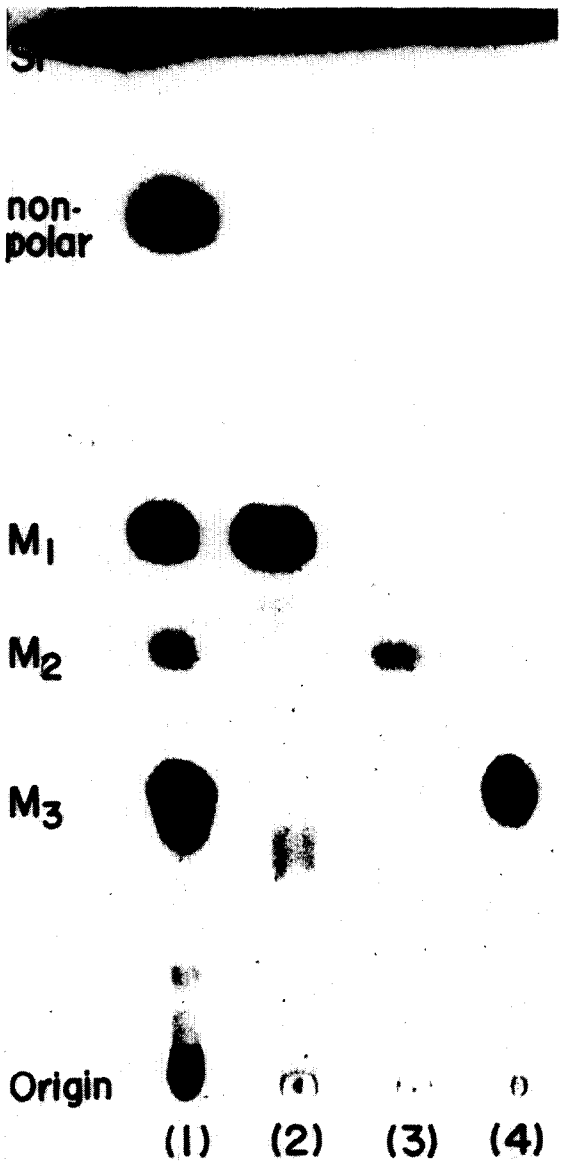


Fig.1. Thin-layer chromatograms of the fatty acid methyl esters from *M. phlei*. The plate was developed with *n*-hexane–diethylether (4:1, v/v). (1) Total fatty acid methylesters from *M. phlei*. (2) α-mycolic acid methylesters (M_1). (3) Unidentified (M_2). (4) Dicarboxy mycolic acid methylesters (M_3).

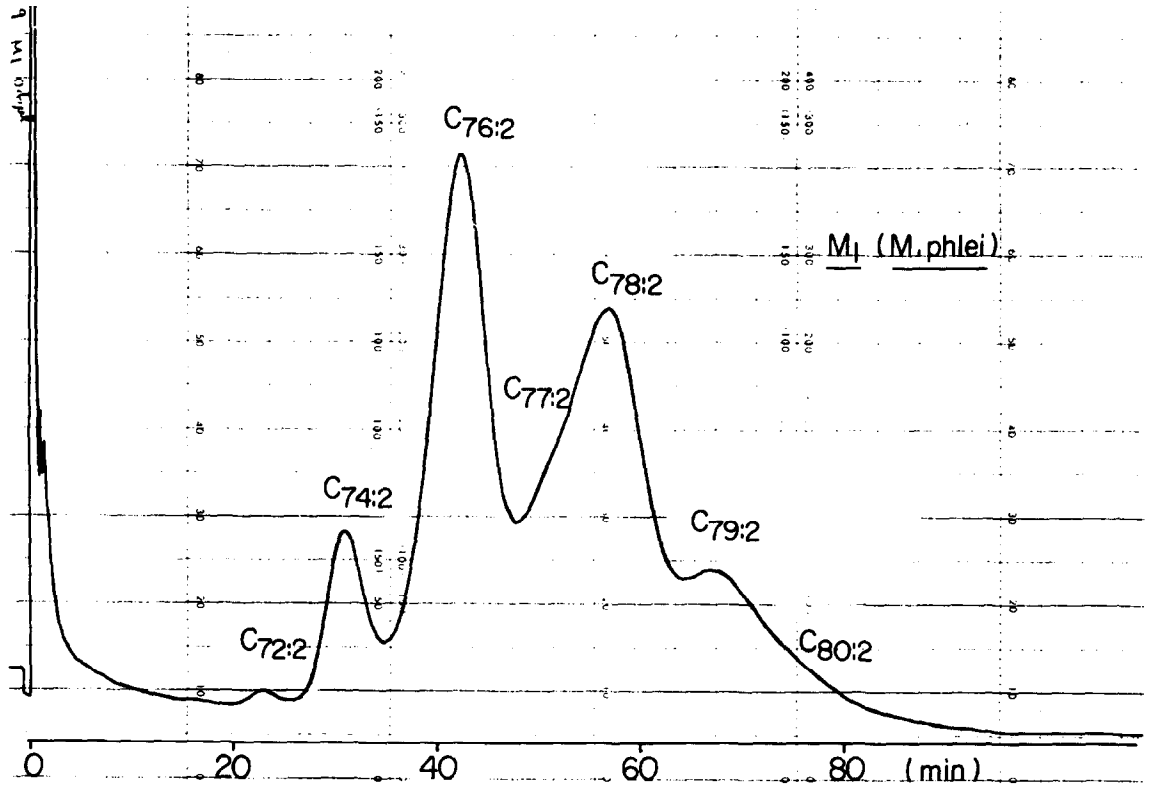


Fig.2. Gas chromatograms of TMS–methyl α-mycolate (M_1) from *M. phlei*. The column (2% OV-1) was maintained isothermally at 345°C. Numbers on the peaks indicate the total carbon and double bond numbers of mycolic acids.

corresponding to C_{56}, C_{57}, C_{58}, C_{59}, C_{60}, C_{61} and C_{62}, judging from the retention times.

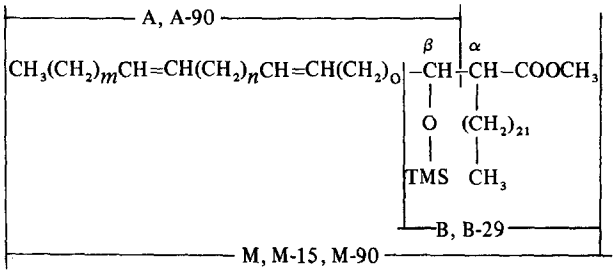
The mass fragmentation patterns of TMS-derivatives of M_3 were similar to those of M_1; however it was noted that the fragment ion [A] contained another carboxy methyl ester structure [12].

For the C_{58} dicarboxy mycolic acids, mass peaks due to $[M]^+$, [M-15] and [M-90] were present at *m/e* 988, 973 and 898, respectively, showing that the M_3 mycolic acids possessed monoenoic (or monocyclopropanoic), dicarboxylic structure. The occurrence of fragment ions [A] at *m/e* 607 and [B] at *m/e* 483 indicated that the straight chain was C_{34} monoenoic (or monocyclopropanoic) and the side-chain at the 2-position was $C_{22:0}$. Other components of M_3 were identified similarly. We have reported the GC–MS analysis of the individual molecular species of nocardomycolic and corynomycolic acids ranging from C_{28}–C_{68} [7–10]. From the above results, it is concluded that the mycolic acids from *M. phlei* ranging from C_{54}–C_{80} can be also analysed by GC–MS.

Since it is also known that bacterial fatty acid compositions can be affected by growth temperatures so as to maintain the proper fluidity of cell membranes [14,15], we compared the composition of mycolic acid subclasses M_1 and M_3 obtained from *M. phlei* grown at different temperature. The results are listed in table 1 (for M_1) and table 2 (for M_3), and in both subclasses, marked effects of growth temperature were observed. It was of great interest that the longer-chain mycolic acids increased on elevating the growth temperature, as in the cases of *iso* and *anteiso* fatty acids from moderately and extremely thermophilic bacteria [16]; this suggests that mycolic acids may also play an important role in regulating the fluidity of the mycobacterial cell membranes. Further-

Table 1
Gas chromatographic and mass spectrometric analysis of TMS–methylmycolate (M_1) from *Mycobacterium phlei*

Peak	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[A]^+$	$[A-90]^+$	$[B]^+$	[B-29]	β-unit	α-unit	% Composition			
										23°C	30°C	40°C	51°C
$C_{72:2}$	1138	1123	1048	785	695	455	426	$C_{50:2}$	$C_{22:0}$	12.6	5.9	0.8	trace
$C_{74:2}$	1166	1151	1076	813	723	455	426	$C_{52:2}$	$C_{22:0}$	46.9	31.3	11.2	5.1
				785	695	483	454	$C_{50:2}$	$C_{24:0}$				
$C_{76:2}$	1194	1179	1104	813	723	483	454	$C_{52:2}$	$C_{24:0}$	31.0	41.4	46.0	17.8
				841	751	455	426	$C_{54:2}$	$C_{22:0}$				
$C_{77:2}$	1208	1193	1118	855	765	455	426	$C_{55:2}$	$C_{22:0}$	6.7	3.6	3.2	4.4
$C_{78:2}$	1222	1207	1132	841	751	483	454	$C_{54:2}$	$C_{24:0}$	2.7	17.8	32.0	51.5
$C_{79:2}$	1236	1221	1146	855	765	483	454	$C_{55:2}$	$C_{24:0}$	trace	trace	2.4	9.9
$C_{80:2}$	1250	1235	1160	869	779	483	454	$C_{56:2}$	$C_{24:0}$	trace	trace	4.4	11.2



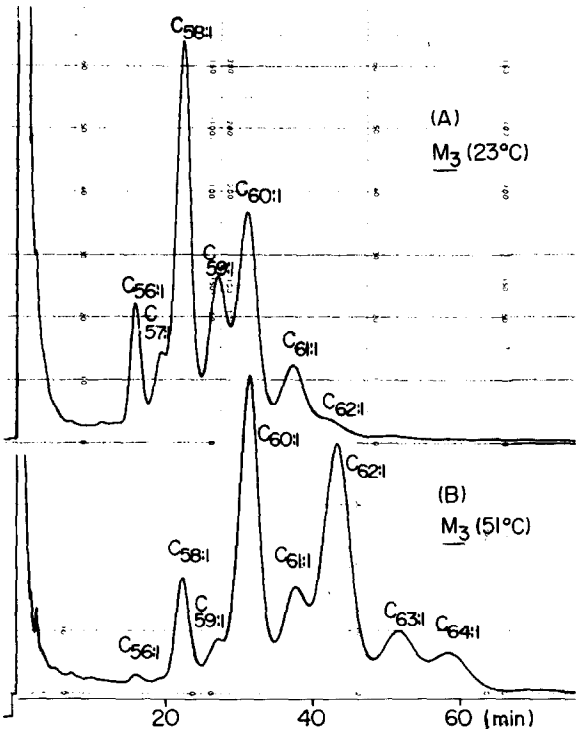
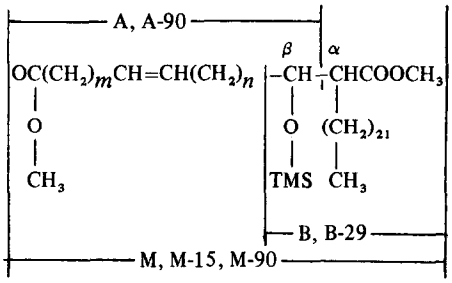


Fig.3. Gas chromatograms of TMS–methyl dicarboxy mycolate (M_3) from *M. phlei*. The column (2% OV-1) was maintained at 320°C. Other conditions are given in fig.2.

more, it was also noted from the mass fragmentographic analysis that even the longest-chain mycolic acid possessed a C_{22} branch at the 2 position, as did in the shorter ones; only the main alkyl chain varied in length. Since the various mycobacteria and related microorganisms possess specific mycolic acids and the composition varied with growth temperatures, the determination of the chain length seems to be controlled both genetically and environmentally. It has been already reported that the mycobacteria possess two types of fatty acid synthetases (I and II), both of which could be affected by growth temperature [17–19]. The former showed a bimodal distribution of products (C_{16} and C_{24}), while the latter enzyme system is involved with the chain elongation of C_{26} or longer chain fatty acids. The mechanism of the elongation steps in mycolic acid biosynthesis is of particular interest and remains to be clarified.

Table 2
Gas chromatographic and mass spectrometric analysis of TMS–methylmycolate (M_3) from *Mycobacterium phlei*

Peak	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[A]^+$	$[A-90]^+$	$[B]^+$	[B-29]	β-unit	α-unit	% Composition			
										23°C	30°C	40°C	51°C
$C_{56:1}$	960	945	870	607	517	455	426	$C_{34:1}$	$C_{22:0}$	10.3	6.0	1.3	0.5
$C_{57:1}$	974	959	884	621	531	455	426	$C_{35:1}$	$C_{22:0}$	2.5	trace	trace	trace
$C_{58:1}$	988	973	898	607	517	483	454	$C_{34:1}$	$C_{24:0}$	35.7	35.8	17.5	9.5
$C_{59:1}$	1002	987	912	621	531	483	454	$C_{35:1}$	$C_{24:0}$	13.3	3.4	3.3	1.3
$C_{60:1}$	1016	1001	926	635	545	483	454	$C_{36:1}$	$C_{24:0}$	28.4	46.5	44.3	34.8
$C_{61:1}$	1030	1015	940	649	559	483	454	$C_{37:1}$	$C_{24:0}$	8.4	3.0	6.7	5.3
$C_{62:1}$	1044	1029	954	663	573	483	454	$C_{38:1}$	$C_{24:0}$	1.5	4.6	25.0	40.4
$C_{63:1}$	1058	1043	968	677	587	483	454	$C_{39:1}$	$C_{24:0}$	–	0.5	2.0	4.8
$C_{64:1}$	1072	1057	982	691	601	483	454	$C_{40:1}$	$C_{24:0}$	–	trace	trace	4.7



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