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Mycolic acid patterns of some species of *Mycobacterium*

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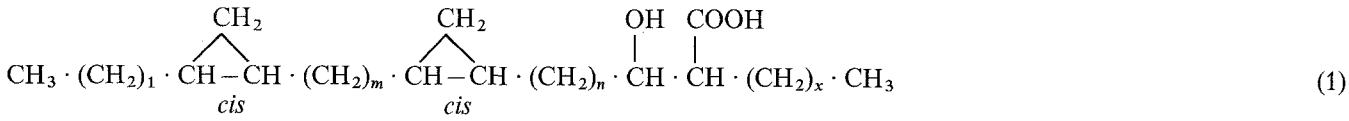
Abstract. Representative strains of some species of *Mycobacterium* were degraded by both acid and alkaline methanolysis. Two-dimensional thin-layer chromatography was used to determine the patterns of mycolic acids and other long-chain components in these methanolysates. Patterns composed of α-, methoxy- and ketomycolates were found in *Mycobacterium asiaticum, Mycobacterium bovis, Mycobacterium gastri, Mycobacterium gordonae, Mycobacterium kansasii, Mycobacterium marinum* and *Mycobacterium tuberculosis;* a representative of *Mycobacterium thermoresistibile* also contained lower molecular weight α′-mycolates in addition to these three acids. In representatives of *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium nonchromogenicum, "Mycobacterium novum", Mycobacterium paratuberculosis, Mycobacterium scrofulaceum, Mycobacterium terrae, Mycobacterium xenopi,* and *Mycobacterium* sp. MNC 165 α- and ketomycolates were accompanied by ω-carboxymycolates and 2-eicosanol and homologous alcohols which are derived from wax-ester mycolates. *Mycobacterium fortuitum* and *"Mycobacterium giae"* contained α′- and epoxymycolates and both serovars of *Mycobacterium simiae* had a very characteristic pattern of α-, α′- and ketomycolic acids. Comparison with data for other mycobacteria showed the chemotaxonomic significance of these mycolic acid patterns.

Key words: Mycolic acids – Mycobacteria – Thin-layer chromatographic analysis – Acid methanolysates – Alkaline methanolysates – Chemotaxonomy

Mycolic acids are high molecular weight 3-hydroxy, 2-alkyl-branched fatty acids found as characteristic components of all strains of *Mycobacterium* investigated so far (Asselineau 1966; Minnikin and Goodfellow 1980; Minnikin 1982). Mycobacterial mycolic acids have between 60 and 90 carbon atoms and the different structural types include the so-called α-mycolates and mycolates having other oxygen functions (keto, methoxy, ω-carboxy, epoxy) in addition to the 3-hydroxy acid unit (Minnikin 1982; Minnikin et al. 1982a). The discontinuous distribution of mycolic acid types has chemotaxonomic potential and special thin-layer chromatographic analysis systems have been developed for comparative purposes (Minnikin et al. 1980). Several types of mycolic acid patterns have been found to be particularly prevalent.



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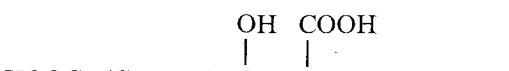

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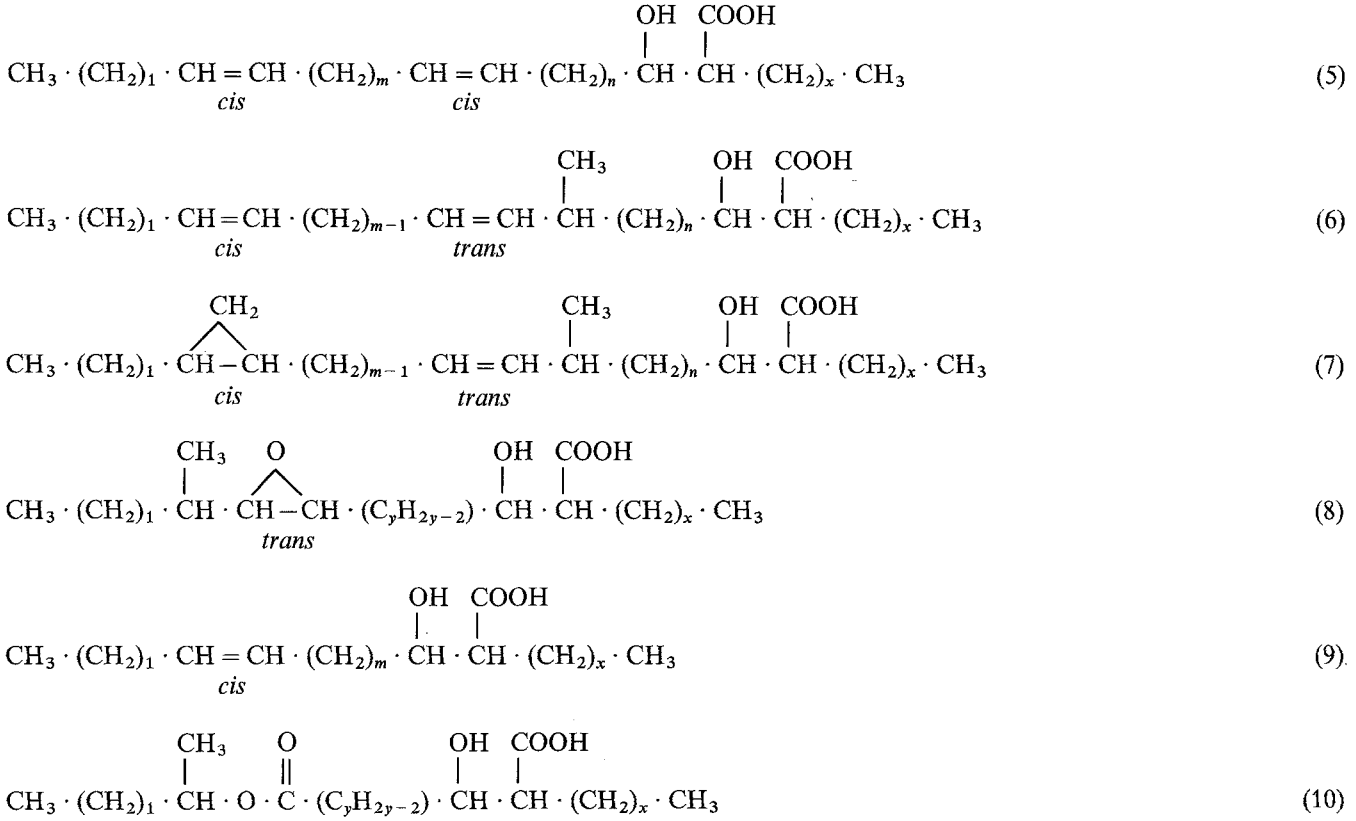
Abbreviations. TLC, thin-layer chromatography; TBDMS, *t*-butyldimethylsilyl

Supplementary data: Copies of the chromatographic patterns of the mycolic acids from all the strains examined can be provided on request from one of the authors (D.E.M.)



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The mycolates of *Mycobacterium tuberculosis*, consisting of α-mycolates (1), ketomycolates (2), and methoxymycolates (3) are an example of one pattern and a second pattern, exemplified by those of *Mycobacterium avium*, comprises α-mycolates (1), ketomycolates (2), and an ω-carboxymycolate (4), co-occurring with 2-eicosanol and homologous alcohols (Minnikin et al. 1980). In a third pattern α-mycolates (5–7) and epoxymycolates (8) are characteristic of, for example, *Mycobacterium fortuitum* (Minnikin et al. 1980, 1982a, 1984a). *Mycobacterium chelonae* shows a fourth pattern having comparable amounts of α-mycolates (5–7) and lower molecular weight α′-mycolates (9) (Goodfellow and Minnikin 1981; Minnikin et al. 1982b) and in strains of *Mycobacterium fallax* and *Mycobacterium triviale* α-mycolates are the principal acids (Lévy-Frébault et al. 1983a). In the present paper mycolic acid patterns of 47 respresentative mycobacterial cultures have been recorded and related to other mycolic acid patterns in order to substantiate the value of these compounds in mycobacterial systematics.

Materials and methods

Organisms. The test strains (Table 1) were inoculated by loop into 180 ml Sauton's medium in a 250 ml flask and incubated at 38°C for 14 to 104 days, depending on the growth rate. From two strains duplicate cultures of slightly different age were studied. The cultures designated 988 and 989 were 7 weeks old, while 988A and 989A were 10-week-old cultures. The cultures were sterilized by heating in flowing steam for 1 h, harvested by paper filtration and dried at about 80°C overnight. Names that do not appear in the Approved Lists of Bacterial Names (Skerman et al. 1980) or subsequent validation lists are written in quotation marks.

Whole-organism methanolysis. Dry biomass (20–50 mg) was degraded by both acid methanolysis (Minnikin et al. 1980) and a newly developed alkaline methanolysis procedure (Minnikin et al. 1982a, 1984b). This latter method involved treatment of biomass with 20% methanolic tetramethylammonium hydroxide (0.5 ml), toluene (0.5 ml) and methanol (1.0 ml) at 37°C overnight. The supernatant was removed and the cell pellet washed with methanol-toluene (2:1 by volume) (1 ml). Dimethylformamide (3 ml) was added to the combined supernatants and the volume reduced under a stream of nitrogen at < 37°C until the level was the same as that before the addition of the dimethylformamide. A 10% solution of iodomethane in dimethylformamide (1 ml) was added and, after mixing, the mixture was extracted with petroleum ether (b.p. 60–80°C) 4 ml and 2 × 2 ml). The combined upper layers were reduced to approximately 1 ml under nitrogen and the residue was again extracted with petroleum ether (1 ml and 2 × 0.5 ml). Evaporation of these latter extracts to dryness gave an alkaline methanolysate.

Thin-layer chromatography. Two-dimensional thin-layer chromatography (TLC) of methanolysates was performed using pieces (6.6 cm × 6.6 cm) of Merck 5554 silica gel 60 F_{254} TLC aluminium sheets. A triple development in the first direction with petroleum ether (b.p. 60–80°C) – acetone (95:5, by volume) was followed by a single development with toluene-acetone (97:3, by volume) in the second direction (Minnikin et al. 1980). The positions of separated components were revealed by spraying with 5% ethanolic molybdophosphoric acid and subsequent heating at 150°C for 15 min (Minnikin et al. 1980).

Derivatization of mycolic acid methyl esters. In order to positively distinguish chromatographically similar methoxy and

Table 1. List of strains used

Laboratory No.	Name	Strains and comment[a]	Source[b]	Habitat
MNC 3	*Mycobacterium tuberculosis*	T 3487	Tuberculosis Dept., SSI[c]	man
MNC 57	*M. tuberculosis*	1568	Tuberculosis Dept., SSI	man
MNC 1394	*M. tuberculosis*	H37Ra	Baess; Sugita	man
MNC 1397	*M. tuberculosis*	E 35497/81	Baess	man
MNC 8	*M. bovis*	V 22250	Tuberculosis Dept., SSI	man
MNC 27	*M. bovis*	T 10620	Tuberculosis Dept., SSI	man
MNC 30	*M. kansasii*	Goss, SSC 225	Engbæk	man
MNC 32	*M. kansasii*	Šula 687A	Engbæk, Šula	man
MNC 170	*M. marinum*	Walker 2893, Arthur Tacderan	Walker	man
MNC 762	*M. gastri*	ATCC 115980-15754, LG Wayne W-417, type strain	Engbæk, Wayne	man
MNC 855	*M. gastri*	Schröder 8842	Schröder	man
MNC 839	*M. asiaticum*	Weiszfeiler N 61, ATCC 25276, tye strain	Weiszfeiler	monkey
MNC 78	*M. thermoresistibile*	Engbæk E 32280, SSC 206	Engbæk	man
MNC 64	*M. gordonae*	Engbæk E 19911, SSC 215	Engbæk	man
MNC 661	*M. gordonae*	SN 719	Bönicke	
MNC 662	*M. gordonae*	SN 720	Bönicke	
MNC 11	*M. avium*	Engbæk E 38686, serovar 4	Engbæk	man
MNC 23	*M. intracellulare*	Engbæk V 22147, SSC 209, serovar 14	Engbæk	man
MNC 86	*M. intracellulare*	Holm 382/49–50, *"Nocardia intracellularis"*, PHS 410	Holm	man
MNC 547	*M. intracellulare*	Runyon P 39, Boone, serovar 14	Engbæk, Runyon	man
MNC 548	*M. intracellulare*	Runyon P 40, Arnold, serovar 20	Engbæk, Runyon	man
MNC 559	*M. intracellulare*	Runyon P 54, Wilson, serovar 17	Engbæk, Runyon	man
MNC 95	*M. scrofulaceum*	526	Masson	man
MNC 16	*M. nonchromogenicum*	Engbæk A 1159, SSC 264	Engbæk	man
MNC 302	*M. nonchromogenicum*	ATCC 25142	ATCC[d]	
MNC 1053	*M. paratuberculosis*	Promise	Berg Jørgensen	
MNC 631	*M. xenopi*	Nassau TB 24, SSC 991	Nassau	man
MNC 165	*Mycobacterium* sp.[e]	3 SA 7	Simon, Weed	man
MNC 336	*M. terrae*	ATCC 15755, LG Wayne W-45, type strain	ATCC[d]	radish (soil)
MNC 763	*M. terrae*	Radish ATCC 15981–15755, type strain, see MNC 336	Engbæk, Wayne	radish (soil)
MNC 919	*M. terrae*	RS 10618/18	Schröder	
MNC 318	*"M. novum"*[f]	ATCC 25269	ATCC[d]	
MNC 988	*"M. novum"*	Tsukamura 1934	Tsukamura	soil
MNC 989	*"M. novum"*	Tsukamura 1950	Tsukamura	soil
MNC 991	*"M. novum"*	Tsukamura 2578	Tsukamura	soil
MNC 992	*"M. novum"*	Tsukamura 2579	Tsukamura	soil
MNC 837	*M. simiae*	Weiszfeiler N 29, ATCC 25275, type strain	Weiszfeiler	monkey
MNC 838	*M. simiae*	Weiszfeiler N 59, serovar 2	Weiszfeiler	monkey
MNC 838A	*M. simiae*	Variant from MNC 838, serovar 2	Weiszfeiler	monkey
MNC 946	*M. simiae*	Weiszfeiler 14, serovar 2	Weiszfeiler	monkey
MNC 1322	*M. simiae*	Weiszfeiler 14, serovar 2, same as MNC 946	Weiszfeiler	monkey
MNC 19	*M. fortuitum*	Engbæk E 32352, SSC 213	Engbæk	man
MNC 20	*M. fortuitum*	Engbæk E 41167, SSC 214	Engbæk	man
MNC 928	*"M. giae"*	ATCC 11440	ATCC	lizard (gia)
MNC 931	*"M. giae"*	ATCC 14467, LF Bojalil 40, *"M. peregrinum"*, type strain	ATCC	man

[a] The serovars of *M. simiae* refer to Meissner and Schröder (1975) and those of *M. avium, M. intracellulare,* and *M. scrufulaceum* refer to Wolinsky and Schaefer (1973)

[b] Dr. I. Baess, Copenhagen, Denmark: Dr. J. Berg Jørgensen, Copenhagen, Denmark; Dr. L. F. Bojalil, Mexico City, Mexico, Dr. R. Bönicke (deceased), Borstel, FRG; Dr. H. C. Engbæk, Copenhagen, Denmark; Dr. P. Holm (deceased), Copenhagen, Denmark; Dr. A. M. Masson, Montreal, Canada; Dr. E. Nassau, Harefield, United Kingdom; Dr. E. H. Runyon, Salt Lake City, Utah, USA; Dr. K. H. Schröder, Borstel, FRG; Dr. C. Simon, Kiel, FRG; Dr. K. Sugita, Yokohama, Japan; Dr. L. Šula, Prague, Czechoslovakia; Dr. M. Tsukamura, Aichi, Japan; Dr. H. H. Walker, Honolulu, Hawaii; Dr. L. G. Wayne, Long Beach, California, USA; Dr. L. A. Weed, Rochester, Minnesota, USA; Dr. G. Weiszfeiler, Budapest, Hungary

[c] SSI; Statens Seruminstitut

[d] ATCC; American Type Culture Collection, Rockville, Maryland, USA

[e] Strain MNC 165 was labelled *M. scrofulaceum* when received; however, by comparative reciprocal intradermal sensitin tests on guinea-pigs (Magnusson 1981) it is clearly distinct from this and from many other species

[f] *"M. novum"* has been considered to be synonymous with *M. terrae* (Meissner et al. 1974); this is despite the fact that the type strain of *M. terrae* (ATCC 15755) had a matching score of 80% or less with the other strains, including strains labelled *"M. novum"* belonging to the same cluster. Strain ATCC 15755 also showed little similarity by comparative reciprocal intradermal sensitin tests to most other strains of the same cluster; see also Tsukamura et al. (1983)

α′-mycolates, mycolic acid methyl esters were converted to *t*-butyldimethylsilyl (TBDMS) ethers. The total mycolates were isolated by preparative TLC and treated at 75° C overnight with *t*-butyldimethylsilyl chloride and imidazole in dimethylformamide-toluene solution (Minnikin et al. 1982a). TBDMS derivatives were analysed by TLC using petroleum ether-toluene (70:30, by volume) as developing solvent in a single direction.

Results

The test organisms were degraded by both acid and alkaline methanolysis procedure and results of the TLC analyses of the latter extracts from representatives of all the species studied are shown in Fig. 1. All chromatograms showed the presence of components corresponding to methyl esters of nonhydroxylated fatty acids and mycolic acids. Representatives of *Mycobacterium tuberculosis, M. bovis, M. kansasii, M. marinum, M. gastri, M. asiaticum* and *M. gordonae* (Fig. 1, Table 1) had qualitatively similar patterns of mycolates, composed of α-mycolates, methoxymycolates and ketomycolates. Patterns of α-mycolates, ketomycolates, ω-carboxymycolates and long-chain alcohols, homologous with 2-eicosanol, were characteristic of strains of *M. avium, M. intracellulare, M. scrofulaceum, M. nonchromogenicum, M. xenopi, M. terrae, "M. novum"* (Fig. 1, Table 1), and *M. paratuberculosis,* and *Mycobacterium* sp. MNC 165 (Table 1). The two duplicate cultures of *"M. novum"* (988 A and 989 A, Table 1) which were cultivated for a 10-week rather than a 7-week period, still have the same overall mycolate pattern, though culture 989 A had some additional minor components when compared with the 7-week culture 989.

Five strains of *M. simiae* (Fig. 1, Table 1) showed a relatively simple pattern of α-, α′-, and ketomycolates and two strains each of *M. fortuitum* and *"M. giae"* (Fig. 1, Table 1) had patterns composed of α-mycolates and epoxymycolates. The presence of epoxymycolates was confirmed by degradation on acid methanolysis to characteristic pairs of polar mycolates (Minnikin et al. 1980, 1982a). The superficial similarity of the patterns of mycolates from *Mycobacterium thermoresistibile* 78 and *M. simiae* (Fig. 1) was investigated by TLC of TBDMS ether derivatives. The TBDMS mycolate derivatives from *M. simiae* had components having R_F 0.75, 0.68, and 0.17 corresponding to derivatives of α-, α′-, and ketomycolate standards. Major spots corresponding to α- and ketomycolate derivatives were also present in *M. thermoresistibile;* the third major component had R_F 0.34 similar to that of the TBDMS ether of methoxymycolate standard but lesser amounts of a TBDMS derivative of an α′-mycolate were also present.

Discussion

The determination of patterns of mycolic acids in providing data of value in the classification and identification of mycobacteria (Minnikin and Goodfellow 1980; Dobson et al. 1984). Mycolic acid patterns appear to be stable under standardized growth conditions and the use of a rapid two-dimensional TLC system allows them to be visualized clearly. The use of an alkaline methanolysis procedure (Minnikin et al. 1984a) has an advantage over acid methanolysis (Minnikin et al. 1980) in that less degradation of mycolates containing cyclopropane rings is observed, clearer patterns being obtained. Acid methanolysis remains of value, however, in confirming the presence of epoxymycolates (Minnikin et al. 1982a) which have chromatographic behaviour very similar to that of ketomycolates.

The patterns of mycolic acid methyl esters recorded for the test strains of *Mycobacterium tuberculosis, M. bovis,* and *M. kansasii* shows the presence of α-, methoxy- and ketomycolates as expected from previous studies (Etémadi 1967; Minnikin and Goodfellow 1980; Minnikin et al. 1980) as is that for the examples of *M. gordonae* (Daffé et al. 1981a). The present study shows that representatives of *M. asiaticum, M. gastri,* and *M. marinum* also share this general type of mycolic acid pattern. *M. thermoresistibile* is unusual in producing small amounts of an α′-mycolate in addition to a methoxymycolate and this characteristic pattern may be of value in the identification of this species. An unknown long-chain component "U" chromatographing between the methyl esters of non-hydroxylated fatty acids and mycolic acids (Fig. 1) is present only in alkaline methanolysates. The unidentified more polar components (Fig. 1) are variable in occurrence and are possibly small amounts of unesterified fatty and mycolic acids.

The mycolic acid pattern characteristic of *M. avium, M. intracellulare,* and *M. paratuberculosis* (Minnikin and Goodfellow 1980; Minnikin et al. 1980), consisting of α-, keto- and ω-carboxymycolate and 2-eicosanol and homologues, is confirmed by the present study and this pattern is shared by *M. nonchromogenicum, M. scrofulaceum, M. terrae, M. xenopi, Mycobacterium* sp. MNC 165, and *"M. novum"*. Extracts of *M. intracellulare* MNC 86 and *"M. novum"* MNC 989A contained additional unidentified minor long-chain components.

The pattern composed of α-, α′- and ketomycolates appear to be very characteristic of both serotypes of *M. simiae* and may be of value in the identification of this species. The presence of α- and epoxymycolates in *M. fortuitum* is in accordance with previous studies (Minnikin et al. 1980, 1982a; Daffé et al. 1981b; Lévy-Frébault et al. 1983b; Minnikin et al. 1984b). The presence of the same pattern of

Fig. 1. Two-dimensional thin-layer chromatography of alkaline methanolysates of **a** *Mycobacterium tuberculosis* MNC 3, **b** *M. bovis* MNC 8, **c** *M. kansasii* MNC 30, **d** *M. marinum* MNC 170, **e** *M. gastri* MNC 762, **f** *M. asiaticum* MNC 839, **g** *M. gordonae* MNC 64, **h** *M. thermoresistibile* MNC 78, **i** *M. avium* MNC 11, **j** *M. intracellulare* MNC 23, **k** *M. scrofulaceum* MNC 95, **l** *M. nonchromogenicum* MNC 302, **m** *M. xenopi* MNC 631, **n** *M. terrae* MNC 336, **o** *"M. novum"* MNC 318, **p** *M. simiae* MNC 838, **q** *M. fortuitum* MNC 19, **r** *"M. giae"* MNC 931. A triple development with petroleum ether (b.p. 60–80°C) – acetone (95:5 v/v) in the first direction was followed by a single development with toluene-acetone (97:3 v/v) in the second direction. Abbreviations: *F* non-hydroxylated fatty acid methyl esters; *A* α-mycolate; *A′* α′-mycolate; *B* methoxymycolate; *C* ketomycolate; *D* ω-carboxymycolate; *E* alcohols homologous with 2-eicosanol; *M* epoxymycolate; *U* unidentified long-chain compound; **?** unknown.

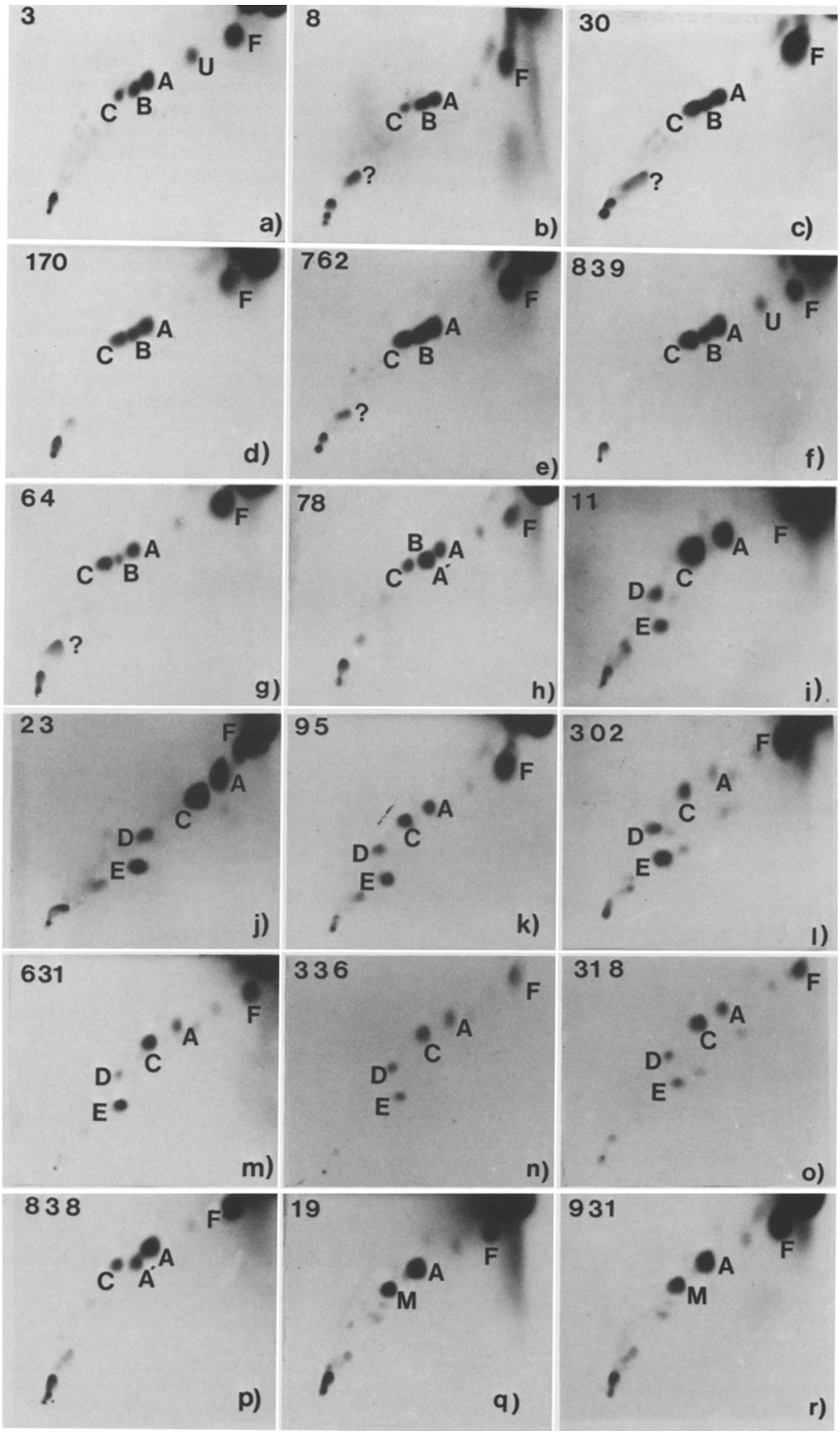
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Fig. 1

Table 2. Distribution of mycobacterial mycoic acids[a]

Pattern of structural types[b]	Mycobacterial species
α only[c]	*M. fallax, M. triviale*
α, α′	*"M. borstelense", M. chelonae*
α, α′, methoxy	*M. agri*[d]
α, α′, epoxy[e]	*M. chitae, M. farcinogenes, M. fortuitum, "M. giae", "M. peregrinum", M. senegalense, M. smegmatis*
α, α′, keto	*M. simiae*
α, α′, keto, wax ester[f]	*M. chubuense*[g], *M. duvalii, M. gilvum, M. parafortuitum, M. vaccae*
α, keto, wax ester	*M. aichiense, M. aurum*[h], *M. avium, M. flavescens, M. gadium, "M. gallinarum", M. intracellulare, M. lepraemurium, M. nonchromogenicum, M. neoaurum*[i], *"M. novum", M. paratuberculosis, M. phlei, M. rhodesiae, M. scrofulaceum, M. terrae, M. tokaiense*[i], *M. xenopi*
α, keto	*M. bovis* BCG, *M. leprae*
α, keto, methoxy	*M. asiaticum, M. bovis, M. gastri, M. gordonae, M. kansasii, M. marinum, M. microti, M. tuberculosis, M. szulgai*[i], *M. ulcerans*
α, α′, methoxy, keto	*M. thermoresistibile*
α, keto[j], methoxy[j], wax ester	*M. komossense*

[a] Adapted from Dobson et al. (1984) to include the results from the present study
[b] See text for explanation of mycolic acid structures
[c] Minor amounts of oxygenated mycolic acids occasionally detected
[d] Traces of ketomycolates and unknown more polar components also may be detected
[e] Proportion of α′-mycolate varies; may be absent in certain cases. A characteristic polar component is sometimes present
[f] Wax ester mycolates (X) are composed of ω-carboxymycolates (IV) esterified to 2-eicosanol and homologues
[g] Trace amounts of a component co-chromatographing with a methoxymycolate were observed
[h] In certain strains α′-mycolates are detected
[i] Characteristic unidentified more polar components are present
[j] Components co-chromatographing with keto- and methoxymycolates require positive identification

mycolates in *"M. giae"* is not unexpected as this taxon has been considered to be a subjective synonym of *M. fortuitum* (Runyon et al. 1974; Goodfellow and Wayne 1982).

The mycolic acid patterns of most approved species of the genus *Mycobacterium* have now been analysed and the distribution of such patterns has been reviewed recently (Dobson et al. 1984). The distribution of mycolic acid types is summarized in Table 2 which also includes the present data. It is interesting that several species that are considered to be related on the basis of biochemical, cultural, immunological and DNA homology data (see Goodfellow and Wayne 1982) have the same pattern of mycolic acids. This applies to the following pairs or groups of species: *M. aurum* and *M. neoaurum; "M. borstelense"* and *M. chelonae; M. kansasii* and *M. marinum; "M. novum"* and *M. terrae; M. parafortuitum* and *M. vaccae; M. avium, M. intracellulare,* and *M. xenopi,* and *M. bovis, M. microti,* and *M. tuberculosis.* In contrast, however, there are many instances where species that show the same mycolic acid pattern are not perceived to be closely related when studied by other methods. Thus, *M. triviale* can readily be distinguished from *M. nonchromogenicum, "M. novum"* and *M. terrae* on the basis of mycolic acid data but in a numerical phenetic survey representatives of these taxa were recovered in a single cluster which appeared to correspond to a single species (Tsukamura et al. 1983). Clearly, further comparative studies are required to determine whether mycobacterial species can be assigned to recognisable aggregate taxa.

The patterns reported in the present paper reinforce the value of mycolic acid analyses in mycobacterial systematics. These data together with those from earlier studies provide good grounds for recommending that minimal descriptions of mycobacterial species should include information on mycolic acid patterns.

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