Pyrolysis Gas Chromatography–Mass Spectrometry of Mycobacterial Mycolic Acid Methyl Esters and Its Application to the Identification of *Mycobacterium leprae*

By TAKASHI KUSAKA¹* AND TATSUO MORI²

¹Biochemistry Department, Kawasaki Medical School, Kurashiki, Japan

²Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

(Received 7 April 1986; revised 14 July 1986)

Pyrolysis gas chromatography–mass spectrometry of methyl mycolates from 32 species of mycobacteria, including *Mycobacterium leprae*, was carried out. The mycobacteria could be classified into four groups in respect of the fatty acid ester patterns detected within the range C₂₀ to C₂₆. The applicability of this pyrolysis-gas chromatographic method for identifying *M. leprae* is discussed.

INTRODUCTION

The mycolic acids from *Mycobacterium leprae* have been analysed in some detail by high-performance liquid chromatography (HPLC), mass spectrometry (MS) and proton nuclear magnetic resonance spectrometry (NMR) (Asselineau *et al.*, 1981; Kusaka *et al.*, 1981; Draper *et al.*, 1982; Minnikin *et al.*, 1985). It was found that *M. leprae* has two homologous series of mycolates, whose main structural characteristic seemed to be in the alkyl branch at the α -carbon having a relatively shorter chain length than for other mycobacterial mycolates. In order to determine whether the structure of the α -branch is specific for *M. leprae*, pyrolysis gas chromatography–mass spectrometry (GC–MS) of mycolates isolated from 32 species of mycobacteria was carried out.

METHODS

Organisms. Foot-pads of nude mice (*nu/nu*) killed one year after inoculation with *M. leprae* (Tai-53) were used as the source of *M. leprae*. All other species of cultivable mycobacteria, grown on 1% Ogawa's media, were supplied from the Laboratory for Culture Collection of the Institute for Microbial Diseases of Osaka University.

Isolation of mycolates. For isolating mycolates directly from the foot-pads of nude mice, the tissues were chopped into small pieces with scissors. Saponification of these tissues or cultivated mycobacterial cells was done by heating at 85 °C for 4 h in more than 10 vols 5% (w/v) KOH in 50% (v/v) ethanol under reflux. The suspension was then acidified (below pH 2) with 6 M-HCl and extracted three times with n-hexane/diethyl ether (1:1, v/v). The extract was evaporated to dryness and the residue containing total fatty acids was dissolved in a small volume of tetrahydrofuran and analysed by HPLC (Shimadzu-LC4), using a gel-permeation column (HSG-15, Shimadzu Co., 79 mm in diameter and 1 m in length) and a differential refractometer detector (Shimadzu RID-4). Various polystyrene standards of known M_r (Waters) were used to obtain a calibration curve. The flow rate of tetrahydrofuran was 2 ml min⁻¹. The eluate corresponding to material of M_r > 1000, assumed to contain total mycolates, was collected and evaporated to dryness.

Pyrolysis GC–MS and pyrolysis GC of methyl mycolates. The total mycolates, obtained as above, were dissolved in a small volume of diethyl ether then esterified with diazomethane. The methyl mycolates were analysed with a GC–MS instrument (Hitachi, M80-B), equipped with a Curie-point pyrolyser (Packard, model 891) fitted with a 3% OV-1 column (3 mm in diameter, 1 m in length), and using the electron-impact mode of MS. Methyl mycolates were also analysed by pyrolysis GC using a Shimadzu GC-8A instrument equipped with the same pyrolyser and column as above for quantitative analysis of the fatty acids.

Fig. 1. Pyrolysis GC-MS of *M. leprae* mycolates. Pyrolysis was performed at 358 °C for 10 s and the GC column (OV-1) temperature was programmed from 180 °C to 300 °C at the rate of 10 °C min⁻¹. The mass spectra shown on the right were scanned at the times marked with arrows in the total ion chromatogram on the left. 'BG' in the mass spectra means background from the column.

Fig. 2. Pyrolysis GC of mycolates from four mycobacterial species. The column (OV-1) temperature was programmed from 180 °C to 300 °C at the rate of 10 °C min⁻¹.

RESULTS

Pyrolysis GC-MS and pyrolysis GC of methyl mycolates

Pyrolysis of methyl mycolates at about 300 °C yields long-chain meroaldehydes and fatty acid methyl esters having two carbon atoms more than the α -branch of the original mycolates (Etémadi, 1967). Accordingly, pyrolysis GC-MS and pyrolysis GC of mycolates from 32 species of mycobacteria (including *M. leprae*) was done to investigate how the α -branches of *M. leprae* mycolates compare with those of other mycobacteria. The results from the pyrolysis GC-MS of the *M. leprae* mycolates are shown in Fig. 1 and those from pyrolysis GC of mycolates from four species in Fig. 2. Summarized results for all species are shown in Table 1.

The α -branches of all the mycobacterial mycolates were straight alkyl chains having from 18 to 24 carbons. The 32 species of mycobacteria could be classified into the following four groups in respect of the fatty acid pattern detected by pyrolysis GC-MS of the mycolates: a '22-group' having fatty acid from 20 to 24 carbons with C₂₂ predominating, a '24-group' having 22 to 24

Table 1. Four groups of mycobacteria on the basis of pyrolysis GC-MS and pyrolysis GC of mycolates

The ratio of fatty acid methyl esters was calculated from the GC peak size, measured by multiplication of the height of the peak by its width at half height, and the mean value was obtained from three GC experiments.

Group	Species*	Ratio of fatty acid methyl ester (C _{24:0} = 1.00)							Time for colony formation on 1% Ogawa's growth medium
		C _{20:0}	C _{21:0}	C _{22:0}	C _{23:0}	C _{24:0}	C _{25:0}	C _{26:0}	
'22'	<i>M. neoaurum</i> (ATCC 25796)	0.40	0.15	9.60	0.30	1.00			<3 d
	<i>M. parafortuitum</i> (ATCC 19686)	0.11	0.06	7.48	0.01	1.00			<3 d
	<i>M. diernhoferi</i> (ATCC 19344)	0.13		5.25	0.04	1.00			<3 d
	<i>M. leprae</i> (Tai 53)			2.68	0.02	1.00			∞
	<i>M. vaccae</i> (ATCC 23014)	0.02	0.01	1.68	0.04	1.00			<3 d
	<i>M. aurum</i> (ATCC 25793)	0.04	0.01	1.20	0.02	1.00			<3 d
'24'	<i>M. phlei</i> (ATCC 23042)			0.82	0.04	1.00			<3 d
	<i>M. rhodesiae</i> (5298)†			0.68	0.07	1.00			<3 d
	' <i>M. kanazawa</i> ' (ATCC 25810)			0.41	0.02	1.00			<3 d
	<i>M. chitae</i> (ATCC 19629)			0.16		1.00			<3 d
	<i>M. smegmatis</i> (ATCC 14468)			0.15	0.01	1.00			<3 d
	' <i>M. peregrinum</i> ' (ATCC 23022)			0.06	0.02	1.00			<3 d
	<i>M. fortuitum</i> (ATCC 19542)			0.04		1.00			<3 d
	<i>M. chelonae</i> (ATCC 19977)			0.03		1.00			<3 d
	<i>M. flavescens</i> (ATCC 23033)			0.19		1.00			<7 d
	<i>M. thermoresistibile</i> (ATCC 19529)			0.06		1.00			<7 d
	<i>M. gastri</i> (ATCC 15754)			0.29	0.03	1.00			2-3 weeks
	<i>M. nonchromogenicum</i> (ATCC 19530)			0.28	0.06	1.00			2-3 weeks
	<i>M. gordonae</i> (ATCC 23285)			0.20		1.00			2-3 weeks
	<i>M. kansasii</i> (ATCC 12478)			0.13		1.00			2-3 weeks
	<i>M. triviale</i> (ATCC 19368)			0.13		1.00			2-3 weeks
	<i>M. terrae</i> (ATCC 15755)			0.05		1.00			2-3 weeks
	<i>M. scrofulaceum</i> (ATCC 19073)			0.04		1.00			2-3 weeks
	<i>M. ulcerans</i> (NCTC 10417)					1.00			4-7 weeks
'24''	<i>M. avium</i> (ATCC 17940)			0.18	0.09	1.00	0.08	0.10	2-3 weeks
	<i>M. intracellulare</i> (ATCC 19077)			0.14	0.06	1.00	0.06	0.06	2-3 weeks
	<i>M. marinum</i> (1160)†			0.05		1.00	0.05	0.12	2-3 weeks
	<i>M. lepraemurium</i> (Hawaiian strain)					1.00	0.10	0.10	>2 months
'26'	<i>M. tuberculosis</i> (H ₃₇ Ra)			0.19	0.16	1.00	0.25	3.94	2-3 weeks
	<i>M. simiae</i> (25)†					1.00		6.40	2-3 weeks
	<i>M. xenopi</i> (ATCC 19250)			0.08		1.00	0.12	3.02	3-4 weeks
	<i>M. bovis</i> BCG (Tokvo strain)					1.00		2.52	3-5 weeks

* Bacterial names that do not appear in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), or subsequent validation lists published in the *International Journal of Systematic Bacteriology*, are written in inverted commas.

† Strain numbers of Laboratory for Culture Collection, Research Institute for Microbial Diseases, Osaka University; these strains were originally from Professor H. Saitō, Department of Microbiology, Shimane Medical School.

carbons with C₂₄ predominating, a '24'-group' having 22 to 26 carbons with C₂₄ predominating, and a '26-group' having 22 to 26 carbons with C₂₆ predominating. The above groups seemed to relate partially to mycobacterial growth rate *in vitro*, since all species of the 22-group except *M. leprae* are rapid growers, requiring less than 3 d for colony formation, while all species of the 26- and 24'-groups are slow growers, requiring 2-3 weeks or more for colony formation. No such relation could be observed for the 24-group.

DISCUSSION

Pyrolysis GC of mycolates has been of value in the characterization of different mycolate-containing taxa such as mycobacteria, bacterionemas, corynebacteria, nocardiae and rhodococci (Lechevalier *et al.*, 1971; Minnikin & Goodfellow, 1980; Daffé *et al.*, 1983). We

found it hard, however, to distinguish small peaks of genuine fatty acid esters from contaminating ghost peaks in this technique; a combination of MS with pyrolysis GC, applied for the first time to mycobacterial mycolates, resolved these difficulties (cf. Fig. 1).

As shown in Table 1, the mycobacteria could be classified into four groups in respect of the pattern of the fatty acid esters released by pyrolysis of mycolates. The reproducibility of the apparent grouping of mycobacteria must be tested by analysing large numbers of strains. Our data from the pyrolysis GC of mycolates coincide completely with those of Daffé *et al.* (1983), who studied 27 species. It was unexpectedly found that our proposed grouping of mycobacteria seemed to have some relation to mycobacterial growth rate, though it is unclear why such a relation exists. In consideration of the known biosynthetic mechanism of mycolates (Shimakata *et al.*, 1984) and the experiential relation between mycobacterial constitutive saturated fatty acid patterns and the length of mycolate α -branches (Kusaka & Izumi, 1983), mycolate α -branches may be derived mainly from fatty acids formed by *de novo* fatty acid synthases in each bacterium. If so, there might be four types of mycobacterial fatty acid synthases or the associated thioesterases in respect of the product specificity.

We have reported previously (Kusaka *et al.*, 1981) that the chain length of the α -branch in *M. leprae* mycolates was 20 carbons; this must now be revised as mainly 20 carbons with lesser amounts of 22 carbons as shown in Table 1. This result is in agreement with previous mass spectrometric studies on mycolates of *M. leprae* harvested from experimentally infected armadillos (Asselineau *et al.*, 1981; Draper *et al.*, 1982).

Our method described here has some value in helping to identify *M. leprae*. The pyrolysis GC of mycolates can be done within 2 d and requires less than 0.5 mg dry weight of the unknown mycobacterial species. If mycolates from infected material produce, on pyrolysis, mainly 22-carbon, with lesser amounts of 24-carbon esters, it is probable that the infecting agent is *M. leprae*, especially if no rapid grower of the 22-group can be cultivated (Table 1).

This study received financial support from the US-Japan Cooperative Medical Science Program and Sasakawa Memorial Health Foundation, for which grateful acknowledgment is made.

REFERENCES

- ASSELINAEU, C., CLAVEL, S., CLÉMENT, F., DAFFÉ, M., DAVID, H., LANÉELLE, M. A. & PROMÉ, J. C. (1981). Constituents lipidiques de *Mycobacterium leprae* isolé de tatou infecté expérimentalement. *Annales de microbiologie* **132A**, 19-30.
- DAFFÉ, M., LANÉELLE, M. A., ASSELINAEU, C., LÉVY-FRÉBAULT, V. & DAVID, H. (1983). Intérêt taxonomique des acides gras des mycobactéries: proposition d'une méthode d'analyse. *Annales de microbiologie* **134B**, 241-256.
- DRAPER, P., DOBSON, G., MINNIKIN, D. E. & MINNIKIN, S. M. (1982). The mycolic acids of *Mycobacterium leprae* harvested from experimentally infected nine-banded armadillos. *Annales de microbiologie* **133B**, 39-47.
- ETÉMADI, A. H. (1967). The use of pyrolysis gas chromatography and mass spectroscopy in the study of the structure of mycolic acids. *Journal of Gas Chromatography* **5**, 447-456.
- KUSAKA, T. & IZUMI, T. (1983). Gas chromatography of constitutive fatty acids in *Mycobacterium leprae*. *Microbiology and Immunology* **27**, 409-414.
- KUSAKA, T., KOHSAKA, K., FUKUNISHI, Y. & AKIMORI, N. (1981). Isolation and identification of mycolic acids in *Mycobacterium leprae* and *Mycobacterium lepraemurium*. *International Journal of Leprosy* **49**, 406-416.
- LECHEVALIER, M. P., HORAN, A. C. & LECHEVALIER, H. (1971). Lipid composition in the classification of nocardiae and mycobacteria. *Journal of Bacteriology* **105**, 313-318.
- MINNIKIN, D. E. & GOODFELLOW, M. (1980). Lipid composition in the classification and identification of acid-fast bacteria. In *Microbiological Classification and Identification*, pp. 189-256. Edited by M. Goodfellow & R. G. Board. London: Academic Press.
- MINNIKIN, D. E., DOBSON, G., GOODFELLOW, M., DRAPER, P. & MAGNUSSEN, M. (1985). Quantitative comparison of the mycolic and fatty acid compositions of *Mycobacterium leprae* and *Mycobacterium goodii*. *Journal of General Microbiology* **131**, 2013-2021.
- SHIMAKATA, T., IWAKI, M. & KUSAKA, T. (1984). *In vitro* synthesis of mycolic acids by the fluffy layer fraction of *Bacterionema matruchotii*. *Archives of Biochemistry and Biophysics* **229**, 329-339.
- SKERMAN, V. B. D., MCGOWAN, V. & SNEATH, P. H. A. (1980). Approved lists of bacterial names. *International Journal of Systematic Bacteriology* **30**, 225-420.