A simple chemical test to distinguish mycobacteria from other mycolic-acid-containing actinomycetes

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Two hundred and fifty-two representatives of the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Tsukamurella* were degraded by alkaline hydrolysis and their mycolic acids extracted as methyl esters following phase-transfer-catalysed esterification. When the mycolic acid methyl esters were treated with a mixture of acetonitrile and toluene all mycobacterial mycolates formed copious white precipitates whereas all but 5 out of the 106 non-mycobacterial mycolates remained in solution. The precipitated methyl mycolates and the dried soluble mycolates were compared by pyrolysis gas chromatography and silica gel thin-layer chromatography. On pyrolysis, the precipitated methyl mycolates from mycobacteria yielded fatty acid methyl esters with 20 to 26 carbon atoms whereas those from the remaining taxa produced shorter-chain esters. Mycobacteria and *Tsukamurella paurometabola* gave multispot mycolic acid patterns on thin-layer chromatography of their methyl esters whereas those from the remaining strains gave single spots. Our results indicate that *Rhodococcus chlorophenolicus* strains contain mycolic acids typical of mycobacteria. It can be concluded that the mycolic acid precipitation test provides a simple and reliable way of distinguishing mycobacteria from all other prokaryotes, notably from other mycolic-acid-containing taxa.

Introduction

Actinomycetes containing mycolic acids (long-chain 2-alkyl 3-hydroxy fatty acids) have many properties in common (Bowden & Goodfellow, 1990; Goodfellow, 1991) and form a distinct suprageneric taxon that encompasses the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Tsukamurella* (Collins *et al.*, 1988; Stackebrandt *et al.*, 1988). Members of these taxa are currently distinguished using a combination of chemical and morphological markers (Bowden & Goodfellow, 1990; Goodfellow, 1991) but improved diagnostic tests are needed, particularly for separating mycobacteria of clinical and veterinary significance from all other mycolic-acid-containing organisms.

Distinguishing mycobacteria from other mycolic-acid-containing taxa presently depends upon the application of chemotaxonomic techniques designed to separate different mycolic acid types in whole-organism methanolysates (Goodfellow, 1991). Mycolic acids vary considerably in structure, ranging from the relatively simple

mixtures of saturated and unsaturated acids found in corynebacteria to the very complicated mixtures characteristic of mycobacteria (Minnikin *et al.*, 1975, 1984*a, b*, 1985). Several simple chemical procedures are available to distinguish between the various types of mycolic acids but none of them unambiguously separates all mycobacteria from the remaining mycolic-acid-containing taxa.

Qualitative evaluation of mycolic acids can be achieved using TLC (Minnikin *et al.*, 1975): methanolysates of mycobacteria give multispot mycolate patterns whereas those of corynebacteria, gordonae, nocardiae and rhodococci give single spots whose mobilities reflect the chain length and structure of the constituent mycolic acids. The patterns of mycolic acid types can also be used for the identification of mycobacterial species (Minnikin *et al.*, 1984*a, b*, 1985). *Mycobacterium fallax* and *Mycobacterium triviale* strains, however, give single mycolic acid spots on TLC analysis (Dobson *et al.*, 1985) and can thereby be confused with gordonae and nocardiae. Moreover, the multispot mycolic acid pattern characteristic of most mycobacteria is also shown by some *Tsukamurella paurometabola* strains (Goodfellow *et al.*, 1978; Collins *et al.*, 1988); the mycolates from strains of this species separate into two components according to the presence or absence of a double bond at the 2-

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Abbreviation: MAME, mycolic acid methyl ester(s).

Table 1. Designation, source and history of test strains

Designation and laboratory number	Source and strain history
Genus <i>Corynebacterium</i>:	
<i>C. bovis</i> *C12	NCTC 3224; A. E. Evans, NIH Washington in 1930; milk
<i>C. cystitidis</i> *C95	NCTC 11863; ATCC 29593; R. Yanagawa, 42; K. Hirato, cow
<i>C. diphtheriae</i> C97†	PW8, A. B. F. Standfast, The Lister Institute of Preventive Medicine, Elstree, UK
<i>C. diphtheriae</i> var. <i>gravis</i> *C96	NCTC 11397; ATCC 27010; L. Barksdale, C7
<i>C. jeikeium</i> *C98 C99 C100, C101	NCTC 11913; D. Pitcher; R. E. Weaver, P. S. Riley; blood NCTC 11914; D. Pitcher; S. Eykyn; blood NCTC 11915, 11917; D. Pitcher; R. Harding, axilla and vagina, respectively.
<i>C. kutscheri</i> *C102	NCTC 11138; ATCC 15677; C. H. Pierce-Chase; lung abscess, mouse
<i>C. matruchotii</i> *C103	NCTC 10254; M. N. Gilmour, 47; oral calculus
<i>C. minutissimum</i> *C104	NCTC 10288; I. Sarkany; erythrasma
<i>C. mycetoides</i> *C63	NCTC 9864; A. Castellani
<i>C. pilosum</i> *C105	NCTC 11862; ATCC 29592; R. Yanagawa 46 Hara; K. Hirato; cow
<i>C. pseudodiphtheriticum</i> *C106	NCTC 11136; ATCC 10700; J. M. Coffey, 153.
<i>C. pseudotuberculosis</i> *C15	NCTC 3450; G. F. Petrie; J. B. Buxton; infected gland of sheep, South America
<i>C. renale</i> *C17	NCTC 7448; B. Weitz, cow
<i>C. striatum</i> *C107	NCTC 764; F. C. Minnett
<i>C. variabilis</i> *C357	NCDO 2097; W. H. J. Crombach (<i>Caseobacter polymorphus</i>), AC920; Meshanger cheese
Genus <i>Gordona</i>:	
<i>G. bronchialis</i> *N654 N1058	NCTC 10677; M. Tsukamura, 3410; H. Kondo; sputum, pulmonary lesion C. K. Campbell, London School of Hygiene and Tropical Medicine, UK, 5424, breast abscess
<i>G. rubropertincta</i> *N4	NCIB 9664; ATCC 14352; R. S. Breed; R. E. Gordon, 154; soil
<i>G. sputi</i> N931	M. Tsukamura, The National Chubu Hospital, Obu, Aichi, Japan, 6130; sputum
<i>G. terrae</i> *N659 N888	NCTC 10669; M. Tsukamura, 3612, soil M. Tsukamura, 3603; soil
Genus <i>Mycobacterium</i>:	
<i>M. abscessus</i> M100	NCTC 10882; C. A. Morris, KH 63330; cervical lymph node
<i>M. aichiensis</i> *M251	NCTC 10820; M. Tsukamura, 49005; soil
<i>M. aurum</i> *M12	NCTC 10437; M. Tsukamura, biotype 1, 358
<i>M. avium</i> M504†	H. B. Lee, Central Veterinary Laboratory, Weybridge, UK, <i>M. avium</i> D4

Table 1. (cont.)

Designation and laboratory number	Source and strain history
<i>M. borstelense</i>	
*M492	M. Tsukamura, 19009; ATCC 19235
M488–M491	M. Tsukamura, 19003, 19004, 19005, 19006; R. N. Bönicke, SN283, SN284, SN275, SN288
M547	U. Krallmann-Wenzel, Institute for Experimental Biology and Medicine, Borstel, Germany, SN287
<i>M. bovis</i>	
M460†	M. Magnusson, Statens Seruminstitut, Copenhagen, Denmark, MNC 27
<i>M. bovis</i> var. BCG	
M461	NCTC 5692; A. S. Griffith; A. Calmette
<i>M. chelonae</i>	
*M350	NCTC 946; F. M. Perry; tortoise tubercle
M438	P. A. Jenkins, PHLS <i>Mycobacterium</i> Reference Unit, University Hospital of Wales, Heath Park, Cardiff, UK, 3330; abscess on arm
M439	P. A. Jenkins, 3622; ulcer on foot
M440, M442	P. A. Jenkins, 7883, 13152; abscesses on hip
M441	P. A. Jenkins, 9920; pus – site unspecified
M444	P. A. Jenkins, 17714; M. Kubica
M445	P. A. Jenkins, 18014; abscess
M446	P. A. Jenkins, 19030; abscess on tibia
M447	P. A. Jenkins, 19410; lymph node
<i>M. chitae</i>	
*M101	NCTC 10485; M. Tsukamura, 2502; soil
<i>M. duvalii</i>	
*M165	NCTC 358
<i>M. fallax</i>	
*M432	V. Lévy-Frébault, Institut Pasteur, Paris, France, CIPT 1390003 (CIP 8139), clinical strain
M428–M431	V. Lévy-Frébault, 54, 80, 81, 206; River Seine
<i>M. farcinogenes</i>	
*M262	NCTC 10955; P. Perreau; G. Chamoiseau, IEMVT 75, bovine farcy, lymph node
M260, M261	M. P. Lechevalier, Rutgers University, New Brunswick, USA: Institut Pasteur (IP), Paris, 736, 740; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
M269, M270	E. H. Runyon, Tuberculosis Research Institute, Pretoria, South Africa, R422, R423; IP735, IP736; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
M271	E. H. Runyon, R415; R. E. Gordon, 1226; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
M272–M277	E. H. Runyon, R425, R414, R426, R427, R428, R429; IP738, IP739, IP740, IP742, IP743, IP744; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
M278	E. H. Runyon, R430; R. E. Gordon, 1224; IP745; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
M280, M281	E. H. Runyon, R147, R457; P. Perreau, MA75, MA938
M284, M285	E. H. Runyon, R416, 417; R. E. Gordon, 1244, 1243; I. E. Mostafa, Khartoum, Sudan
M462–M467	M. Abdulle, Serum and Vaccine Institute, P.O. Box 919, Mogadishu, MA5, MA9, MA30, MA39, MA191, MA192, Somalia; bovine farcy, skin lesions, Somalia
M468–M470	M. Abdulle, MA217, MA612, MA768, bovine farcy, lymph nodes, Somalia
M471	M. Abdulle, MA555, bovine farcy, mammary gland, Somalia
M472	M. Abdulle, MA687, bovine farcy, liver, Somalia
M473	M. Abdulle, MA785, bovine farcy, lung, Somalia
M474	M. E. Hamid, MH15, bovine farcy, skin biopsy, Sudan
M475–M477	M. E. Hamid, MH16, MH62, MH67, bovine farcy, lymph nodes, Sudan
M478–M485	S. M. El-Sanousi, Department of Microbiology, Faculty of Veterinary Science, PO Box 32, Khartoum North, Sudan; MS52 (KHF9), MS54 (KHF1), MS55 (KF8), MS57 (KF6), MS59 (KF16), MS60 (NF6), MS63 (NF10), MS64 (NF12) bovine farcy, lymph nodes, Sudan

Table 1. (cont.)

Designation and laboratory number	Source and strain history
N710†, M711	M. Ridell, Institute of Medical Microbiology, University of Gothenburg, Sweden, GB041, GB042; R. E. Gordon, 1226, 1242; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
N715†, N719, N720	M. Ridell, GB046, GB051, GB052; M. P. Lechevalier, 1224, 1225, 1244; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
N725†	M. Ridell, GA833; M. Magnusson, 749; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
<i>M. fortuitum</i>	
*M204	NCTC 10394; R. E. Gordon; J. C. Cruz 1; cold abscess
M205	NCTC 10395; R. E. Gordon; J. C. Cruz 2; cold abscess
M368	M. Ridell, GA023; Institute Superiore di Sanita, Rome, 456
M369†	M. Ridell, GB178; ATCC 6841
M390	NCTC 2291; A. Stanley Griffith, roe of halibut (<i>Hippoglossus vulgaris</i>)
M506–M512, M514, M516, M522, M528, M531, M534, M538, M539–M542	J. M. Grange, School of Pathology, Middlesex Hospital, London, UK, 6, 9, 11, 13, 14, 15, 17, 21, 29, 47, 58, 67, 79, 86, 93, 94, 98, 101
<i>M. gordonae</i>	
*M495	NCTC 10267; L. F. Bojalil; E. H. Runyon; gastric lavage
<i>M. kansasii</i>	
M496	NCTC 10268; L. F. Bojalil; E. H. Runyon
<i>M. marinum</i>	
*M181	NCTC 2275; J. D. Aronson; salt-water fish
<i>M. microti</i>	
*M497	NCTC 8710; A. Q. Wells, L V 285/303; vole
<i>M. neoaurum</i>	
*M246	NCTC 10818; M. Tsukamura, 3503; soil
<i>M. nonchromogenicum</i> †	
*M498	NCTC 10424; M. Tsukamura, 317; soil
<i>M. obuense</i>	
M499	NCTC 10778; M. Tsukamura, 4388; N. Kita; sputum
<i>M. parafortuitum</i>	
M403	M. Tsukamura, 16001
<i>M. peregrinum</i>	
*M6	NCTC 10264; L. F. Bojalil, 6020; bronchial aspirate of child
M418–M423, M427	P. A. Jenkins, 7678, 7680, 14654, 14657, 14894, 14895, 19774; sputum
M424	P. A. Jenkins, 16788; peritoneal fluid
M425, M426	P. A. Jenkins, 17564; 14462; urine
<i>M. phlei</i>	
M206	NCTC 10266; L. F. Bojalil; W. Steenken Jr
<i>M. rhodesiae</i>	
*M176	NCTC 10779; M. Tsukamura, 5295; L. King; sputum
<i>M. scrofulaceum</i>	
*M500	NCTC 10803; ATCC 19981; P. Hauduroy; F. H. Prissick; cervical lymph node
<i>M. senegalense</i>	
*M263	NCTC 10956; P. Perreau; G. Chamoiseau, IEMVT 378; bovine lymph node
M259	M. P. Lechevalier; M. Goodfellow, W34; NCTC 4524
M264, M265, M266	E. H. Runyon, R397, R408, R409, R410; R. E. Gordon, 1360, 1361, 1362, M2681364; IP369, IP397, IP433C, IP425C; bovine farcy, Dakar, Senegal
M279, M282, M283	E. H. Runyon, R148, R455, R456; P. Perreau, MA378, D1, D2
N714	M. Ridell, GB045; R. E. Gordon, R1363, bovine farcy, Dakar, Senegal
N717†, N718, N721	M. Ridell, GB048, GB049, GB053; M. P. Lechevalier, 1362, 1364, 931; bovine farcy, Dakar, Senegal
N723	M. Ridell, GA816; R. N. Bönicke, SN 5701
N726, N728	M. Ridell, GA881, GA883; M. Magnusson, 874, 876; bovine farcy, Dakar, Senegal
<i>M. smegmatis</i>	
*M216	J. M. Grange, 2; NCTC 8159; R. E. Gordon, Cornell 3
M102†	NCTC 8159; R. E. Gordon, Cornell 3

Table 1. (cont.)

Designation and laboratory number	Source and strain history
M211	NCTC 10265; L. F. Bojalil; S. A. Waksman
M217	J. M. Grange, 77; NCTC 10265; L. F. Bojalil; S. A. Waksman
M218	J. M. Grange, 138; milk
* <i>M. triviale</i>	
M501	ATCC 23292
<i>M. tuberculosis</i>	
M502	NCTC 7416; ATCC 9360, strain H37R _v
M503‡	H. B. Lee, Central Veterinary Laboratory, Weybridge, UK, <i>M. tuberculosis</i> C
<i>M. vaccae</i>	
*M302	NCTC 10916; ATCC 15483; R. N. Bönicke, SN 920
Genus <i>Nocardia</i>	
<i>Nocardia amarae</i>	
*N667	M. P. Lechevalier, Se 6; foaming activated sludge, Andover (Miami), Florida, USA
N787	M. Lechevalier, Se 291; Somerset-Raritan, NJ, USA
<i>N. asteroides</i>	
*N317	ATCC 19247; R. E. Gordon, 727; L. Ajello, M170-6; W. Bowman
N13	NCTC 8595; strain R. H. Gorrill, Salonika, Greece, pus of empyema
N97	R. E. Gordon, N659
N105	R. E. Gordon, 9969; ATCC 9969; C. W. Emmons, 9955; P. Hadley; generalized mycetoma
N201	CBS 51467; plasterwork
N216	J. E. Thiemann, Lepetit, Milan, Italy, S547; PSA 161; IMRU 652; E. N. Azarowicz, 110; A. Gonzáles-Ochoa, 1139; A. L. Carrión
N688–N692, N695	K. P. Schaal, Hygiene Institute, Cologne, Germany, N2, N3, N5, N9, N23, N67
<i>N. brasiliensis</i>	
*N318	ATCC 19296; R. E. Gordon, IMRU 845; J. Schneidau, Jr 381; A. Batista, 631; Institut Pasteur, Paris, 337
<i>N. brevicatena</i>	
*N641	J. Lacey, A 444; M. P. Lechevalier, 1086W
<i>N. carnea</i>	
*N675	R. E. Gordon, 3419; ATCC 6847; NCTC 3527 (<i>Actinomyces carneus</i>); V. Puntoni, 30
<i>N. farcinica</i>	
*N699	ATCC 3318; S. A. Waksman, strain 3318 (Nocard's original strain)
N258†	M. P. Lechevalier, ATCC 3318; S. A. Waksman's 3318
N668	S. G. Bradley, Virginia Commonwealth University, Richmond, VA; Georgia, USA, VAC 462
N669	S. G. Bradley, VAC 300
N671†	S. G. Bradley, VAC 330, ATCC 3318; R. E. Gordon; S. A. Waksman 3318
N687	K. P. Schaal, NI; strain Berlin
N701	M. Ridell, N67; M. Magnusson, 753; ATCC 3399
N704	M. Ridell, N118; M. Magnusson, 45; ATCC 6864; NCTC 1935; rabbit in Sumatra
N705	M. Riddell, N119; M. Magnusson, 844; nocardiosis, Sweden
N707, N708	M. Ridell, N125, N126; M. Magnusson, 878, 952
N900, N901	J. L. Stanford, School of Pathology, Middlesex Hospital, London, UK, N36, N37; generalized nocardiosis
N903	K. P. Schaal, N7
N909†	K. P. Schaal, N66; ATCC 3318
N911	K. P. Schaal, N199; R. E. Gordon, 3419
N912	K. P. Schaal, N228; V. A. Orchard, 255; soil
N963	R. Testa, American Cyanamid Co., Lederle Laboratories, Pearl River, NY, USA, KL1192
NS56	S. M. El-Sanousi, KF9/14, bovine lymph node, Sudan
NS67, NS68	S. M. El-Sanousi, O/80, O/47, cow milk, Sudan
NA488	M. Abdulle, mammary gland, bovine farcy, Somalia
<i>N. otitidis-caviarum</i>	
*N36	NCTC 1934; E. P. Snijders; infected ear of guinea-pig

Table 1. (cont.)

Designation and laboratory number	Source and strain history
<i>N. pinensis</i> N1117	L. L. Blackall, NM102; sewage treatment plant, Burleigh Park, Queensland, Australia
<i>N. seriolae</i> *N1116	JCM 3360; K. Hatai, NA8191; spleen of a yellow tail (<i>Seriola quinqueradiata</i>), Nagasaki, Japan
N1115	JCM 3359; K. Hatai, kidney of a yellow tail (<i>S. quinqueradiata</i>), Nagasaki, Japan
N1117	JCM 3361; K. Hatai; S-1; epidermis of a yellow tail (<i>S. quinqueradiata</i>), Kochi, Japan
N1118	JCM 5849; K. Hatai, NA8231; spleen of a yellow tail (<i>S. quinqueradiata</i>), Nagasaki, Japan
<i>N. transvalensis</i> *N680	R. E. Gordon, 3426; NCTC 2392
<i>N. vaccinii</i> *N682	R. E. Gordon; ATCC 11092; N. R. Smith, BG19; stem galls on blueberry
Genus <i>Rhodococcus</i> :	
<i>R. aichiensis</i> *N934, N938	M. Tsukamura, E9028, E7776; sputum
<i>R. chlorophenolicus</i> *N1053	J. H. A. Apajalahti, Department of General Microbiology, University of Helsinki, Finland, PCP 1; lake sediment
N1131	M. Häggblom, Institute of Environmental Medicine, New York University Medical Center, NY, USA, CP-2; contaminated soil
N1132	M. Häggblom, CG-1; sludge from a wastewater treatment plant
<i>R. coprophilus</i> *N774	T. J. Rowbotham, Department of Biological Sciences, University of Bradford, UK, CUB 687; lake mud, Hawksworth Mere, Guiseley, Leeds, UK
<i>R. equi</i> *C7	D. Jones, Department of Microbiology, University of Leicester, UK, NCTC 1621; R. S. Robinson; H. Magnusson; foal, lung abscess
R39	ATCC 25694
<i>R. erythropolis</i> *N11	NCIB 9158; M. Kocur, BS 277; P. H. H. Gray, O-5
N41	NCIB 8863; M. E. Brown; chalk soil
<i>R. fascians</i> *N1062	NCPPB 3067; ATCC 12974; W. H. Burkholder CF17; P. E. Tilford
<i>R. globerulus</i> *R58	ATCC 25714; R. E. Gordon, 544
R43	ATCC 25710; R. E. Gordon, 494
' <i>R. (Corynebacterium)</i> <i>hoagii</i> ' *C24	NCTC 10673; ATCC 7005; F. S. Orcutt
<i>R. luteus</i> *N1008	O. A. Nesterenko, Institute of Microbiology and Virology, Kiev, Ukraine, IMV 385 soil
<i>R. marinonascens</i> *N1056	NCIMB 2246; H. Weyland, 3438W; marine sediment
<i>R. maris</i> *N1009	O. A. Nesterenko, IMV 195; soil
<i>R. rhodnii</i> *N445	P. Hill, Department of Zoology, University of Edinburgh, UK, B/0; intestinal tract of <i>Rhodnius prolixus</i>
<i>R. rhodochromus</i> *N54	ATCC 13808; R. E. Gordon, 372; R. S. Breed KMRh; Kral collection; W. Migula; W. Zopf
N65	R. E. Gordon, A7698; H. W. Schoening
<i>R. ruber</i> *N361	M. Tsukamura, M-1; I. Uesaka; N. M. McClung

Table 1. (cont.)

Designation and laboratory number	Source and strain history
Genus <i>Tsukamurella</i> :	
<i>T. paurometabola</i>	
*N663	NCTC 10741; M. Tsukamura, 3462; A. Kruse, sputum
M333	R. E. Gordon, IMRU 1283; G. Altmann, 4479; human eye
M334	R. E. Gordon, IMRU 1520; C. McDurmont, R456
M338	R. E. Gordon, IMRU 586; A. J. Kluyver; N. L. Söhngen
M340	R. E. Gordon, IMRU 340; L. K. Georg, 44-45; iceballs, Hong Kong
M347	R. E. Gordon; E. L. Biberstein, 106-11B; pneumonic lung of snail
<i>T. wratislaviensis</i>	
*N802	R. E. Gordon, IMRU 669; N. M. McClung, 33; soil
N800	R. E. Gordon, IMRU 469; R. L. Starkey, 213; soil
N801	R. E. Gordon, IMRU 563; N. M. McClung, 2; soil
N803, N805	R. E. Gordon, IMRU 876, IMRU 878; D. M. Powelson, J-11, J-17
N806, N808	R. E. Gordon, IMRU 1385, IMRU 1396; J. J. Perry, R 22, 35
N809	R. E. Gordon, IMRU 1397; J. J. Perry, P101-W; soil

* Type strain. † Duplicated culture. ‡ Strains received as killed, freeze-dried biomass. Names in inverted commas are not in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980).

Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DSM, German Collection of Micro-organisms, D-3300 Braunschweig, Germany; JCM, Japan Collection of Micro-organisms, RIKEN, Saitama, Japan; NCDO, National Collection of Dairy Organisms, Shinfield, Reading, UK; NCIB and NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; NCTC, National Collection of Type Culture, Central Public Health Laboratories, London, UK.

position in the chain (Goodfellow *et al.*, 1978; Daffé *et al.*, 1988).

Mycobacterial mycolic acids are precipitated from ether solutions by ethanol whereas mycolates from corynebacteria, nocardiae and rhodococci remain in solution or, in some cases, give a sparse precipitate (Kanetsuna & Bartoli, 1972; Hecht & Causey, 1976; El-Sanousi & Tag El-Din, 1986); the behaviour of mycolates from gordonae and tsukamurellae has not been examined in such systems. The aim of the present study was to design an improved mycolic acid precipitation technique to distinguish mycobacteria from all other mycolic-acid-containing actinomycetes.

Methods

Maintenance of test strains. Most of the test strains (Table 1) were maintained on glucose yeast extract agar medium (GYEA; Gordon & Mihm, 1962) at room temperature and as 20% (v/v) glycerol suspensions at -20 °C (Wellington & Williams, 1978). The gordonae, rhodococci and tsukamurellae were maintained on GYEA medium supplemented with thiamin (50 mg l⁻¹), and the corynebacteria on nutrient agar containing Tween 80 (0.3%, v/v).

Cultivation of the test strains. Most of the *Mycobacterium* strains (Table 1) were grown on GYEA plates for 7-14 d at 37 °C, checked for

purity and then used to inoculate 1 litre flasks containing 250 ml modified Saunton's broth (Mordarska *et al.*, 1972). Inoculated flasks were shaken on an orbital shaker (Gallenkamp) at 150 r.p.m. for up to 28 d at 37 °C. After checking for purity, the organisms were killed with formalin (1%, v/v), harvested by centrifugation, washed twice with distilled water, freeze-dried and kept in universal bottles at room temperature. Some mycobacterial strains (Table 1) were obtained as killed freeze-dried biomass. The gordonae, rhodococci and tsukamurellae were grown in shake flasks of modified Sauton's broth at 30 °C for 2-5 d, and the corynebacteria in shake flasks of nutrient broth (Oxoid) supplemented with Tween 80 (0.3%, v/v) for 3-5 d as described by Collins *et al.* (1982).

Extraction of mycolic acids. Dried biomass from the 252 representatives of the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Tsukamurella* (Table 1) was degraded using alkaline methanolysis to obtain crude mycolic acid methyl esters (MAME; Minnikin, 1988). Biomass (50 mg) in an 8.5 ml Corning tube fitted with a polytetrafluoromethane-lined cap was heated with 2 ml 5% (w/v) aqueous tetrabutylammonium hydroxide at 100 °C overnight. After cooling, the mixture was centrifuged, the supernatant transferred to another Corning tube containing dichloromethane (2 ml) and iodomethane (25 µl), shaken for 30 min, centrifuged, and the upper layer discarded. The lower layer, which contained the MAME, was evaporated to dryness under nitrogen at 37 °C.

Precipitation of mycolic acids. The crude dried MAME were dissolved in dichloromethane (0.5 ml), transferred to weighed, 1.5 ml polypropylene microcentrifuge tubes (Treff, Dagersheim, Switzerland) then evaporated to dryness under nitrogen. The residues were shaken with

toluene (0.2 ml) and acetonitrile (0.1 ml) until completely dissolved, when a further 0.2 ml acetonitrile was added to each preparation. Turbidity upon addition of acetonitrile was indicative of the insolubility of the MAME. The contents of the tubes were mixed very gently, and the preparations kept at 4 °C for 1 h, then centrifuged at 2000 r.p.m. for 2–3 min and the supernatants transferred to another tube. Both precipitates and supernatants were dried under a gentle flow of nitrogen, weighed, and kept at 4 °C for further analysis. The procedure was repeated with organisms that gave sparse precipitates.

Thin-layer chromatography of mycolic acids. Analytical one-dimensional TLC was performed using pieces (10 × 10 cm) of aluminium-backed plates (Merck 5554 silica gel 60 F₂₅₄). Dried MAME were dissolved in 100 µl light petroleum (b.p. 60–80 °C); 2 µl solution was applied to the plates, which were then developed twice in light petroleum (b.p. 60–80 °C)/diethyl ether (85:15, v/v; Minnikin *et al.*, 1975). Separated components were revealed by spraying with 5% ethanolic molybdophosphoric acid followed by heating at 180 °C for 15 min.

Pyrolysis gas chromatography. Mycolates from selected strains (Table 2) were examined by pyrolysis gas chromatography using a modification of the procedure described by Lechevalier *et al.* (1971). The representative dried mycolates were dissolved in 100 µl light petroleum (b.p. 60–80 °C) and 5 µl aliquots injected into the system inlet of a Perkin-Elmer F11 instrument fitted with a column (3 m × 3.2 mm) packed with 3% OV-17, on Chromosorb WHP. The oven temperature was 270 °C, the injection port temperature was 300 °C and nitrogen was the carrier gas. Components of the chromatograms were identified by comparing their retention times with those of authentic standards.

Results

Precipitation of mycolic acid methyl esters

The 146 representatives of the 28 *Mycobacterium* species gave copious white precipitates of MAME [0.5–4 mg (50 mg dry biomass)⁻¹] when solutions in acetonitrile/toluene (1:2 v/v) were treated with further acetonitrile (Fig. 1; Table 2). Abundant white precipitates were also consistently recovered from the type strains of *Nocardia seriolae* (N1116) and *Rhodococcus chlorophenolicus* (N1053), and less dense ones from *Gordona sputi* N931, *Nocardia farcinica* N699 and the remaining strains of *Rhodococcus chlorophenolicus* (N1131, N1132). In contrast, The MAME of the 32 representatives of the genera *Corynebacterium* and *Tsukamurella* remained in solution, as did those of the remaining strains of *Gordona* and *Rhodococcus*.

Thin layer chromatography of mycolic acid methyl esters

All the test strains were found to contain MAME with characteristic mobilities on TLC of the crude methyl mycolate preparations; representative results are shown in Figure 2. Multispot mycolate patterns were obtained with all mycobacteria and for the *Tsukamurella paurometabola* strains. The latter gave two spots with *R_F* values similar to those of mycobacteria. In contrast, the

Fig. 1. Precipitation of mycolic acid methyl esters in acetonitrile (0.3 ml) and toluene (0.2 ml). (a) *Mycobacterium farcinogenes* *M262 (note white precipitate in tube) and (b) *Nocardia asteroides* *N317 (absence of precipitate). * Type strains.

Table 2. Dry weight of mycolic acid precipitates derived from representative mycobacteria and some related actinomycetes after addition of a mixture of acetonitrile and toluene (3:2, v/v) to the crude mycolates, together with details of the major fatty acid esters released on pyrolysis gas chromatography of the precipitated mycolates

Organisms	Wt of precipitate [mg (50 mg biomass) ⁻¹]	Major esters released on pyrolysis gas chromatography
<i>Mycobacterium avium</i>		
M504	4.0	C ₂₄
' <i>M. borstelense</i> '		
M491	1.5	C ₂₄
M487	1.5	C ₂₄
M488	2.0	C ₂₄
<i>M. bovis</i> BCG		
M461	3.5	C ₂₆
<i>M. chelonae</i>		
*M350	0.5	C ₂₄
M438	1.5	C ₂₄
M442	1.0	C ₂₄
M441	1.0	C ₂₄
<i>M. chitae</i>		
*M101	2.5	C ₂₄
<i>M. farcinogenes</i>		
*M262	4.0	C ₂₄
M270	3.0	C ₂₄
M272	4.0	C ₂₄
M277	2.5	C ₂₄
M284	3.5	C ₂₄
M466	2.0	C ₂₄
M468	2.5	C ₂₄
M473	3.0	C ₂₄

Table 2. (cont.)

Organisms	Wt of precipitate [mg (50 mg biomass) ⁻¹]	Major esters released on pyrolysis gas chromatography
M474	2.5	C ₂₄
M476	4.0	C ₂₄
M481	2.0	C ₂₄
M482	2.5	C ₂₄
N710	3.0	C ₂₄
N711	2.0	C ₂₄
N720	1.5	C ₂₄
<i>M. fortuitum</i>		
*M204	4.0	C ₂₄
M205	3.0	C ₂₄
M368	2.0	C ₂₄
M369	3.0	C ₂₄
M527	2.0	C ₂₄
<i>M. peregrinum</i>		
*M6	0.5	C ₂₄
M418	0.5	C ₂₄
M419	1.0	C ₂₄
M421	0.5	C ₂₄
M422	0.5	C ₂₄
<i>M. senegalense</i>		
*M263	2.5	C ₂₄
M264	1.5	C ₂₄
M268	2.0	C ₂₄
N718	2.5	C ₂₄
N721	2.5	C ₂₄
N723	2.0	C ₂₄
<i>M. smegmatis</i>		
*M216	3.0	C ₂₄
<i>M. tuberculosis</i>		
M503	3.5	C ₂₆
<i>Gordona sputi</i>		
N931	> 0.5	C ₁₆ -C ₁₈
<i>Nocardia farcinica</i>		
*N699	> 0.5	C ₁₂ -C ₁₈
<i>Nocardia seriolae</i>		
*N1116	2.5	C ₁₂ -C ₁₈
<i>Rhodococcus chlorophenolicus</i>		
*N1053	2.5	C ₂₄

* Type strain.

Tsukamurella wratislaviensis strains gave single mycolic acid spots with R_F values comparable to the single spots produced by *Gordona* (R_F 0.35) and *Nocardia* (R_F 0.35) strains. The corynebacteria and rhodococci also produced single spots, with slightly lower R_F values (0.26–0.29).

Pyrolysis gas chromatography

The major methyl ester obtained from methyl mycolates from most mycobacteria was C₂₄ (Table 2). However, *M.*

Fig. 2. One-dimensional TLC of methyl mycolates of *Mycobacterium tuberculosis* C (lane 1); *Tsukamurella paurometabola* M334 (lane 2); *Gordona bronchialis* N1058 (lane 3); *Nocardia asteroides* *N317 (lane 4); *Rhodococcus rhodochrous* *N54 (lane 5); *Corynebacterium diphtheriae* PW8 (lane 6). Developing solvent: light petroleum (b.p. 60–80 °C)/diethyl ether (85:15, v/v, $\times 2$). Detection agent, 5% ethanolic molybdophosphoric acid followed by charring at 180 °C. Abbreviations: M, MAME; A, α -mycolates; B, methoxymycolates; C, ketomycolates; F, non-hydroxylated fatty acid methyl esters (note the absence of this spot in lane 1 because fatty acid esters were not precipitated). * Type strains.

bovis and *M. tuberculosis* strains released C₂₆ as the predominant methyl ester. Methyl esters corresponding to C₂₀, C₂₂ and C₂₆ components were found in relatively small amounts in several of the mycobacterial strains. *Rhodococcus chlorophenolicus* N1053 gave a pattern of methyl esters characteristic of mycobacteria. In contrast, MAME from *G. sputi* N931, *N. farcinica* N699 and *N. seriolae* N1116, like other representatives of the genera *Gordona* and *Nocardia*, released esters in the range of C₁₂-C₁₈.

Discussion

The mycolic acid precipitation method described above is more reliable and sensitive than existing precipitation procedures designed to separate mycobacteria from the other mycolic-acid-containing taxa (Kanetsuna & Bartoli, 1972; Hecht & Causey, 1976; El-Sanousi & Tag El-Din, 1986). Hecht & Causey (1976), for example, noted that many corynebacteria, nocardiae and rhodococci gave weak precipitates of MAME. The present method also requires smaller amounts of biomass than the procedures described by Kanetsuna & Bartoli (1972) and El-Sanousi & Tag El-Din (1986). The basis of the method is the solubility of all MAME in acetonitrile/

toluene (1:2, v/v) and the insolubility of those from mycobacteria in acetonitrile/toluene (3:2, v/v). These solvent mixtures were found to be superior to the diethyl ether and methanol or ethanol mixtures used previously. There is an advantage in having two relatively high boiling solvents so that no evaporation takes place during the procedure.

The selective insolubility of mycobacterial mycolates in acetonitrile/toluene (3:2, v/v) can be attributed to structural differences between these mycolic acids and those from other mycolic-acid-containing actinomycetes. Mycobacterial mycolic acids have 60–90 carbon atoms with 1–3 double bonds; the corresponding figures for the remaining taxa are: corynebacteria, 22–38 carbons with 0–2 double bonds; gordonae, 48–66 carbons with 1–3 double bonds; nocardiae, 44–60 carbons with 0–3 double bonds; rhodococci, 34–52 carbons with 0–2 double bonds; and tsukamurellae, 64–78 carbons with 1–6 double bonds (Minnikin & Goodfellow, 1980; Goodfellow, 1991; Hartmans & De Bont, 1991). The lower ratio of double bonds to mycolic acid chain length in non-mycobacterial mycolates helps to explain the lower melting points and increased solubility of these components in polar solvents such as acetonitrile.

Mycobacterial mycolic acids also include a range of other functional groups such as cyclopropane rings, methyl branches, keto, methoxy, ester or epoxy groups but only *cis*-double bonds are found in the other taxa. The positioning of the double bonds and other functional groups within the molecule may be an important factor governing the physical properties of these acids. In mycobacteria, the groups are almost equally spaced down the main chain but in members of the other taxa the double bonds are concentrated towards the terminal methyl end of the chain. More data are needed to determine precisely the reasons for the different physical behaviour between mycobacterial mycolates and those from the other taxa but the observed difference provides a practical way of distinguishing the genus *Mycobacterium*.

Pyrolysis gas chromatography may be used to confirm the presence of mycobacterial methyl mycolates in the precipitates as these substances characteristically release C₂₀–C₂₆ fatty acid esters on pyrolysis (Minnikin & Goodfellow, 1980; Hartmans & De Bont, 1991). The pyrolysis products derived from non-mycobacterial methyl mycolates fall within the following ranges: C₈–C₁₈ for corynebacteria, C₁₆–C₁₈ for gordonae, C₂₀–C₂₂ for tsukamurellae, and C₁₂–C₁₈ for nocardiae and rhodococci. The range of pyrolytic products obtained from the mycobacterial and non-mycobacterial methyl mycolates extracted in the present study are in line with the results from previous reports (Lechevalier *et al.*, 1971; Goodfellow *et al.*, 1978; Collins *et al.*, 1982; Luquin

et al., 1991). This simple form of pyrolysis gas chromatography requires only a routine chromatograph with a temperature-controlled inlet system without a special pyrolyser.

It would appear from the precipitation and pyrolysis gas chromatography data that the type strain of *R. chlorophenolicus* contains mycolic acids typical of mycobacteria. This is unexpected since in the original description of this taxon the type strain was reported to have mycolic acids with 33–43 carbon atoms (Apajalahti *et al.*, 1986). Further comparative taxonomic studies are needed to determine the taxonomic status of *R. chlorophenolicus* (or whether cross-contamination has occurred). In contrast, our pyrolysis gas chromatographic data support the current classification of the other non-mycobacterial strains which gave a positive result in the precipitation test, namely *G. sputi* N931, *N. seriolae* N1116 and *N. farcinica* N699.

It is clear from the present investigation that the mycolic acid precipitation method provides a means of distinguishing mycobacteria from other mycolic acid-containing taxa complementing corresponding TLC techniques (Minnikin *et al.*, 1975, 1980, 1984*a, b*, 1985). It was notable that the *M. fallax* and *M. triviale* strains gave dense white precipitates of methyl mycolates; these organisms are unusual amongst mycobacteria in giving single mycolic acid spots on TLC analysis of whole-organism methanolysates (Dobson *et al.*, 1985) so that they may be identified as non-mycobacterial. Conversely, as confirmed in the present study, *T. paurometabola* strains are unusual in producing multispot mycolate patterns on TLC that can be confused with those formed by some mycobacteria (Goodfellow *et al.*, 1978; Collins *et al.*, 1988). However, the MAME of *Tsukamurella* remain in solution in the precipitation test. *Tsukamurella paurometabola* and *T. wratislaviensis* strains can be readily distinguished from each other as the methyl mycolates of the latter form a single spot in TLC analyses.

It can be concluded from the present investigation that the mycolic acid precipitation test provides a reliable and practical means of distinguishing mycobacteria from all other bacteria. The test should be particularly useful in distinguishing mycobacteria from members of other mycolic-acid-containing taxa when more sophisticated methods are unavailable. Improved diagnostic tests are, for instance, needed to distinguish *Nocardia farcinica* from *Mycobacterium farcinogenes* and *Mycobacterium senegalense* strains. The latter are the principal agents of bovine farcy, a chronic infectious disease of zebu cattle that occurs in some parts of the tropics. This disease was originally considered to be caused by *N. farcinica*, but mycolic acid analysis helped to show that the aetiological agent was a *Mycobacterium* (Asselineau *et al.*, 1969). In

the present study all 64 representatives of *M. farcinogenes* and *M. senegalense* gave dense white precipitates of methyl mycolates whereas the corresponding mycolates of 21 of the 22 *N. farcinica* strains remained in solution upon addition of acetonitrile and toluene. The exception, *N. farcinica* N699, gave a weak positive result but was clearly shown to be correctly classified as the precipitated mycolates produced fatty acid esters typical of nocardiae on pyrolysis gas chromatography.

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