Journal of General Microbiology (1986), **132**, 2693–2707. *Printed in Great Britain*

A Cooperative Taxonomic Study of Mycobacteria Isolated from Armadillos Infected with *Mycobacterium leprae*

By F. PORTAELS,[1]* C. ASSELINEAU,[2] I. BAESS,[3] M. DAFFÉ,[2] G. DOBSON,[4] P. DRAPER,[5] D. GREGORY,[5] R. M. HALL,[6]† T. IMAEDA,[7] P. A. JENKINS,[8] M. A. LANÉELLE,[2] L. LARSSON,[9] M. MAGNUSSON,[3] D. E. MINNIKIN,[4] S. R. PATTYN,[1] G. WIETEN[10] AND P. R. WHEELER[6]

[1]*Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2018 Antwerp, Belgium*

[2]*Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS, Université Paul Sabatier, F-31062 Toulouse, France*

[3]*Tuberculosis Department, Statens Seruminstitut, DK-2300 Copenhagen, Denmark*

[4]*School of Chemistry, The University, Newcastle upon Tyne NE1 7RU, UK*

[5]*National Institute for Medical Research, Mill Hill, London NW7 1AA, UK*

[6]*Department of Biochemistry, University of Hull, Hull HU6 7RX, UK*

[7]*University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA*

[8]*Public Health Laboratory, University Hospital of Wales, Cardiff CF4 4XW, UK*

[9]*Department of Medical Microbiology, University of Lund, Lund, Sweden*

[10]*FOM Institute for Atomic and Molecular Physics, 1098SJ Amsterdam, The Netherlands*

(*Received 27 February 1986; revised 11 June 1986*)

Seventeen strains of mycobacteria, recovered from six armadillos experimentally infected with *Mycobacterium leprae*, were examined in ten different laboratories. This collaborative study included use of conventional bacteriological tests, lipid analyses, determination of mycobactins and peptidoglycans, characterization by Py-MS, and immunological, metabolic, pathological and DNA studies. These armadillo-derived mycobacteria (ADM) formed five homogeneous groups (numbered ADM 1 to 5) on the basis of phenetic analyses. However, DNA studies revealed only four homogeneous groups since group ADM 1 and one of the two strains in group ADM 3 showed a high level of DNA relatedness. The phenetic and DNA studies confirmed that the ADM strains differed from all other known mycobacteria. Cultural, biochemical, metabolic and pathogenic properties as well as DNA–DNA hybridizations clearly differentiated these ADM from *M. leprae*.

INTRODUCTION

In 1982, mycobacteria phenotypically distinct from all the presently known species were isolated from the livers of two armadillos which had been experimentally infected with *Mycobacterium leprae* (Portaels *et al.*, 1982). Subsequently, other novel mycobacteria have been recovered from *M. leprae*-infected armadillos (Portaels *et al.*, 1985). These armadillo-derived mycobacteria (ADM) did not fit into the established classification of Jenkins *et al.* (1982) and were temporarily assigned to three new groups: ADM 1, ADM 2 and ADM 3 (Portaels *et al.*,

† Present address: Glaxo Group Research Ltd, Greenford UB6 0HE, UK.

Abbreviations: ADM, armadillo-derived mycobacteria; CRIS testing, comparative reciprocal intradermal sensitin testing; DAP, diaminopimelic acid; MAIS complex, *M. avium–intracellulare–scrofulaceum* complex.

1985). A collaborative study using conventional tests, analysis of lipids, mycobactins and peptidoglycans, characterization by Py-MS, immunology, metabolism, pathogenicity, and DNA–DNA hybridization was undertaken for two main purposes: (1) to clarify the taxonomic status of these ADM and establish their degree of relationship with other mycobacterial species (*M. leprae* included); and (2) to find specific markers in these ADM that might eventually permit their differentiation from *M. leprae*. The results of this study are presented here.

METHODS

Bacterial strains. Strains were isolated according to Portaels *et al.* (1982, 1985) and streaked onto Dubos Oleic Agar for purity. Seventeen strains were obtained, and included in the present study (Table 1). Not all the strains were analysed by all the laboratories; in certain cases, representative strains of the ADM groups were chosen.

Characterization of the ADM. The 17 strains were identified as described by Jenkins *et al.* (1982). The pH range for growth was determined after Portaels & Pattyn (1982) and Portaels *et al.* (1982). The pyridine extractability of acid fastness was tested by the method of Convit & Pinardi (1972). Growth on media containing glucose as sole carbon source and nicotinamide as sole nitrogen source was carried out as described by Tsukamura & Tsukamura (1966).

Lipid analyses. The lipids of all but three strains (i.e. 8837, 8968 and 9091) plus reference cultures were examined by TLC according to Jenkins (1980). This technique did not need any transformation of the native lipids. By using specific reagents (and reference lipid samples), unusual types of lipids were characterized on thin-layer chromatograms of the complex lipids extracted from six strains (8251, 8480, 8507, 8561, 8637 and 9091) using the methods of Portaels *et al.* (1984).

TLC analysis of methyl mycolates required their isolation from complex derivatives (trehalose esters, derivatives of arabinogalactan or of glycerol, or of long-chain secondary alcohols) which occurred in the bacteria. Different methods were used, i.e. acid or alkaline methanolysis (Minnikin & Goodfellow, 1980; Minnikin *et al.*, 1984*a*) or alkaline hydrolysis followed by methylation (Daffé *et al.*, 1983) of the whole cells. These methods gave similar results when applied to the same strain; therefore results concerning mycolate composition are presented as a whole.

The long-chain secondary alcohols (2-octadecanol, 2-eicosanol), which are always present along with the dicarboxymycolates, were characterized either by TLC (during the analysis of methyl mycolates), or by GC as described by Larsson *et al.* (1985).

Lipid profiles were also obtained by Py-GC, as described by Wieten *et al.* (1984). Pyrolysis yields various lipid compounds, including octadecene isomers and eicosene isomers from the esters of long-chain alcohols with dicarboxymycolic acids (wax esters). Py-GC was performed on nine strains (8480, 8483, 8507, 8560, 8563, 8608, 8634, 8637 and 8668) and a comparison was made with Py-GC profiles of other mycobacteria.

Isolation and characterization of the mycobactins. Seven strains (8480, 8634, 8637, 8251, 8563, 8837 and 8968) were examined for possible mycobactin formation. They were cultivated in liquid glycerol/asparagine medium (Ratledge & Hall, 1971) and on glycerol/asparagine medium solidified with 2% (w/v) Lab M agar (London Analytical and Bacteriological Media) (Hall & Ratledge, 1982).

Where mycobactin was formed (as demonstrated by apple-green fluorescence of cells under UV light), bacteria were scraped from solidified medium or harvested by centrifugation (10000 ***g*** for 10 min) from liquid medium. Cell-bound mycobactin was extracted in the usual way using ethanol, with final yields determined as described by Hall & Ratledge (1982).

TLC analysis of mycobactin was done in a single dimension, with a range of adsorbents and solvents (Hall & Ratledge, 1984): system I, silica gel G, 20 × 20 cm (Analtech), developed with petroleum spirit/ethyl acetate/n-butanol (2 : 3 : 3, by vol.); system II, kieselgel 60, 10 × 20 cm with a 2·5 × 10 cm concentrating zone (Merck/BDH), developed as for system I; system III, high performance thin-layer plates 10 × 10 cm with a 2·5 × 10 cm concentrating zone (Merck/BDH), developed as for system I; system IV, alumina GF, 20 × 20 cm (Analtech), developed with cyclohexane/n-butanol (9 : 1, v/v); system V, plates as for system I but using propan-2-ol as solvent.

Peptidoglycan analyses. Wet, 'fixed' cells from four strains (8251, 8507, 8511 and 8480) were dried *in vacuo* over NaOH. Dried cells were extracted twice for 24 h with ethanol/diethyl ether (1 : 1, v/v), then dried. Cells (about 100 mg of each strain) were broken in 5 ml saline for 20 min, using a Dawe Soniprobe ultrasonic generator operating at 80 W. The suspensions were contained in a thin stainless-steel tube cooled in ice-water. Unbroken cells were collected by centrifugation at 3000 ***g*** for 10 min, then the walls were collected by centrifugation at 27000 ***g*** for 1 h. Walls were suspended in 5 ml 0·1 M-HEPES buffer, pH 7·5, containing 1 mM-Mg^{2+}, 0·2% (w/v) sodium azide, RNAase (100 μg ml^{-1}) and DNAase type I (about 50 μg ml^{-1}) and incubated at 37 °C for 24 h. Trypsin and chymotrypsin (each about 100 μg ml^{-1}) were then added and incubation was continued for a further 24 h. Walls were collected by centrifugation at 27000 ***g*** for 1 h, suspended in 1% (w/v) SDS at room temperature

Table 1. *Source of the 17 test strains isolated from armadillos*

Strain no.	Source*	Armadillo no.	Tissue
9091	AFIP	5	liver
8251, 8346, 8480, 8483, 8637	IP	AJ	liver
8507, 8511, 8560, 8561, 8563, 8608, 8698	NIMR	2457/10	liver
8634, 8668	CDC	44	liver
8968	MRI	A49	spleen
8837	MRI	A20	spleen

* AFIP, Armed Forces Institute of Pathology, Washington (Dr G. P. Walsh); IP, Institut Pasteur, Cayenne (Dr Y. Robin); NIMR, National Institute for Medical Research, London (Dr R. J. W. Rees); CDC, Center for Disease Control, Atlanta (Dr C. C. Shepard); MRI, Medical Research Institute, Melbourne (Drs E. E. Storrs & A. M. Dhople).

for 24 h, sequentially washed with water, 1 M-NaCl and water, and finally freeze-dried. Yields for the four strains were between 1 and 2% of the original bacterial suspension.

Walls were hydrolysed in sealed tubes with 4 M-HCl at 105 °C for 16 h (0·5 ml acid, 1–2 mg walls). The hydrolysates were filtered through Whatman no. 50 paper to remove lipids, then evaporated over NaOH *in vacuo* and submitted to automatic amino acid analysis.

Characterization by Py-MS. Curie-point pyrolysis coupled on-line to low-resolution mass spectrometry was used to analyse intact cells from nine strains (8480, 8483, 8560, 8507, 8563, 8608, 8634, 8637 and 8668). Py-MS instrumentation and analysis conditions were as described by Wieten *et al.* (1983). The taxonomic and chemical relationships among the strains were deduced from the Py-MS data by discriminant analysis (Wieten *et al.*, 1983) using the Arthur data handling package, as modified by Hoogerbrugge *et al.* (1983).

Comparative reciprocal intradermal sensitin (CRIS) testing. The ADM strains (8251, 8346, 8563 and 8608) and reference strains tested are listed in Table 5. The experimental procedure was as described previously (Magnusson, 1961, 1971; Magnusson & Mariat, 1968). The data presented here were drawn from a series of 12 studies, comprising a total of 92 guinea-pigs. Male albino guinea-pigs (strain Ssc : A1), each weighing 300–350 g, were obtained from the breeding farm 'Hvidesten', Statens Seruminstitut, Copenhagen.

The immunogens of the ADM strains consisted of heat-killed culture filtrates, emulsified in incomplete Freund's adjuvant. The other strains were injected as suspensions of dried, heat-killed culture (0·4 mg ml^{-1}) in light paraffin oil (Marcol 52). The sensitins used for eliciting skin reactions in the guinea-pigs were heat-sterilized culture filtrates diluted 1 : 5 and 1 : 50 (MNC 461, MNC 579, ADM strains), or 1 : 50 and 1 : 500 (MNC 545), or purified preparations produced by similar methods as used for tuberculin PPD (Magnusson, 1961). The doses of the latter preparations were 0·2 μg and 2 μg. The diluent for the sensitins was PBS (10 mM-phosphate buffer, 0·85% saline, pH 7·2) with 0·01% (w/v) Chinosol and 0·005% (w/v) Tween 80. The results of the skin tests were evaluated by calculating the specificity differences of pairs of sensitins (Magnusson, 1961). The differences are indicated in mm. Each value is the mean difference between eight homologous and eight heterologous reactions.

Metabolic studies. For these studies, ADM strain 8480 was grown in modified Dubos medium. ADM strains 8634, 8251 and 8968 were grown in Middlebrook 7H9 medium except for the experiments involving unbroken ADM, when all were grown in Dubos medium. In all cases, growth was in 25 ml medium contained in 100 ml flasks, which were incubated without shaking for 28 d at 37 °C. Cell-free extracts were prepared by sonicating, in short bursts, for a total of 11 min (Wheeler & Gregory, 1980).

Malate dehydrogenase (EC 1.1.1.37; L-malate : NAD^+ oxidoreductase) was detected spectrophotometrically and on polyacrylamide gels as described by Wheeler & Bharadwaj (1983). Catalase (EC 1.11.1.6; hydrogen peroxide : hydrogen peroxide oxidoreductase) and superoxide dismutase (EC 1.15.1.1; superoxide : superoxide oxidoreductase) were detected by the methods described by Wheeler & Gregory (1980).

Experimental pathogenicity for mice. Seven strains (8251, 8480, 8561, 8634, 8637, 8837 and 9091) were titrated in mouse foot pads as described by Shepard (1960) with 5×10^3, 5×10^2, 50 and 5 acid-fast bacilli. The mice were examined at 6, 9 and 12 months if the counts did not reach 5×10^5 acid-fast bacilli per mouse foot pad. Mice were also intravenously inoculated with 0·25 ml saline containing 10^7 bacteria. The mice were killed after 2 and 6 months and the livers, spleens, lungs and hearts were examined histologically.

DNA–DNA hybridization. DNA was isolated and purified from seven strains (8251, 8346, 8563, 8608, 8634, 8837 and 8968) as described by Baess (1974). The purified DNA (800 μg ml^{-1}) was sheared in a French pressure cell press at 14·06 MPa. A molecular mass of 681 kDa (±5%) for the double-stranded DNA was obtained. For six strains (8251, 8480, 8634, 8637, 8668 and 8698) the DNA was isolated and purified by T. Imaeda (Imaeda *et al.*, 1982).

In the laboratory of one of us (I. Baess), DNA–DNA hybridization was measured optically in a spectrophotometer at a temperature of $T_m - 25$ °C (T_m: temperature at the midpoint of the thermal denaturation

curve of the DNA mixture). The concentration of DNA was 40 μg ml^{-1} in 3 × SSC (1 × SSC is 0·15 M-sodium chloride, 0·015 M-trisodium citrate, pH 7) and 25% formamide. The reassociation of two single bacterial DNAs was compared with the hybridization of the mixture of these, and percentage homology derived from the formula of Baess & Weis Bentzon (1978).

In the other participating laboratory (that of T. Imaeda), the methods used for DNA–DNA hybridization and for determination of $c_0t_{1/2}$ values were described previously (Athwal *et al.*, 1984; Imaeda, 1985).

RESULTS

Cultural, physiological and biochemical properties of ADM

The 17 ADM strains studied were all Gram-positive, strongly acid-alcohol fast and grew optimally at 37 °C. They may be regarded as slow-growing scotochromogens which can be divided into five groups on the basis of their mycolic acid content and their cultural, physiological and biochemical properties (Table 2). Since previous studies suggested relationships between some ADM and *M. avium*, *M. scrofulaceum* or *M. gordonae*, Table 2 also contains data on these three slow-growing mycobacteria.

Five strains isolated from two different armadillos (AJ and 44) were classified in the first group (ADM 1), eight strains isolated from two different animals (AJ and 2457/10) in the second group (ADM 2) and two strains from two different animals (AJ and 5) in the third group (ADM 3). Single strains were classified in groups ADM 4 (8837) and ADM 5 (8968). The five ADM groups differed from *M. avium*, *M. scrofulaceum* and *M. gordonae* by at least two of the characters listed in Table 2. Thus, groups ADM 1 and ADM 4 resembled *M. scrofulaceum* but differed by the sensitivity to isoniazid (10 μg ml^{-1}) and to hydroxylamine hydrochloride (250 μg ml^{-1}). ADM 1 and ADM 4 strains also differed from ADM 2 and ADM 5 strains insofar as they did not hydrolyse Tween 80, used glucose as sole carbon source, and did not use nicotinamide as sole nitrogen source. Conversely, ADM 2 and ADM 5 strains hydrolysed Tween 80, did not use glucose as sole carbon source, used nicotinamide as sole nitrogen source and grew poorly on Löwenstein–Jensen medium in subculture, 10^6 organisms representing 1 c.f.u. Optimal pH ranges for growth also differentiated ADM 1 and ADM 4 strains from ADM 2 and ADM 5 strains, the latter having a very narrow optimal pH range (5·4–5·7 for ADM 2 strains and 5·5–5·7 for the ADM 5 strain 8968).

Many properties distinguished the two ADM 3 strains from the other ADM groups and the species listed in Table 2. These two strains were particularly difficult to grow in subculture; 10^6 organisms corresponded to 1 c.f.u. on both Ogawa and Löwenstein–Jensen media. They were inhibited by malachite green and only grew on Ogawa medium without malachite green. Addition of NaCl (1%, w/v) stimulated growth. The acid fastness of the two ADM 3 strains was extracted by treatment with pyridine. The narrow optimal pH range (5·8–6·1) also differentiated these two strains from the majority of mycobacterial species. A similar pH range for growth has been found only for *M. lepraemurium* (Portaels & Pattyn, 1982).

Lipid analysis

The strains assigned to group ADM 1 gave rise to a single pink spot at the solvent front following TLC according to Jenkins (1980). In the same conditions, the strains assigned to group ADM 2 produced a dark brown spot in the mid-zone of the chromatogram. The ADM 3 strain tested (8637) had a lipid pattern different from those of the other groups or species.

The investigation of glycolipids according to Portaels *et al.* (1984) showed the presence of mycoside C-like compounds in one strain of group ADM 1 (8480), in the two strains of group ADM 3 (8637, 9091), but not in three strains of group ADM 2 (8251, 8507, 8561). However, the latter three strains contained a specific, very polar acidic glycolipid. The glycolipid fractions of the two strains of group ADM 3 exhibited common components (mycosides C), but some more lipophilic glycolipids were observed only in strain 8637.

According to their mycolate patterns, the ADM strains could be separated into three groups (Table 2). All the strains studied, except the two strains of group ADM 3, appeared to contain α-mycolates, ketomycolates and dicarboxymycolates. Strains 8837 (ADM 4) and 8968 (ADM 5) contained only these three kinds of mycolates, identical to those of the MAIS (*M. avium*–

Table 2. *Characteristics of unclassified ADM strains*

	ADM groups							
	ADM 1 8346, 8480, 8483, 8634, 8668	ADM 2 8251, 8507, 8511, 8560, 8561, 8563, 8608, 8698	ADM 3 8637, 9091	ADM 4 8837	ADM 5 8968	*M. avium**	*M. scrofulaceum**	*M. gordonae**
Colony morphology†	SmS	SmS	SmK	SmT	SmT	SmS SmT	SmS SmK	SmS SmK
Growth on Löwenstein–Jensen medium	+	−‡	−‡	+	−‡	+	+	+
Growth on Ogawa medium	+	+	−‡	+	+	+	+	+
Growth on Ogawa medium without malachite green	+	+	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	+	+
Growth at 42 °C	−	−	−	−	−	+	+	−
Growth in presence of:								
Isoniazid (10 μg ml^{-1})	−	−	−	−	−	+	+	−
Thiophene-2-carboxylic acid hydrazide (1 μg ml^{-1})	+	+	+	+	+	+	+	+
Hydroxylamine hydrochloride (250 μg ml^{-1})	−	−	−	−	−	+	+	+
p-Nitrobenzoic acid (500 μg ml^{-1})	+	+	−	+	+	+	+	+
Sodium chloride (5%, w/v)	−	−	+	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	−	−
Presence of pigment	+	+	+	+	+	−	+	+
Catalase >45 mm	+	+	−	+	+	−	+	+
Acid phosphatase production	−	−	−	−	−	−	−	+
Tween 80 hydrolysis	−	+	−	−	+	−	−	+
Urease production	+	+	−	+	+	−	+	−
Putrescine oxidase production	−	−	−	−	−	−	−	−
Niacin accumulated	−	−	−	−	−	−	−	−
Utilization of glucose as sole C source	+	−	−	+	−	F	M	F
Utilization of nicotinamide as sole N source	−	+	−	−	−	M	M	M
Pyridine extractability of acid fastness	−	−	+	−	−	−	−	−
Optimal pH range for growth	5·4–6·5	5·4–5·7	5·8–6·1	5·2–6·3	5·5–5·7	5·4–6·5	5·4–6·5	5·8–7·4
Mycolate types:								
α-Mycolates	+	+	−	+	+	+	+	+
Methoxymycolates	+	+	−	−	−	−	−	+
Ketomycolates	+	+	+	+	+	+	+	+
Dicarboxymycolates	+	+	+	+	+	+	+	−

+, >85% of strains positive; M, 50–85% of strains positive; F, 15–49% of strains positive; −, <15% of strains positive.
* Data from Jenkins *et al.* (1982).
† For colony morphology descriptions, see Jenkins *et al.* (1982).
‡ No growth with $<10^6$ acid-fast bacilli.

intracellulare–scrofulaceum) complex. Strains assigned to groups ADM 1 and ADM 2 also contained methoxymycolates. The mycolate patterns of the two strains assigned to group ADM 3 were unusual in lacking α-mycolate components: only keto- and dicarboxymycolates were observed. However, very small amounts of α-mycolates could be detected by preparative TLC and mass spectrometry. The usual conditions of analytical TLC did not allow their detection.

All 17 strains contained long-chain secondary alcohols, identified as mixtures of 2-octadecanol and 2-eicosanol by GC of the lipid fractions in the case of three ADM 1 strains (8346, 8480, 8634), three ADM 2 strains (8251, 8507, 8561) and the two ADM 3 strains (8637, 9091). The presence of these long-chain secondary alcohols in the hydrolysates or methanolysates of mycobacteria containing dicarboxymycolates is a general feature, since in these bacteria these two kinds of compounds are linked by an ester bond.

The presence of dicarboxymycolic acids was also detected in all nine ADM strains (four ADM 1, four ADM 2 and one ADM 3) which were analysed by Py-GC. Highly specific features were observed in the lipid patterns of all ADM pyrograms, which could be identified by Py-GC-MS as triplets of isomeric alkenes (C_{18}—C_{20}). The distribution of these alkenes correlated with the distribution of these dicarboxymycolates, from which the alkenes can be readily formed by pyrolysis (Wieten *et al.*, 1984). In all nine strains analysed by Py-GC, tuberculostearic acid was observed.

GC analysis of the products obtained by saponification of the lipids revealed methyl esters of mycocerosic or phthioceranic acids in three strains (8251, 8507, 8561) of group ADM 2, but not in two strains (8480, 8634) of group ADM 1; other strains were not examined.

Isolation and characterization of the mycobactins

Growth of ADM 3 strain 8637 was poor throughout, especially on iron-deficient media, giving insufficient cell yield to allow any mycobactin to be isolated. The other ADM strains grew profusely on the different media tested. The mycobactins isolated from these strains grown on both liquid and solidified glycerol/asparagine medium were analysed by TLC. As found by Hall & Ratledge (1984), the mycobactins appeared to be identical irrespective of the growth conditions. Previous studies (Portaels *et al.*, 1982, 1985) had already suggested a close relationship of ADM strains 8480, 8634 and 8837 to strains belonging to the MAIS complex. Some relationships with *M. gordonae* or *M. flavescens* have also been found for ADM strains 8251, 8563 and 8968. Mycobactins isolated from these organisms were therefore compared with those isolated from the ADM strains (Table 3).

ADM 2 strains 8251 and 8563 gave the same typical multi-spot pattern with a four-spot pattern on systems I, II and III. These results clearly differentiated the mycobactins produced by these strains from those of *M. gordonae* (Table 3). Previous examination of the rapidly-growing mycobacteria had shown the mycobactins of *M. flavescens* to be heterogeneous (Hall & Ratledge, 1984). However, none of the four strains which were examined previously showed much resemblance in their mycobactin patterns to the ADM 2 strains 8251 and 8563. Mycobactins isolated from the MAIS complex also had different R_F values from those of strains 8251 and 8563.

The ADM 1 strains 8480 and 8634 and ADM 4 strain 8837 produced a spot pattern similar to strains belonging to the MAIS complex. Strain 8968 (ADM 5) produced spot patterns different from all the other ADM strains and from the reference strains mentioned in Table 3.

Peptidoglycan analysis

The results of the amino acid analyses of walls from some ADM are shown in Table 4. In addition to the amino acids and hexosamines listed, traces of aspartic acid, threonine, serine and leucine were detected ($\leqslant 0{\cdot}1$ molar ratio to DAP).

Characterization by pyrolysis mass spectrometry (Py-MS)

By discriminant analysis of the total batch of ADM mass spectra, subsets of features (discriminant functions) were obtained which gave insight into the relationships among the

Table 3. *Grouping of ADM strains following TLC of their mycobactins, including an examination of other possibly related mycobacteria*

Strain*	ADM grouping	R_F values with selected TLC systems† III	V
8480	1	0·92	0·62
8634	1	0·92	0·62
8563	2	0·77, 0·72, 0·62, 0·55	0·60, 0·52
8251	2	0·77, 0·72, 0·61, 0·55	0·60, 0·54
8637	3	No growth	
8837	4	0·91	0·64
8968	5	0·62, 0·50	0·60
M. avium AM.1	–	0·91	0·62
M. intracellulare AM.12	–	0·91	0·63
M. scrofulaceum AM.22	–	0·92	0·63
M. gordonae NCTC 10996	–	0·86	0·60, 0·53, 0·40
M. flavescens M410	–	–	0·53
M. flavescens M411	–	–	0·49, 0·32
M. flavescens M413	–	–	0·52
M. flavescens M414	–	–	0·54

* Cultures prefixed M were kindly supplied by Dr M. Goodfellow, Department of Microbiology, The Medical School, University of Newcastle upon Tyne, UK; cultures prefixed AM were kindly supplied by Dr A. MacDiamid, Institute for Animal Diseases, Compton, Berkshire, UK.

† See Methods for details of TLC systems III and V. Where more than one R_F value is given, multiple spots were detected.

Table 4. *Amino acid and hexosamine composition of walls of ADM strains*

Composition is expressed as molar ratio relative to diaminopimelic acid (DAP). Figures in parentheses are the actual amounts of DAP found, in nmol mg^{-1}.

	8251 (ADM 2)	8507 (ADM 2)	8511 (ADM 2)	8480 (ADM 1)
Glucosamine	0·69	0·71	0·68	0·78
Ammonia	1·27	1·23	1·76	1·35
Muramic acid	0·61	0·62	0·59	0·63
Glutamic acid	1·13	1·12	2·02	1·10
Glycine	0·24	0·25	0·24	0·21
Alanine	1·47	1·59	1·58	1·64
DAP	1 (161)	1 (182)	1 (149)	1 (148)

strains. Of the four discriminant functions (D1, D2, D3 and D4) that were found to be statistically significant, D1 accounted for 87·3% of the total relevant variance (i.e. that proportion of the total variance in the data set that is meaningful in differentiating between strains). Relative to D1, strains from groups ADM 1 and ADM 3 formed a tight cluster and group ADM 2 strains another cluster, well separated from the former; strain 8507 was intermediate (Fig. 1). On the remaining discriminant functions (D2, D3, and D4), some heterogeneity was apparent among strains of group ADM 2. Among the subset of features that are represented by D1, sulphur-containing fragments were prominent, indicating an important role for sulphur components in differentiating the observed subgroups.

The Py-MS spectra from two strains of *M. gordonae* and one strain of *M. scrofulaceum* were added to the ADM data set to show the relationships between these two species and ADM; for this purpose new discriminant functions were calculated. On each individual (new) discriminant function D1, D2 and D3, a relationship between ADM, *M. gordonae* and *M. scrofulaceum* was observed, whereas on D4, ADM were separated clearly from the latter two species. Notably,

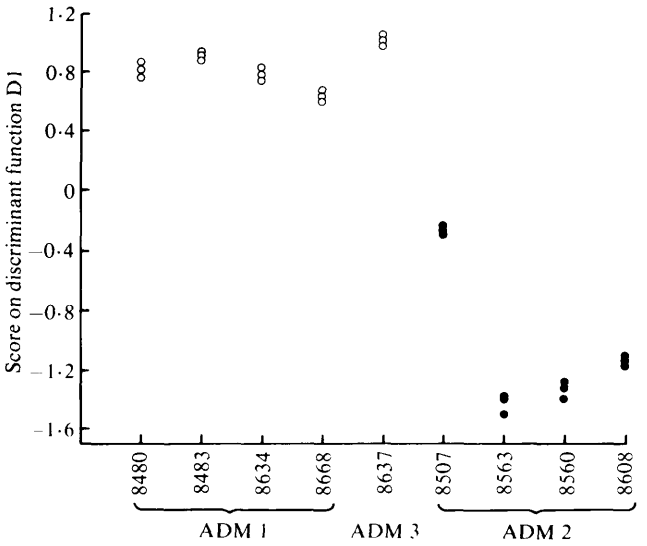

Fig. 1. Characterization of ADM by discriminant analysis of Py-MS data.

combination of the scores on individual discriminant functions in two-dimensional discriminant plots, or non-linear mapping of the scores on all four discriminant functions, indicated that *M. gordonae*, *M. scrofulaceum* and ADM are well separated.

Comparative reciprocal intradermal sensitin (CRIS) testing

The specificity differences of the pairs of sensitins of the strains are shown in Table 5. Specificity differences of <2 mm were found in the comparisons within three ADM 2 strains (8251, 8563, 8608) and specificity differences of >2 mm in most of the others. Generally, the smaller the differences, the more closely related are the immunogenic structures under study. By means of the criteria for the utilization of the values of specificity differences of sensitins in classification reported previously (Magnusson & Mariat, 1968), the three ADM 2 strains 8251, 8563 and 8608 belong to one and the same (unknown) species, and ADM 1 strain 8346 belongs to another unknown species. The ADM strains examined are distinct from *M. avium*, *M. flavescens*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum* and *M. szulgai*, and from the three other unknown species studied. The specificity difference of 2 mm found in the comparison between ADM 8563 and *Mycobacterium* sp. MNC 545 is remarkably small, indicating a close relationship between these two strains.

Metabolic studies

Malate dehydrogenase. NAD-dependent malate dehydrogenase activity was sought in extracts of two ADM strains. In strains 8634 and 8251, activities of 259 mU (mg protein)$^{-1}$ and 370 mU (mg protein)$^{-1}$, respectively, were detected. Corresponding R_F values in polyacrylamide gels were 0·73 and 0·71. The R_F of *M. leprae* malate dehydrogenase in the same gels was 0·63 (see also Wheeler & Bharadwaj, 1983). ADM and *M. leprae* malate dehydrogenases could be distinguished when 10 μg and 25 μg protein extracts, respectively, were run in the same gel.

Catabolism of oxygen free radicals. Superoxide dismutase was detected in all four ADM strains tested (8634, 8480, 8251 and 8968). All these strains had an isoenzyme of R_F 0·67–0·69 in a polyacrylamide gel system in which the R_F of *M. leprae* superoxide dismutase was 0·68 (Wheeler & Gregory, 1980; Wheeler, 1984*a*). Additionally an isoenzyme of R_F 0·35–0·39 (not present in *M. leprae*) was detected in three (i.e. not 8480) of the four ADM strains studied.

Catalase was sought and detected in two ADM strains (Table 6). This enzyme could not be

Table 5. *Specificity differences of sensitins from ADM, from reference strains of M. avium, M. flavescens, M. gordonae, M. intracellulare, M. scrofulaceum and M. szulgai and from three strains belonging to unknown Mycobacterium species*

The differences (mm) are between homologous and heterologous reactions to intradermal tests in guinea-pigs immunized with the strains or with culture filtrates used for the preparation of the sensitins. Italicized figures have been published previously (Magnusson *et al.*, 1961; Magnusson, 1962). Figures followed by + are less precise than other figures, because of weak homologous reactions. Blank spaces indicate that the corresponding pair of strains was not compared. The MNC strain numbers are those used by the laboratory where the work was done.

MNC 1404 *M.* sp. ADM 8251								
−2	MNC 1406 *M.* sp. ADM 8563							
0	1	MNC 1407 *M.* sp. ADM 8608						
11	14	17	MNC 1405 *M.* sp. ADM 8346					
9	10	14	9	MNC 95 *M. scrofulaceum* 526 (A. M. Masson)				
16+	20	24	21	19	MNC 521 *M. flavescens* Bojalil D-25 (ATCC 14475, type strain) (L. Bojalil)			
5+	14	16	15	7	14	MNC 10 *M. avium* Maren Cecilie (SSC314) (H. C. Engbaek)		
3+	10	13	12	*8*	16	*6*	MNC 23 *M. intracellulare* SSC209 (H. C. Engbaek)	
6+	12	16	15	*13*	17	*10*	7	MNC 64 *M. gordonae* E19911 (SSC215) (H. C. Engbaek)
12	9		16			6	10	7
9+	12+		11+	10		8	9	7
8+	9+		7+					3
6+	2+		12+	18	22	18	9	18

MNC 461 *M. szulgai* Stanford 886 (G. Kronvall)

MNC 129 *M.* sp. 2046 (G. Wijsmuller)

MNC 579 *M.* sp. 296 (D. Berman)

MNC 545 *M.* sp. Runyon P36 (H. C. Engbaek)

Table 6. *Catalase in extracts of ADM and M. leprae*

The methods, preincubation conditions and units specified in an earlier study of catalase in *M. leprae* (Wheeler & Gregory, 1980) were used.

	Enzyme asssay		In polyacrylamide gels		
Cell-free extract from	Specific activity [mU (mg protein)$^{-1}$]	Inhibition by 100 mM-3-amino-triazole	R_F	Amount of protein (mg) to detect activity on gel	Activity after NaOH (0·5 M, 30 min) treatment
ADM 8634	144	65	0·10, 0·23	10	retained
ADM 8251	48	55	0·09, 0·25	10	retained
M. leprae	1·35	100	0·27	1000	abolished

detected in *M. leprae* although a host-derived catalase in purified suspensions of *M. leprae* had a very similar R_F value to catalase from ADM (Table 6).

Experimental pathogenicity for mice

None of the seven ADM strains tested multiplied in the mouse foot pads. However, the bacteria remained alive *in situ*, retrocultures being positive one year after inoculation. After intravenous inoculation, ADM 2, ADM 3, ADM 4 and ADM 5 strains were not pathogenic for mice, while two months after the inoculation, ADM 1 strains produced granulomas in the liver with numerous extracellular acid-fast bacilli.

DNA relatedness

ADM strains were separated into four groups on the basis of their DNA relatedness (Table 7). Strains 8346, 8480, 8634, 8668 (ADM 1) and 8637 (ADM 3) showed high levels of relatedness (>90%). The second group consisted of strains 8251, 8563, 8608 and 8698 (ADM 2), which showed 81–97% relatedness to each other, but 24–37% relatedness with the strains of the first group. The third group comprised strain 8837 (ADM 4), which showed about 25% relatedness with the first and second groups. The fourth group comprised strain 8968 (ADM 5), which showed 34% homology with ADM 2 strain 8251, and 23% with ADM 4 strain 8837. All ADM strains showed low levels of relatedness (6–51%) with selected strains of established species of mycobacteria, although 8837 and 8968 were only hybridized with two mycobacterial DNAs in the present study (*M. scrofulaceum* P29 and *M. gordonae* ATCC 14470).

DISCUSSION

The 17 ADM strains formed five homogeneous groups on the basis of cultural, physiological and biochemical properties. Although these strains shared properties with other scotochromogenic mycobacteria, they differed from all other mycobacteria on the basis of the characters listed in Table 2.

The value of mycolic acid analysis in mycobacterial systematics has already been attested (Minnikin & Goodfellow, 1980; Daffé *et al.*, 1983; Minnikin *et al.*, 1984*b*, 1985*a*; Dobson *et al.*, 1985). The patterns reported here emphasize the significance of mycolic acid patterns. Indeed, the patterns of mycolic acid methyl esters recorded for ADM 1 and ADM 2 strains were unusual in showing the presence of α-, methoxy-, keto- and dicarboxymycolates; a similar pattern has only been recorded previously for the saprophytic species *M. komossense* (Minnikin *et al.*, 1985*a*). The two ADM 3 strains also showed a very unusual mycolic acid pattern, with the absence of α-mycolates and the presence of keto- and dicarboxymycolates. The majority of mycobacteria studied so far contain α-mycolates (Daffé *et al.*, 1983; Minnikin *et al.*, 1984*b*; 1985*a*, *b*) but *M. duvalii* strains have minor amounts of α-mycolates (Minnikin *et al.*, 1985*a*). The sizes of the keto- and dicarboxymycolates in ADM 3 strains corresponded to those found in other mycobacteria, confirming that they belong to the genus *Mycobacterium*. The ADM 1 and ADM 4 strains and the ADM 2 and ADM 5 strains differed in their mycolic acid composition

Table 7. *DNA–DNA relatedness between ADM and other mycobacterial species*

Mycobacterial strains†		Percentage homology‡											
		ADM 1			ADM 2			ADM 3	ADM 4	ADM 5			
		8346	8480	8634	8251	8563	8698	8637	8837	8968			
ADM 1	8346	H							25				
	8480		H		24*		31*	98*					
	8634	95	98*	H	25*		26*[8]	100*					
	8668		94*		36*		35*						
ADM 2	8251	37			H	97		20*		34			
	8563					H			23				
	8608				101								
	8698		31*		81*		H	28*					
ADM 4	8837								H	23			
M. avium TMC 701			29*		26*		10*						
M. scrofulaceum	P29, S41	51			38				22	23			
	TMC 1302		39*		35*								
	TMC 1323		29*		6*								
M. intracellulare	TMC1403		14*		17*								
	Mark Roberts S16					33							
M. gordonae	TMC 1324^T		40*		28*								
	ATCC 14470^T	28			27				−3	16			
M. kansasii TMC 1204^T			33*		32*		39*						
M. xenopi TMC 1482^T			35*		30*		26*						
M. tuberculosis TMC 102^T			33*		19*		21*						
M. haemophilum TMC 804^T			32*		10*		25*						
M. marinum TMC 1218^T			29*		28*		25*						
M. asiaticum TMC 803^T			19*		36*		35*						
M. leprae A42[a]			11*		6*		17*						

† TMC, Trudeau Mycobacterial Collection; ATCC, American Type Culture Collection; T, type strain; S, serotype. *a*, Strain supplied by E. E. Storrs through the National Institute of Allergy and Infectious Diseases, USA.

‡Results marked * are from T. Imaeda's laboratory; others are from I. Baess' laboratory. H, homologous.

(Table 2). Similarly, genomic differences were observed between ADM 1 and ADM 4 strains (25% DNA relatedness) and between ADM 2 and ADM 5 strains (34% DNA relatedness). This again emphasizes the importance of mycolic acids as chemotaxonomic characters.

Polars lipid profiles also confirmed that ADM 1, 2 and 3 strains formed three separate homogeneous groups with lipid patterns that do not coincide with any other mycobacteria known at present.

The mycobactins are useful chemotaxonomic markers for the identification and classification of the mycobacteria (Hall & Ratledge, 1985; Hall, 1985). By examination of these compounds, ADM 2 strains (8251 and 8563) were readily differentiated from those species previously thought to be closely related, namely *M. gordonae*, *M. flavescens* and mycobacteria belonging to the MAIS complex. By their mycobactin structures, ADM 1 strains (8480 and 8634) and ADM 4 strain 8837 are related to the MAIS complex and ADM 5 strain 8968 seems to differ from the other ADM strains and reference strains tested so far. A recent HPLC study of mycobactins (Hall & Ratledge, 1985) showed that ADM 1 and 4 strains were indistinguishable from the MAIS group, and that ADM 2 strains synthesize a unique mycobactin different from that of any other species examined.

CRIS testing showed the ADM 2 strains (8251, 8563 and 8608) to be distinct from the ADM 1 strain 8346 and from *M. gordonae*, *M. flavescens*, *M. szulgai* and the mycobacteria of the MAIS complex.

The results of the DNA studies corresponded to those of the phenetic data for all strains except the ADM 3 strain 8637. Although this organism differed from ADM 1 strains in its cultural, physiological and biochemical characters, it showed a high level of DNA relatedness (98–100%) with the ADM 1 strains 8480 and 8634 (Table 7). The results obtained by Py-MS

(Fig. 1), and the presence of mycosides C, also classified 8637 in group ADM 1. However, the difference in the mycolate patterns was mainly quantitative, insofar as the presence of a small amount of α-mycolates was demonstrated in strain 8637. Interestingly, 8637 was isolated from the same armadillo tissue as ADM 1 strains 8346, 8480 and 8483 (Table 1). Strain 8637, like 9091 (ADM 3), was difficult to grow *in vitro*, causing difficulties in obtaining sufficient bacterial cells for analyses. This could have had some influence on the results of the mycolate determination. It was observed that the fast-growing *M. duvalii* showed a mycolate pattern (different from those of ADM) poor in α-mycolate but modified by culture growth conditions. Considering the great discrepancies between the phenotypic and the genomic characteristics, the ADM 3 strains should be studied further. However, discrepancies between genomic and phenotypic characteristics are not unusual and species which differ phenotypically may show high DNA relatedness. This is the case for *M. tuberculosis* and *M. bovis* (Bradley, 1972, 1975; Baess, 1979; Imaeda, 1985), *M. avium* and *M. lepraemurium* (Imaeda *et al.*, 1982; Athwal *et al.*, 1984), *M. scrofulaceum* and *M. xenopi* (Imaeda, 1985), and '*M. lufu*' (Portaels, 1980*a*, *b*) and *M. asiaticum* (Imaeda & Tsukamura, 1985). Conversely, strains classified in groups ADM 1 and 4 or ADM 2 and 5, which were very similar phenotypically, showed little DNA homology. This was also observed with *M. avium*, *M. intracellulare* and *M. scrofulaceum*, which are combined in a single complex (MAIS) on the basis of phenotypic characters but which show low DNA relatedness among themselves (Baess, 1983; Imaeda & Tsukamura, 1985). Phenotypically, the ADM strains might be classified into five homogeneous groups, different from all other known mycobacteria. According to DNA studies, these ADM are classified in four homogeneous groups which differ from all other cultivable mycobacterial species mentioned in Table 7.

Both groups ADM 1 and ADM 2 are composed of strains isolated from different animals (Table 1) and should be regarded as representing two novel species of mycobacteria. Since ADM 4 and ADM 5 are each represented by only one strain, more strains of these groups should be analysed before it can be suggested that they might constitute new species of mycobacteria.

Many characters permit differentiation between the ADM strains and *M. leprae*. Firstly, the ADM are cultivable *in vitro* on conventional media, although some of them require special ingredients (e.g. Ogawa egg yolk medium without malachite green for the ADM 3 strains). Pyridine extraction of acid fastness is not a specific character for the identification of *M. leprae*; indeed, the two ADM 3 strains lost their acid fastness after treatment with fresh pyridine and it has been demonstrated that some other cultivable mycobacteria (*M. vaccae*, *M. phlei*) also conform to this test (Dutta *et al.*, 1983). Conversely, the mycolic acid composition clearly differentiates *M. leprae* from the other mycobacterial species and from the ADM. With the exception of some substrains of *M. bovis* BCG (Minnikin *et al.*, 1984*b*), *M. leprae* is the only species which produces only α- and ketomycolates (Minnikin *et al.*, 1985*b*). The analysis of glycolipids also differentiated the ADM from *M. leprae*. Moreover, in a recent study (Portaels *et al.*, 1984), it has been shown that none of the ADM contained the phenolic glycolipid specific to *M. leprae* (Hunter *et al.*, 1982; Minnikin *et al.*, 1985*c*). Peptidoglycan analyses also differentiated the ADM from *M. leprae*. In *M. leprae* glycine occurs in the peptidoglycan in about the same molar proportion as DAP, while the molar proportion of alanine is less than 1 (Draper, 1976, and unpublished observations). The peptidoglycan of ADM apparently resembles that of other mycobacteria in amino acid composition (Lederer, 1971). It should be noted that 'pure' mycobacterial peptidoglycan should contain about 1 μmol DAP mg^{-1}. The material described here contained only 15% of this amount, largely because, on account of the small quantities obtained, the walls had not been freed from arabinogalactan and mycolic acid. Strain 8511 (ADM 2) contained a molar excess of glutamic acid and ammonia, which would be consistent with the presence of wall-bound, partially amidated polyglutamic acid as described for some other mycobacterial strains (Vilkas & Markovits, 1972). Further analyses with material less contaminated with non-wall amino acids would be needed to confirm this. The ADM were also distinguished from *M. leprae* on the basis of some enzymes sought during the study of the intermediary metabolism of *M. leprae* (Wheeler, 1984*b*). The malate dehydrogenases from *M. leprae*, ADM, and armadillo liver have different mobilities in polyacrylamide gels, so any malate dehydrogenase with $R_F \neq 0{\cdot}63$ is likely to be from a source other than *M. leprae*. Catalase in

ADM was similar in electrophoretic mobility to catalase in armadillo liver, but, in contrast to the liver enzyme, activity could be detected during incubation of ADM catalase with 100 mM-3-aminotriazole. Catalase could not be detected in NaOH-treated *M. leprae* (Wheeler & Gregory, 1980) so its presence in suspensions of bacteria after treatment with NaOH (0·5 M, for 30 min, at 25 °C) would suggest ADM. Taxonomic studies could be developed by comparing immunological distances (Wayne & Diaz, 1979) of ADM with each other, and with other mycobacteria. The absence of multiplication of the ADM in mouse foot pads and the low levels of DNA relatedness with *M. leprae* (6–17%) also confirmed that they are different from *M. leprae*.

Resulting from this study, it is possible to differentiate the ADM studied from *M. leprae* by cultural, biochemical, metabolic and pathogenic properties. Specific chemical markers, absent in *M. leprae*, have been detected in ADM: there are long-chain alcohols associated with the dicarboxymycolates that may be detected by GC as 2-octadecanol or 2-eicosanol after hydrolysis or methanolysis, or as alkenes by Py-GC of their esters. It has recently been demonstrated that the detection of such markers may be used to check the mycobacterial purity of *M. leprae* preparations. In the *M. leprae* suspensions tested up to now and purified from armadillo tissues using the so-called Protocol 1/79 (World Health Organization, 1980), neither eicosene nor eicosanol was detected (Wieten *et al.*, 1984; Larsson *et al.*, 1985).

If the *M. leprae*-infected armadillo tissues are contaminated by mycobacteria which do not produce dicarboxymycolates and long-chain alcohols (as was recently demonstrated by Portaels *et al.*, 1985), other specific markers should be sought in order to detect all possible types of contamination. In view of this, the use of monoclonal antibodies against *M. leprae* and against cultivable mycobacteria was also recently proposed (Kolk *et al.*, 1985). Enzymic markers, especially dehydrogenases – which are easily visualized after separation on electrophoretic gels – may also be useful to detect contaminants in armadillo tissue. It may be expected that more of these enzymes will be partially characterized (like malate dehydrogenase) in armadillo-grown *M. leprae* as studies on metabolism progress.

Since it is now possible to distinguish *M. leprae* from other mycobacterial species by the features analysed in the present study, any organism cultivated from *M. leprae*-infected tissue should be submitted to similar studies in order to evaluate its degree of relatedness to *M. leprae* and before claiming that it is *M. leprae*.

This work was supported in part by the Damien Foundation, Brussels, and by the Leprosy Unit, World Health Organization, Geneva. We also acknowledge LEPRA (British Leprosy Relief Association) for providing funds for maintaining the colony of infected armadillos. R. M. Hall gratefully acknowledges the support of a grant from the Medical Research Council.

We wish to thank Mrs K. De Ridder, Miss K. Fissette and Miss S. Lathwell for excellent technical assistance. We also thank Mrs Y. Baeten, Miss T. Van Doosselaere and Mrs M. Van Nevele for preparation of the manuscript.

REFERENCES

ATHWAL, R. S., DEO, S. S. & IMAEDA, T. (1984). Deoxyribonucleic acid relatedness among *Mycobacterium leprae*, *Mycobacterium lepraemurium* and selected bacteria by dot blot and spectrophotometric deoxyribonucleic acid hybridization assays. *International Journal of Systematic Bacteriology* **34**, 371–375.

BAESS, I. (1974). Isolation and purification of deoxyribonucleic acid from mycobacteria. *Acta pathologica et microbiologica scandinavica* **B82**, 780–784.

BAESS, I. (1979). Deoxyribonucleic acid relatedness among species of slowly-growing mycobacteria. *Acta pathologica et microbiologica scandinavica* **B87**, 221–226.

BAESS, I. (1983). Brief report: deoxyribonucleic acid relationships between different serovars of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. *Acta pathologica, microbiologica et immunologica scandinavica* **B91**, 201–203.

BAESS, I. & WEIS BENTZON, M. (1978). Deoxyribonucleic acid hybridization between different species of mycobacteria. *Acta pathologica et microbiologica scandinavica* **B86**, 71–76.

BRADLEY, S. G. (1972). Reassociation of deoxyribonucleic acid from selected mycobacteria with that from *Mycobacterium bovis* and *Mycobacterium farcinica*. *American Review of Respiratory Diseases* **106**, 122–124.

BRADLEY, S. G. (1975). Significance of nucleic acid hybridization to systematics of actinomycetes. *Advances in Applied Microbiology* **19**, 59–70.

CONVIT, J. & PINARDI, M. E. (1972). A simple method for the differentiation of *Mycobacterium leprae* from

other mycobacteria through routine staining technics. *International Journal of Leprosy* **40**, 130–132.

DAFFÉ, M., LANÉELLE, M. A., ASSELINEAU, 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.

DOBSON, G., MINNIKIN, D. E., MINNIKIN, S. M., PARLETT, J. H., GOODFELLOW, M., RIDELL, M. & MAGNUSSON, M. (1985). Systematic analysis of complex mycobacterial lipids. In *Chemical Methods in Bacterial Systematics*, pp. 237–265. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.

DRAPER, P. (1976). Cell walls of *Mycobacterium leprae*. *International Journal of Leprosy* **44**, 95–98.

DUTTA, A. K., KATOCH, V. M. & SHARMA, V. D. (1983). Effect of pyridine extraction on the acid fastness of mycobacteria. *Leprosy in India* **55**, 299–304.

HALL, R. M. (1985). Mycobactins as taxonomic markers for the mycobacteria. *Actinomycetes* **19**, 92–106.

HALL, R. M. & RATLEDGE, C. (1982). A simple method for the production of mycobactin, the lipid-soluble siderophore, from mycobacteria. *FEMS Microbiology Letters* **15**, 133–136.

HALL, R. M. & RATLEDGE, C. (1984). Mycobactins as chemotaxonomic characters for some rapidly-growing mycobacteria. *Journal of General Microbiology* **130**, 1883–1892.

HALL, R. M. & RATLEDGE, C. (1985). Mycobactins in the classification and identification of armadillo-derived mycobacteria. *FEMS Microbiology Letters* **28**, 243–247.

HOOGERBRUGGE, R., WILLIG, S. J. & KISTEMAKER, P. G. (1983). Discriminant analysis by double stage principal component analysis. *Analytical Chemistry* **55**, 1711–1712.

HUNTER, S. W., FUJIWARA, T. & BRENNAN, P. J. (1982). Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. *Journal of Biological Chemistry* **257**, 15072–15078.

IMAEDA, T. (1985). Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, *Mycobacterium microti* and *Mycobacterium africanum*. *International Journal of Systematic Bacteriology* **35**, 147–150.

IMAEDA, T. & TSUKAMURA, M. (1985). Intraspecies and interspecies DNA relatedness among mycobacteria. *Proceedings of the Twentieth US–Japan Tuberculosis Research Conference, National Institutes of Health, Bethesda, Maryland 15–16th August*, 164–173.

IMAEDA, T., KIRCHHEIMER, W. F. & BARKSDALE, L. (1982). DNA isolated from *Mycobacterium leprae*: genome size, base ratio, and homology with other related bacteria as determined by optical DNA–DNA reassociation. *Journal of Bacteriology* **150**, 414–417.

JENKINS, P. A. (1980). Thin-layer chromatography of mycobacterial lipids as an aid to classification. In *Mycobakterien und mycobakterielle Krankheiten, Teil 1, Systematik der Mycobakterien*, pp. 305–318. Edited by G. Meissner & R. Pfaffenberg. Jena: Gustav Fischer.

JENKINS, P. A., PATTYN, S. R. & PORTAELS, F. (1982). Diagnostic bacteriology. In *The Biology of the Mycobacteria*, pp. 441–471. Edited by C. Ratledge & J. L. Stanford. London: Academic Press.

KOLK, A. H. J., HO, M. L., KLATSER, P. R., EGGELTE, T. A. & PORTAELS, F. (1985). Production of monoclonal antibodies against *Mycobacterium leprae* and armadillo derived mycobacteria. *Annales de l'Institut Pasteur/Microbiologie* **136B**, 217–224.

LARSSON, L., DRAPER, P. & PORTAELS, F. (1985). Use of gas chromatography to differentiate *Mycobacterium leprae* from cultivable armadillo derived mycobacteria, *M. avium–intracellulare* and *M. lepraemurium* by analysis of secondary alcohols. *International Journal of Leprosy* **53**, 451–457.

LEDERER, E. (1971). The mycobacterial cell wall. *Pure and Applied Chemistry* **25**, 135–165.

MAGNUSSON, M. (1961). Specificity of mycobacterial sensitins. I. Studies in guinea-pigs with purified 'tuberculin' prepared from mammalian and avian tubercle bacilli, *Mycobacterium balnei*, and other acid-fast bacilli. *American Review of Respiratory Diseases* **83**, 57–68.

MAGNUSSON, M. (1962). Specificity of sensitins. III. Further studies in guinea-pigs with sensitin of various species of *Mycobacterium* and *Nocardia*. *American Review of Respiratory Diseases* **86**, 395–404.

MAGNUSSON, M. (1971). A comparative study of *Mycobacterium gastri* and *Mycobacterium kansasii* by delayed type skin reactions in guinea pigs. *American Review of Respiratory Diseases* **104**, 377–384.

MAGNUSSON, M. & MARIAT, F. (1968). Delineation of *Nocardia farcinica* by delayed type skin reactions on guinea pigs. *Journal of General Microbiology* **51**, 151–158.

MAGNUSSON, M., ENGBAEK, H. C. & BENTZON, M. W. (1961). Specificity of mycobacterial sensitins. II. Studies in guinea-pigs with purified sensitin prepared from unclassified acid-fast bacilli. *American Review of Respiratory Diseases* **83**, 69–84.

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., MINNIKIN, S. M., O'DONNELL, A. G. & GOODFELLOW, M. (1984*a*). Extraction of mycobacterial mycolic acids and other long-chain compounds by an alkaline methanolysis procedure. *Journal of Microbiological Methods* **2**, 243–249.

MINNIKIN, D. E., MINNIKIN, S. M., PARLETT, J. H., GOODFELLOW, M. & MAGNUSSON, M. (1984*b*). Mycolic acid patterns of some species of *Mycobacterium*. *Archives of Microbiology* **139**, 225–231.

MINNIKIN, D. E., MINNIKIN, S. M., PARLETT, J. H. & GOODFELLOW, M. (1985*a*). Mycolic acid pattterns of some rapidly-growing species of *Mycobacterium*. *Zentralblatt für Bakteriologie, Microbiologie und Hygiene* **A259**, 446–460.

MINNIKIN, D. E., DOBSON, G., GOODFELLOW, M., DRAPER, P. & MAGNUSSON, M. (1985*b*). Quantitative comparison of the mycolic and fatty acid composition of *Mycobacterium leprae* and *Mycobacterium gordonae*. *Journal of General Microbiology* **131**, 2013–2021.

MINNIKIN, D. E., DOBSON, G. & DRAPER, P. (1985*c*). The free lipids of *Mycobacterium leprae* harvested

from experimentally infected nine-banded armadillos. *Journal of General Microbiology* **131**, 2007–2011.

PORTAELS, F. (1980*a*). Unclassified mycobacterial strains susceptible to dapsone isolated from the environment in Central Africa. *International Journal of Leprosy* **48**, 330.

PORTAELS, F. (1980*b*). Study of unclassified dapsone sensitive mycobacteria isolated from the environment in Zaïre. *Annales de la Société belge de médecine tropicale* **60**, 381–386.

PORTAELS, F. & PATTYN, S. R. (1982). Growth of mycobacteria in relation to the pH of the medium. *Annales de Microbiologie* **133B**, 213–221.

PORTAELS, F., FRANCKEN, A. & PATTYN, S. R. (1982). Bacteriological studies of armadillo livers infected with *Mycobacterium leprae*. *Annales de la Société belge de médecine tropicale* **62**, 233–245.

PORTAELS, F., DAFFÉ, M., LANÉELLE, M. A. & ASSELINEAU, C. (1984). Etude de la composition lipidique de mycobacteries cultivables isolées de foies de tatous infectés par *Mycobacterium leprae*. *Annales de Microbiologie* **135A**, 457–465.

PORTAELS, F., DE RIDDER, K. & PATTYN, S. R. (1985). Cultivable mycobacteria isolated from organs of armadillos uninoculated and inoculated with *Mycobacterium leprae*. *Annales de l'Institut Pasteur/Microbiologie* **136A**, 181–190.

RATLEDGE, C. & HALL, M. J. (1971). Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. *Jounal of Bacteriology* **108**, 312–319.

SHEPARD, C. C. (1960). The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. *Journal of Experimental Medicine* **112**, 445–454.

TSUKAMURA, M. & TSUKAMURA, J. (1966). Differentiation of mycobacteria by utilization of amides and amino acids as sole nitrogen sources. *American Review of Respiratory Diseases* **94**, 104–107.

VILKAS, E. & MARKOVITS, J. (1972). Sur la présence d'une acide polyglutamique dans les parois de *Mycobacterium tuberculosis*, souche Peurois. *Comptes rendus hebdomadaires de l'Académie des sciences* **275c**, 913–915.

WAYNE, L. G. & DIAZ, G. A. (1979). Reciprocal immunological distances of catalase derived from strains of *Mycobacterium avium*, *Mycobacterium tuberculosis* and closely related species. *International Journal of Systematic Bacteriology* **29**, 19–25.

WHEELER, P. R. (1984*a*). Variation of superoxide dismutase levels in extracts of *Mycobacterium leprae* from armadillo liver. *International Journal of Leprosy* **52**, 49–54.

WHEELER, P. R. (1984*b*). Metabolism in *Mycobacterium leprae*: its relation to other research on *M. leprae* and to aspects of metabolism in other mycobacteria and intracellular parasites. *International Journal of Leprosy* **52**, 208–230.

WHEELER, P. R. & BHARADWAJ, V. P. (1983). Enzymes of malate oxidation in *Mycobacterium leprae* grown in armadillo livers. *Journal of General Microbiology* **129**, 2321–2325.

WIETEN, G., HAVERKAMP, J., GROOTHUIS, D. G., BERWALD, L. G. & DAVID, H. L. (1983). Classification and identification of *Mycobacterium africanum* by pyrolysis mass spectrometry. *Journal of General Microbiology* **129**, 3679–3688.

WIETEN, G., BOON, J. J., GROOTHUIS, D. G., PORTAELS, F. & MINNIKIN, D. E. (1984). Rapid detection of mycobacterial contamination in batches of whole cells of purified *Mycobacterium leprae* by pyrolysis gas chromatography. *FEMS Microbiology Letters* **25**, 289–293.

WORLD HEALTH ORGANIZATION (1980). UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP). TDR/IMMLEP-SWG (5)/80.3, Annex 4, p. 23. Geneva: WHO.