Use of multilocus enzyme electrophoresis to examine genetic relationships amongst isolates of *Mycobacterium intracellulare* and related species

Mohammad M. Feizabadi,¹ Ian D. Robertson,¹ Debby V. Cousins,² David J. Dawson³ and David J. Hampson¹

Author for correspondence: David J. Hampson. Tel: +61 9 360 2287. Fax: +61 9 310 4144.
e-mail: hampson@numbat.murdoch.edu.au

¹ School of Veterinary Studies, Murdoch University, Murdoch, Western Australia 6150, Australia

² Australian Reference Laboratory for Bovine Tuberculosis, Agriculture Western Australia, South Perth, Western Australia 6151, Australia

³ State Health Laboratory, Brisbane, Queensland 4000, Australia

As part of a larger study investigating diversity and distribution of *Mycobacterium* spp. in Australia, multilocus enzyme electrophoresis was used to assess genetic relationships at 17 enzyme loci amongst a collection of reference strains and isolates initially identified on biochemical and other grounds as *M. intracellulare* (70), 'X' mycobacteria (10), *M. scrofulaceum* (7), *M. avium* (8) and *M. avium* subsp. *paratuberculosis* (2). Two of the isolates initially identified as *M. intracellulare* were shown to be quite distinct from the others. Both gave negative results in a species-specific DNA probe test, whilst one was positive by PCR. These results emphasize the uncertainties involved in identifying members of this group. The other *M. intracellulare* isolates formed a cohesive but diverse group, being divided into 48 electrophoretic types (ETs), with a mean genetic diversity of 0.38. Forty-three of these ETs contained only single isolates. There was no clear relationship between the serovar and ET designation. The index of association calculated for *M. intracellulare* was significantly different from zero, suggesting that it is a clonal species. PFGE was also applied to selected isolates from the ETs containing multiple isolates, and some of these could be differentiated further. The strains of *M. scrofulaceum* and 'X' mycobacteria were distinct from *M. intracellulare*, but themselves were highly heterogeneous, with mean genetic diversities of 0.66 and 0.65, respectively. Each of these groups may represent more than one species. *M. avium* strains were distinct from the two *M. avium* subsp. *paratuberculosis* strains, as well as from the other mycobacteria studied.

Keywords: *Mycobacterium intracellulare*, multilocus enzyme electrophoresis, genetic diversity, pulsed field gel electrophoresis

INTRODUCTION

The *Mycobacterium avium* complex (MAC) is a poorly defined group of slowly growing mycobacteria. It includes the species *M. avium* and *M. intracellulare* (Wolinsky, 1979), but other mycobacteria are genetically or biochemically closely related to members of this complex. For example, *M. avium* subsp. *paratuberculosis* was once thought to be a distinct species, but strains have been shown to share 96% total DNA

sequence homology and 99.9% 16S ribosomal DNA sequence similarity with certain serovars of *M. avium* (Yoshimura & Graham, 1988; Rogall *et al.*, 1990). *M. scrofulaceum* was previously considered to belong to the complex (Schaefer, 1965), but now it is considered to be distinct (Rogall *et al.*, 1990). Strains of the so-called 'X' mycobacteria react with the MAC probe (Accue Probe series) but fail to hybridize with probes specific for *M. avium* or *M. intracellulare* (Cregan *et al.*, 1992). Clearly more precise definitions of these organisms are required if progress is to be made in studying their epidemiology and disease associations.

M. intracellulare is an important pathogen of humans,

Abbreviations: ET, electrophoretic type; MAC, *Mycobacterium avium* complex; MLEE, multilocus enzyme electrophoresis.

causing chronic pulmonary infection, lymphadenitis and occasional disseminated infections (Wolinsky, 1992). It makes up 81–88% of MAC isolates cultured from patients without AIDS in the USA (Drake *et al.*, 1987; Guthertz *et al.*, 1989) and 18% of atypical mycobacterial infections in patients with AIDS (Raszka *et al.*, 1995). Different serovars of *M. intracellulare* have been isolated from granulomas in animals (Corner *et al.*, 1981; Morita *et al.*, 1994), from the environment, including soil and water (Falkinham *et al.*, 1980; Brookes *et al.*, 1984), and from people without predisposing conditions (Brookes *et al.*, 1984; Prince *et al.*, 1989). In parts of Japan, *M. intracellulare* is isolated from more patients than is *M. avium* (Saito *et al.*, 1989). Both species produce similar clinical symptoms and have similar drug susceptibilities (Good, 1985). Biochemically, strains of *M. intracellulare* are difficult to differentiate from *M. avium*, but several other methods have also been used, including the pathogenicity of *M. avium* isolates for chickens and *M. intracellulare* for rabbits, and growth of *M. avium* but not *M. intracellulare* at 45 °C (Thoen & Karlson, 1991). Serotyping has been used by several researchers to subtype MAC strains (Falkinham *et al.*, 1980; Dawson, 1990). The use of nucleic acid probes has also facilitated identification of the two species (Drake *et al.*, 1987; Saito *et al.*, 1990; Thoresen & Saxegaard, 1993). These probes generally identified serovars 1–6, 8–11 and 21 as *M. avium*, and serovars 7, 12–20 and 25 as *M. intracellulare*. The classification of serovars 22–24 and 26–28 is still not settled (Wolinsky, 1992), although some serovars, such as 27, have been identified as *M. scrofulaceum* (Wayne *et al.*, 1993).

Strains of *M. avium* have been subtyped by molecular techniques, including DNA restriction endonuclease analysis, multilocus enzyme electrophoresis (MLEE) and PFGE (Wards *et al.*, 1987; Zhang *et al.*, 1992; Arbeit *et al.*, 1993; Feizabadi *et al.*, 1996a). Little similar work has been conducted with *M. intracellulare* and the nature of the genetic relationships between members of the MAC, isolates of 'X' mycobacteria and *M. scrofulaceum* remains unclear.

In the current study MLEE was used to examine genetic relationships amongst Australian clinical isolates and reference strains identified as *M. intracellulare*. The genetic relationship between these and Australian isolates of 'X' mycobacteria cultured from patients with AIDS, reference strains of *M. scrofulaceum* and other mycobacteria such as *M. avium* and *M. avium* subsp. *paratuberculosis* was also determined. PFGE was used to determine whether related strains could be differentiated further using this technique.

METHODS

Bacterial isolates and their identification. A total of 97 Australian isolates and non-Australian reference strains of different mycobacterial species including *M. intracellulare* (70), 'X' mycobacteria (10), *M. scrofulaceum* (7), *M. avium* (8), and *M. avium* subsp. *paratuberculosis* (2) were analysed (Table 1). These were obtained as pure cultures from the State Health Laboratory in Brisbane, Queensland (40), the Victorian

Infectious Disease Reference Laboratory, Fairfield, Victoria (7), the Mycobacteriology Reference Laboratory, Westmead Hospital, Sydney, New South Wales (1), and the Australian Reference Laboratory for Bovine Tuberculosis, South Perth, Western Australia (WA) (19). Most of the Australian isolates of *M. intracellulare* (49; 94%) were from humans, the other three were from animals. Six of the *M. intracellulare* isolates represented repeated pairs of isolates recovered from three patients at a 3–5 year interval. Reference strains of *M. intracellulare* (18), *M. scrofulaceum* (7) and *M. avium* (5), previously studied by the International Working Group on the Taxonomy of Mycobacteria (Wayne *et al.*, 1993), were also examined. Isolates were identified as *M. intracellulare* if they grew slowly at 22–25 °C and were negative in tests for niacin production, nitrate reduction, urease, NaCl tolerance, Tween hydrolysis and growth at 45 °C; they also were positive for tellurite reduction, catalase and 3-d arylsulfatase activity (Vestal, 1975).

The identification of isolates (15) which were genetically distinct from *M. avium* and *M. intracellulare* in MLEE analysis was repeated using DNA probe tests as recommended by the supplier (Gen-probe). These probes are chemiluminescent-labelled, single-stranded DNA that are complementary to rRNA of the target organism. Isolates were hybridized separately with *M. avium* and *M. intracellulare* probes. Isolates that were biochemically identical to members of the *M. avium* complex, but were negative with the DNA probes for *M. avium* and *M. intracellulare*, were hybridized to the MAC probe (Gen-probe). Positive isolates were identified as 'X' mycobacteria.

A multiplex PCR which differentiates *M. avium* from *M. intracellulare* was used in duplicate as described previously (Wilton & Cousins, 1992) to test two clinical isolates which were biochemically identical to *M. intracellulare*, but were negative with the DNA probes for *M. avium*, *M. intracellulare* and 'X' mycobacteria. This PCR amplifies target sequences of 16S ribosomal DNA and produces product of either 180 (*M. avium*) or 850 (*M. intracellulare*) bp.

The growth of the two bovine isolates identified as *M. avium* subsp. *paratuberculosis* depended on the presence of mycobactin in the medium and they were positive in a PCR test using specific primers to amplify the insertion sequence IS900, as described by Cousins *et al.* (1995). The identification for reference strains including *M. scrofulaceum* has been established previously by Wayne *et al.* (1993).

The Australian isolates were serotyped using the microtube agglutination test as described by Reznikov & Leggo (1972).

All isolates were examined by MLEE, and all 28 isolates of *M. intracellulare* in ETs 4, 8, 9, 10, 23, 27, 32, 33 and three isolates of 'X' mycobacteria from ET 63 were examined by PFGE (see Table 3).

Bacterial culture and enzyme electrophoresis. Isolates other than *M. paratuberculosis* were cultured for 2 weeks at 37 °C in 200 ml Middlebrook 7H9 medium (Difco) supplemented with 10% OAD (0.06% saponified oleic acid, 0.5% bovine serum albumin, 2% glucose). Isolates of *M. paratuberculosis* were grown on ten slopes of Herrold's medium prepared according to the Australian standard protocol (Stephens, 1993) and supplemented with 2 mg mycobactin J l⁻¹ (Allied Monitor). Cells were harvested by centrifugation at 10000 g at 4 °C for 10 min, washed twice in phosphate buffered saline (PBS) and then stored at –20 °C overnight. Approximately 70 µl of packed cells was removed and kept in a microfuge tube for DNA extraction for PFGE and the remainder (approximately 500 µl) was washed twice with PBS and transferred to a glass

Table 1. Species, Australian State of origin and number of isolates examined by MLEE

Species	Australian State*				Reference strains†	Total
	QLD	WA	VIC	NSW		
<i>M. intracellulare</i>	35	17‡			18	70
'X' mycobacteria	2		7	1		10
<i>M. scrofulaceum</i>					7	7
<i>M. avium</i>	3				5	8
<i>M. avium</i> subsp. <i>paratuberculosis</i>		2				2
Total	40	19	7	1	30	97

* NSW, New South Wales; QLD, Queensland; VIC, Victoria; WA, Western Australia.

† Reference strains serotyped by the International Working Group on Mycobacterial Taxonomy (Wayne *et al.*, 1993).

‡ Includes two isolates that were originally identified as *M. intracellulare* by biochemical tests.

bijou bottle containing 1.5 ml sonication buffer (10 mM Tris/1 mM EDTA/0.5 mM NADP, pH 6.8) and 500 mg glass beads (Sigma). These cells were disrupted by four cycles of sonication, each of 1 min duration, at 4 °C, using a 50 W sonic probe (Lab Sonic 1510). Disrupted cells were microfuged at 13000 g for 20 min at 4 °C and the supernatant divided into 100 µl aliquots and stored at -70 °C until used.

The supernatants containing the enzymes were subjected to electrophoresis in 11.4% horizontal starch gels and the electrophoretic mobilities of the following 17 enzymes were determined by staining for specific enzyme activity, as recommended by Selander *et al.* (1986), except for peroxidase which was assayed according to the method of Lygren *et al.* (1986): arginine phosphokinase (APK), esterase (EST), isocitrate dehydrogenase (IDH), fructose-1,6-diphosphate dehydrogenase (FDP), fumarase (FUM), glucose-6-phosphate dehydrogenase (GPD), leucyl-glycyl-glycine peptidase (LGG), leucyl-proline peptidase (LP), leucyl-tyrosine peptidase (LTT) 1 and 2, peroxidase (PER), maleate dehydrogenase (MDH), nucleoside phosphorylase (NP), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (PGD) and superoxidase dismutase (SOD). Four different buffer systems formulated according to Selander *et al.* (1986) were used for electrophoresis as follows: buffers A for PGI, PGM, NP, IDH, MDH, FUM, PER and SOD; B for PGD and GPD; D for APK, FDP and EST; G for LGG, LTT and LP.

DNA extraction and digestion. Seventy microlitres of cells from each isolate was used for DNA extraction. The cells were washed twice in 50 mM EDTA and DNA was extracted by the methods outlined by Lévy-Frébault *et al.* (1989) with minor modifications to the solutions used. Cells were suspended in 400 µl prelysing solution [6 ml 50 mM EDTA, 6 ml 10 mM Tris, 0.1 M sodium citrate, 150 µl β-mercaptoethanol and 2 mg crude lyticase (Sigma)] and the cell suspension was mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad) prepared in 125 mM EDTA (pH 8) and cooled to 55 °C. This mixture was poured into plug moulds. Agarose plugs were kept at 4 °C for 30 min and then transferred to 4 ml tubes containing 0.5 M EDTA plus 7.5% β-mercaptoethanol, and incubated for 24 h at 37 °C in a water bath. Agarose plugs were washed four times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8) for 10 min each and then incubated for 5 h in 3 ml TE buffer containing 3 mg lysozyme. The solution was changed to 0.5 M EDTA containing 2 mg proteinase K ml⁻¹ (Boehringer), 1% sodium lauryl sarcosine (Sigma) and incu-

bated at 55 °C for 48 h. The plugs were washed at room temperature three times for 30 min with TE buffer, and then incubated at 55 °C in TE buffer plus 0.04 mg PMSF ml⁻¹ (Sigma) to inactivate the proteinase K. Agarose plugs were washed three times with TE buffer and stored in 0.5 M EDTA at 4 °C. They were washed extensively with TE buffer before being subjected to restriction endonuclease digestion.

Agarose plugs were cut with a scalpel to fit the size of the combs of the gel casting (4 × 3 mm). They were washed in restriction buffer at 4 °C for 30 min and digested for 24 h with 25 Units *Xba*I (Boehringer) or *Vsp*I (Promega) in the buffer recommended by the supplier, supplemented with 2.5 ml bovine serum albumin (10.27 mg ml⁻¹; Pharmacia).

PFGE. Plugs containing digested DNA were loaded into a 1% agarose gel prepared and run in 0.5 M TBE buffer (1 M TBE contains 0.025 M Tris, 0.5 mM EDTA and 0.025 M boric acid). PFGE was carried out with a contour-clamped homogeneous electric field-DR II system (Bio-Rad) at 14 °C for 24 h at 180 V and 12 A. Pulse time was ramped from 1–30 s for *Xba*I or 1–40 s for *Vsp*I digestion. Gels were stained with 0.5 µg ethidium bromide ml⁻¹ for 30 min and photographed under UV light with polaroid film. Bacteriophage λ DNA (Bio-Rad) was used as a marker.

Analysis. Genetic diversity (*h*) for each enzyme locus was calculated from the formula $h = (1 - \sum P_i^2) / [n / (n - 1)]$, where *P_i* is the frequency of the *i*th allele and *n* is the number of ETs or isolates in the sample (Nei, 1987). Total genetic diversity (*H*) was calculated as the mean diversity at all loci. Genetic diversities among isolates of serovars 14 and 16, which were each represented by four or more isolates, were also calculated. Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred, and the unweighted pair-group method of arithmetic means clustering fusion strategy was used to create a phenogram to show the relationships between isolates (Burr, 1970).

The matrix of coefficients that was used for examining allelic mismatches between each pair of isolates was also used for calculating the index of association (*I_A*). This index, which describes the multilocus linkage disequilibrium in bacterial populations (Maynard Smith *et al.*, 1993) is significantly different from zero for a clonal population.

RESULTS

Information about the isolates and their relationships as determined by MLEE are detailed in Table 2 and in Fig.

Table 2. ET, serovar and source of isolates examined in the study

ET*	Serovar†	Specimen	Origin‡	Species
1	ND	Sputum	WA	<i>M. intracellulare</i>
2	Auto	Sputum	QLD	<i>M. intracellulare</i>
3	14	Sputum	QLD	<i>M. intracellulare</i>
4 (9)	14	Sputum	QLD	<i>M. intracellulare</i>
4 (4)	ND	Sputum	WA	<i>M. intracellulare</i>
5	14	ND	REF	<i>M. intracellulare</i>
6	14	Sputum	QLD	<i>M. intracellulare</i>
7	14	Sputum	QLD	<i>M. intracellulare</i>
8 (2)	14	Sputum	QLD	<i>M. intracellulare</i>
8 (2)	ND	Sputum	WA	<i>M. intracellulare</i>
9 (4)	14	Sputum	QLD	<i>M. intracellulare</i>
10	16	ND	REF	<i>M. intracellulare</i>
11	1	Sputum	QLD	<i>M. intracellulare</i>
12	ND	Sputum	WA	<i>M. intracellulare</i>
13	12	ND	REF	<i>M. intracellulare</i>
14	25	ND	REF	<i>M. intracellulare</i>
15	14	Sputum	QLD	<i>M. intracellulare</i>
16	14	Sputum	QLD	<i>M. intracellulare</i>
17	1	Lung	QLD	<i>M. intracellulare</i>
18	ND	Sputum	WA	<i>M. intracellulare</i>
19	Auto	Sputum	QLD	<i>M. intracellulare</i>
20	1	Sputum	QLD	<i>M. intracellulare</i>
21	13	Sputum	REF	<i>M. intracellulare</i>
22	14	Tissue	QLD	<i>M. intracellulare</i>
23 (2)	14	Sputum	QLD	<i>M. intracellulare</i>
24	ND	Sputum	WA	<i>M. intracellulare</i>
25	13	ND	REF	<i>M. intracellulare</i>
26	15	ND	REF	<i>M. intracellulare</i>
27	14	Sputum	QLD	<i>M. intracellulare</i>
28	16	Sputum	QLD	<i>M. intracellulare</i>
29	14	Sputum	QLD	<i>M. intracellulare</i>
30	7	ND	REF	<i>M. intracellulare</i>
31	16	Sputum	QLD	<i>M. intracellulare</i>
32 (2)	ND	Numbat	WA	<i>M. intracellulare</i>
33	23	ND	REF	<i>M. intracellulare</i>
34	9	Bronchial wash	QLD	<i>M. intracellulare</i>
35	14	Sputum	QLD	<i>M. intracellulare</i>
36	20	ND	REF	<i>M. intracellulare</i>
37	28	ND	REF	<i>M. intracellulare</i>
38	UT	Sputum	VIC	'X' mycobacteria
39	18 A	Sputum	WA	<i>M. intracellulare</i>
40	43	ND	REF	<i>M. intracellulare</i>
41	15	ND	REF	<i>M. intracellulare</i>
42	17	Sputum	REF	<i>M. intracellulare</i>
43	15	ND	REF	<i>M. intracellulare</i>
44	16	Sputum	QLD	<i>M. intracellulare</i>
45	28	ND	REF	<i>M. intracellulare</i>
46	18	Sputum	WA	<i>M. intracellulare</i>
47	ND	Bovine lymph node	WA	<i>M. intracellulare</i>
48	19	ND	REF	<i>M. intracellulare</i>
49	19	ND	REF	<i>M. intracellulare</i>
50	UT	Sputum	QLD	'X' mycobacteria
51	7	Child lymph node	REF	<i>M. scrofulaceum</i>
52 (2)	ND	Bovine faeces	WA	<i>M. a. paratuberculosis</i>
53	2	ND	REF	<i>M. avium</i>
54	9	ND	REF	<i>M. avium</i>

Table 2. Continued

ET*	Serovar†	Specimen	Origin‡	Species
55	5	ND	REF	<i>M. avium</i>
56	3	ND	REF	<i>M. avium</i>
57	9	Sputum	QLD	<i>M. avium</i>
58	6	ND	REF	<i>M. avium</i>
59	1	Blood	QLD	<i>M. avium</i>
60	1	Blood	QLD	<i>M. avium</i>
61	UT	Blood	VIC	'X' mycobacteria
62	15	Blood	VIC	'X' mycobacteria
63 (3)	16, 41	Blood, ND	VIC	'X' mycobacteria
64	15	Sputum-AIDS	VIC	'X' mycobacteria
65	16	Faeces-AIDS	NSW	'X' mycobacteria
66	42	ND	REF	<i>M. scrofulaceum</i>
67	11	Sputum	WA	<i>Mycobacterium</i> sp.
68	9	Sputum	WA	<i>Mycobacterium</i> sp.
69	22	ND	REF	<i>M. scrofulaceum</i>
70	27	ND	REF	<i>M. scrofulaceum</i>
71	43	ND	REF	<i>M. scrofulaceum</i>
72	43	ND	REF	<i>M. scrofulaceum</i>
73	41	ND	REF	<i>M. scrofulaceum</i>
74	14	Bronchial wash	QLD	'X' mycobacteria

* Numbers in parentheses show the total isolates in the ET.

† Auto, Autoagglutinating; ND, not determined; UT, untypeable.

‡ NSW, New South Wales; QLD, Queensland; REF, reference strain; VIC, Victoria; WA, Western Australia.

1. The 97 mycobacterial isolates were clustered into 74 electrophoretic types (ETs). Using MLEE, two isolates submitted for examination as *M. intracellulare*, in ETs 67 and 68, were distinct from the rest of the species. These isolates were negative in all DNA probe tests for members of the *M. avium* complex and 'X' mycobacteria. The multiplex PCR identified the isolate in ET 67 as *M. intracellulare*, but the isolate in ET 68 was negative in the PCR. These two isolates were not included when calculating the diversity of *M. intracellulare*. The remaining 68 isolates of *M. intracellulare* were distributed in 48 ETs (1–37, 39–49), with a mean genetic diversity of 0.38 amongst these ETs. Only five of the 48 ETs (6.2%) contained more than one isolate of *M. intracellulare*, but these five contained 25 isolates (37%). ETs 4, 8 and 9 contained 21 isolates, including 15 isolates of serovar 14 from Queensland and six untyped isolates from WA. Thirteen of these isolates (19.1%) were in ET 4. Thirty-four isolates of *M. intracellulare* from Queensland cultured from 30 patients fell into 21 ETs. The mean genetic diversity amongst these ETs was 2.8. Pairs of isolates that were serially recovered from three patients in Queensland over 3–5 years were found to be identical by MLEE. These were clustered in ETs 4, 9 and 23, respectively.

The mean number of alleles for the 17 enzyme loci was 4.23. The genetic diversity for the 27 isolates of *M. intracellulare* serovar 14, including two reference strains, was 0.16. These isolates were distributed in 14

ETs. The four serovar 16 isolates of *M. intracellulare* (excluding that in ET 65) were more heterogeneous, having a mean genetic diversity of 0.4.

The two reference strains of serovar 19 (ETs 48 and 49) were separated from each other by a genetic distance of 0.2, but were distinct from Australian isolates of *M. intracellulare* by a genetic distance of 0.65. None of the reference strains of *M. intracellulare* shared the same ET as Australian isolates of this species. The two reference strains of serovar 7 (ETs 30 and 51) were separated from each other by a genetic distance of 0.824.

The index of association calculated for the 68 isolates of *M. intracellulare* was 2.17 (SD 0.66), which was significantly different from zero ($P < 0.05$).

Ten isolates identified as 'X' mycobacteria were distributed in seven ETs. One isolate (ET 38) was clustered with the *M. intracellulare* isolates, two (ETs 50 and 74) were distinct and the remaining four (ETs 61–64) were more closely related to each other. In general, isolates of *M. scrofulaceum* and 'X' mycobacteria were more heterogeneous than *M. intracellulare*, having genetic diversities of 0.66 and 0.65, respectively. None of the reference strains of *M. scrofulaceum* shared the same ET, while three isolates of 'X' mycobacteria from Victoria were clustered in ET 63.

The DNA probe test confirmed the identity of the three Australian isolates of *M. avium* in ETs 57, 59 and

Fig. 1. Genetic variation among strains of *M. intracellulare* and other species of mycobacteria estimated using MLEE. The scale shows the genetic distance (expressed as percentage fixed allelic differences) among 74 ETs clustered by the unweighted pair group method with averages strategy. ETs containing 'X' mycobacteria are marked with a filled circle. Isolates of uncertain identity are marked with an open circle.

60. These were separated from the reference strains of *M. avium* by a genetic distance of > 0.5 , however, indicating that this species is also diverse.

ET 52 contained both isolates of *M. avium* subsp. *paratuberculosis* and these were separated from all other isolates by a genetic distance of > 0.7 .

Isolates identified as *M. scrofulaceum* were grouped in three clusters (ETs 51, 66 and 69–73), separated from each other by a genetic distance of 0.824.

The results of PFGE typing of isolates are summarized in Table 3. The nine *M. intracellulare* isolates from Queensland in ET 4 were differentiated into five patterns and these were distinct from the WA isolates in this ET, which formed three other distinct patterns. Four patterns were also found for isolates in ET 8. ET 9 contained four isolates separated into four different PFGE types (Fig. 2). Isolates cultured from the same patient yielded identical PFGE patterns. Two isolates from numbats were also identical by PFGE. Two isolates of 'X' mycobacteria in ET 63, of serovar 41, were identical by PFGE while the third isolate in this ET, of serovar 16,

Table 3. ET, serovar and origin of isolates analysed by PFGE

ET	PFGE pattern	Isolate no.	Serovar*	Origin†
4	4/1	4817	ND	WA
4	4/2	4979	ND	WA
4	4/2	5155	ND	WA
4	4/3	5195	ND	WA
4	4/4	S72/210	14	QLD
4	4/4	S70/87	14	QLD
4	4/5	S72/97	14	QLD
4	4/5	S73/93	14	QLD
4	4/6	S72/122	14	QLD
4	4/7	S90/254	14	QLD
4	4/7	S86/207	14	QLD
4	4/8	S88/144	14	QLD
4	4/8	S85/56	14	QLD
8	8/1	S70/23	14	QLD
8	8/2	S72/35	14	QLD
8	8/3	S082	ND	WA
8	8/4	S370	ND	WA
9	9/1	S73/124	14	QLD
9	9/2	S73/91	14	QLD
9	9/3	S71/15	14	QLD
9	9/4	S79/208	14	QLD
10	10/1	TMC 1406	16	REF
23	23/1	S88/89	14	QLD
23	23/1	S88/17	14	QLD
27	27/1	S90/500	14	REF
32	32/1	92/53	ND	WA
32	32/1	92/52	ND	WA
33	33/1	23393	23	REF
63	63/1	92/128	41	VIC
63	63/1	92/129	41	VIC
63	63/2	92/126	16	VIC

* ND, Not determined.

† WA, Western Australia; QLD, Queensland; REF, reference strain; VIC, Victoria.

was distinct. The remaining four isolates in single ETs were distinct. Since most of the ETs containing *M. intracellulare* contained single isolates, no further analysis by PFGE was performed on these.

DISCUSSION

MLEE was found to be a useful technique for the genetic analysis of isolates of *M. intracellulare* and related mycobacteria. Isolates of *M. intracellulare* showed greater mean genetic diversity (0.38) than previously reported for *M. avium* (0.29) (Feizabadi *et al.*, 1996a). Both species are far more diverse than is the whole *M. tuberculosis* complex (Feizabadi *et al.*, 1996b). The index of association calculated for *M. intracellulare* was significantly greater than zero, suggesting that the population structure of this organism is clonal, with little horizontal gene flow. Similar conclusions have

Fig. 2. DNA polymorphisms among isolates of *M. intracellulare* belonging to ETs 4, 9, 10, 27, 32 and 46 using PFGE. Lanes: a, b ET 32; c, ET 46; d, ET 27; e, ET 10; f, g, ET 9; h, λ DNA marker; i, j and n, o, ET 4 (from Queensland); k–m, ET 4 (from Western Australia).

been made for *M. avium* (Feizabadi *et al.*, 1996a). There appears to be little opportunity for genetic exchange to occur amongst *M. intracellulare* strains in the natural environment.

MLEE demonstrated that the Australian isolates in ETs 67 and 68, which had been biochemically identified as MAC, were genetically distinct from other typical isolates of *M. intracellulare*. This was supported by the results of the DNA probe test, although one of these isolates was positive in the multiplex PCR. On balance these results indicate that the initial identification of these isolates was incorrect and they may belong to a different species. The specificity of the PCR assay should now be reassessed as it was positive with one of these 'atypical' isolates. This PCR may generate DNA fragments from other uncommon species that have similar sizes to those from *M. intracellulare*. The use of a modified protocol with this multiplex PCR on a reference strain of *M. scrofulaceum* generated fragments of similar size to those produced from *M. avium* (Cormican & Jones, 1995). It would be interesting to compare the nucleotide sequences of the PCR products from the 'atypical' *M. intracellulare* strain with those from other typical strains.

The ability to differentiate isolates of *M. avium* from *M. intracellulare* has been listed as an advantage of MLEE over PFGE and other typing techniques (Wasem *et al.*, 1991). Previously, the enzymes GPD, PGM and IDH were reported to be discriminatory for this purpose (Wasem *et al.*, 1991), but in this study three more enzymes (FDP, LP, LGG) were found to distinguish between the two species. Furthermore GPD was shown to have the same banding positions for some isolates of both species. MLEE also had some advantages over rDNA sequencing for characterizing *M. intracellulare*.

For example, the reference strains 13950 (serovar 16), ATCC 25122 (serovar 13) and ATCC 35848 (serovar 15) which could not be differentiated by sequencing the 16S–23S of the internal transcribed spacer of rDNA (Frothingham & Wilson, 1993), were differentiated by MLEE (ETs 10, 25, and 43, respectively). Furthermore two reference strains, 9055 Mathew's (ET 45) and 10409 (ET 69), which previously had not had a consensus on their identification (Wayne *et al.*, 1993), were clearly clustered with *M. intracellulare* and *M. scrofulaceum*, respectively, by MLEE. The technique also demonstrated that reference strains of serovar 7 in ETs 30 and 51 probably belonged to different species as the genetic distance between them was 0.8. The strain in ET 30 was more closely related to other isolates of *M. intracellulare*, confirming the previous identification of this strain (Wayne *et al.*, 1993). The second strain (P-49 in ET 51) was separated from the other two clusters of *M. scrofulaceum* by a genetic distance of 0.8, suggesting that it also was not typical of this species. Large genetic distances (up to 0.8) have also been reported previously between serovars 43 and 42A (Wasem *et al.*, 1991). Other isolates of *M. scrofulaceum* were diverse, being distributed amongst ETs 51–74. The only ET containing two Australian field isolates of *M. avium* subsp. *paratuberculosis* (ET 52) was distinct. The genetic distance observed between these isolates and the MAC was large (0.8), reinforcing the fact that they belong to a distinct genetic group.

As with strains of *M. scrofulaceum*, isolates of 'X' mycobacteria were diverse, being grouped in three clusters, separated from each other by genetic distances greater than 0.8. These strains probably comprise more than one species. Isolates of 'X' mycobacteria cultured from patients in Victoria were found to be more diverse than those from patients in Queensland. ET 63 contained three isolates identified as 'X' mycobacteria cultured from blood taken from patients with AIDS in Victoria. Two isolates of this ET were of serovar 41 and the other was serovar 16. The isolates in this ET were distinct from the only other isolate of serovar 41, which was in ET 73, with the main cluster of *M. scrofulaceum* isolates. Therefore, there appears to be serological cross-reactivity between certain 'X' mycobacteria and *M. scrofulaceum*. As a very genetically diverse group of mycobacteria were found positive with the MAC probe, its specificity requires further definition.

PFGE was found to be very sensitive in differentiating isolates which grouped in the same ETs. The similarity in the DNA patterns of isolates of serovar 14 that were cultured from the same patients at 3–5 year intervals confirms both the genetic stability of the organism and the usefulness of PFGE for subtyping. Isolates of serovar 14 were closely related (genetic distance 0.16, compared to 0.38 for the whole species), suggesting that they may represent a limited number of related clones. This is particularly evident for the large number of isolates in ET 4, which came from different geographical locations and may be a particularly common pathogenic clone.

The DNA polymorphism demonstrated in the genome of such related strains by PFGE may be due to the presence of transposable elements such as IS1141.

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