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Multilocus Enzyme Electrophoresis Analysis of the *Mycobacterium avium* Complex and Other Mycobacteria

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Multilocus enzyme electrophoresis analysis was used to evaluate the *Mycobacterium avium* complex (MAC), *M. paratuberculosis*, and nine other mycobacterial species. The average number of alleles per locus was 2.8 for the 35 MAC and 2 *M. paratuberculosis* strains which represented 24 electrophoretic types (ETs) and two distinct groups. The *M. avium* group was resolved into 17 ETs and contained the *M. paratuberculosis* ET. The *M. intracellulare* group consisted of six ETs. There was complete agreement between Gen-Probe identification and group placement by multilocus enzyme electrophoresis. The mean genetic diversity per locus for the 24 MAC ETs was 0.38. This procedure subdivided some serovars and, if implemented, should prove to be a powerful epidemiologic tool for the MAC. Eleven additional ETs were formed after the data for the other mycobacterial species were pooled with those for the MAC.

Members of the *Mycobacterium avium* complex (MAC) include *M. avium* and *M. intracellulare* and are widely distributed in water (5, 14) and soil (3). Therefore, the environment is presumed to be the major source of human infection by MAC strains (10), although contact with infected birds or animals may be responsible in some instances (25). Infection by the MAC in humans may result in no symptoms, tuberculosislike disease, or as in persons with AIDS, disseminated disease. Good and Snider (11) reported that in 1980 the MAC was second only to *M. tuberculosis* as the most frequent mycobacterial clinical isolate in the United States. The ubiquitous nature of the organism is emphasized by the fact that MAC strains may cause disseminated infection in as many as 53% of patients with AIDS (16).

Epidemiologic surveys of the MAC have been hampered by lack of suitable identification procedures. Although the MAC may be differentiated from other mycobacteria on the basis of biochemical tests (37), this is due to the relative biochemical inactivity of MAC strains. Therefore, further subdivision of the complex by this means has not been accomplished.

Expansion and implementation of serology for identification has resulted in assignment of certain MAC serovars to either *M. avium* or *M. intracellulare*, but these determinations are undergoing revision. Reliance on serologic identification has been impeded by strains that exhibit cross-reactivity, nonreactivity, more than one antigen, or autoagglutination (23, 41).

A recent review by Grange et al. (13) illustrates the lack of consensus on the subdivision of the MAC. Serovar designation was used as a base to compare results obtained via agglutination, immunodiffusion, sensitin type, cultural and sensitin type, or DNA-DNA hybridization. In general, all researchers apply the epithets *M. avium* to serovars 1 to 3 and *M. intracellulare* to serovars 12 through 18; the higher serovars have not been extensively investigated. Thus, the major contentions revolve around serovars 4 through 11, with various designations applied to representative strains. Among the reasons for focus on this group is that it includes serovars 4 and 8, which are a common cause of disseminated MAC disease in patients with AIDS (41). Other related pathogens include *M. scrofulaceum*, environmentally located, and *M. paratuberculosis*, the etiologic agent of Johne's disease in cattle and, possibly, Crohn's disease in humans (4). The spectrum of diseases and source for human infections widens considerably when these are included in the discussion of the MAC. Thus, it is imperative that epidemiologic information on the extent and diversity of strains that make up the MAC and related mycobacteria be obtained.

The Gen-Probe Rapid Diagnostic System for the MAC reliably distinguishes the two MAC species, *M. avium* and *M. intracellulare* (27, 28). This procedure uses either an isotopic (Gen-Probe; being discontinued) or a luminescent (AccuProbe) DNA probe that hybridizes specifically with RNA of the organism. However, it has not been developed for subdivision of the MAC beyond the two species.

Multilocus enzyme electrophoresis (MEE) has recently been successfully used to examine the epidemiology of clinically important bacteria (26, 32, 33). This is because variation in enzyme electrophoretic mobility can detect at least 50% of amino acid substitutions (33) in proteins and, thus, is often more sensitive than nucleic acid homology determinations. The compilation of mobility patterns of several enzymes can be combined to quantitate the overall genetic relatedness of strains under study and may provide a means of distinguishing and identifying different members of the MAC. In the work reported here, MEE was tested for the ability to differentiate MAC strains and evaluated for the ability to distinguish other mycobacterial species.

MATERIALS AND METHODS

Organisms. The mycobacterial strains tested are listed in Table 1. Included were 35 strains designated either *M. avium* or *M. intracellulare* at acquisition; all were placed in the MAC for this study. Among MAC isolates, serovars 1 to 4, 8 to 10, 12, 14, 16, and 19 were represented. Additional strains were obtained from the Veterans Administration reference laboratory (West Haven, Conn.), with identification determined by the Gen-Probe Rapid System for MAC

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TABLE 1. Sources and designations of the mycobacterial strains tested

New Mexico State University strain no.	Species[a]	Source (strain)[b]	Isolation information	Serovar	GP[c]	Medium[d]	ET
LM33	MAC	NJH	Wood duck	12	MI	CM	1
LM40	MAC	NJH (TMC1403; ATCC 35761)	Sputum	14	MI	CM	1
LM115	MAC	VAMC-CT	Sputum; Oklahoma		MI	CM	1
LM117	MAC	VAMC-CT	Sputum; Utah	16	MI	CM	1
LM34	MAC	NJH	Human	14	MI	CM	2
LM35	MAC	NJH	Human	16	MI	CM	2
LM1	MAC	NJH	Sputum; Colorado	1	MI	CM	3
LM135	MAC	VAMC-CT	Sputum; Utah		MI	CM	4
LM139	MAC	VAMC-CT	Sputum; Ohio		MI	CM	5
LM47	MAC	NJH (W552)	Human	19	MI	CM	6
LM51	MAC	CDC (83-529)	AIDS; New York	4	MA	CM	7
LM53	MAC	CDC (83-640)	AIDS; Washington, D.C.	4	MA	CM	7
LM79	MAC	VAMC-AR	AIDS	4	MA	CM	7
LM49	MAC	CDC (83-484)	AIDS; Maryland	4	MA	CM	8
LM131	MAC	VAMC-CT	AIDS; Virginia		MA	CM	9
LM59	MAC	CDC (83-1441)	AIDS; New York	8	MA	CM	10
LM30	MAC	CDC (83-1156)	AIDS; New York	8	MA	CM	11
LM56	MAC	CDC (83-1146)	AIDS; Maryland	8	MA	CM	12
LM107	MAC	VAMC-CT	AIDS; California	9	MA	CM	13
LM125	MAC	VAMC-CT	AIDS; Virginia	10	MA	CM	13
LM129	MAC	VAMC-CT	Sputum; Virginia	10	MA	CM	13
LM133	MAC	VAMC-CT	Bronchial; Virginia	10	MA	CM	13
LM32	MAC	NJH (1784-286)	Human	9	MA	CM	14
LM127	MAC	VAMC-CT	Sputum; Virginia	10	MA	CM	15
LM108	MAC	VAMC-CT	AIDS; California		MA	CM	16
LM109	MAC	VAMC-CT	Sputum; Florida	1	MA	CM	16
LM28	MAC	CDC (84-550)	AIDS; Florida	1	MA	CM	17
LM54	MAC	CDC (83-1085)	AIDS; Arkansas	1	MA	CM	18
LM105	MAC	VAMC-CT	AIDS; California	8	MA	CM	19
LM106	MAC	VAMC-CT	Sputum; California	8	MA	CM	19
LM73	MAC	VAMC-AR (LR113)	Human		MA	CM	20
LM14	MAC	VAMC-AR (LR107)	Human	3	MA	CM	21
DM2	MAC	NJH (B2900)	Bovine	2	MA	CM	22
DM5	MAC	NJH (14141-1395)	Swine	2	MA	CM	22
LM11	MAC	VAMC-AR (LR25)	Human		MA	CM	23
LM121	*M. paratuberculosis*	NVSL (2871)	Bovine			LEMM	24
LM122	*M. paratuberculosis*	NVSL (2930)	Bovine			LEMM	24
LM60	*M. scrofulaceum*	CDC (83-1207)	AIDS	43		CM	25
LM41	*M. kansasii*	NJH (TMC1201; ATCC 35775)	Sputum			7H9	26
LM37	*M. chelonae*	NJH (TMC1542; ATCC 35751)	Eye			CM	27
LM39	*M. gordonae*	NJH (TMC1318; ATCC 35756)	Sputum			7H9	28
LM44	*M. xenopi*	NJH (TMC1482; ATCC 19250)	Toad			7H9	29
LM23	*M. scrofulaceum*	CDC (83-1212)	AIDS	42A		7H9	30
LM36	*M. bovis* BCG	NJH (TMC1011; ATCC 35734)	Bovine			7H9	31
LM43	*M. tuberculosis* H37Ra	NJH (TMC201; ATCC 25177)	Human			7H9	31
LM15	*M. smegmatis*	VAMC-AR (ATCC 607)				BGT	32
LM38	*M. fortuitum*	NJH (TMC1529; ATCC 6841)	Cold abscess			CM	33

[a] MAC strains include the species *M. avium* and *M. intracellulare*.

[b] Strains were obtained from the following sources: NJH, National Jewish Hospital, Denver, Colo. (Anna Tsang); VAMC-CT, Veterans Administration Medical Center, West Haven, Conn. (Jean Hawkins); VAMC-AR, Veterans Administration Medical Center, Little Rock, Ark. (Jack Crawford); CDC, Centers for Disease Control, Atlanta, Ga. (Robert Good, Mitch Yakrus); NVSL, National Veterinary Services Laboratory, Ames, Iowa (Janet Payeur).

[c] Gen-Probe was performed by the Veterans Administration Medical Center, West Haven, Conn. MI strains were positive with an *M. intracellulare* probe, and MA strains were positive with an *M. avium* probe.

[d] Media were prepared and used as described in Materials and Methods.

(Gen-Probe, Inc.). All of the MAC strains from humans were clinical isolates, with about one-half from patients with AIDS. The two strains of *M. scrofulaceum*, serovars 42A and 43, were also from patients with AIDS; the two strains of *M. paratuberculosis* were from cows with Johne's disease. *M. smegmatis* 607 was used as the reference strain in the enzyme analyses, while the other species were from a mycobacterial taxonomic kit and were included for comparative evaluation.

Chemicals. Starch (hydrolyzed potato starch) was purchased from StarchArt Corp. Most of the other chemicals, enzymes, and reagents were purchased from Sigma Chemical Co.

Media and culture conditions. Liquid culture media were prepared by mixing appropriate volumes of concentrated stock solutions in purified water, filtering them through a 0.22-μm-pore-size filter (Millipore Corp.), and dispensing them aseptically. Culture medium (CM) was prepared as described by McCarthy (20) and consisted of B salts (20), 0.5% glycerol, 0.5% bovine albumin (fraction V; Miles

Diagnostic), 0.01% oleic acid, and 0.1% polyoxyethylene sorbitan monooleate (Tween 80). 7H9 medium was prepared from Middlebrook 7H9 broth base (Difco Laboratories) and enriched with 0.5% albumin, 0.005% oleic acid, 0.2% glucose, 0.2% glycerol, 0.085% sodium chloride, and catalase at 4 mg/liter. BGT medium contained B salts (20), 0.5% glycerol, and 0.1% Tween 80. Lowenstein egg medium with mycobactin (LEMM) was prepared from autoclaved Lowenstein medium base (Difco) supplemented with 0.75% glycerol and homogenized eggs prepared as described by Vestal (36). An ethanol solution of mycobactin J (Allied Laboratories, Inc.) was added to a final concentration of 20 mg/liter. The mixture was aseptically dispensed, 100 ml/16-oz (ea. 440-ml) prescription bottle, and the inspissation solidified the medium onto the flat surface (7 by 13 cm) of the bottle. The *M. paratuberculosis* strains were subcultured to small surfaces of LEMM and ultimately to the large surface of the prescription bottle and incubated for 4 to 6 weeks at 37°C until mature colonies appeared. The surface of the LEMM was gently scraped and rinsed with buffer to remove the mycobacteria, which were harvested by centrifugation.

The other mycobacterial stock cultures were prepared from single colony isolates, propagated, concentrated, quickly frozen, and stored at −70°C as described previously (20). For cultivation, a stock was thawed and 50 μl was inoculated into 200 ml of liquid CM, 7H9, or BGT (Table 1) in a 1-liter screw-cap flask and then incubated at 37°C and 150 rpm to approximately 5×10^8 CFU/ml. Purity determinations were made by spotting samples on Middlebrook and Cohn 7H10 agar (BBL Microbiology Systems) and Trypticase soy agar (BBL). The mycobacteria were harvested by centrifugation, and the pellets were stored at −20°C.

Cell extract preparation. The pelleted mycobacteria were suspended in 3 ml of sonicating buffer (50 mM imidazole [pH 7.0] and 10 mM 2-mercaptoethanol in purified water). Each cell suspension, in a sealed atmosphere treatment chamber maintained in an ice bath, was sonicated with a Branson Sonifier cell disruptor for 3 min at 50 W and a 50% pulse. The sonicated preparation was centrifuged at 38,720 × *g* and 4°C to remove cell debris. The supernatant fluid was treated with streptomycin sulfate (2 mg/ml), and the precipitated nucleic acids were removed by centrifugation at 38,720 × *g* and 4°C. Protein determinations were initially estimated by A_{280}, and samples were removed for assay later. Glycerol, at a final concentration of 10%, was added to the remainder of the extract, and aliquots were frozen and stored at −70°C. The assay for the protein content of each extract was determined by the procedure of Lowry et al. (17) with bovine serum albumin as the standard. The protein concentrations of the cell extracts were 3 to 6 mg/ml.

Electrophoresis and enzyme analysis. A 500-ml volume of 11.5% starch with 23 mM Trizma base (Sigma) and 5 mM citric acid (pH 8.0) was prepared as described by Smithies (34), and the preparation was poured into a tray that measured 15 by 18 by 1.6 cm. On the following day, individual thawed extracts were absorbed onto filter paper wicks (Whatman no. 5; 5 by 17 mm) and the latter were inserted into the starch gel. The buffer for both electrodes was 0.687 M Trizma base–0.157 M citric acid (pH 8.0). Electrophoresis was performed at 100 mA and 3°C for the time required to move a marker dye (0.15% bromphenol blue and 10% glycerol) 10 cm towards the anode (about 4.3 h). Gels were sliced 2 mm thick, and each was stained for an enzyme activity (see Table 2).

Electrophoretic variants of each enzyme were assigned allele numbers based on their mobilities on the gel. Each strain was characterized by the profile of alleles for all enzymes, and each unique profile was assigned an electrophoretic type (ET). These determinations were made from results of at least three replicates for each mycobacterial strain.

Statistical analysis. Weighted genetic distances between pairs of strains were obtained for the MAC analyses by using the formula of Selander et al. (32). Unweighted genetic distances were used for evaluation of the MAC with the other mycobacterial species. Dendrograms were developed from dissimilarity matrices by the pair-group method using arithmetic averages (35) and the SAS commercial program (30). Principal-coordinate analysis (12) was performed on similarity matrices by using the IML procedure in the SAS program (29) to determine the genetic relatedness of the MAC strains.

RESULTS

Enzyme activities and loci. The 20 enzyme activities sought and detected are listed in Table 2. Three enzymes—diaphorase, catalase, and peroxidase—exhibited two electrophoretic bands for many of the mycobacterial extracts. Only the diaphorase activities, however, were sufficiently consistent that each band was considered an allele of a separate locus (DP1 and DP2).

The allele profiles of the 47 mycobacterial strains are shown in Table 3. A total of 33 ETs were identified. Detailed information about the individual strains, with their ETs, is shown in Table 1.

All of the enzymes tested were polymorphic. The mean number of alleles per locus for all ETs was 6.1, but it was only 2.8 for the combined MAC and *M. paratuberculosis* ETs (ETs 1 through 24). Among the loci for this latter group, the greatest genetic diversity was observed for esterase, phosphogluconate dehydrogenase, and leucine aminopeptidase. The least genetic diversity occurred with catalase, diaphorase-1, peroxidase, and superoxide dismutase. The mean genetic diversity per locus for the 24 MAC ETs was 0.38.

Relatedness of *M. avium*, *M. intracellulare*, and *M. paratuberculosis*. Attention was focused on the electrophoretic patterns of the strains that ultimately resulted in 24 ETs and included *M. avium*, *M. intracellulare*, and *M. paratuberculosis*. The frequency and distribution of mismatches (dissimilar alleles) were determined by pairwise comparison of these 24 ETs (Fig. 1). There was an average difference of 7.66 loci per pair. All of the mismatches at 1 through 7 loci, from 20 possible loci, were from *M. avium-M. avium* or *M. intracellulare-M. intracellulare* combinations, demonstrating the close intrarelatedness within each cluster. Most (94%) of the mismatches at 8 through 11 loci involved *M. paratuberculosis* (ET 24). The mismatches at 12 through 15 loci were from *M. avium-M. intracellulare* combinations, showing the distinct separation between strains from each of these clusters. Thus, the bimodality shown suggests that at least two groups of organisms were present.

A dissimilarity matrix of coefficients of weighted genetic distance was prepared for ETs 1 through 24. These data were then analyzed by an average-linkage procedure (35) to identify and group related strains. The resulting dendrogram is shown in Fig. 2. The ET assignments (Tables 1 and 3; Fig. 2) for these organisms were based on this grouping.

Two distinct clusters with a genetic distance of 0.54 resulted from this analysis (Fig. 2). The first cluster, the MI cluster, which contained ETs 1 through 6, included strains

TABLE 2. Enzymes tested

EC no.	Abbreviation	Enzyme	Assay reference
1.1.1.37	MDH	Malate dehydrogenase	31
1.1.1.42	IDH	Isocitrate dehydrogenase ($NADP^+$)	31
1.1.1.44	PGD	Phosphogluconate dehydrogenase	31
1.1.1.49	G6P	Glucose 6-phosphate dehydrogenase	31
1.2.1.7	BAD	Benzyl alcohol dehydrogenase	CDC[a]
1.6.99.3	DP1	Diaphorase (NADH)	15
1.6.99.3	DP2	Diaphorase (NADH)	15
1.11.1.6	CAT	Catalase	40
1.11.1.7	PER	Peroxidase	18
1.15.1.1	IPO	Indophenol oxidase (superoxide dismutase)	18
2.4.2.1	NSP	Purine nucleoside phosphorylase	31
2.6.1.1	GOT	Glutamic oxaloacetic transaminase	31
2.7.4.3	ADK	Adenylate kinase	31
2.7.5.1	PGM	Phosphoglucomutase	31
3.1.1.1	EST	Esterase	31
3.1.6.1	ARS	Arylsulfatase	9
3.4.11.1	LAP	Leucine aminopeptidase	31
4.2.1.2	FUM	Fumarate hydratase	31
4.2.1.3	ACO	Aconitate hydratase	31
5.3.1.9	PGI	Phosphoglucose isomerase	31

[a] Assay from the CDC Isoenzyme Assay File (provided by Michael Reeves) contained 0.2 M Tris hydrochloride (pH 8.0), 40 mM benzyl alcohol, 4 mM $MgCl_2$, 0.338 mM dimethylthiazole tetrazolium, 0.326 mM phenazine methosulfate, and 0.269 mM NAD in purified water.

identified as *M. intracellulare* by Gen-Probe and serovars 1, 12, 14, 16, and 19. This group of ETs will be referred to as the MI cluster. Within this cluster, ETs 1 through 4 were closely related, with a genetic distance of 0.06. ETs 5 and 6 joined this cluster at distances of 0.13 and 0.24, respectively. Interestingly, strain LM1 (ET 3), designated *M. avium* in several studies (20–22), was placed in the MI cluster by both MEE and Gen-Probe reactivity. However, LM1 was confirmed as serovar 1 by thin-layer chromatography (35a).

A second cluster, the MA cluster, which contained ETs 7 through 23, included strains identified as *M. avium* by Gen-Probe and serovars 1 to 4 and 8 to 10. This MA cluster contained two groups composed of 13 closely related ETs (ETs 7 through 19) joined at a distance of 0.11. ET 20 was represented by a strain that was previously referred to as *M. intracellulare* (7). Although its serovar has not been determined, ET 20 hybridized with an *M. avium* Gen-Probe and joined the MA cluster at a genetic distance of 0.14. Representatives of serovar 2 (DM2 and DM5) and serovar 3 (LM14), ETs 22 and 21, respectively, formed a cluster at a distance of only 0.09 before being joined to the other MA cluster ETs at a relative genetic distance of 0.15. Among the first MAC clinical strains demonstrated to carry plasmids was LM11 (LR25), reported by Crawford and Bates (6) and referred to as *M. intracellulare*. This strain, assigned to ET 23, hybridized with the *M. avium* Gen-Probe and joined the other MA cluster ETs at a genetic distance of 0.17.

The two *M. paratuberculosis* strains that made up ET 24 joined the MA cluster at a genetic distance of 0.38, which suggests a closer relatedness to *M. avium* than to *M. intracellulare*.

Different methods of analysis can result in a variety of dendrograms with differing genetic distances, and therefore, the results represented here (Fig. 2) should be considered tentative. However, since the MI and MA clusters joined at a genetic distance of 0.54, these results support the conclusions of others (1, 38) that *M. avium* and *M. intracellulare* represent two well-separated mycobacterial species.

Principal-coordinate analysis on a similarity matrix of coefficients of weighted genetic distance, as described by Gower (12), was performed on the *M. intracellulare*, *M. avium*, and *M. paratuberculosis* ETs (Fig. 3). While there was a distinct separation between the MI and MA clusters, close intrarelatedness among the ETs within each cluster was apparent. *M. paratuberculosis* (ET 24) appeared to be distinct from both the MI and MA clusters.

Differentiation of *M. avium* and *M. intracellulare* strains. Multilocus enzyme electrophoresis was capable of distinguishing *M. intracellulare* strains from *M. avium* strains by any of the following enzymes alone or in combination: adenylate kinase, arylsulfatase, diaphorase-2, fumarase, glutamic oxaloacetic transaminase, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, and phosphoglucomutase.

Genetic distance in comparison with serovar. All but one serovar representative clustered within an accepted *M. avium* or *M. intracellulare* group. Serovars 1, 4, 8 to 10, 14, and 16 were further separated into two to four different ETs. ETs 1, 2, and 13 each contained more than one serovar. Thus, MEE and serovar determinations may not be used interchangeably for characterization of MAC strains.

Comparison of the 33 ETs. A second set of data that contained all 33 ETs was analyzed by use of unweighted genetic distances in order not to bias results toward the MAC. The resulting composite dendrogram is shown in Fig. 4. The MI cluster exhibited slightly increased genetic distances among ETs (Fig. 4) compared with the previous analysis (Fig. 2). Some of the ETs in the MA cluster were redistributed with only minor adjustment (Fig. 4) from the previous dendrogram (Fig. 2). The MI and MA clusters (Fig. 4) were joined at a genetic distance of 0.65 rather than 0.54 when weighted values were used (Fig. 2).

The MI cluster joined with one strain of *M. scrofulaceum* (ET 25) at a distance of 0.58, while the MA cluster joined with *M. paratuberculosis* (ET 24) at a distance of 0.49.

M. kansasii (ET 26) joined to a group that contained *M. chelonae*, *M. gordonae*, and *M. xenopi* (ETs 27 to 29, respectively) at a distance of 0.73 and to the *M. avium*, *M.*

TABLE 3. Allele designations of 33 ETs of *Mycobacterium* spp.

ET	Species[a]	No. of strains	Allele at enzyme locus[b]:																		
			ACO	ADK	ARS	BAD	CAT	DP1	DP2	EST	FUM	GOT	G6P	IDH	IPO	LAP	MDH	NSP	PER	PGD	PGI	PGM
1	MAC	4	3	2	2	2	2	5	0	9	3	2	0	4	4	4	2	4	2	2	3	5
2	MAC	2	4	2	2	2	2	5	0	9	3	2	0	4	4	4	2	4	2	2	3	5
3	MAC	1	3	2	2	2	2	5	0	9	3	2	0	4	4	4	2	4	2	2	3	4
4	MAC	1	3	2	2	2	2	5	0	10	3	2	0	4	4	4	2	4	2	2	3	4
5	MAC	1	3	2	2	2	2	5	0	9	3	2	0	4	4	4	2	3	2	2	2	5
6	MAC	1	4	5	2	2	2	5	0	10	3	2	0	4	4	3	3	4	2	2	3	6
7	MAC	3	3	1	3	2	2	5	1	8	2	1	3	1	4	2	2	3	2	3	3	1
8	MAC	1	2	1	3	2	2	5	1	8	2	1	3	1	4	2	2	3	2	3	3	1
9	MAC	1	2	1	3	2	2	5	1	8	2	1	3	1	4	3	2	3	2	3	3	1
10	MAC	1	3	1	3	2	2	5	1	8	2	1	3	1	4	3	2	3	2	1	3	1
11	MAC	1	3	1	3	3	2	5	1	8	2	1	3	1	4	2	2	3	2	3	3	1
12	MAC	1	3	1	3	3	2	5	1	8	2	1	3	1	4	2	2	3	2	2	3	1
13	MAC	4	2	1	3	3	2	5	1	8	2	1	3	1	4	3	2	3	2	3	3	1
14	MAC	1	2	1	3	3	2	5	1	6	2	1	3	1	4	3	2	3	2	3	3	1
15	MAC	1	2	1	3	3	2	5	1	11	2	1	3	1	4	3	2	3	2	3	3	1
16	MAC	2	2	1	3	3	2	5	1	8	2	1	3	1	4	3	2	3	2	2	3	1
17	MAC	1	2	1	3	3	2	5	1	8	2	1	3	1	4	3	2	4	2	2	3	1
18	MAC	1	2	1	3	3	2	5	1	8	2	1	3	1	4	2	2	4	2	2	3	1
19	MAC	2	2	1	3	3	2	5	1	12	2	1	3	1	4	3	2	4	2	3	3	1
20	MAC	1	2	1	0	3	2	5	1	8	2	0	3	1	4	3	2	3	2	3	3	1
21	MAC	1	2	1	3	3	2	5	1	6	2	1	3	1	4	3	2	3	2	2	2	1
22	MAC	2	2	1	3	3	2	5	1	6	2	1	3	1	4	3	2	3	2	0	1	1
23	MAC	1	2	1	3	3	2	5	1	6	2	1	3	1	4	2	2	4	2	4	3	0
24	*M. paratuberculosis*	2	0	2	3	3	2	5	0	0	2	0	3	1	4	2	1	4	2	1	3	2
25	*M. scrofulaceum*	1	4	2	0	4	2	5	0	12	2	3	3	4	5	3	2	3	2	6	3	4
26	*M. kansasii*	1	1	2	2	1	3	1	0	5	2	1	2	1	3	1	1	1	2	1	3	4
27	*M. chelonae*	1	4	6	1	0	3	2	0	2	2	4	6	3	4	5	0	3	2	4	3	3
28	*M. gordonae*	1	4	3	0	3	3	2	2	4	2	5	8	3	4	1	1	2	1	5	3	4
29	*M. xenopi*	1	4	5	0	0	1	4	0	6	2	0	1	5	2	4	1	7	2	7	3	0
30	*M. scrofulaceum*	1	4	4	2	4	4	5	1	7	1	3	4	4	5	5	2	6	4	7	5	3
31	*M. tuberculosis-M. bovis*	2	1	3	0	0	0	3	0	0	3	0	0	6	1	1	3	0	2	0	2	1
32	*M. smegmatis*	1	4	4	4	5	2	2	2	1	4	4	5	2	6	5	0	7	3	5	4	7
33	*M. fortuitum*	1	4	4	0	2	3	1	0	3	2	2	7	2	6	5	3	5	2	4	4	7

[a] MAC strains include the species *M. avium* and *M. intracellulare*.
[b] See Table 2 for enzyme abbreviations.

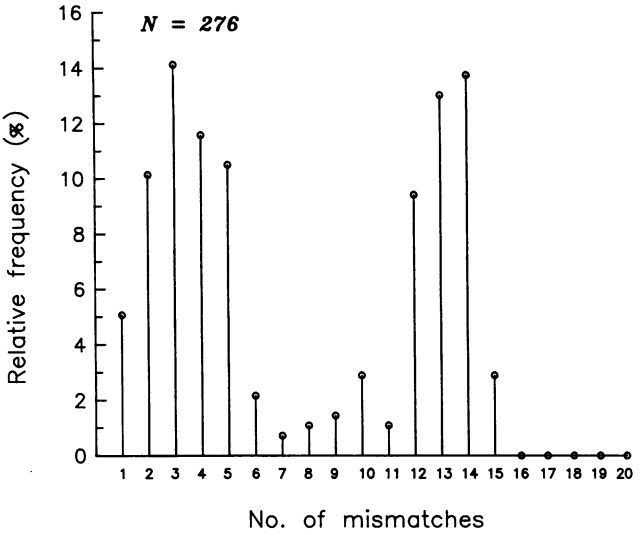

FIG. 1. Distribution of mismatches (dissimilar alleles) in pairwise comparisons of 24 ETs of *M. avium*, *M. intracellulare*, and *M. paratuberculosis* (ETs 1 through 24).

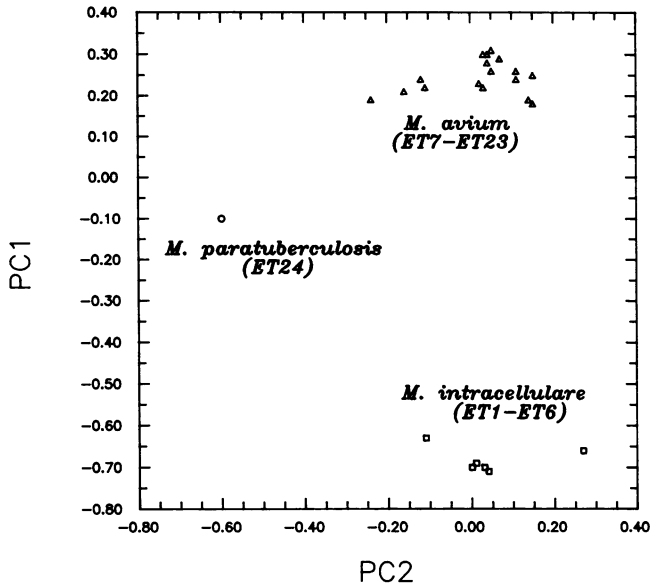

FIG. 3. Distribution of 24 ETs of *M. avium*, *M. intracellulare*, and *M. paratuberculosis* on the first two principal coordinates (PC1 and PC2) generated from principal-coordinate analysis on a matrix of coefficients of weighted genetic distance.

intracellulare, and *M. paratuberculosis* ETs at a distance of 0.79. Another strain of *M. scrofulaceum* (ET 30) and *M. tuberculosis* H37Ra-*M. bovis* (ET 31) were linked to the above ETs at distances of 0.84 and 0.86, respectively. The most distantly formed cluster in this set of data (Fig. 4) contained *M. smegmatis* (ET 32) and *M. fortuitum* (ET 33), which converged with each other at a distance of 0.65 and with the other ETs at a distance of 0.88. The mean genetic diversity per locus for all 33 ETs was 0.58.

DISCUSSION

DNA analyses have demonstrated that the MAC consists of genetically diverse organisms. Crowther and McCarthy (8) reported that the G+C contents of DNAs of MAC strains representing serovars 1 to 4 and 8 ranged from 67.8 to 71.6 mol%. Through DNA-DNA hybridization studies, Baess (1) concluded that MAC serovars 4 to 6 and 8 should be combined with serovars 1 to 3 in *M. avium* rather than in *M. intracellulare*. On the basis of their study of T-catalases from 31 MAC strains by solid-phase immunosorbent assay, Wayne and Diaz (38) concurred. Through restriction fragment length polymorphism analyses, McFadden et al. (24) divided MAC strains into two groups. The avium group contained serovars 2, 5, 6, and 8, as well as *M. paratuberculosis* and *M. lepraemurium*; the base substitution between any of the strains was less than 3%. The intracellulare group contained serovars 11, 16, and 27; the base substitution between these members was greater than 13%, and these strains exhibited less than 85% base homology to the avium group. Saito et al. (27) applied Gen-Probe identification to representatives of MAC serovars 1 through 28. They concluded that serovars 1 to 6, 8 to 11, and 21 should be assigned to *M. avium*; serovars 7, 12 to 20, and 25 should be assigned to *M. intracellulare*; and the other serovars were too disordered to be placed taxonomically.

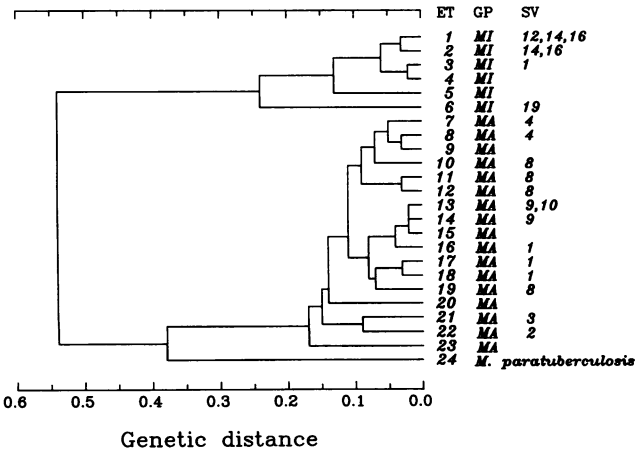

FIG. 2. Genetic relationships among 24 ETs of *M. avium*, *M. intracellulare*, and *M. paratuberculosis* (ETs 1 through 24). The dendrogram was constructed by the average-linkage method of clustering from a matrix of coefficients of weighted genetic distance. GP, Gen-Probe identification as *M. avium* (MA) or *M. intracellulare* (MI); SV, serovar.

The present study revealed that MEE results correlated completely with Gen-Probe identification results for gross separation of the MAC into the MA and MI clusters. In addition, the MAC strains that included serovars 1 to 4 and 8 to 10 were assigned by MEE to the MA cluster, while serovars 12, 14, 16, and 19 were placed in the MI cluster. These results are in agreement with the nucleic acid hybridization results of Baess (1) and the Gen-Probe identification performed by Saito et al. (27). Although the data are not presented, the MEE results for a few colony variants (i.e., transparent versus opaque) were identical to those of their parent strains.

The close intrarelatedness of ETs within each of the MA and MI groups and the clear separation between these two clusters were achieved by use of MEE. In addition, MEE was capable of further delineation of strains beyond the serovar level. A single, unique oligosaccharide side chain of the surface antigen provides the specificity for MAC sero-

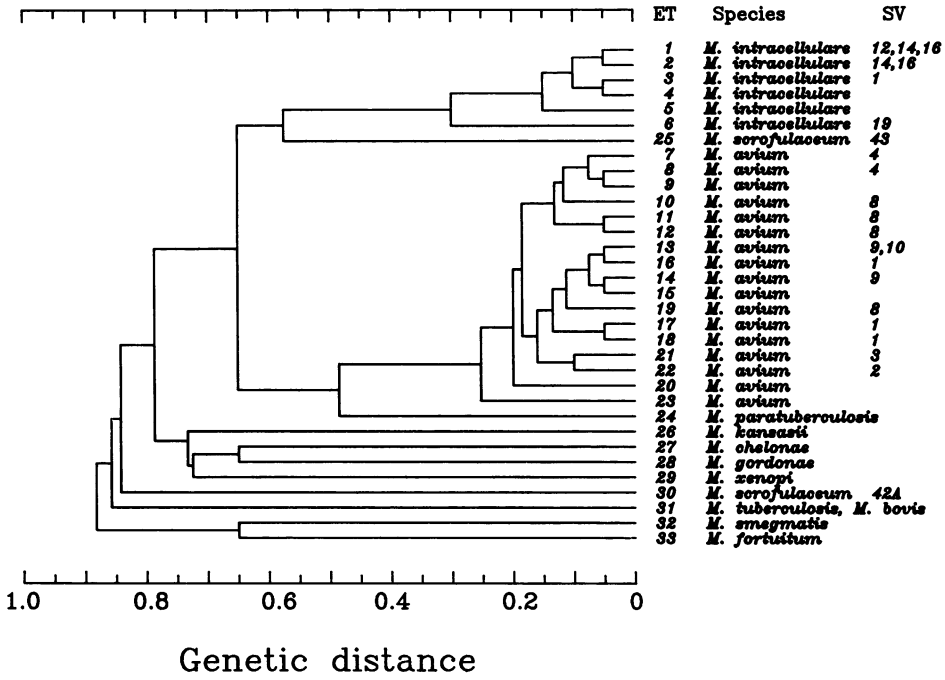

FIG. 4. Genetic relationships among 33 ETs of *Mycobacterium* spp. The dendrogram was constructed by the average-linkage method of clustering from a matrix of coefficients of unweighted genetic distance. SV, Serovar.

vars, while all MAC strains contain an identical peptidoglycolipid core (2). Consequently, through the genetic analysis of enzymes common to all strains and probably chromosomally encoded, MEE provides a greater representation of the genome than serologic typing.

The mean genetic diversity per locus for the 24 MAC ETs was 0.38, which is less than that reported for *Escherichia coli* (39), *Salmonella* spp. (26), and *Treponema* spp. (19) (0.52, 0.56, and 0.64, respectively) and more than that reported for *Shigella* spp. (39) and *Legionella pneumophila* (32) (0.29 and 0.31, respectively).

Nucleic acid hybridization lacks the precision required for measurement of genetic relationships of closely related strains (31). This is, in part, because hybridization, whether total genomic or via a selected probe, provides only information on the similarity of a set of strains to one reference strain (or a few). In contrast, MEE uses the powerful statistical procedures of numerical taxonomy so that each mycobacterial strain may be compared with each other through the coefficients generated.

The genetic similarity between *M. paratuberculosis* and the MAC has not been resolved. Our results show some similarity between the *M. paratuberculosis* ET (ET 24) and the MA cluster (genetic distance, 0.38), although not to the extent suggested by the restriction fragment length polymorphism work of McFadden et al. (24). Further work is needed to determine the relatedness of *M. paratuberculosis* to other MAC strains, including mycobactin-dependent MAC isolates.

Some interesting information was obtained by application of MEE to diverse mycobacterial species. The clustering of these strains correlated somewhat with Runyon groupings (Fig. 4). The distinct difference between the two *M. scrofulaceum* strains (ETs 25 and 30), both isolates from patients with AIDS, demonstrates the need to investigate the overall heterogeneity of this species further.

The results of this study demonstrated the ability of MEE to distinguish between *M. avium* and *M. intracellulare* strains, as well as to differentiate between strains within each group. This method should be a powerful tool in epidemiologic and taxonomic studies of the MAC and related mycobacteria.

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