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# Characterization of Isolates of *Mycobacterium avium* Serotypes 4 and 8 from Patients with AIDS by Multilocus Enzyme Electrophoresis

MITCHELL A. YAKRUS,[1]* MICHAEL W. REEVES,[2] AND SUSAN B. HUNTER[2]

*Respiratory Diseases Branch[1] and Meningitis and Special Pathogens Branch,[2] Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333*

Received 16 December 1991/Accepted 9 March 1992

**Isolates of *Mycobacterium avium* serotypes 4 and 8 originating from patients with AIDS in New York City, Los Angeles, or San Francisco were further characterized by multilocus enzyme electrophoresis. Reference strains used to produce typing antisera were also examined. Thirty-one electrophoretic types (ETs) were found among 58 isolates of serotype 4, while 10 ETs were identified among 21 isolates of serotype 8. One major ET was found within each serotype, and these two ETs were closely related, separated by a genetic distance of only 0.05. Six ETs were found in more than one city. In four cases, isolates of serotypes 4 and 8 shared the same ET. Multilocus enzyme electrophoresis in combination with serotyping should be helpful in locating the specific infection sources of these commonly isolated opportunistic pathogens.**

Disseminated infection caused by *Mycobacterium avium* occurs frequently in the late stages of AIDS. Contaminated food and drink may be major sources of infection since these organisms are found associated with the intestinal mucosa and are often isolated from fecal specimens (3, 9, 11, 14, 21). Seroagglutination has determined that most isolates from patients with AIDS in the United States are either serotype 4 or 8 (11, 14, 26). Restriction enzyme analysis of DNA from isolates of serotypes 4 and 8 suggests that these strains belong to a single, homogeneous group (7).

Multilocus enzyme electrophoresis (MEE) can be used to separate isolates of *M. avium* serotypes 4 and 8 into electrophoretic types (ETs) (24, 27). Different mobilities of an enzyme are due to variations in structure controlled by alleles at a specific locus (19). This indirect genetic approach has also been applied to epidemiologic studies of *Mycobacterium fortuitum* isolates associated with cardiac bypass surgery when specific sources of infection were located (22).

In the present study we used MEE to analyze the genetic diversity among isolates of *M. avium* serotypes 4 and 8 from patients with AIDS and identified enzyme markers for future epidemiologic studies. The geographic distribution of various ETs was also evaluated.

## MATERIALS AND METHODS

**Isolates.** The strains used in this study are listed in Table 1. Seventy-nine isolates of *M. avium* were analyzed by MEE to determine the relationships between strains and the diversity within serotypes of the species. Seventy-five isolates were from patients with AIDS; 56 were identified as serotype 4 and 19 were identified as serotype 8. Two reference strains that were used to produce typing antisera for each serotype were also examined. Isolates were originally submitted to us from medical centers in New York City, Los Angeles, and San Francisco as part of a previous study (26).

**Preparation of extracts.** Each isolate was grown for 2 to 3 weeks in 200 ml of Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) at 35°C. Cells were harvested by centrifugation, and the pellet was suspended in 40 ml of 0.01 M phosphate-buffered saline (pH 7.2). The cell suspension was transferred to a 50-ml Oak Ridge centrifuge tube (Nalge Co., Rochester, N.Y.) and was centrifuged at 15,000 × *g* for 15 min. The washed pellet was stored at −20°C overnight. The pellet was suspended in 2 ml of breaking buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP [pH 6.8]), and 1 ml of glass beads from 75 to 150 μm in diameter (Sigma Chemical Co., St. Louis, Mo.) was added. The tube was tightly sealed, and the cells were sonicated for 5 min in a model 431A Cup Horn accessory attached to a model W-380 cell disrupter (Heat Systems-Ultrasonics, Farmingdale, N.Y.) by using 5-s cycles with a 60% pulse. Cooling was achieved by circulating 50% methanol at −10°C through the Cup Horn accessory. The lysate was centrifuged at 15,000 × *g* for 15 min at 4°C, and the supernatant was filtered through a 0.45-μm-pore-size Millex-HA filter unit (Millipore Corp., Bedford, Mass.). The filtered lysate containing enzymes was stored at −70°C.

**Starch gel electrophoresis.** Enzymes were separated on 11% starch gels and were stained by methods described by Selander et al. (18). We used a Tris-citrate buffer system (pH 8.0) for all our enzymes. After initial screening for the presence of 72 different enzymes, 16 enzyme systems that gave good resolution upon staining were selected for use in the typing of strains. The enzymes are listed in Table 2. Diaphorase (NADH oxidizing form) was stained by the method of Harris and Hopkinson (8). We detected benzyl alcohol dehydrogenase by using our own staining solution containing 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. Esterase 1 was detected by using α-naphthyl acetate as the substrate, and esterase 2 was active by using α-naphthyl proprionate as the substrate. A second prominent band sometimes appeared when we stained for either staining esterase 1 or 2. This enzyme band was considered to be due to expression by another locus; thus, alleles at a total of 17 enzyme loci were used to type the strains. Variations in the mobility of an enzyme were recorded by assigning ascending allele numbers on the basis of increasing migration toward the anode. An absence of activity for an enzyme was recorded as 0 and

* Corresponding author.

TABLE 1. Characteristics of 79 *M. avium* serotype 4 and serotype 8 isolates

| ET | Isolate no. | City | Serotype |
|---|---|---|---|
| 1 | CDC86-8677 | San Francisco | 4 |
| 2 | P55 | Reference strain | 4 |
| 3 | CDC86-8879 | San Francisco | 4 |
| 4 | CDC86-8613 | New York | 8 |
| 5 | CDC86-8812 | Los Angeles | 4 |
| 6 | CDC85-8744 | New York | 4 |
| 7 | CDC84-8748 | San Francisco | 4 |
| 8 | CDC86-8807 | Los Angeles | 8 |
| 8 | CDC86-8878 | San Francisco | 4 |
| 9 | CDC85-8740 | Los Angeles | 8 |
| 9 | CDC85-8743 | Los Angeles | 8 |
| 9 | CDC86-8810 | Los Angeles | 8 |
| 9 | CDC84-8860 | Los Angeles | 4 |
| 10 | CDC85-8795 | San Francisco | 4 |
| 11 | CDC86-8913 | San Francisco | 4 |
| 12 | CDC86-8679 | San Francisco | 4 |
| 13 | CDC86-8814 | Los Angeles | 4 |
| 14 | CDC85-8742 | Los Angeles | 4 |
| 15 | CDC85-8738 | Los Angeles | 8 |
| 15 | 13528-1079 | Reference strain | 4 |
| 16 | CDC86-8698 | New York | 8 |
| 17 | CDC86-8607 | New York | 4 |
| 17 | CDC86-8880 | San Francisco | 4 |
| 18 | CDC86-8750 | New York | 4 |
| 19 | CDC85-8793 | San Francisco | 8 |
| 19 | CDC85-8796 | San Francisco | 8 |
| 19 | CDC86-8676 | San Francisco | 8 |
| 19 | CDC86-8678 | San Francisco | 8 |
| 19 | CDC86-8849 | San Francisco | 8 |
| 19 | CDC86-8907 | San Francisco | 8 |
| 19 | CDC86-8813 | Los Angeles | 8 |
| 19 | CDC86-8984 | New York | 8 |
| 19 | ATCC 23435 | Reference strain | 8 |
| 19 | SJB 2 | Reference strain | 8 |
| 20 | CDC86-8751 | New York | 4 |
| 21 | CDC86-8737 | New York | 4 |
| 22 | CDC85-8773 | New York | 4 |
| 22 | CDC85-8786 | New York | 4 |
| 22 | CDC85-8804 | New York | 4 |
| 22 | CDC85-8806 | New York | 4 |
| 22 | CDC86-8632 | New York | 4 |
| 22 | CDC86-8637 | New York | 4 |
| 22 | CDC86-8640 | New York | 4 |
| 22 | CDC86-8642 | New York | 4 |
| 22 | CDC86-8643 | New York | 4 |
| 22 | CDC86-8646 | New York | 4 |
| 22 | CDC86-8651 | New York | 4 |
| 22 | CDC86-8708 | New York | 4 |
| 22 | CDC86-8742 | New York | 8 |
| 22 | CDC86-8746 | New York | 4 |
| 22 | CDC86-8748 | New York | 4 |
| 22 | CDC86-8749 | New York | 4 |
| 22 | CDC86-8763 | New York | 4 |
| 22 | CDC86-8764 | New York | 4 |
| 22 | CDC86-8897 | New York | 4 |
| 22 | CDC86-8676 | San Francisco | 4 |
| 22 | CDC86-8847 | San Francisco | 4 |
| 22 | CDC84-8864 | Los Angeles | 4 |
| 22 | CDC85-8728 | Los Angeles | 4 |
| 23 | CDC86-8645 | New York | 4 |
| 24 | CDC86-8762 | New York | 4 |
| 24 | CDC86-8823 | New York | 4 |
| 24 | CDC86-8804 | San Francisco | 4 |
| 24 | CDC85-8589 | Los Angeles | 4 |
| 25 | CDC85-8808 | New York | 4 |
| 25 | CDC86-8633 | New York | 4 |
| 25 | CDC86-8760 | San Francisco | 4 |
| 26 | CDC85-8674 | New York | 8 |
| 27 | CDC86-8738 | New York | 4 |
| 28 | CDC86-8740 | New York | 4 |
| 29 | CDC86-8608 | New York | 4 |
| 30 | CDC85-8764 | New York | 4 |
| 31 | CDC86-8739 | New York | 4 |
| 32 | CDC86-8647 | New York | 4 |
| 33 | CDC86-8644 | New York | 4 |
| 34 | CDC86-8653 | New York | 8 |
| 35 | CDC86-8808 | Los Angeles | 8 |
| 36 | CDC86-8609 | New York | 4 |
| 37 | CDC86-8650 | New York | 4 |

was considered to be due to a null allele. Each strain was assigned an ET number on the basis of its profile of allele numbers.

**Statistics.** The variation in mobility of each enzyme was expressed as genetic diversity per locus and was calculated as described by Selander et al. (18). Genetic relationships among ETs were demonstrated by a dendrogram generated by the average-linkage method of clustering from a matrix of coefficients of weighted distance (18, 20) based on 17 enzymes by using a SAS/GRAPH software developed by Jacobs (12).

## RESULTS

On the basis of the mobilities of 17 constituent enzymes, strains of *M. avium* serotypes 4 and 8 were separated into 37 distinct ETs. Each isolate is listed according to ET, geographical location, and serotype in Table 1. Thirty-one ETs were found among 58 isolates of *M. avium* serotype 4, and 10 ETs were identified among 21 isolates of *M. avium* serotype 8. One ET (ET 22) was found among 22 (39%) of the serotype 4 isolates from patients with AIDS. Each of our two serotype 4 reference strains had ETs (ETs 2 and 15) that did not match any ET found among serotype 4 isolates from patients with AIDS. However, one serotype 4 reference strain had the

TABLE 2. Enzymes studied from *M. avium* isolates

| EC no.[a] | Enzyme | Abbreviation |
|---|---|---|
| 1.1.1.37 | Malate dehydrogenase | MDH |
| 1.1.1.42 | Isocitrate dehydrogenase | IDH |
| 1.1.1.44 | Phosphogluconate dehydrogenase | PGD |
| 1.1.1.49 | Glucose-6-phosphate dehydrogenase | G6P |
| 1.2.1.7 | Benzyl alcohol dehydrogenase | BAD |
| 1.6.99.3 | Diaphorase (NADH) | DIA |
| 1.15.1.1 | Indophenol oxidase | IPO |
| 2.6.1.1 | Glutamate oxaloacetic transaminase | GOT |
| 2.7.4.3 | Adenylate kinase | ADK |
| 2.7.5.1 | Phosphoglucomutase | PGM |
| 3.1.1.1 | Esterase 1 | ES1 |
| 3.1.1.1 | Esterase 2 | ES2 |
| 3.1.1.1 | Other esterase[b] | OES |
| 3.4.11.1 | Leucine aminopeptidase | LAP |
| 4.2.1.2 | Fumarase | FUM |
| 4.2.1.3 | Aconitase | ACO |
| 5.3.1.9 | Phosphoglucose isomerase | PGI |

[a] EC no., Enzyme Commission number.
[b] A second prominent band that sometimes appeared when gels were stained for either esterase 1 or esterase 2.

TABLE 3. Enzyme profiles of ETs for *M. avium* serotype 4 and 8 isolates

| ET | Allele no. at locus for enzyme[a]: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MDH | ACO | PGI | PGD | LAP | ES1 | ES2 | OES | BAD | IDH |
| 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 0 | 2 | 2 |
| 2 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 0 | 2 | 2 |
| 3 | 2 | 0 | 2 | 1 | 1 | 1 | 1 | 0 | 2 | 2 |
| 4 | 2 | 2 | 0 | 0 | 2 | 1 | 1 | 0 | 2 | 2 |
| 5 | 2 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 2 | 2 |
| 6 | 2 | 2 | 2 | 3 | 0 | 1 | 1 | 0 | 2 | 2 |
| 7 | 2 | 2 | 2 | 0 | 2 | 1 | 1 | 0 | 2 | 2 |
| 8 | 2 | 2 | 2 | 1 | 2 | 1 | 1 | 0 | 2 | 2 |
| 9 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 0 | 2 | 2 |
| 10 | 2 | 2 | 2 | 3 | 2 | 1 | 1 | 0 | 2 | 2 |
| 11 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 0 | 2 | 2 |
| 12 | 2 | 2 | 2 | 3 | 1 | 1 | 1 | 0 | 2 | 2 |
| 13 | 2 | 2 | 2 | 0 | 2 | 1 | 1 | 0 | 2 | 1 |
| 14 | 2 | 2 | 2 | 0 | 2 | 1 | 1 | 0 | 2 | 3 |
| 15 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 0 | 2 | 2 |
| 16 | 2 | 2 | 0 | 3 | 1 | 1 | 1 | 0 | 2 | 1 |
| 17 | 2 | 0 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 2 |
| 18 | 2 | 1 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 2 |
| 19 | 2 | 2 | 2 | 1 | 2 | 1 | 1 | 0 | 1 | 2 |
| 20 | 2 | 2 | 2 | 3 | 2 | 1 | 1 | 0 | 1 | 2 |
| 21 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 0 | 1 | 2 |
| 22 | 2 | 2 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 2 |
| 23 | 2 | 2 | 2 | 3 | 0 | 1 | 1 | 0 | 1 | 2 |
| 24 | 2 | 2 | 2 | 0 | 1 | 1 | 1 | 0 | 1 | 3 |
| 25 | 2 | 2 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 3 |
| 26 | 2 | 2 | 2 | 0 | 1 | 1 | 1 | 2 | 1 | 3 |
| 27 | 2 | 2 | 2 | 0 | 1 | 1 | 1 | 1 | 1 | 2 |
| 28 | 2 | 0 | 1 | 3 | 1 | 1 | 1 | 0 | 2 | 3 |
| 29 | 2 | 0 | 2 | 2 | 1 | 2 | 2 | 0 | 2 | 2 |
| 30 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 0 | 2 | 2 |
| 31 | 2 | 2 | 2 | 3 | 1 | 2 | 2 | 0 | 2 | 2 |
| 32 | 2 | 2 | 1 | 3 | 2 | 2 | 2 | 0 | 2 | 2 |
| 33 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 2 |
| 34 | 2 | 2 | 0 | 1 | 1 | 2 | 2 | 0 | 2 | 3 |
| 35 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 0 | 2 | 3 |
| 36 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 0 | 2 | 3 |
| 37 | 2 | 2 | 1 | 3 | 1 | 2 | 2 | 1 | 2 | 2 |

[a] See Table 2 for enzyme abbreviations. Enzymes not listed are diaphorase, phosphogluconate dehydrogenase, indophenol oxidase, glucose-6-phosphate dehydrogenase, fumarase, adenylate kinase, and glutamate oxaloacetic transaminase, all of which did not vary in mobility on starch gels.

same ET as a serotype 8 isolate (ET 15). Our two serotype 8 reference strains shared the same ET with eight other serotype 8 isolates from patients with AIDS (ET 19), and this ET was found among 42% of the serotype 8 isolates from these patients. ETs 22 and 19 were found in New York City, San Francisco, and Los Angeles. Together, four ETs (ETs 8, 9, 15, and 22) contained both serotypes, and six ETs (ETs 8, 17, 19, 22, 24, and 25) were found in more than one city.

The individual enzyme profiles for each ET are listed in Table 3. Multiple alleles expressed variation in mobility for 10 enzymes, while 7 enzymes did not have different mobilities on starch gels.

The genetic diversity for serotype 4 and 8 ETs at each enzyme locus (probability of a mismatch between ETs at a given locus) is listed in Table 4. Diversities for all ETs ranged from 0.000 for enzymes that did not vary in mobility to 0.724 for phosphogluconate dehydrogenase. Two enzymes, malate dehydrogenase and aconitase, had genetic diversities of 0.125 and 0.391, respectively, for serotype 4 ETs but did not exhibit diversity for serotype 8 ETs. The

TABLE 4. Genetic diversity at 17 enzyme loci in ETs for *M. avium* serotypes 4 and 8

| Enzyme locus[a] | Probability of a mismatch between ETs | | |
|---|---|---|---|
| | Serotype 4 ($n$ = 31)[b] | Serotype 8 ($n$ = 10) | Total ($n$ = 37) |
| MDH | 0.125 | 0.000 | 0.105 |
| ACO | 0.391 | 0.000 | 0.336 |
| PGI | 0.323 | 0.600 | 0.405 |
| PGD | 0.699 | 0.822 | 0.724 |
| LAP | 0.583 | 0.533 | 0.563 |
| ES1 | 0.361 | 0.356 | 0.378 |
| ES2 | 0.361 | 0.356 | 0.378 |
| OES | 0.125 | 0.200 | 0.156 |
| BAD | 0.426 | 0.467 | 0.429 |
| IDH | 0.333 | 0.600 | 0.429 |

[a] See Table 2 for enzyme abbreviations. Enzymes not listed are diaphorase, phosphogluconate dehydrogenase, indophenol oxidase, glucose-6-phosphate dehydrogenase, fumarase, adenylate kinase, and glutamate oxaloacetic transaminase, all of which did not exhibit genetic diversity.
[b] $n$ = is number of ETs.

mean genetic diversity per locus (probability of a mismatch at the average locus) for all ETs was 0.230.

Genetic distances between ETs are shown in the dendrogram in Fig. 1. All the isolates were closely related, clustering at a genetic distance of less than 0.250. The major ET for serotype 4 (ET 22) and the major ET for serotype 8 (ET 19) were separated by a genetic distance of only 0.05. Most other isolates were scattered throughout the dendrogram, but there were low concentrations of serotype 4 at ET 17 (two isolates), ET 24 (four isolates), and ET 25 (three isolates) and of serotype 8 at ET 9 (three isolates).

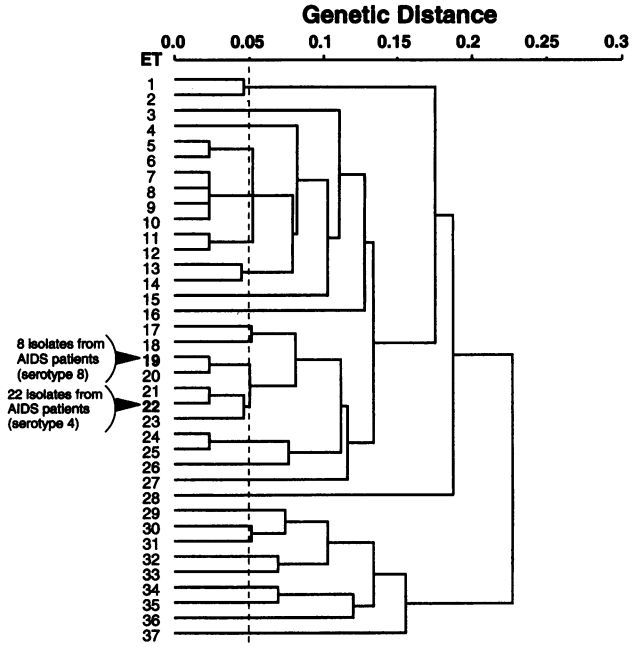


FIG. 1. Dendrogram showing the relationships among 37 ETs for isolates of *M. avium* serotypes 4 and 8. The major ET for serotype 8 isolates (ET 19) and the major ET for serotype 4 isolates (ET 22) were separated by a genetic distance of 0.05 (dashed line).

## DISCUSSION

*M. avium* serotype 4 and 8 isolates from patients with AIDS were further characterized by MEE. Serotypes 4 and 8 each contained a single major ET (ETs 22 and 19, respectively), with other isolates belonging to 35 additional ETs. The low mean genetic diversity per locus (0.230) indicates that all the isolates were closely related. This supports previous observations that almost all serotype 4 and 8 isolates from patients with AIDS belong to a single restriction fragment length polymorphism type (7). MEE can detect differences in enzyme mobility resulting from changes in the primary structure caused by individual amino acid substitutions (18, 19). This ability to detect subtle differences between virtually identical strains makes MEE a powerful epidemiologic tool.

The major ETs for serotypes 4 and 8 were isolated from patients in New York City, San Francisco, and Los Angeles. *M. avium* complex isolates, identified as either serotype 4 or 8, have been found in various aquatic environments along the eastern United States coastline (5). One of our serotype reference strains (SJB 2) was originally isolated from a bull and is ET 19, the predominant serotype 8 ET. Serotypes 4 and 8 have also been isolated from farm animals in Germany (15). It is apparent that these serotypes are not geographically isolated, and the source of human infection could be either food or drink. MEE analysis of serotype 4 and 8 isolates from the environment and food may reveal specific sources of the major pathogenic ETs. Whatever the source of infection, it is evident that these particular strains possess biological factors that give them a pathogenic edge over the other *M. avium* serotypes in colonizing and invading host tissues (1).

Only 10 of 17 enzymes exhibited variation for serotype 4 and 8 isolates. Since each starch gel yields five to six slices for enzyme reactions, at least two gels are needed to determine the ETs of these isolates. Wasem et al. (24) examined 25 strains of *M. avium* by MEE, including representatives of serotypes 1, 2, 3, 4, 8, 9, and 10. In that study, only 9 of 20 enzymes exhibited variation in mobility among the ETs assigned to *M. avium* serotypes. As in our study, more than one serotype shared the same ET. All the *M. avium* strains were found to be closely related, clustering at a genetic distance of 0.17.

*M. avium* serotypes reflect differences in surface antigens under the structural control of genetic loci that code for enzymes which probably are not the same as those analyzed by MEE. It is not surprising that these genetically close strains of serotypes 4 and 8 sometimes shared the same ET. In such cases, serology, when successful, may be useful for separating strains with identical enzyme patterns.

Neither of our serotype 4 reference strains used to produce antisera was ET 22, the most frequently isolated ET from patients with AIDS. At least one isolate of ET 22 should be included as a reference strain in future *M. avium* studies and for the production of additional typing antisera. Both of our serotype 8 reference strains were ET 19, the most common ET for serotype 8, and these remain suitable for future work.

While our observations (unpublished data) agree with those of Wasem et al. (24) that MEE can easily separate *M. avium* from *M. intracellulare*, commercially available probes can also distinguish these species (16, 17). The primary applications for MEE should involve either subtyping of organisms for epidemiologic studies or analysis of the genetic relatedness among similar mycobacterial strains to accurately determine their classification by species clusters. Secondarily, MEE would help define new species of mycobacteria from strains that do not cluster into known groups.

Other methods for typing *M. avium* isolates appear to have limited applications. Although Crawford et al. (2) reported plasmids in all isolates of serotypes 4 and 8 they examined from patients with AIDS, other studies concluded that less than 50% of all *M. avium* isolates from such patients contained plasmids (10, 13). Patterns from restriction enzyme analysis are often difficult to interpret and may be distinguishable neither between nor within serotypes (7, 23, 25). MEE can be used to type strains regardless of plasmid content and may differentiate isolates that appear to be identical by restriction enzyme analysis.

Our results demonstrated that *M. avium* serotype 4 or 8 can be subtyped by MEE into several ETs. The ability of MEE to find markers for these organisms is a prerequisite for epidemiologic studies that seek to match isolates from patients with specific infection sources. *M. avium* serotype 4 has been isolated from several hospital sources, including laboratory reagents used to process specimens, tap water, and hot water systems (4, 6). The application of MEE to characterize this and other serotypes further would help to confirm specific sources of either contamination or infection. Since more than one serotype can share an ET, MEE should be performed in addition to but should not completely replace serologic testing for characterization of *M. avium* isolates. However, MEE will be heavily relied upon for the typing of isolates that either autoagglutinate or fail to react with antisera.

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