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Use of Restriction Fragment Length Polymorphisms Resolved by Pulsed-Field Gel Electrophoresis for Subspecies Identification of Mycobacteria in the *Mycobacterium avium* Complex and for Isolation of DNA Probes

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Mycobacterial strains from the *Mycobacterium avium* complex were compared with each other and with *Mycobacterium phlei* isolates by restriction endonuclease digestion of chromosomal DNA with *Ssp*I and analysis by pulsed-field gel electrophoresis. Characteristic profiles were observed for known typed strains, and five groups were identified. Primary bovine isolates identified as *Mycobacterium paratuberculosis* by classical methods were shown to fall into both the *M. paratuberculosis*- and *M. avium*-like groups. *M. paratuberculosis* 18 was in the latter category. Two *Mycobacterium intracellulare* strains of different Schaefer serotypes had different digestion profiles. In addition, this system was exploited for the preparation of DNA probes by the isolation, digestion, and subcloning of DNA fragments separated by pulsed-field gel electrophoresis. Probe JC12 hybridized only to *M. avium* complex strains, but not to *M. phlei*, showing characteristic hybridization profiles for each of the groups previously identified by pulsed-field gel electrophoresis. The approach taken in the study lends itself to the comparative analysis of members of the *M. avium* complex and to the isolation and characterization of DNA probes with specificity for these mycobacteria.

Organisms of the *Mycobacterium avium* complex are ubiquitous in the environment. These slow-growing mycobacteria include strains of *M. avium* and *Mycobacterium intracellulare* (16, 32, 36). *M. avium* complex strains can cause infections of the human lungs, lymph nodes, skin, bones, soft tissues, and the genitourinary tract (16). In addition, *M. avium* complex infections have become commonplace in immunocompromised populations such as patients with AIDS (12, 16). *Mycobacterium paratuberculosis* is a very closely related organism, although it is not always considered a member of the *M. avium* complex. It is the causative agent of Johne's disease (or paratuberculosis), a debilitating, wasting, and currently untreatable disease of cattle, sheep, and other ungulates (8). Furthermore, *M. paratuberculosis* has been implicated in the etiology of human Crohn's disease (7), although this remains controversial.

Accurate species identification and diagnosis of members of the *M. avium* complex, although extremely important, have proved to be problematic. Traditionally, *M. avium* and *M. intracellulare* were differentiated from each other on the basis of sensitin testing and pathogenicity in chickens, with the former species being virulent while the latter is not (1, 36). An intermediate group of isolates with mild or variable pathogenicity was also recognized, leading to some ambiguity (1, 36). *M. avium* complex strains have also classically been examined by biochemical testing. *M. paratuberculosis* can be differentiated from *M. avium* and *M. intracellulare* by its dependence on an exogenously added iron chelator, mycobactin (8, 16, 41). However, some strains of *M. avium* also require mycobactin on primary isolation, and mycobactin dependence can be lost following serial subculture. All the *M. avium* complex organisms, including *M. paratuberculosis*, however, grow very slowly, and their low metabolic rates complicate biochemical characterizations (5, 8, 16). Growth characteristics and biochemical profiles of large numbers of strains have been subjected to numerical taxonomic analysis, but this has failed to differentiate between members of the *M. avium* complex (26).

Schaefer (36) introduced a seroagglutination test for the characterization of nontuberculous mycobacteria on the basis of the presence of species- or type-specific antigens on the cell surface. In that system, *M. avium* strains fell into serotypes 1, 2, and 3. *M. intracellulare* isolates accounted for an additional 25 serotypes (4 to 28), of which serotypes 4, 5, 6, 8, 9, 10, and 11 belonged to the intermediate group identified in virulence studies (36, 47). However, DNA hybridization (2, 3) and DNA probe (34) analyses suggest other groupings of the serotypes may be more appropriate. Moreover, *M. paratuberculosis* and other mycobactin-dependent strains appear to be missing the serotype antigen and are not typeable by this method (4).

Several groups of investigators have analyzed the relationship among strains of the *M. avium* complex by DNA hybridization (2, 3, 19, 20, 24, 35, 49). A certain degree of hybridization, variously defined as ranging from >60 to >80%, indicates isolates of a single species. By this method, members of the *M. avium* complex could be divided into two groups (2, 3, 20). The first consisted of *M. avium* strains; *M.*

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intracellulare serotypes in the intermediate virulence category; and *M. paratuberculosis* isolates from diverse sources including humans, birds, and animals. The second hybridization group consisted of the remaining *M. intracellulare* serotypes (3, 19, 24, 35, 49).

The genetic relationship between *M. avium* complex strains has also been examined by restriction fragment length polymorphism (RFLP) analysis. This approach appeared to differentiate between *M. paratuberculosis* and *M. avium* serotype 2 strains (21, 28, 44, 46). However, both *M. paratuberculosis* 18 (which has been used for Johne's disease vaccine production) and several *M. paratuberculosis* isolates from wood pigeons displayed greater similarity to *M. avium* profiles than to those of *M. paratuberculosis*. *M. intracellulare* strains appeared to form a separate, but more heterogeneous, group, with there being a number of differences between the various serotypes (21, 44, 46).

In an attempt to simplify the interpretation of restriction digest profiles and in the interest of diagnosis, several groups have developed DNA probes for the *M. avium* complex. Whole chromosomal and large fragment probes which can be used to differentiate the *M. avium* complex from *Mycobacterium tuberculosis* and *M. avium* from *M. intracellulare* have been described (20, 29–31). Radiolabeled probes complementary to rRNA sequences which are specific for *M. avium*, *M. intracellulare*, and *M. tuberculosis* are manufactured commercially by Gen-Probe Corporation, San Diego, Calif. Many studies have confirmed the usefulness of these reagents with clinical samples, although individual serotypes could not be differentiated (13, 14, 34, 38). In addition, the Gen-Probe *M. avium* complex probe reacts with *M. paratuberculosis* (42). Hybridization of the *Escherichia coli* 5S rRNA gene to restriction digests from multiple isolates of *M. paratuberculosis* and several *M. avium* serotypes has also suggested that this group is genetically similar (6).

Several investigators have reported the isolation of *M. paratuberculosis* probes which were derived from repetitive sequences in the genome and which showed some specificity for this organism (10, 11, 18, 23, 25, 45). McFadden and coworkers (23, 25) isolated a number of probes from a isolate from a patient with Crohn's disease, *M. paratuberculosis* Ben, which, in combination with restriction enzyme digestion, could differentiate among *M. paratuberculosis*, *M. avium*, and intermediate serotypes and other serotypes of *M. intracellulare*. One of the probes, pMB22, hybridized to multiple bands in the *M. paratuberculosis* genome. A related oligonucleotide probe has also been reported (17, 45). Collins and coworkers (10, 11) have described a different 0.2-kb probe, pMP103, which was derived from a repetitive sequence and which appeared to be highly specific for *M. paratuberculosis* isolates.

In the present study, we examined a number of mycobacterial strains by restriction endonuclease digestion of DNA prepared in agarose; this was followed by pulsed-field gel electrophoresis (PFGE) with a view to addressing the characterization of *M. avium* complex isolates. This approach allowed the resolution of well-defined RFLP patterns representative of the entire mycobacterial genome and facilitated the comparative analysis of different isolates. In addition, the isolation and characterization of DNA probes derived from the fragments resolved by PFGE are described.

(A preliminary presentation of some of the results reported here was given at the Annual Meeting of the American Society for Microbiology, New Orleans, La., 14 to 18 May 1989.)

TABLE 1. Mycobacterial strains used in this study

Strain	Origin	Source[a]
M. avium ATCC 35712, serotype 2	Avian	1
M. intracellulare ATCC 13950, type strain, serotype 16	Human	1
M. intracellulare ATCC 35771, serotype 8	Bovine	1
M. paratuberculosis ATCC 19698, type strain	Bovine feces	1
M. paratuberculosis ATCC 12227, strain 18	Bovine	2
M. paratuberculosis ATCC 43015, strain Linda	Human ileum	1
M. paratuberculosis C286	Bovine	3
M. paratuberculosis CBM 313, primary isolate	Bovine feces	2
M. paratuberculosis CBM 325, primary isolate	Bovine feces	4
M. paratuberculosis CBM 327, primary isolate	Bovine feces	4
M. phlei ATCC 11758, type strain		1
M. phlei ATCC 35784	Hay	1
M. phlei ATCC 27086		1

[a] Sources: 1, American Type Culture Collection, Rockville, Md.; 2, W. D. Richards, Allied Laboratories Inc., Glenwood Springs, Colo.; 3, E. A. Sugden, Animal Disease Research Institute, Nepeam, Ontario, Canada; 4, this study.

MATERIALS AND METHODS

Mycobacterial strains and growth conditions. The mycobacterial strains used in this study are described in Table 1. Primary culture of mycobacteria from bovine fecal material was performed by a modification of the method of the U.S. Department of Agriculture (43). Briefly, fecal samples were held at room temperature for 48 h and were then treated with 3% benzalkonium chloride (45 ml/g of feces) for 30 min. Particulate material was allowed to settle overnight, and the particulate-free supernatant was used for inoculation. Initial cultures were routinely made on Herrold's egg yolk medium (Remel Laboratories) in the presence and absence of mycobactin J (Allied Laboratories), and cultures were incubated at 37°C for 6 weeks to 6 months. Routine culture of *M. paratuberculosis*, *M. avium*, *M. intracellulare*, and all primary isolates was performed in Middlebrook 7H9 broth (pH 5.9; Difco Laboratories) at depths ranging from 3 to 10 mm or on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 10% (wt/vol) oleic acid-albumin-glucose-catalase (GIBCO/BRL) and 0.05% Tween 80. Mycobactin J was added, when appropriate, to a final concentration of 2 mg/liter. *M. phlei* was grown as outlined above, but media were maintained at pH 6.7 and mycobactin J was omitted. All incubations were performed at 37°C without agitation. Classical biochemical tests for the identification of slow-growing mycobacteria were performed as described previously (5, 9) or according to the kit manufacturers' instructions. For the preparation of DNA, cultures were grown to an optical density at 540 nm of 0.2 to 0.5, at which point D-cycloserine was added to a final concentration of 1 mg/ml. Growth was continued for an additional 18 to 24 h for *M. paratuberculosis*, *M. avium*, *M. intracellulare*, and all primary isolates and for 2.5 to 4 h for *M. phlei*.

Isolation of DNA and agarose insert preparation. DNA was prepared in solution by the procedure of Whipple et al. (46). Large-scale and minipreparations of DNA were made by previously published methods (22). The DNA in agarose

inserts was prepared by a combination of the methods of Whipple et al. (46) and Smith et al. (39). Cells were harvested by centrifugation at 1,400 × *g* for 15 min at 4°C, washed once in 50 mM Tris-HCl (pH 8.0)–100 mM sodium EDTA–150 mM NaCl–0.05% Tween 80 (TEN buffer), and resuspended in the same buffer to a concentration of 100 to 200 mg (wet weight) of cells per ml. Equal volumes of the cell suspension and low-gelling-temperature agarose (1.5% agarose in 0.2 M sodium EDTA; SeaPlaque) were mixed and 100-μl aliquots were dispensed into insert molds. Inserts were suspended in 20 volumes of TEN buffer without Tween 80, lipase (L-4384; Sigma) was added to a final concentration of 20,000 U/ml, and the inserts were incubated at 37°C for 24 h. Removal of cellular components was performed by treatment with detergents, nucleases, lysozyme, and proteinase K as described by Smith et al. (39), except that lysozyme (50,000 U/mg; Boehringer Mannheim) was used at a concentration of 2 mg/ml. Inserts were washed in 50 volumes of 10 mM Tris-HCl (pH 8.0)–100 mM sodium EDTA–1 mM phenylmethylsulfonyl fluoride for 1 h at room temperature, and the wash was repeated three times under the same conditions but by omitting phenylmethylsulfonyl fluoride from the wash buffer.

Restriction endonuclease digestion. Digestion of genomic DNA, which was prepared in solution, with restriction endonucleases was performed by established procedures (22). Restriction enzyme cleavage of the DNA in agarose inserts for PFGE was achieved as follows. Inserts were washed twice in 50 volumes of 10 mM Tris-HCl (pH 8.0)–0.1 mM sodium EDTA for 30 min at room temperature. Digests were performed on single inserts in a final volume of 200 μl by using the buffers described by the enzyme supplier. Digests were incubated at 4°C for 30 min and then for 3 h at the temperature recommended for the restriction endonuclease used. Inserts were loaded directly into slots on agarose gels for electrophoresis. Digests in preparation for Southern transfer were performed as follows. Inserts were washed twice in 10 mM Tris-HCl (pH 8.0)–0.1 mM sodium EDTA for 1 h at room temperature and were then preincubated in the appropriate restriction buffer for 30 min at 4°C. Inserts were then incubated overnight at 37°C in fresh buffer containing the restriction enzyme. After digestion, inserts were melted and loaded into gel slots.

Electrophoretic separation of DNA and Southern hybridization. Horizontal agarose gel electrophoresis of DNA was performed in 0.8% agarose by previously published procedures (22). Southern transfer to nylon membranes (Hybond N; Amersham) was performed by standard methods (22). The DNA in agarose inserts was separated by PFGE by using the LKB 2015 Pulsaphor electrophoresis unit. This apparatus was fitted with a hexagonal electrode array, thus allowing for separation to occur under contour-clamped homogeneous electric field conditions. Bacteriophage lambda DNA concatemers (New England Biolabs) were used as size markers. Gels were cast from 1% (wt/vol) agarose (ultrapure, DNA-grade; Bio-Rad) in 45 mM Tris–45 mM boric acid–1 mM sodium EDTA. Electrophoresis was performed in the same buffer at 6 V/cm with a 10-s pulse time at 12°C for 24 h.

Isolation and labeling of DNA probes. Chromosomal DNA from *M. paratuberculosis* CBM 313 in agarose inserts was digested with *Ssp*I and analyzed by PFGE, and selected DNA fragments were excised from the gel. The DNA was electroeluted (37) and was purified by phenol and chloroform-isoamyl alcohol extractions and ethanol precipitation. The isolated DNA was digested with *Bam*HI, and the resultant fragments were cloned into the vector pUC18 as described previously (22) by using competent *E. coli* DH5α (GIBCO/BRL) as the transformation recipient. Cloned inserts were isolated, labeled with ^{32}P by oligolabeling (15), and used for hybridization to Southern blots.

RESULTS

Analysis of a primary isolate of *M. paratuberculosis* and *M. avium* complex strains by restriction endonuclease digestion and electrophoretic separation of DNA. The original aim of this study was to use PFGE to isolate probes for strains of the *M. avium* complex which might be useful in the diagnosis of infection. Our initial interest was in *M. paratuberculosis*, the causative agent of Johne's disease in cattle. On the basis of the rationale that a primary isolate from a diseased animal would bear the closest resemblance to strains that were subsequently to be detected by a diagnostic probe, *M. paratuberculosis* CBM 313 was chosen as a prototype strain. This strain was isolated from a cow with rampant Johne's disease and was classified as *M. paratuberculosis* on the basis of its source, growth characteristics, mycobactin dependence, and biochemical reactivities.

By using DNA from *M. paratuberculosis* CBM 313, several different enzymes were tested to determine their suitabilities for PFGE. *Dra*I, *Hin*dIII, *Hpa*I, *Nde*I, *Sna*BI, *Spe*I, *Ssp*I, and *Xba*I mostly yielded fragments of >50 kb. The enzymes *Bam*HI, *Not*I, *Eco*RI, and *Sfi*I resulted in fragments of <23 kb. DNAs from known, typed *M. avium* complex strains were examined by using the restriction enzyme *Ssp*I for comparison with the profile of the *M. paratuberculosis* CBM 313 primary isolate. *M. phlei* was included both as a non-*M. avium* complex representative and also because *M. phlei* is common in the environmental milieu of cattle. In addition, it has previously been implicated in complicating the diagnosis of *M. paratuberculosis* infections in these animals (48). DNA samples from the five strains were digested with *Ssp*I and analyzed by PFGE (Fig. 1). Each of the four known typed strains gave a distinct pattern that was easily distinguishable from the profiles of the other organisms. However, the pattern obtained for the primary isolate *M. paratuberculosis* CBM 313 was quite different from that seen for the neotype strain *M. paratuberculosis* ATCC 19698 but was very similar to the profile for *M. avium* ATCC 35712, serotype 2.

Comparison of the DNA digests from *M. paratuberculosis* CBM 313, *M. paratuberculosis* ATCC 19698, and *M. avium* ATCC 35712 using different restriction endonucleases. The discrepancy between the *Ssp*I digestion patterns of DNA from *M. paratuberculosis* CBM 313 and *M. paratuberculosis* ATCC 19698 raised concerns that the differences seen between the typed strains or between the neotype strain and the primary isolate might have an artifactual origin arising from the use of a single restriction endonuclease, *Ssp*I. To address this question, DNAs from the *M. paratuberculosis* neotype strain, *M. paratuberculosis* CBM 313, and *M. avium* serotype 2 were digested with *Hin*dIII, *Hpa*I, *Ssp*I, and *Xba*I (Fig. 2). It is evident that the *M. paratuberculosis* neotype strain represents one group while *M. avium* serotype 2 and *M. paratuberculosis* CBM 313 represent another. This observation was consistent irrespective of the enzyme used for digestion, although different profiles were seen with different endonucleases.

***Ssp*I digestion profiles of other strains and primary isolates and grouping of mycobacterial strains.** On the basis of the observations described above, it was of interest to examine

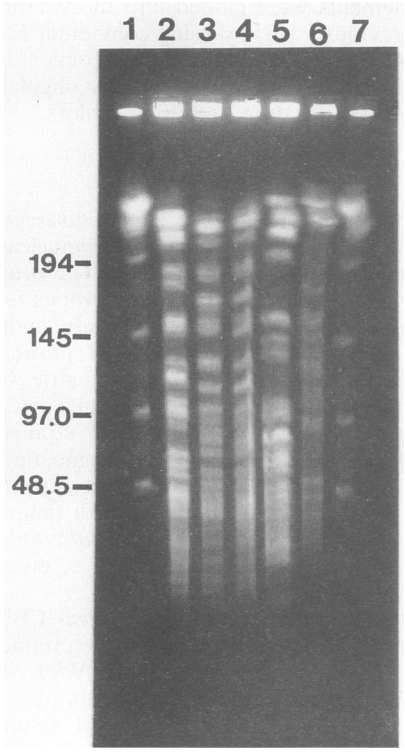

FIG. 1. *Ssp*I digestion profiles of *M. avium* complex strains. Chromosomal DNA was prepared and digested with *Ssp*I in agarose inserts and analyzed by PFGE for 24 h at 175 V with a 10-s pulse time. Lanes: 1 and 7, bacteriophage lambda DNA concatemers (sizes [in kilobases] are indicated on the left); 2, *M. paratuberculosis* ATCC 19698, neotype strain; 3, *M. paratuberculosis* CBM 313, primary isolate; 4, *M. avium* ATCC 35712, serotype 2; 5, *M. intracellulare* ATCC 13950, type strain; 6, *M. phlei* ATCC 11758, type strain.

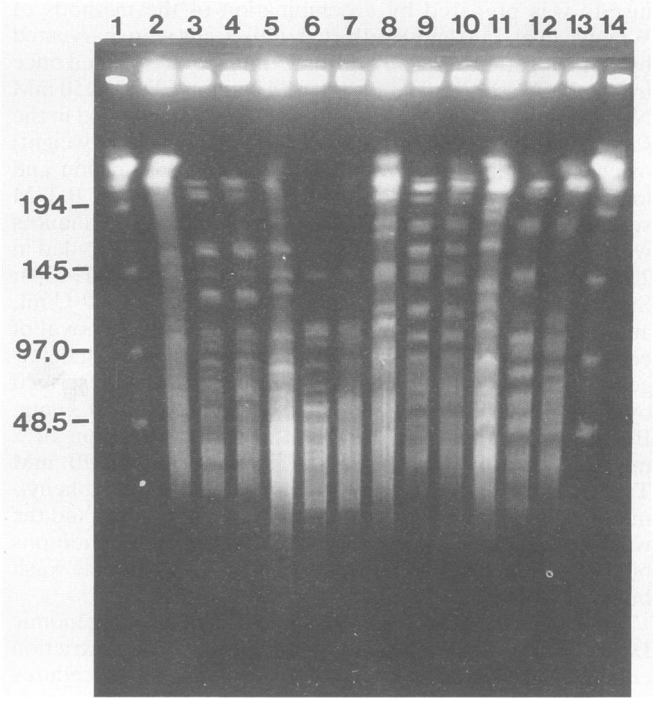

FIG. 2. Comparison of DNA digests from *M. paratuberculosis* CBM 313, *M. paratuberculosis* ATCC 19698, and *M. avium* ATCC 35712 by using different restriction endonucleases. DNA was prepared in inserts and digested with *Hpa*I (lanes 2 to 4), *Hin*dIII (lanes 5 to 7), *Ssp*I (lanes 8 to 10), and *Xba*I (lanes 11 to 13). Digested DNA was run by PFGE for 24 h at 175 V with a pulse time of 10 s. Lanes: 1 and 14, bacteriophage lambda DNA concatemers (sizes [in kilobases] are indicated on the left); 2, 5, 8, and 11, *M. paratuberculosis* ATCC 19698, neotype strain; 3, 6, 9, and 12, *M. paratuberculosis* CBM 313, primary isolate; 4, 7, 10, and 13, *M. avium* ATCC 35712.

the classification of other available strains and, in particular, the identities of isolates characterized as *M. paratuberculosis* by conventional means. DNA in inserts, prepared from several strains of *M. paratuberculosis* and *M. intracellulare*, was also included in the analysis. In all cases, DNA was digested with *Ssp*I and digests were analyzed by PFGE. The profiles that were obtained are shown in Fig. 3.

On the basis of the patterns obtained for the *Ssp*I digests, the mycobacterial strains examined were organized into five groups. These are shown in Table 2. Group 1 consisted of the *M. paratuberculosis* neotype strain, the isolate from a human with Crohn's disease, and two primary bovine isolates cultured during this study (Fig. 3, lanes 5, 6, 7, and 8, respectively). Group 2 was represented by *M. avium* serotype 2. In addition, the primary bovine isolate *M. paratuberculosis* CBM 313 and *M. paratuberculosis* 18 fell into this class (Fig. 3, lanes 2, 3, and 4, respectively). The profiles in lanes 2, 10, and 11 of Fig. 3 are distorted slightly upward because of relative loadings (compare lane 13 which is the same as lane 11 but with less DNA). The *M. intracellulare* type strain formed group 3, while the other *M. intracellulare* strain, serotype 8, made up a separate group, designated group 4 (Fig. 3, lanes 9 and 10, respectively). Surprisingly, *M. paratuberculosis* C286 (40) did not give a profile representative of that of group 1 or 2, but it appeared to be very similar to *M. intracellulare* serotype 8 and was placed in group 4. DNA samples from the two other *M. phlei* isolates gave profiles (data not shown) very similar to that of the *M. phlei* type strain (Fig. 1); the profiles of the two other *M. phlei* isolates were clearly distinct from those of the *M. avium* complex strains. They were classified as group 5.

Isolation of DNA probes and their hybridization to mycobacterial DNA. Since the different mycobacterial strains could be distinguished by their digestion patterns as resolved by PFGE, it seemed reasonable to attempt to isolate a distinguishing fragment with a view to preparing DNA probes. A DNA fragment of 110 kb from the *Ssp*I digest of *M. paratuberculosis* CBM 313 DNA was eluted from the gel and digested with *Bam*HI to give small fragments which were subsequently cloned into pUC18. Two probes, a 0.2-kb fragment designated JC10 and a 0.19-kb fragment designated JC12, were chosen for study. A Southern blot with probe JC12 is shown in Fig. 4. This probe showed no hybridization to *M. phlei* DNA. Hybridization to the *M. intracellulare* isolates was observed, but the patterns seen were distinct both from each other and from those observed for the *M. avium* and *M. paratuberculosis* groups. JC12 highlighted a single *Bam*HI fragment of 0.19 kb, presumably identical to itself, in DNA from *M. avium* ATCC 35712, serotype 2; *M. paratuberculosis* 18; *M. paratuberculosis* C286; and *M. paratuberculosis* CBM 313. The probe hybridized to a single *Bam*HI fragment of 4.4 kb in the digests of DNA from the *M. paratuberculosis* neotype strain, strain Linda, and the primary isolates CBM 325 and CBM 327. In addition, probe

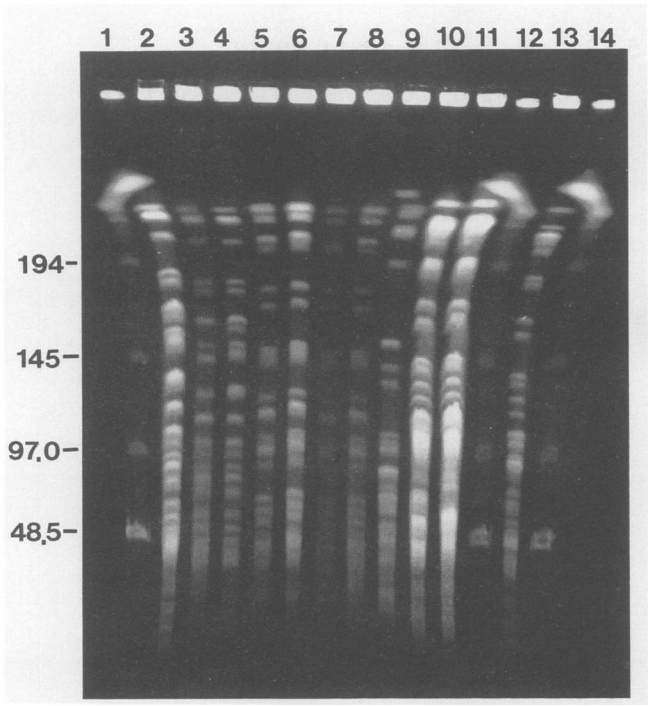

FIG. 3. *Ssp*I digestion profiles of *M. avium* complex strains and primary isolates. DNA was prepared from all strains in agarose inserts and was digested with *Ssp*I. Electrophoresis was for 24 h at 175 V with a 10-s pulse time. Lanes: 1, 12, and 14, bacteriophage lambda DNA concatemers (sizes [in kilobases] are indicated on the left) 2, *M. paratuberculosis* ATCC 12227, strain 18; 3, *M. avium* ATCC 35712, serotype 2; 4, *M. paratuberculosis* CBM 313; 5, *M. paratuberculosis* ATCC 19698, type strain; 6, *M. paratuberculosis* CBM 325, primary isolate; 7, *M. paratuberculosis* CBM 327, primary isolate; 8, *M. paratuberculosis* ATCC 43015, strain Linda; 9, *M. intracellulare* ATCC 13950, type strain; 10, *M. intracellulare* ATCC 35771, serotype 8; 11, *M. paratuberculosis* C286; 13, *M. paratuberculosis* C286.

JC10 hybridized to multiple fragments of DNA from isolates of the *M. avium* complex, resulting in patterns distinct for each group (data not shown), confirming the groupings obtained with JC12.

DISCUSSION

The mycobacteria in general, and the *M. avium* complex in particular, are a group of closely related microorganisms which do not readily lend themselves to identification or differentiation. The question of species identification within this genus and the absolute taxonomic relationship of the myriad isolates to each other would probably remain a curious, but esoteric, question were it not for the significance of these bacteria in human and animal disease. Current evidence suggests that particular strains and serotypes may predominate as causes of particular disease syndromes (18). Because of this, a classification scheme which separates closely related mycobacterial strains in a manner which reflects their natural pathogenicity is desirable. However, such subdivisions may not necessarily coincide with species or subspecies according to rigorous taxonomic definitions. Perhaps the findings of the present study and related reports in the literature should best be reviewed in light of their ability to help address a clinical problem rather than to absolutely resolve a taxonomic dilemma.

TABLE 2. Grouping of mycobacterial strains on the basis of PFGE analysis of *Ssp*I DNA digests

Group	Strain
1	*M. paratuberculosis* ATCC 19698, neotype strain
1	*M. paratuberculosis* ATCC 43015, strain Linda
1	*M. paratuberculosis* CBM 325, primary isolate
1	*M. paratuberculosis* CBM 327, primary isolate
2	*M. avium* ATCC 35712, serotype 2
2	*M. paratuberculosis* ATCC 12227, strain 18
2	*M. paratuberculosis* CBM 313, primary isolate
3	*M. intracellulare* ATCC 13950, type strain, serotype 16
4	*M. intracellulare* ATCC 35771, serotype 8
4	*M. paratuberculosis* C286
5	*M. phlei* ATCC 11758, type strain
5	*M. phlei* ATCC 35784
5	*M. phlei* ATCC 27086

Ideally, the ultimate diagnostic reagents would be a panel of probes with an exquisite sensitivity for particular subgroups of the *M. avium* complex. Probes which could be used directly on clinical samples, in which the number of bacteria is very low, would obviate the necessity for extensive mycobacterial culture, which is both laborious and time-consuming. There are two prerequisites for the development of such an ideal system, the first being a reliable and practical method for subgrouping the *M. avium* complex, to allow the characterization of new isolates and the determination of probe specificity. The second requirement is the isolation and characterization of probes which have specificities for isolates of clinical significance. Moreover, it is desirable that the baseline method of subspecies or subgroup definition be related to the innate properties of isolates and represent a classification which is independent of probes whose specificities have yet to be determined.

Meaningful subspecies identification or grouping of *M. avium* complex strains has not been achieved by either biochemical methods or crude DNA analyses such as G+C content and hybridization (2, 3, 16, 19, 20, 24, 26, 35, 49). For this reason many groups have chosen to look at RFLPs and reactivities with DNA probes (21, 28–31, 44, 46). It must be stated that the interpretation and comparison of RFLPs by conventional technology are complicated by the limitations of the system. Mycobacterial DNA prepared in solution undergoes considerable shearing. It is only practical to use restriction endonucleases which give fragments in the size range of 0.5 to 25 kb. As a consequence, the profiles obtained are exceedingly complex and often smeared. Individual DNA fragments are not usually resolved and thus cannot conveniently be purified for the preparation of probes. Lévy-Frébault and coworkers (21) addressed this problem by examining RFLPs in *M. avium* complex strains using field inversion gel electrophoresis, a method which allows the separation of high-molecular-weight DNA.

In the present study, we used the restriction endonuclease patterns of mycobacterial DNA resolved by PFGE as a baseline method for the subgrouping of *M. avium* complex strains. The use of PFGE overcomes several of the problems of conventional electrophoresis and, like the field inversion method, has the added advantage that large DNA fragments representing a significant proportion of the total cellular DNA are compared. This is particularly useful when very closely related strains are examined. Moreover, the clear resolution of distinct bands of DNA by PFGE allowed the selection and purification of particular restriction endonuclease fragments for probe isolation.

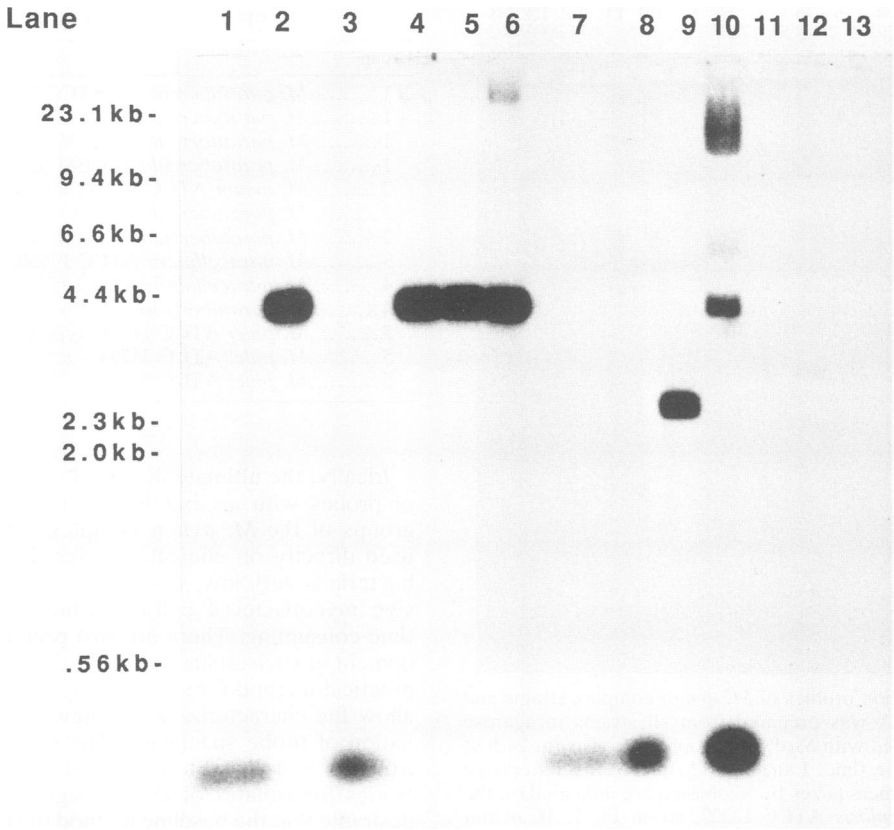

FIG. 4. Hybridization of ^{32}P-labeled probe JC12 to *Bam*HI-digested DNA of mycobacterial strains. DNA was prepared in solution, digested, and run by conventional electrophoresis; and hybridizations were performed as described in the text. Bacteriophage lambda DNA digested with *Hin*dIII was run as a marker, and sizes (in kilobases) are indicated on the left. Lanes: 1, *M. avium* ATCC 35712, serotype 2; 2, *M. paratuberculosis* ATCC 19698, neotype strain; 3, *M. paratuberculosis* CBM 313, primary isolate; 4, *M. paratuberculosis* CBM 325, primary isolate; 5, *M. paratuberculosis* CBM 327, primary isolate; 6, *M. paratuberculosis* ATCC 43015, strain Linda; 7, *M. paratuberculosis* ATCC 12227, strain 18; 8, *M. paratuberculosis* C286; 9, *M. intracellulare* ATCC 13950, type strain, serotype 16; 10, *M. intracellulare* ATCC 35771, serotype 8; 11, *M. phlei* ATCC 11758, type strain; 12, *M. phlei* ATCC 35784; 13, *M. phlei* ATCC 27086.

M. avium complex strains were successfully separated into different groups. Each of the defined neotype or type strains analyzed gave a different, distinguishable restriction enzyme profile. While this was not surprising in the case of *M. phlei* versus *M. avium* complex strains, it confirmed that the method was also capable of differentiating among *M. avium* serotype 2, the *M. paratuberculosis* neotype strain, and *M. intracellulare* serotype 16. *M. paratuberculosis* Linda (the isolate from a human with Crohn's disease) and *M. paratuberculosis* 18 gave profiles similar to those of the *M. paratuberculosis* type strain and *M. avium* serotype 2, respectively. This is consistent with previous documentation in the literature (21, 23, 25, 27, 46) and supports the view that strain 18 is really an *M. avium* isolate, even though it is of bovine origin and has been used as a vaccine strain for Johne's disease.

The observations made on the primary bovine isolates analyzed in this study are interesting, both in their own light and in view of the history of strain 18. We examined three strains which were isolated from bovine feces in essentially identical fashion and identified as *M. paratuberculosis* by conventional means, including growth properties, mycobactin dependence, biochemical reactivities, and antibiotic susceptibilities. Two of these isolates, *M. paratuberculosis* CBM 325 and CBM 327, had restriction patterns on PFGE which were identical to that observed for DNA from the *M. paratuberculosis* type strain. However, the third isolate, *M. paratuberculosis* CBM 313, clearly belonged to the *M. avium* group. Ironically, this strain was deliberately chosen for study because it was a primary bovine isolate. It now seems obvious that strains of both *M. paratuberculosis* and *M. avium* can be isolated with equivalent ease from the feces of cattle suffering from *M. paratuberculosis* infections. However, their identities are easily confused because of misleading classification based on the observation of phenotypic properties such as mycobactin dependence. The significance of *M. avium*-like strains in the diagnosis and management of Johne's disease remains uncertain because of the difficulty of species identification within this group. The role of these organisms in the etiology of bovine and human *M. avium* complex infections will be elucidated only when this problem is resolved.

The grouping of *M. paratuberculosis* C286 and *M. intracellulare* serotype 8 is also of interest, particularly since their apparent similarity to each other has not been reported previously. However, the former strain has not been included in many of the DNA analyses described in the literature, making it difficult to judge the significance of this observation. Disconcertingly, *M. paratuberculosis* C286 behaved as an *M. avium*-like strain with respect to its profile of

hybridization to the probes JC10 and JC12. The reason for this anomaly remains unclear, although it may be that *M. paratuberculosis* C286 possesses some relationship to both groups and may react differentially with different probes. If nothing else, this discrepancy serves to underline the desirability of having a standard subgrouping scheme which reflects the inherent characteristics of the bacterial strains and which is independent of the characteristics of the probe whose specificity is being investigated.

The separation by PFGE of *M. intracellulare* serotype 16 and serotype 8 demonstrates, at least at a superficial level, that the approach of analyzing RFLPs by PFGE is capable of the differentiation of two *M. intracellulare* strains with different Schaefer serotypes. Furthermore, discrimination of the two types was also confirmed by the hybridization patterns obtained with probes JC10 and JC12. These complementary observations are consistent with the reported level of base substitution within the *M. intracellulare* group and support the suggestion that this group may comprise diverse isolates (25). It is clear, however, that many more strains would have to be examined before the utility of this approach as a typing and separation method for all the serotypes of the *M. avium* complex could be confirmed. It also seems likely that additional PFGE subgroups made up of other serotypes of *M. intracellulare* would be identified in such an extended analysis.

Since *M. avium* complex strains could be differentiated by their PFGE restriction endonuclease patterns, we speculated that variant bands in the profiles might serve as a potential source of subgroup-specific DNA probes. Two probes were isolated in this manner and were examined for their interaction with mycobacterial strains. It should be noted that neither of the probes exhibited absolute specificity for an individual subgroup or Schaefer serotype, but this is a goal for continuing investigations. The groups observed with the DNA probes corresponded with the subgroups defined by PFGE with only a single exception, that is, in the behavior of *M. paratuberculosis* C286. The confirmation of the PFGE categories by a second approach serves to strengthen the validity of the subgroup designations.

This study serves as a starting point for a subspecies identification scheme for *M. avium* complex strains. It illustrates the feasibility and potential of achieving this goal by using PFGE of digested mycobacterial DNA. Furthermore, this system lends itself to the easy isolation of DNA probes, whose specificity can then be tested simply by hybridization with DNA from isolates of known PFGE subgroups. Further investigation and exploitation of this combined approach should ultimately lead to the development of reagents which have a significant clinical application and, thus, help to address some of the problems in the diagnosis and management of *M. avium* complex infections in both humans and animals.

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