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Characterization of *Mycobacterium paratuberculosis* and Organisms of the *Mycobacterium avium* Complex by Restriction Polymorphism of the rRNA Gene Region

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Nineteen *Mycobacterium paratuberculosis* strains, including strains of bovine, caprine, ovine, cervid, subhuman primate, and human origins, were compared with organisms of the *M. avium* complex by restriction fragment length polymorphism with a 5S rRNA gene probe as the reference DNA. Mycobacterial DNA was extracted, digested with several restriction enzymes, subjected to electrophoresis and Southern blotting, and then hybridized with a 5S rRNA gene probe from *Escherichia coli*. Hybridizing bands were visualized by autoradiography, and the sizes of the resulting rRNA fragments in kilobases were determined. Base substitutions were calculated on the basis of the number of shared fragments between species and strains. It was determined that *M. paratuberculosis* and the *M. avium* complex possess a single copy of the rRNA genes within their genomes and that the *M. avium* complex and *M. paratuberculosis* are a group of closely related organisms, likely with a common ancestral link. In proximity to the 5S rRNA gene exists a region or regions which display polymorphisms that are capable of species and subspecies differentiation. *M. paratuberculosis* strains isolated from humans, subhuman primates, and animals were found to be genetically identical to each other. *M. paratuberculosis* strains lacked the genetic heterogeneity (restriction fragment length polymorphisms) characteristic of most species, suggesting that this organism has unidirectional genetic selection. It is therefore assumed to be biologically isolated, occupying a unique and specific biological niche. This homogeneity was present in all strains, including those of animal and primate (subhuman and human) origin and strains isolated from different parts of the world.

Mycobacterium paratuberculosis, the causative agent of paratuberculosis (Johne's disease), is a poorly defined species which in recent years has become rather controversial. While long recognized as a pathogen in ruminants (8), it has recently been shown to infect subhuman primates (18), and some suggestions have been made to implicate this organism in some cases of Crohn's disease in humans (5). Despite the interest in *M. paratuberculosis*, current methods of identification are inadequate, particularly with regard to differentiation from its close relatives of the *M. avium* complex (*M. avium* and *M. intracellulare*).

Although a variety of methods have been evaluated as potential diagnostic criteria, including biochemical assays (4) and gas-liquid chromatography (6), identification methods have remained the same over the last 50 years: slow growth (8 to 12 weeks) and a dependence on exogenous mycobactin for in vitro growth (mycobactin dependency). Since some strains of the *M. avium* complex may also be mycobactin dependent (25), no method to precisely or definitively identify *M. paratuberculosis* currently exists.

In recent years, methods for the identification of *M. paratuberculosis* and its differentiation from the *M. avium* complex have focused on the use of genetic analyses. Such methods have included DNA-DNA hybridization (19), whole genomic endonuclease restriction patterns (29), and restriction fragment length polymorphisms (RFLP) using random genomic sequences (20). In the present study, the 5S rRNA gene was selected as a reference DNA to examine changes in restriction fragments of *M. paratuberculosis* and related organisms of the *M. avium* complex.

MATERIALS AND METHODS

Organisms. An authenticated collection of the *M. avium* complex serovars (26) was obtained from Anna Tsang at the National Jewish Hospital and Research Center, Denver, Colo. All *M. avium* complex strains were grown from the lyophilized sample and plated, and isolated colonies were propagated. *M. paratuberculosis* strains were from clinical isolates from my laboratory, except for *M. paratuberculosis* ATCC 19698, which was obtained directly from the National Animal Disease Center, Ames, Iowa.

A total of 19 wild-type *M. paratuberculosis* strains were examined from my laboratory. These were obtained from the following sources: six bovine, four ovine, four caprine, four human, and one subhuman primate. These strains were all previously used in other studies (4–8) except for a single strain of human origin, which was kindly provided by J. Haagsma, Lelystad, The Netherlands. Organisms were identified as *M. paratuberculosis* on the basis of growth rate, mycobactin dependency, biochemical assay, and (for a few) gas-liquid chromatographic analysis (6).

Mycobacteria were grown in Middlebrook 7H9 broth with Tween 80 and Dubos oleic albumin complex at 37°C without added CO_2. Mycobactin J (2 μg/ml) was added to cultures of *M. paratuberculosis*. Cultures were incubated until the stationary phase of growth was obtained, and organisms were harvested by centrifugation at 4,340 × *g*.

Probe. The *Escherichia coli* 5S rRNA gene probe used was originally obtained from H. Liebke at Yale University, New Haven, Conn. (17). This probe contained the entire 5S gene and approximately 200 base pairs of the 23S gene. Thus, the entire probe was approximately 350 base pairs in length. This probe identified all 7 *rrn* operons in Southern blots of *Sal*I-digested *E. coli* DNA. The rRNA gene was excised from the vector by using an appropriate restriction enzyme, and the rRNA gene insert was separated by horizontal electrophoresis in 1% low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, Md.) (10). Radiolabeled rRNA genes were prepared by using [^{32}P]dCTP and [^{32}P]dTTP (3,000 Ci/mmol, 10 μCi/μl) (Dupont, NEN Research Products, Boston, Mass.) by second-strand synthesis as described by Feinberg and Vogelstein (10). Unincorporated radiolabeled nucleotides were removed by Sephadex G50 chromatography or by the use of DNA-RNA purification cartridges (NENSORB 20; Dupont, NEN). Labeled rRNA was suspended in 10 ml of 50% formamide–5× SSPE (20×; 3.0 M NaCl, 0.2 M Na_2HPO_4, 0.04 M EDTA)–1× Denhardt solution (100×; 2.0% Ficoll, 2.0% bovine serum albumin, 2.0% polyvinyl pyrrollidone)–1.0% sodium dodecyl sulfate (SDS). Before being used, probes were incubated overnight at 42°C with blank filters to eliminate nonspecific DNA binding.

DNA extraction. Mycobacterial cells were suspended in 10 ml of an isotonically unstable buffer (0.0132 M phosphate buffer, pH 6.8, 0.05 M EDTA, 0.7% NaCl, and 2,000 μg of lysozyme per ml) and fractionated in a Hughes press (14) at −80°C and 15,000 to 25,000 lb/in^2. Disrupted cells were incubated at 56°C for 60 min in a water bath. An equal volume of phenol (saturated with 100 mM Tris–10 mM EDTA, pH 7.5) was added, followed by a volume of chloroform-isoamyl alcohol (24:1) equal to the volume of phenol added. After mild agitation, the mixture was centrifuged at 2,500 × *g* for 5 min, and the upper aqueous phase was removed. Extraction with phenol and chloroform-isoamyl alcohol was repeated. Nucleic acids were precipitated by the addition of an equal volume of 100% ethyl alcohol. After incubation at room temperature for 10 min, nucleic acids were pelleted by centrifugation at 12,000 × *g* for 20 min. Pellets were briefly air dried and suspended in 2 ml of Tris-EDTA buffer (0.01 M Tris hydrochloride, 0.001 M EDTA). RNase (1,000 μg/ml) was added to a final volume of 50 μg/ml, and the mixture was incubated at 37°C for 30 min. The mixture was then extracted twice with phenol and chloroform-isoamyl alcohol. DNA was precipitated by the addition of 2 volumes of 95% ethyl alcohol and centrifuged at 12,000 × *g* for 20 min. The pelleted DNA was dried in a vacuum oven and then suspended in 2,000 μl of Tris-EDTA buffer. Sodium acetate (3 M) was added to a final concentration of 0.3 M, and this was followed by the addition of 2 volumes of cold (−20°C) 95% ethyl alcohol. Samples were stored overnight at −20°C, and the DNA was pelleted by centrifugation at 12,000 × *g* for 20 min. After the pellet was dried in a vacuum oven, DNA was suspended in 1,000 μl of Tris-EDTA buffer. The quantity of DNA was determined by optical density at 260 and 280 nm and/or by ethidium bromide. Samples were standardized to 2 μg per 10-μl volume and stored at −20°C until use.

Restriction endonuclease digestion and electrophoresis. Restriction enzymes were obtained from Bethesda Research Laboratories and included *Ava*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RII, *Hin*cII, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, *Sst*I, and *Xho*I. Ten microliters of DNA was added to an equal volume of 2× reaction buffer prepared as recommended by the manufacturer, and restriction enzymes were added to a concentration of approximately 4 U per sample. Digests were incubated at 37°C for 2 h, except for *Hin*dIII, which was incubated at 56°C. After incubation, 4 μl of stop dye (1 mM Tris hydrochloride [pH 7.5], 0.1% bromophenol blue, 0.1 M EDTA, 1.5% SDS, 2% Ficoll) was added. Before electrophoresis, samples were heated in a water bath at 65°C for 10 min to eliminate readherence. Agarose gels containing 1% agarose (ultrapure electrophoresis grade; Bethesda Research Laboratories) were prepared in Tris-borate (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) running buffer. Each gel (13.3 by 15.0 cm) was made to contain 17 sample wells or lanes. *Hin*dIII digests of lambda (Bethesda Research Laboratories) precipitated and suspended in 20 mM Tris hydrochloride (pH 8.0)–20 mM NaCl–0.1 mM EDTA with stop dye were used as molecular weight standards. Electrophoresis was conducted at 25 V and 14 mA for 18 to 24 h or until the dye front was 2 to 3 cm from the end of the gel. Gels were stained in ethidium bromide (1 μg/ml) for 30 min and washed in distilled deionized water for an additional 30 min. Photographs were taken with a Polaroid camera under UV light (320 nm) with a ruler adjacent to the lambda lane. The migration of the lambda fragments was recorded in millimeters. Gels were denatured (1.5 M NaCl, 0.5 M NaOH) for 30 min and then neutralized (3.0 M NaCl, 0.5 M Tris) for an additional 30 min.

Southern blot. Transfer to hybridization membranes (Gene Screen Plus; Dupont, NEN) was performed by a modification of the methods of Southern (24). Capillary action through the gel was provided by a 3MM chromatographic paper (Whatman, Hillsboro, Oreg.) wick submerged in 10× SSPE (1.5 M NaCl, 0.1 M Na_2HPO_4, 0.02 M EDTA). The gel was placed directly on the wick and covered with the hybridization membrane and then with several sheets of 3MM paper and paper towels. A weight was placed on top of the paper towels to aid capillary action. Transfer was allowed to proceed for 18 to 24 h. After transfer, membranes were air dried at room temperature, wrapped in plastic wrap (Saran Wrap; Dow Chemical, Indianapolis, Ind.), and stored at 4°C until use.

Hybridization. Membranes were placed in prehybridization solution (50% formamide, 5× SSPE, 1× Denhardt solution, 1.0% SDS, 10% dextran sulfate) at 42°C overnight before hybridization. The prehybridization solution was drained, and the membranes were placed in a sealable plastic bag (Seal-a-Meal; Dazey Corp., Industrial Airport, Kans.). A maximum of four membranes were enclosed in each bag. The probe was added, air bubbles were removed, and the bag was sealed. Hybridization was allowed to proceed for 18 to 24 h at 42°C on a rocker platform. The probe was removed, and the membranes were briefly rinsed in prehybridization solution and then incubated in fresh prehybridization solution for 30 min at 42°C. Membranes were washed in an additional five solutions, each at 42°C for 30 min as follows: wash 2, 2× SSPE–1× Denhardt solution–0.1% SDS; washes 3 and 4, 2× SSPE–0.1% SDS; and washes 5 and 6, 0.1× SSPE–0.1% SDS. Membranes were gently dried between paper towels and wrapped in plastic wrap.

Autoradiographs. A Geiger counter (Ludlum Measurements Inc., Sweetwater, Tex.) was used to examine membranes and to estimate the length of time needed for autoradiograms. Membranes were taped to paper (8 by 10 in. [~20 by 25 cm]) (two filters per paper), the orientation was noted, and they were placed in metal X-ray cassettes (8 by 10 in. [~20 by 25 cm]). Photographic film (X-Omat XAR-5; Eastman Kodak Co., Rochester, N.Y.) and intensifying screens

TABLE 1. 5S rRNA gene region restriction patterns of *M. paratuberculosis* of bovine, ovine, cervid, caprine, human, and subhuman primate origins[a]

Enzyme	Fragment size (kb)
*Ava*I	1.8
*Bam*HI	15.0
*Bgl*II	7.8
*Eco*RI	1.9
*Eco*RII	1.3
*Hin*dIII	17.5
*Hin*cII	1.7
*Pst*I	6.2
*Pvu*II	12.0
*Sst*I	5.9
*Xho*I	8.3

[a] Based on data obtained from 19 strains.

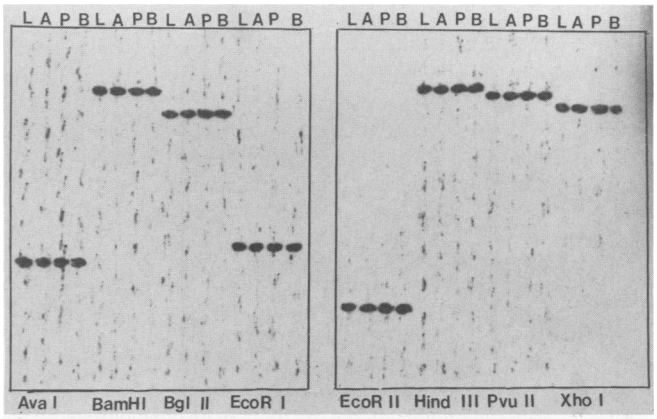

FIG. 1. Autoradiograph of *E. coli* 5S rRNA gene-probed *M. paratuberculosis* digested DNA from strain Linda (lanes L), strain ATCC 19698 neotype (lanes A), a primate wild-type strain (lanes P), and a bovine wild-type strain (lanes B). RFLPs were identical for all strains examined.

(Dupont Co., Wilmington, Del.) were placed on top of the membranes and stored for a predetermined time at −80°C. The photographic film was developed in GBX developer (Kodak) for 5 min and in GBX fixer (Kodak) for 10 min.

Analysis of data. A semilogarithmic graph was constructed by a custom computer program of kilobase versus millimeter migration of the lambda marker. Migration of the mycobacterial rRNA gene was measured from the autoradiogram, and kilobase size was determined from the computer program.

RFLPs were analyzed and compared by the methods described by Upholt (27), Nei and Li (21), and others (1, 12). The determination of phylogenetic relationships on the basis of RFLPs has been widely used, and the detailed mathematical formulas are available from a number of sources (1, 12, 21, 27). The method used in the present study was the phenetic approach, which involves the determination of the proportion of shared restriction sites for any two populations. The degree of genetic divergence between populations is expected to correlate with the proportion of DNA shared by them. These methods are applicable to closely related homologous DNA (>80%) and have been used successfully to measure sequence divergence among *E. coli* strains from laboratory and environmental sources (13, 22).

Briefly, the number of shared fragments in restriction endonuclease digests of DNA was determined as a proportion of the total number of hybridizing fragments to give the fraction of fragments conserved, *F*. From this value, the fraction of substitution bases (*P*) was estimated by the formula (27)

$$P = 1 - \left[\frac{-F + \sqrt{F^2 + 8F}}{2}\right]^{(1/n)}$$

where *n* is the number of bases in the restriction enzyme recognition site.

RESULTS

All wild-type *M. paratuberculosis* strains and ATCC 19698 contained identical restriction fragments of the 5S rRNA gene region (Table 1 and Fig. 1). This pattern was identical regardless of the culture source or origin, including strains of human and subhuman origin which were isolated in different geographic areas of the world, thus suggesting a stability in restriction fragmentation of the 5S rRNA gene region in *M. paratuberculosis*.

Autoradiographs all contained a single band, indicating that only a single copy of the rRNA genes (a single *rrn* operon) exists in *M. paratuberculosis*. Furthermore, the presence of single bands also indicates that restriction recognition sites were not present within any region recognized by the rRNA reference DNA. Thus, the rRNA gene, its spacer region, and the proximal end of the 23S rRNA gene of *M. paratuberculosis* do not contain any sites recognized by the restriction enzymes used, and the fragments produced are the result of cleavages outside these regions. Therefore, data have been presented as restriction polymorphism of the rRNA gene region to indicate that the fragment, but not necessarily the restriction site, contains the 5S rRNA gene.

Organisms of the *M. avium* complex also contained a single rRNA gene restriction pattern, again suggesting the presence of only one *rrn* operon (Table 2). Unlike with *M. paratuberculosis*, a few enzymes did recognize restriction recognition sites within the rRNA gene. Double fragments, suggesting cleavage of the rRNA gene, were observed only with *Ava*I-digested *M. avium* serovar 2 DNA and with

TABLE 2. Comparison between *M. paratuberculosis*, *M. kansasii*, and several members of the *M. avium* complex by RFLP of the rRNA gene region

Enzyme	Size of rRNA gene region (kb) after digestion							
	M. avium complex serovar:						*M. paratuberculosis*	*M. kansasii*
	1	2	3	4	9	13		
*Ava*I	8.0	2.2 1.8	8.0	8.0	8.0	NA[a]	1.8	1.8
*Bam*HI	9.5	9.5	9.5	9.5	9.5	NA	15.0	6.6 5.5
*Bgl*II	17.0	7.8	17.0	7.8	NA	NA	7.8	NA
*Eco*RI	1.8	1.8	1.8	2.8	2.8	2.8	1.8	1.9 1.7
*Eco*RII	1.3	1.3	NA	1.3	1.3	1.3	1.3	3.3 2.5
*Hin*dIII	NA	9.0	9.0	9.0	9.0	9.0	17.5	4.9
*Hin*cII	1.7	1.7	1.7	1.7	1.7	1.7	1.7	3.4 2.5
*Pst*I	6.2	6.2	NA	6.2	NA	6.2	6.2	5.7
*Pvu*II	NA	9.5	7.0	7.0	9.0	9.0	17.0	14.0
*Sst*I	7.0	5.6	12.0	12.0	6.5 10.0	6.5 10.0	5.9	5.6

[a] NA, Data not available.

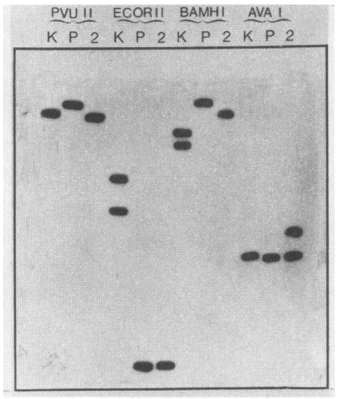

FIG. 2. Autoradiograph of the restriction enzyme-digested 5S rRNA gene region from *M. kansasii* (lanes K), *M. paratuberculosis* (lanes P), and *M. avium* complexes serovar 2 (lanes 2). Various RFLPs were evident between all species, except that no polymorphisms were present between *M. paratuberculosis* and *M. kansasii* with *Ava*I or between *M. paratuberculosis* and *M. avium* complex serovar 2 with *Eco*RII.

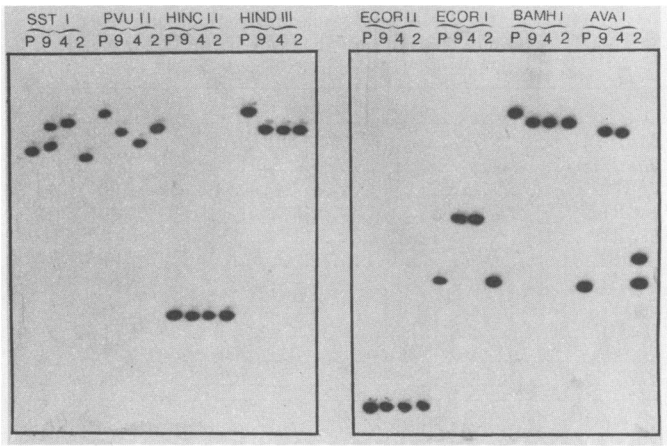

FIG. 3. Autoradiogram showing similarities and RFLPs between *M. paratuberculosis* (lanes P) and *M. avium* complex serovars 2 (lanes 2), 4 (lanes 4), and 9 (lanes 9). Identical fragments between all strains were produced when DNA was digested with *Hin*cII and *Eco*RII. *M. paratuberculosis* and *M. avium* complex serovar 2 shared restriction fragment lengths with *Eco*RI. RFLPs between *M. avium* complex and *M. paratuberculosis* were present with *Hin*dIII, *Sst*I, *Pvu*II, *Bam*HI, and *Ava*I. Polymorphisms were also present between the *M. avium* complex serovars with *Sst*I and *Pvu*II and between *M. avium* complex serovar 2 and other serovars with *Eco*RI and *Ava*I.

*Sst*I-digested DNA from *M. avium* complex serovars 9 and 13. All other digests produced a single band in autoradiographs. Several enzymes produced identical fragments for all *M. avium* complex strains examined. These included DNA digests with *Bam*HI, *Eco*RII, *Hin*cII, *Hin*dIII, and *Pst*I. Other digests, e.g., those with *Ava*I, *Bgl*II, and *Eco*RI, showed minor polymorphisms between strains, and only with *Ava*I digestion was a fragment produced which was unique to a single serovar. These enzymes, at the most, tended to subgroup these organisms into two fragment size classes. DNA digests with *Pst*I and *Sst*I contained enough polymorphisms to provide differentiation between some of these organisms.

Restriction patterns obtained with *M. kansasii* were quite different from those of the *M. avium* complex and *M. paratuberculosis*. Fragments corresponding to those of the other two species were only present with *Ava*I and *Sst*I (Table 2; Fig. 2). Polymorphisms were present with all other enzymes examined. Although identical fragment sizes were produced with *Ava*I digests of *M. kansasii*, *M. paratuberculosis*, and *M. avium* complex serovar 2, that of *M. avium* complex serovar 2 is likely to be a different fragment since the restriction recognition site allowed hybridization with a second fragment, which did not occur with the other digests (Fig. 2). With *Sst*I, shared restriction sites were present between *M. kansasii* and again with the *M. avium* complex serovar 2. The double banding pattern of *M. kansasii* suggests that this species has two copies of the rRNA genes. Only four enzymes (*Ava*I, *Hin*dIII, *Pst*I, and *Sst*I) produced a single fragment, and it is likely that these fragments contained two gene copies. The single fragment produced with *Ava*I digestion is inexplicable; this size fragment is too small to contain two complete rRNA gene copies. Therefore, it is suggested that perhaps two indistinguishable fragments, each approximately 1.8 kilobases (kb) in size, resulted from *Ava*I digestion.

Comparison of restriction fragments produced with the *M. avium* complex and *M. paratuberculosis* digested DNA clearly showed multiple similarities as well as some differences (Table 2; Fig. 3). With the 10 restriction enzymes examined, *M. paratuberculosis* and the *M. avium* complex shared rRNA gene fragments for 5 of them (*Bgl*II, *Eco*RI, *Eco*RII, *Hin*cII, and *Pst*I). Fragments from digests with *Eco*RII, *Hin*cII, and *Pst*I, which are identical in all the *M. avium* complex strains and *M. paratuberculosis* but different in unrelated species, appear to be characteristic of this group of organisms, suggesting a close evolutionary relationship between *M. paratuberculosis* and the *M. avium* complex.

M. avium complex serovar 2, *M. paratuberculosis*, and perhaps *M. kansasii* have an additional *Ava*I restriction site not present in other *M. avium* complex strains which produced the smaller rRNA gene fragment (1.8 versus 8.0 kb). The most apparent distinguishing feature between the *M. avium* complex and *M. paratuberculosis* is the loss of a *Bam*HI and a *Hin*dIII restriction site in *M. paratuberculosis*. With both these enzymes, large restriction fragments of 15.0 and 17.5 kb were obtained with *M. paratuberculosis*, while all of the *M. avium* complex strains produced 9.5- and 9.0-kb fragments, respectively. This is considered a loss of a restriction recognition sequence in *M. paratuberculosis* since it is improbable that all the *M. avium* complex strains could independently acquire an identical restriction site by random mutational events. Although *M. paratuberculosis* lacks a *Pvu*II restriction site, these sites appear to be variable and prone to random mutational events since at least three different fragments were produced by the *M. avium* complex serovars.

Mathematical estimates of base substitution of the rRNA gene region suggest that the *M. avium* complex serovars and *M. paratuberculosis* have genomic rRNA differences ranging from 1.3 to 10.9% base sequence substitution (Table 3). *M. paratuberculosis* and the *M. avium* complex serovar 2 have the least amount of rRNA divergence, with a base sequence difference of only 2.9%. The greatest divergence, i.e., the highest base substitution rate of the rRNA gene region, occurred between *M. paratuberculosis* and *M. avium* complex serovars 3 and 10, each having almost an 11% difference in individual base pairs. Within the *M. avium* complex, base substitution ranged from 1.3% between serovars 4 and 13 to 6.5% between serovar 1 and serovars 9 and 10.

TABLE 3. Percent DNA base substitution of the rRNA gene region between the *M. avium* complex and *M. paratuberculosis*

Organism	% DNA base substitution of rRNA gene region compared with *M. avium* complex serovar:						
	1	2	3	4	9	10	13
M. paratuberculosis	5.4	2.9	10.9	5.5	9.8	10.9	7.4
M. avium complex serovar							
1		2.9	1.6	5.4	6.5	6.5	4.0
2			4.0	3.2	5.4	4.0	2.9
3				2.3	5.4	4.0	4.0
4					2.9	3.3	1.3
9							NA[a]
10							NA

[a] NA, Data not available.

DISCUSSION

Restriction endonuclease polymorphism of specific genes may be used as an indicator of changes in gene sequence. By this method, restriction endonucleases are used to cleave DNA into fragments which are sized by gel electrophoresis. Differences in specific fragment sizes obtained with a given enzyme accurately reflect sequence differences in recognition sites (1, 12, 22). Evolutionary relationships inferred from restriction patterns are thus often considered to be superior to those inferred from other data, excluding direct sequencing (1). RFLP has been widely used to determine relationships of eucaryotic (3) and procaryotic (22) organisms. Although designed originally for the examination of mitochondrial DNAs, since these are evolutionarily related to the procaryotes (30), it was not long before these methods were applied to bacterial populations. This method measures the sizes of chromosomal restriction fragments that contain homologs to selected small segments of the genome of a standard reference DNA (1, 12, 21, 22). Restriction endonuclease profiles of a single or multiple genes within the genome, irrespective of other changes, are thus examined.

The polymorphisms observed were rarely within the rRNA gene itself, since this would have produced more than one band in autoradiographs. Actual restriction sites within the rRNA gene were only detected with *Ava*I digestion of *M. avium* complex serovar 2 and *Sst*I digestion of *M. avium* complex serovars 9 and 13. Therefore, the restriction site differences which account for the observed polymorphisms were located upstream or downstream of the 5S-proximal 23S rRNA gene. Within this vicinity there must exist a region which is highly variable (at least relative to other genes examined) to allow for more polymorphisms than would be expected yet is conserved enough to allow stability of the restriction sites within *M. paratuberculosis* strains. The presence of genus- and species-specific sequences within the rRNA is well known, and these are the basis of commercially available diagnostic probes (9, 16). Such sequences must be reflected in the genome and may have been those detected by the 5S rRNA gene probe.

The RFLP data presented for *M. paratuberculosis* are characteristic of the species and could be used to definitively identify this organism. Because of the polymorphisms with the *M. avium* complex, only a few enzymes would need to be used in order to reach identification. For example, *Eco*RII, *Hin*cII, or *Pst*I would group an isolate into an *M. avium-M. paratuberculosis* complex, and *Ava*I, *Bam*HI, *Hin*dIII, *Pvu*II, or *Sst*I restrictions would provided species identification of *M. paratuberculosis*. The use of only a few restriction enzymes suitable for differentiation greatly diminishes the cost, time, and labor requirements to perform such analyses, and the techniques used, i.e., DNA isolation and restriction, electrophoresis, Southern blot, and DNA probing, are now routine in most laboratories.

All 19 *M. paratuberculosis* strains examined contained the restriction patterns identical to those shown in Table 1; polymorphisms were not observed within the species, even between those separated geographically. Such a finding is not common, as individual strains generally have different genetic forces, i.e., environments, affecting random mutation rates (13). As such, the polymorphisms observed between the *M. avium* serovars were expected, and differences would be expected within the *M. avium* serovars; however, an insufficient number of strains was examined to determine this. Few organisms lack strain polymorphisms, which suggests unidirectional genetic selection, a condition rarely encountered in procaryotic or eucaryotic organisms (11, 30). The lack of restriction polymorphisms within *M. paratuberculosis* has also been shown to occur with random genetic probes (20), which suggests that most of the genome, if not all of it, has remained the same between strains of this species. Therefore, it is suggested that *M. paratuberculosis* must be clonal and biologically isolated, experiencing unidirectional genetic selection on the basis of a unique and specific biological niche. This is in agreement with the biologic and pathogenic characteristics of this species. Other than in vitro, *M. paratuberculosis* has been shown to be capable of growth only within the tissues of the gastrointestinal tract and regional lymph nodes (8). It has never been isolated from the environment, except in feces-contaminated areas where the organism is endemic, and in such environments multiplication does not occur. Environmental material is either bactericidal or bacteriostatic, and *M. paratuberculosis*, even in feces, will eventually die (8). Such a limited growth environment would account for a unique biological niche and unidirectional selection. *Mycobacterium leprae* has also been shown not to have strain polymorphisms, and this organism has a similar biological niche (the skin).

M. paratuberculosis is the etiologic agent of paratuberculosis, a granulomatous ileocolitis primarily affecting ruminants (8). Since this organism, at least as far as we know, is not found in the environment and is only associated with disease (8), it is considered a strict pathogen. Unlike *M. paratuberculosis*, organisms of the *M. avium* complex are ubiquitous and widely distributed in the environment, causing infection as opportunistic pathogens (28). In animals, *M. avium* may cause avian and swine tuberculosis, and on occasion may infect other monogastric animals, but it rarely causes disease in ruminants. On the other hand, *M. paratuberculosis* rarely infects monogastric animals. Clinical recognition of *M. paratuberculosis*, although based on only a few physiologic criteria, i.e., slow growth and mycobactin dependency, appears to be generally accurate. All strains examined here, which were subsequently shown to be identical by RFLP, were originally identified as *M. paratuberculosis* solely on mycobactin dependency and growth rate. Thus, even though clinical identification is based on limited criteria, organisms generally isolated from animals with paratuberculosis correspond to a specific genetic type which is distinguishable from members of the *M. avium* complex.

The species status of *M. paratuberculosis* has been in dispute in recent years on the basis of DNA-DNA homology data, which suggest >90% homology with *M. avium*. Results reported herein support that close relatedness. Organisms

with >70% homologous DNA are often considered to be the same species. Such designations, however, are only applicable to the grouping of organisms which have high genetic diversity, e.g., *E. coli*, and which are not readily distinguishable. Organisms which share >70% homology but contain important phenetic expressions are not appropriate for such designations, and species status is maintained. For example, *Shigella dysenteriae* and *E. coli* have >90% homologous DNA (13) but maintain, and warrant, distinct species status. Additionally, *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum* (the *M. tuberculosis* complex) all contain >70% homologous DNA (15), as do *M. lepraemurium* and *M. avium* (2), and species status is rightfully maintained for each (23). The phenotypic expression of an organism must be, and generally is, considered in the species status. Despite the high homology between *M. paratuberculosis* and *M. avium*, the RFLP data and genetic homogeneity of *M. paratuberculosis*, in addition to the phenotypic differences from the *M. avium* complex, warrant continued species status.

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