Distinction of Species and Strains of Mycoplasmas (Mollicutes) by Genomic DNA Fingerprints with an rRNA Gene Probe

D. YOGEV,[1] D. HALACHMI,[1] G. E. KENNY,[2] AND S. RAZIN[1]*

Department of Membrane and Ultrastructure Research, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel,[1] and Department of Pathobiology SC-38, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195[2]

Genomic fingerprints of *Acholeplasma laidlawii*, *Mycoplasma hominis*, and *Mycoplasma pneumoniae* strains were obtained by Southern blot hybridization of the digested mycoplasmal DNAs with an rRNA gene probe. The hybridization patterns revealed genotypic heterogeneity among *A. laidlawii* and *M. hominis* strains and a remarkable degree of homogeneity among *M. pneumoniae* strains isolated from pneumonia patients during a 10-year period. Genomic fingerprints with the rRNA gene probe can thus serve as indicators of intraspecies genetic homogeneity or heterogeneity and can provide a new, sensitive tool for strain identification with a potential for application in epidemiology.

A novel approach for differentiation and identification of species and strains of mycoplasmas (class Mollicutes) has recently been provided by Southern blot hybridization of digested mycoplasmal DNA with the highly conserved rRNA genes as probes (1, 2, 20). The tested DNA is digested by a restriction endonuclease, electrophoresed, and hybridized by the Southern blot technique with the nick-translated plasmid pMC5 as a probe. This recombinant plasmid carries the 5S and 23S genes and part of the 16S gene of one of the two rRNA operons of *Mycoplasma capricolum* (2, 10). Due to the highly conserved nature of the procaryotic rRNA genes, this plasmid will hybridize with DNA containing rRNA genes of any mycoplasma (1, 19). When mycoplasmal DNA digestion is done with *Eco*RI, *Hind*III, or *Bgl*II (enzymes recognizing six-nucleotide sequences on the DNA), the hybridization patterns consist of a relatively small number of bands because of the presence of only one or two copies of the rRNA genes in the mycoplasmal chromosome and the relatively few restriction sites for these enzymes within the operons (1, 11, 19, 31). The fact that the rRNA operons in the various mycoplasmas may differ in restriction sites within the operons and in their flanking sequences results in hybridization patterns peculiar for the different species. These "fingerprints" were utilized for detection and identification of mycoplasmas infecting cell cultures (20).

A critical question is whether the hybridization patterns are species or strain specific. The serotypes of *Ureaplasma urealyticum* were found to form two genotypically distinct clusters demonstrated by DNA-DNA hybridization (6), restriction endonuclease analysis of genomic DNA (21), and electrophoretic profiles of cell proteins (18). Hybridization of digested DNAs of the various serotypes with pMC5 clearly distinguished strains belonging to the two genotypic clusters (25). In the present study, we applied the new fingerprinting approach to strains of *Mycoplasma hominis* and *Acholeplasma laidlawii*, two species for which DNA-DNA hybridization (4, 28) and DNA cleavage analysis (4, 24) suggested marked intraspecies genotypic heterogeneity. In addition, we included in the study strains of *Mycoplasma pneumoniae* isolated from primary atypical pneumonia patients in Seattle during different epidemics occurring within a 10-year period.

The results presented in this communication show that the fingerprints obtained with the rRNA gene probes are useful indicators of intraspecies genetic homogeneity or heterogeneity. In addition to being useful in taxonomy the new approach may find its application in epidemiological studies, providing a new, sensitive tool for strain identification.

MATERIALS AND METHODS

Organisms and growth conditions. *M. hominis* PG21 and PG25 were obtained from the collection of the late D. G. ff. Edward. Strains V27, DC63, DC63A, and DC63B were obtained from R. M. Chanock (National Institutes of Health, Bethesda, Md.), and strain ATCC 15056 was purchased from the American Type Culture Collection, Rockville, Md. The *M. hominis* strains were grown for 48 to 72 h at 37°C in modified Edward medium (23) supplemented with 8% (vol/vol) horse serum and 2 mM L-arginine. The organisms were harvested by centrifugation at 12,000 × *g* for 30 min in the cold, and the pellet was washed twice in cold 0.25 M NaCl containing 1 mM EDTA. The *M. pneumoniae* strains tested included the high-passage FH strain from our laboratory collection and eight strains isolated from pneumonia patients in Seattle during the period of 1964 through 1974 (29). These strains were cultured in a dialysate broth (14) containing 10% (vol/vol) agamma horse serum (Alpha Gamma Laboratories, Sierra Madre, Calif.). The total number of in vitro passages of these strains did not exceed five (29). Cultures for DNA isolation (1 liter for each strain) were incubated at 37°C in the dialysate broth medium with slow agitation until they became hazy and began to change pH (at about 7 days). The cultures were then harvested by centrifugation, washed three times in 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-buffered saline (pH 7.3), suspended at a 1,000× concentration, and stored at −70°C until DNA extraction. *A. laidlawii* PG8, PG9, SewA, and SewB and the oral strain were from our laboratory collection. *A. laidlawii* B (Conn.), adapted to grow in tryptose broth with no serum supplement, was obtained from M. E. Tourtellotte (University of Connecticut, Storrs, Conn.). Cultures of *A. laidlawii* JA1 were obtained from G. Klotz (University of Ulm, Ulm, Federal Republic of Germany) through S. Rottem and from J. Maniloff (University of Rochester, Rochester, N.Y.). The acholeplasmas were
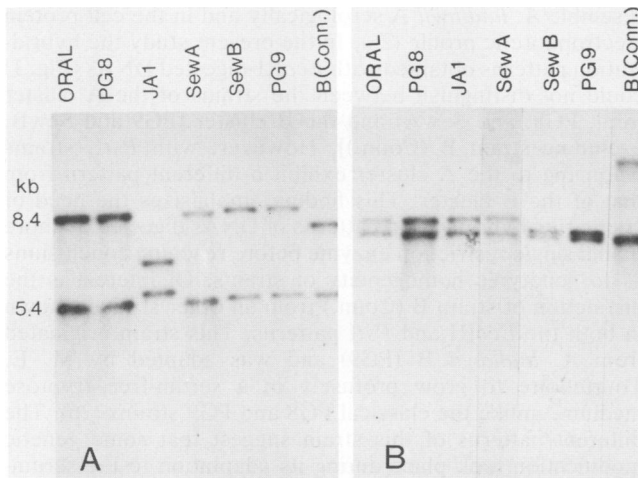
* Corresponding author.

FIG. 1. Hybridization patterns of the DNA of *A. laidlawii* strains digested by *Eco*RI (A) or *Pst*I (B) and hybridized with pMC5 as a probe.

FIG. 2. Hybridization patterns of DNA of *M. hominis* strains digested by *Bgl*II or *Eco*RI and hybridized with pMC5.

grown in the modified Edward medium supplemented with 4% (vol/vol) horse serum and 0.5% glucose for 24 to 48 h at 37°C. The organisms were harvested and washed as described above for *M. hominis*.

DNA preparation and cleavage. The DNA of the washed organisms was extracted and purified by the method of Marmur (17). The DNA was digested by restriction enzymes (New England BioLabs, Inc., Beverly, Mass.) for 2 h at 37°C in buffer solutions recommended by the manufacturer. The digested DNA was electrophoresed at 35 V for 18 h in 0.8% agarose (Seakem; FMC Corp., Rockland, Maine) slab gels in buffer containing 40 mM Tris (pH 8.0), 5 mM sodium acetate, and 1 mM EDTA.

Southern blot analysis. DNA fragments from gels were transferred to nitrocellulose sheets by the method of Southern (27) and hybridized with the nick-translated plasmid pMC5 (1, 2) as a probe. The plasmid was nick translated (30) with deoxycytosine tri[^{32}P]phosphate (New England Nuclear Corp., Boston, Mass.) to a level of approximately 2 × 10^8 cpm/μg of DNA. Hybridization with the DNA fragments on the nitrocellulose sheets was done by a modification of the procedure of Weinstock et al. (30) as described in detail elsewhere (31).

RESULTS

The hybridization patterns of DNAs from the *A. laidlawii* strains digested by *Eco*RI and hybridized to pMC5 (Fig. 1A) consisted of two bands representing two DNA fragments, each carrying a major portion of one of the two rRNA operons present in the genome of this species (1, 2). The sizes of these two DNA fragments were identical in the oral strain and strains PG8, SewA, SewB, and PG9, whereas the sizes of fragments from strains JA1 and strain B (Conn.) differed. Digestion of the same DNAs with *Pst*I (Fig. 1B) also resulted in a two-band pattern for all strains but with sizes different from those obtained with *Eco*RI. The JA1 pattern obtained with *Pst*I could not be distinguished from that of the oral strain, PG8, and SewA, whereas the pattern of strain B (Conn.) obtained with *Pst*I differed from that of all other strains, as was also the case with the *Eco*RI-digested DNAs (Fig. 1A). The hybridization patterns of *Eco*RI- and *Hin*dIII-digested DNAs of the two JA1 strains
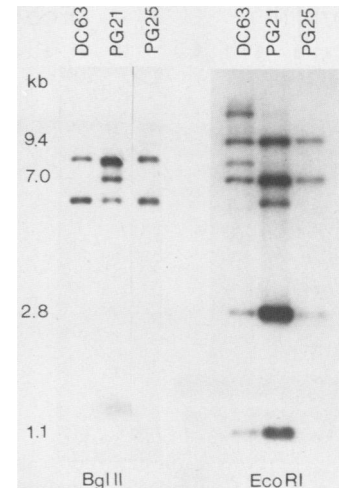
obtained from different laboratories were identical (results not shown).

Although the *M. hominis* strains tested by us revealed a basic hybridization pattern, reflected by the presence of common bands, some differences among the strains were discernible (Fig. 2). Nevertheless, some strains appeared to form clusters exhibiting identical hybridization patterns (Fig. 3). Subclones of the same strain (DC63) had identical patterns (Fig. 3).

The hybridization patterns of the DNAs of the eight clinical isolates of *M. pneumoniae* digested by *Hin*dIII or *Eco*RI were strikingly identical (Fig. 4), as were the cleavage patterns of the DNAs of these strains (results not shown). The hybridization patterns of the clinical isolates resembled the pattern obtained for the laboratory FH strain of *M. pneumoniae* (31).

DISCUSSION

Our data show that Southern blot hybridization patterns of mycoplasmal DNAs digested by restriction endonucleases
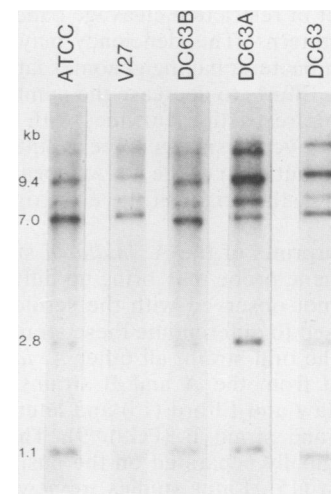

FIG. 3. Hybridization patterns of *M. hominis* strains digested by *Eco*RI and hybridized with pMC5. These strains and subclones of the same strain (DC63) reveal identical patterns.
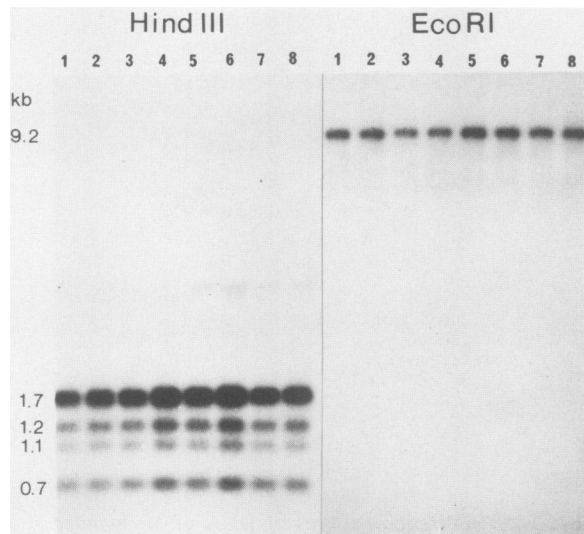
FIG. 4. Hybridization patterns of DNA from eight *M. pneumoniae* strains digested by *Eco*RI or by *Hin*dIII and hybridized with pMC5.

and hybridized with the rRNA gene probe pMC5 differ between strains within certain established species, such as *A. laidlawii* and *M. hominis*. Nevertheless, with a genotypically homogeneous species like *M. pneumoniae*, the patterns may prove to be species specific. The conclusion that the hybridization patterns cannot be used for species identification for genotypically heterogeneous species might be considered disadvantageous, but the present study indicates that these patterns provide a sensitive tool for determining identity and clonality of strains and in this way may be useful in epidemiological studies.

Genomic cleavage patterns by restriction endonucleases have been previously proposed as tools for testing genotypic homogeneity of species in the class Mollicutes (19, 21, 24). The Southern blot hybridization procedure yields genomic cleavage patterns as a by-product, so that these can also be used for comparison of strains. However, the fingerprints produced by the rRNA gene probe are much easier to compare, since they are composed of much fewer bands. The large number of restriction cleavage bands may hamper comparison of patterns. This deficiency may become more pronounced with bacteria having genomes larger than those of mycoplasmas. Efforts to decrease the number of cleavage bands by selecting restriction enzymes with fewer recognition sites in the mycoplasma genome resulted in patterns with fewer bands, but most of the DNA appeared in this case as a heavy band in the origin of the electropherogram (21, 24).

Genomic fingerprints of the *A. laidlawii* strains obtained with the rRNA gene probe may bring up differences among strains that are not observed with the serological and biochemical tests used to differentiate these strains. Apart from strain JA1 and the oral strain, all other *A. laidlawii* strains tested originated from the A and B strains isolated from sewage by Laidlaw and Elford (15) and later designated as strain A (PG8) and strain B (PG9) (9). The two sewage strains were originally separated on the basis of the serum agglutination test (15). Later studies, reviewed by Edward and Freundt (9), were somewhat contradictory as to the degree of relatedness of these strains. The oral strain, isolated from the human oral cavity (22), was found to

resemble *A. laidlawii* A serologically and in the cell protein electrophoretic profile (26). In the present study the hybridization patterns obtained with *Eco*RI-digested DNAs (Fig. 1) could not distinguish between the strains of the A cluster (oral, PG8, and SewA) and the B cluster [PG9 and SewB, excluding strain B (Conn.)]. However, with *Pst*I, strains belonging to the A cluster exhibit a different pattern from that of the B cluster. This finding emphasizes the need of comparing hybridization patterns of DNAs digested by more than a single restriction enzyme before reaching conclusions as to genotypic homogeneity of strains. Of interest is the distinction of strain B (Conn.) from all other strains, shown in both the *Eco*RI and *Pst*I patterns. This strain originated from *A. laidlawii* B (PG9) and was adapted by M. E. Tourtellotte to grow profusely in a serum-free tryptose medium, unlike the classical PG8 and PG9 strains (26). The different patterns of this strain suggest that some genetic modification took place during its adaptation to the serum-free medium.

Another case of interest concerns the JA1 strain. This strain, used as an indicator host for viruses infecting *A. laidlawii*, was originally isolated by Gourlay (12) from bovine nasal secretion and was named BN1, later modified to JA1 (16). The hybridization pattern of the DNA of this strain digested by *Eco*RI distinguishes it from strains A and B. The publication of contradictory results concerning methylation of JA1 genomic DNA by Dybvig et al. (8) and by Honigman et al. (13) prompted us to check this problem with JA1 strains obtained from the two laboratories. Hybridization patterns of the DNAs of the two strains digested by either *Eco*RI or *Hin*dIII with the rRNA gene probe were identical (results not shown). This finding indicated that the discrepancy in results cannot simply be attributed to the use of different *A. laidlawii* strains by the two laboratories. This may serve as an example for the usefulness of the hybridization approach in resolving problems concerning clonality and identity of strains.

Previous indications that *M. hominis* is a genotypically heterogeneous species (3, 4) gain additional support from our present findings with the rRNA gene probe. While our studies on *M. hominis* were in progress, similar observations made by Christiansen et al. (7) but with rRNA gene probes different from pMC5 and with other *M. hominis* strains came to our attention. The results of Christiansen et al. (7) are in accordance with ours in showing heterogeneity of hybridization patterns obtained with different *M. hominis* strains. Moreover, their results as well as ours (D. Yogev, D. Halachmi, and S. Razin, unpublished data) suggest that the *H. hominis* genome carries two rRNA operons.

The identical DNA fingerprints of the eight clinical isolates of *M. pneumoniae* (Fig. 4) indicate a remarkable degree of genotypic homogeneity of this pathogen. The genotypic homogeneity of these clinical isolates was also reflected in their antigenic composition. The same *M. pneumoniae* strains used in the present study exhibited immunoblot patterns that were almost identical to those in the convalescent patients' sera, indicating their remarkable antigenic homogeneity (29). This homogeneity evidently has been retained through several *M. pneumoniae* epidemics occurring in Seattle in the 10-year period during which these strains were collected. Interestingly, another conserved gene probe, the *tufA* gene of *Escherichia coli*, encoding for elongation factor Tu, was recently shown by us (D. Yogev, S. Sela, H. Bercovier, and S. Razin, FEMS Microbiol. Lett., in press) to produce hybridization patterns that are identical to those of digested DNAs of the *M. pneumoniae* strains

examined in the present study, strengthening the conclusion as to the marked genotypic homogeneity of the strains.

As suggested previously (5), it can be speculated that the marked genetic homogeneity of *M. pneumoniae* strains is associated with their rather restricted host and target tissue. In fact, *M. pneumoniae* has so far been isolated only from humans, and its target tissue is restricted to the tracheal epithelial lining. The rather constant environment of this parasite would thus tend to reduce selective pressures, keeping the genotype relatively stable. Moreover, *M. pneumoniae* cultures were found so far to be free of viruses and plasmids, again decreasing chances for genetic modifications (19).

A major advantage of using the rRNA gene probe pMC5 for genome fingerprinting is that it reacts with any mycoplasmal (mollicute) genomic DNA and can provide valuable taxonomic, diagnostic, and epidemiological information. For example, this tool enabled us to distinguish the live vaccine F strain of *Mycoplasma gallisepticum* from virulent field isolates of the avian pathogen and to detect the F strain in areas where vaccination with the F strain took place (D. Yogev, S. Levisohn, D. Halachmi, S. H. Kleven, and S. Razin, Avian Dis., in press). Hence, genomic fingerprinting with rRNA gene probes can be added to the battery of new molecular tools useful in taxonomy and in the epidemiology and diagnosis of mycoplasmal infections.

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