Genomic DNA Analysis of Rodent Mycoplasmas

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Genomic DNA was compared between three typical species of rodent mycoplasmas, *Mycoplasma pulmonis*, *M. arthritidis* and *M. neurolyticum*, and between strains of these species. Each of the three species showed a distinct restriction endonuclease cleavage pattern of genomic DNA. The genetic heterogeneity of these species was revealed by total DNA hybridization as well. In addition, the restriction endonuclease cleavage pattern of genomic DNA was almost identical in three strains of *M. pulmonis* and in two strains of *M. neurolyticum*. The genetic heterogeneity among strains of the same species was revealed by total DNA hybridization as well. These data suggest that the genomic DNA sequence of each rodent mycoplasma species has a high degree of species specificity.

The classification and identification of mycoplasmas are based on morphological, biochemical and serological characteristics. For some mycoplasma species in which serological identification is difficult, a method based on genetic characteristics has also been attempted. To assess genetic relatedness among prokaryotes, including mycoplasmas, DNA-DNA homology studies have generally been used [3, 9, 10]. In recent years, with the spread of restriction endonucleases that recognize specific nucleotide sequences, the comparison of DNA cleavage patterns by these enzymes has proved to be a simple, useful method. DNA analysis based on restriction endonuclease cleavage pattern is highly appropriate for mycoplasmas, which have a very small genome, ca. 500 megadaltons. Although some studies have successfully identified mycoplasma species by this method [1, 8], the properties of genomic DNA of rodent mycoplasmas have not been determined.

In the present study, genomic DNAs were compared between three typical species of rodent mycoplasmas, *Mycoplasma pulmonis*, *M. arthritidis* and *M. neurolyticum*, according to their restriction endonuclease cleavage

patterns and by total DNA hybridization.

Materials and Methods

Organisms and culture conditions: *M. pulmonis* m53 and *M. neurolyticum* Type A were kindly provided by Dr. K. Koshimizu, Division of Animal Research, Faculty of Medicine, University of Tokyo, Japan. *M. pulmonis* M4 and R1, and *M. arthritidis* PG 6 were generously provided by Dr. M. Nakagawa, Department of Veterinary Science, National Institute of Health, Japan. *M. neurolyticum* H322 was originally isolated from the lung of a mouse that was tested in the Central Institute for Experimental Animals, Japan. Mycoplasmas were cultured in Chanock's PPLO broth [2] at 37°C for 3 days. The organisms were harvested by centrifugation at $5,000 \times g$ for 30 min, washed twice with saline-EDTA (0.15M NaCl/0.1M ethylenediamine tetraacetic acid, pH 8.0), and used immediately for DNA isolation.

Isolation of mycoplasmal DNA: Genomic DNA was extracted and purified by a modification of the method of Marmur [5]. Briefly, organisms washed in saline-EDTA were lysed

by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%. The lysates were treated for 30 min at 37°C with ribonuclease A (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 50 µg/ml, then extracted twice with equal volumes of phenol saturated with TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA). The aqueous phase was separated by centrifugation at 5000×g for 10 min, and the DNA was precipitated by adding two volumes of cold ethanol. The DNA precipitates were rinsed with 70% ethanol, and dissolved in TE buffer. The DNA concentration was estimated according to the absorbance measured at 260 nm by a spectrophotometer (LKB, Sweden), assuming that an absorbance of 1.0 at 260 nm corresponded to 50 µg of DNA per ml.

Restriction endonuclease cleavage pattern analysis : All restriction endonucleases used were purchased from Nippon Gene Co. (Toyama, Japan). About 5 µg of mycoplasma DNA was digested at 37°C for 2 hr with 12–24 units of each enzyme under the appropriate buffer conditions recommended by the supplier. The DNA digests were electrophoresed on 1% agarose gels in TBE buffer (89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.2) at 100 V for 3 hr. The gels were stained with ethidium bromide and photographed under a UV transilluminator.

Total DNA hybridization procedure : Mycoplasma DNA dissolved in TE buffer at 100 µg/ml was heat-denatured for 5 min in a boiling-water bath and cooled rapidly on ice. The denatured DNA was serially diluted in 2×SSC (1×SSC is 0.15M NaCl plus 0.015M sodium citrate), and 1 µg to 1 ng of the DNAs was deposited under vacuum on nitrocellulose filters by a dot blot apparatus (ADVANTEC, Tokyo, Japan). The blotted filters were baked at 80°C for 2 hr, and incubated at 42°C for 3 hr in prehybridization buffer [50% v/v formamide, 5×SSC, 5×Denhardt's solution (1×Denhardt's solution contains 0.2 mg/ml each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll-Sigma Chemical Co.), 0.5% SDS, and 200 µg of denatured calf thymus DNA (Pharmacia, Sweden) per ml]. The genomic DNAs of *M. pulmonis* m53, *M. arthritidis* PG 6, and *M. neurolyticum* Type A were labeled with [α -³²P] deoxycytidine triphosphate by the multiprime DNA labeling system (Amersham, England). The labeled DNAs were heat-

denatured and mixed with the prehybridization buffer at a final concentration of 5 ng/ml, and used as the hybridization mixtures. Hybridization was carried out in the hybridization mixtures at 42°C overnight. The filters were then washed twice in 2×SSC/0.1% SDS at room temperature and twice in 0.1×SSC/0.1% SDS at 65°C, dried, and autoradiographed.

Results

Restriction endonuclease cleavage patterns of genomic DNA : Figure 1 shows the genomic DNA cleavage patterns of *M. pulmonis* m53, *M. arthritidis* PG 6 and *M. neurolyticum* type A obtained with various restriction endonucleases. Genomic DNAs of all three species were cleaved to varying degrees by *Eco*RI, *Hind*III, *Pst*I and *Bam*HI, and each of the three species showed a distinct cleavage pattern. The number of cleavage bands obtained with *Pst*I (recognition site CTGCAG) and *Bam*HI (GGATCC), which recognize the guaniner-plus-cytosine (G + C)-rich sequences, was less than that obtained with *Eco*RI (GAATTC) and *Hind*III (AAGCTT), which recognize the adenine-plus-thymine (A + T)-rich sequences. No or very few cleavage bands were found when *Sma*I was used.

The DNA cleavage patterns of the three strains of *M. pulmonis*, the one strain of *M. pulmonis*, the one strain of *M. arthritidis* and the two strains of *M. neurolyticum* were compared by using *Hind*III, which produced the largest number of cleavage bands, and *Pst*I, which produced the characteristic cleavage patterns for each species, with a small number of bands. After digestion with *Hind*III, DNAs from the three strains of *M. pulmonis* and DNAs from the two strains of *M. neurolyticum* showed a remarkably similar cleavage pattern, but it was difficult to determine the details of the differences between strains because of the large number of cleavage bands (Fig. 2-A). When *Pst*I was used, the DNA cleavage patterns of the three strains of *M. pulmonis* were almost identical, showing only minor variation. The cleavage patterns of the two strains of *M. neurolyticum* were identical (Fig. 2-B).

Total DNA hybridization test : Total DNA hybridization was carried out by using the DNA-DNA dot hybridization technique. As shown in Fig. 3-A, the ³²P-labeled total geno-

Fig. 1. Genomic DNA cleavage patterns of three rodent mycoplasma species obtained with the restriction endonucleases *EcoRI* (A), *HindIII* (B), *PstI* (C), *BamHI* (D), and *SmaI* (E) Lane 1: *M. pulmonis* m53 Lane 2: *M. arthritidis* PG 6 Lane 3: *M. neurolyticum* Type A Lane M contained *HindIII*-digests of λ phage DNA as size markers

mic DNA of *M. pulmonis* m53 showed a hybridization signal to as little as 10 ng of homologous *M. pulmonis* m53 DNA. It also showed a hybridization signal of the same intensity to the DNA of *M. pulmonis* M 4 and R 1 strains. In contrast, it showed only weak hybridization with 1 μ g of DNA from *M. arthritidis* and *M. neurolyticum*. The 32 P-labeled total genomic DNAs of *M. arthritidis* PG 6 and *M. neurolyticum* Type A also showed a hybridization signal to as little as 10 ng of DNA from the same species, but only a weak signal to 1 μ g of DNA from different species (Fig. 3-B, C).

Discussion

We compared genomic DNAs of three typical rodent mycoplasmas, *M. pulmonis*, *M. arthritidis* and *M. neurolyticum*, according to their restriction endonuclease cleavage patterns. Selection of the restriction endonuclease used for digestion of DNAs is important in analyzing restriction endonuclease cleavage patterns. Since DNAs of most mycoplasmas have a low C+C content, the DNA cleavage pat-

terns produced with restriction endonucleases that recognize G + C-rich sequences show a small number of bands, providing an easy comparison [1, 7]. In agreement with these findings, our results also showed cleavage patterns that varied distinctly with the species when *PstI* was used, although the number of cleavage bands produced with *BamHI* and *SmaI* was extremely small. On the other hand, there were only slight differences in the restriction endonuclease cleavage patterns of genomic DNAs, despite differences in antigenicity found by the complement fixation test, between the three strains of *M. pulmonis*, m53, M 4 and R 1 [6]. Watson et al. [11] reported that the genomes of *M. pulmonis* strains were very closely related. Our findings are in agreement with their observations.

We also employed total DNA hybridization to examine the genetic relatedness of the three species of rodent mycoplasmas and among strains of the same species. The genetic relatedness among prokaryotes has been conventionally assessed on the basis of the DNA homology value determined by the quantitative

Fig. 2. Genomic DNA cleavage patterns of some strains of rodent mycoplasma species obtained with the restriction endonucleases *Hind*III (A) and *Pst*I (B) Lane 1: *M. pulmonis* m53 Lane 2: *M. pulmonis* M4 Lane 3: *M. pulmonis* R1 Lane 4: *M. arthritidis* PG 6 Lane 5: *M. neurolyticum* Type A Lane 6: *M. neurolyticum* H322 Lane M contained *Hind*III-digests of λ phage DNA as size marers

Fig. 3. DNA-DNA dot hybridization among rodent mycoplasmas. 32 P-labeled total genomic DNA of *M. pulmonis* m53 (A), *M. arthritis* PG 6 (B), or *M. neurolyticum* Type A (C) was used for each hybridization test. The amount of target DNA in a dot is shown on the left side of each row. Lane 1: *M. pulmonis* m53 Lane 2: *M. pulmonis* M 4 Lane 3: *M. pulmonis* R 1 Lane 4: *M. arthritis* PG 6 Lane 5: *M. neurolyticum* Type A Lane 6: *M. neurolyticum* H 322

DNA-DNA hybridization test, but we carried out the DNA-DNA dot hybridization test. This test was simple and provided visually distinct results. Ezaki et al. [4] reported that the results obtained by this procedure were in good agreement with the DNA homology values. The DNA-DNA dot hybridization test revealed only weak cross-hybridization among

the three species of rodent mycoplasmas, indicating considerable heterogeneity of these species. On the other hand, hybridization among the strains of *M. pulmonis* was strong, revealing the high degree of genetic homogeneity within this species. Similarly, *M. neurolyticum* strains showed remarkable genetic homogeneity.

These findings suggest the possibility of identifying the three species of rodent mycoplasmas on the basis of the restriction endonuclease cleavage patterns of genomic DNA or by total DNA hybridization on the basis of the species specificity of their DNA sequences.

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げっ歯類由来マイコプラズマのゲノムDNA解析

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げっ歯類由来の代表的なマイコプラズマである *Mycoplasma pulmonis*, *M. arthritidis*, *M. neurolyticum* の3菌種間およびこれらの菌種の株間におけるゲノムDNAの比較検討を行った。3菌種のDNAは、明らかに異なる制限酵素切断パターンを示した。また、全DNAの交雑試験においても、3菌種は遺伝的に異なることが示された。一方、*M. pulmonis* 3株間およ

び *M. neurolyticum* 2株間においては、DNAの制限酵素切断パターンはほぼ同一であり、全DNAの交雑試験でも、同一菌種の株間における遺伝的均一性が示された。これらの成績は、げっ歯類に由来する各マイコプラズマ種のゲノムDNAの塩基配列が、高い種特異性を有することを示唆している。