Restriction Endonuclease Analyses of Two Porcine Mycoplasma Deoxyribonucleic Acids: Sequence-Specific Methylation in the *Mycoplasma hyopneumoniae* Genome†

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The deoxyribonucleic acids of two porcine mycoplasmas, *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*, were distinguished readily with restriction enzymes. Enzymes that recognized guanine-plus-cytosine-rich sequences, such as *Sma*I and *Bam*HI, were particularly useful since they generated relatively small numbers of deoxyribonucleic acid fragments which could be resolved by gel electrophoresis. In addition, we found that the adenine nucleotide in the GATC sequence of *M. hyopneumoniae* is methylated, whereas that in *M. flocculare* is not.

Identification and classification of mycoplasmas have been difficult due to the scarcity of available biochemical assays, which have been limited to a few serological tests. Recently, Bové and Saillard (3) proposed a new approach to classification of mycoplasmas based on the use of restriction endonucleases. Characterization of bacterial plasmids or viral genomes by using restriction endonucleases has become a standard and indispensable tool in modern molecular biology. Unfortunately, when the much larger genomes of bacteria are digested with restriction enzymes, there are too many deoxyribonucleic acid (DNA) fragments generated for clear separation by gel electrophoresis. The average genome size of mycoplasmas is between 750 and 1,400 kilobases (17), which is smaller than the genomes of most known bacteria, and thus the DNAs of mycoplasmas are amenable to restriction enzyme analysis (20, 21).

In this study, DNAs from *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* were compared by using a number of restriction endonucleases. These two mycoplasma species are common isolates from porcine respiratory tracts; *M. hyopneumoniae* is the primary agent in mycoplasmal pneumonia of swine, whereas *M. flocculare* is considered nonpathogenic. In addition to having the same habitat, *M. hyopneumoniae* and *M. flocculare* are glycolytic and on agar generally form colonies without central zones (27). Although these two species can be distinguished by certain serological tests (25), analysis by crossed-immunoelectrophoresis has shown that they are more closely related to one another than is usual among glycolytic mycoplasmas (L. H. Ro and R. F. Ross, Am. J. Vet. Res., in press). Because of their close serological relatedness, additional characteristics that distinguish these two species would be of considerable taxonomic and diagnostic value. In the work reported here, we found that *M. hyopneumoniae* and *M. flocculare* indeed can be distinguished by restriction enzyme digestion. Moreover, we found that the adenine nucleotide in the GATC sequence of *M. hyopneumoniae* is methylated, whereas that in *M. flocculare* is not.

MATERIALS AND METHODS

Cells. *M. hyopneumoniae* strains J^T (= ATCC 25934^T); T = type strain) (passage 52), 11 (passage 20), 1361A (passage 13), and 1375c (passage 11) and *M. flocculare* ATCC 27399^T were cultivated in Friis medium (9) supplemented with 1% dextrose. The organisms were incubated in stationary cultures at 37°C until color change and turbidity were observed. Samples were examined microscopically and passaged into blood agar to assure that no bacteria were present. The cells were harvested by centrifugation at 16,000 × *g* for 30 min and were washed twice by centrifugation at 12,000 × *g* for 15 min with 0.25 M NaCl–0.01 M MgCl₂. The pellets were frozen at –70°C.

Preparation of mycoplasmal DNAs. Mycoplasmal DNAs were prepared by modifying the procedure of Cerone-McLernon and Furness (4). Briefly, frozen mycoplasma cells were suspended in 25 ml of cold 0.1 M NaCl–0.01 M ethylenediaminetetraacetate buffer (pH 8.5) containing 0.2 µg of ribonuclease per ml. A 1 M tris(hydroxymethyl)aminomethane hydrochloride solution (pH 9.0) containing 10% sodium dodecyl sulfate was added to give a final concentration of 1% sodium dodecyl sulfate. The cells were lysed, and the ribonucleic acid was destroyed by incubating the mixture at 37°C for 30 min. The lysates were then extracted twice with equal volumes of phenol. The mixture was then centrifuged, and the upper aqueous phase was removed. The aqueous phase was extracted twice with chloroform; 2.5 volumes of ice-cold 95% ethanol was added to the aqueous phase to precipitate the DNA. The DNA precipitate was suspended in buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4) and 0.1 mM ethylenediaminetetraacetate.

Preparation of *M. hyopneumoniae*-infected bronchial specimens. Yorkshire-Hampshire cross-bred pigs 8 weeks old were obtained from a disease-free herd maintained at Iowa State University. The pigs were inoculated intratracheally with an *M. hyopneumoniae* pneumonic lung inoculum (W. Amanfu et al., Am. J. Vet. Res., in press); 32 days after inoculation the animals were necropsied. Pneumonia typical of enzootic pneumonia was observed in the lungs of all of the pigs inoculated with *M. hyopneumoniae*. Lung washings were collected by flushing the airways of the left lung

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(bronchus and bronchioles) with 50 ml of saline. DNAs were prepared from the lung washings by a standard sodium dodecyl sulfate-phenol extraction procedure.

Restriction enzyme digestion of DNA. All restriction endonucleases were purchased from New England BioLabs or Bethesda Research Laboratories, Inc. The reaction conditions used were the conditions specified by the suppliers. Restriction fragments were separated by electrophoresis on 0.7% agarose gels. The running tris(hydroxymethyl)aminomethane-borate buffer (pH 8.2) contained 89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, and 2.5 mM ethylenediaminetetraacetate.

Southern blot analyses of DNAs. DNA samples cleaved with the appropriate enzymes were separated by electrophoresis on 0.7% agarose gels. The restriction fragments were then denatured in situ and blotted onto nitrocellulose filters by the procedure of Southern (26), as modified by Jeffreys and Flavell (13). The blotted nitrocellulose filters were soaked in $3 \times$ SSC containing $10 \times$ Denhardt solution (5) for 2 h at 60°C ($1 \times$ SSC is 150 mM sodium chloride plus 15 mM sodium citrate). DNA from an arbitrarily chosen recombinant plasmid, which contained a 9-kilobase *Hind*III fragment insertion of *M. hyopneumoniae* DNA, was radioactively labeled by using a standard nick-translation procedure (22). The filters were hybridized against the radioactively labeled probe and washed as described previously (12). After air drying, the filters were exposed to Kodak XAR-2 X-ray film to detect the hybridized probe.

Molecular cloning of *M. hyopneumoniae* DNA. *M. hyopneumoniae* strain J^T DNA was cleaved with *Hind*III. The DNA

digest was ligated to plasmid pBR322 (2) DNA which had been cleaved with *Hind*III and treated with calf intestine alkaline phosphatase. Competent *Escherichia coli* strain HB101 cells were transformed with the ligation mixture by using the standard CaCl_2 procedure (14). Ampicillin-resistant, tetracycline-susceptible colonies were selected.

RESULTS

Restriction enzyme digestion of *M. hyopneumoniae* and *M. flocculare* DNAs. The results obtained after cleavage of *M. hyopneumoniae* and *M. flocculare* DNAs with *Sma*I (CCCGGG), *Bam*HI (GGATCC), *Hind*III (AAGCTT), *Eco*RI (GAATTC), *Bcl*I (TGATCA), and *Msp*I or *Hpa*II (CCGG) are shown in Fig. 1. Although all of the enzymes generated restriction patterns that readily distinguished the two species, better resolution of low-molecular-weight DNA fragments was obtained with enzymes that recognize guanine-plus-cytosine-rich sequences (e.g., *Sma*I and *Bam*HI) than with enzymes that recognize adenine-plus-thymine-rich sequences (*Eco*RI and *Hind*III). Cleavage with *Msp*I or *Hpa*II (23), enzymes which recognize the tetranucleotide CCGG, also generated DNA fragments of the two species that could be resolved easily.

DNA methylation in *M. hyopneumoniae*. DNA from *M. hyopneumoniae* was completely resistant to digestion by *Bcl*I (Fig. 1), suggesting that it was methylated (1). In contrast, DNA from *M. flocculare* was quite susceptible to *Bcl*I cleavage. Figure 2 shows the patterns obtained by digesting DNAs from *M. hyopneumoniae* strain J^T and *M.*

FIG. 1. Restriction digests of *M. hyopneumoniae* and *M. flocculare* DNAs. DNAs were prepared from mycoplasma cells and subjected to restriction digestion and gel electrophoresis as described in the text. (A) Lane M contained *Hind*III digests of λ DNA; the fragments were 23, 9.6, 6.7, 4.2, 2.2, and 1.9 kilobases long. Lanes 1, 3, 5, 7, and 9 contained *Sma*I, *Bam*HI, *Hind*III, *Eco*RI, and *Bcl*I digests of *M. hyopneumoniae* DNA, respectively; lanes 2, 4, 6, 8, and 10 contained *Sma*I, *Bam*HI, *Hind*III, *Eco*RI, and *Bcl*I digests of *M. flocculare* DNA, respectively. (B) Lanes 1 and 2 contained *Msp*I and *Hpa*II digests of *M. hyopneumoniae* DNA, respectively. Lanes 3 and 4 contained *Msp*I and *Hpa*II digests of *M. flocculare* DNA, respectively. The sizes (in kilobases) of *Hind*III-cleaved λ DNA fragments are indicated on the left.

FIG. 2. Evaluation of DNA methylation in two porcine mycoplasmas by using restriction endonuclease isoschizomers. Uncleaved DNAs (lanes 1 and 5) and *Sau3A* (lanes 2 and 6), *MboI* (lanes 3 and 7), and *DpnI* (lanes 4 and 8) digests of DNAs from *M. hyopneumoniae* (lanes 1 through 4) and *M. flocculare* (lanes 5 through 8) were analyzed by agarose gel electrophoresis. The sizes (in kilobases) of *HindIII*-cleaved λ DNA fragments are indicated on the left.

flocculare with *DpnI*, *MboI*, and *Sau3A* (6, 10, 23), three restriction endonuclease isoschizomers which are particularly useful in evaluating the state of adenine methylation. Consistent with the *BclI* cleavage results, DNA from *M. hyopneumoniae* resisted digestion by *MboI*, an enzyme which cleaves at GATC only when adenine is not methylated. In contrast, cleavage was obtained with both *Sau3A*, which cleaves at GATC regardless of whether adenine is methylated, and *DpnI*, which cleaves only if the adenine in GATC is methylated. These results suggest that the adenine nucleotide in GATC from *M. hyopneumoniae* strain J^T is methylated. On the other hand, *M. flocculare* DNA was equally susceptible to cleavage by *MboI* and *Sau3A*, and thus the GATC sequence is not methylated to any appreciable extent.

To ascertain whether methylation of the adenine nucleotide of the GATC sequence is common among strains of *M. hyopneumoniae*, DNAs from three other strains of this organism (strains 11, 1361A, and 1375C) were prepared and analyzed with *DpnI*, *MboI*, and *Sau3A*. Our results indicate that the DNAs from all four strains of *M. hyopneumoniae* tested appear to contain N⁶-methyladenine in GATC.

***M. hyopneumoniae* cells recovered from infected pigs also contain methylated DNA.** Total DNA was prepared from lung washings of *M. hyopneumoniae*-infected pigs and cleaved

with either *DpnI*, *MboI*, or *Sau3A*. Southern blot hybridization was performed as described above (Fig. 3). Consistent with the results obtained with in vitro-grown cells of *M. hyopneumoniae*, *M. hyopneumoniae* cells recovered from infected animals also contained methylated bases (G^mATC) in their DNA. The radioactive probe used in these experiments also hybridized with *M. flocculare* DNA. Although the DNA fragment sizes were different for the two mycoplasmas, the strong intensity of hybridization with both species indicated that in at least a portion of their genomes, the two species share a high degree of homology in their nucleotide sequences.

DISCUSSION

The small sizes of the genomes of mycoplasmas, as well as their low guanine-plus-cytosine contents (17), facilitates analysis of their DNAs by restriction enzyme digestion. Restriction enzymes which recognize guanine-plus-cytosine-rich sequences are particularly useful since they generate a relatively small number of DNA fragments which can then be resolved by gel electrophoresis.

Sequence-specific methylation is quite common among the procaryotes. The DNA may be methylated at either the

FIG. 3. Hybridization of mycoplasma DNAs to a *M. hyopneumoniae* recombinant DNA probe. (A) DNAs prepared from in vitro-cultured *M. hyopneumoniae* cells (lanes 1 through 3) and *M. flocculare* cells (lanes 4 and 5) were cleaved with *Sau3A* (lanes 1 and 4), *MboI* (lanes 2 and 5), and *DpnI* (lane 3) and analyzed by blot hybridization as described in the text. (B) DNA prepared from lung washings of *M. hyopneumoniae*-infected pigs was cleaved with *Sau3A* (lane 1), *MboI* (lane 2), and *DpnI* (lane 3). Southern blot hybridization was performed as described in the text.

adenine residue (N^6 -methyladenine) or at the cytosine residue (5-methylcytosine) (18). The biological significance of methylation of DNA has not been determined. Although some sequence-specific methylation is clearly part of the restriction-modification phenomenon in bacteria (7, 24), other sequence-specific methylation, such as that induced by the *E. coli* *dam* gene (15) (which methylates adenine in the GATC sequence), is not associated with restriction modification. The *dam* methylase is believed to play a role in postreplication mismatch repair (11).

DNA from some mycoplasmas has been reported previously to contain methylated bases (19). The extent of methylation of adenine residues in mycoplasmal DNA has been reported to range from 0.2 to 2%. It has also been reported that 5.8% of the cytosine residues in *Mycoplasma hyorhinis* DNA are methylated (19). *Acholeplasma laidlawii* strain K2 has also been reported to have methylated cytosine in its GATC sequence (8).

Whether the sequence-specific methylation which we describe in DNA from *M. hyopneumoniae* is associated with a restriction-modification system or is part of a methylation pathway similar to that in *E. coli* *dam* is unclear at this point. One restriction enzyme, *Tha*I, has been isolated from *Thermoplasma acidophilum* (16). *T. acidophilum* is a wall-less procaryote similar to the mycoplasmas but differs substantially from all other members of the *Mollicutes*. Thus, whether *M. hyopneumoniae* or other members of the *Mollicutes* produce restriction enzymes is of great interest and awaits further study.

As suggested by Bové and Saillard (3), restriction enzyme analysis of mycoplasmas provides a useful procedure for additional characterization of mycoplasmas. As illustrated in the present study, *M. hyopneumoniae* and *M. flocculare* can be clearly distinguished by comparing electropherograms of DNA digests. This comparison was suggested recently by the Board of the International Research Program on Comparative Mycoplasmaology.

The distinctiveness of the patterns of digests from *M. hyopneumoniae* and *M. flocculare* supports the serological evidence obtained by metabolic inhibition tests that these two species are indeed distinct. Obviously, DNA-DNA hybridization must be performed before reaching a definite conclusion as to the degree of genetic relatedness between these two mycoplasma species. Although *M. hyopneumoniae* and *M. flocculare* share a common habitat (the porcine respiratory tract), they differ in that *M. hyopneumoniae* is a very important pathogen, whereas *M. flocculare* is apparently without pathogenic potential.

Our ability to detect mycoplasmas in clinical material (i.e., lung washings) by using a nick-translated cloned mycoplasma DNA probe may be of interest to workers in the field. The scarcity of biochemical assays for mycoplasmas renders diagnosis of mycoplasmal infections difficult. Thus, the use of DNA probes offers an easy and sensitive detection method.

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