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Analysis of *Mycoplasma hyorhinis* Genome by Use of Restriction Endonucleases and by Electron Microscopy

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The chromosome of *Mycoplasma hyorhinis* was analyzed by using different restriction endonucleases and electron microscopy. It was found that restriction enzymes *Bst*EII, *Xho*I, and *Sac*I are the enzymes of choice for analysis and characterization of *M. hyorhinis*. The bands resulting from digestion of *M. hyorhinis* DNA with *Bst*EII had apparent molecular weights ranging from 1.2×10^6 to 75×10^6. The apparent total molecular weight of DNA was calculated from the molecular weights of the individual bands and found to be 251×10^6. Electron microscopic contour length measurements of the largest DNA fragments verified the molecular weight values calculated from gel analysis. Electron microscopic contour length measurements of intact DNA of *M. hyorhinis* revealed a molecular weight of $5.4 \pm 5 \times 10^8$. The discrepancy between the values of molecular weight of *M. hyorhinis* DNA as determined by restriction enzyme analysis and contour length measurement is based on the fact that some of the DNA fragments which migrate as an apparent single band in the agarose gel really are double or multiple DNA fragments.

Although mycoplasmas seem to occur ubiquitously, and some are of medical interest because they cause severe disease in humans and other species, limited studies of their genomes have been carried out only in a few cases (15).

In the past the relatively small-sized DNA molecules of viruses, mitochondria, and plasmids have been studied by a combination of restriction enzyme analysis and subsequent fractionation by gel electrophoresis. Large DNA molecules of molecular weight of 500×10^6 or greater, with a few exceptions (2), have not been subjected to this analysis. Attempts to circumvent the low resolution of large DNA molecules have been made by using horizontal slab gels of dilute agarose (6). However, the electrophoretic behavior of large DNA molecules on these dilute gels was found to be different; i.e., they migrate faster than expected and are not well resolved under the conditions usually employed (6). Besides, dilute (0.1%) agarose slab gels are extremely difficult to handle.

Here we report on a combined restriction enzyme and electron microscopic analysis of *Mycoplasma hyorhinis* DNA. *M. hyorhinis* belongs to the group of so-called "noncultivable" mycoplasmas which until recently have not been grown on agar-broth media (1, 5, 9). These strains of *M. hyorhinis* are the only mycoplasmas known to require cell cultures for their growth. Although small numbers of *M. hyorhinis* particles inoculated into cell cultures reach high titers within a few days, their propagation in special synthetic cell-free medium (7) needs a long period of cultivation with progeny of mycoplasma particles. Recently we reported that, among cell cultures, mink lung cells are the cells of choice for rapid propagation of *M. hyorhinis* with very high titer (3); with this system it is possible to achieve the high amounts of this difficult-to-cultivate mycoplasma necessary for molecular biological study of its components. We hope the results presented here will facilitate future studies on this large DNA of 500×10^6 molecular weight, including the construction of a physical map as well as the classification of the different strains of this mycoplasma into subgroups.

MATERIALS AND METHODS

Growth of *M. hyorhinis*. The following *M. hyorhinis* strains were obtained from the American Type Culture Collection: *M. hyorhinis* ATCC 17981, 23839, 25021, 27717, and 29052. A variety of cloned *M. hyorhinis* isolated in this laboratory was also used. The isolate *M. hyorhinis* D3 corresponds to *M. hyorhinis* ATCC 25021. These *M. hyorhinis* strains were propagated and plaque assayed as described previously (3).

Production and purification of *M. hyorhinis*. For the production of *M. hyorhinis*, monolayers of Mv1Lu

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cells were infected with plaque-purified *M. hyorhinis* at a multiplicity of infection of 0.1 PFU per cell. After complete cell lysis had occurred, the supernatants of the infected cultures were centrifuged at 1,000 × *g* for 20 min to remove Mv1Lu cell debris. The supernatants were then centrifuged at 2,500 rpm at 4°C in an SW27 rotor for 60 min. Pellets were suspended in TNE (0.01 M Tris-hydrochloride, pH 7.4, 0.10 M NaCl, 0.001 M EDTA) and pelleted again through a 15-ml cushion of 25% (wt/wt) sucrose at 15,000 rpm at 4°C in an SW27 rotor for 120 min. The pellets were resuspended in TNE (2 ml) and centrifuged by isopycnic centrifugation in a 25 to 60% (wt/wt) sucrose density gradient at 20,000 rpm at 4°C in an SW27 rotor for 19 h. The particles banded at a density of 1.19 g ml^{-1}. This band, which contained 90% of the infectivity, was dialyzed against TNE, layered on a CsCl step gradient (successive 0.5-ml layers of 50, 40, 30, 20, and 5% [wt/vol] CsCl in 1 mM EDTA–0.01 M sodium phosphate [pH 7.5]) in nitrocellulose tubes, and centrifuged at 25,000 rpm at 15°C in an SW41 rotor for 1 h. The band in the lower half of the gradient was collected by piercing the side of the tube with a needle and syringe. The collected material was brought to 36% CsCl (wt/vol) and placed on the bottom of a nitrocellulose tube of an SW41 rotor. The tube was filled with a continuous CsCl gradient (15 to 30%, wt/vol) and centrifuged at 15,000 rpm at 10°C in an SW41 rotor for 16 h. The band in the lower half of the gradient was collected at a density of 1.22 g ml^{-1} and dialyzed. The purified particles contained 85% of the infectivity of the original unpurified *M. hyorhinis* suspension.

Extraction of intact *M. hyorhinis* DNA. Folded intact chromosomal DNA of *M. hyorhinis* was extracted from purified cells of *M. hyorhinis* according to a previously described procedure (8) from which the dialysis step was omitted. DNA concentration was determined by the absorbance at 260 nm and at 280 nm. The preparations had a ratio of absorbance at 260 and 280 nm of 1.9 to 2.0.

Restriction enzyme analysis. The following restriction enzymes were used: *Alu*I, *Ava*I, *Bal*I, *Mam*HI, *Bgl*I, *Bgl*II, *Bst*EII, *Cla*I, *Dpn*I, *Eco*RI, *Hae*II, *Hin*cII, *Hin*dII, *Hin*dIII, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*II, *Mst*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Taq*I, *Xba*I, and *Xho*I. These enzymes were purchased from Biolabs (Beverly, Mass.), BRL (Neu-Isenburg, Germany), or Boehringer (Mannheim, Germany). Incubations were carried out according to a standard procedure for each enzyme, and the resulting DNA fragments were separated on 0.4 or 0.5% agarose slab gels (Seakem, Biomedical, Rockland, Maine) or both. Electrophoresis was performed at 4°C in vertical gels (35 or 40 by 20 by 0.3 cm) which were subsequently stained with ethidium bromide (0.5 μg/ml) and photographed under UV light after electrophoresis with 75 V (constant voltage).

Isolation of restriction fragments from agarose gels. DNA was isolated from agarose gels by using electrophoretic elution as described earlier (10).

Electron microscopy. Cytochrome spreading of *M. hyorhinis* DNA was essentially carried out as described by Davis et al. (4). Contour length measurements on the restriction fragments were made using an *x*, *y*-coordinate measuring stage (Bruehl, Nürnberg). The length of the large circular DNA was determined by computer analysis of an optronics scan of the negatives, using an algorithm described elsewhere (14).

Nick translation. Amounts of the DNA smaller than 0.1 μg were labeled in vitro according to Rigby et al. (13). Each DNA sample (20 μl) contained 2 μCi each of [α-^{32}P]TTP, [α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dGTP (Amersham; specific activity, 2,000 to 3,000 Ci/mmol). The reactions were started by the addition of 0.1 mU of DNase I (Serva, Heidelberg) and 0.2 U of DNA polymerase I from *Escherichia coli* (Boehringer, Mannheim) and were stopped by the addition of EDTA when the ratio of acid-insoluble to acid-soluble radioactivity had reached a plateau.

RESULTS

The analysis of DNAs of different strains of *M. hyorhinis* by a variety of restriction endonucleases revealed characteristic fragment patterns of DNA bands. The results of restriction enzyme analyses are compiled in Table 1. The restriction enzymes tested can be classified into three groups according to the number of the resulting DNA bands and fragments. Group I encompasses those enzymes for which 40 DNA bands or more appeared in the gel. As an example for group I, the pattern of DNA of different *M. hyorhinis* strains after digestion with the restriction endonuclease *Hin*dIII is shown (Fig. 1A). Although the enzymes of group I cut the DNA many times, the resulting cleavage patterns are useful for the reliable identification of different *M. hyorhinis* strains. *Dpn*I cuts *M. hyorhinis* DNA many times. This demonstrates that a relatively high percentage of the adenine residues must be methylated, as also reported by Razin and Razin (12).

The second group (group II) includes restriction enzymes which produce cleavage patterns with a limited number of DNA bands (smaller than 40). Figure 1B presents the DNA cleavage pattern of different *M. hyorhinis* strains after digestion with the *Sac*I enzyme. The clear *Sac*I pattern with its distinct and characteristic DNA bands should be particularly useful for the fine analysis of the *M. hyorhinis* genome. Two other enzymes, namely, *Bst*EII and *Xho*I, as shown in Fig. 1C and D and 2A and B, cleave the *M. hyorhinis* DNA even less often than the *Sac*I enzyme. Since these two endonucleases give rise to 8 to 9 (*Bst*EII) and 10 to 12 (*Xho*I) DNA bands, both of these enzymes can be used for a straightforward restriction analysis. The DNA bands (A, B, C, and D in Fig. 2) of higher molecular weights obtained after *Bst*EII digestion could be separated from each other when the time of electrophoresis was increased (Fig. 2B, lane 5). The same result holds true for the higher-molecular-weight DNA bands after *Xho*I digestion (Fig. 1D, lane 2) and their separation (Fig. 3A, lane 2). Although *Kpn*I also produces a

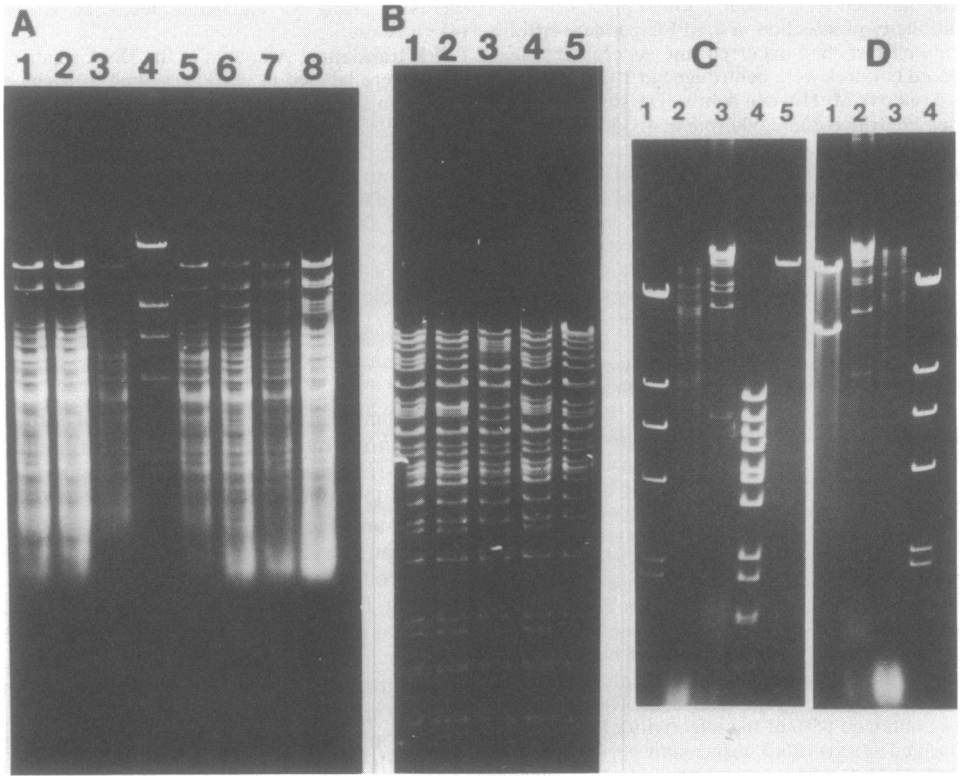

FIG. 1. (A) Cleavage pattern of different *M. hyorhinis* DNAs by restriction endonuclease *Hin*dIII. DNAs: Lane 1, *M. hyorhinis* D5; 2, *M. hyorhinis* D3; 3, *M. hyorhinis* D2; 4, phage lambda DNA as marker; 5, *M. hyorhinis* ATCC 25021; 6, *M. hyorhinis* ATCC 23839; 7, *M. hyorhinis* ATCC 17981; 8, *M. hyorhinis* ATCC 27717. Agarose (0.5%) slab gel electrophoresis; 35 by 20 by 0.3 cm; 4°C; 75 V; 20 h. (B) Cleavage pattern of DNAs of different *M. hyorhinis* strains by restriction endonuclease *Sac*I. *M. hyorhinis* strain DNAs: Lane 1, D3; 2, ATCC 17081; 3, ATCC 23839; 4, ATCC 25021; 5, ATCC 27717. Agarose slab gel electrophoresis; gradient gel (1.0, 0.9, 0.8, 0.6, 0.5%); 35 by 20 by 0.3 cm; 4°C; 75 V; 48 h. (C and D) Cleavage patterns of *M. hyorhinis* DNAs by restriction endonucleases *Bst*EII and *Xho*I. (C) Lane 1, *Hin*dIII digest of lambda DNA; 2, *M. gallisepticum* DNA digested with *Bst*EII; 3, *M. hyorhinis* D3 DNA digested with *Bst*EII; 4, phage lambda DNA digested with *Bst*EII; 5, phage lambda DNA, undigested. (D) Lane 1, phage lambda DNA digested with *Xho*I; 2, *M. hyorhinis* DNA digested with *Xho*I; 3, *M. gallisepticum* DNA digested with *Xho*I; 4, *Hin*dIII digest of phage lambda DNA. Agarose (0.5%) slab gel electrophoresis; 35 by 20 by 0.3 cm; 4°C; 75 V; 24 h.

limited number of DNA bands (Table 1), the bands of high molecular weight migrated too close to each other to allow an unambiguous analysis. Group III endonucleases (Table 1), such as *Sma*I and *Bgl*II, produced DNA bands which migrated close to undigested DNA of *M. hyorhinis*.

To determine the high molecular weight of the intact *M. hyorhinis* chromosome, experiments were performed using the restriction enzyme *Bst*EII and *M. hyorhinis* D3. The molecular weights of the individual bands were estimated by using a variety of standard markers of known molecular weights (native DNA and fragments of DNA of herpes simplex virus and phage lambda) under different electrophoretic conditions. The values found for each individual band (Fig. 2) are summarized in Table 2. To confirm the values estimated from the gel electrophoretic experiments, apparently single DNA bands after *Bst*EII digestion were isolated from the gel and the molecular weights of the eluted DNA molecules were determined by contour length measurement in an electron microscope (Table 2).

The sum of the molecular weights obtained by two independent methods resulted in an apparent total molecular weight of *M. hyorhinis* DNA of approximately 2.5×10^8. However, with respect to the different relative intensities of the DNA bands (e.g., bands A, B, C, D, etc.) of the

*Bst*EII digest (Fig. 1C, lane 3) and of the *Sac*I DNA fragment pattern (Fig. 1B), the above value must be considered an underestimate due to the presence of multiple bands. This value was compared to the result of length measurements on DNA clusters found in cytochrome *c* spreadings of intact DNA which apparently consisted of circular DNA molecules since they did not show free ends. These measurements were obtained by using an algorithm which automatically detects and links all discernible connected line structures in electron microscopic pictures scanned with a drum densitometer (14). This automatic length determination yielded a minimal molecular weight for five molecules of 5.4×10^8 (±10%). This result is in good agreement with contour length measurements described by Teplitz (16), which indicated a molecular weight of apparently 4.4×10^8. Since the total molecular weight of intact DNA of *M. hyorhinis* does not correspond to the molecular weight obtained by summing the individual DNA bands (Table 2), one has to assume that some of the DNA bands contain two or more fragments with similar lengths. This assumption was confirmed as follows. (i) After treatment of the *Bst*EII digest of

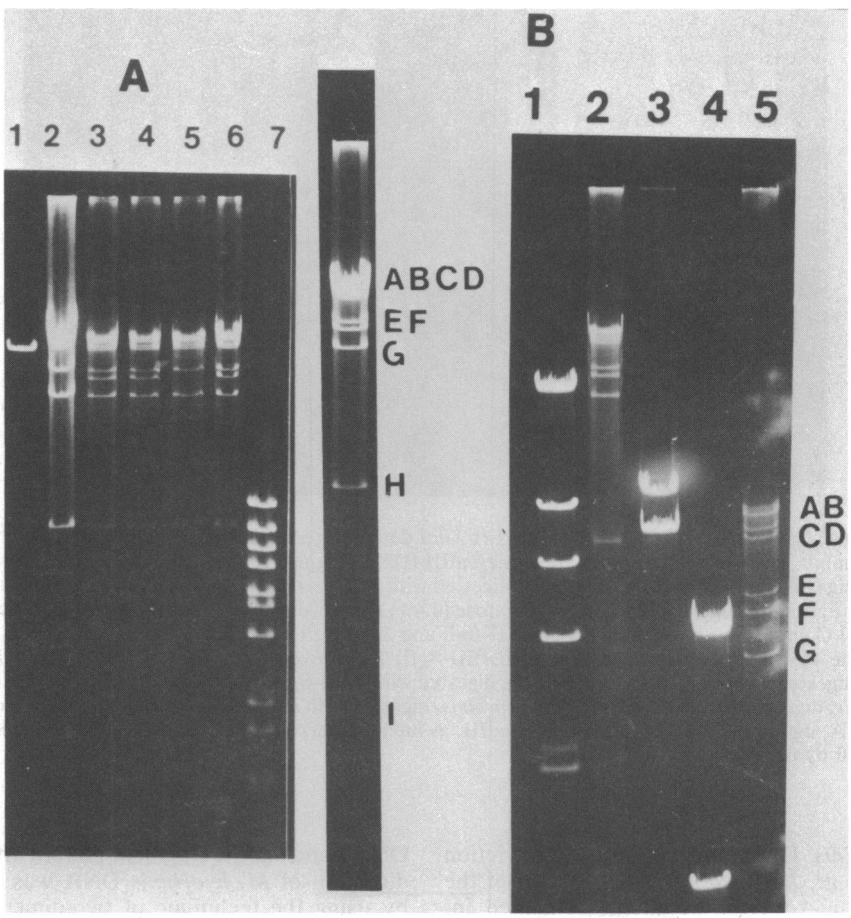

FIG. 2. (A) Cleavage pattern of DNAs of different *M. hyorhinis* strains by restriction endonuclease *Bst*EII. DNAs: Lane 1, phage lambda, undigested; 2, *M. hyorhinis* ATCC 27717; 3, *M. hyorhinis* ATCC 25021; 4, *M. hyorhinis* ATCC 23839; 5, *M. hyorhinis* ATCC 17981; 6, *M. hyorhinis* isolate 2711/79; 7, phage lambda. Agarose (0.5%) slab gel electrophoresis; 35 by 20 by 0.3 cm; 4°C; 75 V; 22 h. (B) Analysis of *M. hyorhinis* DNA using restriction enzyme *Bst*EII under different electrophoresis conditions. DNAs: Lane 1, phage lambda, digested with *Hin*dIII, as marker; 2, *M. hyorhinis* D3, digested with *Bst*EII; 3, herpes simplex virus and phage lambda DNAs, undigested, as internal markers; 4, phage lambda DNA digested with *Hin*dIII (fragments A and B) as internal markers; 5, *M. hyorhinis* D3, digested with *Bst*EII. Agarose (0.4%) slab gel electrophoresis; 40 by 20 by 0.3 cm; 4°C; 75 V; run times for lanes 1 and 2 were 19 h, and those for lanes 3, 4, and 5 were 48 h.

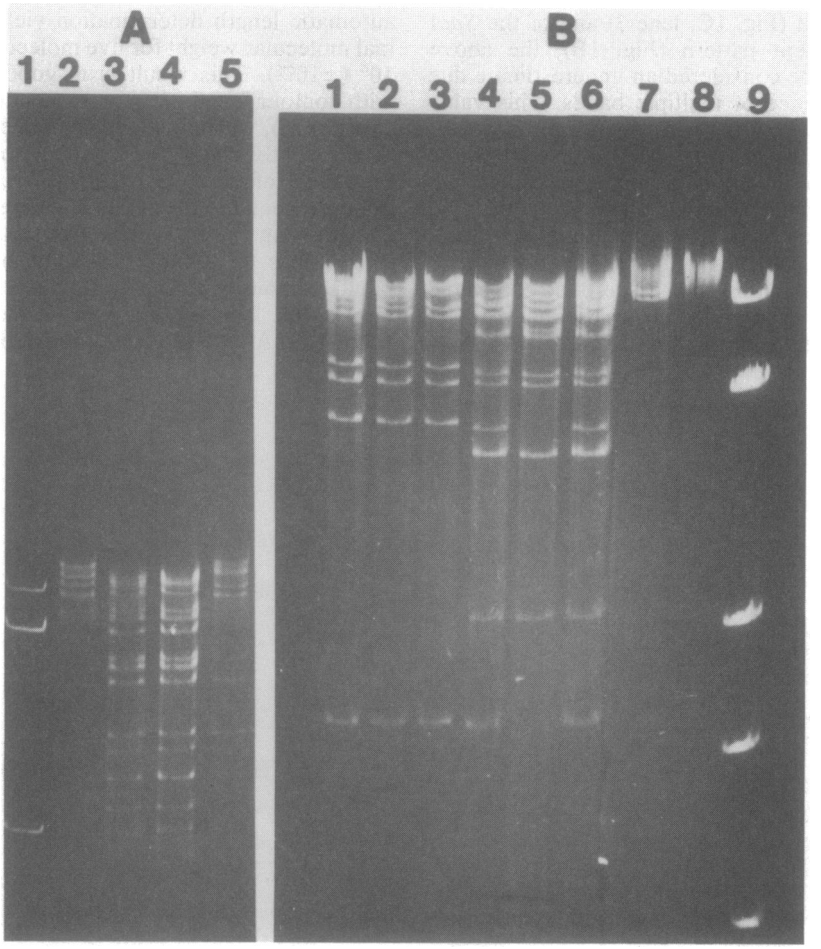

FIG. 3. (A) Composition of fragments in *Bst*EII-*Xho*I double digests of *M. hyorhinis* D3 DNA. DNAs: Lane 1, phage lambda, undigested and digested with *Hin*dIII (DNA fragments A and B), as internal markers; 2, *M. hyorhinis*, digested with *Xho*I; 3, *M. hyorhinis*, digested with *Xho*I-*Bst*EII; 4, *M. hyorhinis*, digested with *Bst*EII-*Xho*I; 5, *M. hyorhinis*, digested with *Bst*EII. Agarose (0.4%) slab gel electrophoresis; 40 by 20 by 0.3 cm; 4°C; 75 V; 52 h. (B) Composition of fragments in *Bst*EII-*Bgl*I and *Xho*I-*Bgl*I double digests of *M. hyorhinis* D3 DNA. DNAs: Lane 1, *M. hyorhinis*, digested with *Bst*EII-*Bgl*I; 2, *M. hyorhinis*, digested with *Bgl*I-*Bst*EII; 3, *M. hyorhinis*, digested with *Bst*EII; 4, *M. hyorhinis*, digested with *Xho*I-*Bgl*I; 5, *M. hyorhinis*, digested with *Xho*I; 6, *M. hyorhinis*, digested with *Bgl*I-*Xho*I; 7, *M. hyorhinis*, digested with *Bgl*I; 8, *M. hyorhinis*, undigested; 9, phage lambda DNA, undigested and digested with *Hin*dIII, as internal markers. Agarose (0.5%) slab gel electrophoresis; 35 by 20 by 0.3 cm; 4°C; 75 V; 43 h.

M. hyorhinis DNA with a second restriction enzyme, e.g., *Bgl*I, *Sac*I, or *Xho*I, most of the high-molecular-weight fragments remained intact, though a large set of smaller fragments was produced (Fig. 3). (ii) Furthermore, *Bst*EII fragments (A, B, and C) of DNA of *M. hyorhinis*, which were isolated as described in Materials and Methods, were nick-translated and treated again with *Sac*I or *Xho*I. The bands of high molecular weight did not disappear, whereas smaller fragments were generated (data not shown). In addition, the presence of multiple DNA bands in the fragment pattern after *Bst*EII digestion of *M. hyorhinis* DNA was confirmed by using the technique of two-dimensional gel electrophoresis for separation of DNAs recently described by Müller et al. (11) (data not shown).

DISCUSSION

The results reported here show that the large chromosomes of certain procaryotes are accessible to DNA analyses analogous to those performed for smaller viral DNAs. Thus the data can be considered as a first step towards the

TABLE 1. Analysis of *M. hyorhinis* DNAs using different restriction endonucleases and electrophoresis on agarose gels

Enzyme group	Restriction enzyme[a]	Apparent no. of DNA bands
I	*Eco*RI (G/AATTC)	>50
	*Dpn*I ($G^{me}A$/TC)	>50
	*Alu*I (AG/CT)	>50
	*Hin*dIII (A/AGCTT)	>50
	*Pvu*II (CAG/CTG)	>50
	*Pst*I (CTGCA/G)	>40
	*Hae*II (PuGCGC/Py)	>40
	*Ava*I (C/PyCGPuG)	>50
	*Taq*I (T/CGA)	>50
	*Cla*I (AT/CGAT)	>50
	*Hin*cII (GTPy/PuAC)	>50
	*Hin*dII (GTPyPuAC)	>40
	*Hpa*I (GTT/AAC)	>40
	*Mbo*II (GAAGA$(N)_8$) ($CTTCT(N)_7$)	>50
II	*Sac*I (GAGCT/C)	32[b]
	*Bal*I (TGG/CCA)	At least 20
	*Mst*I (TGCGCA)	30
	*Hpa*II (C/CGG)	At least 25
	*Xho*I (CTTCGAG)	10–12
	*Kpn*I (GGTAC/C)	At least 9
	*Xba*I (GTT/AAC)	25
	*Bst*EII (G/GTNACC)	8–9
III	*Bgl*II (A/GATCT)	1[c]
	*Bam*HI (G/GATCC)	1[c]
	*Sac*II (CCGC/GG)	At least 2
	*Sma*I (CCC/GGG)	1[c]
	*Sal*I (G/TCGAC)	1[c]
	*Bgl*I (GCC$(N)_4$NGGC)	At least 2

[a] The recognition sequence and site of cleavage are given in parentheses.

[b] Numbers of bands are dependent on the strain of *M. hyorhinis*; bands with an apparently higher intensity were counted as single bands.

[c] At origin close to undigested DNA.

TABLE 2. Molecular weights of DNA bands of *M. hyorhinis* cleaved with restriction enzyme *Bst*EII[a]

Fragment or band	Mol wt × 10^6, determined by:	
	Gel	Contour length measurement
A	76	75
B	52	50
C	34	33.3
D	31	33.3
E	19	18.8
F	17.6	18.3
G	13.8	ND
H	5.1	ND
I	1.2	ND

[a] Determined by restriction enzyme analysis and contour length measurement in an electron microscope. ND, Not done. Sum of DNA molecular weights of individual bands, ~250 × 10^6.

physical map of *M. hyorhinis* DNA. Since some mycoplasmas, including porcine mycoplasmas, are of economic interest in veterinary medicine, cloning and amplification of DNA fragments will be important to search for those necessary for the corresponding antigen expression. We believe that a more extensive use of restriction enzyme analyses of different mycoplasma genomes, such as performed for *M. hyorhinis* in this study, will lead to a new basis for a more meaningful classification of mycoplasmas in general and also to an understanding of their genomic structures. Although the presence of plasmid DNAs and concatemeric forms of DNA might possibly complicate the cleavage patterns, electron microscopy and gel electrophoresis of native DNA would detect such polymorphic forms of DNA. To our best knowledge, a classification of these microorganisms based on DNA cleavage patterns of restriction enzymes has been reported so far only for spiroplasmas by Bové and Saillard (2).

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