Chromosomal Heterogeneity of Various *Mycoplasma hyopneumoniae* Field Strains

JOACHIM FREY,* ANDREAS HALDIMANN, AND JACQUES NICOLET

*Institute for Veterinary Bacteriology, University of Bern, Länggassstrasse 122,
CH-3012 Bern, Switzerland*

Restriction enzyme digestion and field inversion gel electrophoresis were used to analyze the chromosomes of strains of *Mycoplasma hyopneumoniae* and the related organism *Mycoplasma flocculare*. The chromosome size for the *M. hyopneumoniae* type strain was calculated from individual fragments to be $1,011.3 \pm 32.9$ kbp. The chromosomes of *M. hyopneumoniae* field strains were approximately the same size. The restriction patterns obtained for the chromosomes of phenotypically similar *M. hyopneumoniae* strains were quite different. Therefore, the species *M. hyopneumoniae* seems to be very heterogeneous. A field inversion gel electrophoresis analysis of the entire chromosomes allowed us to distinguish *M. hyopneumoniae* strains easily and hence to characterize further the species *M. hyopneumoniae*. The chromosome size for *M. flocculare* was calculated to be 988.3 ± 39.5 kbp. Restriction enzyme *Xho*I, which statistically should cut the *M. hyopneumoniae* chromosome frequently, did not cut the DNA of any of the *M. hyopneumoniae* strains but did digest *M. flocculare* DNA, indicating that there is a site-specific modification at CTCGAG which probably belongs to a restriction modification system in *M. hyopneumoniae* and is absent in *M. flocculare*.

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are commonly isolated from porcine respiratory tracts. *M. hyopneumoniae* is the primary agent of mycoplasmal pneumonia in swine (enzootic porcine pneumonia) (11, 19). This disease is common all over the world and causes large economic losses through retarded growth, poor food conversion, and increased susceptibility of swine to infection by other organisms (27). On the other hand, *M. flocculare* does not seem to be implicated directly in porcine mycoplasmal pneumonia (10). Studies on the taxonomic relationship between *M. hyopneumoniae* and *M. flocculare* have revealed only a few biochemical or cultural differences between these two mycoplasmas (26); the differentiation of these species is based on the results of serological tests, which are hampered by immunological cross-reactions between the species (1, 23). The two species are clearly different at the genetic level, since DNA-DNA hybridization has revealed levels of sequence identity of only approximately 10% (10). No heterogeneity has been reported previously within the species *M. hyopneumoniae*, probably because of the limited tests which are available for differentiation of strains.

Most information on *M. hyopneumoniae* has been obtained from analyses of type strain ATCC 25934 (= NCTC 10110 = J) (11, 26), which has been reported to be indistinguishable from field strains on the basis of the results of growth inhibition, metabolic inhibition, and direct immunoelectrophoresis tests (24, 26). However, very little is known about possible differences in the chromosome structure, immunogenic factors, and virulence of *M. hyopneumoniae* strains. Although immunoblot analyses performed with rabbit hyperimmune serum or serum from experimentally infected pigs do not distinguish *M. hyopneumoniae* strains, Ro and Ross (23) have detected antigenetic diversity among various *M. hyopneumoniae* strains by two-dimensional immunoelectrophoresis. Stipkovits et al. (31) have reported that an early and strongly immunogenic protein, protein P36, is specific to *M. hyopneumoniae* and is conserved in many *M. hyopneumoniae* strains obtained from various geographic

areas. Therefore, this protein is a useful marker for identification of *M. hyopneumoniae* strains and infections. It is essential to have a better understanding of the degree of genetic relatedness or variability among *M. hyopneumoniae* strains for studies on the taxonomy, epidemiology, and pathogenicity of this organism. Any analysis of genetic variability should be practical and reliable. In addition, fundamental knowledge concerning the structure and sizes of the chromosomes of the *M. hyopneumoniae* and the related nonpathogenic organism *M. flocculare* will be necessary to conduct further genetic studies on these species.

Restriction endonuclease fingerprinting analysis is a powerful technique for comparing DNAs of phenotypically and genetically similar organisms (13, 14, 17). However, this method is often hampered by the large number of DNA bands which have to be resolved or by the fact that only bands in a certain size range, representing only a part of the genome, can be analyzed. Field inversion gel electrophoresis (FIGE) (29) is an ideal tool for separating large DNA fragments in analyses of bacterial chromosomes. In this study our objective was to use restriction enzyme digestion and FIGE to analyze the chromosome of the *M. hyopneumoniae* type strain and to use this methodology to compare this type strain with various *M. hyopneumoniae* field strains and *M. flocculare* strains. We took advantage of the relatively low G+C contents (33 mol%) of the chromosomes of these two *Mycoplasma* species (30) and used restriction enzymes with GC-rich recognition sites to cut the chromosomes into approximately 20 large restriction fragments. This analysis permitted us to accurately calculate the sizes of the chromosomes of *M. hyopneumoniae* and *M. flocculare* and to investigate the genetic heterogeneity in various *M. hyopneumoniae* field strains.

MATERIALS AND METHODS

Strains and growth conditions. The porcine *Mycoplasma* strains used in this study are shown in Table 1. The strains were grown in Friis medium (10) at 37°C to the end of the exponential growth phase, which occurred when the pH of the medium was 6.8 (corresponding to a concentration of

* Corresponding author.

TABLE 1. *Mycoplasma* strains

Species	Strain	Origin	Reference or source ^a
<i>M. hyopneumoniae</i>	ATCC 25934 ^T (= J ^T)	United Kingdom	ATCC
<i>M. hyopneumoniae</i>	NCTC 10110 ^T (= J ^T)	United Kingdom	NCTC
<i>M. hyopneumoniae</i>	JF 184a ^T (= J ^T)	United Kingdom	11
<i>M. hyopneumoniae</i>	EP 29	Canada (C. L'Ecuyer)	Field isolate
<i>M. hyopneumoniae</i>	BQ 14	France (M. Kobisch)	Field isolate
<i>M. hyopneumoniae</i>	EP-S 924	Switzerland	Field isolate (our laboratory)
<i>M. hyopneumoniae</i>	EP-S 938	Switzerland	Field isolate (our laboratory)
<i>M. hyopneumoniae</i>	EP-S 939	Switzerland	Field isolate (our laboratory)
<i>M. hyopneumoniae</i>	EP-S 946	Switzerland	Field isolate (our laboratory)
<i>M. hyopneumoniae</i>	EP-S 223	Switzerland	Field isolate (our laboratory)
<i>M. flocculare</i>	NCTC 10143 ^T (= Ms 42 ^T)	Denmark	NCTC

^a ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, United Kingdom.

approximately 10⁸ cells per ml). Chloramphenicol was then added to the growth medium at a concentration of 80 µg/ml, and the cells were incubated for another 2 h before they were harvested in order to let replication forks terminate without the initiation of new rounds of replication (16, 22). The cells were harvested by centrifugation at 20,000 × *g* for 20 min, washed three times in phosphate-buffered saline (10 mM sodium phosphate [pH 7.0], 0.9% NaCl), resuspended in phosphate-buffered saline at a concentration of 3 × 10⁹ cells per ml, and kept frozen until they were used. The field isolates of *M. hyopneumoniae* were identified by using the growth inhibition test described by Clyde (7) and antisera against strain J^T (T = type strain) (11). The strains were also tested for the presence of protein P36, a common and specific antigenic protein of *M. hyopneumoniae* (31).

Southern hybridization of genomic DNA. Genomic DNAs of *M. hyopneumoniae* and *M. flocculare* were isolated as described previously (32). Aliquots (500 ng) of DNA were digested with *Eco*RI, separated on a 0.7% agarose gel in TBE buffer (28), and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.), by using a Pharmacia model LKB 2016 VacuGene system according to the instructions of the supplier. The probes which we used were chromosomal DNAs of *M. hyopneumoniae* NCTC 10110^T and *M. flocculare* Ms 42^T that were digested with *Eco*RI and labeled with [³²P]dCTP (3,000 Ci/mmol; Amersham) by using the random priming method (8). Hybridizations were carried out at 37°C in 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0]) containing 5% polyethylene glycol 6000, 0.5% sodium dodecyl sulfate, 50% formamide, and 100 µg of denatured and sonified salmon sperm DNA per ml. The filters were washed twice in 0.1 × SSC–0.1% sodium dodecyl sulfate at 50°C, a temperature that corresponded to the melting temperature of DNA–DNA duplexes having approximately 85% sequence identity. The filters were exposed for 1 day at –80°C on Fuji RX film by using an intensifying screen.

Preparation of chromosomal DNA in agarose blocks and FIGE. *Mycoplasma* cells were molded into blocks (1.5 by 5.5 by 4 mm) of low-gelling low-melting-point (LGLM) agarose (Biofinex, Praroman, Switzerland) at a concentration of 4 × 10⁹ cells per ml as described previously (22). The *Mycoplasma* cells were then lysed by placing the blocks in ETS buffer (10 mM Tris-HCl, 500 mM EDTA, 1% *N*-lauroylsarcosine [catalog no. L-5125; Sigma]) containing 1 mg of proteinase K (Sigma) per ml (pH 8.0) at 50°C for 18 h, and this was followed by incubation in TEP buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride; pH 8.0) at 4°C for 18 h. The blocks were then stored in

ETS buffer at 4°C. The blocks were washed three times for 2 h in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). DNA restriction digestion was performed by incubating the agar blocks in 250 µl of the appropriate assay buffer (as recommended by the producer) containing 20 U of restriction endonuclease (Boehringer Mannheim). The DNA was methylated in 250 µl of *Taq*I methylase buffer (Biolabs, Beverly, Mass.) containing 2 mM dithiothreitol, 0.1 mM *S*-adenosylmethionine, and 30 U of *Taq*I methylase.

We produced multimers of bacteriophage λ CI857Sam7 DNA (Boehringer Mannheim) to use as molecular size markers for DNA fragments by using the method of Bancroft and Wolk (2), taking care to thaw the DNA only once, just before multimerization and molding into agarose blocks. In addition, intact chromosomes of *Saccharomyces cerevisiae* YP148 (21) which were prepared by using the method of Bellis et al. (3) were used as size standards.

FIGE was performed as described by Carle et al. (5). Agarose gels (0.8%) were run for 40 h in TBE buffer (44.5 mM Tris, 44.5 mM sodium borate, 1 mM EDTA; pH 8.0) cooled to 15°C by using a voltage gradient of 4.0 V/cm. Pulse times were generated by using a model PPI-200 power inverter (MJ Research, Cambridge, Mass.). Preprogrammed pulse time ramps varying from 0.15 to 75 s forward and 0.05 to 25 s backward (according to the size range of DNA fragments to be analyzed) were generated by following the supplier's instructions. After electrophoresis, the gels were stained in ethidium bromide (1 µg/ml) for 30 min, and then they destained in 1 mM MgSO₄ for 60 min before photography under UV light. DNA fragments less than 20 kbp long were analyzed by conventional agarose gel electrophoresis (28), using bacteriophage λ DNA digested with *Hind*III as the size standard.

RESULTS

Chromosome sizes for *M. hyopneumoniae* and *M. flocculare*. Enzymatic digestion of the chromosomal DNA of *M. hyopneumoniae* ATCC 25934^T with 35 restriction enzymes with recognition sequences of 6 bp revealed that the enzymes that have only G and C residues in their recognition sequences generated 12 to 25 different restriction fragments (Table 2), while the numbers of fragments that were generated by the other enzymes were more than 50. Restriction enzyme *Xho*I did not cut *M. hyopneumoniae* DNA. Digestion with *Not*I (recognition sequence, GCGGCCGC) resulted in two fragments. Since the fragments that were produced with *Apa*I were resolved best on our gel systems, we used this enzyme for further studies. The fragments of *M. hyopneumoniae*

TABLE 2. Restriction enzymes that cut infrequently

Enzyme	Site	No. of fragments obtained with ^a :	
		<i>M. hyopneumoniae</i> ATCC 25934 ^T	<i>M. flocculare</i> NCTC 10143 ^T
<i>Apa</i> I	GGGCCC	12	11
<i>Bss</i> III	GCGCGC	18	ND
<i>Eag</i> I	CGGCCG	12	ND
<i>Nae</i> I	GCCGGC	18	ND
<i>Nar</i> I	GGCGCC	16	ND
<i>Sac</i> II	CCGCGG	23	ND
<i>Sma</i> I	CCCGGG	25	ND

^a The G+C content of both strains was 33 mol%. The mean $\pm$ standard deviation number of fragments for *M. hyopneumoniae* ATCC 25934^T was 18 $\pm$ 4. ND, not determined.

ATCC 25934^T and *M. flocculare* NCTC 10143^T chromosomal DNAs that were generated with *Apa*I were subjected to analysis on different FIGE gels by using various FIGE parameters in order to obtain high resolution of the sizes of all of the fragments which are shown in Table 3. The fragment sizes were calculated from the results of 10 independent experiments, and the accuracy of the size determinations was estimated to be 5% or better. Addition of the lengths of the different fragments indicated that the sizes of the *M. hyopneumoniae* ATCC 25934^T and *M. flocculare* NCTC 10143^T chromosomes are 1,011.3 $\pm$ 32.9 and 988 $\pm$ 39.5 kbp, respectively.

Chromosomal divergence. In order to estimate roughly the genetic similarity of the chromosomes of *M. hyopneumoniae* ATCC 25934^T, the field isolates, and *M. flocculare*, chromosomal DNAs of the strains were digested with *Eco*RI, separated by agarose gel electrophoresis, and analyzed by performing DNA-DNA hybridization experiments, using Southern blots and *Eco*RI-digested ³²P-labeled chromosomal DNA of strain ATCC 25934^T as the probe. All of the *M. hyopneumoniae* field strains produced hybridization patterns on autoradiograms that could not be distinguished from the pattern of the homologous type strain. In addition, no clear differences in the *Eco*RI restriction patterns of the various strains were observed because of the high number of bands.

TABLE 3. *Apa*I fragments of *M. hyopneumoniae* and *M. flocculare*^a

Fragment	Size (kbp) in:	
	<i>M. hyopneumoniae</i> ATCC 25934 ^T	<i>M. flocculare</i> NCTC 10143 ^T
A	278.3 $\pm$ 6.2	245.0 $\pm$ 4.1
B	154.0 $\pm$ 4.3	121.7 $\pm$ 6.2
C	144.0 $\pm$ 4.3	106.7 $\pm$ 6.2
D	117.0 $\pm$ 2.2	94.6 $\pm$ 4.1
E	106.6 $\pm$ 2.3	80.7 $\pm$ 4.1
F	72.3 $\pm$ 5.4	78.3 $\pm$ 2.4
G	63.0 $\pm$ 3.7	73.3 $\pm$ 2.3
H	26.0 $\pm$ 1.4	69.3 $\pm$ 3.2
I	16.7 $\pm$ 1.2	49.0 $\pm$ 3.7
J	15.5 $\pm$ 1.0	47.7 $\pm$ 2.1
K	9.6 $\pm$ 0.5	22.0 $\pm$ 1.1
L	8.3 $\pm$ 0.4	

^a The mean $\pm$ standard deviation chromosome sizes of *M. hyopneumoniae* ATCC 25934^T and *M. flocculare* NCTC 10143^T are 1,011.3 $\pm$ 32.9 and 988.3 $\pm$ 39.5 kbp, respectively. The means and standard deviations were calculated from 10 independent experiments for *M. hyopneumoniae* and from 3 independent experiments for *M. flocculare*.

Since *M. flocculare* DNA had only a few bands that hybridized with *M. hyopneumoniae*, the level of sequence identity between the chromosomal DNAs of these two species was estimated to be less than 10%.

A FIGE analysis of chromosomal DNAs that were digested with *Apa*I showed that there were clear differences in the restriction patterns of the various *M. hyopneumoniae* strains (Fig. 1). This method also showed that the restriction pattern for type strain J was identical to the patterns for the two type strains obtained from culture collections, strains ATCC 25934 and NCTC 10110 (Fig. 1). Interestingly, strain JF 184a^T which was a clone of strain J^T (received from P. Whittlestone) but had undergone a different number of passages in culture medium than the culture collection strains, contained an additional 125-kb *Apa*I fragment (Fig. 1) but lacked the 8-kb *Apa*I fragment (not visible on Fig. 1). Digestion with *Bst*EII confirmed that strain JF 184a^T contained additional DNA fragments compared with strains ATCC 25934^T and NCTC 10110^T. The various field strains produced different *Apa*I restriction patterns and could all be distinguished by the FIGE analysis. However, the field strains from Switzerland that were isolated by workers from our laboratory seemed to produce more homogeneous restriction patterns than the strains that had different geographical origins. The chromosome sizes of the different field strains were very similar to the chromosome size of the type strain (close to 1,000 kb) (Fig. 1). The same results were obtained when we used restriction enzyme *Bst*EII.

Site-specific modification of *M. hyopneumoniae* DNA. The DNAs of all of the *M. hyopneumoniae* strains that were tested in our study were completely resistant to digestion by *Xho*I, while *M. flocculare* DNA was fully digested and produced a large number of small fragments. *Xho*I restriction was sensitive to methylation of the C residue in position 3 and the A residue in position 5 (CTC⁺GA⁺G). Therefore, we used the restriction enzymes which contain the same "core" sequence (TCGA) in their recognition sequence as *Xho*I (CTCGAG) and which are sensitive to A residue methylation. Both *M. hyopneumoniae* and *M. flocculare* DNAs were digested with restriction enzymes *Bst*BI (TTCGAA), *Cla*I (ATCGAT), *Sal*I (GTCGAC), and *Taq*I (TCGA). DNAs from *M. hyopneumoniae* and *M. flocculare* that were methylated by using *Taq*I methylase (TCGA^m) were resistant to all of the restriction enzymes mentioned above (data not shown). It is worthwhile to note that an *M. hyopneumoniae* chromosome treated with *Xho*I or a chromosome from *M. hyopneumoniae* or *M. flocculare* treated with *Taq*I methylase and then incubated with any of the restriction enzymes mentioned above on FIGE analysis produced a band at approximately 1,000 kb that corresponded to the full-size chromosome. This might have represented chromosomal DNA that was randomly linearized once.

DISCUSSION

The techniques of pulsed-field gel electrophoresis, including FIGE and contour clamped homogeneous electric field electrophoresis, are suitable for determining the molecular sizes of large DNA fragments, particularly fragments in the range from 20 to 2,000 kbp (4, 5, 13). Therefore, these techniques have been used to analyze and determine the genome sizes of various bacteria. Because of the low G+C contents of mollicute DNAs, there has been concern about the value of these techniques for determining the chromosome sizes of *Mycoplasma* and *Acholeplasma* strains (18).

FIG. 1. Restriction enzyme profiles of *M. hyopneumoniae* strains and the *M. flocculare* type strain. The mycoplasmas were lysed in agarose blocks as described in the text and digested with *Apa*I, and the fragments were separated by FIGE on a 0.8% agarose gel (length, 25 cm) by using model PPI-200 power inverter program 4. The sizes are indicated on the left and were determined by using bacteriophage lambda multimers. The calculated chromosome sizes for the *M. hyopneumoniae* strains were as follows: strain ATCC 25934^T, $1,011.3 \pm 32.9$ kbp; strain NCTC 10110^T, $1,011.0 \pm 41.9$ kbp; strain JF 184a^T, $1,142.4 \pm 38.2$ kbp; strain EP 29, $1,025.0 \pm 58.3$ kbp; strain BQ 14, $1,069.2 \pm 66.2$ kbp; strain EP-S 924, 981.7 ± 31 kbp; strain EP-S 938, 970.0 ± 29 kbp; strain EP-S 939, 970 ± 29.5 kbp; strain EP-S 946, 992.5 ± 33.4 kbp; strain EP-S 223, 955.7 ± 30.6 kbp; and *M. flocculare* NCTC 10143, 988 ± 39.5 kbp. *Mhp*, *M. hyopneumoniae*; *Mf*, *M. flocculare*; Kb, kilobase pairs.

However, so far there have been no experimental data which prove that the low G+C contents affect the migration of DNA fragments during PFGE. In contrast, various workers have successfully used PFGE techniques to estimate the sizes of full-length *Mycoplasma* and *Acholeplasma* chromo-

somes that were linearized by gamma irradiation (20) or by partial restriction enzyme digestion (25).

The chromosome size for *M. hyopneumoniae* J^T (= ATCC 25934^T), as calculated from 12 *Apa*I restriction fragments, is $1,011.3 \pm 32.9$ kbp (Table 3) and corresponds well to the result obtained by Robertson et al. (25), who measured the linearized nonfragmented chromosome of *M. hyopneumoniae* ATCC 25095 (= VMRI-11). The size of the *M. flocculare* chromosome is 988.3 ± 39.5 kbp (calculated from 11 *Apa*I fragments) (Table 3). This is 100 kbp larger than the value determined by Robertson et al. (25) for the nonfragmented *M. flocculare* Ms 42^T (= ATCC 27716^T) chromosome. The difference in the two values could have been due to the fact that the strain used by Robertson et al. (25) (strain ATCC 27716^T) had been propagated and recloned under different growth conditions and for a different number of generations than the strain used in our study (strain NCTC 10143^T [= ATCC 27399^T]); such differences could have caused chromosome rearrangements in one of the strains.

The different restriction enzymes with 6-bp recognition sequences consisting of exclusively G and C residues which we used cut the *M. hyopneumoniae* chromosome an average of 18 times (Table 2). Assuming a G+C content of 33 mol% (30), we expected that the *M. hyopneumoniae* 1,000-kbp chromosome would be cut 18 times by enzymes that recognize a 6-bp sequence that is composed of only G and C residues (number of cuts = chromosome size [in bp] $\times$ $[G+C]^6 \times [0.5]^6$). This value correlates well with the mean number of fragments that were obtained experimentally (Table 2).

The restriction patterns shown in Fig. 1 were different for most *M. hyopneumoniae* strains. Although the strains that were isolated by workers from our laboratory in Switzerland could be distinguished, these strains shared a number of fragments. The differences among the strains isolated in different countries were much larger. The sizes of the chromosomes of most of the *M. hyopneumoniae* strains that were analyzed were close to 1,000 kbp; the exception was strain JF 184a^T, whose chromosome was approximately 100 kbp larger (Fig. 1). It is interesting that the cultures of strain J^T that were obtained from two culture collections (strains ATCC 25934^T and NCTC 10110^T) produced identical restriction patterns, while strain JF 184a^T, which is strain J^T culture that was obtained directly from P. Whittlestone, had a 125-kbp *Apa*I fragment instead of the 8.3-kbp fragment. We assumed that the chromosome of strain JF 184a^T had undergone a duplication of a 117-kbp chromosomal fragment or that the strain J^T cultures that were deposited in the culture collections had undergone a deletion of 117-kb during passage or recloning. A similar chromosomal rearrangement might also have occurred in one of the two culture collection cultures of type strain Ms 42^T, and this could have led to the difference in chromosome sizes described above. The finding that the chromosomes of *M. hyopneumoniae* strains are heterologous is surprising. Some variations, such as the differences among strains NCTC 10110^T, JF 184a^T, and ATCC 25934^T, which all originate from the strain J^T isolate, indicate that the chromosome of *M. hyopneumoniae* undergoes frequent chromosomal rearrangements. These arrangements could be due to repetitive (insertion sequence-like) elements that have recently been detected in *M. hyopneumoniae* (9, 12) and might play a role in control of gene expression and host or medium adaptation. However, other variations in the chromosomal structure seem to be due to larger phylogenetic differences since we observed clonal homogeneity of the strains that were isolated in Switzerland,

a small geographical area, and rather strong heterogeneity of strains that originated from distant places. However, the chromosome differences do not seem to affect the few phenotypes that are currently used for identification of *M. hyopneumoniae*. It should be noted that strains ATCC 25934^T and BQ 14, which produce very different *Apa*I restriction patterns, seem to have very different virulence properties when they are used in experimental infections (13a). A similar situation has been observed with *Leptospira* infections of pigs, in which differences in virulence are associated with differences in the chromosomal restriction patterns of strains which could not be distinguished by other methods (7a).

Restriction enzyme *Xho*I does not cut the chromosomal DNAs of the *M. hyopneumoniae* strains which we analyzed, although on the basis of statistics we expected it to cut the chromosome 114 times. In contrast, *M. flocculare* DNA was digested by *Xho*I. Therefore, believe that *M. hyopneumoniae* DNA is modified (probably methylated) at the *Xho*I site. Our results show that the modification must be specific to the *Xho*I recognition site. Therefore, *M. hyopneumoniae* might have its own restriction modification system. This modification is different from the more frequent site-specific methylation at GATC which has been described in *M. hyopneumoniae* (6) and resembles the methylation induced by the *Escherichia coli* *dam* gene. It should be noted that Pyle et al. (21) have found that the chromosome of *M. hyopneumoniae* is cut only once by *Xho*I, resulting in a 1,140-kbp fragment. On the basis of our results, we believe that the single 1,140-kbp fragment might have been created by linearization of the chromosome at random during an incubation reaction and not by site-specific cleavage.

In summary, we found that the chromosomes of *M. hyopneumoniae* strains are quite different. Restriction enzyme digestion with infrequently cutting enzymes and subsequent analysis by FIGE is a simple and reliable method for distinguishing different *M. hyopneumoniae* strains. This finding is important for studies of the taxonomy of *M. hyopneumoniae*, as well as for epidemiological studies of *M. hyopneumoniae* outbreaks. In view of the genetic diversity of *M. hyopneumoniae* strains, specific diagnostic tools, such as polymerase chain reaction amplification based on nucleotide sequences of randomly chosen DNA fragments, need to be evaluated with a large number of strains before they can be proposed as safe methods for detecting *M. hyopneumoniae* infections. Site-specific modification at CTCGAG, which is probably a component of a restriction modification system, seems to be common to *M. hyopneumoniae* strains and is not found in *M. flocculare*.

ACKNOWLEDGMENTS

We are grateful to Ruud Segers (Boxmeer, The Netherlands) for critical reading of the manuscript, to Jacques Falquet (University of Geneva) for valuable suggestions concerning the FIGE technique, to Lloyd Finch (University of Melbourne) for the gift of *S. cerevisiae* YP148, and to M. Hämmerli (Institute for Mathematical Statistics, University of Bern).

REFERENCES

1. Armstrong, C. M., M. J. Freeman, and L. Sands-Freeman. 1978. Cross-reactions between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*. Practical implications for the serodiagnosis of mycoplasmal pneumonia of swine. *Isr. J. Med. Sci.* **23**:654-656.
2. Bancroft, I., and C. P. Wolk. 1988. Pulsed homogeneous orthogonal field gel electrophoresis (PHOGE). *Nucleic Acids Res.* **16**:7405-7418.
3. Bellis, M., M. Pagès, and G. Roizès. 1987. A simple and rapid method for preparing yeast chromosomes for pulse field gel electrophoresis. *Nucleic Acids Res.* **15**:6749.
4. Cantor, C. R., C. L. Smith, and M. K. Mathew. 1989. Pulsed field gel electrophoresis of very large DNA molecules. *Annu. Rev. Biophys. Chem.* **17**:287-304.
5. Carle, G. F., M. Frank, and M. V. Olson. 1986. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* **232**:65-68.
6. Chan, H. W., and R. F. Ross. 1984. Restriction endonuclease analyses of two porcine *Mycoplasma* deoxyribonucleic acids: sequence-specific methylation in the *Mycoplasma hyopneumoniae* genome. *Int. J. Syst. Bacteriol.* **34**:16-20.
7. Clyde, W. A. 1983. Growth inhibition tests. *Methods Mycoplasma*. **1**:405-417.
- 7a. Ellis, W. A., J. M. Montgomery, and A. B. Thiermann. 1991. Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the australis serogroup of *Leptospira interrogans*. *J. Clin. Microbiol.* **29**:957-961.
8. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
9. Ferrell, R. V., M. B. Heidari, K. S. Wise, and M. A. McIntosh. 1989. A *Mycoplasma* genetic element resembling prokaryotic insertion sequences. *Mol. Microbiol.* **3**:957-967.
10. Friis, N. F. 1973. The pathogenicity of *Mycoplasma flocculare*. *Acta Vet. Scand.* **14**:344-346.
11. Goodwin, R. F. W., A. P. Pomeroy, and P. Whittlestone. 1965. Production of enzootic pneumonia in pigs with a mycoplasma. *Vet. Rec.* **77**:1247-1249.
12. Harasawa, R., K. Koshimizu, O. Takeda, T. Uemori, K. Asada, and I. Kato. 1991. Detection of *Mycoplasma hyopneumoniae* DNA by the polymerase chain reaction. *Mol. Cell. Probes* **5**:103-109.
13. Heller, C., and F. M. Pohl. 1989. A systematic study of field inversion gel electrophoresis. *Nucleic Acids Res.* **17**:5589-6005.
- 13a. Kobisch, M. (Ploufragen, France). Personal communication.
14. Kristiansen, B. E., B. Sørensen, B. Bjorvatn, E. S. Falk, E. Fosse, K. Bryn, L. Frøholm, P. Gaustad, and K. Bovre. 1986. An outbreak of group B meningococcal disease: tracing the causative strain of *Neisseria meningitidis* by DNA fingerprinting. *J. Clin. Microbiol.* **23**:764-767.
15. Kuijper, E. J., J. H. Oudbier, W. N. H. M. Stuif-Bergen, A. Jansz, and H. C. Zanen. 1987. Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of *Clostridium difficile*-induced diarrhea. *J. Clin. Microbiol.* **25**:751-753.
16. Lark, K. G. 1973. Initiation and termination of bacterial deoxyribonucleic acid replication in low concentrations of chloramphenicol. *J. Bacteriol.* **113**:1066-1069.
17. McInnes, J., J. Borr, M. Massoudi, and S. Rosendal. 1990. Analysis of southern Ontario *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* isolates by restriction endonuclease fingerprinting. *Can. J. Vet. Res.* **54**:244-250.
18. Maniloff, J. 1989. Anomalous values of *Mycoplasma* genomes sizes determined by pulse-field gel electrophoresis. *Nucleic Acids Res.* **17**:1268.
19. Maré, C. J., and W. P. Switzer. 1965. *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia. *Vet. Med.* **60**:841-845.
20. Neimark, H. C., and C. S. Lange. 1990. Pulse-field electrophoresis indicates full-length mycoplasma chromosomes range widely in size. *Nucleic Acids Res.* **18**:5443-5448.
21. Pyle, L. E., L. N. Corcoran, B. G. Cocks, A. D. Bergemann, J. C. Whitley, and L. R. Finch. 1988. Pulse field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. *Nucleic Acids Res.* **16**:6015-6025.
22. Pyle, L. E., and L. R. Finch. 1988. Preparation and FIGE separation of infrequent restriction fragments from *Mycoplasma mycoides* DNA. *Nucleic Acids Res.* **16**:2263-2268.
23. Ro, L. H., and R. F. Ross. 1983. Comparison of *Mycoplasma hyopneumoniae* strains by serologic methods. *Am. J. Vet. Res.* **44**:2087-2094.

24. Roberts, D. H., and T. W. A. Little. 1970. Serological studies in pigs with *Mycoplasma hyopneumoniae*. J. Comp. Pathol. **80**: 211–220.
25. Robertson, J. A., L. E. Pyle, G. W. Stemke, and L. R. Finch. 1990. Human ureaplasmas show diverse genome sizes by pulsed-field electrophoresis. Nucleic Acids Res. **18**:1451–1455.
26. Rose, D. L., J. G. Tully, and R. G. Wittler. 1979. Taxonomy of some swine mycoplasmas: *Mycoplasma suis* pneumoniae Goodwin et al. 1965, a later, objective synonym of *Mycoplasma hyopneumoniae* Mare and Switzer 1965, and the status of *Mycoplasma flocculare* Meyling and Friis 1972. Int. J. Syst. Bacteriol. **29**:83–91.
27. Ross, R. F. 1986. Mycoplasmal diseases, p. 469–483. In A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, T. H. C. Penny, and E. Scholl (ed.), Diseases of swine, 5th ed. Iowa State University Press, Ames.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. Schwartz, D. C., and C. R. Cantor. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37**:67–75.
30. Stemke, G. W., D. J. McIntyre, K. L. Roy, M. E. Stemler, and J. A. Robertson. 1985. Guanine-plus-cytosine compositions of and deoxyribonucleic acid hybridization comparisons between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*. Int. J. Syst. Bacteriol. **35**:527–529.
31. Stipkovits, L., J. Nicolet, A. Haldimann, and J. Frey. 1991. Use of antibodies against the P36 protein of *Mycoplasma hyopneumoniae* for the identification of *M. hyopneumoniae* strains. Mol. Cell. Probes **5**:451–457.
32. Strasser, M., J. Frey, G. Bestetti, M. Kobisch, and J. Nicolet. 1991. Cloning and expression of a species-specific early immunogenic 36-kDa protein of *Mycoplasma hyopneumoniae* in *Escherichia coli*. Infect. Immun. **59**:1217–1222.