Phylogenetic Relationships of Three Porcine Mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and Complete 16S rRNA Sequence of *M. flocculare*

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The nucleotide sequence of the 16S rRNA gene of *Mycoplasma flocculare* was determined and was compared with the sequence of a related porcine mycoplasma, *Mycoplasma hyopneumoniae*. While the overall level of DNA-DNA homology was approximately 11%, sequence alignment of the two 16S rRNA genes yielded a homology value of more than 95%, emphasizing the highly conserved nature of the 16S rRNA gene. Multiple sequence alignments with other mollicutes indicated that *M. flocculare*, *M. hyopneumoniae*, and *Mycoplasma hyorhinis* form a subcluster within the fermentans phylogroup, and this subcluster is distinct from the *Mycoplasma pneumoniae* phylogroup. Thus, the three mycoplasmas isolated from porcine respiratory systems exhibit phylogenetic similarities.

The following three mycoplasmal species have been isolated from porcine respiratory systems: *Mycoplasma hyopneumoniae* (*Mycoplasma suis pneumoniae*), *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*. Early work on the taxonomy of *M. hyopneumoniae* and *M. flocculare* was reported by Rose et al. (14). *M. hyopneumoniae* is a recognized etiological agent of porcine mycoplasmal pneumonia, while *M. hyorhinis* is an agent of polyserositis and arthritis (15). *M. flocculare* has not yet been implicated as an agent of naturally occurring diseases. However, antigenic cross-reactivity of *M. flocculare* and *M. hyopneumoniae* has been recognized; there is a weaker and more variable reaction with *M. hyorhinis* (1). The results of DNA-DNA hybridization studies have also indicated that there is some relatedness between *M. hyopneumoniae* and *M. flocculare* (10 to 11%); in contrast, *M. hyopneumoniae* and *M. flocculare* cross-hybridize with nonporcine mycoplasmas at levels of about 3% (17).

Woese (21) and Weisburg et al. (20) have used analysis of 16S rRNA gene sequences to determine the phylogenetic and taxonomic relationships among *Mollicutes* genera and species, as well as between *Mollicutes* and bacteria. We determined the complete 16S rRNA nucleotide sequence of *M. flocculare* and compared it with the previously published complete sequence of *M. hyopneumoniae* (19), as well as with partial sequences of several other *Mollicutes*, including *M. hyorhinis*, obtained from data banks.

MATERIALS AND METHODS

Culture. *M. flocculare* Ms42 was obtained from M. Kobish and was propagated on Friis medium (2) to the first visible color change. Cells were harvested by centrifugation at 14,000 × g for 25 min, washed once, and either used immediately for DNA preparation or stored as a frozen pellet at –80°C until they were used. DNA was purified as described previously (5). *M. flocculare* Ms42 (= ATCC 27716) was grown similarly, and its DNA was prepared.

The authenticity of the strain obtained from M. Kobish was determined by comparing the DNA of this strain with DNA obtained from *M. flocculare* Ms42 (= ATCC 27716); identical restriction enzyme digests were observed with both *EcoRI* and *HindIII*. These patterns differed from those of *M. hyopneumoniae* and *M. hyorhinis*. Furthermore, identical banding patterns for both samples of *M. flocculare* DNA were detected by using a 5S rRNA oligonucleotide probe. In addition, a 16S rRNA gene probe reacted with two *EcoRI* fragments that were approximately 5,700 and 6,500 bp long with both DNA samples, differing also from the sizes of 16S rRNA banding fragments found with either *M. hyopneumoniae* or *M. hyorhinis*.

Data base sequences. Sequences for several *Mollicutes* 16S rRNAs were obtained from the GenBank data base.

Vectors and hosts. Lambda-Dash *EcoRI* (Stratagene, La Jolla, Calif.) (*EcoRI*-cut and alkaline phosphatase-treated arms) was used with the host *Escherichia coli* LE392. We also used plasmid Bluescript (Stratagene) with strain LE392 as the host.

Cloning. In the cloning experiments first the phage arms were mixed with large fragments (9 to 23 kb) of a partial *EcoRI* digest of *M. flocculare* genomic DNA which had been size fractionated on sucrose as described elsewhere (16). The arms and insertion were ligated and packaged into phage heads by using a commercial packaging extract (Gigapack; Stratagene) and the supplier's protocol. This material was then plated with excess host bacteria and incubated until plaques were visible. Nitrocellulose membranes were applied to allow phage to adsorb to the membranes; these membranes were then treated with alkali, neutralized, blocked, and probed for *Mollicutes* 16S rRNA sequences by using an isolated radiolabeled insertion from *Spiroplasma citri* (9). The membranes were exposed to X-ray film and aligned with the phage plaque plate to pick positive plaques; six of these were plaque purified three times, each time verifying the 16S rRNA insertion by using the 16S rRNA probe.

While we encountered difficulties in the preparation of high-titer phage lysates, multiple rounds of plate lysates

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resulted in the preparation of adequate phage to purify and utilize for DNA preparation.

Attempts to directly sequence the lambda DNA insertion by using the double-strand sequencing procedure (see below) gave variable results. The insertion was then subcloned into Bluescript; a partial *EcoRI* digest was directly cloned into the *EcoRI* site of this plasmid. Colonies appearing on Luria broth plates containing 50 µg of ampicillin per ml were picked, and plasmid minipreparations were obtained. These preparations were again probed with the *S. citri* 16S rRNA insertion, and six were chosen for further work. One, pF1-14, was initially chosen for sequencing (see below). When we subsequently found that the entire 16S rRNA gene was not present in the pF1-14 insertion, we used pF1-6, which had a larger insertion. pF1-14 had a 6.5-kb insertion, and pF1-6 had a 12-kb insertion. The sequence which we determined was based upon data from both plasmids.

Sequencing. We used commercial dideoxy sequencing kit (Pharmacia, Uppsala, Sweden) and the Pharmacia protocol for double-strand sequencing. The initial primers were three universal 16S rRNA oligonucleotides (11). The resulting sequence was used to prepare additional primers for primer extension and fill-in, as well as to sequence from the other DNA strand.

Sequencing gels containing 6% acrylamide in 6 M urea were prepared and run as described previously (16). The gels were fixed in methanol-acetic acid for autoradiography with ³⁵S and were read by using an IBI sonic digitizer (International Biotechnologies, Inc., New Haven, Conn.) or a DNA-Parrot cursor and software (T & t Research, Etobicoke, Ontario, Canada).

Analysis. Sequencing files were compared by using the NIH/DCRT analysis system of M. Kanehisa (IBM PC version).

Multiple sequence comparisons were done by using the PCGENE (IntelliGenetics, Inc., Mountain View, Calif.) subroutine Clustal, which also provides a dendrogram output. Sequences need to be truncated to 1,200 nucleotides for this program. The default truncation is from the rRNA 5' end. Qualitatively identical results were obtained when we used files truncated from the 3' end, and the results were not changed significantly by decreasing the gap penalty from default values. The IntelliGenetics Suite program (IntelliGenetics, Inc.) adapted for the UNIX system on a Sun Workstation was used for full-length sequence comparisons.

Nucleotide sequence accession numbers. The nucleotide sequence described below has been deposited in the EMBL data base and is available under accession number X63377. The GenBank nucleotide sequence accession numbers for other *Mollicutes* 16S rRNAs which we used are as follows: *M. hyopneumoniae*, M30378; *M. hyorhinis*, M24658; *Mycoplasma arginini*, M24579; *Mycoplasma fermentans*, M24289; *Mycoplasma hominis*, M24473; *Mycoplasma capricolum*, X00921; *Mycoplasma pneumoniae*, M29061; *Mycoplasma muris*, M23939; *Mycoplasma salivarium*, M24661; *Spiroplasma apis*, M23937; and *Clostridium innocuum*, M23732.

RESULTS AND DISCUSSION

The complete nucleotide sequence of the *M. flocculare* 16S rRNA gene is shown in Fig. 1 along with the sequences of two porcine mycoplasmas, *M. hyopneumoniae* and *M. hyorhinis*, and *Mycoplasma arginini* and *Mycoplasma muris*, two less closely related mycoplasmas. The presumptive starting and terminating nucleotides for the mature 16S

rRNA were chosen on the basis of the sequence of *M. hyopneumoniae* 16S rRNA. This gave a full-length sequence of 1,548 nucleotides, making it among the largest known mycoplasma sequences. The very highly conserved internal *EcoRI* site (4) at position +690 (counting from the mature 16S rRNA 5' site) was noted.

While the overall level of DNA-DNA homology between *M. hyopneumoniae* and *M. flocculare* is only about 11% (17), the sequence analysis program of Kanehisa indicated that there is 95.6% homology with the sequence reported for *M. hyopneumoniae* (19) within the rRNA coding region. Thus, the highly conserved nature of the 16S rRNA gene is further documented. Most of the variations and the variations that were most closely spaced occurred in the region between positions 64 and 103, which represents a non-hydrogen-binding region in the stem-loop structure of *M. capricolum* 16S rRNA (7). Multiple variations also occurred in the regions from position 193 to position 231 and from position 478 to position 500.

The Clustal multiple-alignment program, in which we used the complete 16S rRNA gene of *M. hyopneumoniae* and complete and partial sequences downloaded from the GenBank data base, gave the dendrogram shown in Fig. 2 again indicating the close relationship between *M. flocculare* and *M. hyopneumoniae* but also indicating that these two species represent a subcluster of a larger cluster that includes *M. hyorhinis*. This larger cluster in turn is a portion of the cluster that includes *M. fermentans*, *M. hominis*, and *M. arginini*. This cluster is deeply separated from the cluster containing *M. muris*, *M. pneumoniae*, and *Ureaplasma urealyticum*. The clustering of ureaplasma with *M. pneumoniae* and the clustering of *M. capricolum* with a spiroplasma are in agreement with the results of Laigret et al. (10). *Clostridium innocuum* was used as an outgroup. Thus, our findings both confirm the findings of Woese (21), Weisburg et al. (20), and Laigret et al. (10) and provide support for the relationships described above for the porcine mycoplasmas.

Figure 1 shows the multiply aligned sequences and demonstrates the variations that are indicative of some of the cluster separations. Particularly notable are the *M. flocculare*-*M. hyopneumoniae* sequence AAA(G/A)TA at position 96 and the CT sequence at position 494. The *M. fermentans* cluster, to which these species belong, has a 2-base deletion at position 190 and a 3- or 4-nucleotide deletion compared with the *M. muris*-ureaplasma group at position 835. *M. flocculare* has 3- and 5-base additions at position 79 compared with *M. hyopneumoniae* and *M. hyorhinis*, respectively.

Conclusion. We determined the complete 16S rRNA nucleotide sequence of *M. flocculare* and compared it with the previously published complete sequence of *M. hyopneumoniae*, as well as with partial sequences of several other mollicutes, including *M. hyorhinis*, obtained from data banks. The very close sequence homology of *M. hyopneumoniae* and *M. flocculare* gives very firm evidence of the close phylogenetic relationship of these organisms and explains the antigenic cross-reactions observed between these organisms.

In the 3'-end region of both *M. hyopneumoniae* and *M. flocculare* the identical sequence GAACACCTCCTTTCTA is found. This sequence has been shown experimentally to be the 3'-OH end of the 16S rRNA of *M. hyopneumoniae* (17), and because of the identity of the DNA sequence in *M. flocculare* it may be postulated that the 3' end is the same. This sequence is identical to the sequence in *Bacillus subtilis* (12) and to the sequence in *Mycoplasma iowae* (3). Other

FIG. 1. Nucleotide sequence of *M. flocculare* and sequence alignment with other representative *Mycoplasma* species. An asterisk indicates a nucleotide that was identical to the *M. flocculare* nucleotide; a dash indicates a gap of one nucleotide. N, undetermined nucleotide; Mfl, *M. flocculare*; Mhp, *M. hyopneumoniae*; Mhr, *M. hyorhinis*; Mar, *M. arginini*; Mms, *M. muris*.

FIG. 2. Dendrogram representing the phylogenetic relationships derived from 16S rRNA sequence homologies of *Mollicutes* species. Sequences for comparison with *M. flocculare* were obtained from GenBank. The PCGene multiple-alignment subroutine Cluster was used to generate the dendrogram. All of the sequences were truncated to 1,200 nucleotides for this comparison.

mollicutes belonging to the spiroplasma phylogroup have a slightly longer 3' end.

While the 16S rRNA of *M. flocculare* is larger than that of *M. hyopneumoniae*, it is not immediately evident that *M. flocculare* is ancestral to *M. hyopneumoniae* or vice versa. Thus, the pattern of changes in *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* does not clearly indicate a direction of change. For instance, *M. hyopneumoniae* and *M. hyorhinis* have adenine at position 488, while *M. flocculare* has cytosine; however, *M. flocculare* and *M. hyorhinis* have adenine at position 240, while *M. hyopneumoniae* has guanine.

M. hyopneumoniae has a single set of rRNA genes, and the 5S gene is separated by more than 4 kb from the 16S-23S rRNA genes (19). We found (6) that *M. flocculare* also has only a single set of rRNA genes, on the basis of the results of restriction endonuclease digestion with a variety of enzymes; in no case was 5S and 16S rRNA activity detected on a single fragment with probes specific for these genes. We are in the process of constructing a physical map of the *M. flocculare* genome in which we will show that the 5S rRNA gene of both *M. hyopneumoniae* and *M. flocculare* is at least 100 kb from the 16S gene.

We also found (6) by performing a restriction ribonuclease analysis that *M. hyorhinis* probably has only one set of rRNA genes; also, 5S and 16S gene probes never reacted with the same restriction fragment. These findings further indicate the close genetic relationship between the three mycoplasmas isolated from porcine respiratory systems. In turn, this suggests that these organisms evolved from a common ancestor in a common host. We have reported previously (17) that *M. hyopneumoniae* and *M. flocculare* have identical genomic G+C contents (33 mol%); more recently, Kirchhoff and Flossdorf reported that *M. hyopneu-*

moniae has a G+C content of 27.5 mol% (8). *M. hyorhinis* has been reported to have a G+C content of 28 mol% (13). Redetermination of these values will be required to ascertain whether any of them might be in error or whether the possible variation between *M. hyopneumoniae*-*M. flocculare* and *M. hyorhinis* simply reflects the plasticity of *Mollicutes* genomes.

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