Phylogenetic Analysis of Mycoplasma Strain ISM1499 and Its Assignment to the Acholeplasma oculi Strain Cluster

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A mycoplasma strain designated ISM1499 was used to develop a mycoplasma genetic system (G. G. Mahairas and F. C. Minion, J. Bacteriol. 171:1775–1780, 1989; G. G. Mahairas, C. Jian, and F. C. Minion, Gene 93:61–65, 1990), but phenotypic inconsistencies led to the conclusion that this organism had been classified incorrectly as a member of the species Mycoplasma pulmonis. Studies were initiated to determine the proper taxonomic position of ISM1499, and on the basis of the results of our genetic analysis, this strain was assigned to the Acholeplasma oculi strain cluster. The base composition of strain ISM1499 was identical to the base composition of A. oculi 19L, but not to the base composition of Acholeplasma laidlawii PG8 (28.3 and 30.7 mol% G+C, respectively). The taxonomic position of ISM1499 was examined by performing a parsimony analysis with 16S rDNA sequence data, and the results were compared with previous phylogenetic reconstructions. Our results indicated that ISM1499 is more closely related phylogenetically to A. oculi 19L than to A. laidlawii PG8 and JAI. Heterogeneity in the 16S rDNA sequences of A. oculi 19L and ISM1499 and in the 16S rDNA sequences of A. laidlawii PG8 and JAI may indicate that unusual dissimilarities occur in the 16S rRNA sequences of members of the genus Acholeplasma.

Mycoplasma strain ISM1499 was originally isolated from a Mycoplasma pulmonis culture as a high-frequency transforming isolate. This organism was used to study mycoplasma transformation and to establish a mycoplasma cloning system (9–12). On the basis of the results of these studies and Western blot (immunoblot) analyses performed with antisera raised in rabbits against a M. pulmonis stock strain by Minion and coworkers, strain ISM1499 was assumed to be a M. pulmonis strain, possibly a membrane or restriction mutant with a mutation which conveyed upon it the high-frequency transforming phenotype.

Dybvig and coworkers first noticed differences between ISM1499 and other M. pulmonis strains when they examined recA homology and phage typing data (4, 5). A serological examination by J. G. Tully (Mycoplasmology Section, Laboratory of Medical Microbiology, National Institute of Allergy and Infectious Diseases confirmed that ISM1499 is an Acholeplasma strain rather than an M. pulmonis strain; this resulted in author's corrections being placed in both the Journal of Bacteriology and Plasmid (11, 12). The serological typing results indicated that ISM1499 is antigenically related to Acholeplasma oculi and Acholeplasma laidlawii; the strongest reactivity was obtained with an anti-A. oculi 19L antisera.

The class Mollicutes comprises a unique group of catabacteria that lack cell walls (17). In a newly proposed classification scheme Tully et al. (24) divided these organisms into eight genera on the basis of the phylogenetic data of Weisburg et al. (26) and various other characteristics, including a sterol growth requirement (the genera Mesoplasma, Acholeplasma, and Asteroplasma). There are other distinguishing features, but typically, the mycoplasmas and ureaplasmas are considered to be human and animal pathogens; spiroplasmas, entomoplasmas, and mesoplasmas are insect and plant pathogens or commensal organisms, and the acholeplasmas, anaeroplasmas, and asteroplasmas are generally considered commensal organisms.

The genus Acholeplasma currently includes nine recognized species that have been differentiated by a limited number of biochemical properties and by serological techniques. Serological procedures have proven to be very useful for separating species in the genus Mycoplasma, but the use of such procedures with Acholeplasma species has been problematic. Acholeplasmas are ubiquitous, and low anti-achopleasplasma antibody levels in preimmune rabbit sera are often found (23). In addition, acholeplasmas adsorb serum proteins to their membranes, resulting in the production of antibodies to these proteins and potential serological cross-reactions between species. Both of these phenomena undoubtedly led to confusion in laboratories concerning the correct genus and species of isolate ISM1499. Also, growth-inhibiting antibodies produced in rabbits often have low potency (23). Several investigators have also examined acholeplasmas by performing nucleic acid hybridization experiments (1, 18), and their results have indicated that acholeplasmas species exhibit little interspecific homology at the whole-genome level and that species designations may represent clusters within the genus Acholeplasma that exhibit substantial discontinuities.

Because of the importance of the genetic studies performed with strain ISM1499 to the field of mycoplasma genetics, it was important to establish the identity of this strain conclusively. We performed studies to analyze phylogenetically the 16S rRNA sequence of ISM1499. It was necessary to sequence the 16S rRNA genes of A. oculi 19L (= ATCC 27350) and A. laidlawii PG8 (= ATCC 23206) as well. On the basis of the results of this analysis, the results of an examination of the guanine-plus-cytosine (G+C) contents of these species, and the results of serological typing experiments, strain ISM1499 was assigned to the A. oculi strain cluster.
Anaeroplasma bactoclasticum phoresis followed by treatment with Geneclean. SalI-PstI and PstI-BamHI fragments of cloned 16s rDNAs were then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The ligated into similarly restricted pKS I1 vector DNA (Stratagene, La Jolla, Calif.), methods. Unique SalI and BamHI sites in the primer sequences were restricted, and transformed into Escherichia coli DH5a [supE44 AlcU169(+80 lucZAM15). Mycoplasma mycoides Mycoplasma hyorhinis Mycoplasma haemofelis Mycoplasma lineatus Mycoplasma hominis Mycoplasma hypophyseoneumae Mycoplasma iowae Mycoplasma mycoides Mycoplasma pneumoniae Spiroplasma citri Ureaplasma urealyticum

Materials and Methods

bacterial strains. Table 1 shows the strains used in this study. In some instances, the 16s rDNA sequences were obtained directly from the GenBank database. Standard FPLD broth medium supplemented with 10% agamous horse serum, 2.5% fresh yeast extract, and 0.5% glucose (12) was used to grow all Mycoplasma strains.

Nucleic acid preparation. Genomic DNAs from A. laidlawii PG8 (= ATCC 23206), A. oculi 19L (= ATCC 27350), and ISM1499 were prepared as follows. Cells from a 10 mL culture were harvested by centrifugation at 20,000 x g for 15 min at 4°C, washed once with Tris-saline (0.1 M Tris, 0.14 M NaCl [pH 7.4]), and resuspended in 100 µL of Tris-EDTA buffer (100 µL of a solution containing 1% sodium dodecyl sulfate and 400 µg of proteinase K per mL was added, and the mixture was incubated for 1 h at 37°C). The DNA-containing solution was then extracted twice with phenol-chloroform (1:1) and once with chloroform and then precipitated with 2 volumes of ethanol. The DNA solution was then extracted twice with phenol-chloroform (1:1) and once with chloroform and then precipitated with 2 volumes of ethanol. The DNA was solubilized in Tris-EDTA buffer. Aliquots were electrophoresed in a 0.7% agarose gel and stained with ethidium bromide. DNA concentrations were determined by fluorescence with a model TKO 100 mini-fluorometer (Bio-Rad Laboratories, Richmond, Calif.).

PCR amplification and 16s ribosomal RNA (rDNA) purification. A 0.2- to 0.5-µg portion of DNA was amplified in a 100-µL reaction mixture. Each reaction mixture contained 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.1% Triton X-100, 2 mM MgCl2, primers (each at a concentration of 0.6 µM), each dNTP, plasmid DNA polymerase (Gibco BRL, Gaithersburg, Md.). The primers used were primers F1 (cggctagtgaAGATTGTTACCTGGCTCAG) and RP1 (cggcgccagacACCG TTGTTATCGTGGCGGATCTGCTGCGGATG), which are complementary to the 5' and 3' ends, respectively, of the 16S rRNAs of most eubacteria. Oligonucleotides were synthesized by standard methods with an automated DNA synthesizer (Applied Biosystems, Foster City, Calif.). PCR were performed in an Eppendorf twin-block temperature cycler (Eppendorf, Inc., San Diego, Calif.). The thermal profile involved 30 cycles of denaturation at 93°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 3 min. In the final cycle, primers were extended for 6 min. Aliquots of amplified samples were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The resulting PCR-amplified 1.5-kb fragment was purified by agarose gel electrophoresis followed by treatment with GeneClean.

Cloning. PCR-amplified 16S rDNA fragments were cloned by standard method. Unique SalI and BamHI sites in the primer sequences were restricted, and ligated into similarly restricted pKS II vector DNA (Stratagene, La Jolla, Calif.), and transformed into Escherichia coli DH5a [supE44 lacI]rha17 recA1 endA1 gyrA96 thi-1 relA1. Plasmids were prepared by using the alkaline lysis method of Birnboim and Doly (2). The SalI-EcoRI, EcoRI-BamHI, SalI-KpnI, KpnI-PstI, and PstI-BamHI fragments of cloned 16S rDNAs were then subcloned into the same vector for sequence determinations.

Sequencing. For sequencing, plasmids were purified by using Qiagen columns (Qiagen, Inc., Chatsworth, Calif.). The DNA sequence was determined by using standard dye dispersion sequencing techniques or by automated cycle sequencing. All sequences were obtained in both directions at least three times by using the universal and reverse primers. Some regions were sequenced manually by using T7 polymerase (United States Biochemical Corp., Cleveland, Ohio) and primers T3 and T7. Overlapping sequences were aligned using the DNASIS software program (Hitachi Software Engineering America, Ltd., San Diego, Calif.).

The aligned sequences were analyzed by using the phylogenetic analysis using parsimony package (PAUP version 3.1 [19]). A preliminary analysis was performed to verify the utility of the parsimony method for phylogenetic analysis of 16S rRNA sequence data for mycoplasmas by comparison with previous phylogenetic reconstructions. Twelve of the sequences analyzed in a phylogenetic study based on 16S rRNA sequences (26) (the sequences of the 11 most terminal species in Fig. 1 of Weisburg et al. [26] plus Lactobacillus casei as an outgroup) were obtained and analyzed. Ten replicate analyses were performed by using different randomly determined input orders and global (tree bisection and reconnection) branch swapping. All nucleotide positions were analyzed without the restriction of enriching for less rapidly changing positions (26). A single shortest tree of 2,000 steps was produced (data not shown); this tree had a topology identical to the topology of the tree of Weisburg et al. (26), except that Clostridium ramosum and Clostridium innocuum diverged successively instead of as sister species. Consequently, this method was used for further analysis.

Sequences were obtained for 14 species, and these sequences were analyzed by the branch and bound method. Two methods were used to assess the degree of support of the sequence data for the resulting tree. The first method was a bootstrap analysis in which we used 1,000 subsamples of the data matrix and with different magnesium concentrations (1 to 8 mM MgCl2). The optimal magnesium concentration for ISM1499 was 1 mM, and for A. oculi 19L and A. laidlawii PG8 the optimal magnesium concentration was 2 mM (data not shown). There was no specific signal in the PCR when the annealing temperature was higher than 42°C (data not shown). Additional products of different lengths and having different intensities were produced. Our attempts to eliminate these products by changing the annealing temperature, the time of extension, and the concentration of the template were unsuccessful.

16S rDNA sequencing. To simplify sequencing, PCR-amplified 16S rDNAs of acholeplasma strain ISM1499, A. oculi 19L, and A. laidlawii PG8 were cloned into pKS II, and overlapping fragments were subcloned. There was no difference in the restriction patterns of the 16S rDNAs (data not shown). A comparison of A. laidlawii 3A1 and PG8 sequences obtained during this study revealed a level of homology of 98.5%. The total lengths of the cloned fragments were 1,490 bp for ISM1499 and A. oculi 19L, and 1,476 bp for A. laidlawii PG8.

Sequence alignment and homology searches. A sequence alignment of the rDNA sequences is shown in Fig. 1. We observed two variable regions (bases 165 to 290 and 1140 to 1274) in A. oculi 19L and ISM1499; these regions exhibited levels of homology of 88 and 80.3%, respectively. When the same regions of ISM1499 were compared with the sequences of other Acholeplasma and Mycoplasma species, similar heterogeneity was observed. A. oculi 19L and A. laidlawii PG8 exhibited 95.3% and 93.4% homology with ISM1499 rDNA
sequences, respectively. A. laidlawii JA1 and PG8 were 98.5% homologous (Table 2).

DNA base composition. The melting profiles of ISM1499, A. oculi 19L, A. laidlawii PG8, and E. coli DNAs yielded thermal denaturation values of 65.5, 65.5, 66.3, and 75.5°C, respectively. These values corresponded to G+C contents of 28.3 mol% for the two A. oculi strains, 30.7 mol% for A. laidlawii PG8, and 52.7 mol% for E. coli.

Phylogenetic analysis. A single, shortest tree consisting of 1,725 steps (retention index, 0.714) was produced by the phylogenetic analysis (Fig. 2). We found that the strain identified as ISM1499 exhibited a well-supported sibling relationship with A. oculi 19L (bootstrap value, 100%; decay index value, 15).

DISCUSSION

In the past, classification of the mollicutes has been based largely on oxygen sensitivity and nutritional requirements for sterols. Acholeplasmas are non-sterol-requiring facultative anaerobes, but it is well known that these criteria are poor indicators of phylogenetic relationships. In order to get a more accurate assessment of phylogenetic positions, it is essential that phenotypic groups be confirmed by genetic analyses. DNA-DNA hybridization values and G+C contents have been used to define species, but with acholeplasmas problems occur with the use of this classification technique. In previous studies workers have shown that levels of DNA reassociation among acholeplasma isolates range from 50 to 100% for strains
belonging to the same species and from 2 to 14% for strains belonging to different species (1, 18). Thus, DNA homology values could be used to distinguish between mycoplasma species, but within the genus *Acholeplasma* this type of differentiation is less useful. A wider species concept should be used in order to account for the great differences in homology observed between *Acholeplasma* species. On the other hand, it has been shown by Weisburg and his coworkers that comparative rRNA sequence analysis can be used to reconstruct mollicute phylogeny (26). Therefore, the primary sequences of the 16S rRNA genes of *A. oculi* 19L, *A. laidlawii* PG8, and ISM1499 were obtained and aligned with the *A. laidlawii* JA1 sequence (Fig. 1), and the phylogenetic relationship was determined (Fig. 2).

The data presented above conclusively demonstrate that strain ISM1499 is not a *M. pulmonis* strain as originally reported (9-12). The rRNA sequence data strongly support the hypothesis that strain ISM1499 and *A. oculi* 19L are more closely related to each other than to the other species analyzed (Fig. 2). In addition, the G+C contents of ISM1499 and *A. oculi* 19L are identical (28.3 mol%). The chromosomal map of ISM1499 has been described previously (20) and is much larger than the chromosome size reported for *M. pulmonis* (16) (1,633 and 950 kb, respectively). The pulsed-field gel electrophoresis patterns of the *SmaI*-digested chromosomal DNAs of ISM1499 and *A. oculi* 19L were also indistinguishable (20, 22).

The results of a phylogenetic analysis of our 16S rRNA sequence data and previously published data for other *Acholeplasma* species and strains confirmed that the group is monophyletic (Fig. 2) (1, 18). The 16S rRNA sequences of *A.
discounted. Initially, there was concern about the use of integrative vectors and Tn4001 as genetic tools in the genus *Mycoplasma* because of differences in codon usage. The stop codon UGA is read as a tryptophan-coding codon in the genera *Mycoplasma* and *Spiroplasma*, but not in the genus *Acholeplasma* (15). The usefulness of Tn4001 and the usefulness of integrative vectors have already been established in the genus *Mycoplasma* (7, 8, 21). Since there are few functioning genetic transfer systems available to mycoplasmologists, there is a continual effort to improve the versatility and usefulness of cloning systems in acholeplasmas, spiroplasmas, and mycoplasmas. As the barriers to transformation of many mollicute species fall, the genetic tools developed with ISM1499 should be used more and become more versatile.

### ACKNOWLEDGMENTS

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### REFERENCES


### TABLE 2. Levels of 16S rDNA homology between *Acholeplasma* strains

<table>
<thead>
<tr>
<th>Source of rDNA sequence</th>
<th>% Homology to ISM1499 rDNA sequence</th>
<th>% Homology to PG8 rDNA sequence</th>
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<tbody>
<tr>
<td>ISM1499</td>
<td>94.25</td>
<td>93.2</td>
</tr>
<tr>
<td>A. oculi 19L</td>
<td>94.25</td>
<td>94.25</td>
</tr>
<tr>
<td>A. laidlawii JA1</td>
<td>93.4</td>
<td>98.5</td>
</tr>
<tr>
<td>A. laidlawii PG8</td>
<td>93.3</td>
<td>93.3</td>
</tr>
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</table>

![FIG. 2. Single, shortest tree for selected Acholeplasma, Anaeroplasma, Mycoplasma, and Ureaplasma species. The tree was produced by performing a phylogenetic analysis of small-subunit rRNA sequence data with PAUP software. The horizontal branch lengths are proportional to the number of steps along a branch; the scale bar represents 100 steps. The numbers along the branches indicate the strength of support of the data for each branch. The numbers preceding parentheses are percentages determined from a bootstrap analysis. The numbers in parentheses are decay values.](attachment:image)


22. **Tigges, E., and F. C. Minion.** Unpublished data.


