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 ribosomal DNA 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 JA1. Heterogeneity in the 16S ribosomal DNA sequences of *A. oculi* 19L and ISM1499 and in the 16S ribosomal DNA sequences of *A. laidlawii* PG8 and JA1 may indicate that unusual dissimilarities occur in the 16S ribosomal DNA 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 (Mycoplasma 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 antiserum.

The class *Mollicutes* comprises a unique group of eubacteria 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 *Mycoplasma*, *Ureaplasma*, *Entomoplasma*, *Spiroplasma*, and *Anaeroplasmata*), a positive urease reaction (the genus *Ureaplasma*), helicity (the genus *Spiroplasma*), oxygen sensitivity (the genera *Anaeroplasmata* and *Asteroleplasmata*), and lack of 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-acholeplasma 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 acholeplasma 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.

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TABLE 1. Bacterial strains used in this study

Species	Strain	GenBank accession no.
<i>Acholeplasma laidlawii</i>	PG8 (= ATCC 23206)	U14905 ^a
	JA1	M23932
<i>Acholeplasma oculi</i>	19L (= ATCC 27350)	U14904 ^a
	ISM1499	U14906 ^a
<i>Acholeplasma modicum</i>	PG49 (= ATCC 29102)	M23933
<i>Anaerobaculum abactoclasticum</i>	6-1 (= ATCC 27879)	M25050
<i>Anaerobaculum bacteriostolicum</i>	JR (= ATCC 27112)	M25049
<i>Anaerobaculum varium</i>	A-2 (= ATCC 43167)	M23934
<i>Clostridium ramosum</i>	113-I (= ATCC 25582)	M23731
<i>Mycoplasma flocculare</i>	Ms42 (= ATCC 27399)	X62699
<i>Mycoplasma hominis</i>	PG21 (= ATCC 23114)	M24473
<i>Mycoplasma hyopneumoniae</i>	ATCC 27719	Y00149
<i>Mycoplasma iowae</i>	695 (= ATCC 33552)	M24293
<i>Mycoplasma mycoides</i>	UM30847	M23943
<i>Mycoplasma pneumoniae</i>	Eaton (= ATCC 15531)	M29061
<i>Spiroplasma citri</i>	Maroc (= ATCC 27556)	M23942
<i>Ureaplasma urealyticum</i>	960 (= NCTC 10177)	M23935

^a Determined in this study.

MATERIALS AND METHODS

Bacterial strains. Table 1 shows the strains used in this study. In some instances, the 16S rRNA sequences were obtained directly from the GenBank database. Standard PPLO broth medium supplemented with 10% agamma 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 × 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. A 100-µl portion 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 was solubilized in Tris-EDTA buffer. Aliquots were electrophoresed in a 0.7% agarose gel and stained with ethidium bromide to verify DNA integrity. DNA concentrations were determined by fluorescence with a model TKO 100 mini-fluorometer (Bio-Rad Laboratories, Richmond, Calif.).

PCR amplification and 16S ribosomal DNA (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-HCl (pH 9.0), 0.1% Triton X-100, 2 mM MgCl₂, primers (each at a concentration of 0.6 µM), each deoxyribonucleoside triphosphate at a concentration of 200 µM, and 1 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.). The primers used were primers fd1 (ccgaatt ctgcacacacAGAGTTTGATCCTGGCTCAG) and RP1 (ccccggatccaagcttACGG TTACCTTGTTACGACTT) of Weisburg et al. (25), 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 Ericomp twin-block temperature cycler (Ericomp, 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 methods. Unique *Sal*I and *Bam*HI sites in the primer sequences were restricted, ligated into similarly restricted pKS II vector DNA (Stratagene, La Jolla, Calif.), and transformed into *Escherichia coli* DH5α [*supE44* Δ *lacU169*(ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *grrA96* *thi-1* *relA1*]. Plasmids were prepared by using the alkaline lysis method of Birnboim and Doly (2). The *Sal*I-*Eco*RI, *Eco*RI-*Bam*HI, *Sal*I-*Kpn*I, *Kpn*I-*Pst*I, and *Pst*I-*Bam*HI 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 dideoxy sequencing techniques or by automated cycle sequencing. All inserts were sequenced in both directions at least three times by using the universal and reverse primers. Some regions were sequenced manually by using T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio) and primers T3 and T7. Overlapping sequences were aligned by using the

DNASIS software program (Hitachi Software Engineering America, Ltd., San Diego, Calif.).

Determination of the DNA base composition. The G+C contents of acholeplasma DNAs were determined by the thermal melting method of Marmur and Doty (14). The DNA melting profiles in 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) were determined by using an automatic recording spectrophotometer equipped with a heated cuvette holder. The temperature was increased from 55 to 85°C at a rate of 1.0°C/min.

Phylogenetic analysis. Previously published 16S rRNA sequences were obtained from the GenBank database and were aligned with the sequences determined in this study for ISM1499, *A. oculi* 19L, and *A. laidlawii* PG8 by using the Pileup program of the Genetics Computer Group (6) software system, version 7.3. 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 sibling 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 regional (subpruning and regrafting) branch swapping. The second method involved examination of topological decay in all trees that were up to 10 steps longer than the shortest tree (3).

Nucleotide sequence accession numbers. The GenBank accession numbers for *A. oculi* 19L, *A. laidlawii* PG8, and strain ISM1499 16S rRNA sequences are U14904, U14905, and U14906, respectively. The GenBank accession number for *A. laidlawii* JA1 rRNA is M23932. Other GenBank accession numbers are shown in Table 1.

RESULTS

PCR amplification of 16S rDNA. To determine the optimum PCR conditions for acholeplasma DNAs, reactions were performed at different annealing temperatures (42, 45, and 50°C) 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* JA1 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

FIG. 1. Comparison of ISM1499 (1499), *A. oculi* ATCC 27350 (OCULI), *A. laidlawii* JA1, and *A. laidlawii* PG8 (= ATCC 23206) 16S rDNA sequences. 16S rDNA sequences were obtained either from GenBank (*A. laidlawii* JA1) or by performing a DNA sequence analysis as described in the text and were aligned with the sequence of ISM1499. Only the nucleotides different from the nucleotides in the ISM1499 sequence are indicated.

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, 66.5, 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. Achleplasmas 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 achleplasmas problems occur with the use of this classification technique. In previous studies workers have shown that levels of DNA reassociation among achleplasma isolates range from 50 to 100% for strains

	780	790	800	810	820	830	840	850
1499	CGCCGTAAACGATGAGAACTAAGTGTGGCCCTCAGGGTCAGTGCCTGCAGTTAACGCATTAAGTTCTCCGCCTGAGTAG							
OCULI								
JA1								
PG8								
	860	870	880	890	900	910	920	930
1499	TACGTACGCAAGTATGAACTCAAGGAATTGACGGGACCCCGCACAAAGCGGTGG-ATCATGTTGTTTAAATTCGAAGATA							
OCULI								
JA1								
PG8								
	940	950	960	970	980	990	1000	
1499	CA-CGAAAAACCTTACCAGGTCCTT-GACATACTCTGCAAT-GGCTTAGAAATAAGTTCGGAGGTTAACAGATGTACAGGT							
OCULI								
JA1								
PG8								
	1010	1020	1030	1040	1050	1060	1070	1080
1499	GGTGCACGGTTGTCGTCAGTCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTAATTGCTAGTTA							
OCULI								
JA1								
PG8								
	1090	1100	1110	1120	1130	1140	1150	1160
1499	CCATCAATAAAGTTGGGGGACTCTAGCGAAGACTTGCCAGCTGATAAATTGGGAGGGAAGGGTGAGGGGATGACCTCCAA							
OCULI								
JA1								
PG8								
	1170	1180	1190	1200	1210	1220	1230	1240
1499	ATCATATCGCCCCCTTATGACCTCGGGCCAACCAACGTCGATACAAATGCCTGATAC--AAAGAGAAGCGATGGGGTGACCC							
OCULI								
JA1								
PG8								
	1250	1260	1270	1280	1290	1300	1310	1320
1499	GGAGCGAATCTCATAAAGCAGTCTTAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATC							
OCULI								
JA1								
PG8								
	1330	1340	1350	1360	1370	1380	1390	1400
1499	GCGAATCAGCATGTTCGCGGTGAATACGTTCTCGGGTTTGTACACACCGCCCGTCAAACCCACGAAAGTGAGCAATACCCA							
OCULI								
JA1								
PG8								
	1410	1420	1430	1440	1450	1460	1470	1480
1499	AAGCCGGTGGCCTAACCCGTAAGGGAGGGAGCCGCTCTAAGGTAGGGTTTCATGATTGGGGTTAAGTCGTAACAAGGTAACC							
OCULI								
JA1								
PG8								
	1490							
1499	GT-----A							
OCULI								
JA1	C.ACGGGAACGTGGGGATGGATCACCTCCCTTCT							
PG8	C.-----							

FIG. 1—Continued.

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 *Sma*I-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.*

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

Source of rDNA sequence	% Homology to:	
	ISM1499 rDNA sequence	PG8 rDNA sequence
ISM1499		94.25
<i>A. oculi</i> 19L	95.3	93.2
<i>A. laidlawii</i> JA1	93.4	98.5
<i>A. laidlawii</i> PG8	93.3	

laidlawii JA1 and PG8 were 98.5% homologous, but the levels of homology with *A. oculi* ISM1499 and *A. oculi* 19L were only 94 and 93%, respectively. There was a striking difference between the ISM1499 and *A. oculi* 19L rDNA sequences, however. A level of homology of only 95.3% between the conserved rRNA sequences of these organisms suggests that ISM1499 is a member of a subspecies of *A. oculi* or a new *Acholeplasma* species. More extensive examination of additional *A. oculi* strains is needed to clarify the relationship.

Even though strain ISM1499 has now been conclusively identified as a member of the genus *Acholeplasma* rather than a member of the genus *Mycoplasma*, the genetic systems developed through the use of this organism should not be

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 mycoplasmaologists, 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.

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FIG. 2. Single, shortest tree for selected *Acholeplasma*, *Anaeroplasm*, *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.

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