Phylogenetic Relationships among Members of the Class *Mollicutes* Deduced from *rps3* Gene Sequences

KAREN F. TOTH,^{1†} NIGEL HARRISON,² AND BARBARA B. SEARS^{1*}

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824-1312,¹
and University of Florida, IFAS, Research and Education, Fort Lauderdale, Florida 33314²

A gene for a ribosomal protein, *rps3*, was amplified by PCR and sequenced from representatives of the class *Mollicutes*. Alignments of the deduced amino acid sequences allowed the construction of a phylogeny that is consistent with the phylogenetic trees created from 5S and 16S rRNA comparisons, including the position of the former *Acholeplasma florum* on the *Mycoplasma* branch, rather than with the classical *Acholeplasmataceae*. Additional confirmation of the phylogeny comes from the deduction that the UGA triplet encodes tryptophan in the *rps3* gene from *Mesoplasma florum*, as it does in the mycoplasmas and spiroplasmas. The sequence data from *Acholeplasma axanthum* 743 and *Acholeplasma* sp. strain J233 allow refinements to the phylogenetic tree within the *Acholeplasmataceae*, providing evidence that the sterol requirement of *Anaeroplasmataceae* (order *Anaeroplasmatales*) is a derived trait. It was also evident that the nonhelical plant-pathogenic members of the class *Mollicutes*, referred to as mycoplasmalike organisms or phytoplasmas, are more closely related to the true acholeplasmas (*Acholeplasma laidlawii* and strain J233) than to other members of the *Mollicutes*.

The placement of certain non-sterol-requiring, cell wall-less bacteria from plants and insects into the family *Acholeplasmataceae* of the class *Mollicutes* was brought into question by 16S rRNA sequence data from a large number of these species (13, 31, 34). The 16S rRNA data indicated that two species that were classified as acholeplasmas, *Acholeplasma florum* and the closely related *Acholeplasma entomophilum*, were phylogenetically closer to members of the *Mycoplasmataceae* and *Spiroplasmataceae* than to the other members of the *Acholeplasmataceae*. These data, together with a reassessment of nutritional requirements, led to the suggestion that they be renamed *Mesoplasma florum* and *Mesoplasma entomophilum* and, along with other closely related members of the *Mollicutes*, including all species of *Spiroplasma*, be placed in a new order, the *Entomoplasmatales* (31).

The phylogenetic trees constructed from 5S and 16S rRNA data (27, 34) show that the *Anaeroplasmataceae* are more closely related to the classical *Acholeplasmataceae* than to the other mollicutes, even though species of *Anaeroplasmataceae* require sterols, as do all species of *Mycoplasma* and *Spiroplasma*. The 16S rRNA sequence data from the plant-pathogenic mycoplasmalike organisms (MLOs) indicated a phylogenetic position between the *Acholeplasmatales* and the *Anaeroplasmatales* (9, 10). Considering these relationships, the possibility arose that the difficulty in developing an in vitro culture medium for MLOs could be due to an oxygen-sensitive metabolism in MLOs or to incorrect guesses about their sterol requirements. To provide supplemental data to address the question of phylogeny of the MLOs, we have obtained sequence data from a gene for a ribosomal protein, *rps3*, for which comparable data were already available from *Mycoplasma capricolum* (19), *Acholeplasma laidlawii* (11), and an MLO (11). In this report,

PCR was used to amplify a segment of a ribosomal protein operon from several members of the *Mollicutes* that were isolated from plant surfaces and from isolates of *Acholeplasma axanthum* and *Anaeroplasmataceae* *abactoclasticum*. The *rps3* gene was sequenced directly from the PCR products to allow us to establish a phylogeny that would be independent of the 16S rRNA sequences. These data also provided information about the utilization of the UGA codon for translation termination or to encode tryptophan.

MATERIALS AND METHODS

Bacterial strains. The strains used in this paper are described in Table 1.

Preparation of DNA. The acholeplasma cultures were grown in SP-4 medium (32) at 30°C. Cells were harvested by centrifugation at 20,000 × g for 30 min at 4°C. Pellets were resuspended in 100 mM Tris-HCl (pH 8)–50 mM EDTA–500 mM NaCl, and nucleic acids were extracted as described previously (3). Nucleic acids were precipitated by the addition of sodium acetate and cold isopropanol, washed with 70% ethanol, dried briefly, and then dissolved in 10 mM Tris–1 mM EDTA containing 50 µg of RNase per ml for a 1-h incubation at 37°C. DNA of *Spiroplasma citri* was a gift from Pyung-Ok Lim. Lyophilized cells of *A. abactoclasticum* and *Anaeroplasmataceae* *bactoclasticum* were suspended in 1 ml of 50 mM Tris–20 mM EDTA (pH 8), and sodium dodecyl sulfate was added to a concentration of 1%. Nuclease-free pronase (Sigma, St. Louis, Mo.) was added to a concentration of 1 mg/ml, and the suspensions were incubated at room temperature for 30 min. After three organic extractions, nucleic acids were recovered by the addition of potassium acetate and ethanol to precipitate them. The nucleic acids were washed and dissolved as described above, omitting the RNase treatment.

PCR and DNA sequencing. PCR was performed with a Tempcycler II model 1105 (Coy Corporation, Grass Lake, Mich.), nucleotides from Perkin Elmer Cetus Corp. (Norwalk, Conn.), and *Taq* DNA polymerase from Boehringer Mannheim Biochemicals (Mannheim, Germany). The prim-

* Corresponding author. Phone: (517) 355-0132. Fax: (517) 353-1926. Electronic mail address: 21417bbs@msu.edu.

† Present address: Department of Biological Sciences, University of Iowa, Iowa City, IA 52242.

TABLE 1. *Mollicutes* strains

Strain	Isolated from:	Isolated by:	Received from:	Primary reference(s)
<i>Acholeplasma</i> strain J233	Rotting palm	S. Eden-Green	J. G. Tully	4
<i>Acholeplasma axanthum</i> S743	Cell culture	C. Friend	J. G. Tully	30
<i>A. oculi</i> 19L	Goat eyes	J. M. Al-Aubaidi	J. G. Tully	1
<i>A. florum</i>				
L1	Lemon leaf surface	R. E. McCoy	N. Harrison	14, 15
PP2	Powderpuff leaf surface	R. E. McCoy	N. Harrison	14, 15
GF1	Grapefruit leaf surface	R. E. McCoy	N. Harrison	14, 15
<i>Anaeroplasmabactoclasticum</i> 6-1	Sheep rumen	I. M. Robinson	M. J. Allison	23, 25
<i>Anaeroplasmabactoclasticum</i> JR	Cattle rumen	J. P. Robinson	M. J. Allison	26

ers used for PCR were previously described (11), and their sequences are the first two entries in Table 2.

PCR reactions were done in a 100- μ l total volume and overlaid with mineral oil. The amplification condition consisted of 3 min at 94°C followed by 30 cycles of 1 min at 94°C and 1.5 min at 40 or 45°C and 2 min at 72°C, with a final extension for 10 min at 72°C followed by holding at 4°C. After the oil overlay was removed, the PCR mixture was organically extracted and precipitated with isopropanol. The amplification products were separated from the primers on a 1.5% agarose gel and were extracted from the gel with Qiaex (Qiagen Inc., Chatsworth, Calif.). The amplification products were sequenced directly with Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, Ohio), by the method of Casanova et al. (2), as modified by Carol Bult (1a). Internal primers were synthesized at the Macromolecular Structure Facility at Michigan State University (East Lansing). For the sequencing reactions, Qiagen-extracted DNA was denatured at 100°C for 3 min in the presence of the reaction buffer and

primer (final volume, 10 μ l). This template-primer mixture was then flash-frozen in liquid nitrogen. As the mixture thawed, the labelling mix was added and mixed by pipetting. Sequencing reactions were kept at room temperature (21 to 24°C) for 45 s and then centrifuged for a few seconds and aliquoted to each dideoxynucleotide tube. The termination reactions were kept at 37°C for 2 min before the addition of the stop solution. This protocol allowed us to read at least 400 bases from a primer, but it was difficult to obtain sequence data closer than 30 bases from the end of a primer. Thus, we were able to sequence the *rps3* gene in both directions, except for the last 0 to 34 bases at the carboxy end, which could be read clearly in one direction only, because these sequences were at the very end of the PCR product. Table 2 lists the PCR primers and the primers used to obtain the sequence data.

Alignment of sequences and construction of phylogenetic tree. The amino acid sequences were first aligned in a pairwise fashion by using the "gap" routine in the GCG

TABLE 2. Primers used for PCR and sequencing

Use and organism	Notation for:		
	Forward primer	Primer sequence ^a	Reverse primer
PCR amplifications			
All	PCR1	GGACATAAGTTAGGTGAATTT	NA ^b
	NA ^b	ACGATATTTAGTTCCTTTTGG	PCR2
Sequencing			
<i>A. axanthum</i>		See above ^c	PCR2
	PF12	CTGTAATCGTTG	
	PF2	GAAATGGCTCGTA	PR1
		ATTCAGAAAGGTCAA	PR9
		CTGGTAAACCTGGAG	PR10
<i>Acholeplasma</i> strain J233		See above	PCR2
	PF2	See above	PR1
	PF6	ACTGTGGTAGTAGC	
	PF5	GAATACTTAACT	PR4
<i>Mesoplasma florum</i>		See above	PCR2
	PF2	See above	PR1
	PF4	CATTAGAACTGC	PR3
	PF10	GAGCTCATGGTAGA	
<i>Anaeroplasmabactoclasticum</i>		See above	PCR2
	PF7	AACCTGGTGTAG	PR5
	PF9	GCAATTCAAAGAGC	PR8
	PF11	CTGTAATCGTTG	
	PF3	TAGGCGCGCTGAA	PR2

^a Primer sequence is for the sense strand.

^b NA, not applicable.

^c See primer sequence already listed above.

program (University of Wisconsin computer group); final alignments were done by visual adjustment. Phylogenetic trees were constructed with the Phylogenetic Analysis Using Parsimony (PAUP) program, version 3.0s (Illinois Natural History Survey, Champaign) created by David Swofford for the Macintosh computer (Apple Computers, Inc.). Gaps (except for single amino acid gaps) and uninformative sequences that contribute exactly the same length to all possible trees were excluded from the analysis.

Nucleotide sequence accession numbers. The sequence data have been communicated to GenBank and have been assigned the accession numbers L22464 to L22467, for *Anaeroplasmabactoclasticum*, *A. axanthum*, *Mesoplasma florum*, and *Acholeplasma* strain J233, respectively.

RESULTS

DNA sequences and deduced amino acid sequence alignments. The PCR primers did not amplify DNA from *Anaeroplasmabactoclasticum*, *Acholeplasma oculi*, or *S. citri*. From the other organisms, PCR products of about 1,200 bp were obtained. From *A. axanthum*, a second round of PCR was required to obtain sufficient DNA for sequencing.

Preliminary sequencing of PCR products (400 to 600 bases) from the L1, GF1, and PP2 strains of *Mesoplasma florum* revealed 100% identity. These results indicated that the three strains were probably three separate collections of the same organism. Thus, we completed the *rps3* sequence by using amplified DNA from only the PP2 line. The DNA sequence data are available from GenBank under the accession numbers listed in the Materials and Methods section. The guanine-plus-cytosine (G+C) composition of these genes (33 to 36%) reflects the low G+C content of the *Mollicutes* genomes.

Figure 1 shows an alignment of the deduced amino acid sequences of the *rps3* gene from *Bacillus stearothermophilus* (8) and the following *Mollicutes*: a plant-pathogenic MLO (11), *A. laidlawii* (11), *M. capricolum* (19), *A. axanthum* S743, *Mesoplasma florum* PP2, *Acholeplasma* strain J233, and *Anaeroplasmabactoclasticum* 6-1. As noted previously (11), the carboxy end of the amino acid sequence is highly divergent. In Table 3, pairwise comparisons of the sequence data from the various *Mollicutes* show that amino acid homology ranges from 44 to 78%, with the nucleic acid alignments having 56 to 82% identity. In both sets of comparisons, the most closely related pair is composed of *M. capricolum* and *Mesoplasma florum*, and the next most closely related pair is *A. laidlawii* and *Acholeplasma* strain J233. At the nucleic acid level, the *Mollicutes* genes are consistently more divergent from the homologous gene of the walled gram-positive bacterium *B. stearothermophilus* than from each other. However, this is not apparent in the amino acid comparisons and probably points to convergent evolution within the *Mollicutes* due to A-T pressure (19).

Genetic code. UGA was not used as a termination codon for any of the *rps3* genes of the *Mollicutes* analyzed. The UGA codon was encountered only in the sequence from *Mesoplasma florum*, where an open reading frame exists only if the five UGA triplets encode tryptophan (W residues in Fig. 1). In contrast to the MLO, which uses GUG as the initiation codon for this gene (11), the other members of the *Mollicutes* all use the standard AUG codon.

Phylogeny reconstruction. Possible phylogenies were assembled and tested using the PAUP program, with *B. stearothermophilus* as the out-group. The most parsimonious phylogenetic tree is shown in Fig. 2. The initial branch-

mlo	MGKKTNPNGRLRLGIIRTWESQWCVNDKEIPNLIKEDFLIRKLINNTFKKS
ala	MGKKTNPNGRLRLGIIRTWESKRYADQKDPALIKEDALIREFLINENFSKA
asp	MGKKTNPNGRLRLGIIRSWESKRYANEDKVPALINEDYNIKKYLNKVYKKA
aax	MGQKVNPIGFRVGVIRDWDSKRYADKKIVPALVKEDAVIRKFLNKKYNNNA
aab	MGQKVSFPMGLRVGINREWDAAWYAEKTVQVANLLEDLKKIRKYINEVYKA
myc	MGQKVSFPMGLRVGINRDWENRMYAEKQVVKWLDQDIKIRTAFLKLLKDA
afl	MGQKVSFPMGLRVGINRDWEDTWYAEKQVVKWLDQDIKIREGVLKLLKDA
bst	MGQKVNPIGLRIGIIRDWESRWYAEKD-YADLVHEDLKKIREYINKRLQDA
mlo	ATISQIDIERNKE-KNKNR--ITISVHTAKPGVIGKDGDRNKLVAKLKE
ala	GVSQIEIERVKA-KSKER--VTIKLYVSKPGIALGKEASVKNKAVSNLEY
asp	GVSQIEIERLKG-KAKDRIKVTL--YAKPGVIGRDGGETRNKAVSELEY
aax	AVSHVEIERLKLKLVKKRVKITL--HSGKPGVIGREAAATMKETIASLEK
aab	ATSRVIERVKGPNKDRVKITL--HTAKPGVIGHEAETKNKVVKELGF
myc	AVSKIDIERTTK-----DLTLFIKTARPAIVLGGEGNKIEIVLAVRK
afl	AVSKIKIERTNS-----NITLIIRTAARPAIVLGGEGKNVSIATAVQK
bst	AVSRVEIERAA-----NRVNVTI--HTAKPGMVGKGGSEVALRKALTK
mlo	LT--QXDVN-LNVLEVKNSDKIALLAQNMAEQLENRMFRRVQKMAIGK
ala	LT--KKEVI-LNIEVRRPEKVAVLVAQSIAEQLENRASFRRAQKMAIQK
asp	LT--KKEIV-FNVVEVKRPEKVAELVAQNMAEQLENRASFRRVQKTAIQK
aax	LT--KKEIV-FNVVEVKRPEVVAATLVAQSMAEQLENRASFRKSTKNCYAK
aab	IT--KKNII-LNVVEVRRPELDATLVAKSIAEQLEARASFRRVQKTAIQK
myc	TVKNKLLVMVRVIEIKSPDARSTLVARNIGEISNRASFRTVQKLAIRK
afl	IAKDRNLKVEVKVIEVKNPDADRTLVARNIGEITNRASFRTVQKLAIRK
bst	LT--GKRHI--NIVEIKKPDLDKLVAKENIARQLENRVSFRAQKQAIQK
mlo	ALKAGAKGVKTLISGRLLGGAETARSEGHAEGRVPLHTLRADIDYAAVEAH
ala	ALKSGAGKIRTLVSGRLGGAEMARSEGSEGRVPLHTLRADVDYATAEAS
asp	ALKSGAGKVTLSVSGRLGGAEMARSEGSEGRVPLHTLRADVDYATAEAS
aax	SIKVRAGIKITL-SQRLGGREMARTEGYSEGQVPLHTLRADVDYATAEAS
aab	ALKAGAKGAKTLISGRLLGGAETARSEGYNEGQVPLHTLRADVDYATAEAS
myc	ALKAGAKGKTAIVSGRLGGVEMARTEGYLECSVPLSTLRNNIDYALYEA
afl	ALKAGKVGKIKTSVSGRLGGVEMARTEGYEGSVPLSTLRADIDYALYEA
bst	AMRPGRGKVTMVRRLGGAETARSEGTVPLHTLRADIDYATAEAD
mlo	TTYGVLGKIKVWIEHGNVLPQGQILDRTRKPF-ASQSNTPNRRPRNFKGGN
ala	TTYGILGILVWVYHGEVLPQGQILDRTRKPFAGNQRGQKRRPRNDQPVK
asp	TTYGILGKIKVWVHGEVLPQGQILDRTRKPFASAPRQRNRNKEANNVYN
aax	TTYGILGKIKVWVYHGEVLPQGQSEELRKRQSSASSNHGGGKRRPRSKGP
aab	TTYGKLGKIKVWVYHGEVLPQVPRAFQEEKKPAKFKNKKPVADPANKYEE
myc	TTYGQIGVKVWVNHGEVFKKERMNNSQIMAKPRTNKGGKR*
afl	TTYGQIGVKVWVNHGEVIGGQSQRVSEKAPMNNDRRPNKNNNNRGGKR*
bst	TTYGKIGVKVWVYKGDLPTKKKAEEGGK*
mlo	NGVNNAKKN*
ala	DLNKEKEIARRTNYVNAKKN*
asp	AKKN*
aax	RRSQEDAATTEGGN*
aab	KSSKEVK*
myc	
afl	
bst	

FIG. 1. Amino acid alignments of *rps3* gene sequences from mollicutes. Abbreviations are as in Table 3, except that *Mesoplasma florum* is listed as afl, for its previous name, *Acholeplasma florum*. Amino acid designations use the single-letter system.

ing shows a simultaneous divergence of the *Acholeplasma*-*Anaeroplasmatales* branch, the *Mycoplasma*-*Mesoplasma* branch, and the branch that holds *B. stearothermophilus*, indicating a very deep evolutionary separation of the two major branches of *Mollicutes*.

DISCUSSION

Previous ordinal classifications of the *Mollicutes* have been based on the nutritional requirement or nonrequirement for sterols and oxygen sensitivity (20, 24). Thus, the *Acholeplasmatales* were composed of non-sterol-requiring facultative anaerobes, the *Mycoplasmatiales* encompassed the sterol-requiring facultative anaerobes, and the *Anaeroplasmatales* consisted of obligate anaerobes of the sterol-requiring genus *Anaeroplasmatales* and the non-sterol-requiring genus *Asteroloplasma*. *Asteroloplasma* represents a very deep phylogenetic branch within the class *Mollicutes* according to the analysis of 16S rRNA sequences (34). It has been suggested that the deep branching indicates that *Asteroleplasma* evolved before aerobic bacteria developed, and thus the anaerobic growth habit would be a primitive trait retained in the order *Anaeroplasmatales* (13). On the basis of the 16S rRNA data, the classical *Acholeplasmatales* and the two representatives of the *Anaeroplasmatales* that were analyzed are more related to each other than to any repre-

TABLE 3. Pairwise comparisons of *rps3* gene sequences^a

<i>Mollicutes</i> species or strain	Result for <i>Mollicutes</i> species or strain							
	mlo	ala	asp	aax	aab	myc	mfl	bst
mlo		68	72	66	62	59	58	56
ala	66 (83)		79	74	64	56	60	55
asp	72 (86)	77 (91)		74	69	59	59	58
aax	55 (74)	65 (79)	67 (81)		67	59	60	58
aab	59 (76)	62 (77)	66 (82)	59 (75)		59	59	56
myc	46 (66)	47 (66)	50 (68)	44 (65)	50 (70)		82	53
mfl	48 (65)	52 (69)	51 (66)	46 (65)	50 (70)	78 (87)		54
bst	59 (77)	57 (74)	60 (76)	54 (74)	61 (78)	49 (68)	51 (69)	

^a Numbers at upper right are percent identity at the nucleic acid level and at the lower left are percent identity (percent similarity) at the amino acid level, from the first amino acid or nucleotide of the gene through the end of the *Bacillus stearothermophilus* gene shown in Fig. 1. Abbreviations: mlo, *Oenothera* MLO; ala, *Acholeplasma laidlawii*; asp, *Acholeplasma* sp. strain J233; aax, *Acholeplasma axanthum*; aab, *Anaeroplasmatales*; myc, *Mycoplasma capricolum*; mfl, *Mesoplasma florum*; bst, *B. stearothermophilus*.

representatives of *Ureaplasma*, *Spiroplasma*, or *Mycoplasma* (34). Analyses of sequence data have grouped the plant-pathogenic MLOs as a discrete clade on the *Acholeplasma*-*Anaeroplasmatales* branch of the *Mollicutes* (9–11, 16), but the limited data set did not conclusively resolve the appropriate family or ordinal assignment of the MLOs. The other major phylogenetic branch of the *Mollicutes* holds the *Mycoplasmataceae*, *Spiroplasmataceae*, and *Ureaplasmataceae*, but the 16S rRNA phylogenetic tree placed several species of the *Acholeplasmataceae* among the mycoplasmas. To bring the biosystematic classifications in line with the phylogenetic data, the order *Entomoplasmatales* was created (31). This restructuring also was justified because the *Entomoplasmatales* all inhabit and have evolved within insects and/or plants, are cultivatable, and have fatty acid or lipid requirements that allow them to be clearly differentiated from *Acholeplasma*.

To establish the phylogenetic position of the MLOs, we attempted to gather sequence data from a number of members of the *Acholeplasmataceae* and *Anaeroplasmataceae*. Because very closely related organisms are not easily differentiated by using 16S rRNA data, we examined a protein-coding gene, since wobble in the genetic code permits more variation in protein gene sequences, even of highly conserved genes, than is possible in rRNA sequences. Furthermore, these data would provide information about the genetic code in the organisms under study; this feature is of phylogenetic importance because members of the *Mycoplasmatales* use the UGA codon for tryptophan instead of as a termination signal. The data reported here for the gene for ribosomal protein *rps3* and the deduced phylogeny shown in Fig. 2 include a former *Acholeplasma* species (*Mesoplasma florum*) that is included in the newly created order *Entomoplasmatales* and from which we analyzed three strains

FIG. 2. The most parsimonious phylogenetic tree constructed from the deduced amino acid sequences and alignments shown in Fig. 1. The trivial name phytoplasma is used in place of MLO.

(Table 1). Sequence data were also gathered from several members of the *Acholeplasmatales* and a representative of the *Anaeroplasmatales*. These data supplement the phylogeny based on rRNA sequence data (27, 34) by including two species that were not previously analyzed, *A. axanthum* S743 and an unnamed *Acholeplasma* species, strain J233. Notably, *M. florum* does not lie in the same branch as the true *Acholeplasma* spp. The closer relationship to *Mycoplasma* spp. is reinforced by our deduction that UGA encodes tryptophan at five positions in the *rps3* gene of *Mesoplasma florum* (Fig. 1), as it does in *M. capricolum* (19). This observation confirms a similar deduction regarding the genetic code obtained from a partial sequence of the PTS-II gene from *Mesoplasma florum* (17, 18). Taken together, these data lend support to the reclassification of *Mesoplasma florum* and its close relatives (31). These data also confirm that the recruitment of the UGA codon for tryptophan is a very fundamental difference between the phylogenetic branch to which the *Mycoplasmataceae* and *Spiroplasmataceae* belong and the branch that holds the *Acholeplasmataceae* and the *Anaeroplasmataceae*.

The phylogeny shown in Fig. 2 confirms previous data that place the plant-pathogenic nonhelical *Mollicutes* among the true *Acholeplasmataceae* (9–12, 16). Since it no longer seems appropriate to refer to the plant pathogens as “mycoplasma-like organisms,” and since phylogenetic distance will probably justify a new genus name, we support the well-discussed notion of calling this group of *Mollicutes* by the trivial name “phytoplasmas” (7). Our investigation indicates that *Acholeplasma* strain J233 is more closely related to the phytoplasmas than is *A. laidlawii*. This is an intriguing finding since strain J233 was isolated from the margin of rot on a coconut palm infected with lethal yellowing disease (4). Although isolated from a phytoplasma-diseased tree, this strain was not thought to be the disease agent. While the organism multiplied following injection into likely insect vectors, it failed to be transmitted to plants (5).

The 5S rRNA and 16S rRNA genes from *Acholeplasma* strain J233 and from *A. axanthum* have not been sequenced, and therefore these organisms were not part of the previously published phylogenetic trees (27, 34). Preliminary serology and growth inhibition tests on strain J233, which was established as a clone from an isolate called E847, pointed to a possible relationship to *A. axanthum* or *A. oculi* (4, 6), but protein profiles, hybridization data, and additional serological analyses indicated that strain J233 might represent a unique new species (6, 29, 33). As mentioned above, the *rps3* gene sequence indicates that strain J233 is more

closely related to the phytoplasmas than to other achleplasmas. Consequently, this strain may be the best choice of an *Acholeplasma* strain to use as a control in attempts to develop an in vitro culture system for the phytoplasmas. As seen in other phylogeny analyses (11), the length of the branch leading to the phytoplasmas indicates that they are evolving at a rapid pace. It remains to be determined whether their rapid evolutionary rate is due to the loss of a subset of DNA repair enzymes or some other aspect of DNA metabolism.

The phylogenetic tree shows that *A. axanthum* S743 belongs on a relatively deep branch of the *Acholeplasmataceae*. The parsimony analysis indicates that strain S743 is almost as closely related to *Anaeroplasmatales* as to any of the achleplasmas analyzed. The species *A. axanthum* contains strains isolated from diverse environments such as sewage, plant surfaces, and animal nasal cavities, in addition to the type strain used here that came from a cell culture line (30, 33). The diversity of *A. axanthum* habitats is reflected in a range of DNA relatedness among the strains when they are compared with each other and with *A. laidlawii*, using techniques of DNA-DNA hybridization (30) and DNA restriction fragment patterns (21). Extending the *rps3* analysis to these other strains would likely contribute to a refinement of the phylogenetic relationships of the *Acholeplasmatales* and *Anaeroplasmatales* and would test the validity of the serology that has been used for grouping the various strains as *A. axanthum* (4).

The accumulating data indicate that the nutritional dependency on exogenous sterols is a trait that has arisen several times during the evolution of the *Mollicutes* (31). According to a revised classification of anaerobic *Mollicutes* (24), members of the genus *Anaeroplasmatales* require sterols for growth in vitro, in contrast to the classical *Acholeplasmatales* on this major branch of the *Mollicutes* phylogenetic tree, and in contrast to members of the single species in the genus *Asteroleplasma*, which are also obligate anaerobes and represent a deeper branch point. The sterol utilization of *Anaeroplasmatales* appears to be different from those of the *Spiroplasmataceae* and *Mycoplasmataceae* in several ways: (i) the sterol requirement is not absolute (22, 26); (ii) *Anaeroplasmatales* has a higher tolerance for digitonin than do mycoplasmas (22); and (iii) cells are not sensitive to amphotericin (22). In contrast, mycoplasmas and spiroplasmas require sterols in order to grow; they incorporate the sterols into their plasma membranes and are therefore extremely sensitive to digitonin and amphotericin (28). Although *Mesoplasma* spp. in the newly established *Entomoplasmataceae* (31) fit phylogenetically with the *Mycoplasmataceae* and *Spiroplasmataceae*, they do not share sterol requirement as a trait. Thus, the mollicutes now provide several examples to show that although nutritional requirements may be the most practical traits to assess in routine laboratory tests, they may not always be reliable indicators of phylogenetic relationships. Such a nutritional requirement probably reflects a loss of genetic material, and since different gene functions could be lost but nonetheless result in a similar dependency, caution should apply when using nutritional requirements to establish biosystematic groupings.

ACKNOWLEDGMENTS

We thank J. G. Tully, M. J. Allison, and I. M. Robinson for providing us with strains, references, and helpful comments.

This work was supported by National Science Foundation grant BSR-89068279, USDA grant 9200932, and the Michigan State Agricultural Experiment Station.

REFERENCES

1. Al-Aubaidi, J. M., C. Pannetier, A. H. Dardiri, C. C. Muscopolatt, and E. H. McCauley. 1973. Identification and characterization of *Acholeplasma oculosi spec. nov.* from the eyes of goats with keratoconjunctivitis. *Cornell Vet.* **63**:117-129.
- 1a. Bult, C. (Smithsonian Institution). Personal communication.
2. Casanova, J. L., C. Pannetier, C. Jaulin, and P. Kourilsky. 1990. Optimal conditions for directly sequencing double-stranded PCR products with Sequenase. *Nucleic Acids Res.* **18**:4028.
3. Dellaporta, S. L., J. Wood, and J. B. Hicks. 1983. A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* **1**:19-21.
4. Eden-Green, S., and J. G. Tully. 1979. Isolation of *Acholeplasma* spp. from coconut palms affected by lethal yellowing disease in Jamaica. *Curr. Microbiol.* **2**:311-316.
5. Eden-Green, S. J., and P. G. Markham. 1987. Multiplication and persistence of *Acholeplasma* spp. in leafhoppers. *J. Invertebr. Pathol.* **49**:235-241.
6. Eden-Green, S. J., P. G. Markham, R. Townsend, D. B. Archer, M. F. Clark, and J. G. Tully. 1985. Plant pathogenicity and transmission tests with *Acholeplasma* spp. from coconut palms in Jamaica. *Ann. Appl. Biol.* **106**:439-449.
7. International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Mollicutes. 1993. Minutes of the interim meetings, 1 and 2 August, 1992. *Int. J. Syst. Bacteriol.* **43**:394-397.
8. Kromer, W. J., T. Hatakeyama, and M. Kimura. 1990. Nucleotide sequences of *Bacillus stearothermophilus* ribosomal protein genes: part of the ribosomal S10 operon. *Biol. Chem. Hoppe-Seyler* **371**:631-636.
9. Kuske, C. R., and B. C. Kirkpatrick. 1992. Phylogenetic relationships between the western aster yellows mycoplasma-like organism and other prokaryotes established by 16S rRNA gene sequence. *Int. J. Syst. Bacteriol.* **42**:226-233.
10. Lim, P.-O., and B. B. Sears. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasma-like organisms are evolutionarily distinct from animal mycoplasmas. *J. Bacteriol.* **171**:5901-5906.
11. Lim, P.-O., and B. B. Sears. 1992. Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from several ribosomal protein gene sequences. *J. Bacteriol.* **174**:2602-2611.
12. Lim, P.-O., B. B. Sears, and K. Klomparsens. 1992. Membrane characteristics of a plant-pathogenic mycoplasma-like organism. *J. Bacteriol.* **174**:682-686.
13. Maniloff, J. 1992. Phylogeny of mycoplasmas, p. 549-559. In J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas. Molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
14. McCoy, R. E., H. G. Basham, J. G. Tully, D. L. Rose, P. Carle, and J. M. Bové. 1984. *Acholeplasma florum*, a new species isolated from plants. *Int. J. Syst. Bacteriol.* **34**:11-15.
15. McCoy, R. E., D. S. Williams, and D. L. Thomas. 1979. Isolation of mycoplasmas from flowers, p. 75-80. In: R. E. McCoy and H. J. Su (ed.), *Proceedings of the Republic of China-United States Cooperative Science Seminar, symposium series I. Mycoplasma diseases of plants*. National Science Council, Taipei, Taiwan.
16. Namba, S., H. Oyaizu, S. Kato, S. Iwanami, and T. Tsuchizaki. 1993. Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *Int. J. Syst. Bacteriol.* **43**:461-467.
17. Navas-Castillo, J., F. Laigret, A. Hocquellet, C. J. Chang, and J. M. Bové. Evidence for a phosphoenolpyruvate-dependent sugar-phosphotransferase system in the mollicute *Acholeplasma florum*. *Biochimie*, in press.
18. Navas-Castillo, J., F. Laigret, J. G. Tully, and J. M. Bové. 1992. Le mollicute *Acholeplasma florum* possède un gène du système phosphoenolpyruvate sucre-phosphotransférase et il utilise UGA comme codon tryptophane. *C. R. Acad. Sci.* **315**:43-48.
19. Ohkubo, S., A. Muto, Y. Kawauchi, F. Yamao, and S. Osawa. 1987. The ribosomal protein gene cluster of *Mycoplasma capricolum*. *Mol. Gen. Genet.* **210**:314-322.
20. Razin, S., and E. A. Freundt. 1984. Class I. *Mollicutes* Edward and Freundt 1967, 267^{AL}, p. 7470-755. In N. R. Krieg, and J. G.

- Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. Williams & Wilkins, Baltimore.
21. Razin, S., J. G. Tully, D. L. Rose, and M. F. Barile. 1983. DNA cleavage patterns as indicators of genotypic heterogeneity among strains of *Acholeplasma* and *Mycoplasma* species. *J. Gen. Microbiol.* **129**:1935–1944.
 22. Robinson, I. M., and M. J. Allison. 1975. Transfer of *Acholeplasma bactoclasticum* Robinson and Hungate to the genus *Anaeroplasm* (*Anaeroplasm bactoclasticum* [Robinson and Hungate] comb. nov.): emended description of the species. *Int. J. Syst. Bacteriol.* **25**:182–186.
 23. Robinson, I. M., M. J. Allison, and P. A. Hartman. 1975. *Anaeroplasm abactoclasticum* gen. nov., sp. nov.: an obligately anaerobic mycoplasma from the rumen. *Int. J. Syst. Bacteriol.* **25**:173–181.
 24. Robinson, I. M., and E. A. Freundt. 1987. Proposal for an amended classification of anaerobic mollicutes. *Int. J. Syst. Bacteriol.* **37**:78–81.
 25. Robinson, I. M., and K. R. Rhoades. 1977. Serological relationships between strains of anaerobic mycoplasmas. *Int. J. Syst. Bacteriol.* **27**:200–203.
 26. Robinson, J. P., and R. E. Hungate. 1973. *Acholeplasma bactoclasticum* sp. n., an anaerobic mycoplasma from the bovine rumen. *Int. J. Syst. Bacteriol.* **23**:171–181.
 27. Rogers, M. J., J. Simmons, R. T. Walker, W. G. Weisburg, C. R. Woese, R. S. Tanner, I. M. Robinson, D. A. Stahl, G. Olsen, R. H. Leach, and J. Maniloff. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. *Proc. Natl. Acad. Sci. USA* **82**:1160–1164.
 28. Rottem, S., and S. Razin. 1972. Isolation of mycoplasma membranes by digitonin. *J. Bacteriol.* **110**:699–705.
 29. Stephens, E. B., G. S. Aulakh, R. E. McCoy, D. L. Rose, J. G. Tully, and M. F. Barile. 1981. Lack of genetic relatedness among animal and plant acholeplasmas by nucleic acid hybridization. *Curr. Microbiol.* **5**:367–370.
 30. Stephens, E. B., G. S. Aulakh, D. L. Rose, J. G. Tully, and M. F. Barile. 1983. Intraspecies genetic relatedness among strains of *Acholeplasma laidlawii* and of *Acholeplasma axanthum* by nucleic acid hybridization. *J. Gen. Microbiol.* **129**:1929–1934.
 31. Tully, J. G., J. M. Bové, F. Laigret, and R. F. Whitcomb. 1993. Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. *Int. J. Syst. Bacteriol.* **43**:378–385.
 32. Tully, J. G., R. F. Whitcomb, A. F. Clark, and D. F. Williamson. 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195**:892–894.
 33. Tully, J. G., R. F. Whitcomb, D. L. Rose, K. J. Hackett, E. Clark, R. B. Heneger, P. Carle, and J. M. Bové. 1990. Current insight into the host diversity of acholeplasmas, p. 461–467. In G. Stanek, G. H. Caswell, J. G. Tully, and R. F. Whitcomb (ed.), *Proceedings of the 7th Congress of the International Organization for Mycoplasmaology*, Gustav Fisher Verlag, Stuttgart, Germany.
 34. Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455–6467.