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A Phylogenetic Analysis of the Mycoplasmas: Basis for Their Classification

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Small-subunit rRNA sequences were determined for almost 50 species of mycoplasmas and their walled relatives, providing the basis for a phylogenetic systematic analysis of these organisms. Five groups of mycoplasmas per se were recognized (provisional names are given): the hominis group (which included species such as *Mycoplasma hominis*, *Mycoplasma lipophilum*, *Mycoplasma pulmonis*, and *Mycoplasma neurolyticum*), the pneumoniae group (which included species such as *Mycoplasma pneumoniae* and *Mycoplasma muris*), the spiroplasma group (which included species such as *Mycoplasma mycoides*, *Spiroplasma citri*, and *Spiroplasma apis*), the anaeroplasma group (which encompassed the anaeroplasmas and acholeplasmas), and a group known to contain only the isolated species *Asteroleplasma anaerobium*. In addition to these five mycoplasma groups, a sixth group of variously named gram-positive, walled organisms (which included lactobacilli, clostridia, and other organisms) was also included in the overall phylogenetic unit. In each of these six primary groups, subgroups were readily recognized and defined. Although the phylogenetic units identified by rRNA comparisons are difficult to recognize on the basis of mutually exclusive phenotypic characters alone, phenotypic justification can be given a posteriori for a number of them.

Mycoplasmas are free-living, wall-less procaryotes that are small in size, pass through bacteriologic filters, have unusually small genomes with a low G+C content, and show unusual nutritional needs. These characteristics are the basis for grouping them as *Mollicutes*, a distinct class of procaryotes (29, 30). More than 100 different species have been isolated from humans, animals, plants, and insects. The class *Mollicutes* contains six genera (*Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Mycoplasma*, *Spiroplasma*, and *Ureaplasma*), with generic distinctions resting primarily on differences in morphology, genome size, and some nutritional requirements (30, 31, 38). The class itself appears to be phylogenetically quite broad. Interspecies DNA-DNA hybridizations can detect only relatively small subgroupings within the mollicutes (29, 30).

The genus *Mycoplasma* has the largest number of species, and over 80 of these species have been described. Their major characteristics are a strict need for exogenous sterol and genome sizes of 620 to 780 kilobase pairs (kb) (410 to 510 megadaltons) (1, 3, 30, 40, 41). Members of the genus *Ureaplasma* are similar to *Mycoplasma* species in genome size and sterol requirements, but in addition, they need exogenous urea for growth. Organisms assigned to the other four genera have larger genomes, in the range of 1,360 to 1,830 kb (900 to 1,210 megadaltons) (3, 27, 29, 31). The acholeplasmas and asteroleplasmas are able to grow in the absence of sterols, whereas spiroplasmas and anaeroplasmas require it (or serum) for growth. Anaeroplasmas and asteroleplasmas are strict anaerobes and are found only in the bovine or ovine rumen (31); the other mollicute genera are facultative anaerobes (30). The spiroplasmas, about which our knowledge is rapidly expanding, are helical in shape and occur in plant and insect hosts (39). For several years there have been reports of nonhelical mycoplasmalike organisms also associated with plants and invertebrates (38, 40).

Given their many unusual properties, the origin and phylogeny of mollicutes have aroused considerable interest. Some biologists regarded them as living relics of a primitive type of cell that preceded present-day bacteria in evolution (21); others saw them merely as a phylogenetically heterogeneous collection of wall-less variants of typical bacteria (23).

Early attempts to subdivide the mycoplasmas, by using immunological approaches, DNA compositions, and the like, proved to be taxonomically useful but gave little phylogenetic information (22, 35). On the other hand, rRNA sequences reveal the phylogeny of this group in considerable detail. As a consequence, our understanding of mollicute phylogeny and evolution has increased dramatically during the last decade. The initial characterization of mollicute 16S rRNAs by oligonucleotide cataloging revealed that they are related to the gram-positive bacteria with low G+C DNA compositions; the general phenotype of these bacteria is clostridial. Within that group, the mollicutes are closely related to the bacillus-lactobacillus cluster, but they are related even more so to a particular small subgroup of clostridia represented by *Clostridium innocuum* and *Clostridium ramosum* (45).

Hori and colleagues (11) have sequenced the 5S rRNA of *Mycoplasma capricolum* and have confirmed the close relationship between this species and gram-positive eubacteria. A more extensive phylogenetic analysis of mollicute 5S rRNAs by Rogers and associates (32) was based upon the 5S rRNA sequences of *C. innocuum* and 10 mollicutes. In

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addition to affirming earlier conclusions regarding mollicute phylogeny, the study indicated that the initial divergence of mollicutes from their clostridial ancestors probably involved the *Acholeplasma* branch (in which the chromosome size dropped to 1640 to 1720 kb), with further divergence of this stem leading to the sterol-requiring, anaerobic *Anaeroplasma* branch and a sterol-requiring, helical *Spiroplasma* branch. The study also suggested that the *Spiroplasma* branch further evolved in a series of repeated and independent genome reductions to about 620 to 780 kb, to yield the *Mycoplasma* and *Ureaplasma* lineages.

The present study, based upon the 16S rRNA sequences of more than 40 species of mollicutes and six of their specific walled relatives (M&WR group), confirms, refines, and extends the results of these earlier studies and provides the basis for a phylogenetically valid taxonomy of the group.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains whose 16S rRNA sequences were determined for this study.

Cloning. Nucleic acids were isolated by conventional procedures (17, 46). The 16S rRNA genes for *Acholeplasma laidlawii* and *C. innocuum* were cloned as partial *Sau*3A digests into the *Bam*HI site of lambda phage L47.1 (16). For subcloning into phages M13mp8 and M13mp9 (19), the *Eco*RI site located at position 674 in most eubacterial 16S rRNAs was used. The *Mycoplasma gallisepticum* 16S rRNA gene was cloned as a single *Eco*RI fragment in a lambda gtWES.lambdaB vector (15) and was then subcloned into the M13 system (19). The remaining 16S rRNA sequences were determined by direct sequencing of the RNA, which was purified by ultracentrifugation through cesium trifluoroacetate gradients (Pharamacia Fine Chemicals AB) (5).

Sequencing methods. The dideoxynucleotide chain-termination method was used for both DNA and direct RNA sequencing (2, 14, 33, 50). Direct sequencing of rRNA involved the use of specific reverse primers with lengths of 15 to 18 nucleotides that were designed to be complementary to regions of the 16S rRNA molecule, whose sequence tends to be common to most, if not all, eubacteria (44, 50). In this study eight to nine primers were used; they ended (3′ end) at positions 109, 343, 517, 690, 915, (956, which was used occasionally), 1100, 1392, and 1492. In sequencing the rRNA gene, these primers were used in addition to various forward primers (specific for rRNA) and the customary primers in the M13 system (19, 50).

The actual 16S rRNA sequences determined for this study are not presented here. They have been deposited in GenBank under the accession numbers given in Table 1. Those sequences determined through gene cloning are complete; those determined by direct sequencing of 16S rRNA are 92 to 97% complete, except those for *Acholeplasma florum* (which is a very close relative of *Acholeplasma entomophilum*; about 70% complete) and *Anaeroplasma intermedium* (which is closely related to *Anaeroplasma varium* and *Anaeroplasma bactoclasticum*; about 85% complete). Since the *Acholeplasma florum* and the *Acholeplasma entomophilum* sequences were 99.7% similar, only the more complete of the two, that of *Acholeplasma entomophilum*, was used in this study to represent both.

Data analysis. The sequences were aligned in our sequence editor against a representative collection of eubacterial 16S rRNAs (44). Given the degree of similarity among all sequences, the alignment procedure was a straightforward manual one. Previously aligned near relatives of the new sequences, established secondary structural constraints, and sequence conservation patterns were used to guide the process (44).

Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed from the percent similarities by the correction of Jukes and Cantor (13), as modified by G. J. Olsen (personal communication), to accommodate the actual base ratios. This modification amounted to the replacment of the 0.25 random background term in the formulation of Jukes and Cantor (13) (i.e., their assumption that all four bases are present in equal amounts) by c, where $c = f_{A1}f_{A2} + f_{C1}f_{C2} + f_{G1}f_{G2} + f_{U1}f_{U2}$. The fs are the base ratios (for the positions being considered) in the two sequences in each pair. Dendrograms were constructed from evolutionary distance matrices by the method of De Soete (6).

RESULTS AND DISCUSSION

The mollicutes and their immediate relatives. Figure 1 shows an evolutionary distance tree based on a representative sampling of 16S rRNAs from the clostridial subdivision of the gram-positive eubacteria (7, 42). The phylogenetic grouping defined by the mollicutes also encompassed a small collection of variously named walled bacteria (see below). This combined unit of the M&WR group itself stemmed from the same lineage that spawned the bacillus-lactobacillus cluster. Parsimony analysis (data not shown) yielded this phylogenetic arrangement as well. Both the sister group relationship to the bacillus-lactobacillus lineage and the inclusion of walled bacteria within (or closely linked to) the phylogenetically defined mollicutes were detected in earlier rRNA oligonucleotide catalog studies (45).

Several unusual features of the small-subunit rRNA could be used to identify (define) the relationship between the M&WR group and the bacillus-lactobacillus cluster. One is the composition of the nucleotide pair between positions 52 and 359 in the 16S rRNA secondary structure (44, 45). As a visual guide to the discussion of specific features in the 16S rRNA molecule see Fig. 2, which provides a generalized secondary structural diagram for the gram-positive eubacteria. Among members of the bacillus-lactobacillus and the M&WR groups, the 52 · 359 pair always had the composition U · A. Among the remaining gram-positive lineages, again without exception, its composition is C · G (48). Among cyanobacteria the composition is uniformly C · G, as it is for 98% of the purple bacteria (48; unpublished data). Note that position 52 is covered by the highly conserved eubacterial oligonucleotide CYU(AU)AYACAUG (48) (where Y is a pyrimidine), of which almost 500 examples are now known.

The M&WR group also shares with the bacillus-lactobacillus group the sequence AUAUAUG, which covers position 705 in the 16S rRNA sequence (45). Throughout the entire collection of eubacterial small-subunit rRNAs, the sequence in question was seen only among members of the bacillus-lactobacillus and M&WR groups (with a single exception). It occurred universally within lactobacilli and streptococci but sporadically among the bacilli. It occurred in all members of three of the mollicute groups (see below), all of the walled relatives, and in about half of the members of the hominis group. It was not found in *Asteroleplasma anaerobium*.

The phylogenetically defined M&WR group can be easily distinguished by the composition of a certain few positions in the small-subunit rRNA (45). A U residue occurs at position 888 in all M&WR group sequences, with the exception of a

TABLE 1. Bacterial strains used in this study

Species	Strain designation	ATCC no.[a]	GenBank accession no. of 16S rRNA sequences
Genus *Mycoplasma*			
M. agalactiae	PG2	NCTC 10123	M24290
M. arginini	G230	23838	M24579
M. arthritidis	PG6	19611	M24580
M. bovigenitalium	PG11	19852	M24291
M. californicum	ST-6	33461	M24582
M. capricolum (12)			
M. ellychniae	ELCN-1	43707	M24292
M. fermentans	PG18	19989	M24289
M. gallisepticum	A5969		M22441
M. hominis	PG21	23114	M24473
M. hyopneumoniae (36)			
M. hyorhinis		17981	M24658
M. iowae	695	33552	M24293
M. lipophilum	MaBy	27104	M24581
M. mobile	163K	43663	M24480
M. muris	RIII4	33757	M23939
M. mycoides subsp. *mycoides*	UM30847		M23943
M. neurolyticum	Type A	19988	M23944
M. orale	CH19299	23714	M24659
M. pirum	70-159	25960	M23940
M. pneumoniae	FH	15531	M29061
M. pulmonis	PG34	19612	M23941
M. putrefaciens	KS-1	15718	M23938
M. salivarium	PG20	23064	M24661
M. sualvi	Mayfield B	33004	M23936
Mycoplasma sp.	831-C4	49193	M24479
Mycoplasma sp.	M1	49191	M24478
Genus *Spiroplasma*			
S. apis	B-31	33834	M23937
S. citri	Maroc	27556	M23942
S. mirum	SMCA	29335	M24662
Spiroplasma group II (*Drosophila melanogaster*)	DW1	43153	M24483
Spiroplasma group VI (tick)	Y32	33835	M24477
Spiroplasma group VII (wasp)	MQ-1	33825	M24481
Spiroplasma group IX (beetle)	CN-5	33827	M24474
Spiroplasma group XII (beetle)	DU-1	43210	M24482
S. taiwanense group XXII (mosquito)	CT-1	43302	M24476
Spiroplasma group XXIII (horsefly)	TG-1	43525	M24475
Genus *Acholeplasma*			
A. entomophilum	TAC	43706	M23931
A. florum	L1	33453	
A. laidlawii	JA1		M23932
A. modicum	PG 49	29102	M23933
Genus *Anaeroplasma*			
A. abactoclasticum	6-1	27879	M25050
A. bactoclasticum	JR	27112	M25049
A. intermedium	5LA		
A. varium	A-2	43167	M23934
Asteroleplasma anaerobium	161	27880	M22351
Ureaplasma urealyticum	960	NCTC 10177	M23935
Walled relatives			
Clostridium innocuum	B-3	14501	M23732
Clostridium ramosum	113-I	25582	M23731
Erysipelothrix rhusiopathiae	α-P15	19414	M23728
Lactobacillus catenaforme	1871	25536	M23729
Lactobacillus vitulinus	185	27783	M23727
Streptococcus pleomorphus	60B	29734	M23730

[a] ATCC, American Type Culture Collection, Rockville, Md.

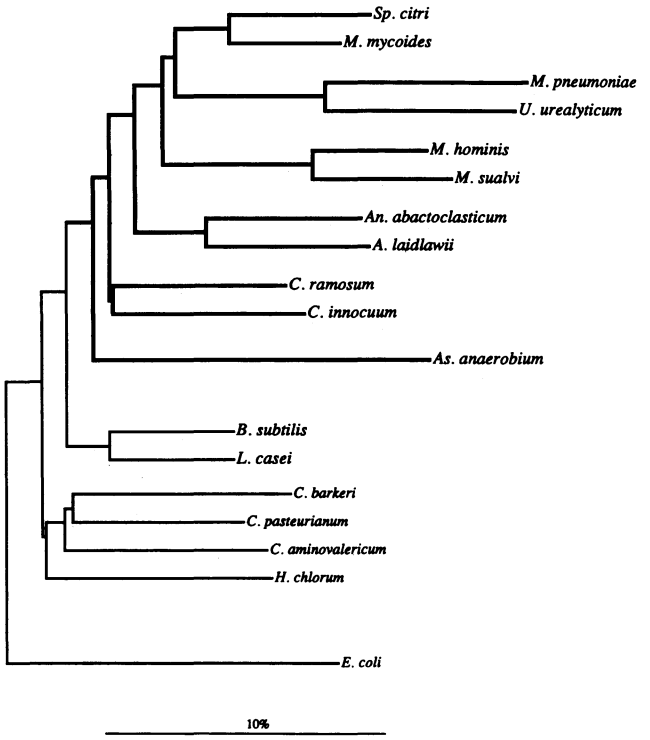

FIG. 1. Phylogenetic tree showing the position of the mollicutes and their walled relatives in the gram-positive eubacterial phylum; see text for details. The sequence of *Escherichia coli* (4) served used as an outgroup, establishing the root of the tree. The other sequences included that were not a direct part of this study were *Bacillus subtilis* (9), *Heliobacterium chlorum* (43), *Clostridium barkeri* (M23927), *Clostridium pasteurianum* (M23930), *Clostridium aminovalericum* (M23929), and *Lactobacillus casei* (M23928). Although the sequences of the last four of these strains are unpublished, they appear in GenBank under the accession numbers indicated in parentheses. Analysis was confined to those positions in the alignment that satisfied the condition that 1 base accounts for at least 50% of the total composition of that position (across the set of gram-positive bacteria used). This procedure enriched for the less rapidly changing positions.

subgroup of the walled relatives that comprises *C. innocuum*, *Erysipelothrix rhusiopathie*, and *Streptococcus pleomorphus* (in which it is a G residue). This contrasted sharply to the purine residue that occupies position 888 in all other eubacteria and archaebacteria (unpublished data). The ancestral eubacterial composition for position 1383 (found in the nearly universal eubacterial [T1] oligonucleotide UUC CCG [48]) was a C residue, with only three (phylogenetically independent) exceptions, i.e., the actinomycetes, the fusobacteria, and (some of) the anaerobic halophiles (48; unpublished data). Among the M&WR group species, position 1383 in 16S rRNA was typically a U residue, with the exception of *Asteroleplasma anaerobium*, *Anaeroplasma varium*, *Streptococcus pleomorphus*, and *Lactobacillus catenaforme*, in which it (independently) reverted to the ancestral C-residue composition.

Perhaps the most convincing synapomorphy (shared derived character) identifying the M&WR group, however, was a higher-order structural feature. In all other known eubacterial 16S rRNAs (including those of the bacillus-lactobacillus cluster), the region of 16S rRNA at positions 1025 to 1036 folds into a helix, whose stalk usually comprises 3 to 5 base pairs and whose terminal loop is capped by a stretch of 4 (rarely 5) nucleotides (10, 44). However, in the M&WR group no helix occurred (with one exception). In this group the entire region consisted of a shorter stretch of 8 seemingly unpaired nucleotides with the general composition R(AU)RRYUA(AU) (where R is a purine and Y is a pyrimidine). The small-subunit rRNA of *L. catenaforme*, the exception mentioned above, formed a helix in this region, but its unique composition and unusual length of 20 nucleotides (unpublished data) strongly suggest that the structure is derived (reevolved), not ancestral.

The major mollicute groups. As shown in Fig. 1, the phylogenetically defined grouping of mollicutes and their walled relatives comprised six distinct clades: (i) the pneumoniae group (6 characterized species), (ii) the hominis group (16 characterized species), (iii) the spiroplasma group (17 characterized species), (iv) the anaeroplasma group (6 characterized species), (v) the asteoleplasma group (1 characterized species), and (vi) the walled relatives (6 characterized species). Detailed phylogenetic trees for the individual groups, shown in Fig. 3 through 7, have been constructed from the evolutionary distance matrices given in Tables 2 through 6, respectively.

The individual mollicute groups that resulted from distance matrix analysis could also be defined in terms of shared derived characters. Because it was represented by a single species only, the asteroleplasma group was omitted from the following characterizations. For the pneumoniae, hominis, spiroplasma, and anaeroplasma groups, the number of positions in 16S rRNA showing a unique characteristic composition was 10 (+8) for the pneumoniae group, 10 (+5) for the hominis group, 2 (+3) for the spiroplasma group, and 4 (+2) for the anaeroplasma group. (The numbers in parentheses indicate the additional signature positions that resulted when transition degeneracy, i.e., U = C and A = G, was imposed on the sequence alignment.) The number of positions was 0 for the walled relatives, however. The composition of a position was considered characteristic in this case when it held for all members of a given group with no more than one exception in that group (except the gram-positive outgroup, *Bacillus subtilis*, *Lactobacillus casei*, *Streptococcus faecalis*, *Clostridium pasteurianum*, *Clostridium aminovalericum*, *Clostridium barkeri*, and *Heliobacterium chlorum*, in which no exceptions were permitted). A characteristic composition was called unique when it was seen in one group only, whereas the remaining groups, including *Asteroleplasma anaerobium*, showed a different characteristic composition (the same in all cases). Given the stringency of their definition, even the small number of characteristic positions (derived characters) shown by the spiroplasma group was considered significant.

In addition to its sequence signature, the pneumoniae group was readily defined by three higher-order structural idiosyncrasies (synapomorphies). The first of these was particularly striking, in that the pneumoniae group was unique in this respect not only among eubacteria but also among archaebacteria and eucaryotes. All members of the pneumoniae group had a C-residue insert following position 915 (AAACGGAA) in an area of 16S rRNA that is otherwise completely constant in length and that has a highly conserved sequence (10, 44). The second synapomorphy involved the elimination of the helix between positions 1126 and 1144 (44). This structure is found in all eubacteria except members of the pneumoniae group (in which it was replaced by a short stretch of 4 to 5 unstructured nucleotides) and members of the green nonsulfur phylum (which similarly truncate it [25]). The third synapomorphy involved the helix

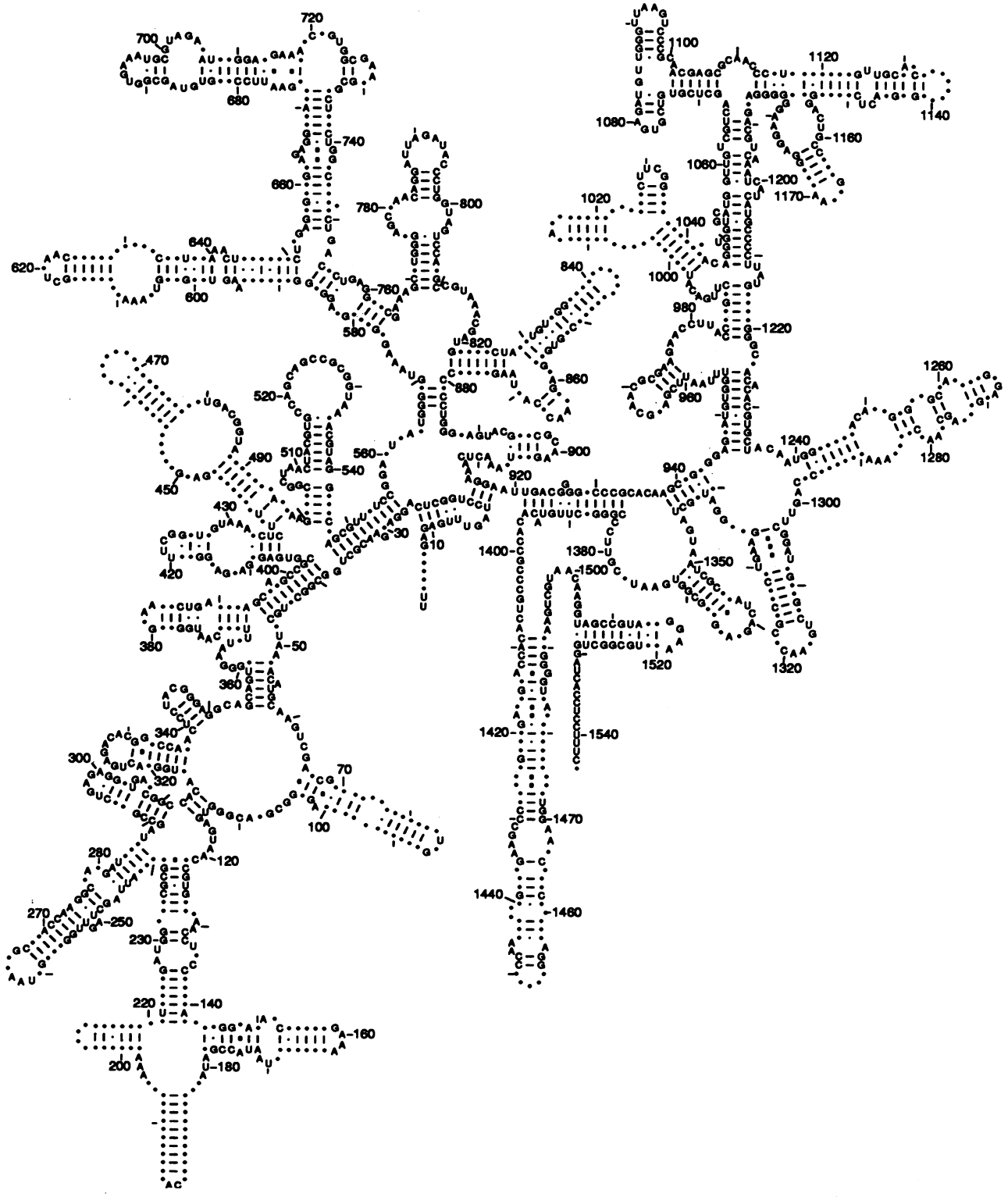

FIG. 2. Secondary structural representation (44) of a generic gram-positive 16S rRNA based on approximately 20 representative gram-positive 16S rRNA sequences (unpublished data). The template for the structure was the *Bacillus subtilis* sequence (9). The numbering of positions, however, followed the *Escherichia coli* convention (4); every 10th sequence position is marked, and (where the structure permits) every 20th position is numbered. The compositions indicated are those that occurred in at least 90% of the 20 sequences at that position. Positions whose compositions did not meet this condition are indicated by dots.

TABLE 2. Evolutionary distances among members of the pneumoniae group[a]

Species	Evolutionary distance					
	1	2	3	4	5	6
1. *M. pneumoniae*						
2. *M. gallisepticum*	11.1					
3. *M. pirum*	9.6	6.0				
4. *M. iowae*	17.0	15.5	14.1			
5. *M. muris*	16.1	14.3	13.0	3.9		
6. *U. urealyticum*	18.0	15.9	14.7	14.4	13.6	
7. *M. mycoides*	25.5	24.2	24.5	23.6	24.0	25.4

[a] The distances were calculated as described in the text. Only positions in the alignment represented by a nucleotide of known composition in all sequences being considered were used in the analysis. *M. mycoides* served as an outgroup. See the text for details.

located between positions 416 and 427 in 16S rRNA, which in eubacteria normally comprises a stalk of 4 pairs capped by a loop of 4 nucleotides; the loop composition is almost always UUCG, and the loop-proximal pair of the stalk is always Y · G (44; unpublished data). All members of the pneumoniae group, however, added what appeared to be a fifth (loop-proximal U · A) pair to the structure. The loop, which contained 3 to 5 bases, comprised exclusively U and A residues. Several members of the hominis group also added an additional loop-proximal pair; in this case it was a C · G or a U · G pair, and the loop size was reduced to 3 nucleotides.

One unusual feature of possible functional significance in the hominis group should be noted. Position 912 in 16S rRNA is covered by the oligonucleotide fragment -AAACUCAAA- (48). No variation in this composition was observed in oligonucleotide catalogs (about 400 species [48]), and the indicated C residue distinguishes eubacteria from archaebacteria and eucaryotes (42), all of which showed a U residue at this position (position 912). It has been reported that a mutation that changes this C residue to a U residue (in *Escherichia coli*) confers streptomycin resistance (20). The only known example of a C residue at this position among the naturally streptomycin-resistant archaebacteria occurs in *Desulfurococcus mobilis*, the only archaebacterium that is sensitive to streptomycin (R. Garrett, personal communication). Position 912 had a U residue in all members of the hominis group for which the sequence is known here (because of technical difficulties, a block terminating the sequencing reaction, the sequence was not determined for this region in a number of cases). Four other phylogenetically independent examples of a U residue at this position occur among the eubacteria: in *M. pirum* (in the pneumoniae group), *Asteroleplasma anaerobium*, the four anaeroplasmas, and *Leuconostoc oenos* (an unusual lactic acid bacterium) (unpublished data).

While the M&WR group as a whole and the six major groups it comprises (except for the walled relatives) are considered to be reliably established phylogenetic units (clades) (they were easily demonstrated by distance matrix analysis, parsimony analysis [data not shown], and signature features [both individual positions in the rRNA sequence and higher-order structural idiosyncrasies]), we did not consider the branching order among the six groups given by distance matrix analysis (Fig. 1) to be established with certainty, except for the specific clustering of the hominis, pneumoniae, and spiroplasma groups to the exclusion of the others. This particular cluster was supported by several stringent synapomorphies. The most convincing of these are the two adjacent pairs 127-128 and 233-234 in the 16S rRNA secondary structure (44), which were of the form YY · RR in every member of these three groups but showed the inverted RR · YY composition in all other members of the M&WR group (including *Asteroleplasma anaerobium*), in all other gram-positive sequences with a low G+C content (more than 100), in all but 1 purple bacterial sequence (approximately 70 known), and in *Anacystis nidulans* (37; unpublished data). A second synapomorphy was the addition of a single nucleotide after position 1361 in 16S rRNA, which occurred in all members of these three groups (and *Asteroleplasma anaerobium*) but nowhere else among the sequences of the M&WR group. Since this addition is not characteristic of the gram-positive eubacteria in general, the purple bacteria, or *Anacystis nidulans* (unpublished data), it appears to be a shared derived character. Similarly, all members of the three groups (and *Asteroleplasma anaerobium*) had a deletion of the nucleotide at position 1167. No other members of the

TABLE 3. Evolutionary distances among members of the hominis group[a]

Species	Evolutionary distance															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *M. neurolyticum*																
2. *M. hyopneumoniae*	12.0															
3. *M. hyorhinis*	9.8	9.7														
4. *M. sualvi*	12.6	16.0	10.6													
5. *M. mobile*	13.7	15.1	12.5	11.9												
6. *M. pulmonis*	13.3	15.8	12.1	14.6	14.4											
7. *M. lipophilum*	14.3	16.3	12.3	14.3	15.2	12.0										
8. *M. bovigenitalium*	15.7	18.6	14.5	15.3	15.4	14.0	7.7									
9. *M. californicum*	14.9	18.0	13.8	15.7	13.9	14.0	8.4	2.6								
10. *M. fermentans*	15.3	18.2	12.8	15.4	14.8	13.9	7.0	5.7	5.6							
11. *M. agalactiae*	14.3	17.8	13.3	14.3	14.8	13.0	6.6	5.9	6.3	5.6						
12. *M. hominis*	13.6	17.4	11.2	12.7	14.5	12.6	12.9	13.4	13.9	13.4	13.5					
13. *M. orale*	13.0	17.6	12.2	13.1	14.4	13.5	13.2	14.6	14.6	13.8	13.4	5.8				
14. *M. salivarius*	13.0	18.2	12.4	13.0	14.7	13.4	13.3	14.5	14.2	13.7	13.1	5.3	2.8			
15. *M. arthritidis*	13.9	18.5	11.8	13.8	14.6	12.7	13.9	14.7	13.9	13.8	14.0	4.5	3.8	3.4		
16. *M. arginini*	14.0	17.6	12.5	13.2	14.3	12.7	14.0	14.8	14.9	14.6	14.9	4.1	4.9	4.6	3.0	
17. *M. mycoides*	21.3	25.1	21.7	21.9	23.0	23.7	23.0	24.0	23.4	24.1	22.8	23.2	22.7	23.5	23.5	24.8

[a] *M. mycoides* served as an outgroup. See the text for details.

TABLE 4. Evolutionary distances among members of the spiroplasma group[a]

Species	Evolutionary distance																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *Spiroplasma* sp. strain Y-32																	
2. *Spiroplasma* sp. *citri*	14.9																
3. *Spiroplasma* sp. strain DW-1	14.3	1.7															
4. *Spiroplasma* sp. *mirum*	13.3	4.6	4.0														
5. *Spiroplasma* sp. strain DU-1	13.1	10.8	10.4	9.9													
6. *Spiroplasma* sp. strain MQ-1	13.4	10.8	10.5	9.9	0.9												
7. *Spiroplasma taiwanense*	13.7	11.3	10.8	10.8	1.7	2.0											
8. *Spiroplasma* sp. strain CN-5	13.4	11.5	11.2	10.3	2.6	2.7	2.3										
9. *Spiroplasma apis*	12.8	10.6	10.1	9.8	2.3	2.5	2.2	2.5									
10. *Spiroplasma* sp. strain TG-1	14.0	11.3	10.9	10.6	2.8	2.7	2.8	3.4	3.2								
11. *Mycoplasma mycoides*	13.5	13.1	13.1	11.9	7.4	7.1	7.8	7.9	8.4	8.4							
12. *Mycoplasma capricolum*	14.1	13.5	13.4	12.5	7.7	7.5	8.2	8.1	8.6	8.8	0.7						
13. *Mycoplasma putrefaciens*	13.1	13.7	13.4	12.1	7.5	7.2	8.1	7.7	8.0	8.5	1.9	2.5					
14. *Acholeplasma entomophilum*	13.6	13.0	12.6	11.8	6.7	6.4	7.7	7.6	7.7	7.5	4.9	5.3	4.5				
15. *Mycoplasma* sp. strain M1	14.1	12.8	12.5	11.6	6.6	6.4	7.6	7.8	7.7	7.5	4.7	5.0	4.5	1.0			
16. *Mycoplasma ellychniae*	14.2	12.7	12.3	11.8	6.6	6.5	7.6	7.7	7.2	7.7	5.0	5.4	4.6	2.9	2.8		
17. *Mycoplasma* sp. strain 831-C4	14.5	13.1	12.7	11.8	7.7	7.7	9.0	9.6	9.2	9.7	8.6	8.9	8.5	7.7	7.3	7.1	
18. *Mycoplasma hominis*	21.9	23.1	22.0	21.5	20.9	21.3	21.8	21.3	21.6	22.1	21.9	22.1	21.4	21.6	21.6	21.6	21.8

[a] *M. hominis* served as an outgroup.

M&WR group exhibit this characteristic (except for the *Lactobacillus catenaforme-Lactobacillus vitulinus* clade). Since the deletion is not characteristic of the gram-positive bacteria, the purple bacteria in general, or *Anacystis nidulans*, it too appeared to be a derived character.

Note that *Asteroleplasma anaerobium* shared two of the three synapomorphies described above with the hominis, pneumoniae, and spiroplasma cluster; this suggests a relationship at variance with that seen in Fig. 1. However, it is not our intention to attempt to resolve the exact branching among the various M&WR groups beyond that described above. Note that the base ratio of *Asteroleplasma* rRNA (56% G+C content) is significantly higher than that of the three groups in question (46 to 49% G+C content). The effect of rRNA composition on phylogenetic branching order is being examined elsewhere (C. Woese and G. Olsen, manuscript in preparation). The branching order among the main M&WR groups will be reexamined in this context at a later time.

Phylogenetic detail in the primary mollicute groups. (i) The pneumoniae group. The pneumoniae group comprises three distinct clusters represented by *M. pneumoniae*, *M. muris*, and (the single species) *Ureaplasma urealyticum* (Fig. 3). All species could be defined by sequence signatures, and some could be defined by higher-order structural synapomorphies as well. A total of 38 positions in the sequence alignment met the condition that their compositions were constant within each of these three clusters, but they were not the same in all three clusters; and 18 of these also showed a compositional constancy across all the outgroups used, i.e, the four remaining mollicute groups, the walled relatives, and the seven other gram-positive species listed above (allowing no more than one exception to constancy in each of these outgroups and allowing no exception for *Asteroleplasma anaerobium*). Of these 18 positions of highly conserved composition, 7, 3, and 4 showed a derived composition that identified the *M. pneumoniae*, the *M. muris*, and the *U. urealyticum* clusters, respectively. Of the remaining 4 of the 18 positions, two suggested that there is a specific relationship between the *M. pneumoniae* and *U. urealyticum* clusters, but 2 others supported a specific relationship between the *M. muris* and *U. urealyticum* clusters, giving an equivocal answer in regard to the branching order among these three clusters. We do not, therefore, consider that the branching order given by the distance analysis (Fig. 3) has been proven.

The members of the *M. pneumoniae* cluster could be distinguished from all remaining members of the M&WR group by the deletion of a nucleotide in the vicinity of position 1286 (in or adjacent to a stretch of 3 to 4 A residues); this deletion is seen only rarely among gram-positive bacteria in general. In the *M. muris* cluster, on the other hand, a nucleotide was added in the loop of the helix covering position 420 (relative to other members of the pneumoniae group; see the discussion of this structure above).

TABLE 5. Evolutionary distances among members of the anaeroplasma group[a]

Species	Evolutionary distance					
	1	2	3	4	5	6
1. *Anaeroplasma bactoclasticum*						
2. *Anaeroplasma varium*	1.4					
3. *Anaeroplasma intermedium*	2.4	2.6				
4. *Anaeroplasma abactoclasticum*	6.1	6.3	5.9			
5. *Acholeplasma modicum*	14.5	14.7	14.4	13.6		
6. *Acholeplasma laidlawii*	14.5	14.5	14.3	13.1	12.6	
7. *Mycoplasma mycoides*	20.7	20.8	20.0	19.3	21.0	20.6

[a] *M. mycoides* served as an outgroup.

TABLE 6. Evolutionary distances among members of the walled relatives of the mycoplasmas[a]

Species	Evolutionary distance					
	1	2	3	4	5	6
1. *Clostridium innocuum*						
2. *Streptococcus pleomorphus*	10.9					
3. *Erysipelothrix rhusiopathiae*	15.5	16.5				
4. *Clostridium ramosum*	18.0	17.4	16.2			
5. *Lactobacillus catenaforme*	21.8	23.9	21.4	19.2		
6. *Lactobacillus vitulinus*	20.5	22.3	18.5	14.1	11.7	
7. *Lactobacillus casei*	19.1	20.2	19.2	19.6	22.7	22.4

[a] *Lactobacillus casei* served as an outgroup.

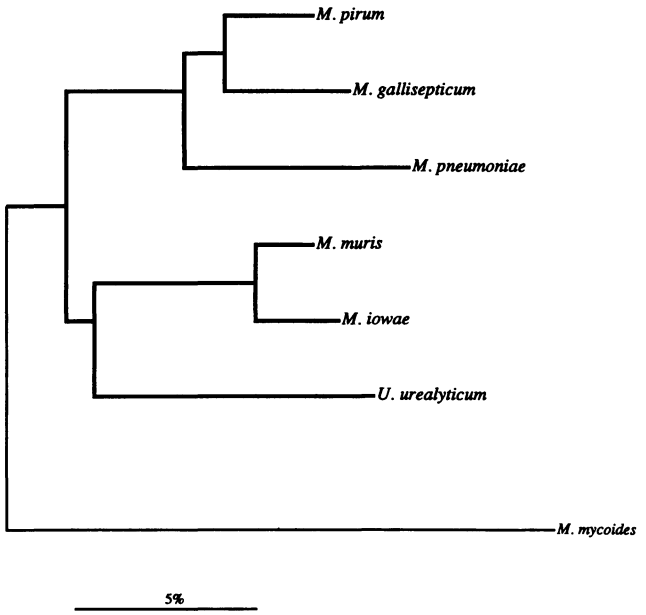

FIG. 3. Detailed phylogenetic tree for the pneumoniae group. Analysis is the same as that described in the legend to Fig. 1, except that it was based on all positions in the alignment that were present in all members of the considered group; the distances used are given in Table 2. *M. mycoides* served as an outgroup. The bar indicates an evolutionary distance of 5%. The branching order among the three subgroups shown here (see text) is not considered to be firmly established.

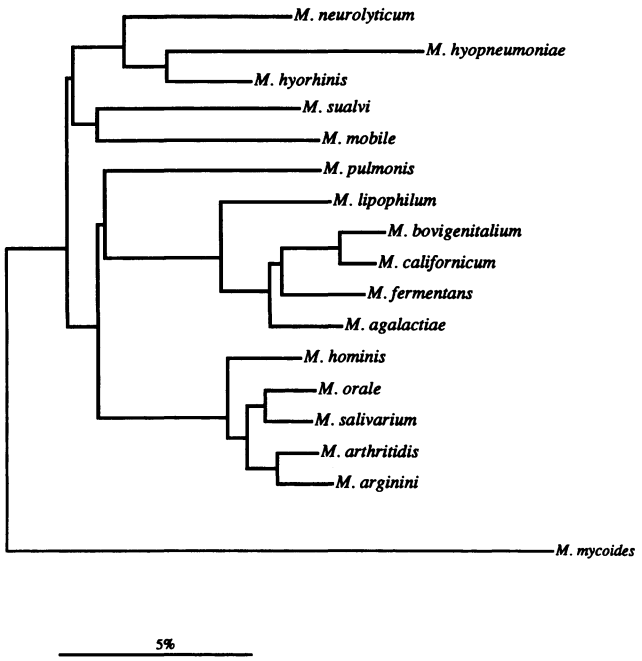

FIG. 4. Detailed phylogenetic tree for the hominis group derived from the distance matrix of Table 3. Conditions for analysis were the same as those described in the legend to Fig. 3. *M. mycoides* served as the outgroup.

(ii) The hominis group. The hominis group comprises five distinct clusters represented by *M. hominis*, *M. lipophilum*, *M. pulmonis* (an isolated species), *M. sualvi*, and *M. neurolyticum* (Fig. 4). As defined above in the strict sense, only the *M. hominis* and *M. sualvi* clusters were supported by signature analysis (two positions in each case); the isolated species *M. pulmonis* was not considered. However, if attention is confined only to the five clusters within this group, the *M. hominis* cluster could be defined by 8 [6] positions in the alignment that showed a unique characteristic composition (i.e., a constant composition in all members of this cluster and a constant but different composition across the remainder of the hominis group; the minimum number of positions that appear to be of derived composition is given in brackets). A similar analysis for the *M. lipophilum*, *M. sualvi*, and *M. neurolyticum* clusters yielded 11 [7], 5 [3], and 3 [3] such positions, respectively.

Two higher-order structural attributes distinguished the *M. lipophilum* cluster. All five of its sequences showed 2 additional nucleotides following position 722, in a locale of 16S rRNA that was otherwise invariant in length among the eubacteria and archaebacteria (with the single exception cited below). All five of these sequences also truncated the helix lying between positions 1435 and 1466, deleting the equivalent of 5 base pairs from the structure. Although truncation of this particular helix was occasionally encountered among other eubacteria (although not among the gram-positive or purple bacteria), the particular form of it seen in the *M. lipophilum* cluster is thus far unique (unpublished data).

The exception mentioned above involved the three members of the *M. neurolyticum* cluster, which added 1 nucleotide following position 722 in 16S rRNA; this too occurs nowhere else among procaryotes.

(iii) The spiroplasma group. The spiroplasma group comprises four distinct clusters, represented by *M. mycoides*, *Spiroplasma apis*, *Spiroplasma citri*, and (the isolated species) *Spiroplasma* sp. strain Y-32 (Fig. 5). As in the case of the hominis group, the more strictly defined sequence signatures provided little evidence either for or against the clusterings shown in Fig. 5. The *M. mycoides* and *Spiroplasma apis* clusters each demonstrated only one position of a common derived sequence, whereas the (smaller) *Spiroplasma citri* cluster had five positions by this criterion. However, by using the less restrictive definition (as in the case of the hominis group), the *M. mycoides* cluster showed 8 [4] positions of unique characteristic composition, the *Spiroplasma apis* cluster showed 4 [3] positions, and the *Spiroplasma citri* cluster showed 19 [15] positions (numbers in brackets are as defined above).

A sister group relationship between the *M. mycoides* and *Spiroplasma apis* clusters was supported by 22 positions of this same type. Even the stricter definition yielded three synapomorphies that supported this particular association. No significant support existed for any of the other possible pairings of these four clusters in the spiroplasma group, except perhaps a distant sister group relationship between the (small) *Spiroplasma citri* and *Spiroplasma* sp. strain Y-32 clusters.

It is clear from the branching shown in Fig. 5 that the spiral shape is undoubtedly the ancestral morphology for this group, justifying its name.

Note the inclusion of a few acholeplasmas, i.e., *Acholeplasma entomophilum* and its relative *Acholeplasma florum*, with the plant- and insect-associated nonspiral mollicutes, which makes the lack of a sterol requirement polyphyletically distributed.

(iv) The anaeroplasma group. In the anaeroplasma group the two *Acholeplasma* species and four *Anaeroplasma* species, respectively, formed the natural clusters (Fig. 6). Each

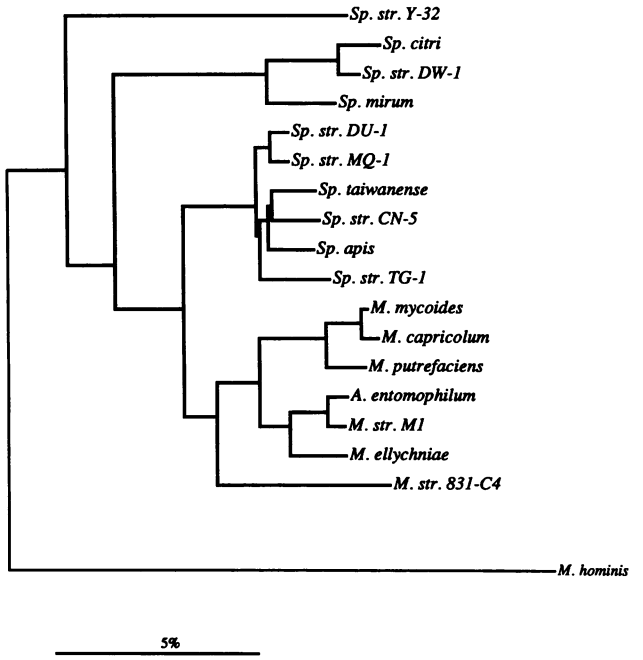

FIG. 5. Detailed phylogenetic tree for the spiroplasma group derived from the distance matrix of Table 4. Conditions for analysis were similar to those described in the legend to Fig. 3. *M. hominis* served as the outgroup.

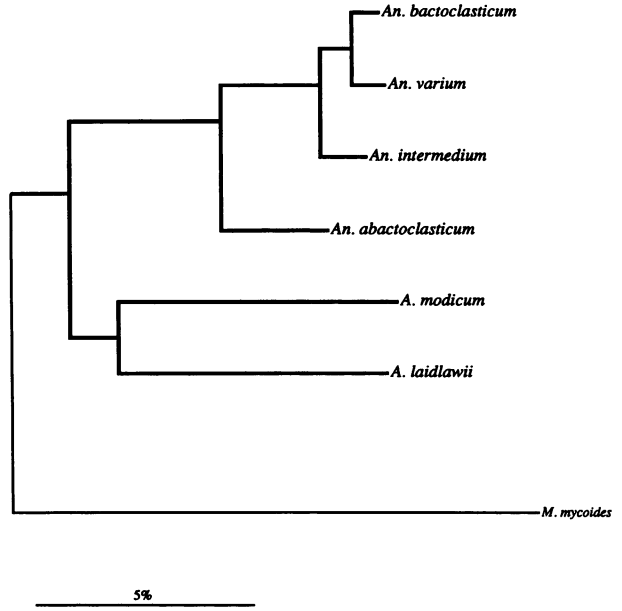

FIG. 6. Detailed phylogenetic tree for the anaeroplasma group derived from the distance matrix of Table 5. Conditions for analysis were similar to those described in the legend to Fig. 3. *M. mycoides* served as the outgroup.

was strongly supported by a sequence signature. Even with the more stringent definition of signature positions (that used for the pneumoniae group), eight positions could be found that distinguished the two. Five were cases in which all members of the *Anaeroplasma* cluster exhibited a common derived composition; in the remaining three, both members of the *Acholeplasma* cluster exhibited a common derived composition.

(v) The walled relatives. Since the monophyletic group of walled relatives suggested by evolutionary distance analysis (unpublished data) is not supported by a significant sequence signature (see above), we do not, at this time, discount the possibility that the walled relatives are paraphyletic. Within the group, however, definite monophyletic units can be recognized (Fig. 7). *C. innocuum* clustered with *Streptococcus pleomorphus*, *C. ramosum* clustered with the two lactobacilli, and *Erysipelothrix rhusiopathiae* represented a third cluster. Cladistic evidence existed for both the *C. ramosum* and the *C. innocuum* clusters. The two were supported, respectively, by six and five positions of derived sequence (as defined above for the pneumoniae group). No significant signature of this type existed to support any other groupings of the walled relatives, except for the two lactobacilli (within the *C. ramosum* cluster), which formed a tight cluster supported by four strong signature positions.

A higher-order structural characteristic consistent with the clusterings within the walled relatives was seen in the helix whose 4-base loop covered position 85 in 16S rRNA. Its stalk contained 15 to 17 pairs in the *C. innocuum* and *Erysipelothrix rhusiopathiae* clusters, but only 8 to 9 pairs in the *C. ramosum* cluster. Almost all other sequences in the M&WR group showed a short version of this helix, while a longer version seems to be characteristic, in general, of the gram-positive bacteria with a low G+C content.

The relationship of *Erysipelothrix rhusiopathiae* to the two defined clusters within the group of walled relatives was considered uncertain. However, it is worth noting that *Erysipelothrix rhusiopathiae* and the *C. innocuum* cluster had a G residue at position 888 (mentioned above), while all other members of the M&WR group had a U residue at this position. Since a pyrimidine residue at this position was found nowhere else among the eubacteria and archaebacteria, the change from ancestral purine (probably a G residue)

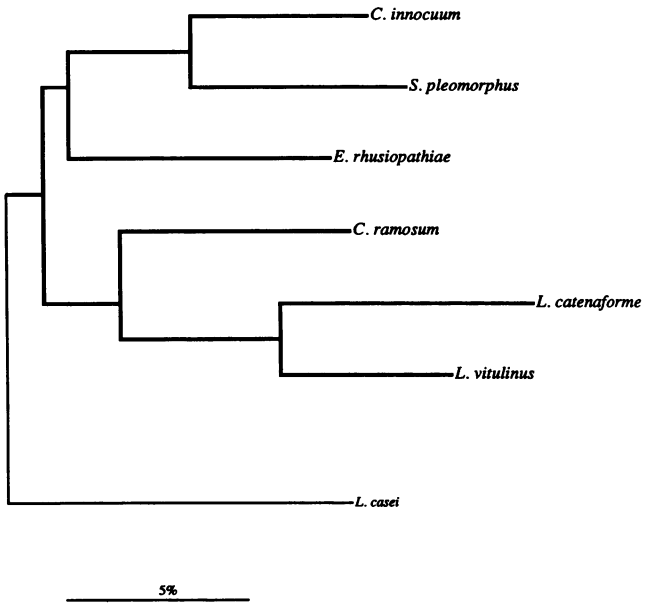

FIG. 7. Detailed phylogenetic tree for the walled relatives derived from the evolutionary distance matrix of Table 6. Conditions for analysis were similar to those described in the legend to Fig. 3. *L. casei* was used as the outgroup.

TABLE 7. Distribution of phenotypic properties among mollicutes[a]

Species	Glucose fermentation	Arginine hydrolysis	Terminal structure	Host	DNA composition (% G+C)	Genome size (kb)
Pneumoniae group						
Mycoplasma pneumoniae	+	−	+	Human	39	720–750
Mycoplasma pirum	+	+	+	?	25	
Mycoplasma gallisepticum	+	−	+	Bird	31	740
Mycoplasma muris	−	+	−	Mouse	25	
Mycoplasma iowae	+	+	−	Bird	25	
U. urealyticum	−	−	−	Animals	26	720
Hominis group						
Mycoplasma hominis	−	+	−	Human	~30	680
Mycoplasma orale	−	+	−	Human	~26	710
Mycoplasma salivarium	−	+	−	Human	~29	710
Mycoplasma arthritidis	−	+	−	Rodent	~31	~720
Mycoplasma arginini	−	+	−	Animals	~29	610
Mycoplasma lipophilum	−	+	−	Human	ND[b]	
Mycoplasma bovigenitalium	−	−	−	Cow	~29	610
Mycoplasma californicum	−	−	−	Cow	32	
Mycoplasma fermentans	−	+	−	Human	~27	730
Mycoplasma agalactiae	−	−	−	Goat	~33	710
Mycoplasma pulmonis	+	−	+	Rodent	28	
Mycoplasma sualvi	+	+	+	Swine	24	
Mycoplasma mobile	+	−	+	Fish (?)	24	780
Mycoplasma neurolyticum	+	−	−	Mice	25	
Mycoplasma hyopneumoniae	+ (?)	−	−	Swine	28	
Mycoplasma hyorhinis	+	−	−	Swine	27	820
Spiroplasma group						
Mycoplasma mycoides	+	−	−	Cow, goat	25	760
Mycoplasma capricolum	+	±	−	Goat	~25	720
Mycoplasma putrefaciens	+	−	−	Goat	25	
Acholeplasma florum	+	−	−	Plant, insect	~26	1,600
Acholeplasma entomophilum	+	−	−	Plant, insect	30	
Mycoplasma M1	+	−	−	Plant	27	860
Mycoplasma ellychniae	+	−	−	Insect	~28	890
Mycoplasma sp. strain 831-C4	+	−	−	Plant	30	870
Spiroplasma citri	+	+	Helical	Plants, insects	26	~1,600
Spiroplasma mirum	+	+	Helical	Ticks	30	
Spiroplasma sp. strain DW-1	+	+	Helical	*Drosophila melanogaster*	26	
Spiroplasma apis	+	+	Helical	Plants, insects	30	
Spiroplasma sp. strain DU-1	+	−	Helical	Beetle	25	
Spiroplasma sp. strain MQ-1	+	−	Helical	Wasp	28	
Spiroplasma sp. strain CN-5	+	+	Helical	Beetle	29	
Spiroplasma sp. strain TG-1	+	−	Helical	Horsefly	25	
Spiroplasma taiwanense CT-1	+	−	Helical	Mosquito	25	
Spiroplasma sp. strain Y32	+	−	Helical	Tick	25	
Anaeroplasma group						
Acholeplasma laidlawii	+	−	−	Animals	~32	~1,680
Acholeplasma modicum	+	−	−	Animals	~30	1,500
Anaeroplasma abactoclasticum	+	−	+ (?)	Pig	29	1,650
Anaeroplasma intermedium	+	−	+ (?)	Pig	33	
Anaeroplasma varium	+	−	+ (?)	Cow	33	
Asteroleplasma group						
Asteroleplasma anaerobium	+	−	−	Pig	40	1,730

[a] Data are from references 1, 3, 22, 27–31, and 38–41 or references cited therein.
[b] ND, Not determined.

to a U residue appeared to be a highly unlikely event that probably occurred only once. If this is true, then either *Erysipelothrix rhusiopathiae* and the *C. innocuum* cluster are sister groups (in whose ancestor the U residue has reverted to a G residue at position 888) or the walled relatives are a paraphyletic group, within which all other members except these three have arisen from a common stem with the mycoplasma groups (a topology that is not in very good agreement with the phylogenetic tree shown in Fig. 1). Additional evidence will be needed to determine whether *Erysipelothrix rhusiopathiae* and the *C. innocuum* cluster are indeed sister groups.

General consideration. As has been known for many years that bacterial phenotypes are poor indicators of phylogenetic relationships. At best, phenotypically defined taxa are incomplete (paraphyletic) and, at worst, are polyphyletic (42). In general, some phenotypic characters are useful, after the fact, to confirm phylogenetic groups established on the basis of genotypic characteristics, such as rRNA sequences.

Several unusual common phenotypic properties provide convincing evidence of the close relationship between the mycoplasmas and their walled relatives. One is the use of PP_i rather than ATP as a cofactor for several enzymes (26). Another is resistance to high levels of rifampin (8; J.-L. Pellegrin, J. Maugein, M.-T. Clerc, B. Leng, J. M. Bové, and C. Bebear, Syst. Appl. Microbiol., in press).

Table 7 shows the distribution of certain phenotypic characteristics among the various phylogenetically defined groups of mollicutes. Arginine catabolism had a largely scattered distribution among the groups. The lack of glucose fermentation was confined, for the most part, to two of the subclusters in the hominis group (but could not be used to define these units, as it was also lacking in two of the members of the pneumoniae group). The terminal structure could be used to define both the *M. pneumoniae* cluster in the pneumoniae group and the *M. sualvi* cluster in the hominis group.

The lack of a requirement for sterol (previously considered a phylogenetically significant marker) manifested itself in two of the groups but was intermingled in both groups with a requirement for sterol. Even genome size did not segregate the mollicutes cleanly. It appears that genome size reductions have occurred more than once in the mollicutes. Indeed, it is possible that the wall-less condition could even have arisen more than once in this general area of the eubacterial tree.

Although only scattered data are available, it appears that the pneumoniae and spiroplasma groups can be distinguished from the anaeroplasma group and (presumably) the walled relatives by a fundamental characteristic, i.e., the way in which the UGA codon is used. *Acholeplasma laidlawii* uses the UGA codon in a normal fashion, as a termination signal (C. Citti, C. Saillard, and J. M. Bové, Syst. Appl. Microbiol., in press; J. M. Inamine, K.-C. Ho, S. Loechel, and P. C. Hu, J. Bacteriol., submitted for publication); however, several members of the spiroplasma group and three members of the pneumoniae group are known to use UGA (in addition to UGG) to encode the amino acid tryptophan (3, 24, 49; Inamine et al., submitted). Data are not available for the remaining groups.

Although the mollicutes no longer present a major taxonomic challenge, they do present an interesting evolutionary one (42, 47). Viewed from a phenotypic perspective, mycoplasmas are very different from normal bacteria. Yet, on the molecular level, they appear normal, and their phylogenetic position (as seen here) is indeed unspectacular. In other words, their abnormal phenotypes do not result from the fact that mycoplamas are phylogenetically remote from other eubacteria.

TABLE 8. Variation of conserved positions in mollicute 16S rRNAs[a]

Species	% Positions varied[b]
Mollicutes and walled relatives	
Mycoplasma hominis	4.9
Mycoplasma lipophilum	5.4
Mycoplasma sualvi	4.7
Mycoplasma hyopneumoniae	6.3
Mycoplasma pneumoniae	6.3
Mycoplasma gallisepticum	6.0
Mycoplasma muris	6.0
Ureaplasma urealyticum	6.4
Mycoplasma mycoides	3.5
Spiroplasma apis	2.8
Spiroplasma citri	3.3
Acholeplasma laidlawii	3.4
Acholeplasma modicum	3.4
Anaeroplasma abactoclasticum	3.8
Asteroleplasma anaerobium	4.8
Clostridium innocuum	1.9
Clostridium ramosum	1.8
Lactobacillus catenaforme	2.7
Outgroup species	
Lactobacillus casei	1.0
Streptococcus faecalis	0.7
Bacillus subtilis	0.6
Clostridium pasteurianum	1.5
Heliobacterium chlorum	0.5
Anabaena nidulans	1.1
Escherichia coli	2.3

[a] A consensus sequence containing only those positions of highly conserved composition was constructed from an alignment of about 20 broadly representative eubacterial 16S rRNA sequences (the exact condition being that 89% or more of the sequences showed the same composition at each position included).

[b] The percentage of such positions in which the listed species showed a composition different from that in the consensus sequence.

Evolutionists studying metazoa (the metazoan fossil record) have long associated atypical phenotypes with a rapid evolutionary pace, the so-called tempo-mode relationship (18, 34). The mycoplasmas and certain other bacteria appear to be examples of this, manifested in molecular terms (42, 47). Mycoplasma lineages are definitely longer than sister lineages represented by normal bacterial (Fig. 1), implying that they have evolved more rapidly than have typical eubacteria. Their rRNAs show another evolutionary peculiarity that seems to accompany a rapid evolutionary pace. Sequence positions whose compositions are normally highly invariant tend to be relatively variable in mycoplasma rRNAs (42, 45, 47) (Table 8). These same characteristics have been seen in *Leuconostoc oenos*, in which case it can be convincingly demonstrated that this form of rapid evolution is manifested at the genetic level in the majority of, if not all, genes, not merely the rRNA genes (D. Yang and C. R. Woese, Syst. Appl. Microbiol., in press). Thus, a quickened evolutionary pace, not phylogenetic uniqueness, somehow seems to be responsible for the idiosyncratic phenotype observed in mycoplasmas (42, 47). These organisms are, therefore, one of the more interesting bacterial groups for evolutionary study. As their molecular characterization expands and deepens, they will provide evolutionists with insights into the role that evolutionary rate plays in the quality of evolutionary change.

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