Proposal for Elevation of the Family *Acholeplasmataceae* to Ordinal Rank: *Acholeplasmatales*

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Species of the *Acholeplasmataceae* differ from species of the *Mycoplasmataceae* and *Spiroplasmataceae* in many respects, including lack of a nutritional requirement for sterol, ability of most species to synthesize saturated fatty acids and polyterpenes from acetate, and several other properties related to lipid metabolism and to the incorporation and location of lipids in the cell membrane. *Acholeplasma* species have also been found to differ from *Mycoplasma* species in possessing a nicotinamide adenine dinucleotide-dependent lactate dehydrogenase that is specifically activated by fructose 1,6-diphosphate and in containing superoxide dismutase, as well as glucose-6-phosphate and 6-phosphogluconate dehydrogenases. In addition, reduced nicotinamide adenine dinucleotide oxidase activity is located in the cell membrane of *Acholeplasma* species and is associated with the soluble cytoplasmic fraction of *Mycoplasma* and *Spiroplasma* species. Finally, significant differences exist between the nucleic acids of the *Acholeplasmataceae* and the *Mycoplasmataceae*. The genome molecular weight for *Acholeplasma* species is about 1.0×10^9 , compared with about 5.0×10^8 for species of the *Mycoplasmataceae*. Moreover, a recent comparison of ribosomal ribonucleic acid oligonucleotide catalogs has demonstrated that *Acholeplasma* species are more closely related phylogenetically to two clostridial species than to the *Mycoplasma* and *Spiroplasma* species tested. Because the characteristics of species of the *Acholeplasmataceae* differ in major respects from those of other families of the *Mollicutes*, we propose elevation of the family *Acholeplasmataceae* to the rank of a new order, *Acholeplasmatales*. We provide a description of the proposed taxon, the second order of the class *Mollicutes*.

At present, three families are recognized in the order *Mycoplasmatales* Freundt 1955 (10), which is in the class *Mollicutes* Edward and Freundt 1967 (6); these are the *Mycoplasmataceae* Freundt 1955 (10), the *Acholeplasmataceae* Edward and Freundt 1970 (8), and the *Spiroplasmataceae* Skripal 1983 (48). Distinction among these three families depends primarily on differences in genome size, morphology, and nutritional requirements for sterol.

At its meeting in Freiburg, West Germany, in 1978 (14), the Subcommittee on the Taxonomy of *Mollicutes* discussed the possibility of elevation of the family *Acholeplasmataceae* to the rank of a separate order. The Subcommittee noted many profound differences that appeared to be suitable for separation of the *Mycoplasmataceae* and the *Acholeplasmataceae* at the ordinal level. Although all of the critical distinguishing tests had not been performed for each of the 71 species of *Mycoplasmataceae*, 4 species of *Spiro-*

plasmataceae, or 10 species of *Acholeplasmataceae*, it was felt that most criteria had been adequately tested and that only very recently discovered criteria or species had not been extensively examined. Fewer but equally robust and adequately tested characteristics appeared to be available for separation of the *Acholeplasmataceae* from the third family of the *Mycoplasmatales*, the *Spiroplasmataceae*. After reviewing this evidence, a majority of the Subcommittee members felt that the unique properties which characterize the *Acholeplasmataceae* deserved recognition by elevation of the family to ordinal rank and decided to prepare a proposal to that effect. We summarize in this paper the properties that distinguish the *Acholeplasmataceae* from the *Mycoplasmataceae* and the *Spiroplasmataceae* and propose elevation of the family *Acholeplasmataceae* to ordinal rank.

The proposal by Edward and Freundt (7, 8) to establish a second genus and family (*Acholeplasma* and *Acholeplasmataceae*) in the order *Mycoplasmatales* was based primarily on the lack of a sterol requirement for growth of *Acholeplasma* species, a characteristic that appeared to be universally present in the species of the family *Mycoplasmataceae* (37, 46). More recently, dependence on sterol for growth has been shown to characterize members of the family *Spiroplasmataceae* (47, 53) and at least some members of the genus *Anaeroplasmata* (41, 42), the latter which has not yet been assigned to a family.

The divergence in the sterol requirements of species of the *Mollicutes* is related to differences in the structure and composition of their cell membranes. Neither *Mycoplasma*

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nor *Acholeplasma* species are capable of synthesizing cholesterol. However, whereas cholesterol is an essential membrane component of *Mycoplasma* and other sterol-dependent species, this is not the case with *Acholeplasma* species (35).

Differentiation between sterol-requiring and sterol-nonrequiring members of the *Mollicutes* can be accomplished by direct (5, 37) or indirect methods. Indirect tests are based on differential susceptibility to amphotericin B (43), digitonin (11, 50), polyanethol-sulfonate (11), lysolecithin (22, 51), and polyene antibiotics (12).

Certain other properties distinguish *Acholeplasma* species from *Mycoplasma* and *Ureaplasma* species. Whereas many *Acholeplasma* species are able to synthesize saturated fatty acids and polyterpenes from acetate, *Mycoplasma* species are not (13, 31, 32, 34, 49). Another difference between *Acholeplasma* and *Mycoplasma* is the positional distribution of fatty acids in their membrane phosphatidylglycerol molecules. In two *Acholeplasma* species the saturated fatty acids are preferentially located at position 1 of glycerol, and the unsaturated fatty acids are located at position 2, as is the rule elsewhere in nature (23, 44). However, in six *Mycoplasma* species, the positional distribution of the fatty acids is the reverse (40, 44, 45). Finally, growing cells of some *Mycoplasma* species have been found to incorporate significant quantities of certain phospholipids (phosphatidylcholine and sphingomyelin), as well as free and esterified cholesterol. In contrast, *Acholeplasma* species fail to take up these phospholipids or esterified cholesterol and incorporate relatively small amounts of free cholesterol (36).

Additional physiological differences between *Acholeplasma* and *Mycoplasma* are of particular interest. For example, a nicotinamide adenine dinucleotide-dependent lactate dehydrogenase that is specifically activated by fructose 1,6-diphosphate is present in *Acholeplasma* but not in *Mycoplasma* species. This regulatory mechanism is uncommon and was previously known to occur only in the *Lactobacillaceae*, especially streptococci (26–28). The demonstration of serological relatedness between aldolases from *Acholeplasma* species and lactic acid bacteria (28) adds further weight to the phylogenetic implications of these observations. Moreover, some *Acholeplasma* species have been shown to differ from *Mycoplasma* species in producing a metalloenzyme, superoxide dismutase, that catalyzes the disproportionation of superoxide anions to H_2O_2 and O_2 (16, 18–20). In one study of multiple isoenzyme expression among *Acholeplasma* and *Mycoplasma* species, two enzymes in addition to superoxide dismutase, viz. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were found in *Acholeplasma* species but not in *Mycoplasma* species (29). These enzymatic activities may also prove to be important markers at the ordinal level. In addition to differences in the enzymatic capabilities of the two genera, there are also differences in the cellular site of certain key enzymatic activities. For example, reduced nicotinamide adenine dinucleotide oxidase and reduced nicotinamide adenine dinucleotide:ferricyanide oxidoreductase activities are associated with the cell membrane of *Acholeplasma* species, but with the soluble cytoplasmic fraction of *Mycoplasma* species (30, 31, 33, 54) and at least one *Spiroplasma* species (15, 24, 25).

There are many significant differences in the molecular genetics of the *Acholeplasmataceae* and the *Mycoplasmataceae*. The demonstration of a genome molecular weight of 10^9 for *Acholeplasma* species compared with 4.5×10^8 for *Mycoplasma* and *Ureaplasma* species (2) was considered by

Edward and Freundt (7) to provide important evidence in support of their proposal to assign the sterol-nonrequiring members of the *Mollicutes* to a separate genus and family.

Recent contributions in nucleic acid research, especially those concerning ribosomal ribonucleic acid (rRNA), have led to deeper insight into the phylogenetic relationships of the *Mollicutes* (9, 56). The special significance of these studies arises from the growing recognition that procaryotic rRNAs were highly conserved during evolution. Several observations indicate that this was also true of mycoplasmal rRNAs. For example, Reff et al. (39) examined the electrophoretic migration of rRNAs from bacteria, L-phase variants, acholeplasmas, and other members of the *Mollicutes* in nondenaturing gels and in formamide-containing gels and found that the migration patterns of mycoplasmal and acholeplasmal RNAs in nondenaturing gels were distinct not only from those of the bacteria examined, but also from each other, indicating some degree of phylogenetic divergence between the two groups. The significance of these studies was clarified considerably by studies involving digestion of 16S rRNA by ribonuclease. In particular, the analyses of Woese et al. (56) and Fox et al. (9) of the base sequences of oligonucleotide catalogs from digested 16S rRNAs have initiated a new era in our understanding of the phylogenetic interrelationships in the *Mollicutes*. Of special interest is the finding of these workers that *Mycoplasma gallisepticum*, *Mycoplasma capricolum*, *Spiroplasma citri*, and *Acholeplasma laidlawii* possess base sequences that are otherwise almost exclusively confined to members of the *Bacillus-Lactobacillus-Streptococcus* cluster. A closer analysis, based on a correction of the new raw data, suggested a specific relationship among *M. gallisepticum*, *M. capricolum*, and *S. citri* and a similar clustering of *A. laidlawii* with two species, *Clostridium ramosum* and *Clostridium innocuum*. According to this interpretation, *Acholeplasma* seemed to be phylogenetically more closely related to the two clostridial species than to species of *Mycoplasma* or *Spiroplasma* (9, 56). However, recent evidence based on studies of 5S RNA suggests a significant degree of relationship between *Acholeplasma* and *Mycoplasma* (55). The most recent proposal (21) for mollicute phylogeny suggests that the clusters consisting of the *Acholeplasmataceae* and the *Mycoplasmataceae-Spiroplasmataceae* group, although deeply divided, comprise a monophyletic branch of procaryote evolution. This branch, of course, corresponds to the class *Mollicutes*.

In summary, the genus *Acholeplasma* is distinguished by many nutritional, biochemical, physiological, and genetic characteristics that differ from those of the genus *Mycoplasma* and, to the extent that pertinent data are available, from those of the genus *Ureaplasma*, the second genus of the *Mycoplasmataceae*.

Fewer taxonomically pertinent characters distinguish the *Acholeplasmataceae* from the *Spiroplasmataceae* than from the *Mycoplasmataceae*. Although the *Spiroplasma* genome resembles the *Acholeplasma* genome in size, there are profound phenotypic differences between the taxa, which probably reflect equally profound differences in their molecular genetics. For example, the helical morphology and motility of members of the *Spiroplasmataceae* clearly distinguish this family from the *Acholeplasmataceae*. Members of the *Spiroplasmataceae*, like members of the *Mycoplasmataceae*, but in direct contrast to members of the *Acholeplasmataceae*, require sterols. Finally, as discussed above, evidence provided by Woese et al. (56) suggests a distant phylogenetic relationship between *Acholeplasma* and

the cluster represented by *M. gallisepticum*, *M. capricolum*, and *S. citri*.

Although a common cytoplasmic antigen has been detected recently in five species of the *Acholeplasmataceae* (17), other observations document considerable heterogeneity in this family. For example, deoxyribonucleic acid-deoxyribonucleic acid hybridization tests between the type strains of eight *Acholeplasma* species (1) revealed, in most instances, less than 8% homology, although a cluster of four species showed 10 to 13% homology. The highest level of homology (21%) was found between the type strains of *A. laidlawii* and *Acholeplasma granularum*. Moreover, the wide range of homology values (between 40 and 100%) observed in comparisons of strains of *A. laidlawii* and *Acholeplasma axanthum* falls into the range observed in reactions among genera of the family *Enterobacteriaceae* (52). Deoxyribonucleic acid cleavage patterns after restriction endonuclease digestion (38) also reflect *Acholeplasma* heterogeneity. The heterogeneity in the *Acholeplasma* genome may be associated with the diversity of habitats (52). In contrast to achleoplasmas, most *Mycoplasma* species have narrow host ranges and, so far as is known, corresponding homogeneity in molecular genetic properties (38). It should be noted that our understanding of mollicute habitats is at present changing dramatically with the discovery of many new strains from plants and invertebrates (3, 4, 52a). These observations, which suggest that the concept of the *Acholeplasmataceae* appears to represent a heterogeneous group of organisms in a wide variety of habitats, are in accord with the decision to elevate the family to ordinal rank.

In consequence of these considerations, we formally propose elevation of the family *Acholeplasmataceae* to the rank of a separate order, *Acholeplasmatales*. The proposed new order becomes the second order of the class *Mollicutes*.

Description of *Acholeplasmatales*, order II of class *Mollicutes* Edward and Freundt 1967. *Acholeplasmatales* (A.cho.le.plas.ma.ta'les. M.L. neut. n. *Acholeplasma* type genus of the family *Acholeplasmataceae*; -ales ending to denote an order; M. L. fam. pl. n. *Acholeplasmatales* the *Acholeplasma* order).

Cells spherical and nonmotile, with a minimum diameter of ca. 300 nm; filamentous, usually 2 to 5 μ m long. Reduced nicotinamide adenine dinucleotide oxidase activity located in the cell membrane. Generally more susceptible to osmotic shock at 37°C than members of the *Mycoplasmatales*. Colonies on solid media have a fried egg appearance and may reach 2 to 3 mm in diameter. Facultatively anaerobic; most strains grow readily in simple media. Sterols are not required for growth. Chemoorganotrophic, most species utilizing glucose and other sugars as the major energy sources. Carbohydrate transport occurs through an active carrier-mediated process different from the phosphoenolpyruvate-dependent phosphotransferase system of some *Mycoplasmatales* species. Strains possess lactic dehydrogenases specifically activated by fructose 1,6-diphosphate. Many strains are capable of fatty acid biosynthesis from acetate. Arginine and urea are not hydrolyzed. Pigmented carotenoids, principally neurosporene, occur in some species. All species are resistant, or only slightly susceptible, to 1.5% digitonin. Saprophytes, parasites, or commensals of vertebrates, insects, or plants. None is known to be pathogenic. The guanine-plus-cytosine content of the deoxyribonucleic acid is approximately 27 to 36 mol% (thermal melting and buoyant density methods). The genome molecular weight is approximately 1.0×10^9 . Other characters are as for the class *Mollicutes*. The type family, *Acholeplasmataceae* Ed-

ward and Freundt 1970, is the only family and is monogenic. The type genus is *Acholeplasma* Edward and Freundt 1970.

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