Proposal for an Amended Classification of Anaerobic Mollicutes

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The taxonomic status of the obligately anaerobic mycoplasmas has remained controversial since their discovery in 1973. For this reason, Anaeroplasma, the single recognized genus, has not been assigned to a higher taxon in the class Mollicutes. Nutritional, biochemical, serological, and genomic data indicate that the anaerobic mollicutes compose a heterogeneous assemblage of organisms, all of which are distinct from facultatively anaerobic mollicutes. A revised and extended classification of these organisms is proposed.

We propose that the genus Anaeroplasma be restricted to obligately anaerobic sterol-requiring organisms. In addition to the two species currently accepted in the genus, Anaeroplasma abactoclasticum and Anaeroplasma bactoclasticum, two new species are proposed: Anaeroplasma varium (A-2T = ATCC 43167) and Anaeroplasma intermedium (7LA T = ATCC 43166). It is further proposed that sterol-nonrequiring, obligately anaerobic strains be assigned a new genus designation, Asteroleplasma, because they differ significantly from Anaeroplasma species in their nutritional, antigenic, and genetic characteristics. The type species, Asteroleplasma anaerobium (161T = ATCC 27880), is the only species recognized in the new genus.

Because of the unique metabolic properties of the obligately anaerobic mollicutes, dependence on strict anaerobiosis should outweigh the sterol requirement as the major taxonomic factor. Therefore, we propose to classify the genera Anaeroplasma and Asteroleplasma in a new family, Anaeroplasmataceae. Since this new family is not referable to either of the two established orders of the Mollicutes, we propose the assignment of Anaeroplasmataceae to a new order, Anaeroplasmatales, as order III of the class Mollicutes.

Two orders are recognized in the class Mollicutes Edward and Freundt 1967AL (2) (Fig. 1). Order I, Mycoplasmatales Freundt 1955AL (5), includes two families, Mycoplasmataceae Freundt 1955AL (4) and Spiroplasmataceae Skripal 1983AL (23). Order II, Acholeplasmatales Freundt, Whitcomb, Barile, Razin, and Tully 1984AL (6), contains only one family, Acholeplasmataceae Edward and Freundt 1970AL (4). Distinction between the two orders and the families assigned to each is currently based on differences in nutritional requirements and metabolism (4, 6); primary importance is attributed to the sterol requirement. Differentiation between the two families included in the order Mycoplasmatales is based on differences in morphology and genome size. The family Mycoplasmataceae is subdivided into two genera, the type genus, Mycoplasma Nowak 1929AL (11), and Ureaplasma Shepard, Lunceford, Ford, Purcell, Taylor-Robinson, Razin, and Black 1973AL (22), whereas only one genus, Spiroplasma Saglio, Hlospital, Laffèche, Dupont, Bové, Tully, and Freundt 1973AL (21) is recognized for the Spiroplasmataceae. The family Acholeplasmataceae, order Acholeplasmatales, is likewise monogeneric; Acholeplasma Edward and Freundt 1970AL (4) is the type genus.

Although obligate anaerobes with the characteristics of the class Mollicutes have been known since 1973 (19), the taxonomic status of these organisms has not been resolved. Anaeroplasma Robinson, Allison, and Hartman 1975AL (16) is included in the class Mollicutes as a "genus of uncertain taxonomic position" by Razin and Freundt (13).

The first described strain (strain JR) of the anaerobic mollicutes was erroneously thought not to require sterol for growth and was originally classified as a new species of the genus Acholeplasma, Acholeplasma bactoclasticum (19). After demonstration of a requirement for sterol, Acholeplasma bactoclasticum was transferred to a new genus, Anaeroplasma Robinson, Allison, and Hartman 1975AL (16), the type species of which is Anaeroplasma abactoclasticum Robinson, Allison, and Hartman 1975AL (16).

In assigning these organisms to a separate genus, major emphasis was placed on the obligate requirement of strain JR and related isolates for anaerobic growth conditions, a property that distinguishes them from all other members of the Mollicutes. The dependence on cholesterol for growth, as exhibited by the type strains of both A. bactoclasticum and A. abactoclasticum, would permit classification of the genus Anaeroplasma as a member of the family Mycoplasmataceae, order Mycoplasmatales. However, classification of Anaeroplasma at the family level was complicated by the proposal of Robinson et al. (16) to classify sterol-nonrequiring strains together with sterol-requiring strains in the same genus. This proposal violated a major principle adhered to in the classification of mycoplasmas since 1969 (3); therefore, the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mycoplasmatales unanimously agreed in 1976 (7), 1982 (8), and 1984 (9) that strains exhibiting properties as widely divergent as A. bactoclasticum and the sterol-nonrequiring strains 161 and 162 should not be placed within a single genus. As a consequence of the recommendations of the subcommittee, Robinson (14) suggested that the sterol-nonrequiring strains should be excluded from the genus Anaeroplasma and that their final classification should await additional studies. Such studies were recently reported by Stephens et al. (24) and Christiansen et al. (1) who analyzed the nucleic acid relationships among the anaerobic mycoplasmas and provided the basis for a rational approach to an amended classification of the anaerobic mollicutes.

Proposal for two new species within the genus Anaeroplasma: Anaeroplasma varium and Anaeroplasma intermedium. At present, two species are recognized in the genus Anaeroplasma, the type species, Anaeroplasma abactoclasticum Robinson, Allison, and Hartman 1975AL (16), and Anaero-

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plasma bactoclasticum (Robinson and Hungate 1973) Robinson and Allison 1975\textsuperscript{AL} (15). The type strains of the two species are strains 6-1 (ATCC 27879) and JR (ATCC 27112), respectively.

Originally, species of the genus Anaeroplasma were classified exclusively on selected phenotypic properties. For example, the possession versus nonpossession of proteolytic and bacteriolytic enzymes distinguished A. bactoclasticum from A. abactoclasticum (15, 16). Moreover, the two species differed, to some extent, with respect to carbohydrates fermented and the products of fermentation (14-16, 19). A serological comparison of the type strains of the two species and a number of closely related strains was carried out in 1977 (17). By means of agglutination, modified metabolism inhibition, and gel immunodiffusion precipitation tests, a total of four serovars could be distinguished. A. bactoclasticum was subdivided into serovar 1 (strain JR\textsuperscript{3}) and serovar 2 (strains 5LA, 5LB, 5LC, and 7LA), whereas A. abactoclasticum (strains 6-1\textsuperscript{3} and 171) constituted serovar 3. The sterol-nonrequiring organisms (strains 161, 162, and 163) were placed in serovar 4. The isolation of additional strains of serovars 1 and 4 was reported in 1982 (18). As a first step in genetic characterization of the species and serovars ascribed to the genus Anaeroplasma, their guanine-plus-cytosine (G + C) contents were determined by the buoyant density (\(B_D\)) method (15-17). The values for the type or representative strains of A. bactoclasticum (serovars 1 and 2) and A. abactoclasticum (serovar 3) were fairly close to each other, viz. 33.7, 32.5, and 29.3 mol\%, respectively. However, the G + C content of serovar 4 (strains 161 and 162) of about 40 mol\% was significantly different from that of the other serovars. Stephens et al. (24) recently examined the genomic relationship between 9 of the 10 strains referred to in the preceding discussion, plus three additional serovar 1 strains (71-B1, A-2, and 10A), by \([\text{\textsuperscript{1}H}]\)deoxyribonucleic acid (DNA)-DNA hybridization. Their data clearly indicated that five genomically distinct groups of anaerobic mycoplasmas existed, each warranting species designation. Surprisingly, two serovar 1 strains (strains A-2 and 10A) that had previously, together with strains JR\textsuperscript{3} and 71-B1, been assigned to A. bactoclasticum showed less than 20\% homology with the latter two strains. However, the three serovar 2 strains (5LA, 5LB, and 7LA) were confirmed as a taxonomic entity, in that they hybridized with each other at the level of 75 to 85\% and showed less than 10\% homology with all other strains tested, including the type strains of A. bactoclasticum and A. abactoclasticum.

The above observations indicate that a number of strains hitherto assigned to one species, A. bactoclasticum serovars 1 and 2, should be reclassified as belonging with three different species, as already suggested by Stephens et al. (24). In consequence, we herewith formally propose the recognition of the following two new species within the genus Anaeroplasma (Fig. 1).

**Anaeroplasma varium** sp. nov. Anaeroplasma varium sp. nov. (va'ri.um. L. neut. adj. varium diverse, varied; intended to mean different from A. bactoclasticum). Strains A-2 and 10A, hitherto classified as A. bactoclasticum serovar 1, are transferred to the species. Strain A-2 (ATCC 43165) is designated as the type strain. Nutritional requirements and physiological properties are essentially as described for A. bactoclasticum. Antigenically related to the latter species as shown by double immunodiffusion precipitation tests in agar gel (I. M. Robinson, unpublished data). The G + C content of the DNA of strain A-2\textsuperscript{T} is 33.4 mol\% (\(Tm\)). Nucleic acid (DNA-DNA) homologies between strain A-2\textsuperscript{T} and strains of A. bactoclasticum and A. abactoclasticum are about 1 and 2\%, respectively (24), whereas the intraspecific percentage DNA homology is about 80\%.

**Anaeroplasma intermedium** sp. nov. Anaeroplasma intermedium sp. nov. (in'ter.me'di.um. L. neut. adj. intermedium intermediate). Strains 7LA, 5LA, and 5LB, hitherto regarded as members of A. bactoclasticum serovar 2 (17, 24), are transferred to this species. Strain 7LA (ATCC 43166) is designated as the type strain. Cultural, nutritional, and physiological characteristics are essentially as reported for A. bactoclasticum. Serologically distinct from other species of the genus by agglutination, metabolism inhibition, and agar gel immunodiffusion precipitation tests (17). The G + C content of strain 7LA\textsuperscript{T} is 32.5 mol\% (\(B_D\)). DNA-DNA homologies between strain 7LA\textsuperscript{T} and the type strains of A. bactoclasticum and A. abactoclasticum are less than 5\%, as in contrast with values of about 80\% intraspecific homology (24).

**Proposal for Asteroleplasma anaerobium** gen. nov., sp. nov. Among the anaerobic mycoplasmas, the group represented by strains 161, 162, and 163 differs from members of the genus Anaeroplasma in not requiring sterol for growth (16, 17, 24). The significance of this group and the need for clarification of their taxonomic status within the Mollicutes is further emphasized by the recent finding that sterol-nonrequiring organisms compose a large proportion of the anaerobic mollicute population of the rumen in cattle and sheep (14, 18). Strains 161, 162, and 163 form a homogeneous and distinct serological group, tentatively referred to as serovar 4 (16, 17). The distinctiveness of the group was further confirmed and strengthened by the study of Stephens et al. (24) who obtained less than 5\% DNA-DNA homology between these strains and all other anaerobic mycoplasmas tested, as opposed to about 80\% homology among the three strains themselves. Furthermore, there was no significant hybridization (<5\%) between a \([\text{\textsuperscript{1}H}]\)DNA probe prepared from strain 161 and unlabelled DNA from each of the nine established Acholeplasma species. Moreover, determination of the G + C ratio was considerably higher for strains 161 and 162 (40.2 and 40.3\%, respectively) than for A. bactoclasticum (6-1\textsuperscript{3}), A. bactoclasticum (JR\textsuperscript{3}), A. varium (A-2\textsuperscript{3}), and A. intermedium (7LA\textsuperscript{T}), viz. 40.3\% versus 29.3, 33.7, 33.4, and 32.5\%, respectively (24). The genome size of strain 161 was about 1,000 megadaltons; this
is similar to the genome size of other anaerobic mycoplasmas (1).

Collectively, the nutritional, antigenic, and genomic characteristics of strains 161, 162, and 163 clearly indicate their unique taxonomic status as compared with anaerobic mycoplasmas of the genus *Anaeroplasma* and with sterol-nonrequiring facultatively anaerobic mollicutes of the family *Acholeplasmataceae*. In accordance with the opinion previously expressed by Stephens et al. (24), these organisms should belong to a new genus, for which we propose the name *Asteroleplasma* (A.ste.ro.l.e.plas'ma. Gr. pref. a not; sterol derived from Gr. adj. stereo's solid, plus Lat. n. ole'um oil, ending of cholesterol, ergosterol, etc., and used as a separate word to denote a class of solid monohydroxy alcohols; e combining vowel; Gr. neut. n. plasma something formed or molded, a form; M.L. neut. n. Asteroleplasma name intended to indicate that sterol is not required for growth).

The three related strains examined most thoroughly, strains 161, 162, and 163, are placed in a single species, *Asteroleplasma anaerobium* (an ae.ro.b'i.um. Gr. pref. an not; Gr. n. aer air; Gr. n. bios life; M.L. adj. anaerobius not living in air). Strain 161 (ATCC 27880) is designated as the type strain of the species.

The characteristics of the new genus and species are as described above.

**Elevation of genus *Anaeroplasma* to family rank, *Anaeroplasmataceae* fam. nov., with *Asteroleplasma* as genus II.** Recognition of the proposed new genus, *Asteroleplasma*, for sterol-nonrequiring anaerobic mollicutes, as well as the existing genus *Anaeroplasma*, poses the problem of classification of both genera at the higher taxonomic levels of family and order. In considering the possible solutions to this problem, major emphasis should be placed on the two most prominent characteristics of the organisms: (i) the requirement of sterol for growth exhibited by members of the genus *Anaeroplasma* but not by *Asteroleplasma* and (ii) the dependence on strict anaerobiosis, a property shared by the two genera.

Since the establishment in 1970 by Edward and Freundt (4) of a separate genus, *Acholeplasma*, and family, *Acholeplasmataceae*, to accommodate the sterol-nonrequiring mollicutes, the requirement versus nonrequirement for sterol has remained an important criterion used to distinguish between the higher taxonomic categories of the class *Mollicutes* (Fig. 1). In consequence of this general principle, it would be logical to place *Anaeroplasma* in order I, the *Mycoplasmatales*, and *Asteroleplasma* in order II, the *Acholeplasmatales*, either as new genera of one of the existing families in each order or—in recognition of the importance of strict anaerobiosis as another taxonomic marker—with the status of separate new families of orders I and II, respectively. However, the question of the decisive weight generally given to the property of sterol requirement is actually still open to discussion. The importance currently attached to this property in *Mollicutes* taxonomy may to some extent be ascribed to the fact that sterol requirement is essentially unique among procaryotes and is a property that can be readily determined (13). Yet, as emphasized by Razin (12), both sterol-nonrequiring and -requiring mollicutes are incapable of synthesizing sterol. In the present context, it may be further argued that the dependence on strict anaerobiosis, a fundamental property most likely to be associated with metabolic pathways that are completely different from those of facultatively anaerobic mollicutes, should outweigh the sterol requirement as the most essential taxonomic characteristic. Consequently, the sterol-requiring genus *Anaeroplasma* and the sterol-nonrequiring genus *Asteroleplasma* should be placed in a single family. This same suggestion was made earlier by Stephens et al. (24). In recognition of this concept, we propose (i) the elevation of genus *Anaeroplasma* to family rank, *Anaeroplasmataceae*, and (ii) the inclusion of *Asteroleplasma* as a second genus of the proposed new family.

**Description of *Anaeroplasmataceae*.** (An ae.ro.plas.ma.ta'ce ae. M.L. neut. n. *Anaeroplasma* type genus of the family; aeae ending to denote a family; M.L. fem. pl. n. *Anaeroplasmataceae* the *Anaeroplasma* family). Cells of young (16- to 18-h-old) cultures are coccoid and may be joined by short filaments; they pass membrane filters with average pore-size designations of 200 to 450 nm; older cells have a variety of pleomorphic forms. Gram-negative and nonmotile. Bound by a single membrane, with no evidence of cell wall. Obligately anaerobic; the inhibitory effect of oxygen on growth is not alleviated during repeated subculture. Prereduced medium maintained in a system for exclusion of oxygen is required for culturing. Optimum temperature, 37°C; optimum pH, 6.5 to 7.0. Subsurface colonies are golden, irregular, and often multilobed. Surface colonies present the “fried-egg” appearance characteristic of most mycoplasmas. Chemoorganotrophic. Strains vary in their ability to ferment various carbohydrates; products of carbohydrate fermentation include acids (generally acetic, formic, propionic, lactic, and succinic), ethanol, and gases (primarily CO₂, although some strains also produce H₂₅). Arginine and urea are not hydrolyzed. Some members produce an extracellular enzyme that hydrolyzes the peptidoglycan layer of the bacterial cell wall, a characteristic that has not been reported for other mycoplasmas (15, 19). The polar lipids of some species have been shown to contain plasmalogens (alk-l-enyl-glycerol ethers) which are commonly found in various anaerobic, but not in aerobic, bacteria. The lipid composition of these species is otherwise similar to that of facultatively anaerobic members of the *Mollicutes* (10). All organisms currently classified in the family are found in the rumens of cattle and sheep.

Two genera are accepted in this family: *Anaeroplasma* Robinson, Allison, and Hartman 1975A² (16) (type genus) and *Asteroleplasma*. Members of *Anaeroplasma* are characterized by requiring sterol for growth. The DNA base composition is within the narrow range of approximately 29 to 34 mol% G + C (Bₒ). Members of *Asteroleplasma* are distinguished by not requiring sterol for growth. The G + C ratio of the DNA is approximately 40 mol% (Bₒ). The genome size of both genera is about 1,000 megadaltons (1).

**Proposal for elevation of the family *Asteroleplasmataceae* to ordinal rank: *Asteroleplasmatales*.** The proposal to place the genera *Anaeroplasma* and *Asteroleplasma* together in one family creates a dilemma. The new family, *Asteroleplasmataceae*, could be placed either in the order *Mycoplasmatales* or in the order *Acholeplasmatales*. Preference should, for phylogenetic reasons, be given to classification of the *Anaeroplasmataceae* as a second family under the order *Acholeplasmatales*. Recent evidence derived by sequencing SS rRNA (20) suggests that *Bacteroides* and *A. abactinolasticum* and *A. abactinolasticum* are phylogenetically closer to the *Acholeplasma* branch of the mollicute tree than to the *Spiroplasma* *Mycoplasma* branch. On the other hand, no data are available to indicate the phylogenetic position of the genus *Asteroleplasma*. Thus, several possibilities exist for the evolutionary origin of members of the family *Asteroleplasmataceae*. They may, as suggested by the data of Rogers et al. (20), have
evolved from an acholeplasmal progenitor. In that case, the *Anaeroplasmataceae* could represent an independent evolution of sterol dependence. Other possible progenitors for members of the *Anaeroplasmataceae* are gram-positive, rumen-inhabiting bacteria or (for the genus *Anaeroplasma*) members of the genus *Mycoplasma*. Tracing the evolutionary origin of these organisms is a laborious task that may not soon be attempted. It would be premature, at present, to classify *Anaeroplasmataceae* in the order *Acholeplasmatales* on the basis of incomplete phylogenetic observations, especially because the current taxonomy of the class *Mollicutes* is not otherwise based on phylogeny. Moreover, objections might be raised against classification of the *Anaeroplasmataceae* in the *Acholeplasmatales* because this would negate the rationale for the recent establishment of the order *Acholeplasmatales* to accommodate sterol-nonrequiring mollicutes (6). In conclusion, as long as we are unable to develop a radical revision of the taxonomy of the *Mollicutes*, based entirely on phylogenetic data, it would not be advisable to totally violate a major principle adhered to for a very long time in the classification of these organisms.

As a logical solution of the problem created by placing the genera *Anaeroplasma* and *Asteroleplasma* in one family, *Anaeroplasmataceae*, we propose elevation of the family to ordinal rank, *Anaeroplasmatales*, as order III of the class *Mollicutes*. Thereby, due attention would be given to both strict anaerobiosis and the sterol requirement as major taxonomic criteria, although priority is given to anaerobic biosynthesis and the sterol requirement as major taxonomic criteria, although priority is given to anaerobic biosynthesis. In doing so, the uniqueness of the obligately anaerobic mollicutes as compared with all other members of the class *Mollicutes* would be stressed. Treatment of the obligately anaerobic mollicutes as a single taxonomic unit at the ordinal level is consistent with existing phenotypic data and is not inconsistent with available phylogenetic observations. It is the most appropriate treatment at this time.

**Description of Anaeroplasmataceae**, order III of class *Mollicutes* Edward and Freundt 1967. *Anaeroplasmataceae* (An.ac.ro.plas.ma.ta’les. M.L. neut. n. *Anaeroplasma* type genus of the family *Anaeroplasmataceae*; -ales fam. pl. n. *Anaeroplasmatales* the *Acholeplasma* order). The characteristics of the order are as described for the family.

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**LITERATURE CITED**


