Supplementary Material

Description of the class *Mollicutes*

There are currently four orders, five families and eight genera assigned to the class *Mollicutes*. Three of the genera (*Mycoplasma*, *Entomoplasma* and *Mesoplasma*) are polyphyletic. Almost all mollicutes are commensals or pathogens of eukaryotic hosts. Their major characteristics are their wall-lessness, the simplicity of their genomes compared with those of most walled bacteria and 16S rRNA gene sequences that clearly identify them as species of the *Mollicutes*. All are referable to a phylogenetic group and/or cluster within an established genus (Weisburg *et al.*, 1989; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). Members of the class are believed to have evolved from ancestors shared with walled, low-G+C-content Gram-positive bacteria such as *Clostridium* or *Erysipelothrix* species (Fox *et al.*, 1980; Woese *et al.*, 1980; Weisburg *et al.*, 1989), but mollicutes lack cell wall and/or muramic or diaminopimelic acid synthesis pathways (Plackett, 1959; Schleifer & Kandler, 1972; Martin *et al.*, 1980). The DNA base compositions of mollicutes resemble those of related bacteria, 23–34 mol% G+C in most cases but as high as 40 mol% G+C in *Mycoplasma pneumoniae*. Their genome sizes are 580–2200 kbp, smaller than those of most walled bacteria. Cells of mollicutes are bounded only by a single membrane (Boatman, 1979; Cole, 1983). Despite the lack of a cell wall, cytoskeletal elements present in many species (Williamson, 1974; Williamson *et al.*, 1984; Stevens & Krause, 1992; Krause, 1996, 1998; Krause *et al.*, 1997; Trachtenberg, 1998; Kürner *et al.*, 2005) permit helicity, motility and various types of polarity. Spiroplasmas are helical mollicutes recognizable by dark-field microscopy in the earliest passages (Davis & Worley, 1973; Williamson & Whitcomb, 1974) or even in host tissue or fluids prior to culture (Whitcomb & Williamson, 1975; Williamson & Poulson, 1979; Bové & Garnier, 1997; Fletcher *et al.*, 1998). Spiroplasmas have structural features that may exhibit considerable change during their life in culture (Williamson *et al.*, 1989; Gasparich *et al.*, 2004). In the pneumoniae group of *Mycoplasma*, the *Mycoplasma sualvi* cluster of the hominis group and perhaps other clusters, cells may have terminal structures or may appear in electron micrographs to be flask-shaped (Del Giudice *et al.*, 1985; Lo *et al.*, 1992; Trachtenberg, 1998; Frasca *et al.*, 2005). The cell surfaces of some *Mycoplasma* species have terminal structures (Biberfeld & Biberfeld, 1970; Del Giudice *et al.*, 1985) composed in part of adhesin proteins that mediate mollicute attachment to surfaces of eukaryotic cells (Razin & Jacobs, 1992). Attachment structures have also been demonstrated in *Spiroplasma* (Ammar *et al.*, 2004).

Some mollicutes with complex cytoskeletal features are motile. Spiroplasmas exhibit rotatory, flexional and/or translational motility readily observed under dark-field microscopy (Williamson & Poulson, 1979; Williamson & Whitcomb, 1974; Tully, 1983; Trachtenberg & Gilad, 2001; Shaevitz *et al.*, 2005). Several *Mycoplasma* species are capable of gliding motility (Bredt, 1968; Bredt & Radestock, 1977; Kirchhoff & Rosengarten, 1984; Kirchhoff, 1992; Miyata *et al.*, 2000, 2002; Shimizu & Miyata, 2002; Wolgemuth & Charon, 2005), which can be observed only with specialized equipment. Motility of spiroplasmas is associated with chemotaxis (Daniels *et al.*, 1980; Daniels & Longland, 1984).

The complex cell structure in the pneumoniae group, which may be the most plesiomorphic (primitive) group in *Mycoplasma*, seems to have been lost in most clusters of the hominis group. This simplification correlates with moderately smaller genome sizes. In the hominis group, contractile movements have been observed in a few species (Bredt *et al.*, 1973), although no cytoskeletal complexity has been noted. 'Rho structures' of unknown function or significance have been observed by electron microscopy in species of the mycoides cluster (Peterson *et al.*, 1973; Rodwell *et al.*, 1975), but most species in that cluster, as viewed in cultures, appear as relatively unstructured filaments.

The absence of a cell wall and apparent lack of cytoskeletal elements in many *Mycoplasma* species is expressed in a general pleomorphism. Structurally simple cells observed by phase (Bredt, 1983) or dark-field (Tully, 1983) microscopy vary in shape: some are coccoidal, others filamentous. In a minority of non-helical species, there is a tendency for filaments to produce branched structures, fragmented filamentous forms and what appear to be buds. Binary fission is sometimes observed and, in some species, particularly acholeplasmas, dyads or tetrads may be observed by light microscopy. Cells of some species appear to be encapsulated (Almeida & Rosenbusch, 1991). Mollicute cells can be very small; the diameters of some viable coccoidal cells are as small as 300 nm and viable helical filaments of *Spiroplasma* species can be as narrow as 200 nm in diameter. The small size of mollicute cells and their lack of a cell wall enable them to pass through 450 and, to a lesser extent, 220 nm filters. Cells of *Mycoplasma neurolyticum* from young cultures often pass 100 nm filter pores, but this is an exception. Cell shape appears to depend on the age of the culture and to some extent on the nutritional qualities and/or the osmotic pressure of the medium (Garnier *et al.*, 1981, 1984; Gasparich *et al.*, 2004). The replication of the genome is not necessarily synchronized with cell division. Details of the mollicute growth cycle may reflect to some extent the presence of cytoskeletal elements (Miyata & Seto, 1999). Although neither spores nor walled stages are known, certain morphological adaptations are thought to represent resting stages (Hackett *et al.*, 1996). All described species can be grown on artificial cell-free

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media of diverse complexity, and cultivation is a fundamental requirement for species description.

The intermediary metabolism of mollicutes is influenced by their wall-lessness and their parasitic or commensal life style (Pollack *et al.*, 1997). Their energy pathways are moderately conserved and provide a character that is significant at the cluster level. Members of the *Entomoplasmatales* and *Acholeplasmatales* invariably utilize glucose as an energy source (Pollack, 1992), but *Mycoplasma* species differ in this respect. Some produce acid from glucose, but others do not. Some *Mycoplasma* species use arginine as an alternative energy source, but a small group of apomorphic (highly evolved) species utilize neither glucose nor arginine (Taylor *et al.*, 1994). *Ureaplasma* species generate ATP through a system coupled to urea hydrolysis by a cytosolic urease (Smith *et al.*, 1993).

Mollicutes have coevolved (Maniloff, 2002) and are closely associated with fish (Kirchhoff *et al.*, 1987; Holben *et al.*, 2002), reptiles (Brown, 2002), birds (Al-Ankari & Bradbury, 1996) and terrestrial and aquatic mammals. The lack of any data from amphibians remains a significant omission in the mycoplasmology of vertebrate hosts. Mollicutes are also associated with invertebrates and plants (Gasparich *et al.*, 2004). As a result, their biological properties (e.g. temperature requirements) tend to reflect their host relationships (Hackett & Clark, 1989). Mollicutes may be commensals or frank or opportunistic pathogens (Razin, 1998; Razin & Barile, 1985). Certain species are proven aetiological agents of diseases of vertebrates or invertebrates and suspected agents of plant diseases (Whitcomb & Tully, 1979, 1989). Certain *Acholeplasma* and *Mycoplasma* species are frequent contaminants in cell culture systems (Del Giudice & Tully, 1996).

Many mollicutes have not yet been cultivated. The majority of these are phytoplasmas (Christensen *et al.*, 2005). Haemotrophic members of the genera *Haemobartonella* and *Eperythrozoon*, originally classified as rickettsias, have been shown by 16S rRNA gene sequencing techniques to be referable to *Mollicutes* (Neimark & Kocan, 1997; Johansson *et al.*, 1999; Neimark *et al.*, 2001, 2002a, b; List Editor, IJSEM, 2002). Some species that are commensals or pathogens in vertebrates (other than the haemoplasmas) have not been cultivated (Neimark *et al.*, 1998). Another group not yet cultivated are insect commensals that have been identified on the basis of PCR-amplified sequences. Some of these induce sex-ratio abnormalities and probably benefit their hosts (Hurst *et al.*, 1999; Hurst & Jiggins, 2000; Jiggins *et al.*, 2000; Fukatsu *et al.*, 2001). Experience has shown that a small fraction of mollicutes thought to be non-cultivable may eventually be cultivated in special media or circumstances (Del Giudice *et al.*, 1980; Hackett & Lynn, 1985; Hackett *et al.*, 1986). The vast majority of plant-pathogenic mollicutes, previously termed 'mycoplasma-like organisms' (Doi *et al.*, 1967; McCoy *et al.*, 1989), are now properly called phytoplasmas (Sears & Kirkpatrick, 1994; IRPCM Phytoplasma/Spiroplasma Working Team, 2004). A few *Spiroplasma* species are also plant pathogens (Chen & Liao, 1975; Williamson & Whitcomb, 1975; Saillard *et al.*, 1987; Bové, 1997). Both of these types of plant pathogens are maintained in biological cycles involving the phloem of their host plants and their homopterous insect vectors. Phylogenetic studies based on the 16S rRNA gene and other genes have determined that phytoplasmas branched from the anaeroplasma-acholeplasma lineage and belong to the class *Mollicutes* (Lim & Sears, 1992; Johansson & Pettersson, 2002). Formal classification of phytoplasmas, using the criteria and methods adopted for other mollicutes, has been constrained by the fact that they have not yet been cultivated *in vitro* despite attempts to do so in many laboratories. Their genomes are much smaller than those of acholeplasmas, and they lack many metabolic capabilities (Oshima *et al.*, 2004). Consequently, phytoplasma characterization is incomplete. Murray & Stackebrandt (1995) proposed provisional '*Candidatus*' taxonomic status for non-cultivable organisms that have been partially characterized and for which specific genomic (chiefly 16S rRNA gene sequence) and phenotypic characteristics have been determined, so the phytoplasmas are presently classified within the provisional genus '*Candidatus* Phytoplasma'. In the last few years, substantial effort has been devoted to the analysis of their genomes and as a result two complete genomes have been sequenced and published (Oshima *et al.*, 2004; Bai *et al.*, 2006). More genome sequences are expected to be completed in the near future. The additional data provided by comparative analysis of the genomes of several organisms in this taxon will be of significant value in confirming unequivocally the position of the genus within the class and in further refining their taxonomic interrelationships. Although the phytoplasmas cannot be classified according to the minimal standards established in this document, their formal recognition as species is imminent, implying that either a broader definition of standards or alternative criteria for description of certain novel members of the class will be required to accommodate these as yet-uncultivated organisms.

(i) The order *Mycoplasmatales* and family *Mycoplasmataceae*. The order *Mycoplasmatales* presently contains the single family *Mycoplasmataceae*. This family and its type genus *Mycoplasma* are polyphyletic. The genus *Mycoplasma* is divided into three phylogenetic groups, the mycoides, hominis and pneumoniae groups (Johansson & Pettersson, 2002). The mycoides group contains the type species of the genus, *Mycoplasma mycoides*, of which the subspecies *mycoides* is an important pathogen of ruminant animals. It is one of six *Mycoplasma* species discovered to date whose 16S rRNA gene sequences cluster within those of the family *Entomoplasmataceae*. Because of that anomaly, the proper taxonomic assignment of these species is a matter of current controversy. The pneumoniae group

is technically paraphyletic but for purposes of classification can be considered to be part of a clade that also includes the hominis group. The orders *Mycoplasmatales* and *Entomoplasmatales* form a clade deeply split from the *Acholeplasmatales*, *Anaeroplasmatales* and '*Candidatus* Phytoplasma' (Woese *et al.*, 1980; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). At present, this split is not recognized by a hierarchical name. Although only a few members of the *Mycoplasmatales* have been examined in this respect, all apparently utilize UGA as a tryptophan (W) codon rather than a stop codon (Yamao *et al.*, 1985; Renaudin *et al.*, 1986; Blanchard, 1990; Citti *et al.*, 1992). UGA is the preferred W codon, but UGG is also used. The ratio between the usage of UGA and UGG tends to differ according to the G+C content of the genome (Rocha *et al.*, 1999). Members of the *Mycoplasmataceae* have, on rare occasions, been isolated from plants (Grau *et al.*, 1991) and insects (Kempf *et al.*, 2000). Similarly, members of the *Entomoplasmatales* have been isolated from vertebrate and invertebrate animals (Wang *et al.*, 2004, 2005; Nunan *et al.*, 2005). Several genera of non-helical mollicutes have been isolated from plants or insects. Therefore, although host association provides an important clue to an organism's identity, it is always necessary to examine the critical properties of a candidate organism to confirm a tentative placement (Pitcher & Nicholas, 2005). The clue to an unexpected occurrence will often be provided by 16S rRNA gene sequence analysis (Grau *et al.*, 1991).

(ia) Genus *Mycoplasma*. The following description applies to all three phylogenetic groups of mycoplasmas. *Mycoplasma* species are aerobic or facultatively anaerobic mollicutes isolated primarily or entirely from vertebrates or fluids of vertebrate origin such as serum. They require cholesterol and/or other sterols for growth, a need that is generally provided by serum in the culture medium. Their genome size is about 580–1360 kbp and the G+C content of their chromosome varies from 23 to 40 mol%. The differential utilization of glucose and arginine is an important feature of *Mycoplasma* species. Some species use one or the other of these two substrates, whereas others use both or neither. These patterns are moderately conserved, so the ability to utilize these substrates tends to characterize some mollicute clusters.

(ib) Genus *Ureaplasma*. The ureaplasmas form a clade that is a terminus in the pneumoniae group of *Mycoplasma*. The vast majority of ureaplasmas have been isolated from the urogenital tract of vertebrates. No other true reservoir is known or suspected. All species possess one or more ureases. Demonstration of urease activity is a minimum requirement for description of a novel *Ureaplasma* species. A standardized technique to demonstrate urea hydrolysis in mollicutes has been described (Razin, 1983). *Ureaplasma* species have general features similar to those of *Mycoplasma* species. Their genome sizes of 760–1170 kbp and G+C contents of 27–30 mol% (Robertson *et al.*, 1990; Glass *et al.*, 2000) are similar to others in the pneumoniae group. Although no other mollicutes are known to hydrolyse urea, this test is mandatory for characterization of all non-helical mollicutes, since this property is a discriminatory phenotypic character.

(ii) The order *Entomoplasmatales*. The order *Entomoplasmatales* presently contains two families and three genera. With *Mycoplasmatales*, it is separated from other mollicute taxa by a deep split not presently recognized by a hierarchical name. As currently defined, the family *Entomoplasmataceae* and the genus *Spiroplasma* are paraphyletic, but this is not reflected in their nomenclature. Nomenclatural changes would be needed to eliminate this, but the reasons why the paraphyletic groups in this order have been tolerated are well substantiated (Weisburg *et al.*, 1989; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004).

(iia) The family *Spiroplasmataceae* and genus *Spiroplasma*. The family *Spiroplasmataceae* is monotypic, so its characteristics are essentially those of the genus *Spiroplasma*. The genus is divided into three well-defined groups on the basis of 16S rRNA gene sequence analysis (Gasparich *et al.*, 2004). These are the ixodetis group, the citri-mirum group and the apis group. Spiroplasmas are helical, aerobic or facultatively anaerobic, usually motile mollicutes associated with invertebrates and/or plant surfaces or phloem. They have well-defined cytoskeletal features (Williamson *et al.*, 1984; Trachtenberg, 2004). They have genome sizes of about 780–2200 kbp. The G+C content of their chromosome varies from 25 to 32 mol% (Williamson *et al.*, 1998). In some clades (e.g. the citri-mirum group), the genome may carry a high content of non-coding, integrated viral or plasmid DNA (Renaudin & Bové, 1994; Melcher *et al.*, 1999). Repeat sequences also make up a significant part of the *Spiroplasma* genome (Nur *et al.*, 1986, 1987). A significant amount of the total DNA of *Spiroplasma* species occurs as free plasmids, which may be involved in gene transfer (Renaudin, 2002) and transmissibility by insects (Berho *et al.*, 2006).

(iib) The genera of the family *Entomoplasmataceae*. Members of the *Entomoplasmataceae* are non-helical mollicutes that are associated with invertebrates or plant surfaces. Members of this family are widely accepted to have evolved from spiroplasmal ancestors and, in so doing, to have lost helicity and motility. Species able to grow in the presence of 0.04% polyoxyethylene sorbitan are placed in the genus *Mesoplasma*, while other species unable to do so are placed in *Entomoplasma* (Tully *et al.*, 1993; Rose *et al.*, 1993). The genomes of known *Mesoplasma* species are about 790–1140 kbp and those of *Entomoplasma* are 870–1100 kbp in size. The G+C content of chromosomal DNA of known *Mesoplasma* species is 27–30 mol%, compared to 27–29 mol% for *Entomoplasma*. Furthermore, the 16S rRNA gene sequences of species of the two genera are very similar

(Weisburg *et al.*, 1989; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). Phylogenetic analyses of those sequences have indicated that both genera are polyphyletic (Weisburg *et al.*, 1989; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). *Mesoplasma* and *Entomoplasma* may thus represent a single taxon and may eventually be combined.

(iii) The order *Acholeplasmatales*. The *Acholeplasmataceae* is the single family in the order *Acholeplasmatales*. The family is monotypic, so its properties are essentially those of the genus *Acholeplasma*. Acholeplasmas are often provisionally recognized early in the characterization process, because the vast majority of them grow in serum-free media. Members of this genus are aerobic or facultatively anaerobic, structurally simple mollicutes from vertebrates, invertebrates or plants. Some cause disease in vertebrate animals. The ecology of *Acholeplasma* isolated from soils and sewage is not clear. Known *Acholeplasma* species grow well at 30–37 °C. They have genome sizes of about 1500–2100 kbp and G+C contents of 27–38 mol%. As far as is known, *Acholeplasma* species always use UGG as a W codon.

(iv) The order *Anaeroplasmatales*. Anaeroplasmas are obligately anaerobic bacteria that have been isolated from the bovine rumen (Robinson *et al.*, 1975; Robinson, 1983; Robinson & Freundt, 1987). Only a single family, the *Anaeroplasmataceae*, containing two genera, has been designated. The genus *Anaeroplasma* currently contains four species, each of which requires sterol. The genus *Asteroleplasma*, according to phylogenetic reconstructions, is not part of the monophyletic tree that comprises the remainder of the mollicutes (Weisburg *et al.*, 1989; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004), but this placement is based on incomplete sequence data, and the true phylogenetic position is yet uncertain. The single known species, *Asteroleplasma anaerobium*, does not require sterol (Robinson *et al.*, 1975). The genome sizes in the two genera are similar (Robinson & Freundt, 1987) and are like those of many *Acholeplasma* species. However, the G+C content of chromosomal DNA in *Asteroleplasma* is about 40 mol%, in contrast to 29–34 mol% in *Anaeroplasma*.

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