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Revised minimal standards for description of new species of the class *Mollicutes* (division *Tenericutes*)

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Abstract

Minimal standards for novel species of the class *Mollicutes* (trivial term, mollicutes), last published in 1995, require revision. The International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Mollicutes* proposes herein revised standards that reflect recent advances in molecular systematics and the species concept for prokaryotes. The mandatory requirements are: (i) deposition of the type strain into two recognized culture collections, preferably located in different countries; (ii) deposition of the 16S rRNA gene sequence into a public database, and a phylogenetic analysis of the relationships among the 16S rRNA gene sequences of the novel species and its neighbours; (iii) deposition of antiserum against the type strain into a recognized collection; (iv) demonstration, by using the combination of 16S rRNA gene sequence analyses, serological analyses and supplementary phenotypic data, that the type strain differs significantly from all previously named species; and (v) assignment to an order, a family and a genus in the class, with an appropriate specific epithet. The 16S rRNA gene sequence provides the primary basis for assignment to hierarchical rank, and may also constitute evidence of species novelty, but serological and supplementary phenotypic data must be presented to substantiate this. Serological methods have been documented to be congruent with DNA–DNA hybridization data and with 16S rRNA gene placements. The novel species must be tested serologically to the greatest extent that the investigators deem feasible against all neighbouring species whose 16S rRNA gene sequences show >0.94 similarity. The investigator is responsible for justifying which characters are most meaningful for assignment to the part of the mollicute phylogenetic tree in which a novel species is located, and for providing the means by which novel species can be identified by other investigators. The publication of the description should appear in a journal having wide circulation. If the journal is not the *International Journal of Systematic and Evolutionary Microbiology*, copies of the publication must be submitted to that journal so that the name may be considered for inclusion in a Validation List as required by the *International Code of Bacteriological Nomenclature* (the *Bacteriological Code*). Updated informal descriptions of the class *Mollicutes* and some of its constituent higher taxa are available as supplementary material in IJSEM Online.

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D. R. B. and J. M. B. are respectively Secretary and Chair of the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Mollicutes*.

Updated informal descriptions of the class *Mollicutes* and some of its constituent higher taxa are available as supplementary material with the online version of this paper.

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Introduction

In 1967, the International Committee on Systematic Bacteriology (ICSB) Subcommittee on taxonomy of *Mycoplasmatales* provided general guidelines for naming species of mollicutes (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1967). In 1972, those guidelines were strengthened and published in the form of minimal standards for description of mollicute species (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972). That document was revised in 1979 and 1995 (ICSB Subcommittee on the Taxonomy of *Mollicutes*, 1979, 1995). Since the last revision of the minimal standards document, many new methods have been introduced into mollicute systematics, mandating a further revision. When the last minimal standards were published, 16S rRNA gene sequence analysis had begun to be used as a definitive indicator of the taxonomic placement of mollicutes. Today, this method has become the benchmark technique for assessment of natural relationships among all microbes (Woese, 1987). The most significant change in this new revision of the minimal standards is the promotion of 16S rRNA gene sequencing to mandatory status, and strong reliance on it to determine hierarchical placement of mollicute species. This character, first applied to mollicutes by Woese *et al.* (1980), was the subject of a major analysis by Weisburg *et al.* (1989) in which a distance algorithm was used for phylogenetic reconstructions of mollicute phylogeny. Mollicute trees were later reconstructed using improved neighbour-joining (Maniloff, 1992; Johansson & Pettersson, 2002) and maximum-parsimony (Gundersen *et al.*, 1994; Gasparich *et al.*, 2004) algorithms to interpret an expanding database of 16S rRNA gene sequences. The status of the 16S rRNA gene sequence as a taxonomic tool for mollicutes was elevated to primacy by the extensive work of K.-E. Johansson and his colleagues on *Mycoplasma* and *Acholeplasma* (Johansson & Pettersson, 2002), Gasparich *et al.* (2004) on *Spiroplasma* and Tom Knight (http://www.broad.mit.edu/annotation/microbes/mesoplasma_florum/background.html) on *Spiroplasma* and the *Entomoplasmataceae*.

The species concept in *Mollicutes*

The first step in proposing minimal standards for new species descriptions is to define the word ‘species’. The nature of bacterial species has received considerable attention in recent years (Rosselló-Mora & Amann, 2001). The need for a universally applicable concept mandates a conservative, practical approach. Wayne *et al.* (1987) advocated a phylogenetic classification, defining species as populations of bacteria whose members share more than 70 % genomic similarity as estimated by heterologous DNA–DNA hybridization (DDH) tests with ΔT_m <5 °C. Phenotypic characters were expected to agree largely with phylogenetic classification. The level of ⩾70 % DDH has been obtained with various prokaryotic species over the years (Johnson, 1980, 1994; Love *et al.*, 1985).

The species concept for *Mollicutes* is compatible with, and is based in part on, the general species concept for other bacteria. From the earliest days of mycoplasmology, species differentiation of mollicutes has been accomplished by serological tests. The diversity of surface antigens in mollicutes ensures that many epitopes are available to be represented in specific antisera. The first serological test to be employed, and the one most commonly used, is the growth inhibition (GI) test (Edward & Fitzgerald, 1954; Clyde, 1964, 1983). Two other tests, metabolism inhibition (MI; Taylor-Robinson, 1983; Taylor-Robinson *et al.*, 1966; Williamson & Whitcomb, 1983) and immunofluorescence (IF; Del Giudice *et al.*, 1967; Gardella *et al.*, 1983; Bradbury, 1998), have been frequently employed for *Mycoplasma* and *Acholeplasma* species. The GI and MI tests have been used with *Spiroplasma* species, but have been largely supplanted by the spiroplasma deformation (DF) test (Williamson *et al.*, 1978, 1979). When evaluated by one or more of these four serological methods, all of which involve surface antigens, most mollicute species exhibit little or no cross-reactivity. Thus, failure to

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cross-react in controlled tests with any of the antisera against previously recognized species provides substantial evidence for species novelty. By the time the Subcommittee first proposed guidelines for species designation (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1967), serology had been established as the most important basis for defining mollicute species. The primacy of serology was affirmed in 1972 in the first minimal standards document, and reaffirmed in later revisions (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972; ICSB Subcommittee on the Taxonomy of *Mollicutes*, 1979, 1995).

Beginning in the late 1960s, DDH was introduced into mycoplasmology (Reich *et al.*, 1966; Somerson *et al.*, 1967). Neimark (1970) used this technique to determine inter-species relationships among *Mycoplasma* species, noting that relationships suggested by the technique were in good accord with serological data. DDH experiments were subsequently used to define the relationships among the members of the mycoides group (*sensu* Weisburg *et al.*, 1989; Johansson & Pettersson, 2002), in defence of the designation of subspecies of *Mycoplasma mycoides* and *Mycoplasma capricolum* (Askaa *et al.*, 1978; Bonnet *et al.*, 1993) and to support elevation of '*Mycoplasma agalactiae* subsp. *bovis*' to full species status. The correlation between DDH and serology was also noted with *Spiroplasma*, and the correlation served in part as a rationale for the designation of serogroups (Junca *et al.*, 1980; Whitcomb *et al.*, 1987). Another series of studies indicated that DDH values and serology were congruent in *Acholeplasma* as well (Aulakh *et al.*, 1983; Stephens *et al.*, 1983a, b). Those studies revealed surprisingly extensive genomic heterogeneity in *Acholeplasma laidlawii* and *Acholeplasma axanthum.* Some strain pairs shared as little as 40 % DDH, differences that in other genera would have justified subdivision of an apparently diverse strain complex into component species. However, no phenotypic or ecological basis was available to support such designations, so the only recourse was to allow the DDH data to defer to the serology supporting the *Acholeplasma* species designations. In summary, with serology justified as a surrogate for DDH to define mollicute species in most cases, the cumbersome reassociation technique could be reserved for special cases in which the affinities of closely related or equivocally cross-reactive strains were in question (Abalain-Colloc *et al.*, 1993; Gasparich *et al.*, 1993).

The introduction of 16S rRNA gene sequence analysis into bacterial systematics provided a third important measure of relationships among mollicute species. Although a general relationship exists between DDH values and 16S rRNA gene sequence similarities, the relationship is imprecise (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001; Rosselló-Mora & Amann, 2001). The phylogenetic separation of group I spiroplasmas (*Spiroplasma citri*, *Spiroplasma insolitum*, *Spiroplasma kunkelii*, *Spiroplasma melliferum* and *Spiroplasma phoeniceum*) also presents some difficulties. Pairs of these organisms have 16S rRNA gene similarity coefficients on the order of 0.98–0.99 (Gasparich *et al.*, 2004). In *Mycoplasma*, one species pair (*Mycoplasma gallisepticum* and *Mycoplasma imitans*) has 16S rRNA gene similarity >0.99, yet shows only 40 % DDH (Bradbury *et al.*, 1993). In another case, *Mycoplasma hominis* strains, which are very heterogeneous (some show as little as 50 % intraspecific DDH), have 16S rRNA gene sequences that show similarities >0.99 (Christiansen *et al.*, 1987a, b; Christiansen & Andersen, 1988; Blanchard *et al.*, 1993). Relationships among strains of highly variable species such as those of the group VIII spiroplasma assemblage cannot be determined by 16S rRNA gene sequence analysis or 16S–23S rRNA intergenic transcribed spacer (ITS) sequence analysis (Regassa *et al.*, 2004). General guidelines were proposed by Stackebrandt & Goebel (1994) for determining the circumstances in which DDH tests were warranted. Those authors proposed an upper limit of 0.97 16S rRNA gene similarity as a threshold, which, if exceeded, would indicate a need for DDH tests to determine whether the strain pair should be regarded as members of a single or separate species. It is presumed that the DDH tests would be performed only if the accumulated character set permitted a novel

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species to be named. For mollicutes, the current minimal standards document would provide the necessary guidelines to make this determination.

In 2002, an ad hoc committee (Stackebrandt *et al.*, 2002) revisited the question of the prokaryote species. They affirmed the usefulness of the criteria set forth by Wayne *et al.* (1987), including the value of DDH as the most important discriminating trait at the species level, and pronounced the systematics that had ensued from this approach sound. However, the committee ultimately deferred to a species definition proposed by Rosselló-Mora & Amann (2001). These authors defined a prokaryotic species as '*a category that circumscribes (preferably) a genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions*'. Although the committee endorsed DDH as the acknowledged standard for species delineation, they refrained from specifying a lower limit of 70 % DDH for intraspecies strain similarity. Rosselló-Mora and Amann actually went a bit further, specifically advising: '*Do not use values of 70 % similarity (or 5 °C ΔT_m)* as absolute limits for circumscribing the species. The current concept allows more relaxed DNA–DNA similarity frontiers, and an internal genomic heterogeneity is permitted.' They urged that a sine qua non requirement for a bacterial species be its coherence as a cluster, even if the encompassed diversity was such that some strain pairs in the cluster showed <70 % DDH. This principle was recently confirmed and expanded (Rosselló-Mora, 2006).

DDH experiments are commonly regarded as laborious, and there is a practical limit on the number of comparisons that can be done. Acknowledging this, the ad hoc committee (Stackebrandt *et al.*, 2002) proposed that tests other than those involving DNA similarities were appropriate for descriptions of bacterial species, as long as they were consistent with DDH data. Unfortunately, there is no genomic test in place today which could serve this purpose. However, the experience of mycoplasmologists over the nearly 40 years of research during which DDH and serology have been compared is sufficient to establish the essential congruence of serology and DDH (Reich *et al.*, 1966; Somerson *et al.*, 1967; Neimark, 1970; Askaa *et al.*, 1978; Junca *et al.*, 1980; Bové *et al.*, 1982, 1983; Aulakh *et al.*, 1983; Stephens *et al.*, 1983a, b; Abalain-Colloc *et al.*, 1993; Bonnet *et al.*, 1993; Gasparich *et al.*, 1993). Serology is an established procedure which serves effectively as a proteomic surrogate for genomic DDH. The practice of mollicute taxonomists in using the combination of 16S rRNA gene sequence analyses, DDH, serology and supplementary phenotypic data to propose novel species is thus consistent with the most recently revisited standards of the microbial systematics community (Stackebrandt *et al.*, 2002).

Steps in the characterization of mollicutes

Early steps in the discovery and characterization of a novel species provide important insights into the organism's biology. Although some procedural details may be taxon-specific, most are applicable to all putative species. Features such as absence of reversion to walled cells, ability to pass ultrafilters, colony morphology and sterol requirement, listed as mandatory tests in the previous minimal standards document (ICSB Subcommittee on the Taxonomy of *Mollicutes*, 1995), are almost always explored during the early processing of all strains. These tests, which formerly provided important evidence for assignment to higher taxic levels, have now been largely supplanted by the extensive use of 16S rRNA gene sequence analyses. However, because these procedures provide supplementary evidence for taxonomic placement, the protocols employed should be included in species descriptions (Stackebrandt *et al.*, 2002).

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(i) Culture media

Many mollicutes are discovered by using standard media, but the availability of a new medium may facilitate cultivation of novel mollicutes. It is common for culture medium variations to be employed in the course of the discovery and characterization process. One of the first variations to be used in early cultural studies of non-helical mollicutes will be one that is serum-free. The first indication that a newly isolated mollicute represents an *Acholeplasma* species often comes by finding that the organism is able to grow sustainably in serum-free media. However, apparent growth in serum-free media can be deceiving (Whitcomb, 1977; Whitcomb *et al.*, 1997). Some mollicutes require very low levels of sterol for growth, which may be supplied in the passage volume when the cultures are passaged (Razin & Tully, 1970). Extended passage in sterol-free media is required for a definitive determination. Conversely, slowly growing acholeplasmas may fail to grow in serum-free media.

(ii) Preliminary identification

The first indication that a strain represents a novel species may be provided by serological screening for other mollicutes associated with the habitat of origin. The definition of habitat is the responsibility of the investigator, who is in a position to predict which known mollicute species may occur, given the circumstances of the isolation. In initial comparisons, the strain should be tested to the extent deemed feasible by the investigator with antisera to all mollicutes known to occur in that habitat. If there are no other known mollicutes in the association, it is logical to proceed directly to cloning. Alternatively, preliminary identification can be by PCR and DNA sequencing using primers specific for eubacterial 16S rRNA genes (Johansson *et al.*, 1998) or the 16S–23S ITS region (Maidak *et al.*, 1997). If a simple BLAST search suggests that the 16S rRNA gene sequence of an isolate may be unique, a similarity matrix (Felsenstein, 1993) relating the candidate strain to its closest neighbours should be constructed. Usually, this will involve species with >0.94 16S rRNA gene sequence similarity. This matrix will suggest an assemblage of related species that should be examined for serological cross-reactivities. These important steps guard against lost time and effort that might be spent studying a strain that is merely an unrecognized representative of a well-known species.

(iii) Triple cloning

A pure culture is essential for determinative studies. Original isolates in many instances are not only mixtures of allelic variants, clones of surviving mutants or cells exhibiting one or another form controlled by a genetic switch, but may also be mixtures of as many as three or more distinct species (Whitcomb & Hackett, 1996). For this reason, cloning of mollicute species is essential. Cloning begins with filtration of a broth culture through a filter with pore sizes that retain clumps (Tully, 1983a). With many mollicutes, this is accomplished by the use of 300 or 450 nm filters but, with group VIII spiroplasmas and a few other mollicutes, a 220 nm filter should be used. The filtrate and dilutions of it are then cultured on solid medium and an isolated colony is subsequently picked from a plate on which only a few colonies have developed. This colony is used to found a new cultural line, which is then expanded, filtered, plated and picked twice more. The filtration and cloning steps are performed at least three times to increase the probability that the clone selected to be the type strain is derived from a single cell. After the final cloning, several colonies are picked and one of the cultural lines derived from this process is chosen as a representative of the putative novel species. An alternative procedure, the limiting dilution method (Fazekas de St Groth, 1982), is now used universally in spiroplasma research. Cloning by this method is performed in liquid media (Whitcomb & Hackett, 1987) in which aliquots of diluted culture are dispensed onto microtitre plates. At some limiting dilution, a microtitre plate will have only a few isolated wells that support organismal growth. Organisms from these wells are ‘picked’ and, as in solid medium cloning,

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one of the cultures grown from single wells is chosen as a representative of the putative novel species. All but a few known *Spiroplasma* species have been cloned by this method.

(iv) Selection of a representative strain

No single strain can embody all of the characters present in the entire cluster of strains that the species name represents. During the early course of cultural passages, some characters may be lost. These include pathogenicity (Williamson & Whitcomb, 1975; Bové, 1997) and/or the ability to negotiate biological passage through one or more hosts (Wayadande & Fletcher, 1995; Fletcher *et al.*, 1998). Such losses, which can occur in fewer than 50 passages, may be inevitable. A possible consequence is that a type strain of a pathogenic mollicute may lose its ability to incite the disease that accounted for its discovery. Because some characters of the wild-type of mollicute species tend to be lost easily during passage *in vitro*, careful observation of organismal morphology and behaviour during early cultivation is essential. Although such characters cannot be documented by the type strain, they are features of the cluster represented by the type and should be meticulously documented. For this reason, cultures from early passages should be preserved periodically by both lyophilization and freezing at −70 °C (Yugi *et al.*, 1973; FAO/WHO Working Group, 1974; Raccach *et al.*, 1975; Leach, 1983). It is common to observe a shift in antigenic properties during early passages of an isolate. This change may reflect resolution of a mixed culture or may be due to shifts in the antigenicity of surface epitopes (Wise *et al.*, 1992; Wise, 1993; Rosengarten & Yogev, 1996). The type strain may or may not retain the ability to switch from one of these variants to another (Watson *et al.*, 1990, 1993; Persson *et al.*, 2002). The strain may retain the potential for further chromosomal modification as profound as a change, usually a reduction, in genome size (Ye *et al.*, 1995). In spiroplasma lineages, integrated plasmid and viral DNA (Renaudin & Bové, 1994) and gene duplications (Nur *et al.*, 1987; McIntosh *et al.*, 1992) render the species prone to chromosomal modification during extended cultural passage. Passage can also result in the loss of free-replicating plasmids (Berho *et al.*, 2006). For those reasons, it is especially important to monitor the changes that occur during cultural passage of citri-mirum group spiroplasmas. However, no matter how early in the passage history one attempts to document the genetic content of the isolated species, one cannot document what is not present, i.e. additional genetic components of other lineages that may exist under the rubric connoted by the specific epithet. It is reasonable to expect that a core set of genes represented by the type is likely to be present in all of the lineages of the novel species (Himmelreich *et al.*, 1997; Lan & Reeves, 2001). Definition of the genes that constitute this core can presently be accomplished only ex post facto by genome annotation (Himmelreich *et al.*, 1997; Pollack, 1997; Dandekar *et al.*, 2000). Thus, the chosen type, imperfect though it may be, should represent the investigator's best effort to select a genotype that has a good likelihood of being genetically stable, and represents the bacterial wild-type to the greatest extent possible. The designation of a single type specimen is in concordance with general practice in systematic biology of eukaryotes (Mayr, 1970), in which it is required that a *holotype* be designated (Winston, 1999). The bacterial type strain (in a sense, a holotype) is designated by a superscript 'T'. For unculturable strains, a reference source which can be maintained, propagated or permanently stored should be cited by a superscript 'R'.

(v) Number of strains

During the course of a study, investigators sometimes accrue multiple isolates or strains. It may be possible to isolate multiple strains from a particular host or habitat in a single geographical locality, and genotypes may differ to some extent in other geographical localities. Additional strains can be thought of as *paratypes*, even though, in general systematics, paratypes are more often members of an initial 'series' taken from the same geographical locality. In the original minimal standards document (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972), two strains were required for characterization of a novel mollicute

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species. However, in 1980 the Subcommittee decided that only a single strain should be required for species characterization (Whitcomb, 1984a, b). Although the International Committee on Systematics of Prokaryotes (ICSP) did not act on a proposal by Christensen *et al.* (2001) to require characterization of no fewer than five strains in species descriptions of bacteria, as many strains of the candidate species as the investigators deem feasible should be studied during characterization (Stackebrandt *et al.*, 2002). Additional strains usually need not be studied as exhaustively as the type strain, but they should be documented by at least one robust test. The investigators should describe briefly the status of any additional lyophilized specimens believed to be part of the microbial population represented by the type strain. Many culture collections are unwilling to accept deposits of material other than the type. However, the International Organization for Mycoplasmology (IOM) Culture Collection presently located at Purdue University (West Lafayette, IN, USA) will accept a reasonable number of lyophilized samples of mollicute strains that document different stages of the research process for a given species.

(vi) Colony morphology

Non-motile mollicutes, or species with only gliding motility, tend to form umbonate ('fried egg-type') colonies on solid media. These are generally noted in the process of cloning non-helical mollicutes. The size and morphology of spiroplasma colonies are variable among species, but a common feature is that satellite colonies are often observed. Because there is no 'typical' colony morphology in *Spiroplasma*, colony morphology need not be demonstrated for putative species of that genus. Under some circumstances, spiroplasmas do form umbonate colonies on solid media. These colonies are an important indication that the motility of the strain is impaired or that the strain is completely non-motile. Umbonate colonies of motile species may sometimes be induced by using exceptionally hard agar media, but there is no taxonomic significance to this observation.

(vii) Cellular morphology

It is important to examine the morphology of a candidate species by light microscopy at the outset of characterization and at various stages during its cultural history. The presence of helical cells throughout the isolation and characterization indicates that the organism, which will have survived continuous exposure to penicillin (ruling out spirochaetes or other helical walled bacteria), is a spiroplasma. Some *Spiroplasma* species form helices only in certain phases of growth (Gasparich *et al.*, 2004). The variations in helicity of spiroplasmas are important characters and must be fully described. Species of *Mycoplasma* are usually observed by dark-field or phase-contrast microscopy, but their morphology is so variable that the variability itself is considered a taxonomic character (Freundt, 1955). If a putative novel species is assigned through 16S rRNA gene sequence analysis to a recognized mollicute clade, all of whose members have been demonstrated to be wall-less, it is not mandatory to prove the status of its limiting membrane by thin-section electron microscopy. However, if a putative novel species is a member of a group known to possess cytoskeletal features, it is necessary to define the complexities of its outer limiting structure. Gliding motility (Bredt, 1979) may be inapparent or may be lost in early passages. For this reason, it is not necessary to make exhaustive attempts to demonstrate motility of *Mycoplasma* species. In contrast, the motility of *Spiroplasma* species can be readily discerned by dark-field microscopy.

(viii) Optimum growth temperature

The optimum growth temperature of a mollicute reflects the usual habitat of the organism. Most *Mycoplasma* species grow best at 37 °C, reflecting their association with homeothermic ('warm-blooded') vertebrates (Tully *et al.*, 1993). Growth at low temperatures is not known or suspected to be a feature of the ecology of *Mycoplasma*, '*Candidatus* Mycoplasma' or

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Ureaplasma species isolated from homeothermic vertebrates. The lower part of the permissive temperature range of *Mycoplasma* species isolated to date from poikilothermic ('cold-blooded') fish and reptiles is only 20–25 °C, although the hosts involved were from temperate or subtropical environments. The investigators will be in the best possible position to justify the growth temperature range they test, based on circumstances of primary isolation. Members of the *Entomoplasmatales*, including species of *Spiroplasma*, *Mesoplasma* and *Entomoplasma*, and some members of the *Acholeplasmatales* usually grow best at 30–32 °C. A few spiroplasmas grow well at 37 °C, but most have optima below this temperature. Procedures for assessing optimum temperature and temperature range of mollicutes have been described (Konai *et al.*, 1996). In general, the ability of members of the *Entomoplasmatales* to grow at temperatures from 10 to 41 °C should be assessed. The growth rate in doubling time can be readily measured by statistical analysis of growth patterns when the temperature requirements are determined (Konai *et al.*, 1996).

(ix) Aerobiosis and anaerobiosis

Many mollicutes are facultative anaerobes. Some mollicute species can be isolated much more readily under anaerobiosis. Mollicute species, even though they may be much more readily isolated under anaerobic conditions, can be adapted to grow reasonably well under aerobic conditions. Mollicutes that have a strict requirement for anaerobic environments and that exhibit sensitivity to oxygen are classified in the *Anaeroplasmataceae*, the single family in the order *Anaeroplasmatales* (Robinson, 1983).

(x) Additional tests

Supporting information useful for mollicute classifications at all taxonomic levels may be obtained from tests other than those mandated herein. For example, sensitivities to rifampicin (Gadeau *et al.*, 1986; Pellegrin *et al.*, 1990; Gaurivaud *et al.*, 1996), digitonin (Freundt *et al.*, 1973; Tully, 1983c) or amphotericin B (Rottem, 1972) characterize some taxa. Tests that detect α-D-glucosidase (Williams & Wittler, 1971; Rose & Tully, 1983), benzyl viologen reduction (Pollack *et al.*, 1996a) or adsorption of erythrocytes to colonies on solid medium (Manchee & Taylor-Robinson, 1968; Aluotto *et al.*, 1970; Gardella & Del Giudice, 1983) are of interest in some species. PAGE of cellular proteins (Razin, 1968; Rodwell & Rodwell, 1978; Mouches *et al.*, 1982, 1983; Saillard *et al.*, 1990) may be an effective technique for special interspecies comparisons. Such data should be reported if they are particularly informative in any respect known to the investigators. It is up to the community of investigators to discover and justify which characters are most meaningful for classification of mollicutes. Additional tests may be included in future standards after they have been shown to be useful in the class.

(xi) Confirmation of novelty

Initial serological indications of the identity of a putative novel mollicute should be confirmed by testing the cloned strains with the antisera used in the initial screening. Confirmatory serological tests are a defence against time and other resources being spent on an originally inappar-ent strain of a known species, a mixture of two or more known species or a serovar that emerged during the cloning process. If the initial discovery was through partial characterization of the 16S rRNA gene or 16S–23S ITS region, such as by restriction endonuclease fingerprinting (Stakenborg *et al.*, 2005), amplified fragment length polymorphism (Hong *et al.*, 2005) or partial sequencing, then, at a minimum, the DNA sequence spanning all variable regions (Woese, 1987) of the 16S rRNA gene must be confirmed for the cloned strains.

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(xii) Genomic and genetic analyses

Genome sizes differ tremendously among *Spiroplasma*, *Mycoplasma* and *Acholeplasma*, ranging from about 580 to 2200 kbp (Neimark & Carle, 1995). The value of genome size for taxonomy of mollicutes (Bak *et al.*, 1969) was considered by some to be reduced when it was discovered that the sizes vary continuously (Pyle *et al.*, 1988; Neimark & Lange, 1990; Barlev & Borchsenius, 1991; Carle *et al.*, 1995) rather than bimodally, as once thought. Yet, because genome sizes are discrete, they provide a distinctive character for morphometric analysis. Mollicute genome sizes can be determined more easily and accurately with PFGE (Pyle *et al.*, 1988; Neimark & Lange, 1990) than by renaturation kinetics (Carle & Bové, 1983). When using this technique, a summation of fragment sizes from PFGE of a related species whose genome size is known precisely from whole genome sequencing should be compared to the value derived for the new genome as a measure of accuracy. Earlier minimal standards recommended that the base composition (G+C content, mol%) of chromosomal DNA be determined for each novel species, but there are frequent non-significant (homoplasic) overlaps in the values. The UGA codon usage appears to offer a sharp distinction between higher mollicute taxa (Yamao *et al.*, 1985; Renaudin *et al.*, 1986; Inamine *et al.*, 1990; Citti *et al.*, 1992; Navas-Castillo *et al.*, 1992). Members of the *Mycoplasmatales* and *Entomoplasmatales*, so far as is known, utilize both UGA and UGG as tryptophan (W) codons, while only UGG encodes W in members of the *Acholeplasmatales* and *Anaeroplasmatales* and phytoplasmas. However, data are available for only a few species, so the universality of this distinction remains to be established. DDH data provided a definitive test for the status of partially related strains (Johnson, 1980, 1994; Love *et al.*, 1985; Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). Cumbersome and difficult to standardize though it may be, this technique remains an important benchmark in assessment of species status in prokaryote taxonomy (Grimont, 1988; Murray *et al.*, 2001; Stackebrandt *et al.*, 2002). DDH experiments may be necessary particularly when designating subspecies or elevating subspecies to species status (Askaa & Ernø, 1976; Bradbury *et al.*, 1993; Leach *et al.*, 1993).

Mandatory requirements for description of a novel species of *Mollicutes*

In consideration of the foregoing reviews of the species concept and usual steps in characterization of *Mollicutes*, the following are required for novel species descriptions.

(i) Assignment to genus and phylogenetic placement using 16S rRNA gene sequences

The hierarchical position of a mollicute can be ascertained with considerable accuracy after the organism is cloned by determining the 16S rRNA gene sequence (Woese, 1987; Weisburg *et al.*, 1989; Maidak *et al.*, 1997). Phylogenetic placements of mollicutes based on 16S rRNA gene sequence analysis are now available for almost all mollicute species (Weisburg *et al.*, 1989; Gundersen *et al.*, 1994; Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). Usually, a simple BLAST search will place the putative novel species in a genus and, in some cases, associate the organism provisionally with one of the major groups, but BLAST is not suitable for a robust analysis of the phylogenetic position in intragroup clades (Koski & Golding, 2001). Although analysis of the 16S rRNA gene sequence of the type strain is the minimum requirement, sequencing of this locus from additional strains, preferably isolated from the same habitat as the type strain, is strongly encouraged (Stackebrandt *et al.*, 2002). This can provide confirmation that the candidate strain is indeed associated with that habitat, and it may provide an opportunity for sequence diversity to be demonstrated.

After the sequence of a putative novel species has been determined, a similarity matrix (Felsenstein, 1993) showing the relationship of the candidate strain to its closest neighbours should be constructed. If the putative novel species is included in a matrix of 16S rRNA gene sequences from its nearest neighbour taxa in the established clades (Johansson & Pettersson,

2002; Gasparich *et al.*, 2004), similarity values <0.94 will constitute evidence of species novelty. If similarity values >0.94 are observed, species novelty must be established by other means. It is the responsibility of the investigator to provide the means by which novel species can be identified by other investigators.

The investigators should produce a cladogram or phylogram depicting the relationships of the species to its nearest neighbours. This is accomplished by analysing the 16S rRNA gene similarity matrix with a phylogenetic algorithm. Although cladists prefer maximum-parsimony (Swofford *et al.*, 1996; Swofford, 1998) or maximum-likelihood (Felsenstein, 1993), many analyses of mollicute phylogeny (Johansson & Pettersson, 2002) have used neighbour-joining (Saitou & Nei, 1987), which is widely accepted in microbial systematics (Kämpfer *et al.*, 2003).

Guindon *et al.* (2005) noted that the development of better likelihood-based approaches, including Bayesian, provides arguably the most successful advance in this area in the last decade. Bayesian analyses of phosphoglycerate kinase sequences including ten mollicutes have been reported (Pollack *et al.*, 2005). Although construction of global phylogenetic trees is beyond the scope of novel species descriptions, sequences from as many established species as the investigators deem meaningful should be included in the analysis. These should include all the species in a cluster indicated by preliminary BLAST comparisons. Broad latitude is given to the investigators to choose outgroup species, but inclusion of sample taxa from several mollicute genera would not be onerous.

With certain strain assemblages (e.g. group VIII spiroplasmas), it may be difficult to establish stable intraspecific taxa as new strains are added to the array. In such cases, it may be wise to regard the assemblage as a single variable species (*sensu* Rosselló-Mora & Amann, 2001). Attempts to force a strain assemblage into a rigid template required by any classification scheme, Linnaean or otherwise, may be ill-advised and ill-fated. Trees themselves may be interpreted as classifications in which the relationships among nested sets of strains can be freely represented.

In the future, bacterial taxonomy will no doubt focus on multiple loci of the bacterial chromosome (Zeigler, 2003). Some workers have used the 16S–23S ITS region for this purpose. This region, because it is less highly conserved than the flanking genes, has the potential to resolve intraspecific relationships or relationships between closely related putative species (Harasawa *et al.*, 2000; Chalker & Brownlie, 2004; Regassa *et al.*, 2004). Various protein-encoding genes have been used to make phylogenetic inferences (Toth *et al.*, 1994; Falah & Gupta, 1997; Gupta, 1998; Teichmann & Mitchison, 1999; Bébéar *et al.*, 2000; Wolf *et al.*, 2004). However, because 16S rRNA gene sequence analysis has provided satisfactory resolution across the entire prokaryotic spectrum (Ludwig *et al.*, 1998), it will probably retain its primacy in the taxonomy of prokaryotes (Ludwig & Schleifer, 1994, 1999). At the time of preparation of this document, at least ten mollicute genomes have been sequenced (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Glass *et al.*, 2000; Chambaud *et al.*, 2001; Sasaki *et al.*, 2002; Papazisi *et al.*, 2003; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Oshima *et al.*, 2004; Westberg *et al.*, 2004), and many more are approaching complete annotation. Genome sequencing is well within the capabilities of current technology (Herrmann, 2002), and the genomes of economically significant species in particular seem certain to be sequenced completely, but it is unrealistic at this time to envision that complete genome sequences will ever become available for more than a minor fraction of known mollicute species.

(ii) Serology

Serology is one of the three pillars upon which mollicute taxonomy is constructed. There are several reasons for this. (i) The mollicute species concept has historically been a serospecies

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concept. It has been used successfully since the first GI tests (Edward & Fitzgerald, 1954). (ii) Serology provides a vital link between mollicute taxonomy and general principles of microbial taxonomy based on the congruence of serological results and those obtained by DDH in *Mollicutes*. (iii) Many mollicutes are not now and never will be studied intensively. Identification of these mollicutes for the foreseeable future requires serological reagents. This is especially true in the *Entomoplasmatales*, in which the discovery rate of novel species greatly exceeds the capacity to develop replacement genotypic methods. (iv) Because antisera potentially represent indices of many loci, they can provide important phenotypic traits otherwise scarcely available for mollicute systematics.

Antiserum is required to perform serological comparisons to previously described strains, including reciprocal tests, and it may be used efficiently to identify future isolates referable to the novel species. The extensive IOM reagent collection presently at Purdue University is a critical resource for mollicute research and identification and is a primary source of previously developed serological reagents. Investigators who are not familiar with the methodology involved in antiserum production should seek guidance from a mycoplasmologist experienced in the procedures. In particular, the investigator should be aware that the choice of adjuvant to be used for preparing the immunogen is critical and that the time required for specific antibodies to appear in rabbit serum is variable. In some cases, booster injections will be required before the levels of specific antibody required for GI, MI or IF tests are reached. After each test bleed, the antiserum should be tested against its homologous species in each of the protocols the investigators wish to use.

(iia) Directed serological testing—Historically, after cloning was accomplished, confirmation that a strain represented a novel species has been achieved by testing the candidate with (ideally) all mollicute antisera (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972; ICSB Subcommittee on the Taxonomy of *Mollicutes*, 1979, 1995). As the number of serologically distinct strains increased substantially over the years, means were found to lighten this burden. To determine whether an isolate represented a novel species, the effort involved was reduced by omitting antisera to species excluded by other tests. For example, spiroplasmas readily recognized by their helicity and penicillin insensitivity were not tested with antisera against non-helical mollicutes. Similarly, if a *Mycoplasma* species fermented glucose, it was tested only with antisera against glucose fermenters. Still, serology has become more and more laborious. The availability of 16S rRNA gene sequence data permits directed serological screening. It is usually necessary to test the strain only with antisera to species with >0.94 16S rRNA gene sequence similarity. The value of 0.94 defines an approximate *clade* boundary, and should not be confused with the value of 0.97 suggested by Stackebrandt & Goebel (1994) as a species frontier which, if exceeded, might require DDH experiments to provide an accurate species circumscription. These will often be located in one of the clusters in published phylogenetic trees (Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). There is one important caveat to the concept of directed testing. In rare instances, there may be reciprocal cross-reactions in some serological tests between species that are not closely related as indicated by the 16S rRNA gene, and presumably therefore not by DDH, e.g. *M. gallisepticum* and *Mycoplasma synoviae* (Yogev *et al.*, 1989). In such cases, it is also necessary to test the strain with antisera to all species known from the habitat of origin. Even if only a one-way reaction exists, this information will vitally affect the interpretation of test results.

(iib) Reciprocal tests and partial cross-reactivity—If a positive reaction is obtained, it is necessary to perform the reciprocal reaction, since many one-way cross-reactions occur in mollicute serology, especially in *Ureaplasma* and the apis clade of *Spiroplasma*. If a negative screening reaction is obtained when a putative novel species is tested with an antiserum to an established species, it is unnecessary to do the reciprocal test, inasmuch as only reciprocal

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reactions are thought to indicate true relationship (Williamson *et al.*, 1979, 1998). Not infrequently, strains will be encountered that exhibit partial serological cross-reactivity. Partial cross-reactions usually suggest that the strain being compared shares a limited amount of DNA similarity with the type (<80 % DDH). In such cases, DDH measurements may be required to resolve the status of a candidate strain. Species that are distantly related genetically may show a significant serological cross-reaction. In this case, the misleading serological cross-reaction is one of the important features of a novel species that must be described.

(iic) Species-level tests—Three serological tests have been shown to provide species-level sensitivity for non-helical mollicutes. The GI test is used most frequently (Edward & Fitzgerald, 1954; Clyde, 1964, 1983; Black, 1973; Poveda & Nicholas, 1998). If the candidate organism cannot be grown easily on agar, the MI test may be used (Taylor-Robinson *et al.*, 1966; Taylor-Robinson & Berry, 1969). This procedure has been used successfully in serological analyses of *Ureaplasma* (Robertson & Stemke, 1979), *Spiroplasma* (Williamson *et al.*, 1979; Williamson & Whitcomb, 1983) and *Mycoplasma* strains. With *Spiroplasma*, the DF test (Williamson *et al.*, 1978) is universally used. The MI test (Williamson *et al.*, 1979) has served as a second test for spiroplasma species descriptions. With non-helical mollicutes, the agar plate IF method can be performed as either a direct or indirect fluorescent antibody test (Del Giudice *et al.*, 1967; Black & Krogsgaard-Jensen, 1974; Gardella *et al.*, 1983; Bradbury, 1998). In general, the GI, MI and IF tests have yielded congruent results.

(iid) Intra- and interspecific relationships—Other tests provide more sophisticated analyses of interspecies relationships, particularly those in which there is considerable DNA similarity (e.g. spiroplasma subgroups; the mycoides group of *Mycoplasma; Mycoplasma bovis* and *M. agalactiae; M. gallisepticum* and *M. imitans*). Interspecific relationships between *Mycoplasma alkalescens* and its allies are revealed by immunodiffusion and growth precipitation tests. These species are closely related members of a single cluster in the hominis group of *Mycoplasma* (Pettersson *et al.*, 2000). Tests such as agar-gel double diffusion (Lemcke, 1965) and crossed immuno-electrophoresis, which permit visualization of discrete antigen bands, are more specific and may be better suited for the study of interspecies relationships (Archer & Best, 1980; Archer & Townsend, 1981; Johansson & Wróblewski, 1983). Western immunoblot tests have also proved useful for the study of interspecies relationships (Rikihisa *et al.*, 1997; Brown *et al.*, 2006). Serological tests using monoclonal antibodies may be useful at intraspecific levels (Yoshida *et al.*, 2000).

(iie) Number of tests required—Serological testing in mollicutes has classically involved at least two serological methods. However, experience has indicated that, in the vast majority of cases, the serological tests regularly employed have been mutually supportive. Furthermore, because the 16S rRNA gene sequence of the new strain will have been determined before definitive serology is performed, it is unlikely that a strain that is simply a member of a previously described species will be mistaken for a strain of novel one. Thus, the use of two or more serological tests should be reserved for cases in which the initial results are ambiguous.

(iif) Polyvalent screening techniques—Recommendations concerning serological techniques are based on an extensive background of use and on a unique record of sensitivity and specificity of these techniques for distinguishing mollicute species. However, some species, e.g. *M. hominis* (Lin & Kass, 1974), *Mycoplasma iowae* (Al-Ankari & Bradbury, 1996) and group VIII spiroplasmas (Regassa *et al.*, 2004), exhibit substantial serological heterogeneity. As a result, an occasional isolate may not react in some serological tests with antiserum to the type strain of its species. For such mollicutes, it is necessary to use an expanded set of antisera as screening reagents. Artificially polyvalent typing antisera have been used extensively in spiroplasma systematics and in certain *Mycoplasma* studies (Ernø, 1977).

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Extensive research on multiple chromosomal loci may eventually make it possible to reduce or eliminate the role of serology in identification of important species. For example, *Ureaplasma urealyticum* (*sensu* Shepard *et al.* 1974) was eventually subdivided into two species, each of which can be identified on the basis of multiple chromosomal loci (Robertson *et al.*, 2002). This work and the careful supporting research leading to it (Harasawa *et al.*, 1991; Robertson *et al.*, 1994; Knox *et al.*, 1998; Kong *et al.*, 1999, 2000) is an example of a successful transition from serology to molecular genetics, which now permits refined analyses of ureaplasmas (Kong & Gilbert, 2004).

(iii) Correlation with phenotype

After a tentative hierarchical placement has been obtained as a result of 16S rRNA gene sequence and serological analyses, an attempt should be made to assemble phenotypic data to test the assumptions generated by the phylogenetic placement. Properties expected to be consistent with the 16S rRNA gene placement include cellular and colonial morphology, optimum growth temperature and sterol and oxygen requirements. Many of these properties will have been determined to some extent in the course of preliminary characterization.

(iiia) Cellular ultrastructure—Previous versions of the minimal standards document required that electron microscopy be performed to show that the organism is bounded by a single membrane and that it lacks a cell wall (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972; ICSB Subcommittee on the Taxonomy of *Mollicutes*, 1979, 1995). However, when the 16S rRNA gene sequence of a novel species is determined, the candidate can usually be placed in one of the clusters of published phylogenetic trees of mollicutes (Johansson & Pettersson, 2002; Gasparich *et al.*, 2004). In the majority of such cases, it can be safely inferred that the organism lacks a cell wall, because others in that cluster will have been shown to be solely membrane-bound. This does not imply that electron microscopy has no value, because it may reveal unusual cellular features such as the presence of virions (Cole, 1979; Renaudin & Bové, 1994). Further, although placements permitted by the 16S rRNA gene sequence obviate the requirement for electron microscopy for the sole purpose of demonstrating the absence of a cell wall, it is mandatory to describe the ultrastructural details of species that are members of clusters with cytoskeletal features (e.g. all of the clusters of the pneumoniae group and the *Mycoplasma sualvi* cluster of the hominis group). A flask-shaped morphology is common in these lineages (Trachtenberg, 1998). Other features, such as terminal structures or blebs, must be documented. Cell shape and differentiated structures are best studied by negative staining (Cole, 1983), scanning electron microscopy (Carson & Collier, 1983) or techniques involving cryofixation of the cells (Henderson & Jensen, 2006).

(iiib) Substrate metabolism—The species description should give full information concerning formulation of the media chosen for primary isolations and extended study, including both medium composition and method of formulation. Any problems associated with growth of the putative novel species in artificial media should be reported. It is important to describe selective inhibitors (Tully, 1983b) included in the media. In particular, insensitivity to penicillin provides important evidence of the lack of a cell wall. With most putative novel species, penicillin insensitivity, in conjunction with 16S rRNA gene sequence data, will be sufficient to infer wall-lessness. The ability of the putative novel species to metabolize relevant substrates should be assessed (Poveda, 1998). Experience has revealed difficulties and pitfalls encountered with metabolic tests performed in complex growth media. One of these pitfalls is the tendency for substances other than the substrate to be metabolized, with a concomitant change in pH. It is advisable to perform metabolic tests in the simplest medium possible. In particular, it is helpful to omit yeast extract and/or serum from the medium. One useful strategy is to replace the serum with bovine serum fraction (Whitcomb, 1983). Repeated subculture in the presence of the substrate may be required in order to detect catabolism of certain substances.

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It is also essential that tests for pH change be carefully controlled by observations of uninoculated medium with substrate, inoculated medium without substrate and positive and negative control organisms with known requirements. The temperature and length of incubation used in the test should also be defined.

Mollicutes have diverse metabolic pathways (McElwain *et al.*, 1988; Pollack *et al.*, 1989, 1996b, 1997; Miles, 1992; Pollack, 1992), but the implementation of tests reflecting comparative metabolism of mollicutes has posed many challenges (Pollack *et al.*, 1997). Improvements in test protocols (Miles & Nicholas, 1998) may now make it possible to utilize certain metabolic characterizations more fully in mollicute taxonomy. Catabolism of substrates which the investigator deems to be of special interest or importance to the putative species or to its neighbours may be included in the species description. For example, utilization of fructose should be determined for plant-pathogenic mollicutes (Gaurivaud *et al.*, 2000).

Glucose fermentation: The ability to ferment glucose with the production of acid must be assessed (Aluotto *et al.*, 1970; Edward & Moore, 1975; Razin & Cirillo, 1983). This test is especially critical with *Mycoplasma* species because, in this genus, glucose may or may not serve as a primary energy source (Miles, 1992; Pollack, 1992).

Urea hydrolysis: It is extremely important to determine the ability of non-helical mollicutes from vertebrate urogenital tracts to hydrolyse urea (Razin, 1983). Because *Ureaplasma* species are occasionally isolated from sites other than the urogenital tract, the possibility that a non-helical mollicute of vertebrate origin is a ureaplasma must be seriously considered. The placement of such an organism in *Ureaplasma*, however, should be readily accomplished by 16S rRNA gene sequence analysis. The inability of *Spiroplasma* species to utilize urea has been demonstrated for each of the known species.

Arginine hydrolysis: The ability of the candidate to hydrolyse arginine with the production of ammonia should be determined (Barile, 1983). Results of this test have been variable and at times confusing (Hackett *et al.*, 1996). A modified test performed with arginine concentrations that range from 2 to 10 g l^{-1} is sometimes used, inasmuch as some organisms are inhibited by relatively high concentrations of arginine (Leach, 1976). It should also be emphasized that arginine hydrolysis in some *Spiroplasma* species can be demonstrated only when glucose or another energy source is supplied at the same time (Townsend, 1976). This can be accomplished by adding small amounts of glucose (0.1 %) to arginine-containing broth. If the test is positive, an initial decrease in pH will be followed by an elevation of pH. If test results appear to be negative, it is important to perform repeated subcultures in the presence of arginine. This is especially important if the organism also metabolizes glucose.

(iv) Ecology

The following kinds of ecological information often obtained in the course of characterization, if known to the investigators, should accompany a species description: (i) circumstances of isolation, including the medium, selective inhibitors and details of filtration procedures used; (ii) common and binomial name of the host; (iii) geographical location of the original isolation, including Global Positioning Satellite coordinates; (iv) data on known interactions between the candidate mollicute and the host, including the status of the mollicute as a pathogen, commensal or epiphyte; and (v) in the case of disease, signs induced and evidence for specific aetiology.

(v) Adherence to taxonomic rules and procedures

Finally, it is important that workers who propose new taxonomic descriptions for mollicutes have a familiarity with bacterial nomenclature and its specific historical application to the

Mollicutes. For example, when naming new *Spiroplasma* species, workers must assign the novel species a place in the group system, which encompasses both named and putative species (Whitcomb *et al.*, 1987). Investigators should also be familiar with the Rules and Recommendations of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992; Trüper, 1996, 1999). A Latin reference that can provide guidance in naming scientific taxa should be consulted (MacAdoo, 1993; Trüper, 2007).

In *Mollicutes*, the subspecies rank should be reserved for important strains that differ consistently but are too closely related, by serological properties and/or >70 % DDH, to warrant species rank (Johnson, 1980, 1994; Love *et al.*, 1985). The extent of DDH, although recently demoted as an absolute arbiter of species status (Rosselló-Mora & Amann, 2001), may provide essential justification for promoting a strain group to subspecies status (Leach *et al.*, 1993) or elevation of a subspecies to species rank (Askaa & Ernø, 1976). A subspecies should not be proposed if serological and supporting phenotypic or ecological evidence is limited or if the strain has few cohorts. For strains that do not merit subspecies designations, temporary use of infrasubspecific ranks (strain, serovar, pathovar, biovar, etc.) is much preferred, since the use of such designations provides more flexibility than Latin tertiary combinations (i.e. subspecies names) and encourages the accumulation of additional data relevant to classification.

Two important alternative classifications of mollicutes other than the hierarchical Linnaean system exist. As mentioned above, '*Candidatus* Phytoplasma' organisms are classified in a system maintained by the IRPCM Phytoplasma/Spiroplasma Working Team (2004). In the case of spiroplasmas, the characterization process may be truncated, resulting in the designation of 'group' status (Junca *et al.*, 1980; Williamson *et al.*, 1998).

Many factors other than rRNA gene sequences and/or serology are of potential importance in making taxonomic decisions. Thus, species descriptions and or taxic revisions should always be, to some extent, polyphasic (Colwell, 1970; Rosselló-Mora & Amann, 2001). Recent rediscoveries of polyphasic taxonomy underscore its importance (Vandamme *et al.*, 1996). No prescription can be given for these tests, because they differ among genera and species. The investigator is responsible for justifying which specifics are most meaningful for the part of the mollicute tree in which a novel species is located. This responsibility is shared by the reviewers and editors who endorse novel species descriptions. Good species descriptions should be polyphasic to the extent that they describe meaningful idiosyncrasies of novel species as well as common parameters. The present document contains a list of procedures that will be common to all species descriptions. The investigator may discover properties of certain organisms that should be documented that would be inappropriate for other species. Failure to document case-specific details known to the investigators should not be excused on grounds that they are not part of the minimal standards, and reviewers of papers should take into consideration the adequacy of descriptions with special respect to the clade or cluster in which the novel species is placed. On the other hand, investigators who have otherwise satisfied the mandatory requirements are not expected to search for as-yet unknown idiosyncrasies on grounds that they would be meaningful if discovered.

In the years following the publication of the first minimal standards documents (ICSB Subcommittee on the Taxonomy of *Mycoplasmatales*, 1972), adherence to the spirit of the standards has been excellent. Increasing emphasis is being placed on adherence to the standards (Stackebrandt *et al.*, 2002; Kämpfer *et al.*, 2003), and authors should expect manuscripts to be judged in part on fulfilment of the standards, since, after nearly 50 years of conformance, no other standards exist. Investigators are expected to document the procedures they use, for example through the provision for supplementary material in the *International Journal of Systematic and Evolutionary Microbiology*. It is understood that circumstances vary among mollicute strains and that, in some cases, a particular test or procedure may yield equivocal

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results. Authors are encouraged to explain briefly the circumstances that prevented recommended tests from being performed. Reviewers and editors are expected to defer to authors who offer such explanations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DDH
: DNA–DNA hybridization

DF
: deformation

GI
: growth inhibition

IF
: immunofluorescence

MI
: metabolism inhibition

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