Evolution and devolution of minimal standards for descriptions of species of the class *Mollicutes*: analysis of two *Spiroplasma* descriptions

In 1966, the newly formed International Committee on Systematic Bacteriology (ICSB) Subcommittee on the taxonomy of *Mycoplasmatales* [now the International Committee on Systematics of Prokaryotes (ICSP) Subcommittee on the taxonomy of *Mollicutes*], concerned with the absence of published standards for descriptions of 'mycoplasmas' (now mollicutes) and fearful that 'chaos' could ensue in the absence of such standards, set forth some general guidelines for species descriptions (Edward *et al.*, 1967). In 1972, these guidelines were updated and were presented as a formal proposal for description of species of *Mycoplasmatales* (ICSB Subcommittee, 1972). The guidelines were later amended and updated (ICSB Subcommittee, 1979, 1995). The high desirability of minimal standards has been recognized in recent discussions of the bacterial species concept (Stackebrandt *et al.*, 2002; Kämpfer *et al.*, 2003).

In the first 30 years or so after the first minimal standards had been proposed, the standards served as a widely respected guide for species descriptions, and papers describing more than 160 novel species were published, almost all of which deferred to the proposed standards as reasonable and compelling. Indeed, since previous species descriptions have conformed to the published standards, no other standards exist. For some reason, however, in the past few years, a significant decay in standards has been permitted to enter into the mollicute systematic literature. If this situation is not corrected, it will rapidly lead to the 'chaos' feared by the earlier Subcommittee.

Further details of the deficiencies of the two descriptions discussed and details relating to compliance with the minimal standards are available as supplementary material in IJSEM Online.

The current minimal standards document

Some of the provisions of the current minimal standards document published in 1995 are listed in Supplementary Table S1, available in IJSEM Online. Briefly, detailed biological information must be supplied, especially with respect to the ecological interactions of the organism. The ability of the organism to grow at various temperatures must be assessed, as must its ability to grow under aerobic and anaerobic conditions. The organism must be examined by light microscopy, and details of its morphology and motility (if any) must be documented.

A requirement for detailed serological analyses for species delineations has been accorded special importance in all minimal standards documents for mollicutes, including the updated version submitted to the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) in 2006. Despite recent advances made in molecular taxonomy of mollicutes, serology remains the centrepiece of mollicute species taxonomy. This emphasis is not misplaced. Over the years, serological results have proved to be congruent with results from DNA–DNA reassociation, which are currently recognized as the 'gold standard' in determination of bacterial species (Wayne *et al.*, 1987; Johnson, 1994; Stackebrandt *et al.*, 2002). This congruence has been demonstrated in *Mycoplasma* (Reich *et al.*, 1966a, b; Somerson *et al.*, 1966; Neimark, 1970), *Spiroplasma* (Junca *et al.*, 1980; Bové *et al.*, 1982, 1983) and *Acholeplasma* (Aulakh *et al.*, 1983; Stephens *et al.*, 1983a, b).

Today, an opinion is sometimes conveyed informally among mycoplasmologists to the effect that serology is outdated. This opinion has no basis in fact. Serology is needed to define the mollicute species and to provide a specific identification tool

for mollicute identification. A second common misconception is that 16S rRNA gene sequence technology is adequate for species definition. However, this idea is strongly and explicitly negated in many commonly cited papers (e.g. Fox *et al.*, 1992; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). These papers make it clear that 16S rRNA gene sequences are too highly conserved to be definitive in cases where the 16S rRNA gene sequence similarity is greater than 97%.

Conformity to the minimal standards documents in *Spiroplasma* descriptions, 1973–1999

In this letter, I discuss *spiroplasma* taxonomy. In Supplementary Table S1, I have summarized the degree to which species descriptions of *Spiroplasma* have conformed to the minimal standards. In general, conformity to the standards has been good in the description of the first 34 groups, 11 additional subgroups and 29 named species published before 2000.

Devolution of standards

Unfortunately, adherence to the historical minimal standards for descriptions of mollicute species has substantially (and rather suddenly) eroded. I would like to use a recent paper as an illustration; this paper describes the novel species *Spiroplasma leucomae*, and appeared in the November 2005 issue of IJSEM (Odouri *et al.*, 2005).

This paper has many serious deficiencies. These include:

(1) Lack of description of methodology of sequence determination and incorrect attribution of sequence source. A 16S rRNA gene sequence is reported in the *S. leucomae* paper but (i) its origin is not described, (ii) no method is given by which the sequence was obtained, (iii) there is no description of

the sequence length and/or position number and (iv) the authors claim that the sequence was included in a dataset described by Gasparich *et al.* (2004). However, there is no mention of a sequence of strain SMA^T in the 2004 study. All but one of the previously unreported sequences reported in that study were determined in the Perkin Elmer facility (Foster City, CA, USA), by a team led by Dr D. E. Dodge (Dodge *et al.*, 1998). Thus, the origin of the sequence, and the methods used to determine it, are not only undescribed but are also misrepresented. It is important that the correct source of the sequence in the *S. leucomae* paper be reported and that the methods used to obtain it should be described.

(2) Inadequacy of serological data.

The serological data in this paper are inadequate in all respects. (i) In the abstract, the authors state that there were serological cross-reactions in spiroplasma deformation (DF) tests with *two* group I spiroplasmas, but in the text they state that there were cross-reactions with *four* group I spiroplasmas. These cross-reactions were determined by using a bank of 40 different spiroplasmas that represent established type strains and group and subgroup reference strains (Williamson *et al.*, 1998). This statement is on the face of it incorrect, inasmuch as 45 representative strains are listed in the cited paper. (ii) The authors claim to have produced an antiserum specific for *S. leucomae* and cite a previous report of antiserum preparation (Williamson *et al.*, 1979) as describing their procedures in full. Particularly because the Towson laboratory has not previously prepared antisera, the failure of the authors to describe the procedures is unacceptable. Procedures have changed drastically over the years, and it is almost certain that the procedures used in the *S. leucomae* paper were not identical to those used in the 1979 study. (iii) Although the authors claim to have performed reciprocal cross-reactions with the anti-*S. leucomae* sera that they prepared, no data from these reciprocal reactions are given. The sole serological reaction reported using the anti-*S. leucomae* serum is a homologous deformation end point of 5120. (iv) No homologous titre (or, for

that matter, heterologous titre) is reported for the metabolism inhibition (MI) test. Because non-specific one-way cross-reactions (many of which are substantial) are common in *Spiroplasma* serology (Whitcomb *et al.*, 1997; Williamson *et al.*, 1998), cross-reactions involving a candidate novel species and antisera to other species are utterly meaningless in the absence of reciprocal tests. (v) The specific homologous DF titre of one of the group I antisera employed, *Spiroplasma citri*, is given as 2560. Oddly, a literature citation (Hackett *et al.*, 1996) is given for this number, suggesting that it was not determined directly. (vi) Even more oddly, the reference cited is a species description of an unrelated spiroplasma, *Spiroplasma leptinotarsae*. Homologous titres with the other three group I species employed presumably were neither determined nor gleaned from the literature.

There are other major problems with the serological results presented. (vii) On the basis of the authors' descriptions of their methods and results, the serological methodology in both MI and DF tests is questionable. The authors reported no positive reactions in their MI tests, even in homologous crosses. This is extremely odd on the face of it, in that almost all spiroplasmas in the extensive MI matrices produced in the development of the serological classification (Williamson *et al.*, 1998) showed cross-reactions when tested against at least a few heterologous antigens. Also, cross-reactions with other members of group I and group VIII were the rule. These data, which were described in part by Williamson *et al.* (1998), are summarized in Supplementary Table S2 (available in IJSEM Online).

(viii) A further problem exists with respect to partial serological cross-reactions involving *S. leucomae*. In all previous spiroplasma serological tests, DF and MI end points showed proportionality, with MI titres consistently much higher than DF end points. This impression was developed in studies involving a matrix of more than 5000 cross-reactions (see Supplementary Table S2) determined in the course of defining spiroplasma groups and subgroups (Whitcomb *et al.*, 1982, 1983; Tully *et al.*, 1987; Williamson *et al.*, 1998). Especially

within (but not between) group VIII and group I strains, extensive cross-reactions occur, a circumstance associated with the congruence of serological and general genomic relatedness as measured by DNA–DNA reassociation (Junca *et al.*, 1980; Bové *et al.*, 1982, 1983). However, universally, within these two groups, the results from DF and MI serology were highly correlated and, in all cases (ix), the MI titres were much higher than the DF titres. It is therefore extraordinary to find, in the *S. leucomae* description, that only negative results were obtained in MI tests when DF titres as high as 80 were observed in the same cross. Results such as those described (complete lack of cross-reactions, non-specific and/or specific) would be expected if the MI antigen was incorrectly pre-titrated, so that the concentration of viable cells was high enough to overwhelm all antiserum dilutions and, in so doing, to mask non-specific (or even specific) cross-reactions. One would have hoped that results as unusual as these would have incited some comment, but on this matter the authors are silent.

(x) The presentation of the authors also raises doubts concerning the validity of the spiroplasma deformation end points. The authors state that 'positive results for the DF test were confirmed by the presence of grape-like clusters on the spiroplasmas'. This is an incorrect description of the morphological status of spiroplasma cells at the end point of spiroplasma deformation tests. The interpretation of the end point was discussed in the initial paper describing the DF test (Williamson *et al.*, 1978) and later with extensive details (Whitcomb & Hackett, 1996), including diagrammatic sketches of positive and negative reactions. The antigen:antibody ratio at which grape-like clusters are formed by attacked spiroplasmas involves a high level of antibody. In contrast, at the deformation end point as classically defined (Williamson *et al.*, 1978; Williamson & Whitcomb, 1983), the antibody attack level is much lower than that in which numerous blebs and agglutinated cells are noted. At the end point, 50 % of the cells are apparently unaffected by antibody, and affected cells show only one, two or rarely three blebs, separated by unaffected cell lengths. Thus, the antigen:antibody ratio in the 'grape cluster' phase of deformation tests is at least

several twofold dilutions lower than at the end point. (xi) The authors further state that the 'presence of normal, helical/spiral-shaped spiroplasmas in the reaction mix indicates that the reaction is negative'. This statement is alarming, in that, by definition, half of the spiroplasma cells at DF end points are unaffected (Williamson *et al.*, 1978; Williamson & Whitcomb, 1983). These two descriptive statements in the *S. leucomae* paper, taken together, suggest that the reported end points may have been determined incorrectly by a considerable order of magnitude. If the end points were, in fact, wrongly interpreted, a much higher level of relationship between strain SMA^T and group I spiroplasmas would be indicated.

In summary, the observed low-level cross-reactions in DF serology between strain SMA^T and group I spiroplasmas claimed by the authors are not supported by reciprocal DF tests, are not confirmed by MI tests and represent a test matrix that is poorly defined, in that the composition of the bank of tested strains is incorrectly represented. The highly unusual results are not interpreted or discussed in any way. Further, the described criteria used by the authors for determination of DF end points are incorrect and suggest that a large systemic error may have occurred. Also, the MI results, which lack a homologous control, are without precedent in the apparent lack of any positive cross-reaction, even in crosses that produced moderate DF titres. Thus, the claim that *S. leucomae* has been meaningfully tested serologically against other *Spiroplasma* species lacks evidential support and, because serological evidence is critical in spiroplasma taxonomy, the novelty of strain SMA^T has not been demonstrated.

(3) Lack of congruence between serological and phylogenetic data and among other tested properties. The interpretation of the serological reactions of *S. leucomae* is critical. The authors, despite a general failure to provide discussion and/or interpretation of unusual results, commented on these cross-reactions, which they regarded as 'interesting' because 'initial' observations (no data presented) had 'suggested increased [over what?] insect mortality [no locale or scenario specified]'. However, the source and verity of the sequence of strain

SMA^T is very important when the phylogenetic results are considered. Despite the serological cross-reactions indicating affinities of strain SMA^T with group I, the phylogenetic reconstructions suggest that the strain belongs in the group VIII cluster. However, a group VIII affinity is contraindicated by the observed properties of the organisms. The G + C content reported (24 mol%) is much more in line with group I spiroplasmas than those of group VIII, since group I spiroplasmas have G + C contents of $25-28 \pm 1$ mol%, whereas the G + C contents of group VIII spiroplasmas are in the range $28-31 \pm 1$ mol%. The cells of the vast majority of group VIII strains tend to be short, so much so that they often pass 220 nm filter pores almost quantitatively. There is little or no difficulty in distinguishing the long, wide cells of most group I strains from established strains of group VIII at the first microscopic glance.

The thrust of the above difficulties is to cast serious doubt on the correctness of the phylogeny of *S. leucomae* presented in the published paper. One would have supposed, with a novel species showing an unprecedented relationship, phylogenetic affinity with group VIII but serological affinity with group I, that the authors would have discussed the discrepancy. But this extraordinary circumstance provokes no discussion at all. Surely the authors are aware of the general correlation between 16S rRNA gene sequence similarities, DNA-DNA reassociation and serology (Gasparich *et al.*, 2004). All known spiroplasma species sets exhibiting serological interrelatedness have 16S rRNA gene similarity values of 97–99% and higher. Thus, one would expect strains exhibiting serological cross-reactivity (as strain SMA^T seems to have done, assuming that reciprocal serological results are available) to have high levels of 16S rRNA gene sequence similarity (>97%). On this issue, the authors are unhelpful. Despite the increasing use of the 16S rRNA gene sequence similarity benchmark as an indication of the status of candidate isolates vis-à-vis established species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002), the authors do not present any data on 16S rRNA gene sequence similarity between *S. leucomae* and established group I or group

VIII spiroplasmas. However, group VIII and group I representatives have been shown to have similarities of <97.5% (G. E. Gasparich and R. F. Whitcomb, unpublished data). Thus, there is an apparent uncharacteristic lack of congruence among these parameters with strain SMA^T. When the basis for the phylogenetic tree is examined, at least one difficulty becomes evident. *The essential problem with the published phylogeny is that the 'sequence' deposited consists of only about 200 bases!* This is grossly insufficient. The corresponding author is well aware, as a result of her authorship of a major phylogeny paper (Gasparich *et al.*, 2004), that the deposited sequence is unacceptably short for any phylogenetic work. Thus, the phylogenetic data, like the serological data, are completely unacceptable.

(4) The description of the filter-cloning technique is incomplete or misleading.

(i) The authors state that they utilized 'conventional filter-cloning procedures' (Tully, 1983) to clone strain SMA^T. Conventional cloning of mollicutes involves plating the organism on solid medium (Tully, 1983). However, no mention of solid medium is made in the manuscript, and no description of colonies is provided. (ii) Neither is there any mention of the liquid cloning method (Whitcomb & Hackett, 1987; Fazekas de St Groth, 1982). Because all or almost all spiroplasmas described since the mid-1980s have been cloned by the liquid cloning method (see Supplementary Table S1 in IJSEM Online), it would be extremely odd if *S. leucomae*, which in early passages grew very slowly, would have been cloned on solid medium. As a result, the burden of proof remains with the authors of the *S. leucomae* paper to present data showing that the organisms were in fact cloned.

(5) The identity of the deposited strain has not been affirmed, either in terms of the original isolate or vis-à-vis the field infections. In general, the identity and passage history of strain SMA^T is incompletely or erroneously described. The authors report that, after a 1 month adaptation period (starting point not specified), the strain grew rapidly, requiring daily passes. This circumstance in itself should arouse suspicion. No previously studied slow-growing

spiroplasmas have shown the adaptiveness reported for *S. leucomae*. In Supplementary Table S3 in IJSEM Online, I list the various slow-growing spiroplasmas that we have encountered in our studies and the doubling times they achieved after long adaptation in media. An alternative explanation for the results with the organism described as *S. leucomae* exists. A sudden apparent 'adaptation for rapid growth' may indicate that a bench contaminant has overwhelmed a slower-growing organism. No statement is made concerning the position in the cultural passage history when the presumed adaptation occurred. My recollection is that this strain was sent to the USDA laboratory at Beltsville, MD, USA. After an unknown number of passages, it was then studied in the Towson laboratory. It seems likely that some of the early work on the strain, including adaptation, which involved the use of complex media, was done in the Beltsville laboratory. Oddly, there is no mention of the Beltsville history in the *S. leucomae* paper. However, during the period in which the CPBS media such as DCCM were employed in the Beltsville laboratory, *Spiroplasma insolitum* appeared as a contaminant in *Spiroplasma leptinotarsae* cultures (R. F. Whitcomb, unpublished data). The key observation that enabled us to detect the contamination was a sudden rapid growth, requiring daily passages of the strain, a circumstance very similar to the reported rapid growth of strain SMA^T.

Given the above circumstances, there is substantial doubt about the identity of strain SMA^T. The authors report no confirmatory serology or any other evidence to affirm that the strain eventually chosen for study was actually derived from the originally isolated strain. Such confirmation could be easily obtained by DF serology on early-passage isolates of the spiroplasma, if any still exist.

The confusion concerning strain identity extends to the original field circumstances. Data are provided for only a single spiroplasma strain, SMA^T. Claims in the paper (paragraph 1) that *S. leucomae* had a natural infection rate of 43 % in Poland are unsubstantiated. Mixed infections of insect hosts with spiroplasmas are common

(Hackett & Clark, 1989; Whitcomb & Hackett, 1996). Tabanid flies in Georgia (USA) have been found to carry at least 13 species of *Spiroplasma*. Even single insect species can carry two or more spiroplasma species (F. E. French and R. F. Whitcomb, unpublished observations). Thus, in the absence of confirmatory evidence of any sort, the identity of the deposited strain SMA^T vis-à-vis infection of white satin moths in Poland is in question, as is the identity of other spiroplasmas reported to occur in white satin moths in nature.

(6) Temperature requirements. The authors of the *S. leucomae* paper cite a paper of Konai *et al.* (1996), claiming that the requirement for determination of temperature requirements has been fulfilled. However, the strain seems to have been grown at a single temperature, and none of the approaches used by Konai *et al.* (1996) were implemented. Thus, the (essentially non-existent) temperature data in the *S. leucomae* paper are misrepresented. Details of this and other serious errors or omissions are documented in Supplement A available in IJSEM Online.

The above comments are directed to major systemic flaws in the research presented that raise serious questions concerning not only fulfilment of the minimal standards, but also the validity of strain SMA^T as a representative of a unique species. However, there are numerous other errors in the paper, some of which are gross. Especially in the context of the concerns expressed above, these cannot be ignored. Five examples are: (i) no group designation is provided; (ii) it is claimed (with no supporting data) that comparisons of growth patterns of other spiroplasmas had been performed; (iii) it is claimed, incorrectly, that *S. leucomae* is the first spiroplasma to be isolated from a lepidopteran; (iv) some literature citations are grossly incorrect; (v) ecological information provided is vague and misleading. These errors are analysed in detail in Supplement A in IJSEM Online.

'Fulfilment of the minimal standards' in the description of *S. leucomae*

Given the above circumstances, the claim that the minimal standards have been

fulfilled for *S. leucomae* is unwarranted, and the paper should be retracted. Failing retraction, the problems revealed in the above analysis must be clarified.

Clarification can be achieved only by presentation of the missing data, which could be published as an electronic erratum in IJSEM Online. A list of suggested points that should be addressed is presented in Supplement B in IJSEM Online. By revealing what they actually did (or did not do), the authors would satisfy a general need in species descriptions identified by Stackebrandt *et al.* (2002). In the words of these authors, providing full details of procedures used in taxonomic papers 'would be neither onerous nor expensive', given the opportunity to publish supplementary material in IJSEM Online. If it is not possible to resolve these problems, a Request for an Opinion may be placed with the Judicial Commission of the ICSP contesting the validity of the name *S. leucomae*.

Another recent paper in spiroplasma systematics

The paper in which *S. leucomae* was described is so inadequate that a search for the roots of its errors, omissions and misrepresentations is in order. One such root can be found in the paper describing *Spiroplasma atrichopogonis* (Koeber *et al.*, 2005). In this paper, many of the difficulties outlined above also apply. These difficulties are summarized in Supplement C in IJSEM Online. Thus, for reasons similar to those given for *S. leucomae*, the authenticity of *S. atrichopogonis* as a novel species, like *S. leucomae*, has not been established.

The current condition of spiroplasma taxonomy, as a result of the two papers critiqued herein, is not good. It is reasonable to ask how this unsatisfactory position came about. The authors of the above papers have published some good papers. What is it about microbial systematics that encourages papers as unacceptable as those discussed herein? Is it the existence of a formulaic but limited list of tasks, many of which can seemingly be ignored without stirring the ire of editors or reviewers? Once allowed essentially to ignore the minimal standards document, some of the authors of the *S. atrichopogonis* paper have now come back with an even more inadequate paper, replete with

omitted tests, major errors and misrepresentations. The fact that these two papers somehow found their way into print acts as an invitation to future authors of mollicute descriptions to ignore the minimal standards altogether. I have heard the idea expressed that adherence to prescribed minimal standards need not be demanded of authors. These papers provide evidence that relaxation of prescribed standards for species descriptions is a process that is apt to be limitless at the bottom end and that serious breaches of the established standards can rapidly lead to abandonment of all standards.

A characterization well done

Given the problems outlined above with some recent papers, it is important to stress that fine species descriptions are still being produced by some authors in mollicute taxonomy. A particularly good example is the recent description of *Mycoplasma amphoriforme* (Pitcher *et al.*, 2005). These authors clearly stated their intent to fulfil the currently applicable minimal standards (see Table 1 of Pitcher *et al.*, 2005) in the absence of the forthcoming revision. They not only adhered to these standards but also used many supplementary procedures to strengthen their description. Non-required tests that were reported in this paper included haemadsorption, polyacrylamide gel analysis of cell protein, sequence analysis of the 16S–23S rRNA spacer region and specific PCR analyses (see Supplementary Table S4 in IJSEM Online). Their paper establishes a standard well worth emulating.

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