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Taxonomic Note

Agrobacterium is a definable genus of the family *Rhizobiaceae*

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Members of the genus *Agrobacterium* constitute a diverse group of organisms, all of which, when harbouring the appropriate plasmids, are capable of causing neoplastic growths on susceptible host plants. The agrobacteria, which are members of the family *Rhizobiaceae*, can be differentiated into at least three biovars, corresponding to species divisions based on differential biochemical and physiological tests. Recently, Young *et al.* [*Int J Syst Evol Microbiol* **51** (2003), 89–103] proposed to incorporate all members of the genus *Agrobacterium* into the genus *Rhizobium*. We present evidence from classical and molecular comparisons that supports the conclusion that the biovar 1 and biovar 3 agrobacteria are sufficiently different from members of the genus *Rhizobium* to warrant retention of the genus *Agrobacterium*. The biovar 2 agrobacteria cluster more closely to the genus *Rhizobium*, but some studies suggest that these isolates differ from species of *Rhizobium* with respect to their capacity to interact with plants. We conclude that there is little scientific support for the proposal to group the agrobacteria into the genus *Rhizobium* and consequently recommend retention of the genus *Agrobacterium*.

In their recent paper, Young *et al.* (2001) addressed the difficult and controversial question of the taxonomy of two genera, *Agrobacterium* and *Allorhizobium*, within the family *Rhizobiaceae*. Over the past three decades this has become a recurrent issue and arises in part from the differentiation of the genus *Agrobacterium* from the genus *Rhizobium* as the group of nitrogen non-fixing species of rhizobia that produce 'other types of hypertrophies' (Jordan, 1984). It is clear from a current understanding of a large body of descriptive work that species within the genus *Agrobacterium* do not form a monophyletic group. This conclusion is not restricted to *Agrobacterium*; it also applies to other genera in the family *Rhizobiaceae*, and led recently to the division of the genus *Rhizobium* into several genera including *Rhizobium*, *Mesorhizobium* and *Sinorhizobium*. Proposals on how to resolve the issue of *Agrobacterium* taxonomy have appeared from time to time, but they have had little impact on how the members of this genus are described and named in the scientific literature. In their paper, Young *et al.* (2001) explore the history of this taxonomic issue and concluded by proposing that all members of the genera *Agrobacterium* and *Allorhizobium* be included in the genus *Rhizobium*. While we appreciate the efforts of Young *et al.* (2001), in this letter we explain why we cannot lend our support to their proposal.

There is no doubt that the genus *Agrobacterium* is polyphyletic. There also is no doubt that the agrobacteria and the rhizobia constitute a paradoxically diverse group of related members of the *α-Proteobacteria*. Based on biochemical and phenotypic analyses, Keane *et al.* (1970) suggested that the genus *Agrobacterium* be subdivided into two biovars. Subsequently, a third group, biovar 3, was described and includes isolates from grapevine (Kerr & Panagopoulos, 1977). It is remarkable how accurate and useful this set of divisions is, and for the purposes of this discussion we will use these biovar designations for the three major groupings of the genus *Agrobacterium*, precluding the need for species names. More recently, 16S rRNA sequence analysis supports, in our opinion, this subdivision. The biovar 1 isolates all group together, and cluster with *Allorhizobium undicola* and several atypical *Rhizobium* species, including *Rhizobium galegae* and *Rhizobium huautlense*. Significantly, this group correlates well with the *Agrobacterium tumefaciens* group of Holmes & Roberts (1981), defined by numerical taxonomy, and also with the divisions proposed by Tighe *et al.* (2000), based on analysis

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Abbreviation: RIME, rhizobium-specific intergenic mosaic element.

of fatty acid profiles. 16S rRNA analyses place isolates of *Agrobacterium rubi* into this group. Based on phenotypic analyses, *Agrobacterium rubi* is atypical but again, is most closely related to biovar 1 isolates (Tighe *et al.*, 2000). The biovar 2 agrobacterial isolates form a second group and, on the basis of 16S rRNA sequence analysis, cluster with several members of the genus *Rhizobium*, including *Rhizobium etli*, *Rhizobium leguminosarum* and *Rhizobium tropici* (Young *et al.*, 2001). The biovar 2 group corresponds to the *Agrobacterium rhizogenes* group of Holmes & Roberts (1981). The position of the biovar 3 isolates remains uncertain. Based on biochemical and metabolic characteristics, Ophel & Kerr (1990) reclassified this group as a new species, *Agrobacterium vitis*. Most published studies that use 16S rRNA sequences place *Agrobacterium vitis* in or at the periphery of the cluster containing the biovar 1 agrobacteria or in a cluster between the biovar 1 and biovar 2 agrobacteria. On the other hand, 23S rRNA sequence analysis places *Agrobacterium vitis* in its own branch, along with the type strain of *Rhizobium galegae* (Pulawska *et al.*, 2000). In summary, virtually every inclusive study based on 16S rRNA sequence supports the division of the family *Rhizobiaceae* into at least four clades, one containing the biovar 1 agrobacteria and *Agrobacterium rubi*, one containing the biovar 2 agrobacteria, *Rhizobium leguminosarum* and *Rhizobium tropici*, one containing *Sinorhizobium meliloti*, and one containing *Mesorhizobium loti* (for examples, see Sawada *et al.*, 1993; Willems & Collins, 1993; Yanagi & Yamasato, 1993; de Lajudie *et al.*, 1994; Wang *et al.*, 1998).

From this summary, it is clear that isolates of *Agrobacterium* spp. and *Rhizobium* spp. are related but comprise a large group of diverse bacteria. Since there is such diversity among these groups, there is, in our opinion, insufficient reason to place all of these different species into a single genus, *Rhizobium*. In this regard we disagree with the statement by Young *et al.* (2001) that no discriminating characters differentiate species within the genera *Agrobacterium* and *Rhizobium*. The biovar 1 agrobacteria exhibit phenotypic traits that clearly differentiate them from members of the genus *Rhizobium*, as well as from the other agrobacteria. Allen & Allen (1950) published a table listing as many as 18 traits culled from the literature of the time by which the fast-growing rhizobia and members of the genus *Agrobacterium* could be differentiated. This conclusion is further supported by more recent studies (Holmes & Roberts, 1981; de Lajudie *et al.*, 1994), and is nowhere made clearer than in the auxanographic dendograms of de Lajudie *et al.* (1994). Their phenotypic cluster analysis supports the inclusion of the biovar 1 and 2 agrobacteria, as well as *Sinorhizobium meliloti*, into a group that is separate from all of the rhizobia examined, clearly a conclusion that is inconsistent with the proposals of Young *et al.* (2001). Even the data presented by Young *et al.* (2001) in their Table 1 support the existence of characters that define and differentiate the agrobacteria from the genus *Rhizobium*. Moreover, even when traits appear to be identical, caution is warranted because the physiology and biochemistry, and therefore the genetic structure underlying these characters, may be different. For example, virtually all members of the family *Rhizobiaceae* catabolize lactose, making this trait seemingly non-discriminating. However, the biovar 1 agrobacteria catabolize this sugar and certain other disaccharides such as sucrose by a pathway quite different from that used by other members of the family (Bernaerts & DeLey, 1963). If we were to apply the criteria used by Young *et al.* (2001) to the genera *Rhizobium* and *Sinorhizobium*, then the phenotypic data of de Lajudie *et al.* (1994) provides no support for separating these two genera. In fact, we note with some puzzlement that while Young *et al.* (2001) dismiss the absence of such phenotypic support for the division of *Rhizobium* and *Sinorhizobium*, claiming that 'pending' information supports the separation, they place defining weight on a minimalistic and itself incomplete set of phenotypic traits as shown in their Table 1 to combine the agrobacteria with the rhizobia. Nevertheless, even the comparisons shown in their Table 1 clearly define the biovar 1 agrobacteria in comparison with the rhizobia.

Young *et al.* (2001) use the lack of congruence between results from several types of analyses (DNA hybridization patterns, biochemical traits, fatty acid profiles) as evidence that the genus *Agrobacterium* has no legitimacy. Beyond the fact that these analyses do indeed provide defining features, this argument is itself specious. The problem of congruence, or lack thereof, between datasets is not specific to the *Rhizobium*/*Agrobacterium* cluster. Rather, lack of congruence can be inherent to the data type and to the algorithms used to analyse the data. Inconsistencies among datasets also may reflect a lack of informative characters among results of different approaches (Moreira & Philippe, 2000). Incongruities also can reflect the degree to which two organisms have acquired horizontally transferred DNA from different sources (Brochier *et al.*, 2000) and therefore be poor measures of speciation. Given these difficulties, more weight should be accorded to similarities than to dissimilarities when grouping organisms into phylogenetic relationships. By this criterion of similarities, there exist sets of like traits among members of the genus *Rhizobium* and other sets of like traits among members of the genus *Agrobacterium*, and the two groups do not share these sets in common.

Young *et al.* (2001) claim that the high relatedness of 16S rRNA sequences, less than 7 % mismatch, warrants regrouping the agrobacteria and the rhizobia into the single genus *Rhizobium*. However, the authors note that such a comparison cannot be used as the sole criterion, otherwise members of the genera *Brucella* and *Bartonella* must be transferred into the genus *Rhizobium* since the three share 16S rRNA sequences that differ by less than 7 %. A consistent application of this criterion would also void the separation of the genus *Sinorhizobium* from the other rhizobia, since the 16S rRNA sequences of these organisms are more than 97 % identical. The reasoning used by Young *et al.* (2001) also conflicts with the recognition of *Salmonella*

and *Escherichia* as separate genera even though the 16S rRNA sequences among species of these genera share more than 95 % identity. These examples illustrate the point that 16S rRNA sequence homologies cannot be used as the predominant criterion for the separation or consolidation of different groups at the genus level. Although Young *et al.* (2001) make this point, they chose to ignore it in the case of *Agrobacterium* and *Rhizobium* by placing considerable and undue weight on comparative 16S rRNA sequence analysis, which in our opinion is the only new data they bring to their paper. From these points, namely the phenotypic diversity of the family coupled with the inappropriate reliance placed on 16S rRNA homologies, we argue that Young *et al.* (2001) have failed to make a compelling case for combining species of *Agrobacterium* and *Rhizobium* into a single genus.

We believe that there are valid and compelling scientific reasons to retain *Agrobacterium* and *Rhizobium* as separate genera. First, as detailed above, the agrobacteria exhibit phenotypic characteristics that clearly set them apart from other members of the family *Rhizobiaceae*. Second, the genome structure of certain members of the genus *Agrobacterium* differs profoundly from that of other members of the family. Most notably, the chromosomal complement of the biovar 1 agrobacteria and of at least one isolate of *Agrobacterium rubi* is composed of two chromosomes, one circular and one linear (Jumas-Bilak *et al.*, 1998). This organization is quite different from that of the other members of the family *Rhizobiaceae*, which contain one or two circular chromosomes, depending upon the species and isolate (Jumas-Bilak *et al.*, 1998). Third, although no complete genome sequence is available for any member of the genus *Rhizobium*, the genome of the biovar 1 *Agrobacterium tumefaciens* strain C58 (Goodner *et al.*, 2001; Wood *et al.*, 2001) is now completely known at the nucleotide sequence level. We predict that while there may indeed be large regions of similarity between the circular chromosome of biovar 1 agrobacteria and one of the circular elements of *Rhizobium leguminosarum*, the linear chromosome of the biovar 1 agrobacteria will differ significantly in its coding capacity from the other large circular elements found in most species of *Rhizobium*. This point will be resolved only after comparative analysis of the complete genome sequences of selected members of the genera *Agrobacterium* and *Rhizobium*. However, what is certain is that these differences in gene complements will express themselves as differences in phenotypes, that is, taxonomically differentiatable traits, if only one knew the traits to examine. Fourth, the chromosomes of *Rhizobium* spp. and also of *Sinorhizobium meliloti* contain characteristic nucleotide repeat elements called RIMEs (rhizobium-specific intergenic mosaic elements), which are not present in the genome of the biovar 1 *Agrobacterium tumefaciens* strain C58 (Østerås *et al.*, 1995). Nor does there seem to exist an *Agrobacterium*-specific RIME-type element in the genome of strain C58. These elements may well be involved in genome rearrangements and evolution, and their absence from the genomes of biovar 1 agrobacteria is striking in its contrast to the rhizobia. Thus, the phenotypic and genotypic evidence indicate that the biovar 1 agrobacteria are significantly different from other members of the family *Rhizobiaceae*. Fifth, an analysis of current available data suggests that the characteristics of the plant–microbe interaction should not be ignored when evaluating differences among species of *Agrobacterium* and *Rhizobium*. For example, transconjugants of biovar 1 *Agrobacterium* strains carrying sym plasmids from several biovars of *Rhizobium leguminosarum* produced morphologically atypical nodules that failed to fix nitrogen (Hooykaas *et al.*, 1981, 1982). Similar atypical reactions were observed in plants infected with *Agrobacterium* harbouring a sym plasmid from *Sinorhizobium meliloti* (Truchet *et al.*, 1984). Nor do the rhizobia necessarily become tumorigenic upon acquisition of a Ti plasmid. *Sinorhizobium meliloti*, into which a Ti plasmid from *Agrobacterium tumefaciens* had been introduced, failed to induce tumours on any plant species tested (Van Veen *et al.*, 1989). From these observations it seems likely that *Agrobacterium* and *Rhizobium* carry on their chromosomes genus-specific gene sets that characterize the nature of their interactions with plants, irrespective of the determinants carried on sym or Ti plasmids. In our opinion, the agrobacteria and the rhizobia are diverging along their own evolutionary paths and these paths are tied, in part, to the specific characters of their interactions with host plants.

As noted by Young *et al.* (2001), there clearly exist problems in the taxonomies of the genera *Agrobacterium* and *Rhizobium*. However, these difficulties cannot be resolved simply by renaming the agrobacteria. We propose that the genus *Agrobacterium* as described by Kersters & De Ley (1984) be retained for the present, and that this genus descriptor be used certainly for the biovar 1 isolates, and also for species *Agrobacterium rubi*. As noted above, there is no compelling reason to include these bacteria in the genus *Rhizobium*, and genome structure as well as classical taxonomic measures support their division into a separate genus.

The issue is less clear with respect to the biovar 2 agrobacteria; these isolates appear to be more closely related overall to members of the genus *Rhizobium* than they are to the biovar 1 agrobacteria. Nevertheless, we propose to retain provisionally the biovar 2 agrobacteria within the genus *Agrobacterium*, as described above, for two reasons. First, given the polyphyletic nature of the family, there is no pressing need to redefine the genus status of the biovar 2 agrobacteria. This issue can be more thoroughly and definitively addressed when complete genome sequences are available for representative members of the relevant groups. Second, there is the issue of how we presently define these organisms as agrobacteria or rhizobia; namely a combination of phenotypic and genetic traits in conjunction with their interactions with host plants. Leaving aside the problems of plasmids and the traits they confer, it is quite possible that biovar 2 agrobacteria, in their pathogenic and

non-pathogenic forms, represent a group that is diverging from that which has evolved as plant symbionts, the rhizobia. Consistent with this interpretation, biovar 2 agrobacteria into which sym plasmids have been introduced induce atypical nodules (Paul Hooykaas, personal communication). Moreover, not all non-nodulating isolates classified within the genus *Rhizobium* gain the ability to nodulate host plants upon acquisition of a sym plasmid (Jarvis *et al.*, 1989). Such isolates may represent bacteria that have evolved along the agrobacterial lineage, i.e. the biovar 2 agrobacteria. One could conclude from these two observations that the biovar 2 agrobacteria do differ from the fast-growing rhizobia.

Clearly, more studies are required to resolve these issues, and until such definitive studies are available, we propose retention of the taxonomic status quo. It is clear from several published analyses of the rhizobia, including isolates unable to nodulate host plants, that these bacteria can be differentiated easily from the biovar 1 agrobacteria (Jarvis *et al.*, 1989; Soberón-Chávez & Nájera, 1989; Segovia *et al.*, 1991). The opposite is also true; isolates of agrobacteria, even those of biovar 2, can be differentiated from the rhizobia using selective media and standard keys (for example, see Schroth *et al.*, 1965; Du Plessis *et al.*, 1984; López *et al.*, 1988; Bouzar *et al.*, 1993, 1995).

With respect to species designations, we remain in a quandary. Young *et al.* (2001) contend that the epithets *tumefaciens* and *rhizogenes* are formally untenable because they describe traits that have as their genetic bases transmissible plasmids. But perhaps we should remember one purpose of taxonomy and that is to provide stable, meaningful names by which to refer to an organism in comparison with and in contrast to other organisms. The names should be designed for easy recognition and recollection. And if we are to do away with *tumefaciens* (tumour-inducing) as proposed by Young *et al.* (2001), why retain *rhizogenes* (root-inducing), also as proposed by Young *et al.* (2001)? Both species names describe a pathology, and to make matters worse, a variable trait conferred by a transmissible plasmid. In our opinion, retention of one but not the other cannot be excused on the basis of established rules for assigning species names. Moreover, we contend that these species names may in fact be tenable. It is becoming evident that the genomes of the agrobacteria, and perhaps also those of *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* are evolving in concert with their plasmids and their host plants (see for example: Jarvis *et al.*, 1989; Soberón-Chávez & Nájera, 1989; Segovia *et al.*, 1991; Bouzar *et al.*, 1993; Otten *et al.*, 1996; Pionnat *et al.*, 1999; Ridé *et al.*, 2000). While it is true that the large defining plasmids of one group can, on occasion, confer their specific phenotype on a non-cognate chromosomal background, the opposite also may be the case; there often is a required specificity, usually in the quantitative sense, between the bacterium, its plasmids and its host plants.

The goal in taxonomy is to identify the dividing lines in the continuum of bacterial genotypes that meaningfully describe and delineate genera. In our opinion the polyphasic differences between the rhizobia and the agrobacteria, which include chromosomal structure, presence or absence of RIMEs, auxanographic differences, differences in fatty acid profiles, and even divergences in 16S rRNA sequences set the two groups of bacteria apart at the genus level. These sets of criteria in themselves constitute reason for caution, and caution, which equates to stability, in our opinion dictates retention of the genus *Agrobacterium*. Consistent with our proposal, from their detailed study of the family *Rhizobiaceae*, de Lajudie *et al.* (1994) conclude that, although in need of revision at the species level, the genus *Agrobacterium* should be retained. Moreover, the microbial physiologists, geneticists and molecular biologists, as well as the plant scientists who work with *Agrobacterium* spp. know and use these organisms by their classical genus name. We believe that, in the absence of compelling and meaningful taxonomic weight, there is little to be gained by changing this terminology. Certainly, the scientific arguments to do so are not compelling. On the other hand, given the wide use of *Agrobacterium* species in disciplines ranging from basic bacteriology, genetics and molecular biology, through microbial physiology and enzymology, to plant molecular biology and biotechnology, there is the certainty of much confusion attendant to the unnecessary and unwarranted changes in taxonomic nomenclature proposed by Young *et al.* (2001).

This paper is co-signed by the following individuals, all of whom have communicated to the Editor of IJSEM their agreement with the positions of the authors concerning the taxonomic validity of the genus *Agrobacterium*. Some of the co-signatories have contributed to the Editor additional information in support of the position of the authors.

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Note Added in Proof

Two recent publications (Weller *et al.*, 2002; Van Berkum *et al.*, 2003) present results from the phylogenetic analysis of nucleotide sequences that are consistent with our position that there is no sound scientific evidence that warrants combining the members of the genus *Agrobacterium* into the genus *Rhizobium*.

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