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Short communication

Phenotypic tests in *Rhizobium* species description: An opinion and (a sympatric speciation) hypothesis

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ABSTRACT

Rhizobia seem to have large degradative and metabolic capabilities that allow them to grow on diverse soil and rhizospheric substances, many of which are still unknown. Rhizobial genome sequences encode numerous transporters for unknown substrates, and transcriptomic studies have revealed genes with unknown functions that are highly expressed in roots or rhizospheres. It is proposed here that some of these unknown-function genes may have roles in the assimilation of root or soil substances and that rhizobial speciation avoids nutrient competition. Phenotypic tests, as currently performed in taxonomy (mainly for carbon and nitrogen usage), seem to underestimate rhizobial catabolic capabilities and the differences among species. Furthermore, considering that many *Rhizobium* transporter and catabolism genes are plasmid-borne, the value of phenotypic results in taxonomic studies is questionable. Genomo-taxonomy could soon become a robust basis for proposing novel rhizobial species.

Rhizobia are best known for forming nitrogen-fixing nodules in legumes, but they are soil bacteria, endophytes and also successful rhizosphere colonizers of legumes and non-legumes [18,29]. The ancestral lifestyle of rhizobia was probably rhizospheric, and the degradative capabilities of rhizospheric bacteria have been highlighted [41]. Other soil bacteria, such as burkholderias, *Pseudomonas* or *Streptomyces*, are also known to have large degradative capabilities [22,25,29,41].

In soil and rhizosphere, rhizobia are nourished on root exudates, mucilage, decaying organic matter and minerals that the rhizobia may help to solubilize. There are over 10,000 different flavonoids identified from plants [12] which correspond to a large proportion of exudates [9]. In soil or rhizosphere, degradation of polyphenols, humic acids, lignin, or oligosaccharides derived from arabinogalactans or other complex molecules, as well as flavonoids, opines, calystegine, rhizopines, mimosine, betaines and polyamines [5–7,15,26,33,42,46,47,49] may support rhizobial growth. It is proposed here that differential degradation of soil and rhizospheric substances may avoid sympatric bacterial competition and our hypothesis is that divergence to avoid competition for nutrients is a driver of rhizobial speciation and also of intraspecific variation. To date, such natural molecules have not been normally tested in the laboratory as some of them are unknown, not commercially available or are very expensive. Consequently, the spectrum of rhizobial degradative capabilities is largely unknown. Simple molecules, such as homoserine excreted by peas, may allow the growth of the specific symbiont [53], as in a similar case observed with mimosine exuded by *Leucaena* plants [7]. *Rhizobium leguminosarum* has an unexpectedly large diversity of carbohydrases and can use pea root mucilage as a sole carbon source [24].

Analyses of rhizobial genomes have revealed a large number of transporters [31] and other genes without known function, many of which could be responsible for the degradation of a plethora of natural substances or their intermediates produced by diverse microorganisms including fungi in soil or rhizospheres. Transcriptomic analysis on roots has shown that many unknown rhizobial genes are highly expressed [28,39,56], as are many transporters whose substrates are unknown [28,39,45]. However, we suppose that some of these genes are involved in soil, root and rhizosphere substance assimilation.

The outstanding degradative capabilities of rhizospheric bacteria are shown when they are used in bioremediation (rhizoremediation). Rhizobia may degrade dibenzothiophene [13], phenanthrene [23], benzopyrene [16], *m*-toluate [48], acenaphthylene [38] and bisphenol A (our unpublished data). Interestingly, multiple bacterial species may participate in bioremediation [14].

We argue here that the phenotypic tests currently performed in taxonomic studies of rhizobia have a small value, although, unfortunately, a better method of characterization seems hard to achieve at present, since natural substrates, as mentioned above, are not easily available. Common phenotypic tests in the laboratory normally include the usage of amino acids (and some derivatives) and simple carbohydrates, as carbon or nitrogen sources. Virtually all protein amino acids are found in all root exudates, although

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there are significant quantitative differences of amino acids even within the same plant species. It has been suggested that the large numbers of ABC transporters encoded in rhizobial genomes have roles in amino acid transport [20]. Proline is exuded by legume and non-legume roots [51,54] and rhizobial strains that use it more efficiently are more competitive for nodule formation [52]. Often, only the type strain is tested in phenotypic analysis and only against other type strains, in spite of the fact that differences among strains from a single species could be as large as between species. Important phenotypic characteristics are patchily found in some bacterial species, for example, nitrogen fixation that is a very costly biochemical process may be easily lost in some strains from a single species or may be transferred between bacteria.

Phenotypic results depend on the growth medium and conditions used. In some cases degradation may depend on inducers, such as flavonoids [24,37], on substrate-related molecules or on a team of bacteria that contribute sequentially to natural product degradation (syntrophy).

It is worth considering that some degradative capabilities may be plasmid or genomic-island encoded in rhizobia (reviewed in [28]), thus, losing or gaining plasmids or islands may affect phenotypic tests. With almost half of the genome in extrachromosomal replicons in *Rhizobium* and *Sinorhizobium* [29], it is evident that many metabolic capabilities reside on them. By using Biolog phenotypic arrays, we found that *Rhizobium etli* strains cured of different plasmids moved to different positions relative to the wild type strain in phenotypic dendrograms (unpublished). Rhizobial genes involved in transport and/or catabolism of opines, calystegin, ribitol and most amino acids [2,11,17,36,55] are coded in plasmids, while those for glycerol, rhamnose and erythritol are located in newly described types of plasmids, designated chromids, which are recognized by their stability, similar GC content to the chromosome and by containing putative essential genes [19].

Other phenotypic characteristics, such as melanin production, are plasmid-borne in many rhizobial species [8,21,32] and are lost in cured derivative strains. Motility (swarming) may be plasmid mediated as well [34], as are other important processes, such as hydrogen uptake or trifolitoxin production that are found in only some rhizobial strains within a species [27,44].

By phenotypic analysis, *Rhizobium tropici* type A strains (now reclassified as *Rhizobium leucaenae* [43]) were clearly separated from *R. tropici* type B strains, and other rhizobial species strains were placed in between them [30]. Such a phenotypic dendrogram is clearly incongruent with genetic data that has clearly shown both *Rhizobium* species belong to a single "tropici group" [43]. Phenotypic tests have, in some cases, misplaced isolates and led to erroneous taxonomical results. When the *Sinorhizobium* genus was first proposed based on phenotypic characteristics it did not include *Sinorhizobium meliloti* [10]. Nowadays, phenotype-based taxonomy is validated or rebutted by genetic or genomic analysis. Nevertheless, in consideration of current phenotypic requirements that exist for new species proposals, long lists of substrates used by rhizobia are published in descriptions of novel species and they have very little practical use, thus being a waste of time and effort. Therefore, we should consider eliminating the requirement for phenotypic tests in novel rhizobial species descriptions. Furthermore, there are few reported phenotypic differences among some closely related species [40] that are easily differentiated by genotypic methods [1,40].

Experimental evolution studies with *Escherichia coli* have shown that rapidly evolving lineages diverged to use carbon sources such as acetate, which was a waste product from the main bacterial group [50]. In fact, an *E. coli* mutant strain that evolved in the laboratory to use citrate may even be considered as a different species on the basis of discriminative phenotypic rules [3,4].

In conclusion, many phenotypic tests in rhizobial studies that describe new species are questionable, as they are limited to available substrates, and they seem to underestimate the phenotypic differences among species. However, does it make sense to test more degradation capabilities in the taxonomic context of a species definition if these characters are mostly plasmid-borne or in genomic islands? If they belong to the accessory genome and are potentially unstable over time, they would not be of taxonomic interest. In contrast, metabolic capacities coded in chromosomes or chromids do have taxonomic value. Interestingly, many genes expressed in plant rhizospheres have been found in chromids [28], as well as some genes involved in antibiotic resistance [35]. Probably, in the future, genomic-based studies (genomotaxonomy) will represent the basis for proposing novel species, phenotypes could perhaps be inferred from such studies (for example, Ormeño-Orrillo et al. [35] deduced antibiotic and stress resistance from genomic analysis in *R. tropici* and related species), and their replicon location (on plasmids, chromids or the chromosome) could be assigned.

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