Short Communication

RELATEDNESS AMONG *RHIZOBIUM* SPECIES

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The current classification of symbiotically nitrogen-fixing root-nodule bacteria (16) divides these organisms into two genera, *Rhizobium* and *Bradyrhizobium*. The separation at the generic level of the two groups of bacteria that are traditionally referred to as fast-growing and slow-growing is based upon cultural (10, 18, 19, 24), biochemical (17, 23) and genetic (7–9, 11) differences. On the other hand, the classification merges the former species *Rhizobium leguminosarum*, *Rhizobium trifolii* and *Rhizobium phaseoli* together into a single species and recognizes them as biovars of *Rhizobium leguminosarum* due to their broad similarities (16). *Rhizobium meliloti* is still recognized as a distinct separate species. The fast-growing *Rhizobium* of *Lotus* spp., *Rhizobium loti* has been added to the list of fast-growing species based on previously published information (14). Since the publication of the latest edition of Bergey's Manual of Systematic Bacteriology (16) the fast-growing bacteria nodulating soybean have been described and named *Rhizobium fredii* (21). Yet there are many strains of *Rhizobium* which have never been designated by a species name. In the current classification, bacteria belonging to the genus *Bradyrhizobium* is represented by a single species *Bradyrhizobium japonicum* and the former species *Rhizobium lupini* has been eliminated.

Here we describe the DNA-DNA homology of strains of *Rhizobium* and *Bradyrhizobium* including some unnamed *Rhizobium* isolated from local legume hosts. The results are consistent with known genetic relationships. A *Rhizobium* strain isolated from *Dolichos lablab* was found to be closely related to *Rhizobium meliloti*.

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Table 1. Bacterial

Name as received	Strain No. ^a	Plant origin
<i>Rhizobium leguminosarum</i>	SU 391 (= 175G11 ^b)	Not known
	CC 308 (= 175F1 ^b)	Not known
	CC 306 (= 128C53 ^b)	Not known
	BICC 601	<i>Pisum sativum</i>
<i>Rhizobium trifolii</i>	CC 224	Not known
	T 19	Not known
	UNZ 29	<i>Trifolium subterraneum</i>
<i>Rhizobium phaseoli</i>	CC 365	<i>Phaseolus vulgaris</i>
	CC 511 (= 3I6C15 ^b)	<i>Phaseolus vulgaris</i>
	BICC 602	<i>Phaseolus vulgaris</i>
	BICC 603	<i>Phaseolus vulgaris</i>
<i>Rhizobium meliloti</i>	SU 216	Not known
	CC 169	<i>Medicago rugosa</i> cv. paragosa
	BICC 604	<i>Melilotus alba</i>
<i>Rhizobium loti</i>	NZP 2213 ^T (= ATCC33669 ^T)	<i>Lotus corniculatus</i>
<i>Rhizobium</i> sp. (<i>Arachis hypogaea</i>)	BICC 606	<i>Arachis hypogaea</i>
<i>Rhizobium</i> sp. (<i>Dolichos lablab</i>)	BICC 607	<i>Dolichos lablab</i>
<i>Rhizobium</i> sp. (<i>Cassia sophera</i>)	BICC 609	<i>Cassia sophera</i>
<i>Rhizobium japonicum</i>	CC 409	<i>Vigna radiata</i>
	CC 709 (= 61A77 ^b)	<i>Glycine max</i>
	SB 16	Not known
<i>Rhizobium lupini</i>	RL 3001	Not known

^a Abbreviations: SU, University of Sydney, Australia; CC, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organizations, Canberra City, Australia; BICC, Department of Microbiology, Bose Institute, Calcutta, India; UNZ, Plant Diseases Division, Department of Scientific and Industrial Research, Mont Albert, New Zealand (designated thus after transfer to Uruguay and Australia); NZP, Applied Biochemistry Division, Department of Scientific and

The bacterial strains studied are listed in Table 1, together with their strain numbers and sources. The cultures were maintained on yeast extract mannitol (25) agar slopes. For DNA preparations all the organisms were grown in yeast extract mannitol broth at 28°C under shake condition.

Labeling of cell DNA with H₃³²PO₄ and isolation of labeled as well as unlabeled DNA have been described previously (4, 5). For DNA-DNA hybridization DNA was sheared by sonication in a Braun sonicator at 100 W for a total of 10 min allowing a probe rest for 1 min intermittently after each 30 s pulse. DNA was hybridized in a solution adjusted to 0.42 M Na⁺ at 80°C to an equivalent Cot of 200 (2). The fraction of labeled DNA reassociated was quantitated by S₁ endonuclease treatment and application on a DE81 filter disc (Whatman) then washed with 5% Na₂HPO₄ as described (20). Radioactivity was measured by

strains studied.

Geographic origin	Effective for	Source
Australia	<i>Pisum sativum</i> , <i>Vicia hirsuta</i>	CC
Not known	<i>Pisum sativum</i> , <i>Vicia hirsuta</i>	CC
Not known	<i>Pisum sativum</i> , <i>Vicia hirsuta</i>	CC
India	<i>Pisum sativum</i>	BICC
Not known	Ineffective for every species of <i>Trifolium</i> on which tested	CC
Not known	<i>Trifolium</i>	HU
Uruguay	Most species of <i>Trifolium</i> of Mediterranean origin	CC
Brazil	<i>Phaseolus vulgaris</i>	CC
Australia	<i>Phaseolus vulgaris</i>	CC
India	<i>Phaseolus vulgaris</i>	BICC
India	<i>Phaseolus vulgaris</i>	BICC
Not known	<i>Medicago sativa</i>	HU
Australia	Most species of <i>Medicago</i>	CC
India	<i>Melilotus alba</i>	BICC
Not known	<i>Lotus corniculatus</i> , <i>Lotus tenuis</i> , <i>Lupinus densifloras</i>	NZP
India	<i>Arachis hypogaea</i>	BICC
India	<i>Dolichos lablab</i>	BICC
India	<i>Cassia sophera</i>	BICC
India	<i>Vigna radiata</i>	CC
Not known	<i>Glycine max</i>	CC
Not known	<i>Glycine max</i>	HU
Not known	<i>Lupinus</i>	HU

Industrial Research, Palmerston North, New Zealand; ATCC, American Type Culture Collection, Rockville, Md.; HU, Haryana Agricultural University, Haryana, India. Whole forms of T, SB and RL are not known to us.

^b Designations of Nitragin Co., Milwaukee, Wisconsin, U.S.A.; [†] Type strain.

Cerenkov Counting (6).

Reference DNAs from *Rhizobium leguminosarum* biovar *viceae* SU 391, *Rhizobium leguminosarum* biovar *trifolii* CC 224, *Rhizobium meliloti* SU 216 and *Rhizobium loti* NZP 2213 were hybridized with DNA from each organism examined and the percentages of hybridizations are given in Table 2. The values involving the reference strains SU 391 and CC 224 were plotted against that of the reference strain SU 216 to produce the similarity maps in Fig. 1 in which the interrelationships of the strains of *Rhizobium* are plotted. Representative DNAs from four strains of *Bradyrhizobium* were also included.

Crow *et al.* (7) determined DNA homologies among 122 strains of root-nodule bacteria, to confirm and extend the results reported by Jarvis *et al.* (12) who earlier established that all rhizobia which effectively nodulated *Trifolium repens*, *T.*

Table 2. Relative hybridization of DNA from strains of *Rhizobium* and *Bradyrhizobium* with DNA from reference strains of *Rhizobium*.

Source of unlabeled DNA	Source of reference DNA			
	<i>R. leguminosarum</i> biovar <i>viceae</i> SU 391	<i>R. leguminosarum</i> biovar <i>trifolii</i> CC 224	<i>R. meliloti</i> SU 216	<i>R. loti</i> NZP 2213
<i>R. leguminosarum</i> biovar <i>viceae</i>				
SU 391	100	46	42	11
CC 308	78	41	47	11
CC 306	56	31	40	16
BICC 601	46	32	44	10
<i>R. leguminosarum</i> biovar <i>trifolii</i>				
CC 224	45	100	40	12
T 19	32	54	42	10
UNZ 29	46	46	45	12
<i>R. leguminosarum</i> biovar <i>phaseoli</i>				
CC 365	44	42	42	10
CC 511	34	40	40	10
BICC 602	38	33	52	9
BICC 603	30	36	55	8
<i>R. meliloti</i>				
SU 216	36	41	100	11
CC 169	29	30	58	15
BICC 604	24	40	70	10
<i>R. loti</i>				
NZP 2213	19	15	20	100
<i>Rhizobium</i> sp. (<i>Arachis hypogaea</i>)				
BICC 606	16	18	40	9
<i>Rhizobium</i> sp. (<i>Dolichos lablab</i>)				
BICC 607	24	28	58	11
<i>Rhizobium</i> sp. (<i>Cassia sophora</i>)				
BICC 609	18	22	45	10
<i>Bradyrhizobium japonicum</i>				
CC 409	16	18	22	15
CC 709	18	19	19	12
SB 16	16	18	24	14
<i>Bradyrhizobium</i> sp. (<i>Lupinus</i>)				
RL 3001	20	22	24	17

subterraneum, *T. ambiguum* (*R. leguminosarum* biovar *trifolii*) and *Vicia hirsuta* (*R. leguminosarum* biovar *viceae*) formed one population with an average relatedness of 70% (range 49 to 94%). Two strains from African *Trifolium* species and two strains from a northern Asiatic species were less closely related. The average relatedness of strains from *Phaseolus vulgaris* to clover rhizobia was 46% (range 37 to 50%). These authors proposed that the former species *Rhizobium trifolii* and *Rhizobium leguminosarum* should be combined under the name *Rhizobium leguminosarum* and strains of *Rhizobium phaseoli* should be examined in more detail. Crow *et al.* (12) by DNA homology identified four genetic groups of fast-growing strains. Group I of these authors included strains which originated from *Trifolium*,

Fig. 1. Interrelationship map of strains of *Rhizobium* studied. The percent relative hybridization of DNA from each strain with labeled DNA from *R. meliloti* SU 216 is plotted against the percent relative hybridization with labeled DNA from (a) *R. leguminosarum* biovar *viceae* SU 391 and (b) *R. leguminosarum* biovar *trifolii* CC 224 on log₁₀ scales. Organisms belonging to the same taxon are joined by lines. Δ , *Rhizobium meliloti*; $\blacktriangle$, *Rhizobium leguminosarum* biovar *phaseoli*; $\circ$, *Rhizobium leguminosarum* biovar *trifolii*; $\bullet$, *Rhizobium leguminosarum* biovar *viceae*; $\star$, *Rhizobium loti*; $\blacklozenge$, A = *Rhizobium* sp. (*Arachis hypogaea*), C = *Rhizobium* sp. (*Cassia sophora*), D = *Rhizobium* sp. (*Dolichos lablab*); $\square$, *Bradyrhizobium* sp., 3001 = *Bradyrhizobium* sp. (*Lupinus*) RL3001.

Pisum, *Vicia*, *Lathyrus* and *Phaseolus vulgaris* including 2 strains from *Carmichaelia* sp. and 2 from *Neptunia gracilis*. All strains belonging to the alfalfa cross-inoculation group (*Rhizobium meliloti*) were assigned to group 3. These authors found little (3 to 17%) homology of the members of this group with reference DNAs from other groups such as strains from *Trifolium*, *Phaseolus* and *Lotus*. In our experience this level of hybridization appears to be low. Measurements of intra- and intergeneric similarity between rRNA cistrons of *Rhizobium* show very close genetic relationship between *R. fredii* and *R. meliloti* (13). The dendrogram presented in this work (13) shows that the *R. meliloti*-*R. fredii* branch has *R. leguminosarum* (as per current classification) as its closest neighbour. At the other end of the group, in their dendrogram, is *R. loti*, a separate branch and the most remote taxon. They reported a six to 26% homology value of *R. fredii* to *R. loti* (26), which belongs to a very remote taxon, and 11 to 40% to *R. leguminosarum*. From the close genetic similarity of *R. fredii* and *R. meliloti* we expected a higher level of DNA-DNA homology between *R. meliloti* and strains of *R. leguminosarum* biovars than Crow *et al.* reported (7). Figure 1, a log-log plot of the clustering of strains of *Rhizobium* and *Bradyrhizobium* according to their relative hybridization values, shows that *R. meliloti* strains form a distinct cluster separate from strains of *Rhizobium leguminosarum* biovars, although the levels of hybridization with respect to reference strains SU 391 and CC 224 were relatively high. This supports the separate species status of the strains of *R. meliloti* proposed here.

Using a large number of unweighted characters including morphological and physiological ones in numerical taxonomy, Graham (10) observed strains of *R. meliloti* exhibiting greater than 70% similarity with strains belonging to biovars of *R. leguminosarum*, while the members of the latter were grouped together with similarity values in the range of 80 to 97%. Graham (10) pointed that similarity values obtained from computer survey may be related to taxonomic rank, but since the number and variety of tests used may vary, no absolute criterion can be set for the different taxonomic levels. Brenner (1) considered that a genus should comprise phenotypically similar species and phenotype should take precedence over DNA relatedness at the genus level while a species may be defined as a collection of more or less similar clones tied into a bundle. How small or large the bundle is depends upon the scientific insight of the individual taxonomist. Hence, *Rhizobium leguminosarum*, *R. trifolii* and *R. phaseoli* are independent species in the approved lists of bacterial names (22) but they are recognized as biovars in Bergey's Manual of Systematic Bacteriology (16).

Plant specificity in *Rhizobium* is a plasmid-borne character and involves a relatively short DNA base sequence and nodulating ability is transferred between its members at high frequency (15). Strains do not have to be genetically similar to fall in the same inoculation group as is evident from the paired combination of *Rhizobium* and *Bradyrhizobium* for *Cicer* and *Lotus*. This argues that those factors which determine some inoculation groups can also be expressed in diverse genetic backgrounds. Strains of *R. leguminosarum* biovar *phaseoli* are considered to be diverse by protein fingerprinting analysis. Using nucleic acid hybridization Gibbins and Gregory (9) also observed a close relationship between *R. meliloti* and *R. phaseoli*, although with less certainty. These reports confirm the findings of 't Mannetje (24) who also observed a diverse genetic background of the strains labeled as *R. phaseoli* by inoculation criterion. In the present investigation, two out of the four strains of *R. leguminosarum* biovar *phaseoli*, BICC 602 and BICC 603, seem to occupy a position between *R. meliloti* and the rest of the strains of biovars of *R. leguminosarum*. The strains BICC 602 and BICC 603 originated in India whereas the other two strains CC 365 and CC 511 were isolated from plants of Brazil and Australia, respectively. It is possible that strains originating from diverse geographic locations may have diverse genetic background while having the same inoculation determinant. Crow *et al.* (7) also reported that strains designated *Rhizobium phaseoli* are a genetically diverse group which contains members that are not closely related to *Rhizobium trifolii* and *Rhizobium leguminosarum*, although the authors recommended their placement together with the biovars of *Rhizobium leguminosarum*.

The position of *R. loti* as a separate species and as a taxon remote from *R. leguminosarum* and *R. meliloti* has been confirmed in the present investigation using the lone strain NZP 2213, which is the type strain. Of the three unnamed isolates included in this study the isolate from *Dolichos lablab* was found to be a close relative of *R. meliloti* and with further evidence could perhaps be included in the *R. meliloti* cluster.

Any of the four strains of *Bradyrhizobium* used in this study was found to be distantly related to the *Rhizobium* strains used as references. Our earlier work placed the three *Bradyrhizobium japonicum* strains in a homology group different from the strain of *Bradyrhizobium* sp. (*Lupinus*) (3).

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