NOTE

Phylogenetic and DNA–DNA hybridization analyses of *Bradyrhizobium* species

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The 16S rDNA sequence of *Bradyrhizobium liaoningense* was determined and analysed together with sequences of other *Bradyrhizobium* species and related taxa. In addition, DNA–DNA hybridizations were performed between representative strains of the three *Bradyrhizobium* species. *Bradyrhizobium liaoningense* is genotypically highly related to *Bradyrhizobium japonicum*, whereas *Bradyrhizobium elkanii* is more distantly related to these two species. The fact that *Afipia*, *Agromonas*, *Blastobacter*, *Nitrobacter* and *Rhodopseudomonas* are phylogenetically more closely related to *Bradyrhizobium japonicum* than to *Bradyrhizobium elkanii* is discussed.

Keywords: *Bradyrhizobium*, 16S rDNA phylogeny, DNA–DNA hybridizations

The genus *Bradyrhizobium* was created for the slow-growing members of the genus *Rhizobium* (Jordan, 1982) and initially contained one species, *Bradyrhizobium japonicum*, for isolates from *Glycine*. Other isolates and groups were included in the genus, but not given a formal species status. They were referred to as *Bradyrhizobium* sp., with the host plant usually listed in parentheses. Using DNA–DNA hybridizations, Hollis *et al.* (1981) described three homology groups among *Rhizobium japonicum* strains. Groups I and Ia correspond to the current species *Bradyrhizobium japonicum*. For group II, a separate species, *Bradyrhizobium elkanii*, was later proposed on the basis of genotypic and phenotypic results (Kuykendall *et al.*, 1992). Finally, a third species, *Bradyrhizobium liaoningense*, was proposed for a group of extra-slow-growing *Glycine* isolates which were both phenotypically and genotypically distinct from the other two species (Xu *et al.*, 1995). This report included the partial 16S rDNA sequence (260 bp, positions 44–337) of *Bradyrhizobium liaoningense* strain 2062, which was identical to the sequence of *Bradyrhizobium japonicum* USDA 110. In view of these findings, assessment of the phylogenetic position of *Bradyrhizobium liaoningense* requires at least the determination of its complete 16S rDNA sequence.

Phylogenetic relationships

The 16S rRNA gene sequences of *Bradyrhizobium liaoningense* LMG 18230^T and LMG 18231 were

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The EMBL accession number for the 16S rDNA of *Bradyrhizobium liaoningense* LMG 18230^T is AJ250813.

determined as described by Molouba *et al.* (1999). Both were identical and thus only the type strain sequence (AJ250813) was used in further phylogenetic analyses. Using the computing facilities of the Belgian EMBnet Node of the Brussels Free University Computing Centre, sequences of related organisms in the α -subclass of the *Proteobacteria* were retrieved from the EMBL database and aligned together with the new sequence data using the program PILEUP from the Genetics Computer Group package version 10.0 (Devereux *et al.*, 1984). A continuous stretch of 1301 aligned base positions was used for further analysis using the TREECON program (Van de Peer & De Wachter, 1994). Kimura-2 distances between the aligned sequences were calculated and an unrooted tree was calculated using the neighbour-joining algorithm (Saitou & Nei, 1987). The stability of the groupings was assessed by performing a bootstrap analysis with 500 replications. Sequence similarity values were calculated by transforming uncorrected distance values as calculated using TREECON. In addition, a parsimony analysis was performed using the program Bionumerics (Applied Maths, Kortrijk, Belgium).

All *Bradyrhizobium* sequences with a length of at least 1350 bp available from EMBL were included in the analysis. A large number of these were isolated from shrubby legumes in southern Australia and are referred to as separate genospecies as proposed by Lafay & Burdon (1998). In addition, representatives of the following taxa, known to be phylogenetically close to *Bradyrhizobium*, were included: *Afipia* and *Blastobacter denitrificans* (Willems & Collins, 1992), *Nitro-*

Fig. 1. Dendrogram obtained by neighbour-joining analysis of distance values, showing the phylogenetic relationships of *Bradyrhizobium* strains and related organisms. Sequence accession numbers are listed in parentheses. Bootstrap values of more than 70 % are listed at the nodes.

bacter and *Rhodopseudomonas palustris* (Seewaldt *et al.*, 1982; Orso *et al.*, 1994) and *Agromonas oligotrophica* (Saito *et al.*, 1998). Four soil isolates reported to be closely related to *Bradyrhizobium* (Saito *et al.*, 1998) were also included in the analysis. The resulting phylogenetic tree is shown in Fig. 1. *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Mesorhizobium loti* were included as an outgroup. A parsimony analysis, performed with the program Bionumerics, produced a nearly identical grouping (data not shown).

The tree topology revealed two main clusters that comprise bradyrhizobia. Both are well supported by

bootstrap probabilities. In the largest of these main clusters, overall sequence similarities ranged from 96.5 to 100 %, but the limited sequence divergence among the *Bradyrhizobium* strains in this cluster (0.1–2.0 %) resulted in the poor resolution of most branches. Nevertheless, a few subclusters are apparent: *Rhodopseudomonas palustris*, *Nitrobacter* and *Afipia* strains form three subclusters, respectively, supported by high bootstrap values. A further well-supported subcluster consists of the photosynthetic *Bradyrhizobium* strains BTAi1, LMG 12187 and USDA 4377, *Agromonas oligotrophica* and *Blastobacter denitrificans*. These sequences showed a divergence of less than 0.7 %. A separate analysis was performed, which included

shorter sequences for additional photosynthetic strains reported by Wong *et al.* (1994), and here again photosynthetic strains grouped together in one subcluster (data not shown), indicating that the photosynthetic bradyrhizobia seem to represent a separate phylogenetic group. Similar data were reported by So *et al.* (1994) and Molouba *et al.* (1999) and it has been suggested that the photosynthetic strains may represent a separate species (So *et al.*, 1994; Fleischman & Kramer, 1998). *Agromonas oligotrophica* is a nitrogen-fixing oligotrophic soil bacterium (Ohta & Hattori, 1983) and *Blastobacter denitrificans* is a surface water isolate from a lake (Hirsch & Müller, 1985). Both produce budding cells and are not reported to be plant-associated. The fact that they grouped in the same subcluster as the photosynthetic bradyrhizobia and have very similar 16S rDNA sequences would suggest that they may contain a photosynthetic apparatus or the remains of it. This close relationship between symbiotic and non-plant-associated organisms is similar to the close relationship described between symbiotic and non-symbiotic isolates from the rhizosphere of *Lotus corniculatus* (Sullivan *et al.*, 1996). Both *Agromonas oligotrophica* and *Blastobacter denitrificans* are validly published species, although the status of the genus *Blastobacter* is unclear because no strains are available of the type species *Blastobacter henricii* (Hugenholtz *et al.*, 1994; Sly & Cahill, 1997). DNA–DNA hybridizations are required to resolve relationships within this subcluster in more detail.

In addition to the above-mentioned subclusters, the largest cluster contains strains representing *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense*, together with a large number of *Bradyrhizobium* species strains and four soil isolates. They form a large group of highly related sequences (at least 98.0% sequence similarity) among which further subdivision is not clearly supported by the data. Six recently described strains from *Arachis hypogaea* (Zhang *et al.*, 1999) for which a partial (780 bp) 16S rDNA sequence is available also cluster in this large group (data not shown). This large group of *Bradyrhizobium* strains shows 96.5–98.8% sequence similarity with strains of the *Afipia*, *Nitrobacter* and *Rhodopseudomonas palustris* subclusters. *Bradyrhizobium japonicum* strain USDA 110 [representing DNA group Ia of Hollis *et al.* (1981)] groups separately from other *Bradyrhizobium japonicum* strains, but the significance of this is unclear because of the low sequence divergence. *Bradyrhizobium japonicum* DNA group I (Hollis *et al.*, 1981) can not be distinguished clearly from *Bradyrhizobium liaoningense* and several *Bradyrhizobium* sp. strains. Several authors have previously noted the limited use of 16S rDNA sequences at species and subspecies level for fast-growing rhizobia, a group of organisms where lateral gene transfer is known to occur and the 16S rRNA genes show relatively little divergence (Sullivan *et al.*, 1996; Haukka *et al.*, 1996). Similarly, among the bradyrhizobia, this study and others (Urtz & Elkan, 1996; Barrera *et al.*, 1997) demonstrate that 16S rDNA

sequences provide little phylogenetic depth, and alternative approaches, for example studying molecules with a higher divergence rate or DNA–DNA hybridizations, are needed to assess relationships.

Incidentally, two sequences, accession numbers D11345 and D12781, were available for *Bradyrhizobium japonicum* strain IAM 12608^T. Barrera *et al.* (1997) pointed out that the first of these was different from the sequences for other subcultures of the type strain (LMG 6138^T and USDA 6^T). In our analysis, only sequence D12781 grouped with the sequences for LMG 6138^T and USDA 6^T (Fig. 1) and therefore sequence D11345 should not be regarded as representing the type strain (eight differences with LMG 6138^T). The sequence for a fourth subculture of the type strain, DSM 30131^T, also grouped separately from the other type strain sequences (10 base differences with LMG 6138^T) and therefore does not seem to represent the same type strain.

The second and smallest of two main clusters contains two named *Bradyrhizobium elkanii* strains and seven *Bradyrhizobium* sp. strains from various host plants. Again, because of the very small sequence divergence (less than 1.2%) within the cluster, the branches are poorly resolved and it is thus not clear whether this cluster completely or only in part represents *Bradyrhizobium elkanii*. DNA–DNA hybridizations are needed to establish this. The separate phylogenetic position of this second *Bradyrhizobium* cluster relative to the other bradyrhizobial strains and the *Afipia*, *Nitrobacter* and *Rhodopseudomonas palustris* subclusters (Fig. 1) may be interpreted as an indication that this cluster represents a separate genus. However, at present the internal relationships within this cluster are unclear and phenotypic differentiation of the other large *Bradyrhizobium* cluster is not possible, precluding a clear conclusion on its status. In addition, the *Bradyrhizobium elkanii* cluster shows a sequence divergence of only 2.1–4.4% with the *Bradyrhizobium* strains from the larger cluster (Fig. 1) and a divergence of 3.3–5.4% with the *Afipia*, *Nitrobacter* and *Rhodopseudomonas palustris* subclusters inside the same large cluster. The divergence of these three genera from the *Bradyrhizobium* strains of the large cluster is 1.2–3.5%. Thus, purely on phylogenetic grounds, an alternative proposal of merging all these genera can be considered. For now, we would not favour such a proposal because (i) the genera, though highly related, group separately with the support of high bootstrap values and (ii) the phenotypic considerations that have led to the creation of these separate groups remain valuable for identification and differentiation of these organisms from others in the same ecosystem. It is also clear that in the past, organisms that were not thought to be related have not been studied in a comparative way (Young *et al.*, 1991). Now that a close phylogenetic link has been clearly demonstrated by several authors, phenotypic and chemotaxonomic studies should be taken up again and extended in a comprehensive way. For the time

being, we join the opinion voiced by Wong *et al.* (1994) that a polyphasic approach to reconciliation of phenotype and phylogeny (Wayne *et al.*, 1987) is needed for this group as a whole.

DNA–DNA hybridization

The very high 16S rDNA sequence similarity between *Bradyrhizobium liaoningense* and *Bradyrhizobium japonicum* [e.g. four positions (including one gap) sequence difference between both type strains] is in line with the finding that the type strains of both species belong to the same 16S amplified rDNA restriction analysis cluster (Doignon-Bourcier *et al.*, 1999). On the other hand, both species are phenotypically distinct (Xu *et al.*, 1995) and DNA–DNA hybridizations of *Bradyrhizobium liaoningense* strains with two *Bradyrhizobium japonicum* strains [representing DNA groups I and Ia of Hollis *et al.* (1981)] showed low levels of DNA homology (9–38%; Xu *et al.*, 1995). In view of the high 16S rDNA sequence similarity, we considered it useful to extend the DNA hybridization data, using more strains.

We carried out DNA–DNA hybridizations between four strains of *Bradyrhizobium japonicum* [LMG 4252 (= Bonnier 3.1), LMG 4271 (= USDA 59), LMG 6138^T (= NZP 5549^T) and USDA 110] and two strains of *Bradyrhizobium liaoningense* [LMG 18230^T (= 2281^T) and LMG 18231 (= 2062)], as well as two strains of *Bradyrhizobium elkanii* [LMG 6134^T (= NZP 5531^T) and LMG 6135 (= NZP 5532)]. The strains of *Bradyrhizobium japonicum* represent DNA groups I (LMG 6138^T, LMG 4252 and LMG 4271) and Ia (strain USDA 110) of Hollis *et al.* (1981). Cells were grown in liquid cultures of 800 ml yeast mannitol medium (Vincent, 1970) and harvested by centri-

fugation. For strains that produced large amounts of exopolysaccharides, extra washing steps or a mild alkaline hydrolysis step (incubation for 10 min in 0.05 M NaOH at 60 °C) were necessary, after which cells could be harvested by centrifugation. DNA was extracted and purified by a modification of the procedure of Marmur (1961): before lysis, cells were suspended in at least 100–150 ml saline-EDTA per 2 g of cells and proteinase K (Merck) was added to a concentration of 8 µg per 100 ml. After lysis, to separate nucleic acids from proteins, NaCl was used instead of sodium perchlorate. DNA hybridizations were carried out using a microplate method in which unlabelled DNA, non-covalently linked to a microplate, is hybridized with biotinylated probe DNA (Ezaki *et al.*, 1989). The main advantage of this method for use with bradyrhizobia, which often give low DNA yields, is that only small amounts of DNA are required as compared to, for example, the classical initial renaturation rate method (De Ley *et al.*, 1970). The microplate method was shown to produce results comparable to those of the initial renaturation rate method for several groups of bacteria (Goris *et al.*, 1998).

Hybridization results are presented in Table 1. Each value is a mean of four replicate experiments. Mostly differences between reciprocal values (i.e. values obtained by hybridization of fixed DNA of strain A with probe DNA of strain B and vice versa) are less than 10%. However, for hybridizations between group Ia and group I strains a larger difference of up to 20% was obtained. A similar, although smaller, difference between reciprocal hybridizations can also be observed in the original paper describing these groups (Hollis *et al.*, 1981). One possible explanation for this phenom-

Table 1. Results of DNA–DNA hybridizations, expressed as percentage DNA homology

Strain providing microplate-bound DNA	Strain providing probe DNA							
	USDA 110	LMG 4252	LMG 4271	LMG 6138 ^T	LMG 18230 ^T	LMG 18231	LMG 6134 ^T	LMG 6135
<i>B. japonicum</i> USDA 110 (group Ia)	100	53		58	54	52	25	23
<i>B. japonicum</i> LMG 4252 (group I)	73	100	91	100	53	48	26	28
<i>B. japonicum</i> LMG 4271 (group I)		80	100	97		43	22	
<i>B. japonicum</i> LMG 6138 ^T (group I)	73	87	82	100	56	46	25	30
<i>B. liaoningense</i> LMG 18230 ^T	45	45	41	40	100	94	28	
<i>B. liaoningense</i> LMG 18231	50	48		48	100	100	36	
<i>B. elkanii</i> LMG 6134 ^T (group II)	30	20		23	28	27	100	87
<i>B. elkanii</i> LMG 6135 (group II)	33	25		26	28	26	90	100

Table 2. Comparison of percentage DNA homology values between *Bradyrhizobium* species or groups

Numbers in parentheses represent the number of hybridizations that were used to calculate the mean DNA homology values. ND, Not determined.

<i>Bradyrhizobium</i> species/group	Hybridization method used	<i>B. japonicum</i> I	<i>B. japonicum</i> Ia	<i>B. elkanii</i> II
<i>B. japonicum</i> Ia	Microplate method*	64.0 ± 10.0 (4)		
	Hydroxyapatite method, 65 °C†	51.3 ± 6.7 (14)		
	Hydroxyapatite method, 80 °C‡	34.6 ± 9.9 (14)		
	Renaturation rate method§	61.7 ± 23.7 (6)		
<i>B. elkanii</i> II	Microplate method*	24.9 ± 2.8 (9)	27.8 ± 4.6 (4)	
	Hydroxyapatite method, 65 °C†	24.1 ± 6.3 (11)	28.0 ± 7.8 (14)	
	Hydroxyapatite method, 80 °C‡	10.5 ± 3.3 (11)	10.9 ± 5.4 (14)	
	Renaturation rate method	ND	ND	
<i>B. liaoningense</i>	Microplate method*	46.8 ± 5.0 (10)	50.3 ± 3.9 (4)	28.8 ± 3.6 (6)
	Hydroxyapatite method, 65 °C†	ND	ND	ND
	Hydroxyapatite method, 80 °C‡	ND	ND	ND
	Renaturation rate method	20.5 ± 10.1 (5)	30.0 ± 9.6 (5)	19.9 ± 9.1 (5)

* Hybridization at 50 °C in the presence of 50 % formamide.

† Non-restrictive conditions; data from Hollis *et al.* (1981).

‡ Restrictive conditions; data from Hollis *et al.* (1981).

§ Data from Scholla *et al.* (1990).

|| Xu *et al.* (1995).

enon is a difference in genome size between these groups, with group Ia having a larger genome than group I and therefore generating a lower signal when used as fixed DNA. For the interpretation of the data, the means of reciprocal experiments were used, for example, leading to values of about 63 % DNA homology between group I and Ia strains.

From our data (Table 1), it is clear that there is a significant level of DNA hybridization between *Bradyrhizobium japonicum* DNA groups I and Ia and between *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense*. These two species show levels of DNA homology of 40–56 %. These values are not above the 70 % level seen as indicative of a single species (Wayne *et al.*, 1987), but may be seen as indicative of closely related species. In view of the phenotypic differences reported between *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense* (Xu *et al.*, 1995), it seems justified to regard them as separate, but closely related species. In agreement with previous reports (Hollis *et al.*, 1981; Xu *et al.*, 1995), *Bradyrhizobium elkanii* forms a separate species with a mean DNA homology value of below 30 % with the other two species.

Table 2 provides an overview of hybridization values between *Bradyrhizobium* species reported in the literature and our own data. In such a comparison, hybridization conditions have to be taken into account when evaluating results. Our hybridizations were performed at a temperature of 50 °C in 2 × SSC (standard saline citrate or 1 × SSC is 0.15 M NaCl

plus 0.015 M trisodium citrate, pH 7.0 ± 0.2), in the presence of 50 % formamide. The optimal renaturation temperature in 2 × SSC ($T_{OR[2SSC]}$) can be calculated by the formula $T_{OR[2SSC]} = 1.24 \times T_{m[1SSC]} - 38.8$, with $T_{m[1SSC]}$ the midpoint of the thermal denaturation curve in 1 × SSC (Gillis *et al.*, 1970). Thus $T_{OR[2SSC]}$ for organisms with a G + C content of approximately 64 mol % is 79.6 °C. Taking into account that 0.72 °C should be subtracted per 1 % formamide, the presence of 50 % formamide results in an optimal renaturation temperature of 43.6 °C. Our use of 50 °C as hybridization temperature was based on the observation that hybridization values were little or not affected within an interval of optimal temperature ± 5 °C and our conditions can therefore be regarded as optimal. Hollis *et al.* (1981) reported data from non-restrictive (65 °C) and restrictive conditions (80 °C) using the hydroxyapatite method. They used 0.28 M phosphate buffer (equimolar amounts of NaH_2PO_4 and Na_2HPO_4) for hybridizations. There is no formula equivalent to the one used above for calculating the optimal renaturation temperature under these conditions. A rough estimate can be obtained from the formula $T_{OR} = 1.24 T_m - 43.76$, with T_{OR} and T_m in the same salt concentration. This formula is obtained by replacing $T_{m[1SSC]}$ by $(T_{m[2SSC]} - 4)$ in the formula given above (Gillis *et al.*, 1970). T_m can be calculated as $T_m = 0.41 \times \%G + C + 78.7 + 13.3 \log C_{Na}^+$, with C_{Na}^+ the molar concentration of sodium ions (Gillis *et al.*, 1970). In 0.28 M phosphate buffer, an estimate for T_m would thus be 99.9 °C and T_{OR} would be 80.1 °C. If this approximation is valid, it would indicate that the

temperatures used by Hollis *et al.* (1981) were non-stringent (60 °C) and optimal (80 °C). However, their homology values are clearly lower than our own between *Bradyrhizobium* groups I and Ia (Table 2). We cannot explain this discrepancy. In the lower homology range, between *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* there is a better correspondence of values. Scholla *et al.* (1990) used the initial renaturation rate method at 70 °C in $6 \times$ SSC and in the presence of 25% formamide. Using the same formulas as above, under these conditions T_m would be 105.8 °C and T_{OR} would be 62.0 °C. Again, these values should be regarded as a rough estimate only, because the formulas used were originally deduced for the buffer range $0.5\text{--}2 \times$ SSC (Gillis *et al.*, 1970). They indicate that these conditions were close to optimal. The values reported by Scholla *et al.* (1990) between *Bradyrhizobium japonicum* groups I and Ia are similar to our own data using the microplate method (Table 2). Xu *et al.* (1995) used the initial renaturation rate method in $2 \times$ SSC, but the temperature used is not explicitly stated and therefore comparison is not possible. Their results are slightly lower than those obtained with the microplate method (Table 2). Overall, although the different methods used are not always completely comparable, literature data are generally in agreement with our own results.

In conclusion, on the basis of 16S rDNA similarities and total DNA homology values, *Bradyrhizobium elkanii* is clearly a separate species from *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense*. The latter two species are phenotypically distinct, but genotypically related. The fact that other genera (*Afipia*, *Agromonas*, *Blastobacter*, *Nitrobacter* and *Rhodopseudomonas*) are phylogenetically closer to *Bradyrhizobium japonicum* than to *Bradyrhizobium elkanii* leaves the current classification of *Bradyrhizobium* looking rather unsatisfactory. In view of the phylogenetic data, *Bradyrhizobium elkanii* and related strains probably represent a separate genus. The photosynthetic *Bradyrhizobium* strains may represent a further genus, which could also include *Blastobacter denitrificans* and *Agromonas oligotrophica*. However, until a comprehensive phenotypic comparison of these genera becomes available and can provide supportive evidence, it seems most appropriate to maintain the current situation.

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