Bradyrhizobia isolated from root nodules of *Parasponia* (Ulmaceae) do not constitute a separate coherent lineage

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Rhizobial bacteria almost exclusively nodulate members of the families Fabaceae, Mimosaceae and Caesalpiniaceae, but are found on a single non-legume taxon, *Parasponia* (Ulmaceae). Based on their host-range, their nitrogen-fixing ability and strain competition experiments, bacterial strains isolated from *Parasponia* were thought to constitute a separate lineage that would account for their exceptional host affinity. This hypothesis was investigated by focusing on four isolates that are representative of the morphological and cultural types of *Parasponia*-nodulating bradyrhizobia. Their evolutionary relationships with other rhizobia were analysed using 16S rRNA gene sequences and their nodulation properties were explored using the *nodA* gene as a proxy for host-range specificity. Phylogenetic analyses of the 16S rRNA and *nodA* gene sequences revealed that bacterial isolates from *Parasponia* species are embedded among other bradyrhizobia. They did not cluster together in topologies based on the 16S rRNA or *nodA* gene sequences, but were scattered among other bradyrhizobia belonging to either the *Bradyrhizobium japonicum* or the *Bradyrhizobium elkanii* lineages. These data suggest that the ability of some bradyrhizobia to nodulate species of the genus *Parasponia* does not represent a historical relationship that predates the relationship between rhizobia and legumes, but is probably a more recent host switch for some rhizobia.

INTRODUCTION

Rhizobium-like bacteria nodulate almost exclusively members of the families Fabaceae, Mimosaceae and Caesalpiniaceae. The only known non-leguminous plants on which rhizobial bacteria form nodules belong to the genus *Parasponia*, one of 18 genera comprising the Ulmaceae (Trinick, 1973; Akkermans *et al.*, 1978). Species of the genus *Parasponia* are small woody trees up to 15 m high with a tropical distribution that is restricted to the Malay Archipelago (Soepadmo, 1977). They are pioneer plants in mountainous areas of Indonesia, Malaysia and Papua New Guinea (Akkermans & van Dijk, 1981) where they appear indifferent to soil fertility or type (Soepadmo, 1977; Trinick & Hadobas, 1988). Nodulation by rhizobia-like bacteria was first reported on *Parasponia rugosa* Bl. in the highlands of Papua New Guinea (Trinick, 1973). Nodulation has also been described on other species within the genus *Parasponia*

(Trinick, 1976, 1980; Trinick & Hadobas, 1988), but appears to be restricted to the genus (Akkermans *et al.*, 1978).

The *Parasponia*–rhizobia association can be highly effective and levels of nitrogen fixation comparable with those observed in legume–rhizobia symbioses have been detected (Trinick, 1980). Both fast- and slow-growing rhizobia are capable of nodulating *Parasponia* species (Trinick & Galbraith, 1980; Trinick & Hadobas, 1988), but all isolates from field-collected nodules from *Parasponia* species correspond to slow-growing *Bradyrhizobium* species (Trinick, 1976; Trinick & Hadobas, 1988). They form a group distinct from other bradyrhizobia insofar as they differ in their cultural characteristics and host-range and, notably, are generally ineffective in forming associations with legume species (Becking, 1983; Trinick & Hadobas, 1988, 1989). Conversely, most of the legume-derived *Bradyrhizobium* and *Rhizobium* (*Sinorhizobium*/*Ensifer*) strains tested usually form poorly effective or non-effective nodules on various *Parasponia* species (Trinick & Galbraith, 1980; Trinick & Hadobas, 1988) and, even when the association is effective, nodule formation and the onset of nitrogenase activity (nitrogen fixation) are delayed (Trinick, 1988). Based on morphological and cultural criteria, *Bradyrhizobium* strains

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *nodA* gene sequences reported in this paper are AJ920034–AJ920045.

isolated from *Parasponia* species have been classified into five types (Trinick, 1988; Trinick & Hadobas, 1988), but no formal taxonomic or molecular identification has been performed to ascertain the evolutionary origin of bradyrhizobia isolated from *Parasponia*.

Here, we address the question of the evolutionary relationships of *Parasponia*-derived isolates of rhizobia using the 16S rRNA gene as a taxonomic index and the *nodA* gene as a host specificity marker.

METHODS

Rhizobial strains. The four *Bradyrhizobium* strains studied are those referenced in Trinick & Hadobas (1988): NGR 231 (type 1), CP 299 (type 2), CP 315 (type 3) and CP 283 (type 5). For comparison purposes, *nodA* gene sequences were obtained for Australian *Bradyrhizobium* strains BDV5028, BDV5040, BDV5329 and BDV5111 that were nodulating Australian native legumes isolated in south-eastern Australia (Lafay & Burdon, 1998). These correspond to genotypes A, B, H and P, respectively, as designated by Lafay & Burdon (1998).

Small-subunit rRNA gene amplification. Bacterial DNA was prepared following a previously described method (Sritharan & Barker, 1991). Primers corresponding to positions 8–28 and 1498–1509 in the *Escherichia coli* 16S rRNA gene sequence (GenBank accession no. J01695) were used for amplification of the 16S rRNA genes by PCR. PCR was carried out as described by Lafay & Burdon (1998).

***nodA* gene amplification.** PCR products corresponding to nearly complete *nodA* genes were obtained using primers *nodA1f/nodA1r* as described by Chaintreuil *et al.* (2001) following the touch-down procedure used by Hannibal *et al.* (2000).

PCR product sequencing. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.0 cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems) and the products were analysed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sense and anti-sense synthetic primers complementary to conserved eubacterial domains (Lane *et al.*, 1985) were used to sequence both strands of the 16S rRNA gene. Primers used in the amplification reaction were used in the sequencing reactions of the *nodA* genes.

Phylogenetic analyses. The four strains isolated from *Parasponia* species were compared with taxa for which both 16S rRNA and *nodA* gene sequences were available. These included a number of *Bradyrhizobium* strains as well as strains of nodulating taxa outside the *Bradyrhizobium* group. Among these outgroups, two strains have

been shown to be able to nodulate *Parasponia* species, albeit ineffectively. Additionally, sequences most similar to those of the four isolates from *Parasponia* species [assessed by sequence similarity searches against the GenBank database using the MEGA BLAST program on the NCBI BLAST website (<http://www.ncbi.nlm.nih.gov/blast/>)] and sequences for the type strains of each *Bradyrhizobium* species were included in the 16S rRNA gene sequence analyses. Australian *Bradyrhizobium* isolate WU425 and strain ANU289 (a streptomycin-resistant derivative of the *Parasponia* *Bradyrhizobium* strain CP 283) for which no 16S rRNA gene sequences were available were included in the *nodA* gene phylogeny reconstruction only. For both 16S rRNA and *nodA* genes, sequences were aligned manually. In the case of *nodA*, aligned translated amino acid sequences served as a guide to place gaps in the DNA sequences with respect to the codon structure of the sequences. Nearly full-length 16S rRNA gene or *nodA* gene aligned sequences were used in the analyses. For the former, the extreme 5'- and 3'-ends of the alignment were excluded, leaving 1410 nucleotides. For the latter, 594 nucleotide sites were included in the analysis with only the extreme 5'-ends being omitted, as their high variability prevented unambiguous alignment between taxa. Phylogenetic analyses were performed using PHYML (Guindon & Gascuel, 2003). Maximum-likelihood phylogenies were reconstructed using the generalized time reversible model of nucleotide substitution and a discrete gamma distribution to account for variable substitution rates among sites with eight rate categories. Nucleotide frequencies, nucleotide change rate and gamma distribution shape parameters were estimated from the data. The starting tree was obtained using BIONJ. Five hundred bootstrap replications were performed to assess confidence in the topologies.

RESULTS AND DISCUSSION

We obtained sequence data for the 16S rRNA and *nodA* genes of four *Bradyrhizobium* strains isolated from *Parasponia* corresponding to four of the five types described by Trinick & Hadobas (1988). Strain CC330, representative of the fifth type (type 4) defined by Trinick and Hadobas, has been lost and was not available from any culture collection.

Molecular identification based on 16S rRNA gene sequence comparisons confirmed that the four bacterial isolates capable of nodulating the non-legume genus *Parasponia*, CP 283, CP 299, CP 315 and NGR 231, belong to the genus *Bradyrhizobium* (Fig. 1). However, in contrast to previous assumptions (Trinick & Hadobas, 1988, 1989), these isolates do not constitute a separate lineage that accounts for their exceptional host affinity. Indeed, our results show that the four isolates representing the morphological and cultural types of *Parasponia*-nodulating bradyrhizobia (Trinick,

Fig. 1. Phylogenetic characterization of bradyrhizobia isolated from the root nodules of *Parasponia* species (bold) based on (a) the 16S rRNA gene and (b) the common nodulation *nodA* gene. Taxa indicated by filled diamonds are capable of nodulating *Parasponia* species. Taxa highlighted by open diamonds were isolated from root nodules of Australian-native legume species. The three major divisions among bradyrhizobia, *B. japonicum*, *B. elkanii* and photosynthetic bradyrhizobia, are indicated by J, E and P, respectively. The identification of the *nodA* clusters follows that of Moulin *et al.* (2004). The bradyrhizobia 16S rRNA gene tree was rooted using *Methylobacterium nodulans* ORS 2060^T (GenBank accession number AF220763), *Mesorhizobium huakuii* bv. *loti* MAFF303099 (BA000012), *Rhizobium etli* CFN 42^T (U28916), *Ensifer meliloti* 1021 (AL591688), *Ensifer* sp. NGR 234 (AJ301628), *Ensifer fredii* USDA 257 (AY260150), *Azorhizobium caulinodans* ORS 571^T (D11342), *Cupriavidus taiwanensis* LMG 19424^T (AF300324), *Burkholderia caribensis* TJ182 (AJ505301) and *Burkholderia tuberum* STM678^T (AJ302311) as outgroups (not shown). Percentage bootstrap supports for internal branches based on 500 replications >50 are shown. Bar, 0.01 (a) or 0.1 (b) substitutions per site.

(a) 16S rRNA gene

(b) *nodA*

1988; Trinick & Hadobas, 1988) can be separated into *Bradyrhizobium japonicum*-related and *Bradyrhizobium elkanii*-related genospecies. Isolates in the former group (NGR 231, CP 299 and CP 315) are as closely related to one another as they are to any species in that group, whereas the 16S rRNA gene sequence of the remaining isolate (CP 283) is virtually identical to the sequence of a number of *Bradyrhizobium* strains (e.g. strain tpma1.5 in our phylogeny). Hence, according to this analysis of variation in the 16S rRNA gene, which remains to date one of the most reliable indices for organism identification and classification (Woese, 2000), *Bradyrhizobium* strains derived from *Parasponia* are indistinguishable from those derived from a range of legume species.

What separates the former strains is that they were first isolated from nodules on the roots of the only non-legume host known to associate symbiotically with rhizobia. This raises the question as to how these bradyrhizobia acquired the ability to nodulate the exceptional host of *Parasponia* species. This property is not restricted to bradyrhizobia isolated from this host. A number of bradyrhizobia isolated from legumes have formed symbiotic associations with species of the genus *Parasponia* in glasshouse experiments (Trinick & Hadobas, 1989). Thus, irrespective of how well they form such associations under natural conditions, they do possess the relevant genetic determinants. Such determinants also appear to exist outside the genus *Bradyrhizobium*. Particularly well-studied examples are found in *Ensifer* (formerly *Sinorhizobium*) sp. NGR 234, isolated from *Lablab purpureus* nodules in Papua New Guinea (Trinick, 1980), and *Ensifer fredii* USDA 257, isolated from *Glycine soja* in China (Keyser *et al.*, 1982). These bacteria are characterized by exceptionally broad host-ranges (including the genus *Parasponia*), a state which is thought to constitute an ancestral feature in rhizobial evolution (Pueppke & Broughton, 1999).

In rhizobia, host-range is determined by the 'nodulation genotype', i.e. the combination of *nod* genes present in the chromosomal or plasmid genomes (Ueda *et al.*, 1995; Dénarié *et al.*, 1996; Mergaert *et al.*, 1997). Upon activation by plant flavonoids, the products of these genes interact to synthesize lipo-chitooligosaccharides, the Nod factors, which act as signals for plant tissue to differentiate and initiate nodule formation (Dénarié *et al.*, 1996; Esseling & Emons, 2004). Overall, host-range and nodulation gene relationships do not correlate with rhizobial phylogeny (Young & Johnston, 1989; Dobert *et al.*, 1994; Young & Haukka, 1996), suggesting that horizontal transfer has played a significant role in the evolution of rhizobial symbiosis (Mergaert *et al.*, 1997; Suominen *et al.*, 2001). Indeed, a closer examination of nodulation gene phylogenies suggests a multi-scale evolutionary history where geographical isolation, historical descent and lateral transfer interplay with differing intensities depending on the rhizobial group under consideration (Haukka *et al.*, 1998; Wernegreen & Riley, 1999; Moulin *et al.*, 2004).

Under a lateral transfer hypothesis, isolates from members of the genus *Parasponia* should exhibit closely related, if not identical, nodulation genes despite their divergent taxonomic positions. We investigated whether isolates from *Parasponia* species possessed a unique type of *nodA* gene. This gene belongs to the class of common *nod* genes which are found in all nodule-forming bacteria (Dénarié *et al.*, 1996) and is more specifically involved in host-range determination (Ritsema *et al.*, 1996; Roche *et al.*, 1996). Whereas no such data had yet been obtained for any of the four types of isolates from *Parasponia* species, the sequence of *nodA* was available for strain ANU289, a streptomycin-resistant derivative of strain CP 283 (Scott, 1986) (Fig. 1). We therefore expected CP 283 and ANU289 *nodA* gene sequences to be identical and indeed they almost were, grouping together within the *nodA* cluster III that includes the vast majority of bradyrhizobia sequences (Moulin *et al.*, 2004). We found a single discrepancy at positions 235–236 where the strain CP 283 dinucleotide was GC instead of CG in the reported sequence for the strain ANU289 *nodA* gene. The discrepancy probably arose from a sequencing error, since all other available *nodA* gene sequences possess GC in these positions.

The *nodA* gene sequences of the three remaining isolate types from *Parasponia* species were quite different from that of strain CP 283, although amongst themselves they exhibited a high level of gene sequence similarity, with strain NGR 231 and strain CP 315 *nodA* gene sequences differing by a single nucleotide at position 392 relative to the full-length bradyrhizobial-like *nodA* gene sequence (corresponding to an amino acid difference at position 127 between the translated protein sequences). They also differed from the closely related *nodA* genes obtained for two broad-host-range *Ensifer* strains that induce the formation of ineffective nodules on *Parasponia* species. Compared with a representative set of *nodA* gene sequences, they were found, nevertheless, to be bradyrhizobial in type and to form a well-supported phylogenetic group with sequences obtained from our four Australian isolates (isolated from native legumes) as well as two strains isolated elsewhere in the world from Australian-native *Acacia* species (Fig. 1). These latter two isolates formed a separate lineage (cluster I) in a study of bradyrhizobia *nodA* gene phylogeny (Moulin *et al.*, 2004). Strains NGR 231 and CP 315 form a separate group diverging at the base of this cluster, whereas strain CP 299 branches well within that cluster, with its *nodA* gene being most similar to those of the six legume-derived strains constituting that group. The *nodA* genes of the bradyrhizobia within this cluster are characterized by an additional nucleotide triplet (TTG or GTG), which translates into an additional amino acid (Leu or Val) at position 184 in the corresponding NodA protein sequence. Interestingly, the only other available *nodA* gene sequence obtained from an Australian isolate (strain WU425, isolated from a non-Australian legume species) did not possess this molecular signature and grouped within *nodA* gene cluster II as defined by Moulin *et al.* (2004).

Whereas lateral transfer may explain the near identity of the *nodA* genes of strains NGR 231 and CP 315, it cannot account for all the available data regarding nodulation of *Parasponia* species. This suggests that non-legume nodulation probably represents a plesiomorphic character among rhizobia. Strain CP 283 provides some evidence of this. In addition to the respective phylogenetic positions of its 16S rRNA and *nodA* genes, strain CP 283 stands apart from other isolates obtained from *Parasponia* species because of its ability to nodulate a wide range of tropical legumes effectively that are normally nodulated by slow-growing root-nodule bacteria. In contrast, the other isolates appear to be more specialized towards nodulation of *Parasponia* species (Trinick & Hadobas, 1988). Consequently, Trinick & Hadobas (1988) proposed that this strain nodulated *Parasponia* species by accident in the presence of other more competitive legume-nodulating strains. This hypothesis is further supported by data obtained for strains isolated from legume nodules. *Ensifer* sp. strain NGR 234 and *Ensifer fredii* USDA 257, both of which are capable of nodulating species of the genus *Parasponia*, have rhizobial-like *nodA* genes only remotely related to those of the novel *Parasponia* isolates. Most of the bradyrhizobial strains isolated from legume hosts and capable of nodulating *Parasponia* species studied by Trinick & Hadobas (1989) were from Papua New Guinea, but others were from Brazil, USA and Zimbabwe. Human-mediated introduction of these strains cannot be ruled out, but it is most likely that the ability to nodulate species of the genus *Parasponia* is present in the genomes of isolates outside the host's geographical range.

Host-range specificity does not generally correlate with nodulation genotypes and, even though *nod* genes are crucial to the nodulation process, other elements participate in the specification of the host-range of rhizobia. In regard to the existence of legume-nodulating rhizobia capable of nodulating *Parasponia* species, our results suggest that the nodulation of *Parasponia* species constitutes an accident in the life history of primarily legume-nodulating rhizobial lineages. Such events probably occur because of host plant predisposition to symbiosis and nodulation. Insight into the evolution of this process requires a dual approach, combining an investigation of the dispersal and biogeography of rhizobia populations in order to discriminate between the effects of ancestry and host transfer, and an in-depth exploration of the host side of interspecies communication.

ACKNOWLEDGEMENTS

We are grateful to Dr M. J. Trinick for providing us with the *Parasponia*-derived *Bradyrhizobium* isolates characterized in this study and to Dr John Brockwell for critical reading of the manuscript.

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