ORIGINAL ARTICLE

Insights learned from pBTAi1, a 229-kb accessory plasmid from *Bradyrhizobium* sp. strain BTAi1 and prevalence of accessory plasmids in other *Bradyrhizobium* sp. strains

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In silico, physiological and *in planta* analyses were used to characterize pBTAi1, a 229-kb accessory plasmid from *Bradyrhizobium* sp. strain BTAi1, and assess its potential ecological function under free-living and symbiotic growth conditions. Sequence analysis revealed the presence of an uptake hydrogenase system, a *repABC* family plasmid replication module and open reading frames encoding type IV secretion system, TraI and TraR autoinducer proteins and several copper resistance-related proteins. *Bradyrhizobium* sp. BTAi1 was capable of growing in 200 mg l^{-1} CuCl$_2$. In contrast, the closely related, plasmid-free *Bradyrhizobium* sp. strain ORS278 could not grow at copper concentrations exceeding 100 mg l^{-1}. The plasmid-localized hydrogenase genes were phylogenetically distinct from those typically found in other rhizobial species, and were most related to *hup* genes from *Thiobacillus denitrificans*. The induction of the plasmid-borne hydrogenase genes during symbiosis was significantly lower than the two chromosomal-borne hydrogenase clusters. CHEF-pulsed-field gel electrophoresis was used for a comprehensive analysis of the diversity, abundance and genetic composition of accessory plasmids in other *Bradyrhizobium* strains. Plasmids were detected in 11 of 46 (23.9%) geographically diverse *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains, isolated from the United States, China and Thailand. Plasmid size was heterogeneous, ranging from 75 to 330 kb, with only two strains (DASA01244 and DASA01265) harboring plasmids with identical (240 kb) size. None of the plasmids harbored nodulation or hydrogenase genes. Taken together, our results indicate that while plasmids having ecologically significant functions may be detected in *Bradyrhizobium* sp. strains, they lack genes necessary for symbioses with legumes.
The ISME Journal (2008) **2,** 158–170; doi:10.1038/ismej.2007.105; published online 24 January 2008

Introduction

The primary source of biologically-fixed nitrogen (N$_2$) in agricultural systems is produced via the symbiotic interaction between legumes and the root- and shoot-nodulating soil bacteria, collectively referred to as the rhizobia. The rhizobia can be divided into three broad groups based on their speed

of growth on laboratory media: the fast growers, which consist of strains in the genera *Rhizobium*, *Sinorhizobium* and *Azorhizobium*; the intermediate growth rate strains in the genus *Mesorhizobium* and the slow growers, which comprise strains in the phylogenetically distinct genus *Bradyrhizobium* (Sawada *et al.*, 2003).

Fast- and slow-growing rhizobia are also unique with respect to the location of genes involved in nodulation and symbiotic nitrogen fixation. Generally speaking, the structural genes for the nitrogenase enzyme (*nifHDK*) and genes essential for the production of lipochitooligosaccharide nodulation factors (*nodABC*) in the fast-growing species are located on large, high-molecular weight,

plasmids (pSym; Merrick and Edwards, 1995). Plasmids can vary significantly between and within species of rhizobia, ranging from 180 kb in *Rhizobium leguminosarum* bv. *trifolii*, to 1.35 Mb in *Sinorhizobium meliloti* (Mercado-Blanco and Toro, 1996; Barnett *et al.*, 2001). In contrast, genes encoding nod factors and nitrogenase in *Bradyrhizobium* species and most mesorhizobia are located on the chromosome in regions termed 'symbiosis islands' (Sawada *et al.*, 2003). In the soybean symbiont *Bradyrhizobium japonicum*, symbiosis-related and hydrogenase genes are localized to on a 611-kb chromosomal-borne symbiosis island that displays a distinct distribution of insertion sequence elements relative to the rest of the chromosome (Gottfert *et al.*, 2001; Kaneko *et al.*, 2002). Interestingly, while *Mesorhizobium loti* has a chromosomal-borne symbiosis island, the moderately slow-growing *Mesorhizobium amorphae* was found to carry a 930-kb pSym (Wang *et al.*, 1999).

Several *Rhizobium* and *Bradyrhizobium* species contain uptake hydrogenases, which reduce energy loss associated with symbiotic nitrogen fixation (Albrecht *et al.*, 1979; Evans *et al.*, 1987). Generally, the hydrogen uptake (*hup*) genes are arranged in at least three operons with conserved gene composition and organization (Baginsky *et al.*, 2002). The *hupS* and *hupL* genes encode for hydrogenase structural subunits, whereas the remaining *hup* and *hyp* gene products encode for maturation proteins, which are responsible for the recruitment and incorporation of nickel and other metals into the hydrogenase active site (Casalot and Rousset, 2001). While the *R. leguminosarum* and *B. japonicum* hydrogenase systems are highly homologous, they differ in gene regulation and in the presence of specific genes. In *Rhizobium tropici* and *R. leguminosarum* the *hup* genes are encoded on the symbiotic plasmid, whereas in *B. japonicum*, *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*), the *hup* genes have been presumed to be on the chromosome, since no plasmids could be detected in the strains examined by using standard plasmid isolation procedure (Baginsky *et al.*, 2002; Brito *et al.*, 2005).

In addition to symbiotic plasmids, several rhizobial strains also contain one or more smaller, accessory plasmids, also termed non-symbiotic plasmids (Mercado-Blanco and Toro, 1996; Gonzalez *et al.*, 2006; Stiens *et al.*, 2006; Young *et al.*, 2006). Some of these plasmids have been shown to transfer between populations of indigenous rhizobia (Mercado-Blanco and Toro, 1996). The acquisition of some accessory plasmids has been correlated with enhanced symbiotic performance (Martínez *et al.*, 1987), while others have been shown to enhance growth under specific environmental conditions (Mercado-Blanco and Toro, 1996). Both symbiotic and accessory plasmids require replication control mechanisms that ensure proper segregation and efficient maintenance of the low-copy number

DNA replicons in their host bacterium (Ramirez-Romero *et al.*, 2000).

In contrast to the significant number of reports of plasmids in the fast-growing rhizobia, there have been relatively few studies concerning the presence of plasmids in *Bradyrhizobium* strains, perhaps due to methodological limitations. While a few studies have reported the presence of two to four plasmids in *B. japonicum* strains, ranging in size from 74 to 294 kb (Gross *et al.*, 1979; Masterson *et al.*, 1982), the taxonomic status of some of the strains was not verified using molecular, phylogenetic tools (Gross *et al.*, 1979; Masterson *et al.*, 1982). Although Masterson *et al.* (1982) showed that *nod* genes were not found on any plasmids they detected, no information was presented regarding the genetic composition of these plasmids, and thus the physiological role of plasmids in bradyrhizobia could not be inferred. In addition, due to changes in the taxonomy of soybean-nodulating bacteria, some strains have since been reclassified as belonging to *Sinorhizobium fredii*, a fast-growing species. To date, the functional role of plasmids in the bradyrhizobia has not been explored, and until now (Giraud *et al.*, 2007) there have been no reports on the complete sequence and analysis of plasmids from any *Bradyrhizobium* strain.

The *Bradyrhizobium* strain BTAi1 belongs to a novel group of phototrophic bradyrhizobia that form a symbiotic association with several species in the legume genus *Aeschynomene*. Members of this bacterial group although taxonomically related to other bradyrhizobia, are unique in their ability to couple photosynthesis to nitrogen fixation (Giraud and Fleischman, 2004). Recently, it was reported that *Bradyrhizobium* sp. strain BTAi1, and other bradyrhizobia capable of forming nodules on *Aeschynomene indica*, are taxonomically related to *Blastobacter denitrificans*, and it has been proposed that the later bacterium be transferred to the genus *Bradyrhizobium* (van Berkum *et al.*, 2006). To date, however, *Bradyrhizobium* strains capable of nodulating *Aeschynomene* have not been given specific species status.

A comprehensive description of the structural and functional genomics of strain BTAil was recently described, revealing the presence of a 229-kb accessory plasmid (Giraud *et al.*, 2007). The presence of plasmids in *Bradyrhizobium* sp. strains capable of nodulating *Aeschynomene* appears not to be a common feature of this group of bacteria, as no plasmid was identified in the closely related strain ORS278 (Giraud *et al.*, 2007). In this study we used *in silico*, physiological and *in planta* analyses to understand the mode of regulation, phylogenetic affiliation and potential environmental role of the accessory plasmid pBTAi1 from *Bradyrhizobium* sp. strain BTAi1. This is, to our knowledge, the first comprehensive sequence analysis of a plasmid from a member of the genus *Bradyrhizobium*. We also report here on the presence of other accessory

plasmids in genetically diverse *B. japonicum* and *B. elkanii* strains isolated from three geographically distinct regions of the world. These plasmids, however, do not contain symbiosis-related nodulation and uptake hydrogenase genes.

Materials and methods

Bacterial strains, media and growth conditions
The strains used in this study are listed in Table 1. All *B. japonicum* strains were re-isolated from soybean nodules by plant infection assay (Somasagaran and Hoben, 1994), and streaked several times for single colonies onto yeast extract-mannitol agar medium containing either bromthymol blue or congo red as indicators (Somasagaran and Hoben, 1994). All strains were grown at 30 °C.

Sequence analysis of nearly full-length 16S rDNA was performed to determine the species status of the Thai *Bradyrhizobium* strains used in these studies. Polymerase chain reaction was performed using 16S rRNA gene primers 27F, 5′-AGAGTTTGATCMTGGC TCAG-3′, and 1525R, 5′-AAGGA GGTGWTCCARCC-3′ (Lane, 1991). The following reaction conditions were used: 95 °C for 5 min, followed by 30 cycles consisting of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. After final extension at 72 °C for 5 min, PCR reaction mixtures were stored at 4 °C. Electrophoresis was performed at 100 V for 2.5 h. DNA fragments about 1.5 kb in size were recovered from gels, purified using a QIAquick Kit (Qiagen, Valencia, CA, USA) and directly used as templates for sequencing using primers 27F and 1525R. Sequences of 16S rDNA were aligned and identified by using the BLASTN algorithm (http://www.ncbi. nlm.nih.gov/BLAST/).

Preparation of high-molecular-weight total of genomic DNA
High-molecular-weight total genomic DNA from *Bradyrhizobium* strains was isolated using an embedded agarose method (Birren and Lai, 1993). Strains were cultivated in Arabinose-Gluconate (AG) medium (Sadowsky *et al.*, 1987) to OD_{600} of about 0.5. Cells were harvested by centrifugation at 8000 g at 4 °C, washed twice with TE buffer (20 mM Tris and 50 mM ethylenediamine tetraacetic acid, pH 8.0) and resuspended in TEN buffer (10 mM Tris, 20 mM NaCl and 50 mM ethylenediamine tetraacetic acid, pH 7.2) to an OD_{600} of 0.6–0.7. Cell suspensions were combined with an equal volume of 2% (w/v) low-melting-point SeaPlaque GTG Agarose (BMA, Rockland, ME, USA) prepared in TE buffer containing 1% sodium dodecyl sulfate, and plugs were prepared using a plug mold (BioRad, Hercules, CA, USA). Plugs were treated with Lysozyme buffer, Proteinase K buffer, and wash buffer containing 1 mM phenylmethyl sulforyl fluoride (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 30 min as previously described (Birren and Lai, 1993). The agarose plugs were stored in TE buffer at 4 °C until use.

Plasmid profiling
Plasmid profiles were obtained using a combination of S1-nuclease digestion and pulsed-field gel electrophoresis (PFGE)-contour clamped homogenous electric field (CHEF) as previously described (Sajjaphan *et al.*, 2006). DNA plugs were incubated at 37 °C for 10 min with 5 U of *Aspergillus oryzae* S1 nuclease (Invitrogen, Carlsbad, CA, USA) as described (Barton *et al.*, 1995). PFGE-CHEF was performed using a CHEF-DRII apparatus (BioRad) and gels were run for 22 h at 14 °C in $0.5 \times$ TBE buffer at 6 V cm^{-1} (45 mM Tris base, 45 mM boric acid and 1 mM ethylenediamine tetraacetic acid, pH 8.0), with switch times ramped from 1.0 to 40.0 s. A λ-PFG ladder (New England Biolabs, Ipswich, MA, USA) served as the molecular weight marker. Following electrophoresis, gels were stained for 20 min with ethidium bromide (1 μg ml^{-1}) in TBE buffer, destained for 30 min in distilled water and plasmid bands were visualized using a UV transilluminator.

Southern hybridization analyses
Cosmid pR32 (Sadowsky *et al.*, 1991), which was used as a nodulation (*nod*) gene probe, was isolated as described by Itoh *et al.* (1984). Plasmid pR32 was digested to completion with *Hind*III, releasing the nodulation genes *nolA*, *nodD2*, *nodD1YABC*, *nodSUIJ* and *nodZ* and 50 ng of probe DNA was

Table 1 *Bradyrhizobium* strains used in this study

Strains	Source
USDA 4, USDA 46, USDA 76[a], USDA 94, USDA 110, USDA 122, USDA 135, USDA 138, USDA 146. USDA 148, USDA 162, USDA 170, USDA 177, USDA 181, USDA 183, USDA 222, USDA 224, USDA 225, USDA 227, USDA 231, USDA 235, USDA 241, USDA 249, USDA 254	USDA[b]
THAI (USDA 325)[c], THA2 (USDA 326), THA3 (USDA 327), THA4 (USDA 328), THA5 (USDA 329), THA6 (USDA 330), THA7 (USDA 313), DASA01148, DASA01150, DASA01152, DASA01155, DASA01156, DASA01182, DASA01196, DASA01207, DASA01214, DASA01238, DASA01244, DASA01264, DASA01264, DASA01265, DASA01304, DASA01311	DOA[d]
BTAi1	IRD-INRA[e]

[a]USDA 76 is type strain for *B. elkanii* = ATCC 49852.
[b]United States Department of Agriculture, Beltsville, MD, USA.
[c]Numbers is parentheses refer to strain synonyms.
[d]DOA, Thailand Department of Agriculture, Bangkok.
[e]Institut de Recherche pour le Développement and Institut National de la Recherche Agronomique, Montpellier, France.

[32]P-labeled using the Rediprime II random prime labeling system (Amersham Biosciences, Piscataway, NJ, USA). DNA from *B. japonicum* USDA110 served as the positive control for all hybridization reactions. Total genomic DNA was isolated as previously described (Sadowsky *et al.*, 1987), digested with *Eco*RI and separated by agarose gel electrophoresis as described (Sadowsky *et al.*, 1991). Intact (undigested) plasmid profiles obtained from PFGE-CHEF were transferred to Nytran immobilization membranes (Schleicher&Schuell, Keene, NH, USA) and hybridized to probes as previously described (Sadowsky *et al.*, 1987).

Plasmid sequencing and phylogenetic analyses

Genomic DNA was isolated from *Bradyrhizobium* sp. BTAi1 as previously described (Sadowsky *et al.*, 1987), and sequencing was performed at the DOE Joint Genome Institute (Giraud *et al.*, 2007). Genes on pBTAi1 were annotated using the Mage Microbial Genome Annotation System (Vallenet *et al.*, 2006) as previously described (Giraud *et al.*, 2007). Plasmid sequence data have been deposited in GenBank under accession number NC_009475.

Amino-acid (AA) sequences were aligned by using ClustalW (http://align.genome.jp/), and bootstrap analyses and phylogenetic trees were generated using the maximum likelihood method from the Phylip phylogenetic package (Felsenstein, 1989).

Plant assays

A. indica seeds were surface sterilized in concentrated sulfuric acid for 1 h, thoroughly rinsed and soaked overnight in sterile water and seeds were transferred to water agar (8 g l^{-1}) plates for 20 h at 35 °C for germination. Plants were grown in Leonard jar assemblies, two plants per jar, as previously described (Sadowsky *et al.*, 1995) and watered with sterile N-free plant nutrient solution (Summerfield *et al.*, 1977). Plants roots were inoculated with 1 ml of approximately 10^8 cells per ml of *Bradyrhizobium* sp. strain BTAi1. Seeds were covered with a 1-cm layer of sterilized paraffin-coated sand (Sadowsky

et al., 1995) and plants were grown 30–45 days in a growth chamber, with 16 h of light and day and night temperatures of 28 and 23 °C, respectively. After three weeks of growth, plant stems were inoculated by carefully applying ∼10^8 cells in sterile water to the stems using a sterile cotton-swab applicator.

RNA extraction, reverse transcription and quantitative real-time PCR

RNA was extracted from AG-grown, log-phase *Bradyrhizobium* sp. BTAi1 cultures and *A. indica* root and stem nodules using the RNeasy purification kit (Qiagen) according to the manufacturer's instructions, with slight modification. RNA from nodule tissue (0.2 g) was extracted using glass beads and lysosyme solution (0.5 mg ml^{-1}) and a Fast Prep bead beating system (Bio101, Vista, CA, USA).

Reverse transcription was performed using 0.5 µg of total RNA, 1 mM dNTP's, 1 × first-strand buffer, 15 mM DTT and 400 units Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reactions were incubated for 3 h at 42 °C followed by an inactivation step of 65 °C for 20 min. RNA samples were treated with RNAse-free DNAse before reaction. Quantitative real-time PCR of the three BTAi1 hydrogenases, under both free-living and symbiotic *in planta* conditions, was performed using the primer sets listed in Table 2. Specificity of the primer sets was confirmed using negative controls consisting of DNA extracted from the phylogenetically similar *Bradyrhizobium sp.* strain ORS278 and from *A. indica* tissue. Primers targeting the *hupL* genes for each of the three *Bradyrhizobium* sp. BTAi1 hydrogenase clusters, and the housekeeping gene *parA* (BBta_0167), were designed using the online version of Primer 3 software (Rozen and Skaletsky, 2000). Each 25-µl reaction contained 0.5 µg of total cDNA, 8.75 µl dH$_2$O, 12.5 µl *Power* SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.25 µg µl^{-1} BSA and 0.2 µM each of forward and reverse gene-specific primers. The quantitative real-time PCR reactions were run on an Applied Biosystems 7500 real-time PCR system,

Table 2 Quantitative real-time PCR primers used in this study

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Target and location
BBta_0167F	ACCTGTCCAATCAGGTCGTC	165	Chromosome-partitioning protein A (*parA*)
BBta_0167R	CGAGCTTCAGATAGGCTTCG		
BBta_1997F	ATCGAGTTCGTGCAGAAGGT	188	*hupL* (typical, chrom type II)
BBta_1997R	AGATTGCCGTTGAGGATCAC		
BBta_0461F	GATCATTACGCCAACGACCT	156	*hupL* (atypical, chrom type I)
BBta_0461R	CAGACGAGGCACGAGTCATA		
BBta_p0179F	TTCGTACACGACCACGTCAT	206	*hupL* (plasmid)
BBta_p0179R	GTAAAGATGCCGAGCTGACC		

Abbreviation: PCR, polymerase chain reaction.

using Sequence Detection System software (version 1.3; Foster City, CA, USA). The PCR program was as follows: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Expression values for three biological replicates for each treatment were normalized to the expression level of the BTAi1-encoded *parA* gene. The expression of *parA* in BTAi1 was previously shown to be stable under a variety of physiological conditions (Chang *et al.*, unpublished observations; Cytryn *et al.*, unpublished observations).

Hydrogenase activity

Hydrogenase activity was determined using a modification of a previously described method (Keyser *et al.*, 1982). Fresh root or stem nodules (0.2–0.3 g) were homogenized in 5 ml HEPES buffer, pH 7.0, placed in 20 ml glass vials sealed with butyl rubber stoppers, and H_2 was injected into the bottles to a final concentration of 5% (v/v). Bottles were incubated at 30 °C and hydrogen concentration in the headspace was periodically measured using a Hewlett-Packard 5890 Series II gas chromatograph containing a 6 ft stainless steel, 1/8th inch inner diameter, 5A matrix 60/80 Molecular Sieve column (Supelco, Bellefonte, PA, USA) and a thermal conductivity detector. Hydrogenase activity was determined by measuring the rate at which the injected H_2 was consumed in test vials in relation to control vials without added nodule tissue.

Results and discussion

Sequence analysis of accessory plasmid pBTAi1 from Bradyrhizobium *sp. strain BTAi1*

Complete sequencing of the *Bradyrhizobium* sp. strain BTAi1 genome revealed the presence of a circular plasmid, pBTAi1. The plasmid was 228 826 bp in length, had a mean $G + C$ content of 60.71% (compared with 64.9% for the *Bradyrhizobium* sp. strain BTAi1 chromosome) and contained 257 predicted open reading frames (ORFs), of which 66.2% (174) are presently characterized as encoding hypothetical proteins. The plasmid contained 29 pseudogenes, and seven and 10 putative insertion elements and transposons, respectively. A full description of all ORFs present on pBTAi1 is shown in Supplementary Table 1.

The circular map of plasmid pBTAi1 is shown in Figure 1. Six principal functional regions on pBTAi1 were defined based on AA identity and synteny to known genes in other microorganisms. The functional characterization of genes in these regions and the closest identity of corresponding ORFs in other microorganisms are summarized in Table 3. Region I (from nucleotides 192 to 3864) contained a *repABC* gene cluster and region II (nucleotides 34 100–38 204) encoded for a probable ABC-type AA transport system. Genes likely involved in heavy metal and copper resistance (nucleotides 84 302–
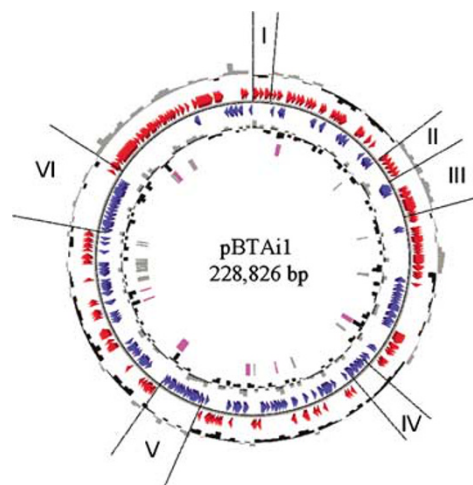

Figure 1 Circular map of the *Bradyrhizobium* sp. strain plasmid pBTAi1. Functional regions are as described in Table 3. Regions: I, a RepABC replicon module; II, AA transport and metabolism proteins; III and IV, copper and metal resistance proteins; V, uptake hydrogenase and accessory proteins and VI, type IV secretion system and TraI/R quorum-sensing plasmid transfer system. The two circles outside and inside the red and blue arrows indicate the GC skew (positive and negative) of the genome regions transcribed in the clockwise and counterclockwise directions, respectively. Red and blue arrows represent predicted ORFs transcribed in the clockwise and counterclockwise directions, respectively. Pink and gray rectangles in the inner circle represent transposable elements and pseudogenes, respectively. AA, amino acid; ORF, open reading frame.

89 201) were identified in regions III and IV, and region V (nucleotides 126 219–136 790) contained a gene cluster encoding an uptake hydrogenase. The region VI (nucleotides 180 987–192 253) contained genes encoding a type IV secretion system, the synthesis of autoinducer and the transcriptional activator proteins TraI and TraR, respectively. Details of principal genes in these regions are presented below.

The repABC replicon of plasmid pBTAi1

The ORFs BBta_p0001 to BBta_p0003 encode for a RepABC-type replicon. These three ORFs form a monophyletic cluster with the *repABC* genes from a diversity of previously described α-proteobacterial plasmids. The *repA* and *repB* genes encode homologues of the ParAB family of replicon partitioning proteins (Bignell and Thomas, 2001), and *repC* is thought to encode for the replication initiation (replicase) protein (Ramirez-Romero *et al.*, 2001). The pBTAi1 *repA* and *repB* gene products each shared 72% AA identity to *repA* and *repB* from the *Agrobacterium rhizogenes* plasmid pRi1724 (accession number AP002086), and *repC* shared 89% AA identity to the *repC* gene of plasmid X14 (accession number YP_571716) from *Nitrobacter hamburgensis*. A large A + T-rich intergenic sequence, commonly found in other rhizobial plasmids, was found between *repB* and *repC* on the pBTAi1 plasmid

Table 3 Principal functional regions of plasmid pBTAi1

Gene ID	Functional region	Gene name	Closest AA identity (%)	Functional role
BBta_p0001	I	Putative replication protein A	*A. rhizogenes* pRi1724 (72%)	*repABC* replicon module
BBta_p0002		Putative replication protein B		
BBta_p0003		Putative replication protein C	*N. hamburgensis* plasmid 2 (89%)	
BBta_p0051	II	Cystathionine β-lyase	BTAi1 chromosome (43%)	Amino-acid transport and metabolism
BBta_p0052		General L-amino-acid-binding protein	BTAi1 chromosome (44%)	
BBta_p0053		Amino-acid ABC transporter permease	BTAi1 chromosome (37%)	
BBta_p0054		Amino-acid ABC transporter permease	BTAi1 chromosome (43%)	
BBta_p0055		L-Amino-acid transport ATP binding	BTAi1 chromosome (76%)	
BBta_p0056	III	Copper-transporting P-type ATPase	*N. hamburgensis* plasmid 2 (45%)	Copper resistance
BBta_p0058		HP, putative signal/membrane protein	*N. hamburgensis* plasmid 2 (45%)	
BBta_p0059		HP, putative signa protein	*N. hamburgensis* plasmid 2 (45%)	
BBta_p0060		*hylD* secretion protein	*N. hamburgensis* plasmid 2 (45%)	
BBta_p0061		*CzcA*-like copper (silver) drug transporter	*N. hamburgensis* plasmid 2 (45%)	
BBta_p0062		Sigma-24 ECF-type σ-factor	*N. hamburgensis* plasmid 2 (45%)	
BBta_p0114	IV	Copper-resistance protein D	*R. palustris* BisB5 (57%)	Copper resistance
BBta_p0115		Copper-resistance protein C	*R. palustris* BisB5 (62%)	
BBta_p0116		Putative cation efflux system protein	*R. palustris* BisB5 (60%)	
BBta_p0117		Copper-resistance protein A	*R. palustris* BisB5 (81%)	
BBta_p0118		Copper-resistance protein B	*R. palustris* BisB5 (71%)	
BBta_p0170	V	Hydrogenase expression/formation	*T. denitrificans* (41%), cBTAi1[a] (25%)	Hydrogenase gene cluster
BBta_p0171		*hypE*—hydrogenase expression/ formation	*T. denitrificans* (63%), cBTAi1 (48%)	
BBta_p0172		*hypF*—hydrogenase maturation protein	*T. denitrificans* (50%), cBTAi1 (41%)	
BBta_p0173		*hupD*—hydrogenase maturation protease	*T. denitrificans* (51%), cBTAi1 (41%)	
BBta_p0174		*hypD*—hydrogenase expression/ formation	*T. denitrificans* (65%), cBTAi1 (38%)	
BBta_p0175		*hypB*—hydrogenase nickel incorporation	*T. denitrificans* (55%), cBTAi1 (0%)	
BBta_p0176		*hypA*—hydrogenase nickel incorporation	*T. denitrificans* (46%), cBTAi1 (28%)	
BBta_p0177		*hupF*—hydrogenase expression/ formation	*T. denitrificans* (61%), cBTAi1 (37%)	
BBta_p0179		*hupL*—uptake hydrogenase large subunit	*T. denitrificans* (67%), cBTAi1 (54%)	
BBta_p0180		*hupS*—uptake hydrogenase small subunit	*T. denitrificans* (57%), cBTAi1 (45%)	
BBta_p0248	VI	VirB11 type IV secretion protein	*S. meliloti* 1021 pSymA (59%)	Type IV secretion system gene cluster
BBta_p0249		VirB10 type IV secretion protein	*S. meliloti* 1021 pSymA (52%)	
BBta_p0250		VirB9 type IV secretion protein	*S. meliloti* 1021 pSymA (56%)	
BBta_p0251		VirB8 type IV secretion protein	*S. meliloti* 1021 pSymA (65%)	
BBta_p0252		VirB6 type IV secretion protein	*S. meliloti* 1021 pSymA (38%)	
BBta_p0254		VirB5 type IV secretion protein	*S. meliloti* 1021 pSymA (45%)	
BBta_p0255		VirB4 type IV secretion protein	*S. meliloti* 1021 pSymA (60%)	
BBta_p0256		VirB3 type IV secretion protein	*S. meliloti* 1021 pSymA (61%)	
BBta_p0257		VirB2 type IV secretion protein	*S. meliloti* 1021 pSymA (52%)	
BBta_p0258		VirB1 type IV secretion protein	*S. meliloti* 1021 pSymA (26%)	
BBta_p0259		*traI*—autoinducer synthesis	*M. loti* R7 symbiosis island (55%)	Plasmid transfer
BBta_p0260		*traR*—autoinducer binding activator	*M. loti* R7 symbiosis island (35%)	

Abbreviation: AA, amino acid; HP, hypothetical protein.
[a]cBTAi1, typical *Bradyrhizobium* BTAi1 chromosome-borne hydrogenase gene cluster, chrom type I, found at nucleotide positions BBTa_1993 to 2012.

(Ramirez-Romero *et al.*, 2000). The *repABC* replicon family is required for ensuring proper segregation and efficient maintenance of low-copy DNA repli-cons (Ramirez-Romero *et al.*, 2000), and has been extensively characterized and is genetically con-served in the α-proteobacteria. The *repABC* replicon

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has also been shown to control the maintenance of rhizobial symbiotic megaplasmids (Ramirez-Romero et al., 2000; Barnett et al., 2001), the Ti plasmids in Agrobacterium sp. strains (Li and Farrand, 2000) and the second chromosome in the animal pathogen Brucella (Paulsen et al., 2002). The repABC genes also appear to be widely distributed on accessory plasmids in several rhizobial species (Cevallos et al., 2002; Stiens et al., 2006; Watson and Heys, 2006).

Amino-acid sequence analysis revealed that two ORFs, BBta_p0014 and BBta_p0015, located downstream of the repABC replication module, were 42% and 35% identical to ParA-like ATPase and ParB-like partition protein from S. meliloti plasmid pMBA19a (accession number AY914874) and the Rhodobacter sphaeroides plasmid pRSPA04 (accession number NC_009432), respectively. The parA and parB, which are required for efficient plasmid and chromosome partitioning, are found on many low-copy-number plasmids and bacterial chromosomes (Bignell and Thomas, 2001). The existence of an alternative replication-control region in tandem to repABC has been previously described in other rhizobial plasmids, and is thought to broaden host range (Stiens et al., 2006; Watson and Heys, 2006).

Conjugation and gene transfer systems
Gene cluster VI (Table 3; Figure 1) contained 10 ORFs encoding VirB1 to VirB11 components of the TraG/VirD4 family type IV secretion system (T4SS). Although not detected in the original annotation, re-examination of the plasmid genome revealed the presence of a Vir B7 homologue (BBta_p0296) located between BBta_p0251 and BBta_p0252. Sequences from these ORFs formed a monophyletic clade with T4SS genes from plasmids in other members of the Rhizobiaceae, and were most similar to T4SS genes from S. meliloti 1021 pSymA (Table 3). Although presumably involved in inducing conjugative horizontal DNA transfer, very little data currently exist regarding the functional role of the plasmid-encoded T4SS in rhizobia. Stiens et al. (2006) however reported that the T4SS on pSmeS-M11a from S. meliloti was not involved in mobilization of this plasmid, and S. meliloti strains containing virB operon deletions were not affected in nodulation or symbiotic nitrogen fixation (Barnett et al., 2001).

Plasmid pBTAi1 also contained traI and traR quorum sensing-related genes directly upstream from the T4SS genes. In some cases, repABC expression, and therefore plasmid copy number control is subject to TraR-mediated quorum sensing (He et al., 2003; Pappas and Winans, 2003). Control of the conjugal transfer of pBTAi1 may also be mediated by ORF BBta_p0279, which encodes a putative ArdC-like conjugal transfer antirestriction protein. This ORF forms a monophyletic clade with ArdC proteins from other α-proteobacterial plasmid, and is most closely related (74% AA identity) to ArdC from N. hamburgensis X14. The Ard proteins are thought to protect plasmid DNA from host restriction endonuclease activity during conjugal transfer (Belogurov et al., 2000).

Copper-resistance genes
The pBTAi1 regions III and IV (Table 3) contained several ORFs (BBta_p0056, BBta_p0061, BBta_p0114 to p0118) with high AA identity to copper-resistance proteins from other, closely related α-proteobacteria, including Rhodopseudomonas, and Nitrobacter species. These included components of a putative copABCD (pcoABCD) copper-resistance operon (BBta_p0114 to p0118) conferring structural resistance to high concentrations of copper (Rensing and Grass, 2003). At least seven additional ORFs that are putatively involved in copper resistance and transport were also detected throughout the pBTAi1 genome. These included additional copA- (BBta_p0190) and copB-like (BBta_p0189) ORFs located upstream of region II, and three putative copper-transporting P-type ATPases (BBta_p0056, BBta_p0121 and BBta_p0125). Copper resistance assays showed that Bradyrhizobium sp. BTAi1 has a relatively high level of copper resistance, with growth at concentrations of up to $200 \, \text{mg} \, \text{l}^{-1}$ $CuCl_2$. In contrast, the closely related plasmid-free, phototrophic Bradyrhizobium sp. ORS278 could not grow at concentrations of $CuCl_2$ exceeding $100 \, \text{mg} \, \text{l}^{-1}$, indicating a potential ecological role of the Bradyrhizobium sp. BTAi1 plasmid-borne copper-resistant genes. Although BTAi 1 was isolated from a submerged stem nodule on greenhouse-grown A. indica, the original inoculum originated from quartz sand rooting medium obtained from an open pit mine in West Virginia (Stowers and Eaglesham, 1983). Thus, enhanced heavy metal resistance, conferred by pBTAi1, might have provided an evolutionary advantage in this metal-rich environment. Plasmid-borne Pco/copABCD-type copper-resistance operons have been described in detail for Escherichia coli (Rensing and Grass, 2003) and Pseudomonas syringae pv. tomato (Mills et al., 1994). Moreover, genomic analysis has revealed a large number of similar genes on bacterial plasmids and chromosomes for a variety of other microorganisms (Mergeay et al., 1995; Francki et al., 2000; Monchy et al., 2006). The pco/copABCD operon, transcribed from a copper-inducible promoter, is controlled by the two-component regulatory module pco/copRS (Mills et al., 1994). Although no copRS homologues were detected on pBTAi1, a two-component-system response regulator (BBta_3078/3079) similar to copRS was found on the Bradyrhizobium sp. BTAi1 chromosome, along with two copCD homologues (BBta_2513/4 and BBta_5430/1). This result suggests that transcription of copper-resistance genes is controlled by chromosomal regulators in Bradyrhizobium sp. BTAi1. Lim and

Cooksey (1993) previously proposed that there is a linkage between chromosome- and plasmid-borne copper-resistance genes in *P. syringae* pv. tomato.

Plasmid-borne hydrogenase genes

Sequence analysis also revealed that pBTAi1 had a [Ni–Fe] hydrogenase gene cluster (ORFs BBta_p0170 to p0180) containing the large (*hupL*) and small (*hupS*) hydrogenase subunits, and various *hup*- and *hyp*-like maturation genes required for hydrogenase activity. Two additional hydrogenase gene clusters were previously shown to be localized to the *Bradyrhizobium sp.* BTAi1 chromosome (ORFs BBta_0458 to 0482, and ORFs BBta_1993 to 2012; Giraud *et al.*, 2007). One of the chromosome-borne hydrogenase clusters (ORFs BBta_1993 to 2012), type II, had almost identical gene synteny and high sequence similarity to hydrogenase genes found in other *Rhizobium* and *Bradyrhizobium* sp. strains. The large and small subunits showed 99/97%, 92/96%, 90/84% and 91/87% AA sequence identity to *hupS* and *hupL*, respectively, from *Bradyrhizobium* sp. ORS278, *B. japonicum* USDA 110, *R. leguminosarum* bv. *viciae* and *Rhodopseudomonas palustris* BisB5, respectively. We refer to these ORFs as comprising the 'typical hydrogenase genes (chrom type II). In contrast, a second 'atypical' chromosomal-borne hydrogenase gene cluster (ORFs BBta_0458 to 0482), chrom type I, was only distantly related to typical rhizobial hydrogenases, and shared much greater gene synteny and sequence similarity (81% and 84% AA sequence identity for the large and small hydrogenase subunits, respectively) to plasmid-borne genes from *Sphingopyxis*

alaskensis strain RB2256, a heterotrophic bacterium prominent in oligotrophic marine environments. Phylogenetic analysis indicated that the atypical *Bradyrhizobium.* sp. BTAi1 and the *S. alaskensis* hydrogenase genes phylogenetically-cluster with cyanobacterial uptake hydrogenases from the genera *Anabaena*, *Nostoc*, *Cyanothece* and *Nodularia* (Figure 2).

Sequence and synteny analyses revealed that the pBTAi1 plasmid-borne hydrogenase genes had a closer relationship to ORFs from the sulfur-oxidizing bacterium *Thiobacillus denitrificans* (Beller *et al.*, 2006), than to either of the chromosomal-borne hydrogenase gene clusters in strain BTAi1 (Table 3; Figures 2 and 3). While the BTAi1 *hupS* and *hupL* had 67% and 57% AA sequence identity to the same genes in *T. denitrificans*, they only had only 54% and 45% AA identity to orthologues of the same genes in the 'typical' chromosomal-borne hydrogenase gene cluster in strain BTAi1. In addition, both the pBTAi1 and *T. denitrificans* hydrogenase clusters each lacked *hupC*, *hupG*, and *hupI* and *hupJ*, which are typically found in the hydrogenase gene clusters in other rhizobia (Baginsky *et al.*, 2002). Despite this similarity, the pBTAi1 hydrogenase gene cluster did not contain the *isp1* and *isp2* genes that separate the large and small hydrogenase genes in the *T. denitrificans* (Figure 3).

Both the BTAi1 plasmid-borne (ORFs BBta_p0170 to p0180) and the atypical chromosomal-borne (BBta_0458 to 0482) hydrogenase genes were flanked by transposable elements, suggesting that they were acquired by means of lateral gene transfer from a non-rhizobial donor. Horizontal transfer of hydrogenase gene clusters between prokaryotes has
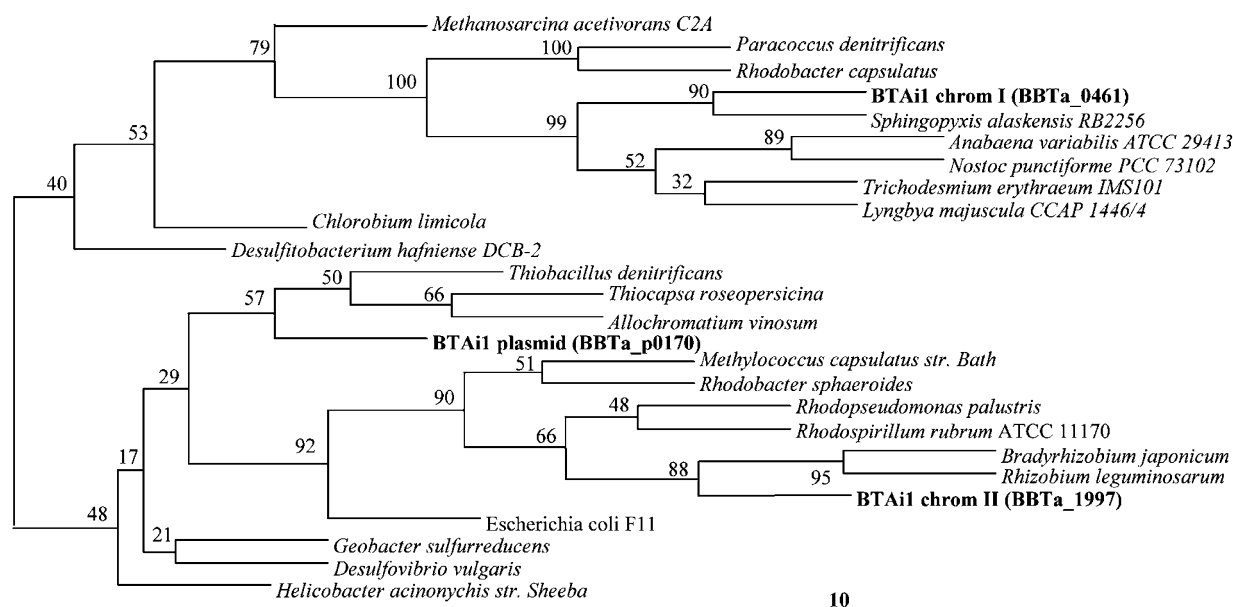

Figure 2 Phylogeny of the three distinct *Bradyrhizobium* sp. BTAi1 uptake hydrogenase large subunit (*hupL*) genes relative to closely related reference species. Bootstrap values (100 replicates) are shown at branch nodes. Bar represents 10% sequence divergence. Phylogenetic trees were generated using the maximum likelihood method of the Phylip phylogenetic package (Felsenstein, 1989).
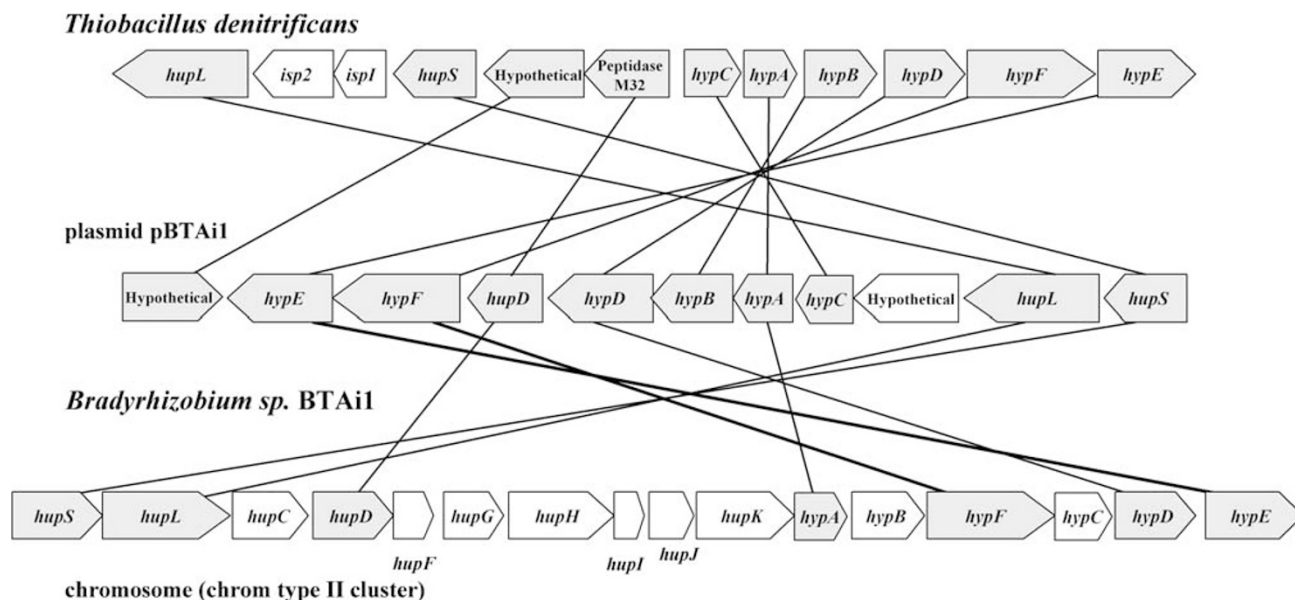
Figure 3 Synteny analysis of hydrogenase gene clusters from pBTAi1, the *Bradyrhizobium* sp. BTAi1 plasmid, *Bradyrhizobium* sp. BTAi1 chromosome (chrom type II) and *Thiobacillus denitrificans* chromosome. Synteny is based on a ⩾30% AA gene identity cutoff threshold. AA, amino acid.

been previously postulated (Calteau *et al.*, 2005), and past indirect evidence suggested that *hup* sequences from specific *Bradyrhizobium* sp. (*Vigna*) strains may have been acquired via horizontal transfer from a non-rhizobial source (Baginsky *et al.*, 2002).

Comparative expression of BTAi1 hydrogenase genes
Some rhizobia induce uptake hydrogenases during symbiotic growth in order to reduce energy losses associated with nitrogen fixation (Albrecht *et al.*, 1979). The multiplicity of phylogenetically distinct uptake hydrogenases, and the presence of a plasmid-borne hydrogenase gene cluster in strain BTAi1, warranted assessment of the functional capacity of these enzymes and their potential role in symbiosis. Gas chromatographic analyses confirmed that both root and stems nodules of *A. indica* plants, inoculated with *Bradyrhizobium* sp. strain BTAi1, expressed hydrogenase activity (Figure 4). Quantitative real-time PCR analyses of mRNA from root and stem nodules, performed using specific primers targeting each of the three *hupL* genes, indicated that transcriptional levels of the two chromosome *hupL* homologues were significantly induced relative to free living BTAi1 cultures (Figure 5). In contrast, expression of the plasmid-borne *hupL* (BBta_p0179) in nodule extracts was considerably lower than the two chromosomal *hupL* homologues. While the small level of induction of the plasmid-encoded *hupL* in stem nodules suggests that plasmid-borne hydrogenase may be induced under symbiotic conditions, the relatively low level of expression implies that it does not play a significant role in the symbiotic process. Thus, the specific environmental role of this plasmid-borne hydrogenase still remains unclear.
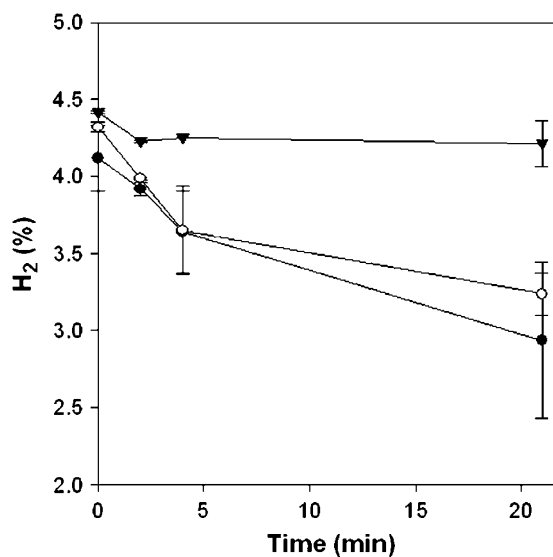

Figure 4 Hydrogenase activity in nodule extracts from *A. indica* inoculated with *Bradyrhizobium* sp. strain BTAi1. Legend: ●, root nodules; ○, stem nodules and ▼, control extracts from bacteria-free plant tissue.

Detection of accessory plasmids in diverse
Bradyrhizobium *sp. strains*
Reports of plasmids in other *Bradyrhizobium* strains have been inconsistent and infrequent, and results of these studies have been confusing due to changes in the taxonomy of soybean-nodulating bacteria. To determine whether the presence of accessory plasmids in the bradyrhizobia is relatively common, we used PFGE-CHEF to screen 46 *Bradyrhizobium* strains, isolated from the United States, China and Thailand. These strains were chosen due to their taxonomic and geographic diversity, and because
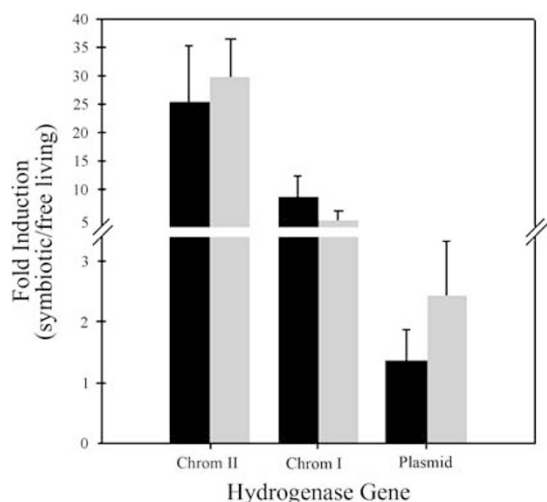
Figure 5 *In planta* gene expression of the typical- (chrom type II), atypical-chromosomal (chrom type I) and plasmid-borne large hydrogenase subunits (*hupL*) of *Bradyrhizbium* sp. strain BTAi1 performed using quantitative real-time RT-PCR. Relative gene expression values for *hupL* under free-living and symbiotic (root and stem nodules) conditions were normalized to the *parA* housekeeping gene. Legend: ■, root nodules; (▨), stem nodules.
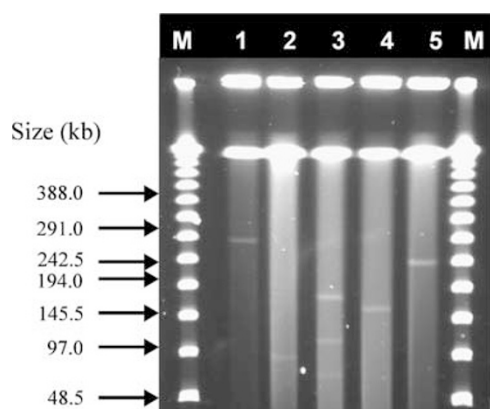

Figure 6 Plasmids isolated from selected genetically and geographically diverse *Bradyrhizobium* sp. strains using pulse-field CHEF gel electrophoresis. Lanes: M, λ-PFG marker ladders; 1, *B. elkanii* USDA 46; 2, *B. elkanii* USDA 94; 3, *B. japonicum* USDA 135; 4, *B. japonicum* USDA 146 and 5, *B. japonicum* USDA 225. PFG, pulsed-field gel.

Table 4 Other *Bradyrhizobium* strains harboring accessory plasmids

Strain	Source or orgin	Plasmid size (kb)
Bradyrhizobium japonicum		
USDA 135	USDA[a]	75, 115, 170
USDA 146[b]	USDA	160
USDA 177[b]	USDA	255
USDA 225[b]	USDA	230
DASA01196	DOA[c]	145, 215
Bradyrhizobium elkanii		
USDA 46	USDA	280
USDA 94	USDA	90
USDA 227[b]	USDA	285
DASA01244	DOA	270
DASA01265	DOA	265
DASA01304	DOA	220

[a]USDA, United States Department of Agriculture.
[b]Strains isolated from soybeans in the People's Republic of China.
[c]DOA, Thailand Department of Agriculture, Bangkok; strains isolated from soybean in Thailand.

many were isolated from the geographic origins of soybeans, China and Southeast Asia. In addition, many of the strains with USDA prefixes represent members of the main serogroups of *B. japonicum* and *B. elkanii* strains. Eleven of the tested strains (23.9%), from all three geographic locations, were found to harbor large, high-molecular-weight plasmids of varying sizes (75–330 kb; Figure 6). Sequence analysis of 16S rDNA indicated that these strains were either *Bradyrhizobium elkani* or *B. japonicum* (Table 4). Five strains of *B. japonicum* (USDA 135, USDA 146, USDA 177, USDA 225 and DASA01196) were found to possess plasmids,

whereas six plasmid-containing strains (USDA 46, USDA 94, USDA 227, DSA01244, DSA011265 and DSA011304) were found to be *B. elkanii*. As has been previously reported in several other studies, the sequenced *B. japonicum* strain, USDA 110, was not found to contain any plasmids. Interestingly, to our knowledge, this is the first report of the presence of plasmids in *B. elkanii* strains.

Among the plasmid-containing bradyrhizobia, three strains originated from the United States and four each from China and Thailand. Thus, there does not appear to be a relationship between geographic origin of the bradyrhizobia and the presence of plasmids. While the majority of the strains contained a single plasmid, two strains, *Bradyrhizobium japonicum* strains USDA 135 DASA01196, harbored three and two plasmids, respectively. Our results showing multiple accessory plasmids in some strains, obtained using a highly effective CHEF-PFGE technique, are in contrast to those reported by Masterson *et al.*, 1982 in which only one plasmid was found in each of the strains they examined. However, compared with the fast-growing rhizobia, such as *R. leguminosarum*, which can harbor 2–11 different plasmids, the bradyrhizobia appear to contain relatively few plasmids. Plasmid size in the various strains was found to be highly heterogeneous, with only two strains (DASA01244 and DASA01265) containing plasmids of identical size (260 kb). None of the plasmids identified in these studies hybridized to ^{32}P-labeled *nolA*, *nodD2*, *nodD1YABC*, *nodSUIJ* or *nodZ*; or *hup* (pLNJ1) gene probes from *B. japonicum* strain USDA 110. Taken together, these results indicate that accessory plasmids are more commonly present in the bradyrhizobia than was previously thought, but none contain symbiosis-related *nod* or *hup* genes.

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Conclusions

While previous studies have reported the sequence and in-depth analysis of symbiotic and accessory plasmids in *Rhizobium, Mesorhizobium* and *Sinorhizobium* strains, this is the first report of comprehensive sequence and analysis of a plasmid from a *Bradyrhizobium* sp. strain. Here we also demonstrated the presence of heterogeneously sized plasmids in geographically and genetically diverse *Bradyrhizobium*. Moreover, we report for the first time the presence of plasmids in *B. elkanii*. Complete sequence analysis of plasmid pBTAi1 from one of these strains, *Bradyrhizobium sp.* BTAi1, revealed the presence of a *repABC* replication module and several conjugation and gene transfer mechanisms that are characteristic of other rhizobial plasmids and α-proteobacteria. In contrast, the majority of coding sequences on pBTAi1 were not significantly related to any sequences on previously described rhizobial plasmids or chromosomes. The most notable functional genes identified on pBTAi1 encoded for proteins involved in copper resistance and hydrogen oxidation. Enhanced copper resistance relative to the closely related, plasmid-free phototrophic *Bradyrhizobium sp.* ORS278 suggests that the plasmid-borne copper-resistance genes may provide this strain an environmental advantage in metal-rich environments under free-living conditions. Sequence analyses of the plasmid-borne hydrogenase genes indicated that they were likely acquired from a non-symbiotic organism via horizontal gene transfer. The low level of induction of pBTAi1-encoded hydrogenase genes during symbiotic growth with *A. indica*, relative to two additional chromosomal-borne hydrogenase enzymes, suggests that the plasmid-borne hydrogenase is not significantly involved in symbiotic growth. While the phylogenetic affiliation of the pBTAi1-borne uptake hydrogenease with an enzyme system from a non-rhizobial species suggests that it may have a role in free-living growth, this question remains currently unresolved.

Acknowledgements

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)