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ORIGINAL ARTICLE

The *Rhizobium leucaenae* CFN 299 pSym plasmid contains genes expressed in free life and symbiosis, as well as two replication systems

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Abstract The alpha-protobacterium *Rhizobium leucaenae* CFN 299 is able to nodulate and fix nitrogen in symbiosis with a wide range of legumes, including *Phaseolus vulgaris* (common bean). Strain CFN 299 contains a 500-kb symbiotic plasmid (pSym) that encodes genes required for nodulation and nitrogen fixation as well as many genes whose function is unknown. In this work, we characterized the transcriptional expression of 16 pSym genes in common bean nodules and in free-living cells grown in culture. A functionally diverse group of genes were expressed during discrete stages of the symbiosis or in free-living cells. These included genes whose products are involved in nodulation and nitrogen fixation, carbon metabolism, vitamin synthesis, sulfur utilization, conjugation, transposition and DNA replication. We also examined the functionality of two replication systems encoded on pSym and found that *repABC*, but not *repC2*, is required for pSym replication.

Keywords *Rhizobium leucaenae* CFN 299 · Symbiotic plasmid · Nodulation · Gene expression · Replication systems · *repABC*

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Introduction

Rhizobia are a group of Gram-negative bacteria that form nitrogen-fixing nodules on the roots of legume host plants. *Rhizobium tropici* Type A CFN 299, recently renamed as *Rhizobium leucaenae* CFN 299 (Ribeiro et al. 2012), is a soil bacterium that establishes symbiosis with at least 22 legume species, including *Leucaena leucocephala* and *Phaseolus vulgaris* (common bean) (Martínez-Romero et al. 1991; Hernández-Lucas et al. 1995b; Acosta-Durán and Martínez-Romero 2002). The CFN 299 genome comprises an approximately 3-MB chromosome, a 1.5-MB megaplasmid with genes for exopolysaccharide synthesis, plasmid A (185 kb) containing genes for the transport of compounds present in bean root exudates, plasmid B (240 kb) that participates in nodulation competitiveness and the 500-kb symbiotic plasmid (pSym) (Martínez et al. 1987; Geniaux et al. 1995; Rosenblueth et al. 1998; Ormeño-Orrillo et al. 2016). The pSym contains genes required for nodulation, nodulation competitiveness, nitrogen fixation, phytohormone biosynthesis and multiple functional insertion sequence (IS) elements (Mavingui et al. 1997; Hernández-Lucas et al. 2006; Ormeño-Orrillo et al. 2012).

We have previously reported that transfer of the pSym from CFN 299 to *Agrobacterium tumefaciens* and *Ensifer adherens* allowed the latter two species to nodulate and fix low levels of nitrogen in combination with *P. vulgaris* (Martínez et al. 1987;

Rogel et al. 2001). Transfer of the pSym to *Brucella melitensis*, a human pathogen, resulted in transconjugant cells able to form non-nitrogen-fixing pseudonodules on common bean (Martínez-Romero, unpublished results). The CFN 299 pSym is not easily cured, and we have been able to delete only a 300-kb region of the plasmid that includes genes involved in nodulation and nitrogen fixation (Pardo et al. 1994). The sequences of the pSym of *R. leucaenae* CFN 299, *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 are highly conserved (Ormeño-Orrillo et al. 2012) and, interestingly, all of these strains have a wide nodulation host range and are efficient at fixing nitrogen in tropical climates.

Rhizobium leucaenae CFN 299 is able to utilize diverse carbon sources (Romanov et al. 1994) and contains additional copies of some chromosomal metabolic genes on the pSym. We previously characterized pSym-encoded second gene copies of isocitrate lyase and citrate synthase. Mutants in the plasmidic citrate synthase gene were adversely affected in symbiosis (Hernández-Lucas et al. 1995a), while mutants lacking either or both the chromosomal and pSym-encoded isocitrate lyases were not (Ramírez-Trujillo et al. 2007). Apart from these studies, the free-living and symbiotic roles of most of the genes encoded on the CFN 299 pSym remain unknown. To address this gap in our knowledge, we have evaluated the expression of several CFN 299 pSym-encoded genes in bean nodules and in cultures using transcriptional fusions. We found that this replicon contains a functionally diverse group of genes that are expressed at different symbiotic stages or in the free-living state. We also characterized the functionality of the *repABC* and *repC2* replication system encoded on the pSym.

Materials and methods

Bacterial strains, plasmids, culture conditions and matings

The bacterial strains and plasmids used in this work are listed in Electronic Supplementary Material (ESM) Table S1. Rhizobia were grown in PY medium (0.5% peptone, 0.3% yeast extract, 7 mM $CaCl_2$) (Beringer 1974) supplemented with the following antibiotics when required: kanamycin (Km; 40 mg l^{-1}), nalidixic acid (Nal; 20 mg l^{-1}), neomycin (Neo; 30 mg l^{-1}) and/or rifampicin (50 mg l^{-1}). *Escherichia coli* strains (Hanahan 1983; Simon et al. 1983) were grown in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) (Sambrook et al. 1989) supplemented with ampicillin (100 mg l^{-1}), Km (40 mg l^{-1}), Nal (20 mg l^{-1}), and streptomycin (25 mg l^{-1}), when needed. The antibiotics were acquired from Sigma-Aldrich (St. Louis, MO). *Escherichia coli* and rhizobia were grown at 37 °C and 30 °C respectively. *Escherichia coli* HB101 containing genes cloned in plasmid pUX19 or pVO155 were mated with *Agrobacterium tumefaciens* (Rosenberg and Huguet 1984) or *R. leucaenae* CFN 299 using pRK2013 as the helper plasmid (Figurski and Helinski 1979). Strains were grown in liquid medium to stationary phase, mixed in a donor–recipient ratio of 1:2 on PY plates and incubated at 30 °C overnight. Cells were resuspended in PY medium, and serial dilutions were plated on the appropriate selective medium (Charles and Finan 1990).

DNA manipulations

Plasmid purification and genomic DNA extraction were done according to published protocols (Sambrook et al. 1989). Primers used for PCR amplifications are shown in ESM Table S2. Restriction enzymes, ligase, nucleotides and polymerases were acquired from New England Biolabs (Ipswich, MA) or Thermo Fisher Scientific (Walham, MA). For sequencing, double-stranded DNA was purified with the High Pure Plasmid Isolation Kit (Roche Applied Science, Penzburg, Germany) and sequenced with an automated sequencer (model 3130xl; Applied Biosystems, Foster City, CA). Plasmid profiles were obtained by the Eckhardt method as modified by Hynes and McGregor (1990). For hybridization, total DNA was digested with restriction enzymes and transferred from agarose gels to nylon membranes. Probes were labeled with ^{32}P by polymerase extension using random primers, and hybridization was carried out under high stringency conditions (Southern 1975).

Transcriptional gene fusions

To generate transcriptional gene fusions, 500 kb of the *R. leucaenae* CFN 299 pSym was cloned into a bacterial artificial chromosome (BAC) vector by an in vivo cloning procedure (Hernández-Lucas et al. 2002). The BAC DNA was then purified, nebulized, size-fractionated into 0.8- to 3-kb fragments and cloned into plasmid pVO155 (Oke and Long 1999), which contains the β-glucuronidase (*gusA*) reporter gene and a Neo resistance cassette. Individual pVO155 plasmids, harboring an internal fragment of the pSym, were mated into *R. leucaenae* CFN 299, and Neo-resistant transconjugants were selected. Plasmid pVO155 cannot replicate in strain CFN 299, so Neo-resistant colonies can arise only after recombination between the gene fragment on plasmid pVO155 and the genomic copy

on the pSym via two types of recombination events: (1) transcriptional fusions in which gene function is maintained and (2) transcriptional fusions that disrupt the corresponding pSym gene.

Measurement of β-glucuronidase activity

β-Glucuronidase assay activity was evaluated qualitatively on solid PY medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu) substrate. β-glucuronidase activity was also measured in cells from separate cultures grown in PY medium to an OD_{595} of 0.5 or 1.0. A 1-ml sample of culture was centrifuged and resuspended in a salt wash solution containing chloramphenicol (100 μg ml^{-1}), and quantitative β-glucuronidase assays were performed with *p*-nitrophenyl β-D-glucuronide as substrate (Wilson et al. 1992). Data were normalized to total cellular protein as determined by the Bradford method (Bradford 1976). The activity values were corrected for the low background expression of β-glucuronidase activity in the wild-type strain. To detect β-glucuronidase activity in nodules, nodulated *Phaseolus vulgaris* roots obtained 10, 15 and 21 days post-inoculation (dpi) were incubated with the X-glu substrate and visually analyzed (Shamseldin 2007).

Nodulation and nitrogen fixation assays

Phaseolus vulgaris cv. Negro Jamapa nodulation assays were performed in flasks with agar (Martínez et al. 1987), and nitrogenase activity was measured by acetylene reduction 21 days after inoculation (Burris 1972).

Construction of *repC* and *repABC* plasmid derivatives

The following plasmids containing PCR-amplified gene inserts were constructed to characterize the pSym replication system. The *repC2* insert in pUX1 was obtained by PCR amplification with the REP5 and REP6 primers (ESM Table S2). For pUX2, pUX3, and pUX4, the *repABC* regions were amplified with REP1, REP2 and REP3, respectively, as forward primers and with REP4 as reverse primer (ESM Table S2). All the PCR fragments were cloned in pCR2.1, digested with *Bam*HI–*Xba*I and ligated into pUX19.

Phylogenetic analysis of RepC2 and RepABC1 replicator proteins

The RepC2 and RepABC1 protein sequences of *R. leucaenae* CFN 299 were compared with those in the GenBank database using the BLAST program (Johnson et al. 2008). The concatenated protein sequences were aligned using CLUSTAL X software (Larkin et al. 2007). Phylogenetic relationships of RepC2 and RepABC1 proteins were estimated by the maximum likelihood method using MEGA 7 software (Jones et al. 1992; Kumar et al. 2016). The significance of internal branches of the phylogenetic trees was estimated with 1000 bootstrap replicates.

Results and discussion

Identification of pSym genes expressed under free-living and symbiotic conditions

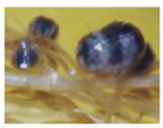
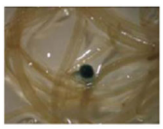
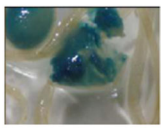
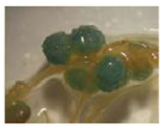
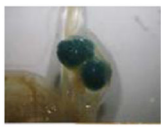
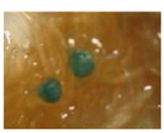
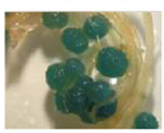
To identify genes on the pSym that are expressed in nodules and under free-living conditions, we constructed an *R. leucaenae* CFN 299 β-glucuronidase fusion library by recombining a pSym region cloned in suicide reporter plasmid pVO155 with the corresponding genomic region on pSym. The recombinant strains were analyzed in Eckhardt gels, and 136 transconjugants showed no deletions or rearrangements of their plasmid profiles (ESM Fig. S1). Hybridization experiments using the pVO155 plasmid as a probe against the genomic DNA of several recombinants showed that a single insertion was present in each clone (ESM Fig. S2).

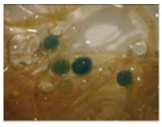
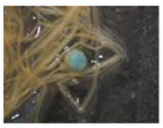
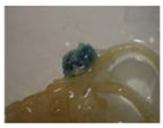
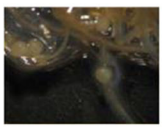
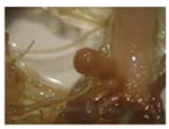
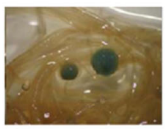
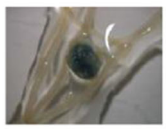
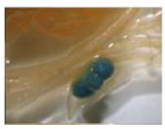
We then characterized gene expression in the 136 transconjugants under free-living conditions and in nodules (Table 1). The strains were evaluated in solid Tully's medium (Tully 1985) supplemented with the X-gluc substrate and 10 mM glucose, sucrose, succinate, glyoxylate or glycolate as carbon source. These carbon sources were selected to determine if the pSym expresses genes involved in sugar, C_4 and C_2 metabolism. Of the 136 transconjugants, only five strains, namely RH257, RH260, RH363, RH387 and RH404, displayed transcriptional activity exclusively under free-living conditions. These transconjugants also showed β-glucuronidase activity on plates independently supplemented with all of the carbon sources tested (data not shown) as well as on the rich medium (PY). These results indicate constitutive expression of the genes fused to *gusA* in these strains under the conditions tested. The β-glucuronidase activity of these strains was quantified by growing them in liquid PY medium to OD_{595} values of 0.5 and 1.0, corresponding to mid-exponential and stationary phase, respectively. Clone RH363 expressed β-glucuronidase activity at OD_{595} of 0.5 and 1.0, clone RH404 produced activity only at an OD_{595} of 0.5 and clones RH257, RH260 and RH387 had activity only at an OD_{595} of 1 (Table 1). To identify the genes fused to *gusA*, the pV0155 plasmids from the *E. coli* strains containing the corresponding pSym genes were sequenced using primers pVO155-1, pVO155-2 and pVO155-3 (ESM Table S2); the results are shown in Table 1. The transcriptionally fused gene

in clone RH363, expressed in both log and stationary phase, encodes a hypothetical protein with a twin-arginine translocation (TAT) amino terminal secretion signal sequence. Proteins secreted by the TAT system are involved in diverse physiological processes, including cellular homeostasis (Berks et al. 2003). The product of the gene fused in clone RH404 has DNA-helicase and endonuclease domains, and its expression exclusively in log phase is consistent with a role in DNA replication. The gene products of the clone RH257, RH260, RH387 fusions, expressed only in stationary phase, corresponded to the TrbL, TraB and TrbI gene products. *trbL* and *trbI* are in the same transcriptional unit, and *traB* are in a divergent contiguous operon. These type IV secretion system (T4SSs) family proteins are involved in bacterial conjugation mediated by quorum sensing (Cook et al. 1997; Wilkinson et al. 2002), and their expression only at the higher cell density (OD_{595} of 1.0) is consistent with this function. Expression of T4SS genes could promote horizontal gene transfer, which would lead to the spread of the symbiovar tropici plasmid to other bacterial species. In agreement, the symbiotic plasmid from the symbiovar tropici is found in *Rhizobium lusitanum*, *Rhizobium freirei*, and *R. tropici* in addition to *R. leucaenae* (Ormeño-Orrillo et al. 2012).

To reveal some of the molecular events occurring during nodule development, we analyzed the dynamics of pSym gene expression during different stages of symbiotic development. Five *Phaseolus vulgaris* (bean) plants were independently inoculated with each of the 136 *R. leucaenae* fusion strains, and nodules were collected and stained to detect β-glucuronidase activity at 10, 15 and 21 dpi (Table 1). The gene fusions in clones RH191, RH245 and RH253 were expressed in nodules at all three time points. In RH191, the expression of the isocitrate lyase gene (*aceA*), encoding the first enzyme of the glyoxylate cycle for C_2 carbon utilization, was detected. Isocitrate lyase activity is induced in rhizobia grown on acetate or fatty acids; it is generally present at only trace levels in bacteroids (Dunn 1998) and is dispensable for the *R. leuecaenae* CFN 299–bean symbiosis (Ramírez-Trujillo et al. 2007). Therefore, deeper transcriptional studies with *aceA* are necessary to determine the meaning of its expression

Table 1 pSym transcriptional activity of *Rhizobium leucaenae* CFN 299 transconjugants under free-living conditions and in nodules

Strain	Insert size (nt) cloned in pVO155	Free-living β-glucuronidase activity in mid-exponential‡ and stationary phase*	β-glucuronidase activity in symbiosis			Nitrogen fixation†	Gene fused to β-glucuronidase (Accession number)
			10 DPI	15 DPI	21 DPI		
WT	0	ND				+	
RH191	500	ND				+	*aceA* (AGB73680)
RH245	336	ND				-	*nifQ* (AGB73564)
RH246	3015	ND				+	*xre* (AGB73770)
RH253	2871	ND				+	*thiO* (AGB73512)
RH256	1279	ND				+	*hipB* (AGB73727)
RH257	3137	1595*	-	-	-	+	*trbL* (AGB73806)
RH260	3821	2009*	-	-	-	+	*traB* (AGB73796)

RH261	2193	ND				+	Hypothetical protein (AGB73775)
RH340	3500	ND				+	Transposase IS30 (ENN86839)
RH354	1192	ND				+	Hypothetical protein (WP_041679003)
RH361	1606	ND				+	Putative alkanesulfonate monooxygenase (AGB73350)
RH363	1732	2048‡ 1470*	-	-	-	+	Secretion hypothetical protein (AGB73347)
RH370	1994	ND				+	Helicase (WP_047525531)
RH387	1419	1099*	-	-	-	+	*trbI* (AGB73802)
RH404	1908	1052*	-	-	-	+	Hypothetical helicase (ENN87158)
RH406	540	ND				+	Hypothetical protein (WP_026477726)

Cultures grown in PY medium and assayed at mid-exponential‡ and stationary phase*. Values are in nmol PNG min^{-1} mg $protein^{-1}$. ND, not determined.

†Presence (+) or absence (-) of acetylene reduction activity at 21 dpi.

Values are in nmol PNG min^{-1} mg $protein^{-1}$

ND, not determined

‡ Cultures grown in PY medium and assayed at mid-exponential

* Cultures grown in PY medium and assayed at stationary phase

† Presence (+) or absence (-) of acetylene reduction activity at 21 dpi

in bean nodules. In RH245, *gusA* is fused to *nifQ*, whose product is required for the biosynthesis of the iron–molybdenum cofactor (FeMo-co) in the active site of nitrogenase (Fumeaux et al. 2011). In RH253 the fusion is to *thiO2*, which encodes a glycine oxidase required for thiamine biosynthesis (Miranda-Ríos et al. 1997). The synthesis of this vitamin by rhizobial microsymbionts is important for nitrogen fixation, and colonization of plant roots can be limited by the availability of thiamine (Streit et al. 1996; Buendia-Clavería et al. 1998).

In nodules at 15 and 21 dpi, β-glucuronidase activity was detected in strains RH246, RH256, RH261, RH340, RH361, RH370 and RH406. In RH246, the *gusA* gene is fused to *xre*, a putative transcriptional regulator involved in the xenobiotic response. Xre proteins also have an as yet undefined role in allowing the conjugative transfer of a *Rhizobium etli* megaplasmid from *A. tumefaciens* following its introduction into that non-native host (López-Fuentes et al. 2014). Strain RH256 expresses a fusion to *hipB*, whose product is part of a toxin–antitoxin system encoded by the *hipAB* cluster. HipB is a negative regulator of HipA, which encodes a serine-threonine kinase that inhibits cell growth and allows survival in the presence of antibiotics (Germain et al. 2013; Kaspy et al. 2013). In *Sinorhizobium meliloti* NGR234, HipA and HipB are postulated to function in preventing the loss of the pSym from the cells (Falla and Chopra 1999). This could be relevant to the finding that the pSym is not easily cured from *R. leucaenae* strain CFN 299 (Pardo et al. 1994). Strain RH261 expressed a fusion to a hypothetical gene encoding a 110-amino acid protein that is also present in some

Agrobacterium and *Mesorhizobium loti* strains (Mitchell et al. 2015). In RH340, *gusA* is fused to a IS30 family transposase with a helix–turn–helix domain; the expression of this IS elements support previous results obtained in our laboratory showing that IS elements encoded in the pSym plasmid are functional (Hernández-Lucas et al. 2006). RH361 expresses a fusion to an alkanesulfonate monooxygenase gene, whose product is involved in sulfur acquisition under conditions where this element is limiting (Ellis 2011). The significant expression of two functionally characterized alkanesulfonate monooxygenase genes in *Bradyrhizobium japonicum* bacteroids indicates that this organism metabolizes organic sulfur compounds in nodules (Sugawara et al. 2011), and the product of the corresponding gene in CFN 299 could have a similar function. Strains RH370 and RH406 contained fusions to a helicase and a hypothetical gene, respectively. The gene fusions in strains RH354 were only expressed at 21 dpi (Table 1). In RH354, a hypothetical protein was expressed.

As mentioned, the fusions integrated in the pSym have the potential to create mutants affected in the target or a neighboring gene. We thus evaluated the nitrogen-fixing ability of the 136 transconjugants by independently inoculating eight bean plants with each strain. We found that strain RH245 at 10, 15 and 21 dpi was unable to fix nitrogen in two independent experiments since no acetylene reduction activity was detected compared with the wild-type strain at 21 dpi (Table 1). In RH245 the *gusA* gene is under control of the *nifQ* promoter, but the reporter gene interrupts the divergently transcribed *rpoN* gene encoding a σ^{54} transcriptional factor. The symbiotic phenotype obtained with strain RH245 agrees with results reported in our studies showing that rhizobial σ^{54} mutants are unable to fix nitrogen (Kullik et al. 1991; Michiels et al. 1998).

Our results show that in rich medium the *R. leucaenae* CFN 299 pSym expresses genes involved in protein secretion, conjugation and DNA replication and that these genetics elements are dispensable for nodulation and nitrogen fixation in bean plants (Table 1). In nodules at 10, 15 and 21 dpi genes for acetate metabolism, nitrogen fixation and thiamine biosynthesis were expressed. Analysis of the fusion RH253 in the thiamine gene showed no disruption on *thiO2*; therefore our strain nodules are able to fix nitrogen similarly to the wild type strain (Table 1). In nodules obtained at 15 and 21 dpi, a gene encoding an IS element, a hypothetical gene, the HipB-encoding component of a toxin antitoxin system and a regulator involved in the xenobiotic response were expressed. Nodules at 21 dpi expressed a gene of unknown function. Interestingly, independent helicases were expressed in free-living conditions and in nodules. Overall, the data show that the pSym temporally expresses both genes known to be important in nodulation and nitrogen fixation as well as genes that likely have accessory symbiotic roles.

Isolation and functional characterization of the pSym *repC2* and *repABC* replication systems

The origin of replication is critical for the maintenance of *Rhizobium* plasmids. The CFN 299 sequencing project showed the presence of two replication systems in its pSym plasmid, *repC2* and *repABC1* (Ormeño-Orrillo et al. 2012). We evaluated the functionality of both systems.

Previous studies of the *R. leucaenae* CFN 299 pSym plasmid showed that it contains a *repC2* replication system (Ormeño-Orrillo et al. 2012), and a similar (66% identical) functional *repC* replicator is found in the pRmeGR4a plasmid of *Sinorhizobium meliloti* GR4. Experimental evidence indicates that the pRmeGR4a replicator is able to independently replicate in *A. tumefaciens* (Izquierdo et al. 2005). Therefore, to determine the function of the *repC2* replicator of CFN 299, we PCR-amplified a genomic region that contains the bacterial conjugation factor *psiB* (without promoter), open reading frame 1 (ORF1), the regulatory region of *repC2* and the entire *repC2* (Fig. 1). All of these genetic elements were cloned into plasmid pUX19, which cannot replicate in *A. tumefaciens* (Zhang et al. 2001), to generate plasmid pUX1. As a positive control, a 2402-bp PCR fragment containing ORF1, the regulatory region of *repC* and the entire *repC* of the *S. meliloti* plasmid pRmeGR4a was cloned in pUX19 to generate plasmid pXKR4 (Fig. 1). These plasmids were independently introduced by mating into the plasmid-less *A. tumefaciens* strain GMI9023. The *S. meliloti* replicator of plasmid pXKR4 allowed it to replicate autonomously in *A. tumefaciens*, as reported previously (Izquierdo et al. 2005). In contrast, the *repC2* construct derived from the pSym of CFN 299 was unable to replicate in *A. tumefaciens* GMI9023 since we were unable to obtain *Agrobacterium* transconjugants.

Sequence analysis using the InterPro database (Mitchell et al. 2015) revealed structural differences between the non-functional *repC2* of the *R. leucaenae* pSym and the functional *repC* of *S. meliloti* pRmeGR4a. The RepC protein of plasmid pRmeGR4a has a N-terminal helix–turn–helix (HTH) DNA binding motif (from amino acid 83 to 126; Fig. 1), and this class of motif is required to initiate plasmid replication by interacting with the DNA at the origin of replication (Han et al. 2007; Pinto et al. 2012). In contrast, the RepC2 protein of the *R. leucaenae* pSym lacks this motif and, presumably, DNA binding activity (Fig. 1), which likely explains the inability of plasmid pUX1 to replicate autonomously in *A. tumefaciens*. Pardo et al. (1994) reported that the pSym plasmid with a 300-kb deletion was able to replicate autonomously in *R. leucaenae* CFN 299–10. Using Southern hybridization, we found that this strain does not contain the *repC2* gene (Fig. 2a), indicating that it is not required for pSym replication.

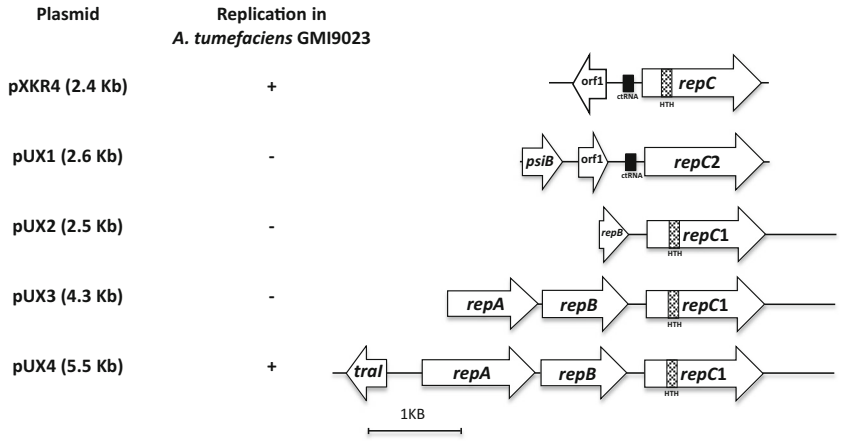


Fig. 1 Schematic representation of the *repC2* and *repABC1* replication systems of the *Rhizobium leucaenae* CFN 299 pSym plasmid. The *repC* replicator of the *Sinorhizobium meliloti* plasmid GR4pRmeGR4a (pXKR4), the *repC2* replicator of CFN 299 (pUX1), and subclones of the *repABC1* operon (pUX2, pUX3 and pUX4) are shown. *repC2* and *repABC1* fragments were cloned into the pUX19 vector and mated into *Agrobacterium tumefaciens* to evaluate their autonomous replication. *orf1* Open reading frame 1

To determine the functionality of *repABC1*, two plasmid constructs were used: the first contained *repA*, *repB* and *repC1* (pUX3; Fig. 1) and the second contained *repB* and *repC1* (pUX2; Fig. 1). Both constructs were independently cloned in plasmid pUX19. Introduction of these plasmids separately into *A. tumefaciens* showed that neither was able to replicate. This result indicated that *repC1* alone and *repC1* together with *repB* did not allow replication in *A. tumefaciens*. Based on this result, we cloned a 5.5-kb fragment that included the *repABC1* genes into vector pUX19, generating plasmid pUX4 (Fig. 1). When pUX4 was introduced into *A. tumefaciens*, it was able to replicate, resulting in the generation of 3.8×10^4 transformant colony forming units per milliliter. Hybridization experiments showed that the *repABC1* operon is present in the pSym deletion strain *R. leucaenae* CFN 299–10 (Fig. 2b), suggesting that in this strain the pSym replicates under the control of the *repABC1* replicator. Studies performed in our laboratory demonstrated that the symbiotic plasmid is also able to replicate in *Brucella melitensis* and *Ochrobactrum anthropi* (Martínez-Romero, unpublished results), and it is probable that the *repABC1* operon encodes the genetic information needed to replicate in these bacteria and other alpha-Proteobacteria.

We performed phylogenetic studies to determine the distribution and conservation of replication systems similar to that of the *R. leucaenae* CFN 299 pSym and found orthologs that were identical to RepC2 and RepABC1 encoded only on the pSym of *R. tropici* CIAT 899, and *R. freirei* PRF 81. Thus, these replicators are distributed in *Rhizobium* species belonging to symbiovar tropici (Ormeño-Orrillo et al. 2012), all of which have a wide host range of nodulation and are efficient nitrogen fixers (Fig. 3) (Hernández-Lucas et al. 1995b; Hungria et al. 2000; Acosta-Durán and Martínez-Romero 2002). While *Rhizobium* species such as *R. gallicum* bv. gallicum R602, *R. etli* bv. mimosae IE4771 and *R. etli* bv. mimosae Mim1 (Accession Number: PRJNA227036; PRJNA230502; Rogel et al. 2014) also encode RepC and RepABC, their identity with the replication systems of *R. leucaenae* is only 66 and 69%, respectively. Therefore, the *repC2* and

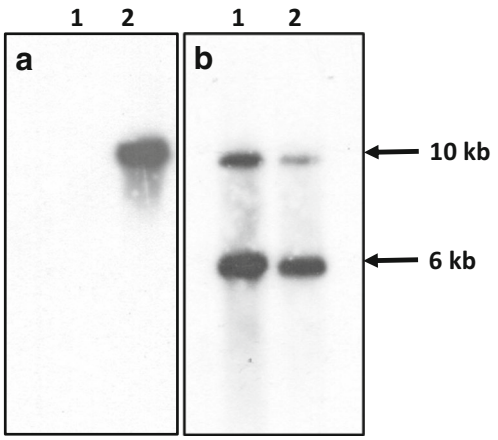


Fig. 2 Presence of *repC2* and *repABC1* replication systems in the pSym plasmid of *Rhizobium leucaenae* CFN 299. **a** Autoradiogram of *Sac*I-digested genomic DNAs hybridized with *repC2*, where the 10-kb band corresponds to this gene. *Lanes*: *1 Rhizobium leucaenae* CFN 299-10, *2 R. leucaenae* CFN 299. **b** Autoradiogram of *Eco*RI-digested genomic DNAs hybridized with the entire *repC1* gene. The 10-kb band corresponds to part of *repC1*, and the 6-kb band corresponds to *repAB* plus part of *repC1*. *Lanes*: *1 R. leucaenae* CFN 299–10, *2 R. leucaenae* CFN 299

repABC1 genes of the pSym plasmid can be used as molecular markers to identify *Rhizobium* species belonging to symbiovar tropici.

In summary, the results of this study show that the *R. leucaenae* CFN 299 pSym contains a functional *repABC* replication system. We also demonstrate the temporal free-living or symbiotic expression of genes encoded in this replicon, several of which are known to be important in, or required for, nodulation, nitrogen fixation, gene regulation or metabolism. These data support other results showing that rhizobial symbiotic plasmids have fundamental roles in plant–microbe interactions and in adaptation to different environments (Barnett et al. 2001). Interestingly, our data suggest that relatively few genes on the pSym are expressed in the *Phaseolus vulgaris–R. leucaenae* CFN 299 interaction, since 8% of the 136 gene fusions evaluated were expressed in symbiosis. It would be of interest to evaluate our fusion and mutant strains in the interaction of strain CFN 299 with other legumes such as *Leucaena leucocephala*, *Gliricidia maculata* or *Lotus corniculatus*. This approach might allow the identification of pSym genes that are expressed in response to specific hosts and increase our understanding of the mechanisms used by rhizobia that establish successful symbioses with multiple legumes. We believe that this information will contribute to a fuller understanding of the symbiosis and allow the rational design of improved rhizobial inoculants important for sustainable agriculture.

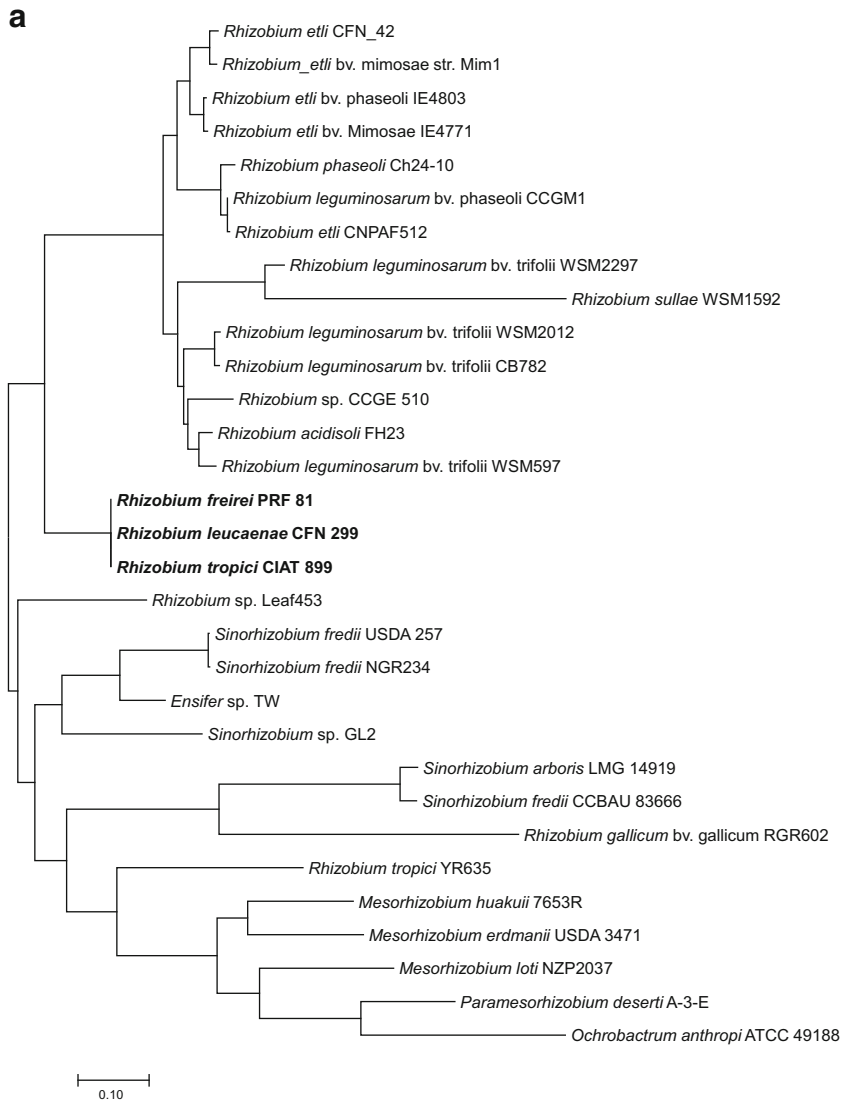


Fig. 3 Maximum likelihood tree showing the phylogenetic relationship of RepC2 (**a**) and RepABC1 (**b**) proteins of *Rhizobium tropici* CIAT 899, *R. leucaena* CFN 299, *R. freirei* PRF 81 and related species. *Bar*: Estimated amino acid substitutions per site

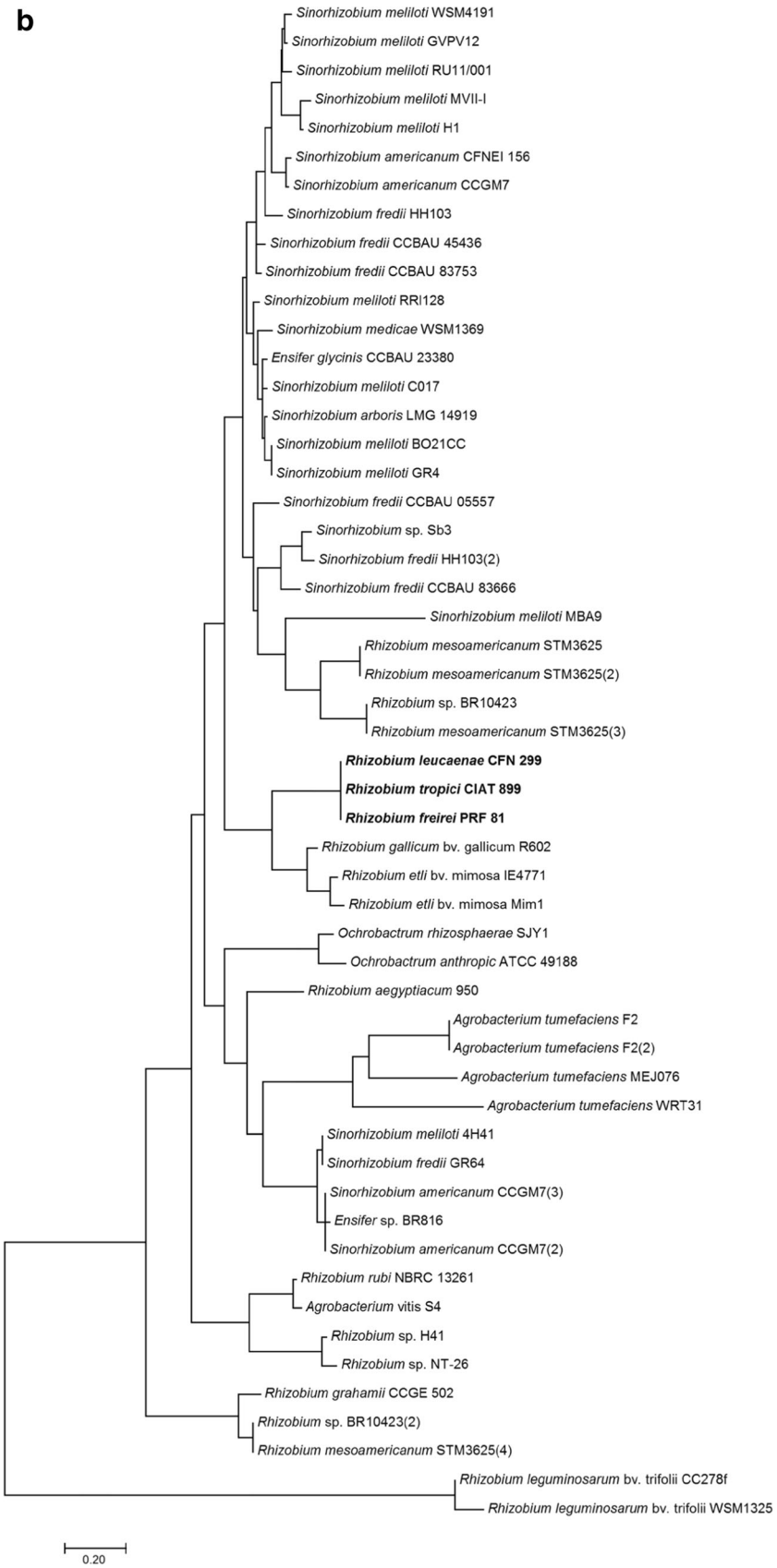


Fig. 3 (continued)

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