Isolation and characterization of functional insertion sequences of rhizobia

Ismael Hernandez-Lucas1, Jose Augusto Ramirez-Trujillo1, Miguel Angel Gaitan1, Xianwu Guo2, Margarita Flores2, Esperanza Martinez-Romero2, Ernesto Perez-Rueda1 & Patrick Mavingui3

1Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México; 2Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México; and 3UMR CNRS 5557, Ecologie Microbiennne, IFR41 Bio-Environnement et Santé, Université Lyon 1, Villeurbanne, France

Correspondence: Ismael Hernandez-Lucas, Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Av. Universidad 2001, Cuernavaca, Morelos 62210, México. Tel.: +52 777 329-1627; fax: +52 777 313-8673; e-mail:ismaelh@ibt.unam.mx

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Abstract
Rhizobia are a group of bacteria that form nodules on the roots of legume host plants. The sequenced genomes of the rhizobia are characterized by the presence of many putative insertion sequences (IS) elements. However, it is unknown whether these IS elements are functional and it is therefore relevant to assess their transposition activity. In this work, several functional insertion sequences belonging to the IS1256, IS3, IS5, IS166, and IS21 families were captured from Rhizobium tropici, Rhizobium sp. NGR234 and Sinorhizobium meliloti, using pGBG1 as a trapping system. In silico analysis shows that homologs of rhizobia mobile elements are present in distantly related genomes, suggesting that Rhizobium IS elements are prone to genetic transfer.

Introduction
Since the original discovery of transposable elements by Barbara McClintock in the late 1940s, many mobile elements have been identified in euukaryotes and prokaryotes. These genetic elements mediate numerous molecular and genetic phenomena such as gene activation, repression, duplication, deletion, inversion, and transfer. These DNA rearrangements can be advantageous, deleterious, or neutral for the host organism. Among transposable elements, insertion sequence (IS) elements are the most widespread. Their sizes range from 200 bp to more than 2500 bp. IS elements share a common structure consisting of short inverted repeats at their ends, and at least one ORF coding for a transposase (Galas & Chandler, 1989). The transposase binds to both inverted repeats and the target DNA. It then performs DNA breakage to remove the element and insert it into its new site. The inserted IS element is flanked by short duplicated sequences at the target site. While transposition of most IS elements occurs by this cut and paste mechanism, several bacterial transposons move by replicative transposition during which two copies of the transposable element are generated. IS elements have been found in different bacterial genomes including those of rhizobia. For instance, the genomes of Agrobacterium tumefaciens (Wood et al., 2001), Bradyrhizobium japonicum (Kaneko et al., 2002), Mesorhizobium loti (Kaneko et al., 2000), and Sinorhizobium meliloti (Galibert et al., 2001) contain IS elements on both chromosome and plasmids. Comparative genomic studies have inferred that these IS elements have shaped the mosaic structure of Rhizobium replicons (Freiberg et al., 1997; Galibert et al., 2001; Gonzalez et al., 2003). In some cases, participation of IS elements in recombination leading to genomic rearrangements has been evidenced by experimental studies on distinct Rhizobium species (Mavingui et al., 1998, 2002; Flores et al., 2000). Although several works have reported the presence of IS elements in rhizobia (Wheatcroft & Watson, 1988; Kosier et al., 1993; Selbitschka et al., 1995, 1999; Rochepeau et al., 1997; Schneiker et al., 1999), transposition activity was demonstrated for only some of them (Priefer et al., 1981, 1989; Ruvkun et al., 1982; Gay et al., 1985; Dusha et al., 1987; Simon et al., 1991; Zekri & Toro, 1996). In this work, we report several functional IS elements in R. tropici CFN299, Rhizobium sp. NGR234, and S. meliloti 1021.
Materials and methods

Bacterial strains, plasmids, culture conditions and matings

The bacterial strains and plasmids used are listed in Table 1. Rhizobia were grown on PY medium (0.5% peptone, 0.3% yeast extract, 10 mM CaCl$_2$) supplemented with the following antibiotics (µg mL$^{-1}$) when required: chloramphenicol (Cm, 10), tetracycline (Tc, 10) and nalidixic acid (Nal, 20). *Escherichia coli* strains were grown in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) supplemented with chloramphenicol (10), kanamycin (Km, 25) and ampicillin (Amp, 100) when needed. *Escherichia coli* and rhizobia were grown at 30 °C. Conjugation experiments were performed in the presence of the helper strain *E. coli* HB101 containing pRK2013 (Figurski & Helinski, 1979) as described previously (Charles & Finan, 1991).

IS entrapment procedure

To isolate active IS elements from rhizobia, the plasmid pGBG1 was used as a trap (Schneider et al., 2000). pGBG1 confers resistance to chloramphenicol and contains the mutagenesis target that consists of a *tetA* gene under the control of the *pR* promoter from bacteriophage λ which is repressed by λ CI repressor. Insertion of mobile elements in the repressor activates the *tetA* gene and allows the positive selection of tetracycline-resistant colonies.

The plasmid pGBG1 was introduced by triparental mating into rhizobia. Transconjugants were obtained at frequency of c. 10$^{-5}$ on PY medium supplemented with chloramphenicol. The presence of pGBG1 was ascertained by plasmid profiles. For *Rhizobium* sp. NRG234 and *Sinorhizobium meliloti* 1021, five individual Cm$^R$ clones containing pGBG1 were selected and were grown separately to late log phase in liquid PY medium supplemented with chloramphenicol. For *Rhizobium tropici* CNF299, one Cm$^R$ colony of RH165 bearing pGBG1 was grown overnight in liquid minimal medium (MM) (Romanov et al., 1994) supplemented with 10 mM succinate. To isolate derivative clones bearing IS inserted into the target, each culture was diluted and spread on the corresponding agar plate medium containing tetracycline. To isolate IS elements from bacteria inside nodules, seeds of *Phaseolus vulgaris* were inoculated with RH165. Nodules from two plants, harvested 18 days after inoculation, were crushed, diluted and 0.1 mL was spread on PY plates containing tetracycline. To estimate the frequencies of transconjugant appearance, cultures were also titered by plating on PY agar containing chloramphenicol alone.

DNA manipulations

Plasmid purification and genomic DNA extraction were performed according to published protocols (Sambrook et al., 1989). Plasmid profiles were obtained by the Eckhardt method as modified by Hynes & McGregor (1990). For hybridization, DNA was digested with restriction enzymes then transferred from agarose gels to nylon membranes. Probes (internal fragments of IS elements) were labelled with $^{32}$P by polymerase extension using random primers and hybridization was carried out under high stringency conditions (Southern, 1975). For sequencing, double stranded DNA was purified with the High Pure Plasmid Isolation Kit (Roche, Germany) and sequencing was performed with an automatic Perkin Elmer/Applied Biosystems 377-18 or Genome Express (Meylan, France) sequencer.

PCR amplification

Primers G11 (5'-TAT CAG CTA TGC GCC GAC CAG AAC-3') and G12 (5'-GCC AAT CCC CAT GGC ATC GAG TAA-3') were used to amplify IS elements inserted into the trapping vector pGBG1 (Schneider et al., 2000). PCR assays were carried out in a 25 µL reaction containing the template genomic DNA (250 ng) in 1 × polymerase...
reaction XL buffer II (Perkin-Elmer), 1.1 mM Mg(OAc)$_2$, 200 µM dNTPs, 5 pmol of each primer, and 1 U of Tth polymerase (Perkin-Elmer). PCR amplifications were performed in a 9700 Thermocycler (Perkin-Elmer) with the following conditions: an initial denaturation at 94°C for 1 min; then 35 cycles of denaturation (94°C, 15 s), annealing and extension (65°C, 3 min); and a final extension at 72°C for 7 min. PCR samples were electrophoresed through 0.8–1% agarose gels in TAE buffer, and stained with ethidium bromide.

**Sequence analysis**

ORF identification was performed with the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/). In order to determine the domain organization of the insertion sequences, predicted ORFs were scanned against databases SUPER-FAMILY v1.63 (Gough et al., 2001) and PFAM. To identify homologs of the IS elements reported in this work, a total of 152 genomes, 129 bacteria, 15 archaea, and eight eukarya (Entrez genome database ftp://ftp.ncbi.nlm.nih.gov/genomes/bacteria) were analyzed. The novel IS elements identified in this work were registered in the IS database at http://www-is.biotoul.fr/is.html

**Nodulation assays**

Plant nodulation assays were performed in flasks containing agar as described previously (Martínez et al., 1985). Seedlings of Phaseolus vulgaris cv. Negro Jamapa were inoculated with $10^5$ Rhizobium cells.

**Results and discussion**

**Isolation of IS elements from rhizobia**

Active IS elements were isolated from rhizobia using the plasmid pGBG1 as a trap (see Materials and methods). The frequency of Tc$^R$ transconjugants among Cm$^R$ colonies was between $10^{-5}$ and $10^{-6}$ for both *Rhizobium* sp. NGR234 and Sinorhizobium meliloti 1021 strains. The plasmid profiles of a total of 100 Tc$^R$ clones (20 per culture) from each strain were analyzed. For *Rhizobium* colonics, colonies resistant to tetracycline appeared at a frequency of around $10^{-6}$ from culture-based selection, without significant differences between PY and MM-succinate media, whereas those from nodules emerged at a frequency of $3 \times 10^{-7}$. A total of 46 Tc$^R$ clones obtained from nodules and 85 Tc$^R$ clones selected in culture media were screened by plasmid profiles. Presence of IS elements in the target was inferred by an increase in size of pGBG1 in plasmid patterns of Tc$^R$ transconjugants. The proportion of resistant transconjugants harboring larger sized-pGBG1 plasmid was 22% for *Rhizobium* sp. NGR234 and 15% for *S. meliloti* 1021, whereas for *R. tropici* it was 6.5% from synthetic media and 8.5% from nodules. Plasmid patterns of the remaining Tc$^R$ clones showed no detectable changes in the size of pGBG1, indicating point mutations, and microdeletion or micro-insertion events. All positive Tc$^R$ clones bearing potential mobile elements were further analyzed by PCR amplification using primers G11 and G12 (Schneider et al., 2000). Sequence analyses of the PCR products revealed eight distinct IS elements, five from *R. tropici*, designated as ISRt1, ISRt2, ISRt3, ISRt4 and ISRt5, two from *Rhizobium* sp. NGR234 (Freiberg et al., 1997; Perret et al., 1997) designated here as ISNGR3 and ISNGR4, and one from *S. meliloti* 1021 previously reported as ISRm1 (Watson & Wheatcroft, 1991). ISRt1, ISNGR3, ISNGR4 and ISRm1 were isolated from bacteria grown in PY medium, and their sequences were identical to the published data. ISRt2 and ISRt3 were obtained from bacteria grown in MM-succinate. ISRt4 and ISRt5 were isolated from bacteria recovered from nodules inoculated with the *R. tropici* RH165 transconjugant.

**Characterization of functional IS elements from rhizobia**

A structural analysis of the isolated IS elements was performed. The relevant characteristics such as length (bp), number of ORFs, transposase size, G+C content, IS family, copy number and distribution of rhizobia IS elements in different genomes are shown in Table 2. As the functional IS elements from *Rhizobium* sp NGR234 and *S. meliloti* 1021, as well as the ISRt1 of *R. tropici* isolated here were previously reported, only novel mobile IS obtained from *R. tropici* will be further described. In addition to the salient characteristics mentioned in Table 2, we found other relevant features of *R. tropici* IS elements. For instance, ISRt2 contains a DDE motif typical of the IS3 family (Mahillon & Chandler, 1998). ISRt2 is highly similar (81%) to IS686 of *Agrobacterium tumefaciens* biotype III (Paulus et al., 1991). Furthermore, the GC content of ISRt2 and IS868 are similar, 59.2% and 59.3%, respectively, suggesting a common origin. By sequencing the region adjacent to ISRt2 located on the symbiotic plasmid of *R. tropici* CFN299, we determined that this mobile element is flanked by genes for citrate synthase and isocitrate lyase, with a GC content of 56.4% and 57.7%, respectively. This result implies that ISRt2 may have an external origin. The copy numbers (i.e. number of hybridization bands) of ISRt2 in *R. tropici* was determined by Southern hybridization of EcoRI-digested genomic DNA (Fig. 1). As no cleavage site for EcoRI is present in the IS element the number of hybridizing bands reflect the copy number. The resulting IS profiles of CFN299 and CFN299-10 (a derivative of 299 lacking plasmid B and 300 kb fragment of the symbiotic plasmid) shows that
Table 2. Mobile IS elements trapped from rhizobia

<table>
<thead>
<tr>
<th>Strain</th>
<th>IS isolated</th>
<th>Length (bp)</th>
<th>G+C %</th>
<th>ORFs</th>
<th>IR/</th>
<th>Tnp size (bp)</th>
<th>IS family</th>
<th>Copy number</th>
<th>Gene Bank accession number</th>
<th>Similarity (%) with other organisms</th>
</tr>
</thead>
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<tr>
<td>Rhizobium tropici CFN299</td>
<td>ISRr1</td>
<td>1364</td>
<td>61</td>
<td>2</td>
<td>28/4</td>
<td>172</td>
<td>IS1256</td>
<td>6</td>
<td>AF041379</td>
<td>45–85% α, Gram-</td>
</tr>
<tr>
<td></td>
<td>ISRr2</td>
<td>1321</td>
<td>59</td>
<td>2</td>
<td>26</td>
<td>301</td>
<td>IS3</td>
<td>1</td>
<td>AY751757</td>
<td>46–81% α, β, γ, Gram-</td>
</tr>
<tr>
<td></td>
<td>ISRr3</td>
<td>933</td>
<td>57</td>
<td>1</td>
<td>28/7</td>
<td>273</td>
<td>IS5</td>
<td>1</td>
<td>AY753543</td>
<td>37–85% β, γ, Gram-</td>
</tr>
<tr>
<td></td>
<td>ISRr4</td>
<td>932</td>
<td>58</td>
<td>1</td>
<td>28/12</td>
<td>291</td>
<td>IS5</td>
<td>6</td>
<td>AY753544</td>
<td>44–87% α, β, Cyanobacteria,</td>
</tr>
<tr>
<td></td>
<td>ISRr5</td>
<td>2699</td>
<td>60</td>
<td>3</td>
<td>27</td>
<td>451</td>
<td>IS166</td>
<td>1</td>
<td>DQ499058</td>
<td>38–70% α, β, Cyanobacteria,</td>
</tr>
<tr>
<td>Rhizobium sp. NGR234</td>
<td>INGR3</td>
<td>2625</td>
<td>62</td>
<td>2</td>
<td>31/2</td>
<td>316</td>
<td>IS21</td>
<td>3</td>
<td>U00090</td>
<td>38–65% α, β, Cyanobacteria,</td>
</tr>
<tr>
<td></td>
<td>INGR4</td>
<td>3324</td>
<td>63</td>
<td>4</td>
<td>0</td>
<td>694</td>
<td>ND</td>
<td>9</td>
<td>U00090</td>
<td>49–81% α, β, Gram+</td>
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<tr>
<td>Sinorhizobium meliloti 1021</td>
<td>ISRm1</td>
<td>1321</td>
<td>60</td>
<td>2</td>
<td>23/3</td>
<td>276</td>
<td>IS3</td>
<td>9</td>
<td>X56563</td>
<td>54–60% α, β</td>
</tr>
</tbody>
</table>

*Based on protein and catalytic domain analyses.
1Determined by hybridization.
IS, insertion sequence; ND, not determined; Tnp, transposase; IR, inverted repeats.

Fig. 1. Presence of the IS elements in Rhizobium tropici. Autoradiogram of Southern blot of digested genomic DNA hybridized with IS probes. In lane A, CFN299 shows a 5 kb signal. In lane B, no signal was detected in CFN299-10. In lane C, CFN299 shows a 2.2 kb hybridization band. In lane D, no hybridization band was detected in CFN299-10. In lane E, CFN299, and lane F, CFN299-10 identical hybridization profile was obtained. In lane G, CFN299 shows six bands ranging from 3.8 to 9 kb. In lane H, CFN299-10 present two bands of 4.5 and 6.5 kb. Genomic DNAs were restricted with endonucleases EcoRI (lanes A and B) and PstI (lanes C–H). Lanes A–B, C–D, E–F, and G–H correspond to DNA pattern hybridized with probe ISRr2, ISRr3, ISRr5, and ISRr4, respectively.

CFN299 presents a single hybridization band, while no signal was obtained with CFN299-10, suggesting that the mobile element is located on a plasmid. Furthermore, the sequencing project of the R. tropici symbiotic plasmid confirms that ISRr2 is located in this plasmid. No hybridization signal was detected in the genome of Agrobacterium, sp. K-Ag-3, Agrobacterium sp. Ch-Ag-4, Mesorhizobium huakui CCBAU2609, Rhizobium etli CFN42, Rhizobium sp. CFN234, Sinorhizobium terangae USDA4102, and Mesorhizobium mediterraneum USDA 3392 (data not shown). The scarcity of ISRr2 among rhizobia suggests that this mobile element was recently acquired in its host genome.

ISRr3 encodes a putative transposase of 273 aa which is highly similar (85%) to the IS element ISRIF7-2 of R. leguminosarum (W. Selbitschka et al., unpublished results), and it contains the N3 (DGGY) and C1 (LPRRWVVERT) domains, typical of the IS family (Rezsohazy et al., 1992; Zekri et al., 1998). In order to determine the copy number of ISRr3 in R. tropici, PstI restricted genomic DNA was hybridized with an internal fragment of ISRr3 as a probe (Fig. 1). One hybridizing band of 2.2 kb was detected in CFN299, however no signal was observed in CFN299-10. This result indicates that ISRr3 is present in R. tropici plasmid B or in the symbiotic plasmid.

ISRr4 is predicted to encode a transposase of 291 aa that is similar (87%) with ISRm4-1 from S. meliloti GR4 (Soto et al., 1992; Zekri et al., 1998). ISRr4 contains the N3 and C1 domains, typical of the IS family (Mahillon & Chandler, 1998). The copy number (Fig. 1) of this element was determined and the result shows that it is present in a single copy in both CFN299 and CFN299-10, therefore the exact localization of ISRr4 remains to be established.

ISRr5 presents the features typical of the IS66 family, such as the presence of multiple ORFs. ISRr5 encodes products of 159, 117 and 451 aa, respectively. The 159 and 117 aa proteins are 67% and 92% identical to ynhF and ynl of A. tumefaciens (Winans et al., unpublished results), whereas the 451 aa protein is 70% identical to a transposase from Nitrobacter hamburgensis (Copeland et al., unpublished...
results). It is important to mention that \textit{ynH} and \textit{ynI} ORFs overlap by 17 bp that include the termination codon of \textit{ynH} and the initiation codon of \textit{ynI}, suggesting that translational coupling is involved in expression of \textit{YnI}; this genetic signature is common in members of the IS66 family (Chang-Gyun et al., 2001). Southern hybridization experiments show that IS\textit{Rtr5} was present in six copies in the genome of \textit{R. tropici} CFN299 (Fig. 1). The hybridization profiles of CFN299-10 suggest that at least four copies of IS\textit{Rtr5} are located on the symbiotic plasmid or in plasmid B.

In this work, the mobility of the IS elements from rhizobia was evaluated in minimal and rich media as well as in nodules using a plasmid trapping system. The percentage of IS capture was similar in free-living conditions and in nodules. The mobile elements isolated have a smaller size (932–3324 bp) and present conservative properties of IS elements such as inverted repeats and the presence of at least one ORF encoding for a transposase. Some of the mobile elements captured in this work seem to be specific for each rhizobial strain, since as they are not present in the completely sequenced genomes of other members of the \textit{Rhizobiaceae} family. Therefore, these mobile elements can be used as a molecular tool to identify strains belonged to \textit{R. tropici}, \textit{S. meliloti} and \textit{Rhizobium} sp. NGR234. It is important to mention that a moderate number of IS elements were trapped, even though some of them not detected here are known to be present in high copy numbers (e.g. in the genome of \textit{S. meliloti} 1021 IS\textit{Rm5} is present in 10 copies and IS\textit{Rm11} in 12 copies). The reason could be that such elements have a low frequency of transposition, or that under the physiological conditions tested the mobilization of these elements is not occurring, or these elements have preferential target sequences, or a conditional function. It is also possible that some of these IS are no longer functional. However, their ability to move should be tested by other methods.

IS elements are involved in several processes such as transposition and rearrangements. IS elements may confer a selective advantage to their host. For instance, it was reported that increased motility in \textit{Escherichia coli} is obtained by spontaneous insertion of an IS1 upstream of the \textit{flhD} operon (Barker et al., 2004). It is also known that IS150 mediates deletions of the ribose operon, and such deletions are beneficial to \textit{E. coli} in glucose minimal medium (Cooper et al., 2001). Previously we reported that several nodulation and nitrogen fixation genes are delimited by two IS\textit{Rtr} elements and recombination between these elements generate a circular 60 kb fragment. \textit{Agrobacterium tumefaciens} containing the 60 kb region acquires the ability to produce nod factors and to nodulate \textit{Phaseolus vulgaris}. In this example, a biological role of IS\textit{Rtr1} was demonstrated (Mavingui et al., 1998). However many important issues such as the functional role of rhizobial IS’s in free-living cells and in symbiosis, as well as their transposition mechanism remain to be established. In this regard, preliminary results obtained in our laboratory suggest that the IS66 family represented by IS\textit{Rtr5} move by replicative transposition whereas the IS5 family represented by IS\textit{Rtr3} move by a cut and paste mechanism in \textit{R. tropici}.

In conclusion, we demonstrated the functionality of six IS elements (IS\textit{Rtr2}, IS\textit{Rtr3}, IS\textit{Rtr4}, IS\textit{Rtr5}, IS\textit{NGR3} and IS\textit{NGR4}) and confirmed the transposition activity of two previously reported mobile elements (IS\textit{Rtrl} and IS\textit{Rm11}). The mobile elements isolated are present in single or multiple copies in host genomes and homologous transposases were detected in several other bacterial genera as well as in archaea branch suggesting that rhizobia IS elements are prone to genetic transfer. In addition to their mobility and potential associated mutator phenotype, IS elements are sites for homologous recombination that generate genomic rearrangements, thus participating in shaping the overall structure of rhizobia genomes.

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**References**


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