The conjugative plasmid of a bean-nodulating Sinorhizobium fredii strain is assembled from sequences of two Rhizobium plasmids and the chromosome of a Sinorhizobium strain

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Abstract

Background: Bean-nodulating Rhizobium etli originated in Mesoamerica, while soybean-nodulating Sinorhizobium fredii evolved in East Asia. S. fredii strains, such as GR64, have been isolated from bean nodules in Spain, suggesting the occurrence of conjugative transfer events between introduced and native strains. In R. etli CFN42, transfer of the symbiotic plasmid (pRet42d) requires cointegration with the endogenous self-transmissible plasmid pRet42a. Aiming at further understanding the generation of diversity among bean nodulating strains, we analyzed the plasmids of S. fredii GR64: pSfr64a and pSfr64b (symbiotic plasmid).

Results: The conjugative transfer of the plasmids of strain GR64 was analyzed. Plasmid pSfr64a was self-transmissible, and required for transfer of the symbiotic plasmid. We sequenced pSfr64a, finding 166 ORFs. pSfr64a showed three large segments of different evolutionary origins; the first one presented 38 ORFs that were highly similar to genes located on the chromosome of Sinorhizobium strain NGR234; the second one harbored 51 ORFs with highest similarity to genes from pRet42d, including the replication, but not the symbiosis genes. Accordingly, pSfr64a was incompatible with the R. etli CFN42 symbiotic plasmid, but did not contribute to symbiosis. The third segment contained 36 ORFs with highest similarity to genes localized on pRet42a, 20 of them involved in conjugative transfer. Plasmid pRet42a was unable to substitute pSfr64a for induction of pSym transfer, and its own transfer was significantly diminished in GR64 background. The symbiotic plasmid pSfr64b was found to differ from typical R. etli symbiotic plasmids.

Conclusions: S. fredii GR64 contains a chimeric transmissible plasmid, with segments from two R. etli plasmids and a S. fredii chromosome, and a symbiotic plasmid different from the one usually found in R. etli bv phaseoli. We infer that these plasmids originated through the transfer of a symbiotic-conjugative-plasmid cointegrate from R. etli to a S. fredii strain, and at least two recombination events among the R. etli plasmids and the S. fredii genome. As in R. etli CFN42, the S. fredii GR64 transmissible plasmid is required for the conjugative transfer of the symbiotic plasmid. In spite of the similarity in the conjugation related genes, the transfer process of these plasmids shows a host-specific behaviour.

Background

Bacterial species belonging to the Rhizobiaceae are common inhabitants of the soil and the rhizosphere. Most of them are able to establish a symbiotic relationship with the roots of leguminous plants through the formation of nodules, where bacteria differentiate into nitrogen fixing bacteroids [1]. The genomes of these bacteria contain a circular chromosome. Some, like Agrobacterium tumefaciens, also contain a linear chromosome, in addition to a variable number of plasmids, which may carry up to 50% of the genomic sequence. The bacterial genetic information required for the establishment of the symbiosis is usually localized on large plasmids, or in genomic islands [2].
Conjugative transfer is thought to be the most relevant mechanism that contributes to the dissemination and diversification of genetic information, particularly that localized on plasmids. Conjugation systems are constituted by a DNA transfer and replication (Dtr) component, encoded by tra genes and a cis-acting oriT site, and a mating pair formation (Mpf) component, encoded by trb genes [3]. Information on the conjugative transfer mechanisms of rhizobial plasmids is still scarce. However, two groups of plasmids containing a functional set of conjugative transfer genes have been described: Type I, which are regulated by quorum-sensing [3-5], and Type II, which have permanently RctA-repressed transfer genes [3,6]. A third type of conjugative plasmids has been recently proposed, represented by the largest plasmids of *R. leguminosarum* bv *viciae* strains [3]. Some plasmids are mobilizable in the presence of transmissible plasmids, either by cointegration (conduction) [7], or by classical (trans) helper mechanisms [8,9]. Specifically in the bean nodulating type strain *Rhizobium etli* CFN42, we have previously shown that it contains a quorum-sensing regulated self-transmissible plasmid (pRet42a) [5], and that transfer of the symbiotic plasmid (pRet42d) occurs only in the presence of pRet42a. The event requires cointegration of both replicons. This may be achieved through IntA-dependent site-specific recombination between *attA* and *attD* sites, or through RecA-dependent homologous recombination among large sequence segments shared between the replicons. The cointegrate is able to transfer, using the pRet42a-encoded machinery. In the transconjugants, the cointegrate is usually resolved to regenerate the wild-type plasmids, but in a few cases, resolution of the cointegrate leads to the formation of recombinant plasmids that contain segments of each plasmid, pRet42a and pRet42d [7]. Mesoamerica has been identified as the place of origin of bean plants and *Rhizobium etli* bacteria [10], while soybean and its nodulating bacteria (*Sinorhizobium fredii*) originated in East Asia [11]. In the early XVIth century, common beans and their symbionts were transported to Europe and other parts of the world. A survey of bean-nodulating strains in Granada, Spain, showed the presence of strains belonging to five different species: *R. etli*, *R. gallicum*, *R. gardineri*, *R. leguminosarum* and *S. fredii* [12]. The usual host of *Sinorhizobium fredii* strains is soybean (*Glycine soja*), not common bean (*Phaseolus vulgaris*). Nevertheless, the bean-nodulating strains classified as *S. fredii*, were unable to nodulate cvs. Williams or Peking of *Glycine max*. Hybridization of digested genomic DNA with *nodB* and *nifH* genes from *R. etli*, showed a very weak signal [12]. *R. etli* bv phaseoli symbiotic plasmids (pSym) are characterized by the presence of three copies of *nifH*. The bean-nodulating *S. fredii* strains showed only one copy of this gene [12]. While conjugative transfer may explain the acquisition of new symbiotic features by strains belonging to diverse species, the relationship between *R. etli* and bean-nodulating *S. fredii* is not so easily established. In order to gain further insight into the mechanisms and pathways leading to the generation of new rhizobial strains, in this work we present the analysis of the bean-nodulating *S. fredii* strain GR64, isolated from the soil in Granada. The results indicate that the plasmids present in GR64 likely derived from conjugative transfer and rearrangement events among sequences localized in at least three different replicons, including two different plasmids and a chromosome.

**Results**

**Plasmid pSfr64b is required for symbiosis but pSfr64a is dispensable**

Strain GR64 contains two plasmids: pSfr64a (183 kb) and pSfr64b (~400 kb) (Figure 1A, Table 1). A band corresponding to a megaplasmid (~1300 kb), has been visualized [13], but is not always clearly apparent in the gels. Plasmid pSfr64b was identified as the symbiotic plasmid [13], because it hybridizes with the *nifH* gene. Nodulation assays confirmed that the genetic information in pSfr64b is necessary and sufficient to establish symbiosis. Table 2 shows that all derivatives carrying pSfr64b, were able to form nodules (GR64, CFN2001-1, GMI9023/pSfr64b), and that the construct lacking pSfr64b (GR64-4) was unable to nodulate beans. Consistent with previous findings [14,15], the number of nodules was decreased in an *Agrobacterium* genomic background. On the other hand, lack of pSfr64a had no effect on the symbiotic process (GR64-2), and its presence in *Agrobacterium* did not confer nodulation capacity to the receptor, indicating that pSfr64a encodes none of the essential symbiotic genes.

**Plasmid pSfr64a shares sequences with the *R. etli* pSym, pRet42a, and with the chromosome of *Sinorhizobium fredii* NGR234**

We sequenced plasmid pSfr64a (GenBank accession number: CP002245). The main features of this plasmid are shown in Figure 2 and Additional File 1. Plasmid pSfr64a is 183 612 bp long. The genetic organization of this plasmid clearly reveals its chimeric nature, since 38 (23%) of the 166 ORFs encoded in the plasmid presented highest similarity to sequences of the chromosome of *Sinorhizobium fredii* NGR234, while 87 (52%) were most similar to ORFs encoded in *R. etli* CFN42 plasmids pRet42a (36 ORFs, 22%) and pRet42d (51 ORFs, 31%).

The functional assignment of the 166 ORFs (Figure 2, Table 3) shows that the plasmid is largely involved in metabolic, transport and conjugative functions.

Among the ORFs shared between pSfr64a and pRet42a, the self-transmissible plasmid of CFN42, most are related to conjugative transfer (20 ORFs), only two were ascribed to macromolecular metabolism. Interestingly, both are
Figure 1. Eckhardt type gel showing the plasmid profile of S. fredii strain GR64 and derivatives, in comparison to R. etli CFN42. Panel A. Ethidium bromide stained Eckhardt gel. Lane 1: CFN42, lane 2: wild type GR64, lane 3: GR64-2, lane 4: GR64-3, lane 5: GR64-4, lane 6: GR64-5, lane 7: GR64-6, lane 8: GM10023/pSfr64a, lane 9: GM10023/pSfr64b, lane 10: CFN2001, lane 11: CFN2001-1, lane 12: CFN2001-2, lane 13: CFN2001-3. Panel B. Ethidium bromide stained Eckhardt gel (lanes 1 and 2), and Southern blot of the plasmid profiles probed with pSfr64a (lanes 3 and 4). Lanes 1 and 3: GR64-1 (GR64/pSfr64a::Tn5-GDYN, pSfr64b::Tn5mob), lanes 2 and 4: GR64-2 (pSfr64a-, pSfr64b::Tn5mob).

Table 1. Strains and plasmids used in this study

| Strain          | Relevant characteristic                                      | Source                  |
|-----------------|-------------------------------------------------------------|-------------------------|
| **Rhizobium**   |                                                             |                         |
| CFN42           | wild type R. etli (pRet42a to pRet42f)                      | [58]                    |
| CFN2001         | CFN42 lacking pRet42a and pRet42d                           | [37]                    |
| CFNX195         | CFN42 derivative cured of pRet42a, pRet42d::Tn5mob          | [32]                    |
| GR64            | wild type bean-nodulating S. fredii (pSfr64a, pSfr64b)      | [12]                    |
| GR64-1          | GR64/pSfr64a::Tn5-GDYN, pSfr64b::Tn5mob                      | This work               |
| GR64-2          | GR64 cured of pSfr64a, pSfr64b::Tn5mob                       | This work               |
| GR64-3          | GR64-2 with pRet42a::Tn5-GDYN                               | This work               |
| GR64-4          | GR64 cured of pSfr64a and pSfr64b, RifR                      | This work               |
| GR64-5          | GR64-4/ppRet42a::Tn5-GDYN                                   | This work               |
| GR64-6          | GR64-4/pSfr64a::Tn5-GDYN                                    | This work               |
| CFN2001-1       | CFN2001/pSfr64b::Tn5mob                                     | This work               |
| CFN2001-2       | CFN2001/pSfr64b::Tn5mob, pRet42a::Tn5-GDYN                   | This work               |
| CFN2001-3       | CFN2001/pSfr64b::Tn5mob, pSfr64a::Tn5-GDYN                   | This work               |
| **Escherichia coli** |                                                        |                         |
| DH5a            | Receptor for transformation                                 | [59]                    |
| S17-1           | C600:RP-4::Tn5-GDYN                                          | [60]                    |
| S17pOR21        | Source of Tn5-GDYN                                          | [17]                    |
| **Agrobacterium tumefaciens** |                                                      |                         |
| GM10023         | C-58 cured of its native plasmids                            | [35]                    |
| UIA143          | recA pTi derivative of C58                                   | [61]                    |
| GM10023/pSfr64a | GM10023 with pSfr64a::Tn5-GDYN                              | This work               |
| GM10023/pSfr64b | GM10023 with pSfr64b::Tn5mob                                 | This work               |
| **Plasmids**    |                                                             |                         |
| pSUP5011        | Tn5mob                                                      | [60]                    |
| pRK2013         | Conjugation helper                                          | [36]                    |
related to DNA metabolism, one was classified as a putative nuclease, and the other as a probable DNA methylase. In Figure 3, it can be appreciated that the genomic region shared between pRet42a and pSfr64a is markedly colinear. Colinearity is disrupted by the absence of an homolog to the regulatory gene cinR of pRet42a, and the presence of pSfr64a ORFs 147 and 148, which encode hypothetical proteins. The correspondence between pSfr64a and pRetCFN42 ORFs is presented in Additional File 1. Figure 2 shows that the segment of pSfr64a shared with pRet42a has a high GC content, compared to the rest of the plasmid. This feature is also present in the similar pRet42a sequence.

The ORFs similar to the pSym of CFN42 (pRet42d) include the repABC genes (Figure 2, Table 3). This is congruent with our finding that pSfr64a and pRet42d

![Figure 2 Structure of plasmid pSfr64a](image)

Table 2 Nodulation assay of bean-nodulating strains

| Strain                  | Relevant features | N° nodules/ plant |
|-------------------------|-------------------|-------------------|
| CFN42 wild type R. etli |                   | 57.3 (31.0)       |
| GR64 wild type bean-nodulating S. fredii | | 30.6 (5.3) |
| CFN2001-1 pSfr64a: Tn5mob |   | 31.6 (13.1) |
| GR64-2 pSfr64a, pSfr64b: Tn5mob | | 24.7 (4.7) |
| GR64-4 pSfr64a, pSfr64b | | 0 |
| GM9023/ pSfr64a | GM9023 with pSfr64b: Tn5mob | 4.6 (3.2) |
| GM9023/ pSfr64a | GM9023 with pSfr64a: Tn5-GDYN | 0 |
| GM9023 wild type | | 0 |

* Average of three plants.
* Standard deviation.

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http://www.biomedcentral.com/1471-2180/11/149
Table 3 Functional assignment of pSfr64a ORFs

| Function                     | Total ORFs | p42d | p42a | NGR234 |
|------------------------------|------------|------|------|--------|
| Small Molecule Metabolism    | 48         | 25   | 0    | 15     |
| Macromolecule Metabolism     | 5          | 0    | 2    | 1      |
| Chemotaxis                   | 4          | 2    | 0    | 1      |
| Transport of small molecules | 28         | 13   | 0    | 11     |
| Transposon-related           | 4          | 0    | 0    | 0      |
| Replication                  | 3          | 3    | 0    | 0      |
| Conjugation                  | 20         | 0    | 20   | 0      |
| Transcriptional regulation   | 14         | 5    | 0    | 4      |
| Conserved hypothetical       | 38         | 3    | 14   | 6      |
| Unknown                      | 2          |      |      |        |
| Total                        | 166        | 51   | 36   | 38     |

a Number of pSfr64a ORFs assigned to each functional category.
b Number of pSfr64a ORFs in each category, with highest similarity to ORFs from pRet42d.
c Number of pSfr64a ORFs in each category, with highest similarity to ORFs from pRet42a.
d Number of pSfr64a ORFs in each category, with highest similarity to ORFs from the chromosome of NGR234.

are incompatible (data not shown). The pSfr64a-pRet42d-shared ORFs are mainly involved in small molecule metabolism (26 ORFs), and carbohydrate transport (13 ORFs). It is noteworthy that, in spite of the fact that pRet42d carries genes engaged in symbiotic functions, none of these are present in pSfr64a.

Within the region similar to pRet42d (ORFs 46 to 110), the colinearity is restricted to small segments, some of them in inverse orientation. (Figure 3, Additional file 1). The repABC genes (pSfr64a ORFs 164 to 166) were adjacent to the transfer region, separated from the other pRet42d genes. It has been amply documented that plasmid pRet42d is subject to frequent genomic rearrangements, due to the presence of repetitions and a high density of insertion sequences [16-20].

R. etli ORFs encoding transposon-related proteins located near to the sites where colinearity is disrupted are indicated in Figure 2 (purple arrows) and Additional File 1. For example, pSfr64a ORFs 122 to 146 are colinear with pRet42a ORFs 139 to 162. The adjacent ORF on pRet42a (ORF 138) encodes a transposon-related protein. It is possible that these sequences are related to the generation of rearrangements, causing the interruptions in colinearity. ORFs 114, 115, 116, 117, 118 and 121 show homology to ORFs encoded in another Rhizobium etli strain; IE4771 [21]. The genome of this strain has been sequenced, but not assembled, so we cannot assign them to the pSym, although that is their most probable localization.

The majority of the ORFs shared between pSfr64a and the chromosome of NGR234 are related to small molecule metabolism (15 ORFs), and to the transport of small molecules (11 ORFs). As shown in Figure 3 and Additional File 1 this region is also highly colinear with the corresponding genes on the chromosome of NGR234. Data presented in this section suggest that pSfr64a was assembled during evolution as a chimeric structure, harboring segments from two separate R. etli plasmids and the chromosome of a Sinorhizobium strain, such as NGR234.

Plasmid pSfr64a is transmissible and required for transfer of pSfr64b

The structural conservation on pSfr64a of genes involved in conjugation, raised the possibility of self-transmissibility of this replicon; therefore, the conjugative capacity of GR64 plasmids was studied. The results (Table 4) show that plasmid pSfr64a is transmissible at a high frequency. The symbiotic plasmid pSfr64b was also able to perform conjugative transfer, but only when pSfr64a was present. We conclude that pSfr64a provides transfer functions to pSfr64b. The process could be similar to what we described for CFN42, where pRet42a induces pSym transfer by cointegration. Alternatively, pSfr64b mobilization could be induced in trans. Interestingly, the transfer frequency of this pSym was found
Table 4 Transfer frequency of self-transmissible and symbiotic plasmids

| Donor         | Relevant genotype | Transfer Frequency |
|---------------|-------------------|--------------------|
|               |                   | STP<sup>a</sup>   |
| CFN42         | wild type R. etli | 10<sup>−2</sup>   |
| CFN195        | CFN42 derivative: pRet42a, pRet42d: Tn5mob | -<sup>d</sup> ND<sup>d</sup> |
| GR64          | wild type S. fredii | 10<sup>1</sup> 10<sup>4</sup> |
| GR64-2        | GR64/pSfr64a, pSfr64b: Tn5mob | - ND |
| GR64-3        | GR64-2/pRet42a: Tn5-GDYN | ND ND |
| GR64-5        | GR64/pSfr64a, pSfr64b, pRet42a: Tn5-GDYN | ND - |
| GR64-6        | GR64/pSfr64a, pSfr64b, pSfr64a: Tn5-GDYN | 10<sup>1</sup> - |
| CFN2001-1     | CFN2001/pSfr64b: Tn5mob | - ND |
| CFN2001-2     | CFN2001-1/pRet42a: Tn5-GDYN | 10<sup>4</sup> 10<sup>6</sup> |
| CFN2001-3     | CFN2001-3/pSfr64a: Tn5-GDYN | ND ND |

<sup>a</sup> Strain GM19023 was used as receptor. All crosses were repeated at least three times.

<sup>b</sup> Expressed as the number of transconjugants per donor.

<sup>c</sup> STP: Self Transmissible Plasmid

<sup>d</sup> Not done

<sup>e</sup> not detected (transfer frequency < 10<sup>−9</sup>).

to be two orders of magnitude higher than that of R. etli CFN42 pSym.

**Genomic background determines functionality of conjugal plasmids**

In order to assess the specificity of pSym transfer induction, we constructed derivatives containing diverse plasmid combinations, in either R. etli or S. fredii genomic backgrounds, as described in Materials and Methods, and determined the transfer frequency of the self-transmissible and symbiotic plasmids (Table 4). Analysis of a derivative containing the R. etli self-transmissible plasmid pRet42a in S. fredii background (GR64-3) showed a dramatic decrease in the transfer ability of the plasmid as well as no transfer of the GR64 pSym. These results suggest that the genome of GR64 contains an inhibitor of pRet42a transfer. The decrease in pRet42a transfer could mask its function as helper for pSym transfer induction. The fact that pRet42a transfer is also decreased in a derivative lacking the pSym of GR64 (GR64-5), points to a chromosomal location of the putative inhibitor locus. Similarly, S. fredii pSfr64a was unable to perform conjugal transfer or induce transfer of pSfr64b in R. etli genomic background (CFN2001-3). Only R. etli pRet42a was still able to induce pSfr64b transfer in the R. etli background (CFN2001-2).

The pSym of GR64 differs from the typical R. etli pSym

To further analyze the bean-nodulating S. fredii strain GR64, we performed a phylogenetic analysis with chromosomal genes (recA, rpoB), and with the plasmid-encoded genes nifH and repB. The results (Figure 4) show that, based on the phylogeny of the chromosomal genes, GR64 clusters within the fredii clade, while nifH and repB genes group strain GR64 with other bean-nodulating Sinorhizobium strains isolated from the South of Spain (Granada and Sevilla) [22,23] and from the North of Africa (Tunisia) [24] (Figure 4C). The data obtained indicate that GR64 has a S. fredii chromosome but carries a pSym that allows nodulation of Phaseolus. However, this plasmid differs from typical R. etli pSyms in its replication genes, allowing it to coexist with plasmid pSfr64a, which does share its replication genes with the R. etli pSym. Another feature that differentiates this pSym is the presence of a single copy of the nifH gene.

**Discussion**

Genomic comparisons of *S. meliloti*, *A. tumefaciens*, and R. etli [25], and between *Rhizobium leguminosarum* bv. *vicea* and *Rhizobium etli* [26], have shown that chromosomes are well conserved both in gene content and gene order, whereas plasmids presented few common regions and lacked synteny, except for some pairs of plasmids whose features indicate that they were part of the ancestral genome, and may be considered as secondary chromosomes [26,27]. In R. etli, the symbiotic and self-transmissible plasmids are the less conserved replicas [25] with fewer collinear blocks [26].

In this paper we show that a conjugative plasmid from a bean nodulating *S. fredii* strain is formed by large segments of replicons found in strains belonging to different species from diverse geographic origins. These replicons include two plasmids of *R. etli*, and a S. fredii chromosome. In GR64, bean-nodulation is provided by pSfr64b. Although the phylogenetic relationship of the GR64 nifH gene shows that it is closely related to the *R. etli* gene (Figure 4), pSfr64b differs from the typical *R. etli* pSym in other features (see above).

We have previously reported that *R. etli* pRet42a is able to form a cointegrate with the pSym, and thus promote its conjugal transfer, and that in some cases (10% of the events), resolution of the cointegrate leads to the generation of recombinant plasmids containing segments of both pRet42a and the pSym [7]. Also, the occurrence of frequent genomic rearrangements in rhizobial species has been amply documented [19,20,25,28].

Integrating these data, we propose that the *R. etli* plasmids were transferred to a S. fredii strain and recombination events among the plasmids, the chromosome, and possibly another endogenous S. fredii plasmid, led to the generation of plasmids pSfr64a and pSfr64b. This would indicate that pSfr64a is an evolutionary "new" plasmid of chimeric origin, that was generated after *R. etli* strains arrived to Europe, following the discovery of America, when bean seeds coated with bacteria were most likely introduced to that continent [29]. It is noteworthy that pSfr64a, in spite of carrying a large segment...
of chromosomal origin, would not be considered as a secondary chromosome, as it can be cured without affecting the saprophytic phenotype of the strain (data not shown). It is possible that such a plasmid is an “intermediate” in the formation of secondary chromosomes. Other plasmids with a structure similar to that of pSr64a, have yet to be described. The finding of such a plasmid in a natural environment may be a living example of a pathway that allows shuffling of the repABC genes, which has been proposed as a strategy to explain the plasmid diversity of Rhizobium [26]. Also, the fact that the repABC genes are located adjacent to the transfer region that is similar to that of pRet42a, and separate from the other sequences that are similar to the R. etli pSym, highlights the impact of evolutionary forces leading to this arrangement, which is highly conserved in many plasmids, and must have evolved in a relatively short time period.

Strain NGR234 was isolated in 1965 by M. J. Trinick, from Lablab purpureus nodules in Papua New Guinea [11]. The complete genome of strain NGR234 has been sequenced [30]. Very recently, the classification of NGR234 was changed from Rhizobium sp to Sinorhizobium fredii. However, no genomic sequence of a type strain of S. fredii is available at present. Genome analysis of other S. fredii strains, both, typical and bean-nodulating, would help to define if the sequence migrated to a plasmid in a S. fredii ancestor, or in a more recent event.

Figure 4 Phylogeny of S. fredii GR64. Maximum likelihood phylogenetic trees based on chromosomal: (A) recA, (B) rpoB, and plasmid: (C) nifH and (D) repB gene fragments. Arrows indicate the localization of S. fredii GR64, and R. etli CFN42.
The segment containing sequences similar to the R. etli transmissible plasmid pRet42a includes the genes involved in conjugal transfer. Conjugative transfer of Agrobacterium tumefaciens pTi and other rhizobial plasmids is subject to quorum-sensing regulation [3,4,31]. In pRet42a, transcription of tra and trb genes is activated by the autoinducer TraI and the transcriptional regulators TraR and CinR. The repressor encoded by traM is not active [5]. Plasmid pSfr64a contains similar regulatory genes, indicating that its transfer is probably regulated by quorum-sensing. Some differences, such as absence of cinR may account for specific responses to different host-related or environmental conditions. Preliminary data indicate the participation of new elements for the activation of the conjugative transfer of pSfr64a.

A comprehensive study of the regulatory mechanisms governing pSfr64a transfer will be addressed in the future.

We have shown that the pSym of GR64 is able to perform pSfr64a-dependent conjugal transfer. The process could be similar to what occurs in CFN42, where pRet42a forms a cointegrate with the pSym, allowing its transfer. Alternatively, pSfr64b mobilization could be induced in trans. The analysis of this process will be pursued in the future.

R. etli plasmid p42a was defined as self-transmissible because it may be transferred from diverse genomic backgrounds, such as Agrobacterium, containing no other plasmids [5,32]. The conjugation experiments performed in this work, show that pRet42a transfer is significantly decreased in GR64 background, suggesting the presence of host-specific elements that interfere with the transfer function. Regarding pSfr64a, conjugation occurs at high frequency when the donor is the native strain. Transfer has not been determined from plasmid-less strains, so that the lack of transfer from R. etli background could be due to the presence of an inhibitor, or to the lack of a required factor, encoded in the chromosome or pSfr64b. These data suggest that a plasmid may be “sequestered” by a host, and imply that the plasmid needs to adjust the appropriate expression of conjugal transfer functions to the new host environment.

Conclusions

Bean-nodulating S. fredii strain GR64 carries a conjuga-
tive plasmid (pSfr64a) that has a large segment similar to the R. etli pSym, including replication, but not symbiosis-related genes, another segment similar to pRet42a, containing the transfer region, and a third segment, similar to the S. fredii NGR234 chromosome. The generation of this plasmid can be explained by the transfer of a symbiotic-conjugative-plasmid cointegrate from R. etli to a S. fredii strain; at least two recombination events among the R. etli plasmids and the S. fredii genome need to be invoked to explain the chimeric composition of plasmid pSfr64a. The structure of the symbiotic plasmid of GR64 could also be the result of these recombination events. Plasmid pSfr64a is required for conjugative transfer of the symbiotic plasmid. In spite of the similarity among pSfr64a and R. etli pRet42a conjugation related genes, the transfer process of these plasmids shows a host-specific behaviour.

Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are described in Table 1. R. etli strains were grown at 30°C on PY medium [33]. Escherichia coli and Agrobacterium tumefaciens strains were grown on Luria-Bertani (LB) medium [34] at 37°C and 30°C respectively. When required, antibiotics were added at the following concentrations (in μg ml⁻¹): nalidixic acid (Nal) 20, spectinomycin (Sp) 75, kanamycin (Km) 15, neomycin (Nm) 60, rifampicin (Rif) 100, streptomycin (Str) 50, tetracycline (Tc) 2 for Rhizobium and 10 for E. coli, gentamicin (Gm), and erythromycin (Ery) 100.

Genetic manipulations

Conjugation experiments were performed on PY plates at 30°C, using overnight cultures grown to stationary phase. Donors and recipients were mixed in a 1:2 ratio and incubated overnight. The mixtures were collected and suspended in 1 ml of 10 mM MgSO₄·0.01% Tween 40 (vol/vol). Serial dilutions were plated on suitable selective media. The transfer frequency was expressed as the number of transconjugants per donor.

A derivative of GR64 carrying a Tn5mob-labeled pSym was constructed by mating GR64 with strain S-17/ pSUP5011 and selecting for resistance to Nal and Nm. Tagged plasmids were mobilized to A. tumefaciens GMI9023 [35] in triparental crosses, using pRK2013 [36] as helper, and selecting for RifR NmR transconjugants. Transconjugants carrying the tagged pSym (pSfr64b) were identified using Eckhardt type gels.

To determine the presence of transmissible plasmids, we randomly labeled strain GR64 with Tn5-GDYN, by mating it with E. coli S17/Tn5-GDYN [17] and selecting NalR SpR transconjugants. The labeled transconjugants were used as donors in conjugations with A. tumefaciens strain GMI9023. As the transposon integrates randomly into the chromosome or plasmids present in a strain, its integration into a transmissible plasmid confers a selective marker to the plasmid. Plasmids present in the selected transconjugants were visualized with Eckhardt gels.

The Tn5-GDYN element contains the sacR-sacB genes, which confer sucrose sensitivity in several gram-negative bacteria, so that selection of sucrose-resistant
colonies allows the isolation of plasmid-less derivatives [17]. Plasmid-curing was carried out by plating overnight cultures of the transposon-labeled strains on PY plates containing 12.5% sucrose. Sucrose-resistant colonies were selected and verified as Sp<sup>+</sup>. Plasmid profiles of such colonies were analyzed in Eckhardt type gels.

**Construction of *S. fredii* and *R. etli* derivatives with diverse plasmid content**

We constructed various derivatives of GR64 (Table 1): GR64-1 has pSfr64a labeled with Tn<sub>5</sub>-GDYN and pSfr64b with Tn<sub>5</sub>mob. This construct allowed us to obtain a derivative cured of pSfr64a (GR64-2). The absence of pSfr64a in GR64-2 was confirmed by Southern type hybridization of plasmid profiles probed with purified pSfr64a (Figure 1B), and of total restricted DNA (data not shown). Tn<sub>5</sub>-GDYN-labeled-pRet42a from *R. etli* CFN42 was introduced into GR64-2 to generate GR64-3. A derivative of GR64-2 with a Tn<sub>5</sub>-GDYN inserted in pSfr64b was constructed. This strain was used to generate GR64-4, cured of both plasmids. Tn<sub>5</sub>-GDYN-labeled-pRet42a from *R. etli* CFN42 was introduced into GR64-4 to generate GR64-5. To construct GR64-5mob, Tn<sub>5</sub>-GDYN-labeled-pRet42a was introduced into GR64-4. CFN2001 is a derivative of GR64, while that ofrepB used in the BLASTP searches were those from the sequenced PCR amplicons from strain GR64, while that of repB was obtained from the sequence of pSfr64a. Nucleotide sequences were translated and aligned using muscle 3.7 [45]. The resulting protein multiple sequence alignments were used as masks to generate the underlying codon alignments using custom Perl scripts.

**Plasmid profiles**

Plasmid profiles were visualized by the Eckhardt technique [38], as modified by Hynes and McGregor [39].

**Filter blot hybridization and plasmid visualization**

For Southern-type hybridizations [40], Eckhardt type gels, or 1% agarose gels where restricted DNA was electrophoresed, were blotted onto nylon membranes, and hybridized under stringent conditions, as previously reported [41], by using Rapid-hyb buffer. Probes were linearized by digesting them with appropriate restriction enzymes and were labeled with [α<sup>32</sup>P]dCTP by using a Rediprime DNA labeling system. All restriction endonucleases, [α<sup>-32</sup>P]dCTP, hybridization buffer, and labeling systems were purchased from Amersham Pharmacia Biotech.

**Nodulation assays**

Overnight cultures were used to inoculate surface-sterilized *Phaseolus vulgaris* cv. Negro Jamapa seeds. Plants were grown in 250-ml Erlenmeyer flasks with Fahraeus agar medium [42], without added nitrogen, at 28°C. Nodulation was scored at day 15 after inoculation. Surface-sterilized nodules were crushed on PY plates, and the plasmid pattern of single colonies was checked on Eckhardt type gels.

**Amplification and sequencing of recA, rpoB, and nifH gene fragments**

Partial *nifH*, *recA* and *rpoB* fragments were amplified with the primer pairs *nifH*F40F/*nifH*817R, *recA*41F/*recA*640R and *rpoB*454F/*rpoB*1364R as previously described [43,44]. All amplifications were performed with *Taq* polymerase (USB-Amersham). Amplification products were purified using Roche’s PCR product purification system. Both strands were commercially sequenced by Macrogen, Korea.

**Phylogenetic inference**

Reference *nifH*, *recA*, *rpoB* and *repB* sequences were retrieved via BLASTP searches from a locally maintained BLAST database containing all fully sequenced Rhizobiales genomes, and via remote BLASTP searches against NCBI’s non-redundant database. The query sequences for *nifH*, *recA* and *rpoB* were included in the BLASTP searches were those obtained from the sequenced PCR amplicons from strain GR64, while that of *repB* was obtained from the sequence of pSfr64a. Nucleotide sequences were translated and aligned using muscle 3.7 [45]. The resulting protein multiple sequence alignments were used as masks to generate the underlying codon alignments using custom Perl scripts.

Models of nucleotide substitution were selected by the Akaike information criterion (AIC), using MODELT-EST3.7 [46]. Among-site rate variation was modelled by a gamma distribution, approximated with 4 rate categories, each category being represented by its mean. Maximum likelihood (ML) trees were inferred under the AIC-selected models of nucleotide substitution for each data set using PhyML v3.0.1 [47]. The robustness of the ML topologies was evaluated using a recently developed Shimodaira-Hasegawa-like test for branches implemented in PhyML v3.0.1 [47]. For the sake of clarity, a small selection of the most relevant sequences was performed to show herein, based on the results of the phylogenetic analysis with the full set of homologous sequences.

**Sequencing of plasmid pSfr64a**

Plasmid pSfr64a was purified by the Hirsch method [48], and used to construct a shotgun library with inserts of approximately 1-2 kb. A total of 1970 high-quality readings were collected by using the ABI3730XL automatic DNA sequencing machine (Applied Biosystems, Foster City, CA). Gaps were filled in by performing appropriate
PCR amplification. Assemblages were obtained by the PhredPhrap-Consed software [49-51]. The quality of the final assembly was less than 1 error per 100,000 bases and had an average coverage of 6.5X.

Annnotation
Open reading frames were predicted by using GLIMMER 3.0 [52,53] and annotation was carried out with the help of BLASTX [54] comparisons against the GenBank nonredundant database [55], INTERPRO [56] searches, and manual curation by using ARTEMIS [57]. To compare partial genomic sequences with the nonredundant database of GenBank, BLASTX searches were performed, and the top hits were classified with respect to organisms with which they matched.

Nucleotide sequence accesion number
Plasmid pSfr64a accession number is GenBank: CP002245. GR64 nifH, recA CP002245. GR64

Acknowledgements
We are grateful to José Luis Fernández, Javier Rivera and Nadya Chaíra for excellent technical assistance, and to Paul Gaytán and Eugenio López for synthesis of oligonucleotides. This work was partially supported by grant IN203109 from DGAPA, UNAM.

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Authors’ contributions
LG carried out most of the molecular genetics experiments. PB assembled the sequence, performed annotation and sequence alignments. LG participated in the design and performed some of the molecular genetics experiments. SB obtained the sequence, and participated in the annotation and preparation of some illustrations. GD designed the sequencing strategy, participated in its analysis and prepared some of the illustrations. PV performed the phylogenetic analyses. DR participated in the design of the study and in the discussion of results. SB conceived the study, participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 30 March 2011 Accepted: 25 June 2011

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Additional material

Additional file 1: Similarity of pSfr64a ORFs to genes located in the chromosome of NGR234, pRet42a and pRet42d plasmids. Lists all the ORFs of pSfr64a, their predicted function, e-value and % of identity to the corresponding ORFs, with highest similarity, located on the chromosome of S. fredii NGR234, and R. etli plasmids pRet42a and pRet42d.
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