Genes encoding conserved hypothetical proteins localized in the conjugative transfer region of plasmid pRet42a from Rhizobium etli CFN42 participate in modulating transfer and affect conjugation from different donors

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INTRODUCTION

The availability of sequenced genomes has increased exponentially in the last years. At present, there are 168 complete sequences of archaea, 2788 of bacteria, and 222 of eukarya, according to NCBI. Analyses of the sequences usually show the presence of genes similar to others with known functions, but, invariably, genes with unknown function are present. Some of them are “orphans” found only in a specific strain, while others are shared among various organisms, encoding conserved hypothetical proteins (chp).

Bacteria belonging to the rhizobia are able to form nitrogen-fixing symbiosis with the roots of leguminous plants (Masson-Boivin et al., 2009). The genomes of these bacteria are usually composed of a chromosome and various plasmids of sizes ranging between 150 and 1800 Kb. The plasmids may carry up to 40% of the total genomic content, including the information allowing the establishment of the symbiosis (Romero and Brom, 2004). Also, some of the plasmids have been shown to carry genes involved in other bacterial functions, such as LPS biosynthesis (García de los Santos and Brom, 1997), metabolic functions (Villasenor et al., 2011) vitamin synthesis, and even some functions essential for bacterial maintenance (Landeta et al., 2011). A characteristic commonly ascribed to plasmids is the ability to perform conjugative transfer (CT). The elements required for CT are a set of genes involved in the processing of DNA (Dtr, DNA transfer and replication), a set of genes involved in formation of the mating pair.
(Mpf, Mating pair formation), and an oriT site, where transfer is
initiated (de la Cruz et al., 2010). Various rhizobial plasmids
have been shown to have this capacity. They have been grouped
according to their transfer genes into four types (Ding and Hynes,
2009; Giusti et al., 2012) those regulated by: (I) quorum-sensing,
(II) the RctA-repressor, (III) those lacking a Mpf system, and
(IV) those containing other regulators. Accordingly, these plas-
mids contain segments with the Dtr, Mpf, oriT and regulatory
genes. Additionally, some of them also contain genes encoding
conserved hypothetical proteins. As these chp-encoding genes are
intercalated between transfer related genes, we hypothesized that
they may be involved in this function. To analyze this, we stud-
iied the participation of the chp- encoding genes localized in the
transfer region, in the CT ability of plasmid pRet42a of Rhizobium
etli strain CFN42.

MATERIALS AND METHODS
BACTERIAL STRAINS AND PLASMIDS
The bacterial strains and plasmids used in this work are described
in Supplementary Table 1. Rhizobium and Agrobacterium strains
were grown on PY medium (peptone-yeast extract medium sup-
plemented with CaCl2 at a final concentration of 4.5 mM) at
30°C (Noel et al., 1984). Escherichia coli strains were grown in
LB medium (Miller, 1972), at 37°C. When required, antibi-
otics were added at the following concentrations: nalidixic acid,
gentamicin, 20 μg/ml; kanamycin, 15 or 30 μg/ml; gentamicin, 30 μg/ml;
rifampin, 50 or 100 μg/ml; erythromycin, 25 μg/ml; spectinomycin,
100 μg/ml; neomycin, 60 μg/ml; streptomycin, 100 μg/ml; and
tetracycline, 2 μg/ml.

BACTERIAL MATINGS
Conjugation between E. coli and R. etli was done biparentally,
using E. coli S17-1 (Simon, 1984) as the donor. Transconjugants
were selected with the appropriate antibiotics. Conjugation
experiments were performed on PY plates at 30°C, using
overnight cultures grown to stationary phase. Donors and recipi-
ents were mixed in a 1:2 ratio and incubated overnight. The mix-
tures were collected and suspended in 1 ml of 10 mM MgSO4–
0.01% (vol/vol) Tween 40. Serial dilutions were plated on suitable
selective media. The transfer frequency was expressed as the
number of transconjugants per donor cell.

PCR
All oligonucleotides used (Table 1) were synthesized at the
Unidad de Síntesis Química IBT-UNAM. PCR amplification was
carried out with Taq polymerase (Invitrogen). PCR conditions
consisted of 30 cycles of 94°C for 1 min, 56–64°C for 1 min and
72°C for 1 min.

CONSTRUCTION OF MUTANT DERIVATIVES
RHE_PA00163 and RHE_PA00164 mutants were constructed by
interrupting the genes with pK18mob (Schafer et al., 1994) intro-
duced by recombination. RHE_PA00163 was mutagenized with
plasmid pK18mob-163 (pK18mob with a 332 bp EcoRI-BamHI
internal fragment of RHE_PA00163). Recombination creates two
incomplete copies of the gene. One of them lacks 41 bp of the
3’ end, while the other lacks 57 bp of the 5’ end. RHE_PA00164
was mutagenized with plasmid pK18 mob-164 (pK18mob with a
326 bp EcoRI-BamHI fragment of RHE_PA00164). One of the
copies lacks 173 bp of the 3’ end, ending at nucleotide position
459, while the other lacks 200 bp of 5’ end.

To construct a mutant in RHE_PA00165, an internal fragment
was cloned with EcoRI-BamHI in the pK18-mob-sacB suicide
vector (Schafer et al., 1994), using the molecular techniques
from Sambrook et al. (1989). The sacB gene confers lethal sus-
ceptibility to sucrose, allowing for positive selection of double
recombinants. The pK18-mob- sacB-165 plasmid was digested
with EcoRV, and a Sp cassette was introduced in this site, gen-
ernating pK18-mob- sacB-165::Sp. This plasmid was used to obtain
a mutant in RHE_PA00165 by double recombination, selecting

| Gene       | Sequence                  | Position   | PCR product |
|------------|---------------------------|------------|-------------|
| RHE_PA00163 | F: 5’ GCTGAAATCCAGGCCACGAGATGTCTT 3’ | 176724–176740 | 332 bp |
| RHE_PA00164 | F: 5’ TGGATACTCCAGCAAGCGGCTGA 3’ | 177208–177226 | 316 bp |
| RHE_PA00165 | F: 5’ AAGGATCCACAGCGCCTGCTCT 3’ | 177735–177751 | 175 bp |
| Complete RHE_PA00163 | F: 5’ GCTGAAATCCAGGCCACGAGATGTCTT 3’ | 176329–176346 | 506 bp |
| Complete RHE_PA00164 and RHE_PA00165 | F: 5’ GCTGAAATCCAGGCCACGAGATGTCTT 3’ | 178063–178092 | 1798 bp |

* These products were used to construct the mutants. The F oligonucleotides contained a PstI site, and the reverse contained a BamHI. The introduced bases are underlined.

* This product was used to clone the complete RHE_PA00163, a PstI site was introduced in the F oligonucleotide, and a BamHI restriction site in the R. The introduced bases are underlined.

* This product was used to clone the complete RHE_PA00163, RHE_PA00164 and RHE_PA00165. An EcoRI site was introduced in the F oligonucleotide, and a BamHI restriction site in the R. The introduced bases are underlined.
for spectinomycin-resistant, sucrose-resistant colonies. All the
constructs were checked by PCR.

CLONING OF THE WILD-TYPE GENES

The pTE3-163 plasmid, containing the entire RHE_PA00163, was
constructed by cloning a 506 bp fragment (generated with Taq
polymerase High Fidelity (Invitrogen) and engineered to contain
the appropriate cloning sites), into the PstI/BamHI in the muli-
tple cloning site of the vector pTE3 (Egelhoff and Long, 1985),
which contains a strong constitutive promoter.

Plasmid pWR, containing the three hcp genes RHE_PA00163, 
RHE_PA00164 and RHE_PA00165 was constructed by cloning a
1798 bp fragment, containing their own promoters, into the EcoRI/BamHI sites of pBR1MCS-5 (Kovach et al., 1995).

MEASUREMENT OF β-GLUCURONIDASE ACTIVITY

Cultures of R. etli harboring a transcriptional fusions
were grown to stationary phase. Quantitative uidA activity was
measured in 1 ml culture samples with p-nitrophenyl glucoronide
as a substrate, as described by Girard et al. (2000).

BIOINFORMATICS ANALYSES

For the construction of the XRE phylogenetic trees, the proteins
were aligned with the module of Clustal implemented in MEGA5
(Tamura et al., 2011). The models of protein evolution for our
sequences were selected with ProtTest 2.4 (Abascal et al., 2005).
The model selected was LG +1+G. Maximum likelihood (ML)
trees were inferred under the selected model using PhyML v3.1
(Guindon and Gascuel, 2003). The robustness of the ML topolo-
gies was evaluated by bootstrap analysis implemented in PhyML
v3.1 (100 replicates). We employed the best of NNIs and SPRs
algorithms to search the tree topology and 100 random trees as
initial trees. The accession numbers are indicated in the figure.

BLASTP analysis on the NCBI and https://img.jgi.doe.gov
servers were used to get homologs and examine the neighborhood
of the selected genes.

RESULTS

THE TRANSFER REGION OF pRet42a CONTAINS CONSERVED
HYPOTHETICAL GENES AND AN XRE-TYPE REGULATOR

Rhizobium etli strain CFN42 (Quinto et al., 1982) contains 6
plasmids, named pRet42a to pRet42f, ranging in size from 185
to 650 Kb. Plasmid pRet42a is a conjugative plasmid, whose transfer genes are regulated by quorum-sensing (Tun-Garrido
et al., 2003). Plasmid pRet42d corresponds to the symbiotic plas-
mid (pSym) this plasmid is able to perform conjugative transfer
to xenobiotic elements. Only SFGR64a_00147 showed no con-
served domains, but we have determined that it is required for
efficient conjugative transfer of plasmid pSfr64a (unpublished
results).

Regarding the organization of the genes, RHE_PA00163, 
RHE_PA00164, and RHE_PA00165, as well as SFGR64a_00149
are transcribed divergent to traH, while SFGR64a_00147 and
SFGR64a_00148 are in the same direction as traH gene
(Figure 1B).

The fact that hypothetical genes are present in the transfer
regions of the two plasmids shown above, led us to question if
the similar genes present in other bacteria are also localized next
to transfer regions.

GENES SIMILAR TO RHE_PA00163, RHE_PA00164 AND RHE_PA00165
ARE LOCALIZED IN THE TRANSFER REGIONS FROM PLASMIDS
PRESENT IN DIVERSE ORGANISMS

To determine the range of organisms showing similar gene clus-
ters, we analyzed the distribution and diversity of homologs of
the hypothetical proteins. Initially we performed a BLASTP
analysis for each protein against the nr database. A high num-
ber of matches were found, and thus a minimum of 30%
of identity was set to reduce the number of hits. This per-
centage of identity is the usually accepted cut-off to define
orthologs (Rost, 1999). With this threshold, we obtained 114,
448 and 9070 hits for R. etli hypothetical proteins RHE_PA00163, 
RHE_PA00164, and RHE_PA00165, respectively; and 356, 126
and 4838 hits for S. fredii hypothetical proteins SFGR64a_00147,
SFGR64a_00148, and SFGR64a_00149 (Figure 2, Supplementary
Table 2).

RHE_PA00163 hits were mostly distributed in Proteobacteria
(68.42% of the hits), with a few in Cyanobacteria and two hits in Archaea. RHE_PA00164 had 33.93% hits in
Proteobacteria and 41.29% in Cyanobacteria. RHE_PA00165
presented the highest diversity: hits were found in Archaea,
Bacteria, Eukaryota and Viruses. Among Bacteria, Firmicutes
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and Proteobacteria harbored most of the hits (38.19 and 40.83%, respectively), while in the Proteobacteria Phylum, Gammaproteobacteria (19.63%) carried most of the homologs.

In the case of the *S. fredii* hypothetical proteins, homologs to SFGR64a_00147 and SFGR64a_00148 were mostly present in Gammaproteobacteria (49.72 and 46.04%), with some hits in Alphaproteobacteria (12.36 and 19.05%) and Betaproteobacteria (15.45 and 8.73%), remarkably, some hits were found in Eukaryota. For SFGR64a_00149, homologs were distributed among Firmicutes and Proteobacteria, and in this Phylum, Gammaproteobacteria were the most represented (18.91%), this gene also had hits in Archaea Eukaryota.

These results display the wide distribution of the chp-encoding genes from the transfer region of pRet42a. It is probable that horizontal gene transfer events could be related to their presence in very diverse organisms, including Archaea, Virus and Eucaryota.

Even if both, SFGR64a_00149 and RHE_PA00165, possess a XRE domain and are similarly located upstream of a *traM* regulator, a phylogenetic analysis showed that they are not closely related (Figure 3).

In addition to being widely distributed in diverse genomes, these chp-encoding genes are located in the neighborhood of Dtr and Mpf gene clusters of several bacteria, such as *Rhizobium etli* bv. *mimosae* IE4771 (pA), *Ensifer* sp. TW10, *Rhizobium* sp. LPU83
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FIGURE 2 | Organisms that present homologs to hypothetical orfs. (A) From *R. etli* CFN42. (B) From *S. fredii* GR64. Homologs were searched for by BLASTP. The number of hits and the percent over the total hits are shown in parenthesis.

THE HYPOTHETICAL GENES FROM pRet42a AFFECT CONJUGATIVE TRANSFER OF THE PLASMID

In order to determine if the chp-encoding genes localized in pRet42a participate in conjugative transfer, we constructed mutant derivatives, and analyzed their phenotype. RHE_PA00163 and RHE_PA00164 were interrupted with pK18mob, as described in Materials and Methods.

Mutation of RHE_PA00163 increases CT frequency in wild-type background

The derivative carrying a mutation in RHE_PA00163 showed a 10-fold increase in conjugative transfer frequency compared to the wild type strain (Table 2). Interestingly, this phenotype was only observed when the donor carried all the other endogenous plasmids of the strain. It was not observed when the donor

(pLPU83b) (Wibberg et al., 2014), *R. leguminosarum* bv viciae 8401(pRL11) (Danino et al., 2003), *Rhodopseudomonas palustris* CGA009 (Larimer et al., 2004), *Rhizobium tropici* CIAT 899 (pA) (Ormeño-Orrillo et al., 2012), *Rhizobium etli* bv. *mimosae* Mim1 (pRetNIM1c), *Rhizobium leucanet* USDA 9039, *Rhizobium freirei* PRF 81 (pPRF81b), *Rhizobium mesoamericanum* STM3625, *Rhizobium grahamii* CCGE 502 (pRg502a) (Althabegoiti et al., 2012) and *Gluconobacter oxydans* H24 (Figure 1A), the genomic island of *E. coli* Nissle 1917 (Grozdanov et al., 2004) and the symbiosis island of *Mesorhizobium loti* R7A (Ramsay et al., 2013). The orthologs of RHE_PA00163 located near transfer genes, and/or next to RHE_PA00164 orthologs are indicated in Supplementary Table 2.

The broad distribution and conserved position of these chp-encoding proteins hints that they may participate in the conjugative transfer phenomena.
lacked the symbiotic plasmid pRet42d, or pRet42f, or from an Agrobacterium donor (data not shown). This suggests that the modulation effect caused by RHE_PA00163 may involve elements localized in these plasmids. Also, the transfer frequency varies with different recipient strains; compare lines 1 vs. 3, and 2 vs. 4 in Table 2. We see that transfer frequency is higher when UIA143 (Farrand et al., 1989) is used as recipient. However, the increase in transfer frequency of the mutant in RHE_PA00163 compared to the wild type is maintained, indicating that, in addition to the effect of the mutation on the transfer frequency of pRet42a, there is also an effect of the recipient.

Expression levels of tral decrease in the RHE_PA00163 mutant
Previously, we determined that conjugative transfer of pRet42a depends on quorum-sensing regulation mediated by tral, traR and cinR (Tun-Garrido et al., 2003). To determine if the increase in transfer frequency of the RHE_PA00163 mutant was due to an increase in the expression level of tral, we introduced plasmid pCT7 (pBBMCS3/tral-uidA) carrying a transcriptional fusion of tral (Tun-Garrido et al., 2003), into the mutant in RHE_PA00163 and determined the β-glucuronidase activity, in comparison to the wild-type strain. Surprisingly, the results showed that the expression level of tral decreased in the mutant.
background (Table 3). This indicates that the increase in transfer frequency in the mutant depends on elements different from the TraI quorum-sensing regulator.

**Complementation of the RHE_PA00163 mutant with the wild-type gene**

We cloned the complete RHE_PA00163 mutant in a vector able to replicate in Rhizobium, as described in Material and Methods. This clone was introduced into the RHE_PA00163 mutant containing pCT7 (pBBMCS53/traIp-uidA). We determined the β-glucuronidase activity and the results showed that, although it did not reach the level of the wild-type, the complemented strain partially restored the expression level of tral (Table 3). Also, the transfer frequency decreased in the complemented strain (Table 2). A possible explanation for this is that RHE_PA00163 has a dual role in conjugative transfer, with a positive effect on tral expression, and a negative effect on some unidentified participant, able to induce a fine-tuned increase in transfer.

**RHE_PA00164 AND RHE_PA00165 ARE REQUIRED FOR TRANSFER FROM DIFFERENT AGROBACTERIUM DONORS**

The derivatives lacking functional RHE_PA00164 or RHE_PA00165 showed transfer frequencies similar to those of the wild-type strain from rhizobial donors (Table 2), but a different phenotype was observed when the plasmid carrying the mutation was transferred from Agrobacterium donors (Table 4). The strain carrying a mutation in RHE_PA00164 was unable to generate transconjugants when the donor was Agrobacterium strain GM19023 (Rosenberg and Huguet, 1984), a derivative that lacks its endogenous pTi and pAT plasmids, however, the mutant is still able to conjugate from an Agrobacterium donor that lacks the pTi, but conserves the pAT (strain UIA143). The plasmid with a mutation in RHE_PA00165 also lost its ability to transfer from GM19023. Additionally, its transfer frequency from UIA143 was lower than that of the wild-type plasmid. Both mutants acquired the wild-type phenotype when a plasmid carrying the three hcp-encoding genes was introduced. This plasmid did not alter the transfer frequency of the wild-type plasmid, although it carries the whole region, possibly because, as mentioned earlier, the effect of RHE_PA00163 is not observed from Agrobacterium donors (Table 4). These data suggest that RHE_PA00164 and RHE_PA00165 do participate in conjugative transfer, but their activity is masked in their native background.

**DISCUSSION**

The analyses presented in this paper, regarding the distribution of hypothetical protein-encoding genes and XRE-type regulators similar to those localized in the transfer region of plasmid pRet42a from R. etli C5842, show that these genes are widely distributed among bacteria, and even some archaea and eukaryotic organisms (Figure 2). The highest proportion of homologs to RHE_PA00163 and RHE_PA00164 was found in Alphaproteobacteria (60.53 and 22.99% of them in rhizobiales, respectively). At least 45 of the species detected contained both, RHE_PA00163 and RHE_PA00164, usually localized close to each other. Interestingly, in plasmids of various organisms such as Rhizobium etli cv. mimosae IE4771 (pA), Ensifer sp. TW10, Rhizobium sp. LP83 (pLP83b), Rhodopseudomonas palustris CGA009, Rhizobium tropici CIAT899 (pA), Rhizobium etli cv. mimosae Mimm1 (pRET42a), Rhizobium leucaena USDA9039, Rhizobium freeri PRF81 (pPRF81b), Rhizobium mesoamericanum STM3625, Rhizobium grahamii CCGE 502 (pRG502a) and Gluconobacter oxydans H24 the genes were localized next to Dtr and/or Mpf clusters, as exemplified in Figure 1A.

Regarding SFRG64a_00147 and SFRG64a_00148 from S. fredii GR64, they showed a similar distribution of homologs, mostly among proteobacteria, with the highest proportion present in Gammaproteobacteria (45%) many of these were found in Escherichia genera. Also in some of these strains, the homologs were localized in genomic islands (e.g., Nissle 1917) or plasmids.

The XRE-type regulators presented the highest number of homologs, 9070 for RHE_PA00165 and 4838 for SFRG64a_00149 (Figure 2). Although both contain an XRE-type domain, these two orfs are phylogenetically distant (Figure 3). All these data suggest that these genes may participate in the conjugative transfer of bacterial plasmids, and even of genomic islands.

The functional studies of the chp-encoding orfs from pRet42a showed that RHE_PA00163 participates as a fine-tuning modulator of transfer, possibly through components encoded in plasmids pRet42d and pRet42f, as donors lacking these plasmids

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**Table 2 | Conjugation frequencies from rhizobial donors**

| Donor Characteristics | Transfer frequencyb |
|-----------------------|---------------------|
| (1) CFNX187 Wild type, labeled pRet42a (Brom et al., 1992) | 1.4 ± 1.6 × 10⁻¹ |
| (2) CE3-163::pK18mob RHE_PA00163 mutant | 3.7 ± 3.0 × 10⁰ |
| (3) CFNX187 Wild type, labeled pRet42a | 4.1 ± 4.0 × 10⁻³ |
| (4) CE3-163::pK18mob RHE_PA00163 mutant | 1.6 ± 2.0 × 10⁻¹ |
| (5) CE3-163::pK18mob/pTE3-163 RHE_PA00163 mutant complemented with cloned RHE_PA00163 | 1.3 ± 0.2 × 10⁻¹ |
| (6) CE3-164::pK18mob Mutant in RHE_PA00164 | 1.1 ± 0.01 × 10⁻¹ |
| (7) CE3-165::Sp Mutant in RHE_PA00165 | 5.27 ± 0.74 × 10⁻¹ |

*The recipient in crosses 1, 2 5, 6, and 7 was UIA143, and CFN2001 (Leemans et al., 1984) in crosses 3 and 4.

*bTransfer frequency is expressed as number of transconjugants per donor cell, and is the average of at least three experiments.

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**Table 3 | Expression levels of tral**

| Strain Characteristics | Expression levela |
|------------------------|------------------|
| CE3/traIp-uidA Wild type, labeled pRet42a, tral fusion | 11.9 ± 1.19 |
| CE3-163::pK18mob, tral-uidA RHE_PA00163 mutant, tral fusion | 4.2 ± 0.65 |
| CE3-163::pK18mob, tral-uidA, pTE3-163 RHE_PA00163 complemented with cloned RHE_PA00163 tral fusion | 75 ± 0.81 |

*aExpression level is expressed as β glucuronidase specific activity (nm/min/mg of prot), it is the average of at least three experiments and the SD is shown.
did not show the increase in transfer frequency. Additionally, we found that a mutation in this orf leads to a decrease in the expression of _traI_. These results suggest that RHE_PA00163 differentially affects elements involved in transfer, having a positive effect on _traI_, and a negative one on other elements, which are able to induce a slight increase in transfer in the absence of RHE_PA00163. How does RHE_PA00163 achieve its effects? It could be a directly interacting with the different elements or it could be an indirect effect. Another open question for further research is if the protein product of the gene is responsible, or if the effect is mediated through RNA.

RHE_PA00164 and RHE_PA00165 also showed a role in conjugative transfer, although in this case the effect was only revealed in conjugation from non-native _Agrobacterium_ donors. It is possible that these orfs are only expressed in the heterologous background. The fact that the RHE_PA00164 and RHE_PA00165 mutants only were able to transfer from the donor containing plasmid pAT, suggests that the conjugative ability is probably due to their interaction with genes encoded in plasmid pAT.

Due to their organization, it would be possible that RHE_PA00163, RHE_PA00164, and RHE_PA00165 form an operon. However, our experimental data shows that mutation in each of the genes presents an independent phenotype. Also, we performed a search for putative promotors using the BPROM program for prediction of bacterial promoters (Solovyev and Salamov, 2011). The results indicate the presence of a putative promoter for each gene (Supplementary Table 3). A recent paper by López-Leal et al. (2014) shows that the transcription levels of the three genes differ greatly among them. In another paper (Vercruyse et al., 2011) it can be seen that RHE_PA00163 is regulated by (p)ppGpp, while RHE_PA00164 and RHE_PA00165 are not affected. All these data suggest that these genes are transcribed independently, and do not form an operon. However, it is still possible that under some conditions the genes could be transcribed as an operon.

Some data have begun to emerge regarding the participation in conjugative transfer of genes similar to those described in this paper. In plasmid pReVF39b of _R. leguminosarum_ by viciae strain VF39 it was shown that a XRE-type regulator encoded close to the Dtr genes functions as a repressor of conjugative transfer (Ding et al., 2013). In the symbiosis island of _M. loti_ R7A, the gene named qseC, encoding a XRE type regulator was shown to participate in regulation of excision and transfer of the island (Ramsay et al., 2013). In _S. meliloti_ strain LPU88, plasmid pLPU88a mobilizes pLPU88b, inactivation of a hypothetical encoding protein gene localized in pLPU88a resulted in its inability to promote transfer of pLPU88b from _S. meliloti_ strain 2011, but was dispensable from the native LPU88 background (Pistorio et al., 2013). In _S. fredii_ GR64, we have found that a mutation in SGR64a_00147 impairs transfer of pSr64a (our unpublished results).

The examples from the literature, in conjunction with the data presented in this work implicate the participation of genes with unknown function localized near transfer regions in this process. Their mode of participation seems to be variable, some as positive effectors, others as repressors, some acting as modulators, and others showing an absolute requirement. Additionally, they seem to depend on interaction with elements encoded in other replicons.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [http://www.frontiersin.org/journal/10.3389/fmicb.2014.00793/abstract](http://www.frontiersin.org/journal/10.3389/fmicb.2014.00793/abstract)
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