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Conjugative Transfer of p42a from *Rhizobium etli* CFN42, Which Is Required for Mobilization of the Symbiotic Plasmid, Is Regulated by Quorum Sensing

Cristina Tun-Garrido,[1]* Patricia Bustos,[2] Víctor González,[2] and Susana Brom[1]

Programa de Genética Molecular de Plásmidos Bacterianos[1] and Programa de Evolución Molecular,[2] Centro de Investigación Sobre Fijación de Nitrógeno, UNAM, Cuernavaca, Morelos, Mexico

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***Rhizobium etli* CFN42 contains six plasmids. Only one of them, p42a, is self-conjugative at high frequency. This plasmid is strictly required for mobilization of the symbiotic plasmid (pSym). To study the transfer mechanism of p42a, a self-transmissible cosmid clone containing its transfer region was isolated. Its sequence showed that most of the *tra* genes are highly similar to genes of *Agrobacterium tumefaciens* pTiC58 and other related plasmids. Four putative regulatory genes were identified; three of these (*traI*, *traR*, and *cinR*) belong to the LuxR-LuxI family. Mutagenesis of these genes confirmed their requirement for p42a transfer. We found that the conjugative transfer of p42a is dependent on quorum sensing, and consequently pSym transfer also was found to be similarly regulated, establishing a complex link between environmental conditions and pSym transfer. Although *R. etli* has been shown to produce different *N*-acyl-homoserine lactones, only one of them, a 3-oxo-C_8-homoserine lactone encoded by the *traI* gene described here, was involved in transfer. Mutagenesis of the fourth regulatory gene, *traM*, had no effect on transfer. Analysis of transcriptional fusions of the regulatory genes to a reporter gene suggests a complex regulation scheme for p42a conjugative transfer. Conjugal transfer gene expression was found to be directly upregulated by TraR and the 3-oxo-C_8-homoserine lactone synthesized by TraI. The *traI* gene was autoregulated by these elements and positively regulated by CinR, while *cinR* expression required *traI*. Finally, we did not detect expression of *traM*, indicating that in p42a TraM may be expressed so weakly that it cannot inhibit conjugal transfer, leading to the unrepressed transfer of p42a.**

Rhizobium species are gram-negative soil bacteria that are able to fix nitrogen and to establish a symbiotic relationship with leguminous plants. Besides the chromosome, their genome is usually constituted by large plasmids which carry genetic material relevant for very diverse functions, such as utilization of plant metabolites, aromatic compounds, and diverse sugars (14), in addition to nodulation and nitrogen fixation, which is the best-studied role of rhizobial plasmids.

Rhizobium etli CFN42 induces the formation of nitrogen fixing nodules in roots of *Phaseolus vulgaris* (bean) plants. In this strain, the genomic material is distributed between one chromosome and six plasmids (p42a to p42f). One of these plasmids, p42d, has been identified as the symbiotic plasmid (pSym), because it carries most of the genes required for nodulation and nitrogen fixation (43). Some functional characteristics have previously been ascribed to these plasmids (4). The conjugative abilities of the six plasmids have been evaluated by using an *Agrobacterium tumefaciens* strain lacking the Ti plasmid as a recipient. It was found that p42a is self-transmissible at a high frequency (10^{-2}). Transfer of pSym was also detected but was found to be fully dependent on the presence of p42a. Thus, p42a functions as a helper for the transfer of the pSym, which is a mobilizable but not self-transmissible plasmid. The mechanism for pSym transfer requires its cointegration with p42a, and this cointegration is apparently accomplished through two mechanisms: one dependent on and another independent of *recA*. Transfer of the other plasmids of *R. etli* CFN42 has not been detected (5).

Distribution of symbiotic plasmids is a fundamental issue in the *Rhizobium* soil population. Some transfer genes have been found in rhizobial plasmids such as pNGR234a, the symbiotic plasmid of *Rhizobium* sp. strain NGR234. These genes were identified through homology to the conjugal transfer genes of *Agrobacterium* Ti plasmids (13); however, clear data regarding the transfer frequency of this plasmid have not been published. The horizontal transfer of genetic material between bacteria of the genus *Rhizobium* is a frequent event and has been demonstrated previously under laboratory conditions. Indeed, self-transmissible symbiotic plasmids have been observed in *Rhizobium leguminosarum* (28). Indirect evidence also suggests pSym transfer in soil (16), and recently, several studies have demonstrated the presence of similar plasmids in different genomic backgrounds (31). An identical organization of plasmid-borne symbiotic genes was found in isolates belonging to four distinct 16S ribosomal DNA species (22). These studies suggest the occurrence of horizontal transfer during the diversification of natural populations of rhizobia. From an applied perspective, it has been possible to increase the effectiveness of the *Rhizobium*-legume symbiosis with an *R. leguminosarum* strain through selective transfer of symbiotic plasmids (10).

In *Agrobacterium*, another genus of the *Rhizobiaceae*, the tumor-inducing plasmid (pTi) can be transferred between bac-

* Corresponding author. Mailing address: Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Ap. P. 565-A, Cuernavaca, Morelos, Mexico. Phone: 52 (777) 3 29 16 91. Fax: 52 (777) 3 17 55 81. E-mail: cristina@cifn.unam.mx.

terial populations, which remain in the soil after infection of the plant tissues. Opines produced during the infection by the plant are the signal that turns on the expression of the genes required for their utilization. Additionally, they also induce the expression of the transcriptional regulator of the transfer process, *traR*, which activates *traI* expression. This regulatory effect is mediated by the AccR repressor (2, 20). The TraR and TraI proteins are LuxR-LuxI-type regulators that activate the expression of target genes in a quorum-sensing-dependent manner. The TraI protein is an acylated homoserine lactone (acyl-HSL) synthase that synthesizes 3-oxo-C_8-HSL, and TraR is the autoinducer-responsive transcriptional regulator (40, 25). Both control the expression of at least five promoters of genes involved in plasmid transfer, and consequently, pTi transfer is quorum sensing dependent. A third regulatory protein, TraM, antagonizes TraR activity (26, 35).

The tumor-inducing plasmid pTiC58 of *A. tumefaciens* seems to be phylogenetically close to p42a. Previous studies from our laboratory have shown that these plasmids are incompatible (15). In addition, Bittinger et al. have identified a region in p42a that contains genes with similarity to *vir* genes of pTi (3).

Recent data suggest that the *cinI* locus, belonging to the LuxI family, is involved in conjugative transfer of pRL1JI in *R. leguminosarum*. Surprisingly, insertion mutations in *cinI* in both the donor and the recipient are required to decrease conjugation frequency (34). BisR and TriR, two LuxR-type transcriptional regulators, seem to participate in conjugal transfer gene expression, through an elaborate regulatory cascade (53). Also, two quorum-sensing systems have been identified in *Sinorhizobium meliloti* (36). Based on sequence homologies, those authors propose that a *traR-traM* locus is involved in conjugation. Nevertheless, data regarding the transfer mechanism for *Rhizobium* plasmids are still scarce.

In this paper we present the structural and functional characterization of the mobilization region of p42a of *R. etli*. Although the arrangement of the mobilization region is highly similar to that of pTi, there are important differences among the regulatory mechanisms. Knowledge regarding the regulation of p42a transfer will be an important factor to better understand the distribution of symbiotic information, due to its fundamental role in the conjugative transfer of this plasmid.

MATERIALS AND 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 (39). *Escherichia coli* and *A. tumefaciens* strains were grown on Luria-Bertani medium (38) at 37 and 30°C, respectively. When required, antibiotics were added at the following concentrations: nalidixic acid, 20 μg/ml; spectinomycin, 75 μg/ml; kanamycin, 15 μg/ml; neomycin, 60 μg/ml; rifampin, 100 μg/ml; streptomycin, 50 μg/ml; tetracycline, 2 μg/ml for *Rhizobium* and 10 μg/ml for *E. coli*; gentamicin, 15 μg/ml; ampicillin, 200 μg/ml; and carbenicillin, 100 μg/ml.

When conditioned medium was required, 3-ml cultures of wild-type *R. etli* were grown to stationary phase. The cultures were centrifuged, and the supernatants were filtered through a 0.22-μm-pore-size Millipore filter and added to cultures of other strains.

Bacterial matings. Conjugation between *E. coli* and *R. etli* was done biparentally, using *E. coli* S17-1 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 recipients were mixed in a 1:2 ratio and incubated overnight. The mixtures were collected and suspended in 1 ml of 10 mM $MgSO_4$–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 output donor.

Transfer experiments to determine dependence on cellular density were performed with donor cultures grown to low density (10^4 cells/ml) and recipient cultures grown to high density (10^6cells/ml). Cultures were mixed, and samples of 100 μl of the mixtures were plated on PY and incubated at 30°C. Every 4 h, two samples were collected and suspended in 1 ml of 10 mM $MgSO_4$–0.01% (vol/vol) Tween 40. Serial dilutions were plated on appropriate media to select either transconjugants containing p42a, transconjugants containing pSym, or the donors.

Hybridization experiments. p42a was purified by the method of Hirsch et al. (23), digested with *Eco*RI, and used as a probe in colony hybridization experiments (21). A total of 2,200 colonies from a cosmid library of the complete genome of *R. etli* CFN42 (24) were analyzed. Forty-two colonies which showed homology to the probe were selected for further analysis.

Southern hybridizations. Cosmid DNA was digested with *Eco*RI as specified by the manufacturer (Amersham Ltd.). Digested DNA was separated by electrophoresis in 1% agarose gels and transferred to nylon (Hybond N+) as described by Southern (50). Seven bands purified from several vectors containing the transfer genes of pTiC58 (Table 1) were labeled with [α-^{32}P]dCTP by random priming with Amersham's Rediprime system and used as probes. Hybridizations were done as previously described (17).

***N*-Acyl-HSL detection.** Autoinducers were detected through thin-layer chromatography (TLC) analysis with the reporter plasmid pZLR4 (48). This plasmid contains the *traR* gene and the *traG*::*lacZ* reporter fusion from pTiC58, independently cloned into the broad-host-range vector pBBR1MCS5 (7). Extracts of *R. etli* derivatives were prepared from 5-ml cultures grown in YM medium (51).

PCR amplification and cloning. The oligonucleotides used in this study are listed in Table 2. All of the primers were purchased from Unidad de Síntesis Química IBT-UNAM. PCR amplification was carried out with recombinant *Taq* DNA polymerase as specified by the manufacturer in a Mastercycler 5330 (Eppendorf). PCR conditions consisted of 30 cycles of 92°C for 1 min, 56°C for 1 min, and 72°C for 1 min. PCR products were purified with a Geneclean III kit (Bio101). Vectors were purified by standard protocols (47). T4 polynucleotide ligase was used as indicated by the manufacturer (Amersham Ltd.).

Transcriptional fusions. A PCR product containing the complete *traI* gene was obtained with oligonucleotides +traI-E and −traI-E (Table 2) and cloned in pSK+ (Table 1). A 991-bp *Eco*RI-*Cla*I fragment from this clone was subcloned into plasmid pBBMCS53 (Table 1) to construct a *traI*::*uidA* reporter fusion containing the first 15 amino acids (aa) of *repA*, the intergenic region between *repA* and *traI*, and the first 195 aa of *traI*.

A *traR*::*uidA* fusion was generated by cloning a PCR product obtained by using the oligonucleotides +traR-E and −traR-S (Table 2). This product, containing the complete *traR* gene, was cloned in pSK+ (Table 1). A 514-bp *Eco*RI-*Hin*dIII fragment from this clone, containing 59 bp of noncoding region and the first 150 aa of *traR*, was subcloned into plasmid pBBMCS53 (Table 1).

A *traM*::*uidA* fusion was constructed by cloning a 747-bp *Eco*RI-*Sal*I fragment, obtained from a PCR product generated by using the oligonucleotides −traM-E and +traM-S, into plasmid pBBMCS53 (Table 1). This fragment contains 580 bp of noncoding region and the first 49 aa of *traM*.

A *cinR*::*uidA* fusion was constructed by cloning a PCR product obtained by using oligonucleotides +cinR-S and −cinR-E (Table 2). This product, containing the complete *cinR* gene, was cloned in pSK+ (Table 1). A 469-bp *Sma*I-*Cla*I fragment from this clone, containing the last 6 aa of *trbI*, the noncoding region between *trbI* and *cinR*, and the first 150 aa of *traR*, was subcloned into plasmid pBBMCS53 (Table 1).

Measurement of β-glucuronidase activity. Cultures of *R. etli* derivatives harboring transcriptional fusions were grown to stationary phase. Quantitative *uidA* activity was measured in 1-ml culture samples with *p*-nitrophenyl glucuronide as a substrate (18).

Construction of mutant derivatives. *traM* and *traI* mutants were constructed by interruption with pSUPΩSp (Table 1) introduced by single recombination. *traM* was mutagenized by using plasmid pCT1 (pSUP202ΩSp with a 254-bp *Eco*RI fragment of *traM* [Table 1]). Recombination creates two incomplete copies of the gene. One of them lacks 43 bp of the carboxy-terminal end, ending at nucleotide position 296, while the other lacks 44 bp of the amino-terminal end, starting at nucleotide position 44. *traI* was mutagenized by using plasmid pCT2 (pSUP202ΩSp with a 359-bp *Eco*RI fragment of *traI* [Table 1]). One of the interrupted copies lacks 78 bp of the carboxy-terminal end, ending at nucleotide position 543, while the other lacks 258 bp of the amino-terminal end, starting at nucleotide position 258 (Table 1).

The *traR* and *cinR* mutants were generated by insertion of a *lox*::*P*Sp cassette by using plasmids pCT64 and pCT53, respectively, to generate double recombi-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference or source
R. etli		
CFN42	Wild type, contains 6 plasmids (p42a to p42f)	44
CFN2001	CFN42 derivative (p42a$^-$ p42d$^-$)	32
CFNX182	CFN42 derivative (p42a$^-$)	4
CFNX187	CFNX182 complemented with p42a::Tn*5mob*	4
CFNX668	CFN42 derivative, *traM*::pSUPΩSp	This work
CFNX669	CFN42 derivative, *traI*::pSUPΩSp	This work
CFNX670	CFN42 derivative, *traR*::*loxP*Sp	This work
CFNX671	CFN42 derivative, *cinR*::*loxP*Sp	This work
CFNX672	CFN42 derivative containing pCT7	This work
CFNX673	CFN42 derivative containing pCT8	This work
CFNX674	CFN42 derivative containing pCT9	This work
CFNX675	CFN42 derivative containing pCT10	This work
CFNX676	CFNX669 derivative containing pCT7	This work
CFNX677	CFNX669 derivative containing pCT8	This work
CFNX678	CFNX669 derivative containing pCT9	This work
CFNX679	CFNX669 derivative containing pCT10	This work
CFNX680	CFNX670 derivative containing pCT7	This work
CFNX681	CFNX670 derivative containing pCT8	This work
CFNX682	CFNX670 derivative containing pCT9	This work
CFNX683	CFNX670 derivative containing pCT10	This work
CFNX684	CFNX671 derivative containing pCT7	This work
CFNX685	CFNX671 derivative containing pCT8	This work
CFNX686	CFNX671 derivative containing pCT9	This work
CFNX687	CFNX671 derivative containing pCT10	This work
CFNX688	CFN42 containing p42a::Tn*5mob* and p42d::Tn*5*GDYN1	This work
CFNX689	CFNX669 derivative containing p42d::Tn*5mob*	This work
CFNX690	CFNX670 derivative containing p42d::Tn*5mob*	This work
CFNX691	CFNX671 derivative containing p42d::Tn*5mob*	This work
CFNX692	CFN42 derivative containing pC-13 and p42d::Tn*5*GDYN1	This work
Agrobacterium		
GM19023	C-58 cured of its native plasmids	46
NT1	C-58 harboring plasmid pTiC58Trac::Tn*517*-52	1
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	47
HB101	*supE44 hsd20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	47
S17-1	C600::RP-4-2 (Tc::Mu) (Km::Tn*7*)	49
S17-1/pSUP5011	Source of Tn*5mob*	49
S17-1/pDR21	Source of Tn*5*GDYN1	12
Plasmids		
pLAX	pBluescript derivative carrying an 8.5-kb *Apa*I-*Xba*I fragment containing from the middle of *trbC* to the end of *trbI* of pTiC58 (Ap^r)	S. K. Farrand, unpublished data
pUCM	pCU18 derivative carrying the *traM* gene of pTiC58 (Ap^r)	26
pSEP-1	pUC18 derivative carrying the *traI* gene of pTiC58 (Ap^r)	S. K. Farrand, unpublished data
pBADTR3	pBAD22 derivative carrying a *Nco*I-*Hin*dIII fragment containing the *traR* gene of pTiC58 (Ap^r)	S. K. Farrand, unpublished data
pTraC1-DG	pET-14b derivative carrying a 2.6-kb *Nde*I-*Eco*RI fragment containing the *traC*, *traD*, and *traG* genes of pTiC58 (Ap^r)	11
ptraAF	pET-14b derivative carrying a 4.7-kb *Nde*I-*Bam*HI fragment containing the *traA*, *traF*, and *traB* of pTiC58 (Ap^r)	11
pSK+	Sequencing vector (Ap^r)	Stratagene, La Jolla, Calif.
pBBMCS53	Δ*placZ*pBBRIMCS-5 derivative carrying the promoterless *uidA* gene from pWM5 (Gm^r)	18
pJQ200SK+	Suicide cloning vector (Gm^r)	42
pJMS2	pMS102 derivative, *loxP*Sp interposon (Sp^r)	37
pSUP202ΩSp	pSUP202 derivative containing an ΩSp cassette in *Hin*dIII site	L. Girard, unpublished data
pBBRIMCS5	Broad-host-range cloning vector (Gm^r)	30
pZLR4	PBBR1MCS5 derivative (Gm^r) containing *traG*::*lacZ* reporter fusion and the *traR* gene from pTiC58	7
pRK7813	Broad-host-range cosmid vector, *oriV-oriT* from RK2, *lacZ*α (Tc^r)	29
pC-13	pLAFR1 derivative containing the complete mobilization region of p42a	This work
pCT1	pSUP202Ωsp derivative carrying a 254-bp *traM Eco*RI fragment obtained by PCR with oligonucleotides UtraM-E and LtraR-E	This work

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant features	Reference or source
pCT2	pSUP202ΩSp derivative carrying a 359-bp *traI Eco*RI fragment obtained by PCR with oligonucleotides UtraI-E and LtraI-E	This work
pCT3	pSK+ derivative carrying a 0.9-kb *Xho*I-*Eco*RI fragment (generated by PCR) containing a fragment of the wild-type *traR* gene	This work
pCT4	pSK+ derivative carrying a 1.1-kb *Hin*dIII-*Xho*I fragment containing the complete *cin*R gene and a fragment of *traR*	This work
pCT5	pSK+ derivative carrying 1.1-kb *Hin*dIII-*Xho*I fragment containing *cinR*::*loxP*Sp in the *Sal*I site and a fragment of wild-type *traR*	This work
pCT6	pSK+ derivative carring a 0.9-bp *Xho*I-*Eco*RI (generated by PCR) fragment containing *traR*::*loxP*Sp in the *Sal*I site	This work
pCT53	pJQ200SK+ carrying a 2-kb *Spe*I fragment containing *cinR*::*loxP*Sp and wild-type *traR*, constructed by ligation of the vector with *Spe*I-*Xho*I fragments from pCT5 and pCT3	This work
pCT64	pJQ200SK+ carrying a 2-kb *Spe*I fragment containing *traR*::*loxP*Sp and wild-type *cinR*, constructed by ligation of the vector with *Spe*I-*Xho*I fragments from pCT6 and pCT4	This work
pCT7	pBBMCS53 *traI*::*uidA*	This work
pCT8	pBBMCS53 *traR*::*uidA*	This work
pCT9	pBBMCS53 *traM*::*uidA*	This work
pCT10	pBBMCS53 *cinR*::*uidA*	This work
pCT11	pBBRIMCS5 derivative carrying 143-bp *Hin*dIII-*Eco*RI fragment containing the *oriT*	This work
pDCKE 7ΔB	pRK415K derivative carrying a 250-bp *Bam*HI-*Eco*RI fragment containing the active *oriT* of pTiC58	8
pCT12	pBBRIMCS5 derivative carrying the complete *traI* gene	This work
pCT13	pRK7813 derivative carrying the complete *traR* gene	This work
pCT14	pRK7813 derivative carrying the complete *cinR* gene	This work
pCF424	pBBRIMCS5 derivative containing *traM* of pNGR234a	He and Fuqua, submitted for publication

nants (Table 1). *cinR* was interrupted at the *Sal*I site at nucleotide position 414 (Fig. 1). *traR* was interrupted at the *Sal*I site at nucleotide position 248 (Fig. 1).

Nucleotide sequence accession number. The sequence of the complete *tra* region of p42a of *R. etli*, cloned in pC-13, was deposited in the GenBank database under accession number AF528525.

RESULTS

Identification of a self-transmissible cosmid clone from p42a. Plasmid p42a has been shown to be self-transmissible (5). Its high transfer frequency remains constant, even if it is mobilized from a different background such as *A. tumefaciens* GMI9023 (Table 3), thus indicating that no additional transfer regulatory elements are located in a replicon other than p42a. To identify the transfer region of p42a, a collection of clones from this plasmid was isolated from a cosmid library covering the whole *R. etli* CFN42 genome (24) through the selection of clones showing a positive signal in Southern blot colony hybridization with the complete p42a as a probe. Forty-two clones were selected, digested with *Eco*RI, and hybridized against transfer genes of pTiC58 from *A. tumefaciens* (Table 1) under low-stringency conditions. Most of the probes (*traAFB*, *traCDG*, *trbC-I*, and *traI*) showed positive signals with a 27.1-kb cosmid clone. This cosmid was called pC-13. We did not find hybridization signals for *traM* and *traR*.

TABLE 2. Oligonucleotides used in this work

Primer[a]	Sequence[b]
−traI-E	TAGAATTCCTTGCGGTTCGGGTTT
+traI-E	TCGAATTCACGGCTGTCTCCTT
−traR-E	TGGAATTCTCGGTTCGGGGGATTTA
+traR-S	CCGTCGACCATCGCCGTTTCAGGTG
−traM-E	CTGAATTCGTGCATGGCACCGATTA
+traM-S	ATGTCGACGAGGAGCCGACGGTGTT
−cinR-E	CTGAATTCTATCGACCGTCGCAACT
+cinR-S	GCGTCGACAAAGCCCTCCCGAATGA
UtraM-E	TTGAATTCGTCCAGGATTTCCGAGA
LtraM-E	TTGAATTCGCAGACGAAACAAACGG
UtraI-E	TTGAATTCGCTGCTCCCCGAGGGC
LtraI-E	TCGAATTCAGGATGCCAGCGACGG

[a] E corresponds to *Eco*RI sites, and S corresponds to *Sal*I sites.
[b] Nucleotides corresponding to the restriction sites are underlined.

The conjugative ability of pC-13 from different backgrounds was tested. This cosmid showed the same transfer frequency as the complete p42a, either from *R. etli* derivatives lacking one or more plasmids or from *A. tumefaciens* GMI9023 (Table 3). These experiments show that pC-13 carries all of the genes necessary for p42a transfer. As mentioned above, we have previously shown that p42a is able to mobilize the pSym by a cointegration-mediated mechanism (5). In this work we tested whether pC-13 could also do that. Our results showed that pC-13 is also able to mobilize pSym (Table 3). Analysis of the transconjugants indicated that the pC-13-mediated transfer of pSym in all cases involved a cointegration event, similar to what happens with p42a. In contrast, we did not detect transfer of pC-13 from *E. coli* HB101 even though several different recipients were utilized. This suggests that the transfer genes contained in pC-13 are not efficiently expressed in an *E. coli* background.

Sequence analysis of the *tra* genes contained in pC-13. The complete DNA sequence of pC-13 was determined. It revealed 21 open reading frames (ORFs) whose predicted products are

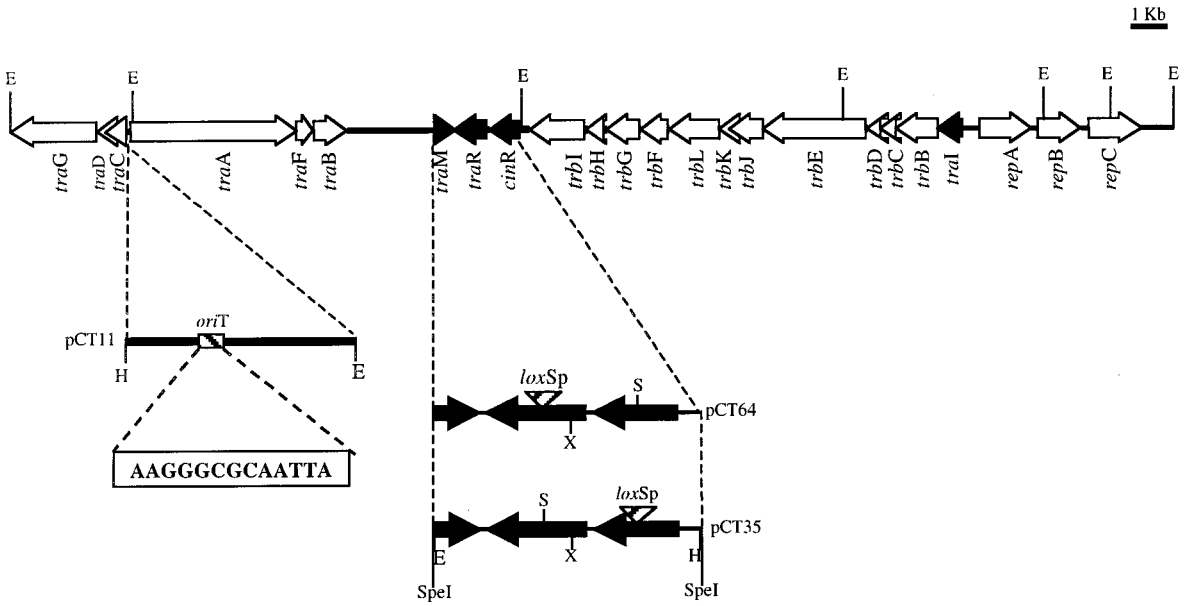

FIG. 1. Physical map of pC-13, containing the transfer region of p42a. Arrows indicate the transcriptional directions of the identified genes. The regulator genes *traI*, *traR*, *cinR*, and *traM* are shown by black arrows. pCT11 contains the 143-bp mobilizable region, carrying the *oriT* of p42a. The 13 bp of the *nic* site sequence are shown. pCT53 and pCT64 contain the regions used to generate mutations in the *traR* and *cinR* genes. H, *Hin*dIII; E, *Eco*RI; X, *Xho*I; S, *Sal*I.

similar to known transfer proteins of *A. tumefaciens* pTiC58 and related plasmids (Fig. 1). The gene arrangement in pC-13 was similar to that found in pTiC58 (11, 33), except that no opine catabolism-related genes were identified. The transfer origin (*oriT*) was located between *traCDG* and *traAFB*, which are transcribed divergently from this site. The *oriT* was identical to the *oriT* of pNGR234a and also to the *oriT* of pTiC58.

Four ORFs encoding regulator proteins were identified in pC-13. Homologous proteins for three of them have been previously characterized for the pTiC58 transfer system. Their

TABLE 3. Transfer frequencies of *R. etli* plasmids p42a, pSym, and pC-13 from different donors[a]

Transfer	Donor	Relevant genotype	Recipient	Transfer frequency[b]
p42a	CFNX187	p42a::Tn*5mob*	GMI9023	1.0×10^{-2}
	GMI9023/p42a	p42a::Tn*5mob*	GMI9023	1.0×10^{-2}
	CFNX187/pCF424	p42a::Tn*5mob, traM* (pNGR234a)	GMI9023	6.3×10^{-4}
	CFNX668	*traM*::pSUPΩSp	CFN2001(p42a$^-$ pSym$^-$)	1.0×10^{-2}
	CFNX669	*traI*::pSUPΩSp	CFN2001(p42a$^-$ pSym$^-$)	ND[c]
	CFNX670	*traR*::*loxP*Sp	CFN2001(p42a$^-$ pSym$^-$)	ND
	CFNX671	*cinR*::*loxP*Sp	CFN2001(p42a$^-$ pSym$^-$)	ND
	CFNX669/pCT12	*traI*::pSUPΩsp, pBBR1MCS5/*traI*	CFN2001(p42a$^-$ pSym$^-$)	8.7×10^{-3}
	CFNX670/pCT13	*traR*::*loxP*Sp, pRK7813-*traR*	CFN2001(p42a$^-$ pSym$^-$)	7.3×10^{-3}
	CFNX671/pCT14	*cinR*::*loxP*Sp, pRK7813-*cinR*	CFN2001(p42a$^-$ pSym$^-$)	8.5×10^{-3}
p42a *tra* region, cloned in pC-13	CFN42/pC-13	Cosmid with all p42a *tra* genes	GMI9023 or DH5α	1.2×10^{-2}
	GMI9023/pC-13	Cosmid with all p42a *tra* genes	GMI9023 or DH5α	1.0×10^{-2}
Cloned *oriT* from p42a	CFN42/pCT11	pBBRIMCS5::*oriT* of p42a	DH5α	1.0×10^{-2}
	CFN42/pBBRIMCS5	pBBRIMCS5 (vector)	DH5α	ND
	CFNX182/pCT11	p42a$^-$/pBBRIMCS5::*oriT* of p42a	DH5α	ND
Cloned *oriT* from pTiC58	CFN2001/p42a+ pDCKE.7ΔB	p42a::GDYN1, *oriT* of pTiC58	HB101	2.5×10^{-4}
	CFN2001/pDCKE.7ΔB	pDCKE.7ΔB	HB101	ND
p42d (pSym)	CFNX689	*traI*::pSUPΩSp, pd::Tn*5*GDYN1	CFN2001(p42a$^-$ pSym$^-$)	ND
	CFNX690	*traR*::*loxP*Sp, pd::Tn*5*GDYN1	CFN2001(p42a$^-$ pSym$^-$)	ND
	CFNX691	*cinR*::*loxP*Sp, pd::Tn*5*GDYN1	CFN2001(p42a$^-$ pSym$^-$)	ND
	CFNX692	pC-13, pd::Tn*5*GDYN1	CFN2001(p42a$^-$ pSym$^-$)	3×10^{-6}

[a] All crosses were repeated at least twice.
[b] Expressed as transconjugants per donor.
[c] ND, not detected

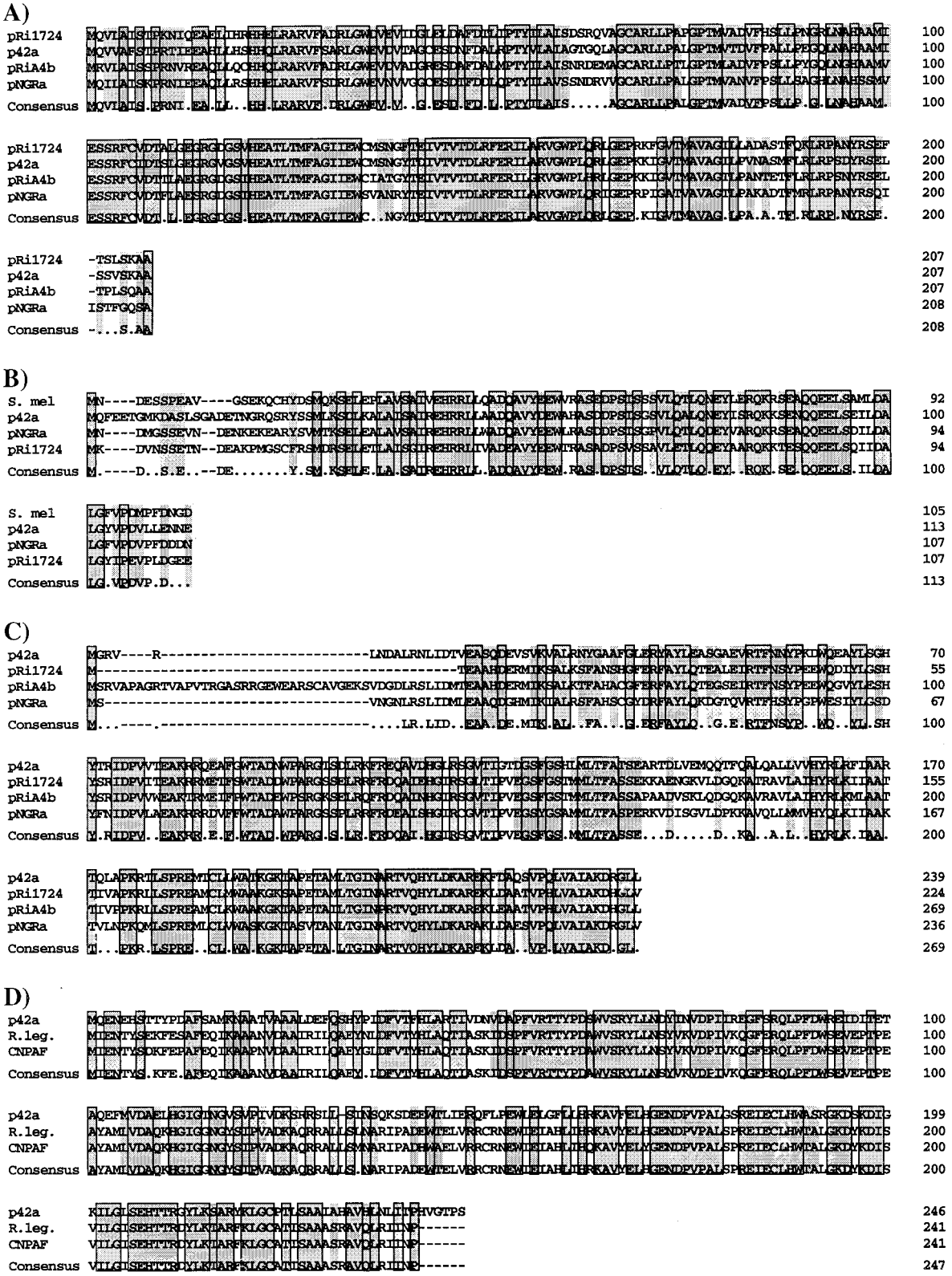

FIG. 2. Alignment of p42a transfer regulator proteins with the corresponding proteins of related systems. S. mel., *S. meliloti*; pNGRa, plasmid pNGR234a; R. leg., *R. leguminosarum*. Identical amino acids are boxed, and conserved amino acids are shown in the consensus. (A) TraI; (B) TraM; (C) TraR; (D) CinR.

products, TraR, TraM, and TraI, are highly conserved with those from other related plasmids (Fig. 2). However, the identity of the pC-13 genes to those from pTiC58 was low (31% for TraR, 29% for TraM, and 50% for TraI). This could explain why we were not able to detect signals for *traR* and *traM* genes in our hybridization experiments (see above). TraR of pC-13 is a 239-aa protein, showing 60% identity to the LuxR family quorum-sensing transcriptional factor of pRiA4b (a plasmid of *A. tumefaciens*) (AB050904), 62% identity to the TraR protein of pRi1724 of *Rhizobium rhizogenes* (NC_002575), and 58% identity to TraR of pNGR234a of *Rhizobium* sp. strain NGR234 (AE00069).

We found another regulator gene, *cinR*. CinR from pC-13 is a 246-aa protein that is 59% identical to CinR of *R. leguminosarum* bv. Viciae (AF210630) and 57% identical to CinR of *R. etli* CNPAF512 (AF393621). Both CinR and TraR are clearly proteins belonging to the LuxR family of transcriptional regulators.

TraI of pC-13 is a 207-aa protein with 75% identity to TraI of pRi1724 (NC_002575), 74% identity to an autoinducer synthase of pRiA4b (AB050904), and 70% identity to TraI of pNGR234a (AE000068). Therefore, TraI is evidently an acylated HSL synthase member of the LuxI family proteins. In the intergenic region between *traI* and *repA*, a *tra* box with the sequence GTTGTAGAATCCTACAAG was found 59 bp upstream of *traI*, suggesting that the transcription of *traI* is dependent on TraR. Another *tra* box was localized upstream of *traC*.

The fourth regulator, TraM, is a 113-aa protein homologous to an antiactivator that inhibits TraR activity through interaction with this protein in domains located at their respective C termini. It shows 69% identity to TraM of pNGR234a (AE000069), 65% identity to TraM of pRiA4b (AB050904), and 57% identity to TraM of pRi1724 (NC_002575). All of these proteins show low identity to transfer regulator proteins of pTiC58 (NC_003065).

ORFs similar to *trbB*, *-C*, *-D*, *-E*, *-J*, *-K*, *-L*, *-F*, *-G*, *-H*, and *-I*, involved in mating-pair formation (33), were also found between *traI* and *cinR*; all of them are transcribed in the same direction as *traI*. Replication genes *repABC* are located upstream of *traI* (Fig. 1).

Characterization of *oriT*. The *oriT* of pC-13, identified through similarity with transfer origins of pNGR234a and pTiC58, was localized between *traA* and *traC*. The sequence analysis revealed a motif identical to that present in the *oriT* region of pTiC58, which is common to the region where nick sites are located in pTF1 and RSF1010. However, the inverted repeats near this motif (8) seem to be absent. To test the functionality of this transfer origin, a fragment of 143 bp containing the *oriT* was cloned in pBBR1MCS5 (pCT11) (Fig. 1) and introduced by conjugation into different strains. pCT11 could be mobilized from wild-type CFN42 but not from CFNX182, a strain lacking p42a (Table 3). As a control we tested the mobilization ability of the vector pBBR1MCS5. As can be seen in Table 3, pBBR1MCS5 could not be transferred. These results indicate that the fragment cloned in pCT11 carries a functional *oriT* dependent upon p42a-encoded proteins. Similar results were found with plasmid pDCKE.7ΔB, which contains the *oriT* of pTiC58 (Table 3); it can be mobilized from a strain containing p42a but not from a strain lacking this plasmid.

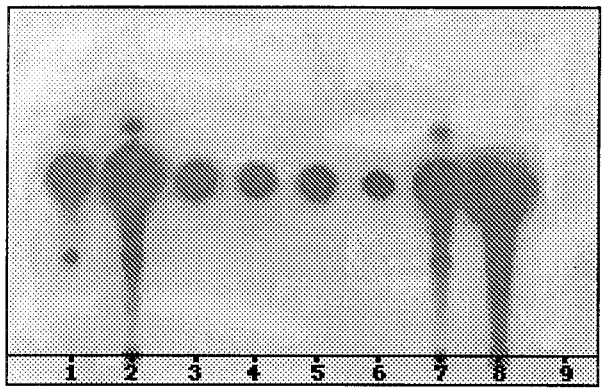

FIG. 3. Thin-layer chromatogram of the acyl-HSL produced by *R. etli* CFN42 and derivatives. Lane 1, *A. tumefaciens* NT1(pTiC58Δ*accR*); lane 2, CFN42; lane 3, CFNX182 (cured of p42a); lane 4, CFNX671 (*cinR*::*loxP*Sp); lane 5, CFNX670 (*traR*::*loxP*Sp); lane 6, CFNX669 (*traI*::pSUPΩSp); lane 7, CFNX668 (*traM*::pSUPΩSp); lane 8, 3-oxo-C_8-HSL; lane 9, YM medium control.

***traI* is responsible for the production of an autoinducer in *R. etli* CFN42.** In pTiC58, TraI is responsible for the production of a conjugal factor or autoinducer, which is a member of a family of substituted HSLs (acyl-HSLs) (25). To determine if an acyl-HSL is involved in the transfer of p42a, the production of these compounds by *R. etli* CFN42 and some derivatives was determined by using the TLC system described by Shaw et al. (48).

Acyl-HSLs can differ with respect to the length of the acyl side chain, the presence or absence of one or more unsaturations, and the nature of the substitution at the carbon 3 position. In some acyl-HSLs, carbon 3 is hydroxylated (3-hydroxyl); it also may carry a carbonyl group (3-oxo) or a fully reduced methylene. The 3-oxo derivatives characteristically produce tailing spots with diffuse edges, whereas the 3-unsubstituted forms produce circular spots with sharp edges (48). To determine whether the *traI* gene found in pC-13 is responsible for production of one of the autoinducers, we analyzed the autoinducer production in strain CFNX669 (*traI*::pSUPΩSp). The results showed that there is one tailing reactive spot which comigrates with a 3-oxo-C_8-HSL. This 3-oxo-C_8-HSL is detected in the wild-type strain but disappears in the *traI* mutant (Fig. 3, lanes 2 and 6) and in the p42a-cured derivative (Fig. 3, lane 3). There is another reactive spot that comigrates with the 3-oxo-C_8-HSL, and its circular form indicates that it is a 3-OH-C_8-HSL. This spot is present in strain CFNX182 (cured of p42a) (Fig. 3, lane 3), indicating that it is encoded in the chromosome or in a plasmid different from p42a. Two unidentified spots were detected in the wild type at low levels; they may be contaminants, similar to those previously detected with this technique (7).

In order to determine the role of the 3-oxo-C_8-HSL in conjugative transfer, the mobilization of p42a containing the interrupted *traI* was tested and found not to be detectable (Table 3). Complementation with the wild-type gene (CFNX669/pCT12) restored the transfer ability of the strain (Table 3). Also, when the *traI* mutant CFNX699 was grown in a condi-

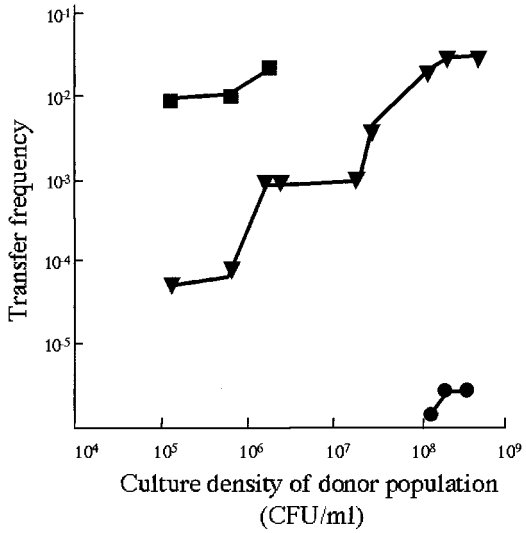

FIG. 4. Effect of culture density on the transfer of p42a and pSym from CFNX688. Transfer frequencies of p42a (triangles), of p42a with conditioned medium added before mating (squares), and of pSym (circles) are shown. Strain CFN2001 (cured of p42a and pSym) was always used as the recipient.

tioned medium consisting of the supernatant of a CFN42 culture grown to stationary phase or in a medium supplemented with 5 μl of 3-oxo-C_8-HSL, the transfer frequency of p42a *traI*::pSUPΩSp was similar to that of the wild-type strain (1.7×10^{-2} and 0.77×10^{-2}, respectively). Our conclusion from these experiments is that TraI is a positive regulator of the transfer process through the synthesis of a 3-oxo-C_8-HSL. On the other hand, in spite of the fact that CFN42 produces at least two acyl-HSLs at readily detectable levels, only the one encoded by the *traI* localized on p42a seems to be involved in conjugative transfer; no other product can substitute for its function, indicating very specific regulation.

Mobilization of p42a, and consequently of pSym, depends on quorum sensing. Acyl-HSLs are molecules involved in quorum-sensing processes, processes that are cell density and growth phase dependent. We have shown that one of these compounds is necessary for p42a mobilization. We also know that the transfer of pSym depends on the presence of p42a (5). Thus, to test if transfer of *R. etli* plasmids is a quorum-sensing-dependent process, we constructed an *R. etli* derivative harboring both p42a and p42d (pSym) labeled with different markers (CFNX688). We determined the transfer frequencies of both plasmids at different growth stages of the donor strain (Fig. 4). The results showed that transfer of p42a initiates when the donor population density is approximately 10^5 and the frequency increases as the donor population augments its density. Furthermore, when the donor strain is pregrown for 6 h in a conditioned medium, transfer is induced at an earlier stage (Fig. 4). These results clearly indicate that the transfer of p42a is dependent on quorum sensing.

The transfer of pSym initiates only after the p42a transfer frequency reaches its highest level (at a population density of 10^8 to 10^9), and it also increases in accordance with the donor population (Fig. 4). Therefore, the transfer of pSym is also dependent on quorum sensing, albeit indirectly.

Two regulators of the LuxR family are activators of p42a transfer. To analyze the role of *cinR* and *traR* in p42a mobilization, we tested the transfer ability of p42a in derivatives containing mutations in these genes (Table 3). Transfer of p42a was completely abolished in derivatives containing mutations in *traI*, *cinR*, or *traR* and was restored when complemented with the respective wild-type genes (CFNX669/pCT12, CFNX670/pCT13, and CFNX671/pCT14) (Table 3). Therefore, the three genes are necessary and sufficient for p42a transfer. Since mobilization of pSym depends on the p42a transfer system, we confirmed that these mutations also eliminate pSym transfer (Table 3).

To further understand the regulation of the p42a transfer genes, we constructed derivatives containing fusions of each of the regulatory genes to a reporter gene: pCT7 (*traI*::*uidA*), pCT8 (*traR*::*uidA*), and pCT10 (*cinR*::*uidA*). Expression of these fusions was tested in the wild-type and *traI*, *traR*, and *cinR* mutant backgrounds. The analysis (Table 4) showed that *traI* expression is reduced when *traR* is mutated (CFNX680). A more dramatic effect was observed in *cinR* or *traI* mutants (CFNX684 and CFNX676), where *traI* expression was com-

TABLE 4. Expression of *traI*::*uidA*, *traR*::*uidA*, and *cinR*::*uidA* gene fusions in *R. etli* wild-type and mutant backgrounds

Strain	Strain genotype	Mean β-glucuronidase sp act ± SD[a]
CFNX672	Wild type, *traI*::*uidA*	116.53 ± 16.62
CFNX676	*traI*::ΩSp *traI*::*uidA*	13 ± 3.03
CFNX680	*traR*::*lox*Sp *traI*::*uidA*	19.15 ± 3.26
CFNX684	*cinR*::*lox*Sp *traI*::*uidA*	ND[b]
CFNX673	Wild type, *traR*::*uidA*	15.39 ± 5.15
CFNX677	*traI*::ΩSp *traR*::*uidA*	15.035 ± 1.9
CFNX681	*traR*::*lox*Sp *traR*::*uidA*	20.69 ± 4.91
CFNX685	*cinR*::*lox*Sp *traR*::*uidA*	18.98 ± 2.79
CFNX675	Wild type, *cinR*::*uidA*	20.59 ± 2.073
CFNX679	*traI*::ΩSp *cinR*::*uidA*	ND[b]
CFNX683	*traR*::*lox*Sp *cinR*::*uidA*	24.54 ± 2.35
CFNX687	*cinR*::*lox*Sp *cinR*::*uidA*	20.94 ± 3.57
CFNX182/pCT7+pCT13	p42a$^-$, pRK7813-*traR*, *traI*::*uidA*	ND[b]
CFNX182/pCT7+pCT14	p42a$^-$, pRK7813-*cinR*, *traI*::*uidA*	ND[b]

[a] Values are expressed as nanomoles per minute per milligram of protein and are means from two independent experiments performed in duplicate.
[b] ND, not detected.

pletely abolished. These results led us to predict that production of the *traI*-dependent acyl-HSL should be affected in these mutants. TLC detection analysis demonstrated that in CFNX669, CFNX670, and CFNX671, the reactive-spot profile was similar to the profile of strain CFNX182 (cured of p42a), while in CFNX668 (*traM* mutant), the acyl-HSL profile was identical to that of the wild type (Fig. 3). Regarding *cinR*, its expression was reduced only in a *traI* mutant background (CFNX679) (Table 4), while the other mutations had no effect on its expression. Finally, *traR* expression was similar in all of the different backgrounds (Table 4), suggesting that it is constitutively expressed.

Since *cinR* and *traR* mutants affect *traI* expression and this gene is responsible for 3-oxo-C_8-HSL production, we performed an experiment to determine whether 3-oxo-C_8-HSL allows *cinR* and *traR* mutants to perform conjugative transfer. The results showed that transfer ability was recovered only at a low frequency in the *traR* mutant (1.2×10^{-6}), and in the *cinR* mutant (3.7×10^{-5}). These results indicate that in addition to their role in *traI* expression, both regulators participate directly in regulation of *tra* genes expression.

The *traI* fusion (pCT7) was introduced into a derivative of the p42a-cured strain CFNX182 carrying the clone containing the *traR* gene (pCT13) or the *cinR* gene (pCT14). The β-glucuronidase activity in cultures of these strains grown in conditioned medium was determined. It was found that *traI* is not expressed in these backgrounds (Table 4). These results suggest that both genes are required for *traI* expression and that they function in parallel. This is in agreement with previous results (see above), since the wild-type strain carrying mutations in either *traR* or *cinR* shows a decrease in *traI* expression and transfer, in spite of the fact that *traR* has no effect on *cinR* expression and vice versa.

TraM does not repress conjugative transfer in p42a. TraM has been shown to be a negative regulator of pTiC58 conjugative transfer through interaction with TraR in domains located at their respective C termini (26, 27, 41). To verify whether the *traM* is an antiactivator in p42a, we constructed a derivative (CFNX668) containing a mutation in *traM* (see Materials and Methods) and tested its conjugative ability. Surprisingly, the transfer frequency was not affected (Table 3). Since we knew by sequence analysis that the C terminus of TraR was conserved with those of other homologous proteins of related pTi plasmids, we questioned whether it could be inactivated by an heterologous TraM repressor. To this end, pCF424 (Table 1), a pBBR1MCS5 derivative containing *traM* of pNGR234a, was introduced into CFNX187 by conjugation. In this strain, we found that the p42a transfer frequency decreased by 2 orders of magnitude (Table 3). This indicates that *traR* of p42a is able to interact with TraM but, for some unknown reason, this interaction is not carried out with the endogenous TraM. To further explore this issue, a transcriptional fusion of *traM* with the β-glucuronidase reporter (pCT9) was constructed. We did not detect expression in any of the backgrounds tested, i.e., wild-type CFN42 or the mutants CFNX669 (*traI*::pSUPΩsp), CFNX670 (*traR*::*loxP*Sp), or CFNX671 (*cinR*::*loxP*Sp) (data not shown). We concluded that TraR in CFN42 is not inactivated because the *traM* gene is not expressed, and thus the antiactivator is not produced, at least under the experimental conditions tested.

DISCUSSION

In the last few years, it has been found that quorum-sensing-dependent processes are widespread among gram-negative bacteria, including plant-associated bacteria (52). Production of multiple autoinducers and complex regulatory networks has been reported for *R. etli*, *R. leguminosarum*, and *S. meliloti* (19, 36, 45, 53, 54). *R. etli* CNPAF512 produces seven different acyl-HSLs (45). Three of these autoinducers have been shown to be produced inside the nodules by bacteroids; mutations in one of them result in a substantial decrease in acetylene reduction activity, indicating that they are involved in nodulation and nitrogen fixation (9). In *R. etli* CFN42 we detected the production of only two acyl-HSLs. Strains CNPAF512 and CFN42 also differ in other plasmid-encoded features, such as the presence of a FixJ homolog in CNPAF512, which is absent in CFN42, and the presence of a novel FixL in the latter strain (18). The organization of these genes was analyzed in 24 *R. etli* strains from different geographic origins. Seventeen (70%) were similar to CFN42 (6). All of these data support the suggestion that in *R. etli* there is intraspecies variability regarding different plasmid-encoded traits.

In *R. leguminosarum*, acyl-HSLs have been related to inhibition of bacterial growth through the production of a small bacteriocin (19, 53, 54). An intricate regulatory cascade involving various *luxI-luxR*-type regulatory genes (*cinR*, *bisR*, and *triR*) has been shown to participate in regulation of the conjugative transfer of a symbiotic plasmid in *R. leguminosarum* (34, 53). In this cascade, TriR positively regulates conjugative transfer genes in response to the 3-oxo-C_8-HSL synthesized by *traI*. *triR* expression is positively controlled by BisR; this induction requires 3OH-$C_{14:1}$-HSL synthesized by the chromosomally encoded CinI. Also, BisR plays a role in repression of *cinI* expression. Although a *trb* operon was identified, localization of the transfer origin, *oriT*, and other transfer genes (*traAFB* and *traCDG*), as well as analysis of *traI*, will surely contribute to complete the regulatory scheme for transfer of this plasmid. In addition, a recently described *traR-traM* locus from *S. meliloti* has been proposed to be involved in transfer (36); however, experimental confirmation is still required.

In this work, we performed a comprehensive analysis of the conjugative transfer mechanism of p42a of *R. etli* and distinctly showed that it is quorum sensing dependent. Interestingly, as pSym transfer depends on p42a, we find that the transfer of the symbiotic plasmid also depends on cell density, in an even more restricted manner than for p42a.

Based on the expression assays of regulatory gene fusions in different backgrounds, we propose a regulatory model for p42a transfer (Fig. 5). Analysis of β-glucuronidase activity demonstrated that *traI* is positively autoregulated and is upregulated by TraR and CinR. According to the proposed model, a complex should be formed by TraR and the 3-oxo-C_8-HSL synthesized by TraI. This complex would be able to bind to the promoters of transfer genes where *tra* boxes have been identified. This complex would also mediate the autoregulatory control of *traI*, where a *tra* box was also found. A mutation in *traR* still allows a low level of *traI* expression, but TraI activity is not high enough to synthesize the autoinducer required to promote transfer of p42a, or even to be detected by TLC analysis. Also, CinR was found to be a positive regulator of *traI*

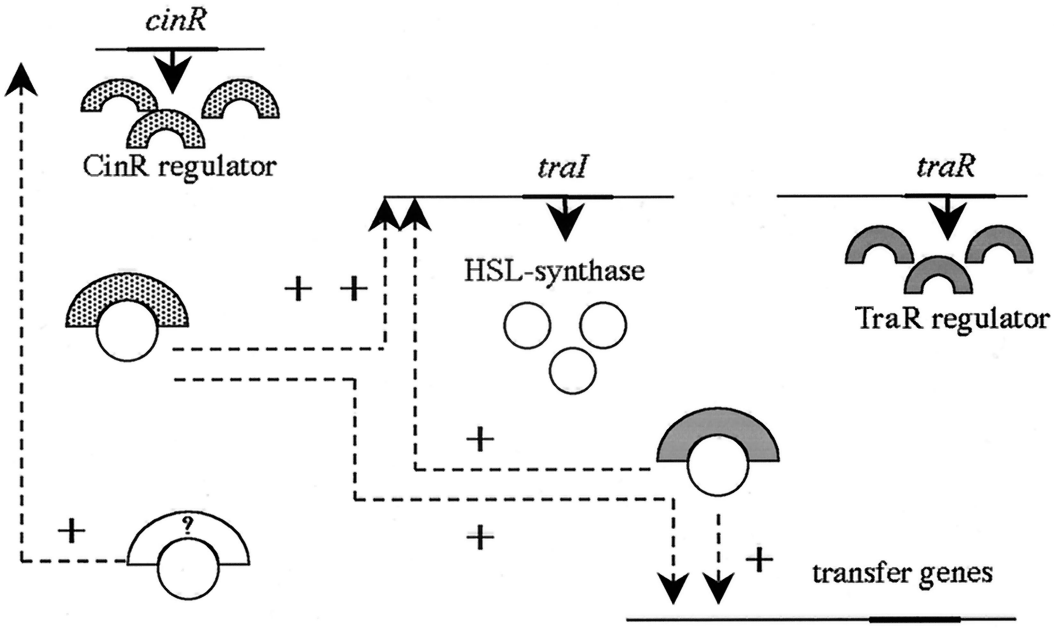

FIG. 5. Model for the regulation of transfer gene expression in p42a. +, positive regulation; ?, unknown regulatory protein from the LuxR family.

independently of TraR, because a mutation in *cinR* abolishes *traI* expression but has no effect on *traR*. As mentioned above (see Results), the transfer frequency of pC-13 is similar to that of the complete plasmid p42a. This indicates that no other regulators are involved in transfer of p42a. Therefore, we predict that the 3-oxo-C_8-HSL produced by TraI is able to form a complex with CinR and to upregulate *traI* (Fig. 5).

Regarding *cinR*, its expression is upregulated by TraI and decreases when *traI* is mutated. As CinR is a positive regulator for *traI*, we would expect a decrease of *cinR* expression in a *cinR* mutant. However, the results show that *cinR* expression remains unaltered in a *cinR* mutant. We propose that *traI* is transcribed at a basal level independent of TraR and CinR, allowing the accumulation of enough 3-oxo-C_8-HSL to activate *cinR*. In the *traI* mutant, this basal level produces an inactive TraI, completely abolishing autoinducer production. The transcriptional activator for *cinR* has not been defined. This hypothetical activator could be either TraR or CinR, acting separately and being able to replace each other, or an unknown and yet-to-be-described regulator. Finally, *traR* expression was found to be constitutive under all conditions tested.

Regulation of p42a transfer resembles that of pTiC58 in many aspects. Nevertheless, they differ in some important aspects. In pTiC58, opines secreted by the plant are the first signal for transfer. In contrast, p42a mobilization does not require its symbiotic host. Another important difference among the transfer systems of these plasmids concerns TraM activity. According to Piper and Farrand (41), in pTiC58 TraM serves to inactivate the small amount of TraR which is constitutively produced in the absence of the conjugal opines. As a consequence, conjugal transfer of this plasmid requires not only the accumulation of autoinducer but also expression of *traR* at levels that allow it to overcome the activity of TraM. In contrast, we found no expression of TraM in p42a. As opines are not required to initiate conjugal transfer, we believe that *traM* might be a relic of an acquired transfer machine in *R. etli*. The absence of the inhibitory effect of TraM, as well as the fact that p42a is able to initiate transfer at a lower culture density than pTiC58, may explain the high frequency of p42a transfer (compare Fig. 4 with Fig. 2 of reference 41). However, we cannot rule out the possibility that TraM might be expressed and have a regulatory role under other experimental conditions.

From the results presented in this paper, it is evident that there is a regulatory cascade controlling conjugative transfer of p42a and an even more complex regulation for pSym transfer. To complete the regulatory scheme, it will be necessary to identify other controlling elements, such as the transcriptional regulator of *cinR*.

We have previously shown both that pSym transfer depends on the presence of p42a and the feasibility of cointegration among both plasmids (5). Here, we show the existence of another level for pSym transfer regulation, indirectly linked to the environmental conditions, which may have an effect on the cellular density. Although p42a transfer is not restricted to the presence of the symbiotic host (see above), we expect that the cellular population is more likely to achieve the level required for plasmid transfer in a closed environment, such as the nodule or infection thread, than in soil.

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