Sequence, localization and characteristics of the replicator region of the symbiotic plasmid of *Rhizobium etli*

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The replicator region of the symbiotic plasmid of *Rhizobium etli* CFN42 was cloned and sequenced. A plasmid derivative (pH3) harbouring a 5.6 kb *HindIII* fragment from the symbiotic plasmid was found to be capable of independent replication and eliminated the symbiotic plasmid when introduced into a *R. etli* CFNX101 strain (a *recA* derivative). The stability and the copy number of pH3 were the same as that of the symbiotic plasmid, indicating that the information required for stable replication and incompatibility resides in the 5.6 kb *HindIII* fragment. The sequence analysis of this fragment showed the presence of three ORFs similar in sequence and organization to *repA*, *repB* and *repC* described for the replicator regions of the *Agrobacterium* plasmids pTiB6S3 and pRiA4b and for the *R. leguminosarum* cryptic plasmid pRL8J1. Hybridization studies showed that p42d-like replicator sequences are found in the symbiotic plasmids of other *R. etli* strains and in a 'cryptic' plasmid of *R. tropici*.

Keywords: symbiotic plasmid, plasmid replication, *Rhizobium etli*

INTRODUCTION

The bacterial genera *Rhizobium* and *Sinorhizobium* belong to the family *Rhizobiaceae* and are able to form nitrogen-fixing nodules with several legume species. Members of the genus *Agrobacterium* of the same family also interact with plants but induce pathogenic responses in plants, such as crown-gall or hairy-root diseases. A distinctive characteristic of these organisms is the huge amount of genetic information encoded in large plasmids of low copy number. Calculations based on assumed sizes of chromosomes indicate that the plasmid pool may represent between 25 and 50% of the genome (Martínez *et al.*, 1990; García-de los Santos *et al.*, 1996). It is well established that many of the bacterial genes governing plant-microbe interactions are plasmid-borne. In *Rhizobium* spp. many of the genes involved in nodulation and nitrogen fixation lie on a single plasmid, the symbiotic plasmid or pSym. How-

ever, pSym coexists with 1–10 so-called 'cryptic' plasmids. Information accumulated over many years indicates that cryptic plasmids may harbour genes involved in basic metabolic functions, utilization of nutrients and synthesis of cellular components (García-de los Santos *et al.*, 1996). It has been shown that some *Rhizobium* plasmids are transmissible or mobilizable by other plasmids under laboratory conditions or in media resembling soil (Johnston *et al.*, 1982; O'Connell *et al.*, 1987; Mercado-Blanco & Olivares, 1993; Kinkle & Schmidt, 1991). Studies of population genetics of rhizobia based on chromosomal and plasmid characteristics suggest that horizontal gene transfer occurs in natural populations (Schofield *et al.*, 1987; Young & Wexler, 1988).

Incompatibility has been observed between *Rhizobium* plasmids (including symbiotic plasmids) (Beynon *et al.*, 1980; O'Connell *et al.*, 1984; Hooykaas *et al.*, 1985) and between *Rhizobium* and *Agrobacterium* plasmids (including pTi and pRi plasmids) (O'Connell *et al.*, 1987; Hynes *et al.*, 1985). Some *Rhizobium* strains containing two symbiotic plasmids with the same or different host-range determinants have been constructed, indicating that the symbiotic plasmids can belong to different incompatibility groups (Hooykaas *et al.*, 1981; Geniaux

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The GenBank accession number for the nucleotide sequence reported in this paper is U80928.

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics*	Reference or source
<i>Rhizobium</i> spp.		
CIAT895	Wild-type, nodulates <i>Phaseolus vulgaris</i>	Pinero <i>et al.</i> (1988)
<i>R. etli</i>		
VIKING1	Wild-type, nodulates <i>P. vulgaris</i>	Pinero <i>et al.</i> (1988)
TAL182	Wild-type, nodulates <i>P. vulgaris</i>	Pinero <i>et al.</i> (1988)
BRA5	Wild-type, nodulates <i>P. vulgaris</i>	Pinero <i>et al.</i> (1988)
NITRAGIN8251	Wild-type, nodulates <i>P. vulgaris</i>	Pinero <i>et al.</i> (1988)
CFN42	Wild-type (type strain), nodulates <i>P. vulgaris</i>	Quinto <i>et al.</i> (1982)
CE3	Sm ^r derivative of CFN42	Noel <i>et al.</i> (1984)
CFNX101	<i>recA</i> ::Ω, Sp ^r Sm ^r derivative of CE3	Martínez-Salazar <i>et al.</i> (1991)
CFNX107	<i>recA</i> ::Ω, Sp ^r Sm ^r , p42a ⁻ , p42d ⁻ derivative of CE3	Martínez-Salazar <i>et al.</i> (1991)
<i>R. huakuii</i>		
CCBAU2605	Wild-type, nodulates <i>Astragalus sinicus</i>	N. Novikova, St Petersburg, Russia
<i>R. loti</i>		
CIAM1801	Wild-type, nodulates <i>Lotus</i>	N. Novikova, St Petersburg, Russia
<i>R. leguminosarum</i> bv. <i>viciae</i>		
VF39	Wild-type, nodulates <i>Vicia faba</i>	Hynes <i>et al.</i> (1988)
<i>R. tropici</i>		
CFN299	Wild-type, nodulates <i>P. vulgaris</i>	Pinero <i>et al.</i> (1988)
<i>S. meliloti</i>		
2011	Wild-type, Sm ^r , nodulates Alfalfa	Casse <i>et al.</i> (1979)
<i>A. tumefaciens</i>		
GM19023	C58, plasmid-less derivative	Rosenberg & Huguet (1984)
<i>E. coli</i>		
HB101	Host strain for plasmids	Boyer & Roulland-Dussoix (1969)
DH5α	Host strain for plasmids	Hanahan (1983)
Plasmid		
pBluescript II SK +	Ap ^r , cloning vector, ColE1 origin of replication	Stratagene
pSUP202	Ap ^r Cm ^r Tc ^r , ColE1 origin unable to replicate in <i>Rhizobium</i> spp.	Simon <i>et al.</i> (1983)
cGD47	Lorist 3B cosmid derivative containing <i>Bam</i> HI fragments 72–80 of the structural map of p42d	Girard <i>et al.</i> (1991)
pB79	pSUP202 derivative containing <i>Bam</i> HI fragment 79 of the structural map of p42d; contains the replicator region of p42d	This work
pH3	pSUP202 derivative containing a <i>Hind</i> III fragment of 5.6 kb containing the replicator region of p42d	This work
pRK2013	Helper plasmid, Km ^r	Ditta <i>et al.</i> (1980)

* Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

& Amarger, 1993). Incompatibility is linked to common replication functions and should reflect a close ancestral relationship between plasmids (O'Connell *et al.*, 1987).

Diverse types of rearrangements within plasmids (amplifications, deletions and translocations) and recombination between different plasmids have been described for *Rhizobium* (Romero *et al.*, 1997; Johnston *et al.*, 1982). These phenomena, coupled to the transmissibility of plasmids between different *Rhizobium* species, are partially responsible for the large genetic diversity observed within the *Rhizobiaceae*.

The study of the molecular basis of incompatibility, stability and replication of plasmids may help us to

understand one important factor directing the flux of information between members of the *Rhizobiaceae*. With this aim, we report here the isolation and molecular characterization of the replicator region of the symbiotic plasmid of *Rhizobium etli* CFN42. This constitutes the first description of a symbiotic replicator within the *Rhizobiaceae*.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed on Table 1. *Rhizobium* strains were grown at 30 °C in PY medium (Noel *et al.*, 1984). *Escherichia coli* strains were grown at 37 °C in Luria-Bertani medium. Antibiotics were supplemented at the following

Fig. 1. Plasmid profiles of CFNX107 (lane 1), CFNX107 (pH3) (lane 2), DH5α(pH3) (lane 3), CFNX101(pH3) (lane 4) and CFNX101 (lane 5). The arrow labelled 'S' indicates the position of the symbiotic plasmid p42d. The arrow labelled '*' indicates the position of pH3.

concentrations ($\mu\text{g ml}^{-1}$): nalidixic acid, 20; tetracycline, 10; kanamycin, 30; chloramphenicol, 25; ampicillin, streptomycin, rifampicin or spectinomycin, 100.

Plasmid profiles. High molecular mass plasmids were visualized by the procedure described by Wheatcroft *et al.* (1990) (see Fig. 1).

Mobilization of cosmids to *Rhizobium* strains. To mobilize a set of cosmids derived from Lorist 3B, a non-mobilizable vector unable to replicate in *Rhizobium*, it was necessary to make a co-integrate between each cosmid and a mobilizable vector. For this purpose a pSUP202 derivative containing a 3.8 kb *Pst*I fragment of Lorist 3B was constructed to provide regions of homology between pSUP202 and the cosmids. This construction was transformed into *E. coli* strains containing the cosmid and the co-integrates were mobilized to *Rhizobium* in triparental matings using pRK2013 as helper plasmid (Ditta *et al.*, 1980).

DNA sequencing. *Xho*I, *Eco*RI, *Sal*I and *Pst*I fragments of the DNA insert of clone pH3 were subcloned in the pBluescript II SK+ phagemid vector (Stratagene) and transformed into *E. coli* DH5α. Double-stranded DNA was isolated and sequenced with a combination of universal primer and custom-made oligonucleotide primers using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA Sequencing System (Applied Biosystems).

Analysis of nucleotide and amino acid sequences. Nucleic acid sequence data and deduced amino acid sequences were analysed with the GeneWorks package (release 2.5; IntelliGenetics). Similarity searches with the GenBank and EMBL databases were made using the FASTA, TFASTA, BLASTN and BLASTX programs, which are part of the Wisconsin Sequence

Analysis Software Package (Genetic Computer Group Package, version 8).

Plasmid stability. Plasmid stability was calculated according to the procedure described by Durland *et al.* (1987). Briefly, stationary phase cultures were diluted in fresh medium without selection to give an initial OD_{620} of 0.001 and cultivated for 9, 18 and 31 generations. Samples taken at these times were serially diluted and plated on solid media in the absence of selective drugs. One hundred colonies were chosen and picked onto plates with and without the selective antibiotic. The percentage of plasmid loss per generation was calculated with the formula: percentage plasmid loss per generation = $[1 - (F_n/F_0)^{1/n}] \times 100$, where F_n is the antibiotic-resistant cell fraction after n generations and F_0 is the initial fraction of resistant cells.

DNA isolation, manipulation and hybridization. Genomic and plasmid DNA were isolated as described in Sambrook *et al.* (1989). DNA was restricted and ligated under the conditions specified by the enzyme manufacturer (Amersham). DNA restriction fragments were separated for hybridization by electrophoresis in 1% agarose gels, transferred onto Hybond-N membranes (Amersham) and cross-linked in vacuum at 80 °C. Hybridizations were performed overnight using [α - ^{32}P]dCTP-labelled probes (Megaprime Kit, Amersham) and high stringency conditions (65 °C in sodium phosphate buffer). Hybridization signals were detected on X-OMAT-K film (Kodak) in the presence of intensifying screens.

Determination of plasmid copy number. A DNA blot containing genomic DNA of strains CE3, CFNX101(pH3) and CFNX107(pH3), restricted with *Hind*III, was hybridized simultaneously with a 1 kb fragment of the chromosomally encoded gene *recA* and with an internal 1 kb fragment of pH3. Both probes were labelled at the same specific radioactivity. The *recA* probe and pH3 probe hybridized with a 1.2 kb and a 1.4 kb *Hind*III fragment, respectively. Hybridization signals were integrated by scanning the autoradiographs with a GS300 scanning densitometer using the GS365W data system (Hoefer Scientific Instruments), taking care that measurements were within the linear range of the film. Plasmid copy number was calculated as the ratio of the integrated hybridization signal of the symbiotic plasmid (pH3) and the integrated hybridization signal of the chromosome probe.

RESULTS AND DISCUSSION

Cloning of the replicator region of symbiotic plasmid p42d

R. etli CFN42 (type strain) contains six large plasmids. The symbiotic plasmid (p42d) of 390 kb contains many of the *nif*, *fix* and *nod* genes and confers nodulation proficiency when transferred to *Agrobacterium tumefaciens* (Martínez *et al.*, 1990). A structural map of this plasmid has been assembled from an ordered cosmid collection, comprising 85 *Bam*HI fragments, which were numbered consecutively on the circular map from an arbitrary start in the *nifHDK* region 'a' (Girard *et al.*, 1991) (Fig. 2). To isolate the replicator region of p42d, a set of cosmids covering the entire symbiotic plasmid (Girard *et al.*, 1991) was transferred by conjugation to strains CFNX107 and CE3 as described in Methods. CFNX107 was utilized to reduce stabilization of the cosmids by integration, since plasmids p42a and p42d

Fig. 2. (a) Circular map of plasmid p42d. The lines radiating from the circle represent *Bam*HI restriction sites. Restriction fragments were numbered successively in a clockwise direction, starting with the fragment containing the *nifHa* gene. Arrows indicate the location of some *nod* and *nif* genes. The black box indicates the position of the replicator region. The map is drawn to a scale of 1 kb = 0.93°. (b) The enlargement shows a restriction map of the pH3 insert. The orientation of the three ORFs is indicated by shaded arrows. Vertical black bars indicate the position in which putative Shine-Dalgarno sequences were found.

share regions of extensive homology. Tetracycline-resistant transconjugants were recovered with the complete set of cosmids when CE3 was the recipient strain but such transconjugants were obtained only with cosmid cGD47 when CFNX107 was used as receptor. Analysis of the plasmid profiles of CFNX107 containing cosmid cGD47 showed the presence of an additional small plasmid. Southern blot analysis confirmed that the new small replicon was indeed cGD47. When the same analysis was applied to transconjugants of CE3 two observations were made. Firstly, transconjugants containing cGD47 maintained the cosmid as an independent replicon but with the concomitant loss of the symbiotic plasmid, and secondly, transconjugants with the other cosmids were the result of co-integration events.

These results demonstrate that cGD47 contains the DNA regions involved in replication and incompatibility. Supported by the fact that only one cosmid was capable of independent replication, we concluded that the symbiotic plasmid contains only one replicator region in contrast to the megaplasmid pSym-b of *Sinorhizobium meliloti* which contains more than one origin of replication (Margolin & Long, 1993).

Characterization of the replicator region of symbiotic plasmid p42d

On the basis of the restriction map, the estimated length of cGD47 is 39.8 kb comprising nine *Bam*HI fragments, from fragment 72 to fragment 80 of the physical map of plasmid p42d (Girard *et al.*, 1991). Each of these fragments was subcloned in vector pSUP202 and transferred to strains CFNX107 and CE3. Only clone pB79, which carries *Bam*HI fragment 79 (16.8 kb), was capable of independent replication in the CFNX107 background. However, when plasmid profiles of CE3 transconjugants containing pB79 were analysed, no new replicon was found, indicating that pB79 had integrated into the genome of the recipient strain. These data demonstrate that the replicator region of p42d resides in *Bam*HI fragment 79 but suggest that DNA sequences that lie outside of this fragment are required to avoid integration in a wild-type background and that *recA* plays an important role in the establishment of incoming plasmids.

The insert DNA of plasmid pB79 has six *Hind*III fragments and each was subcloned into pSUP202 and introduced by conjugation into strains CFNX107 and CFNX101. Plasmid pH3, containing a *Hind*III fragment of 5.6 kb, was capable of independent replication in both CFNX107 and CFNX101. Moreover, a plasmid profile analysis of the CFNX101 transconjugants showed that the symbiotic plasmid was absent. The different *Eco*RI, *Sal*I and *Xho*I fragments of pH3 were tested individually and none were able to confer independent replication to pSUP202. From these experiments we concluded that the replicator and incompatibility functions of the symbiotic plasmid are restricted to the 5.6 kb *Hind*III fragment contained in pH3. Fig. 2(a) shows the location of the 5.6 kb *Hind*III fragment and the position of some *nif* and *nod* genes already sequenced on the circular map of p42d. The *Hind*III restriction site downstream of *repC* is located approximately 2.5 kb from the *Bam*HI site boundary of fragments 79 and 80. The *repA*, *repB* and *repC* genes are transcribed in a clockwise direction (as for the *nifHDK* operons of regions 'a' and 'b'). Plasmid p42d is subject to frequent tandem amplifications in defined regions (amplicons). Usually boundaries of amplicons are reiterated sequences. The replicator of p42d is located in a region characterized by the presence of multiple reiterated sequences and the lack of tandem amplifications (Girard *et al.*, 1991; Romero *et al.*, 1995). As pointed out by Romero *et al.* (1995), this situation can be explained because a duplication of a sector containing the replicator may induce a delay in plasmid replication, due to a copy number control mechanism.

Stability of cGD47, pB79 and pH3 plasmids

By growing the cells in the absence of selective antibiotics at 30 °C, the stability of cGD47, pB79 and pH3 could be examined in the CFNX107 and CFNX101 genetic backgrounds. The results indicated that cosmid cGD47 and plasmids pB79 and pH3 are as stable as the

symbiotic plasmid because no plasmid loss was observed in either background after 18 generations.

Copy number of plasmid pH3

To demonstrate that the replicator region contained in plasmid pH3 has the same properties as that of the symbiotic plasmid, the copy number of pH3 per chromosome of p42d was evaluated. Briefly, a Southern blot containing total DNA of strains CE3, CFNX101(pH3) and CFNX107(pH3) digested with *Hind*III was hybridized simultaneously with an internal fragment of the chromosome-encoded gene *recA* and with an internal fragment of the pH3 insert as described in Methods. Each lane of the exposed film was evaluated by densitometry. In all three cases the plasmid/chromosome ratio fluctuated between 1.3 and 1.6, indicating that both replicons existed in the same copy number per chromosome.

The nucleotide sequence of the 5.6 kb insert of pH3

To examine the gene organization of the replicator region of the symbiotic plasmid, the complete sequence of the pH3 insert was determined. The sequence of the pH3 insert contained 5615 nt and had three large potential ORFs on one strand (Fig. 2b). The DNA sequence and the putative translation products of the ORFs showed significant identity with *repA*, *repB* and *repC* as described for the replicator regions from *R. leguminosarum* cryptic plasmid pRL8JI (Turner & Young, 1995), *A. tumefaciens* plasmid pTiB6S3 (Tabata *et al.*, 1989), *A. rhizogenes* plasmid pRiA4b (Nishiguchi *et al.*, 1987) and *Paracoccus versutus* plasmid pTAV1 (GenBank U60522). Two putative ribosome-binding sites were found, one preceding *repA* and the other preceding *repC*. Regions containing the *E. coli* promoter consensus [TTGACA(N₁₇)TATA C/A A/T] were not found, suggesting that these genes are not expressed in *E. coli* (Greener *et al.*, 1992).

The four replicators described above and the *R. etli* replicator described here have the same genetic organization: the *repA* gene precedes *repB* and *repB* precedes *repC*. Two intergenic sequences were found, one between *repA* and *repB* and the other between *repB* and *repC*. Comparisons between the sequence of the replicator regions mentioned above showed that the replicator region of p42d is very closely related to that of plasmid pRiA4b.

Studies of the replicator region of plasmid pTiB6S3 demonstrated that genes *repA* and *repB* encode proteins that are required for the stability of the plasmid but are not essential. BLAST analysis showed that the *repA* and *repB* gene products have some similarity with proteins required for the partitioning of P1 and F plasmids (Williams & Thomas, 1992). Sequence comparisons with the complete sequence of other *repA* gene products showed that RepA of p42d is closely related to the *repA* gene product of pRiA4b with an identity of 92%.

Sequence comparisons indicate that *repB* gene products are also conserved but the highest degree of identity was

found in the central region of the proteins, suggesting that this region contains the functional domain for the stability function.

Surprisingly, the product of the *repC*, the principal product associated with the replication function, is less conserved. Comparison of the different complete RepC sequences showed identities between 27 and 73% with pRmeGR4a and pRiA4b, respectively. The hydrophobicity patterns (Kyte & Doolittle, 1982) of the different RepC proteins were similar, suggesting that the three-dimensional structure of these proteins is conserved (data not shown). The wide variation observed for *repC* gene products is perhaps required to allow compatibility as suggested by Turner *et al.* (1996).

It has been suggested that the intergenic sequence between *repB* and *repC* participates as an incompatibility determinant (Tabata *et al.*, 1989). Sequence alignments of the intergenic sequence, including that between ORF2 and *repC* of plasmid pRmeGR4a of *S. meliloti*, showed regions of sequence conservation, including an AAAGAAA motif present in all intergenic sequences. This motif may be important in the transcription of *repC* or as a recognition sequence for a protein involved in incompatibility functions. The highest similarity was found with the intergenic sequence of pRiA4b of *A. rhizogenes* with 93% similarity in a 152 bp overlap.

Occurrence of p42d-like replicator sequences in other *Rhizobium* species

Plasmid profiles of *R. huakuii*, *R. leguminosarum* bv. *viciae*, *R. loti*, *R. tropici*, *S. meliloti* and several *R. etli* strains having different geographic origins were hybridized against the pH3 insert to assess whether related sequences are present in plasmids of other species (Fig. 3). Only six of the twelve strains tested contained a plasmid with homology to the replicator region of symbiotic plasmid p42d, and in five cases the detected plasmid corresponded to a *R. etli* symbiotic plasmid (previously determined to be symbiotic plasmids by hybridization against a *nifH* probe; data not shown). In *R. tropici* the hybridization signal was detected in the smallest non-symbiotic plasmid. Moreover, the symbiotic plasmid of *R. tropici* CFN299 contains a *repC* type A gene similar to those described for *S. meliloti* (Burgos *et al.*, 1996). Plasmid pH3 is autonomously replicated in the plasmid-less *A. tumefaciens* GMI9023, indicating that the replicator of p42d is functional in other species (data not shown).

The fact that p42d-like sequences were always found in symbiotic plasmids of *R. etli* strains of different geographic origins together with the high degree of homology detected in several *R. etli* symbiotic plasmids when hybridized against cosmids of the p42d symbiotic plasmid covering the whole plasmid suggests a monophyletic origin for the symbiotic plasmids of *R. etli* (Rodríguez *et al.*, 1993). The study of the genetic basis of replication, mobilization and incompatibility of the plasmids will give us a better understanding of the rules

Fig. 3. Autoradiogram of plasmid profiles hybridized with pH3 insert demonstrating the presence of DNA homologous to the p42d replicator region in different *Rhizobium* strains. Lanes: 1, *R. etli* CE3; 2, *R. etli* BRA5; 3, *Rhizobium* spp. CIAT895; 4, *R. etli* NITRAGIN8251; 5, *R. etli* TAL182; 6, *R. etli* VIKING1; 7, *R. huakuii* CCBAU2605; 8, *R. leguminosarum* bv. *viciae* VF39; 9, *R. loti* CIAM1810; 10, *R. tropici* CFN299; 11, *S. meliloti*, 2011; 12, *R. etli* CFNX107 (a pSym-cured derivative).

governing horizontal plasmid transfer and hence a better picture of how the bacterial populations evolve.

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