Short Technical Reports

In Vivo Cloning Strategy for *Rhizobium* Plasmids

BioTechniques 33:782-788 (October 2002)

ABSTRACT

We have developed a simple system to clone indigenous Rhizobium *plasmids into* E. coli. *The strategy consists of three matings: the first is to insert Tn5 in the plasmid to be cloned, the second incorporates the integrative vector into the inserted Tn5 in the native* Rhizobium *plasmid, and the last mating transfers the target plasmid directly into* E. coli. *This mating-based system was successfully used to clone plasmids of* Rhizobium *species with sizes ranging from 150 to 270 kb. In addition, a 500-kb fragment of a 600-kb megaplasmid was also cloned. This strategy could be used for cloning indigenous replicons of other Gram-negative bacteria into a different host.*

INTRODUCTION

Bacteria of the genus *Rhizobium* fix nitrogen within nodules that they form in symbiotic association with legumes. Many genes involved in the symbiotic process are encoded on plasmids, whose sizes range from 100 to 1700 kb (11). Usually, the production of an overlapping collection of cosmids or BAC clones is the first step in generating physical and genetic maps of such large plasmids (18). Unfortunately, the clones obtained are often not large enough and are so random that a large collection of clones is needed to completely cover an entire plasmid. In a previous report (3), we developed a procedure for cloning large fragments from the *Sinorhizobium meliloti* pExo megaplasmid. Regions cloned were flanked by the RK2 *oriT* gene in direct orientation. Although amenable to cloning large DNA regions from *S. meliloti*, this method requires a genetic map of the plasmid to isolate the desired regions, a transduction system, and other genetic traits (3).

Here we describe a general system to clone plasmids of *Rhizobium* species. This in vivo system has several advantages with respect to the traditional in vitro systems, such as a direct isolation of specific plasmids, fast cloning, and coverage of plasmids or chromosomes with only a few BAC clones. The system is based on three single matings. The principal components of the system are the *oriT* (RK2) to transfer DNA and the F origin to stably maintain large replicons in *E. coli* (7). Using this system, we were able to clone different plasmids from various *Rhizobium* species. Our system could be useful in producing large replicons that could then be used for sequencing or evaluating gene expression in different genetic backgrounds.

Table 1. Bacterial Strains and Plasmids Used

Strains or Plasmids	Relevant Characteristics	Source or Reference
R. tropici		
CFN299	Wild-type strain Nal^r	14
CFNE68	pSym::Tn5 *mob*	This study
CFNE84	pSym::Tn5 *mob*	This study
CFNE87	pSym::Tn5 *mob*	This study
CFNE130	CFN299, *pcsA*::Tn5 *mob*	16
CFNE299-19	CFN299, *teu*::Tn5 *gusA*	20
CFNE299-10	CFN299, Sym plasmid deleted	13
R. etli		
CFN42	Wild-type Nal^r	22
CFNX187	pCFN42*a*::Tn5 *mob* Nal^r Km^r	1
CFN037	pCFN42*b*::Tn5 *mob* Nal^r Km^r	1
CFNX191	pCFN42*c*::Tn5 *mob* Nal^r Km^r	1
CFNX2001	CFN42 cured of pCFN42*a* and pCFN42*b*	10
S. meliloti		
GRM6LR	pRmeGR4*a*::ΩSp^r/St^r	2
A. tumefaciens		
GMI9023	Plasmid-less Rif^r strain	19
GMI9023/pCFN42*a*	pCFN42*a*::Tn5 *mob* Km^r Rif^r	6
E. coli		
DH5α		Invitrogen (Carlsbad, CA, USA)
Plasmids		
pTH509	IS50, *oriV*, ΩSp^r, *oriT*, Cm^r pBAC F origin	3
pRK2013	ColE1 replicon with RK2 transfer region Km^r Nm^r	5

Sp/St, spectinomycin and streptomycin, respectively.
Rif, rifampicin.

MATERIALS AND METHODS

The bacterial strains and plasmids used are listed in Table 1. *Rhizobium* strains were grown on PY medium (0.5% peptone, 0.3% yeast extract, 10 mM $CaCl_2$) supplemented with the following antibiotics: 25 µg/mL kanamycin, 10 µg/mL chloramphenicol, 25 µg/mL spectinomycin, 30 µg/mL streptomycin, 20 µg/mL rifampicin, and 10

µg/mL nalidixic acid. *E. coli* strains were grown in LB medium (1% peptone, 0.5% yeast extract, and 1% NaCl) supplemented with 25 µg/mL kanamycin, 10 µg/mL chloramphenicol, 25 µg/mL spectinomycin, and 100 µg/mL ampicillin. *E. coli* and *Rhizobium* were grown at 30°C. Conjugation experiments were performed in the presence of pRK2013 as a helper (5) as previously described (4).

Plasmid purification and genomic DNA extraction were performed according to published protocols (21). For hybridization, DNA was purified, digested with *Bam*HI, and transferred from agarose gels to nylon membranes. Probes were labeled with ^{32}P by random primer, and hybridization was carried out under high-stringency conditions (21). Plasmid profiles were obtained by a modified Eckhardt agarose gel electrophoresis method (9). For sequencing, dsDNA was purified with the High Pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany), and sequencing was performed in an automatic ALF™ DNA sequencer (Amersham Biosciences, Uppsala, Sweden).

Plant nodulation assays were performed in flasks containing agar as previously described (12). Seeds of *Phaseolus vulgaris* cv. Negro Jamapa were inoculated with 10^5 *Rhizobium* cells. Nitrogenase activity was measured by acetylene reduction 21 days after inoculation.

RESULTS AND DISCUSSION

In general, the technique is as follows. (*i*) The first mating is carried out with *E. coli* harboring a Tn5 (23) that is resistant to kanamycin (Km^r), while the recipient *Rhizobium* strain should be resistant to nalidixic acid (Nal^r). *Rhizobium* transconjugants, which are resistant to both kanamycin and nalidixic acid, and thus contain the transposon in the replicon to be cloned, are selected. (*ii*) In the second mating, these Km^r Nal^r *Rhizobium* transconjugants are mated with *E. coli* harboring the integrative vector pTH509, which contains genes for resistance to chloramphenicol (Cm^r) and spectinomycin (Sp^r). During this second mating, a single crossover occurs between IS50 of the integrative vector and that of target Tn5 that is inserted in the *Rhizobium* plasmid. Then, *Rhizobium* recombinants resistant to kanamycin, chloramphenicol, and nalidixic acid are selected and purified. (*iii*) In the third mating, *Rhizobium* transconjugants containing the recombinant cointegrate (i.e., integrative vector pTH509 plus target plasmid) are mated with *E. coli* DH5α. *E. coli* exconjugants are selected on LB medium supplemented with chloramphenicol, spectinomycin (integrative vector), and kanamycin (target *Rhizobium* plasmid). *Rhizobium* donors are counterselected either by the inability to grow on LB or by a difference in growth rate in comparison to the recipient *E. coli*. A schematic representation of the cointegrate structure that is generated between the integrative vector pTH509 and the IS50 of the target *Rhizobium* Tn5, as well as the resultant plasmid in *E. coli*, is shown in Figure 1.

We applied our system to clone plasmids of *R. etli* (22), *R. tropici* (14), and *S. meliloti* (2). The integrative vector pTH509 was found to insert in the IS50 at a frequency of 10^{-7}. However, when the *Rhizobium* plasmid is targeted with Tn5 *mob* recombination of pTH509 occurred between either IS50 or *oriT* (data not shown). Transfer of the cointegrate molecules from *Rhizobium* to *E. coli* occurred at a frequency of 10^{-6}.

The presence of cointegrated cloned plasmids in *E. coli* was visualized by Eckhardt gels. Sizes of the cloned replicons ranged from 150 to 270 kb (Figure 2A). They included pCFN42*a* (180 kb),

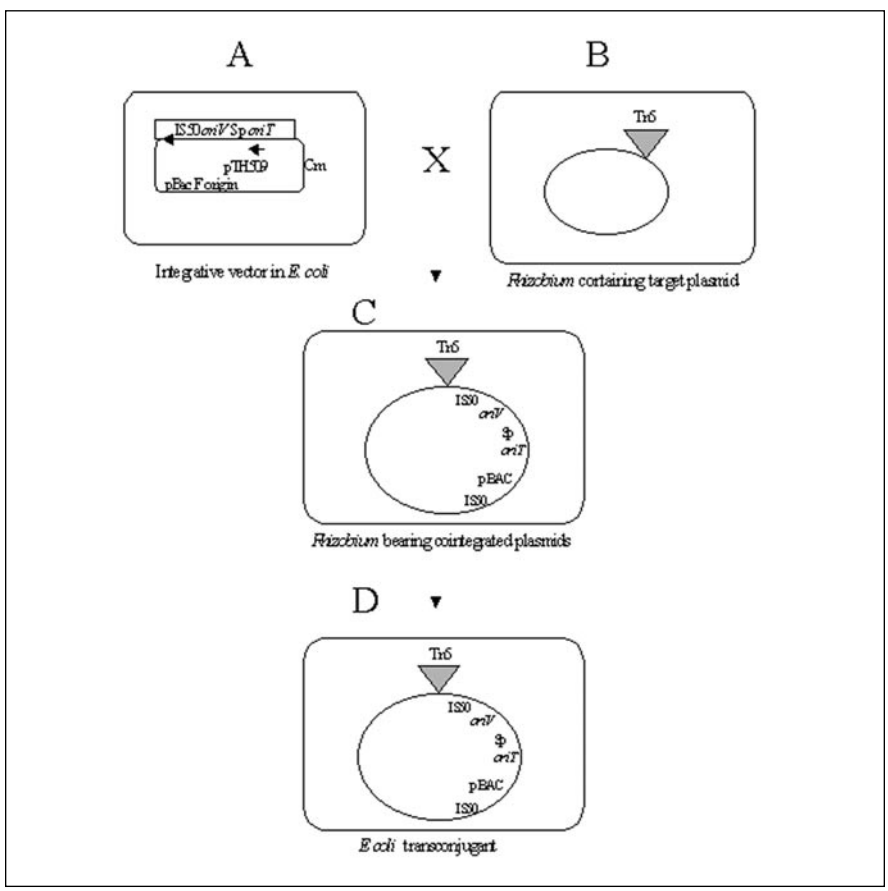

Figure 1. Diagram of the in vivo cloning system. *Rhizobium* containing a Tn5 in the target plasmid was mated with *E. coli* harboring the integrative vector. *Rhizobium* transconjugants were used as donors for the transfer of the resultant cointegrated molecule in *E. coli* (see text for details). (A) Schematic representation of the integrative vector pTH509, which contains 296 bp of the IS50 element of Tn5, the *oriT* of RK2, the F origin of replication, and the Sp^r and Cm^r cassettes. (B) Schematic representation of the target *Rhizobium* plasmid labeled with Tn5. (C) The vector pTH509 is integrated into the *Rhizobium* plasmid by single recombination with the IS50 of Tn5. (D) A third mating between *Rhizobium* donor and the recipient *E. coli* is necessary to isolate the *Rhizobium* replicon in *E. coli*.

pCFN42*b* (180 kb), and pCFN42*c* (270 kb) of *R. etli*, as well as pCFN299*a* (185 kb) of *R. tropici* and pGR4*a* (150 kb) of *S. meliloti* (Figure 2A, lanes 4, 3, 2, 6, and 10, respectively). These replicons were stably maintained in *E. coli* (after 400 generations without antibiotic) and were easily purified by alkaline lysis (21).

To ascertain whether the replicons cloned in *E. coli* originated from *Rhizobium*, pCFN42*a* of *R. etli* rescue from *E. coli* was used as a probe against the *R. etli* CFN42 wild-type strain, *R. etli* CFN42 cured of pCFN42*a*, *Agrobacterium tumefaciens* GMI9023 carrying pCFN42*a*, and *A. tumefaciens* plasmidless strain GMI9023 (Figure 2B). The results showed that the *R. etli* CFN42 wild-type and *A. tumefaciens* containing pCFN42*a* presented the same pattern (Figure 2B, lanes 1 and 4, respectively). *R. etli* devoid of pCFN42*a* presented a few weakly hybridizing bands that may result from reiterated sequences elsewhere in the genome (Figure 2B, lane 2). In contrast, no hybridization signals were detected in the *A. tumefaciens* plasmid-less strain (Figure 2B, lane 3). This experiment confirms that *R. etli* pCFN42*a* was successfully isolated from *E. coli*. Furthermore, the advance in the sequencing project of this replicon revealed that most of the information of pCFN42*a* is present in this BAC clone and that the DNA did not suffer major rearrangements during the cloning process (Gonzalez and Davila, unpublished results).

The system is moderately efficient for cloning plasmids of medium size (150–270 kb), since, from 213 clones tested in 18 experiments, 70% were positives. The remaining 30% negative clones are found to contain both pTH-509 and pRK2013 plasmids. pTH509 resulted by excision from the *Rhizobium* replicon through homologous recombination between directed repeats generated during the cointegration event and then was transferred together with pRK2013 in the *E. coli* recipient strain.

When we tried to clone the *R. tropici* CFN299 symbiotic plasmid (pSym) of approximately 600 kb (8), only fragments, in the range of 200–500 kb, were obtained (Figure 2A, lanes 7–9, 11, and 12). The regions obtained were overlapping and covered most of the pSym of *R. tropici* CFN299, as tested by PCR and sequence analyses of several genetic markers. For instance, a region containing metabolic genes such as succinate-semialdehyde dehydrogenase, citrate synthase (16), isocitrate lyase, and one insertion sequence (IS) element similar to *A. tumefaciens* IS3 (17) were identified (data not shown). The presence of these genes on the pSym was also confirmed by hybridization experiments (data not shown). Moreover, to determine the presence of

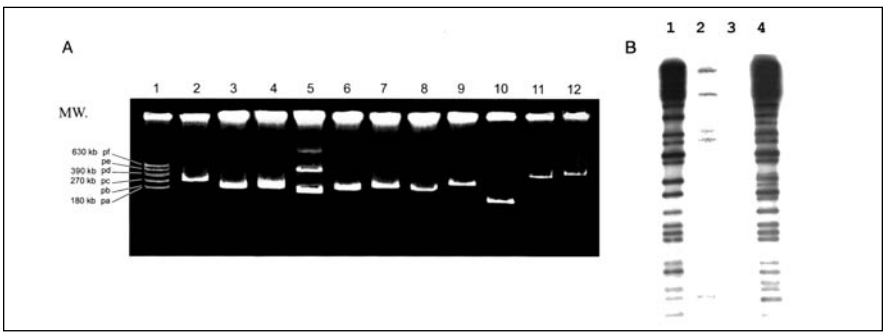

Figure 2. *Rhizobium* replicons isolated with the three-mating system. (A) Ethidium bromide-stained Eckhardt gel showing *Rhizobium* replicons cloned in *E. coli*. Lanes 2, 3, and 4 are plasmid pCFN42*c*, pCFN42*b*, and pCFN42*a* of *R. etli*, cloned from CFNX191, CFN037, and CFNX187, respectively. Lane 6, pCFN299*a* of *R. tropici* obtained from CFNE299-19. Lanes 7–9, 11, and 12 are different regions of the pSym of *R. tropici* isolated from CFNE130, CFNE87, CFNE68, and CFNE84, respectively. Lane 10, plasmid pRmeGR4*a* obtained from *S. meliloti* GRM6LR. Lane 1, *R. etli* CFN42, pa to pf are indigenous plasmids of *R. etli*, and lane 5, *R. tropici* CFN299, were used as size standards. MW, molecular weight. (B) Autoradiogram of Southern blot of *Bam*HI-digested genomic DNAs, hybridized with the rescue pCFN42*a* of *R. etli* purified from *E. coli*. Lane 1, *R. etli* CFN42; lane 2, *R. etli* cured of pCFN42*a* (CFNX2001); lane 3, *A. tumefaciens* GMI9023 plasmid-less strain; and lane 4, *A. tumefaciens* GMI9023 containing pCFN42*a* of *R. etli*.

nod and *fix* genes in the isolated CFN299 pSym fragments, complementation experiments were performed. For this purpose, each of the different fragments was transferred to the CFNE299-10 strain that has a deletion in the Sym plasmid and is unable to form nodules (13). With this approach, we identified a fragment of 200 kb on CFNE299-10 that conferred the ability to form nodules and to fix nitrogen in *Phaseolus vulgaris*. This result suggests that the corresponding region contains genes involved in nodulation and nitrogen fixation. Another isolated fragment appeared to contain the origin of replication of the *R. tropici* CFN299 pSym. This suggestion is based on the fact that this locus is able to autonomously replicate in *A. tumefaciens*. One reason why we were unable to clone the whole replicon may be that, in *Rhizobium* cells, deletions are generated by homologous recombination between repeated sequences that are present on the pSym. In previous work (15), *R. tropici* pSym was shown to contain IS elements that participate in the generation of rearrangements such as amplifications and deletions. Another possible reason for obtaining only fragments of the pSym would be the inability of the F origin to sustain large regions as the entire pSym. However, we were able to obtain clones containing large fragments (500 kb) that carry most of the information of this replicon.

The cloning procedure presented here is very accessible because few elements are required: the target transposon or cassette in the molecule to be isolated, the integrative vector pTH509 that contains 300 bp of the IS50, an *oriV*, *oriT* of RK2, as well as two antibiotic-resistant cassettes Ω spectinomycin and chloramphenicol. Potentially, all these genetic elements can be used to recombine in the target plasmid. We chose to integrate pTH509 in the Ω spectinomycin cassette contained in plasmid pRmeGR4a, as well as in the *mob* site and IS50 element of the transposon Tn5 *mob*. The integration of our vector in three different sites and the success of the cloning showed the versatility of our system. In principle, the cloning of plasmids with this method can be applied to a broad range of bacterial species and would be useful to clone gene clusters for subsequent use in the generation of organisms expressing new genetic features.

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We thank J.A. Ramírez-Trujillo, M.A. Gaitan, J.A. Gama, V. González, M.A. Rogel-Hernández, M. Rodriguez, J. Caballero, and M. Dunn for technical help and discussion, as well as Susana Brom who kindly supplied strains. Address correspondence to Ismael Hernández-Lucas, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos, México. e-mail: ismael@cifn.unam.mx

Received 6 May 2002; accepted 16 July 2002.

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Yeast tRNA as Carrier in the Isolation of Microscale RNA for Global Amplification and Expression Profiling

BioTechniques 33:788-796 (October 2002)

ABSTRACT

The characterization of global gene expression patterns of microscale samples is important in many areas of biological and clinical research. The choice of carrier is critical for the efficient isolation and successful amplification of RNA at the nanogram level. Here we show that recovery of nanograms of RNA is significantly higher when carrier linear polyacrylamide is supplemented with carrier tRNA. Reverse transcription and in vitro transcription reactions remain efficient and specific in the presence of carrier tRNA. Finally, comparison of GeneChip™ array hybridization patterns demonstrates that the presence of carrier tRNA does not cause detectable distortion in global amplification. Taken together, tRNA is a superior carrier for the isolation and global amplification of microscale RNA.

INTRODUCTION

Genome-wide expression profiling using cDNA microarrays or oligonucleotide microarrays (GeneChips™; Affymetrix, Santa Clara, CA, USA) has become an important tool in biomedical studies (13,18,23). Expression data have been obtained from a wide variety of samples including bacteria, fungi, mammalian cells, and tissues (8,19–22,24). The application of array technology generally requires the isolation of RNA from millions of cells containing micrograms to milligrams of total RNA. This is usually achievable only in samples that can be easily propagated in vitro, such as yeast and bacteria. In higher organisms, expression analysis is often crippled by the fact that samples in need of analysis come in only hundreds of cells.