Research Note

Stable RK2-Derived Cloning Vectors for the Analysis of Gene Expression and Gene Function in Gram-Negative Bacteria

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The construction of several stable RK2-derived cloning vectors for the analysis of gene expression and function in gram-negative bacteria is reported. Plasmid stability is conferred by the RK2 *par* locus or by insertion of the *spsAB* or *spsCD* symbiotic plasmid stability loci from pNGR234a of *Rhizobium* sp. NGR234. The vectors carry multiple cloning sites with protection against read-through transcriptional activity of vector sequences. Vector derivatives with the constitutive *nptII* promoter or a promoterless *gusA* gene are suitable for the study of gene function or regulation in bacteria.

Additional keywords: *Azospirillum brasilense* Sp245, post-segregational killing, pTR102, *Rhizobium etli* CNPAF512, y4dLM, y4mFE.

RK2-derivatives (IncPα) such as pVK101, pLAFR3, and pRK415 are widely used as cloning vectors in the study of gram-negative, plant-associated bacteria (Keen et al. 1988; Knauf and Nester 1982; Staskawicz et al. 1987). These plasmids, however, are not always maintained stably in bacteria in the absence of antibiotic selection. This is illustrated by the plasmid loss in *Rhizobium* spp. bacteroids recovered from legume nodules, making the analysis of gene expression during symbiosis troublesome. Weinstein et al. (1992) countered this problem by cloning the RK2 broad-host-range plasmid stability locus (*par*) in a mini-RK2 plasmid. The resulting plasmid pTR102 was stably maintained in *Sinorhizobium meliloti* bacteroids. The same *par* locus also confers plasmid stabilization in *Escherichia coli*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Azotobacter vinelandii* (Roberts and Helinski 1992), and *Rhizobium etli* CE3 (Taté et al. 1999). Plasmids use different mechanisms for their stable maintenance in bacteria such as active partitioning, multimer resolution, and postsegregational killing (Helinski et al. 1996). The RK2 *par* locus consists of two operons. The *parCBA* operon stabilizes plasmids in *E. coli* and several other gram-negative bacteria and encodes ParA, a resolvase; ParB, a nuclease; and ParC, a protein of unknown function (Easter et al. 1998; John-

son et al. 1999). The *parDE* operon encodes a postsegregational killing system, consisting of the ParD antitoxin and the ParE toxin (Johnson et al. 1996; Roberts et al. 1994). Many bacteria use postsegregational killing systems to retain their plasmids (Gerdes 2000; Rawlings 1999).

Major drawbacks of these RK2-derivatives are the relative absence of suitable cloning sites and the lack of protection against read-through transcriptional activity of vector sequences as experienced, for instance, in pLAFR3. In addition, pTR102 is not maintained stably in all species tested (see below). To overcome these problems, we modified pTR102 (Weinstein et al. 1992) (Fig. 1). Before introducing a multiple cloning site (MCS), several restriction sites were removed from the vector. DNA preparation and recombinant DNA techniques were performed according to standard procedures (Sambrook et al. 1989). *R. etli* and *S. meliloti* were grown routinely at 30°C in TY supplemented with 7 mM CaCl₂ and CM79 media (Michiels et al. 1998b), *Azospirillum brasilense* at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgCl₂, and *E. coli* at 37°C in LB medium. First, the *EcoRI* site in pTR102 was removed by *EcoRI* digestion, blunting with Klenow DNA polymerase in the presence of the four deoxynucleoside triphosphates and subsequent ligation, yielding pTR102-ΔE. Next, the 0.2-kb *KpnI*-*PstI* fragment was removed by *KpnI*-*PstI* digestion, blunting with T4 DNA polymerase and subsequent ligation, yielding pTR102-ΔEKSP (Fig. 1A). To introduce the MCS, primers Rhi-42 (5'-AGCTAGCCCGCCTAATGAGCGGGCT TTTTTTGGCGCGAAGCTTTCTAGAGGATCCGGCGCG CCGCGGCCGCGTTAACGGCCGGCCGTTTAAACCTGC AGGAATTCGAGCTCGGTACCAACAAAAAAGCCCCG CTCATTAGGCGGGCT-3') and Rhi-43 (5'-GATGAGCCCCG CTAATGAGCGGGCTTTTTTTTGTGGTACCGAGCTC GAATTCCTGCAAGTTTAAACGGCCGGCCGTTAACGC GGCCGCGGCGCGCCGATCCTCTAGAAAGCTTCGCG CCAAAAAAAGCCCCGCTCATTAGGCGGGCT-3') were annealed at room temperature, and the product was cloned in *HindIII*-*BamHI* digested pTR102-ΔEKSP, resulting in pFAJ1700 (Fig. 1B). The MCS of pFAJ1700 contains the following restriction sites: *HindIII*, *XbaI*, *BamHI*, *AscI*, *NotI*, *HpaI*, *FseI*, *PmeI*, *PstI*, *EcoRI*, *SacI*, and *KpnI* (Fig. 2A). The MCS is bordered by two *trpA* transcriptional terminators orientated on both sides toward the MCS. The use of these ter-

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minators in plasmids for gram-negative bacteria was reported previously by Wilson et al. (1995). pFAJ1700 was introduced in several bacterial species, as described by Michiels et al. (1998a), and the stability of the plasmid was assessed in the absence of antibiotic selection. Stability tests were carried out (Fig. 3). pFAJ1700 is retained 100% after 72 generations in *A. brasilense* Sp245, *S. meliloti* Rm1021, and *R. etli* CE3 (data not shown), but not in *R. etli* CNPAF512 (Fig. 3). These results confirm previous data obtained for *R. etli* CE3 (Taté et al. 1999) and *S. meliloti* (Weinstein et al. 1992) and adds *A. brasilense* Sp245 to the list of bacteria that stably maintain plasmids carrying the RK2 *par* locus. Strain variation in plasmid stability within the same species, as observed here for

the *R. etli* strains CE3 and CNPAF512, was reported previously by Smith and Rawlings (1998) for the *pas* system of pTF-FC2 in different *E. coli* backgrounds. The reason for this variation is unknown, but it was noticed that in those strains where the *pas* system was most effective the PasB toxin was the most toxic. Furthermore, Roberts et al. (1994) observed that plasmids carrying the *parDE* operon lose their stability in the presence of the *parD* antitoxin gene in *trans* on a compatible plasmid. Thus, the presence of a *parD* like antitoxin gene on the chromosome of CNPAF512 or on a plasmid present in CNPAF512, but not in CE3 (Brom et al. 1992; F. A. Janssens Laboratory of Genetics, *unpublished*), could account for the instability of pFAJ1700 in *R. etli* CNPAF512.

Fig. 1. Schematic representation of plasmid constructions, as described in text. Plasmid size (base pairs) is indicated between parentheses. Multiple cloning sites of plasmids are shown in detail in Figure 2. All plasmids shown encode resistance against tetracycline and ampicillin. *bla* encodes beta-lactamase (ampicillin resistance); *tetA* and *tetR* encode tetracycline resistance; *trfA* encodes replication initiation protein; and *gusA* encodes beta-glucuronidase. *oriV*: origin of vegetative replication (IncPα); *oriT*: origin of transfer; *trpA*: transcriptional terminator; *PnpIII*: *nptII* promoter.

In order to obtain plasmid stability in *R. etli* CNPAF512, two proposed postsegregational killing systems, y4dLM and y4mFE, from the symbiotic plasmid pNGR234a of *Rhizobium* sp. NGR234 (Falla and Chopra 1999; Freiberg et al. 1997) were envisaged. The loci were amplified by polymerase chain reaction (PCR) from total DNA of NGR234 with the Pwo polymerase, as described (Michiels et al. 1998b). The y4mFE genes were produced with primers BD-50 (5'-CTG AGGATCCGACGTGGAAGACAGCGGCTAAATCG-3'; the *Bam*HI restriction site is underlined) and BD-51 (5'-TTCAGCATGCCCTCCCCGACGATC-3'; the *Sph*I restriction site is underlined) and the y4dLM genes with primers BD-52 (5'-TTCAGCATGCGGTCTGCGCAGGTTGATCTG C-3'; the *Sph*I restriction site is underlined) and BD-53 (5'-CTGAGGATCCCGAGCCGCAAGGCACTACTTCG-3'; the *Bam*HI restriction site is underlined). The PCR products obtained, 2.0 kb (y4dLM) and 1.9 kb (y4mFE), were adenylated with Taq polymerase and dATP and subsequently cloned in pCRII-TOPO with the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands), resulting in pFAJ1704 and pFAJ1705, respectively. The correct DNA sequence of the PCR products was controlled by DNA sequencing on an AL-Fexpress sequencer (Pharmacia, Uppsala, Sweden) with fluorescein-labeled oligonucleotide primers. Finally, the 2.0-kb y4dLM and 1.9-kb y4mFE *Bam*HI-*Sph*I fragments from the respective pCRII-TOPO vectors were cloned into the corresponding sites of pFAJ1700, yielding pFAJ1706 and pFAJ1707, respectively (Fig. 1C). Stability tests were carried out in *R. etli* CNPAF512 (Fig. 3). y4dLM and y4mFE conferred 100% stability to the plasmids after 72 generations in free-living growth conditions and 3-week-old bacteroids, whereas pTR102 and pFAJ1700 (both containing the *par* locus from RK2) and pLAFR3 (lacking any stabilization locus) are not maintained stably. In view of these results and considering the homology of Y4dM and Y4mE to the HipA toxin (Falla and Chopra 1999), we propose the following acronym for these loci: *sps* (symbiotic plasmid stability locus), namely

spsAB (y4dLM) and *spsCD* (y4mFE) (Fig. 1). So far there has been no clear evidence on how rhizobia retain their symbiotic plasmids without any obvious selection system in the absence of the plant. *Rhizobium* sp. NGR234 can be cured of pNGR234a yielding strain ANU256, which is viable, but not capable of symbiosis (Morrison et al. 1984). *spsAB* and *spsCD* are good candidates for postsegregational killer loci that ensure stable maintenance of pNGR234a. pFAJ1702 was derived from pFAJ1706 by cloning the annealing product of primers BD-59 (5'-AGCTTTCTAGAGGCGCGCCGTTAAC GGCCGGCCGTTTAAACGGATCCGGTACCAACAAAAA AAAGCCCGCTCATTAGGCGGGCT-3') and BD-60 (5'-GATCAGCCCGCCTAATGAGCGGGCTTTTTTTTGTGGTACCGGATCCGTTTAAACGGCCGGCCGTTAACGGCGCG CCTCTAGAA-3') into *Hind*III-*Bam*HI digested pFAJ1706 (Fig. 1D). The MCS of pFAJ1702 contains the following restriction sites, *Hind*III, *Xba*I, *Hpa*I, *Fse*I, *Bam*HI, and *Kpn*I, and is bordered, as observed in pFAJ1700, by *trpA* transcriptional terminators (Fig. 2B).

gusA-fusion derivatives of pFAJ1700 and pFAJ1702 were constructed. A *gusA* gene preceded by a ribosome binding site was amplified by PCR from pRAJ260 (Metcalf and Wanner 1993) with the Pwo DNA polymerase and primers Rhi-44 (5'-ATGCAAGCTTGTGATTGTTTGCCTCC-3'; the *Hind*III restriction site is underlined) and Rhi-45 (5'-ATGC TCTAGATGACTAGCTAAGGAGGAGTCCCTTATGTTAC GTCCTG-3'; the *Xba*I restriction site is underlined, the ribosome binding site is in bold, and the start codon of *gusA* is italicized). The 1.8-kb PCR product was digested with *Hind*III and *Xba*I, and the fragment was cloned into *Hind*III-*Xba*I-digested pFAJ1700 and pFAJ1702, yielding pFAJ1701 and pFAJ1703, respectively (Fig. 1E). To test the functionality of these vectors, the promoter of the *R. etli* CNPAF512 y4vC homolog was amplified by PCR with Pwo polymerase and primers Rhi-108 (5'-TCGAGGCGCGCCGGCGGCATTCTC TGTGAGC-3'; the *Asc*I restriction site is underlined) and Rhi-107 (5'-ATGAGGATCCCTCGTCCACG-3'; the *Bam*HI

Fig. 2. Multiple cloning sites (MCS) of **A**, pFAJ1700, pFAJ1701, and pFAJ1708 and **B**, pFAJ1702, pFAJ1703, and pFAJ1709. Arrows indicate *trpA* transcriptional terminators. *oriT* borders the *Hind*III site, stabilization loci border the *Kpn*I site in both MCS. *gusA* and *PnptII* are cloned as *Hind*III-*Xba*I fragments in the MCS.

restriction site is underlined) and subsequently cloned *AscI*–*Bam*HI pFAJ1703. The resulting *y4vC*–*gusA* fusion was conjugated to wild-type *R. etli* CNPAF512 and its *nifA* mutant background, Rp1000 (Michiels et al. 1994). Expression tests were carried out basically as described by Michiels et al. (1998a). The microaerobical expression of the *R. etli* *y4vC* homolog in wild-type background amounted to approximately 1,200 Miller units, whereas the aerobical expression in CNPAF512 and Rp1000 as well as microaerobical expression in Rp1000 remained at a background level of approximately 20 Miller units.

pFAJ1700 and pFAJ1702 derivatives, allowing the overexpression of target genes, were constructed with the promoter of the *nptII* gene from Tn5. The suitability of the promoter of the neomycin phosphotransferase gene for overexpression in rhizobial background was described previously by Labes et al. (1990) and Xi et al. (1999). The *nptII* promoter was amplified by PCR from pUC18-2 (Michiels et al. 1991) with Pwo DNA polymerase and primers Rhi-94 (5′-ATGCAAGCTTCACGCTGCCGCAAGCACTCAGG-3′; the *Hind*III restriction site is underlined) and Rhi-95 (5′-CTGATCTAGATCCTGTCTCTTGATCAGATCTTG-3′ the *Xba*I restriction site is underlined). The 0.35-kb PCR product was digested with *Hind*III and *Xba*I and cloned in *Hind*III–*Xba*I-digested pFAJ1700 and pFAJ1702, yielding pFAJ1708 and pFAJ1709, respectively (Fig. 1F). The functionality of these constructs was assessed by cloning of the *gusA* PCR product of Pwo polymerase and primers Rhi-61 (5′-ATGCGGATCCTGACTAGCTAAGGAGGAGTCCCTTATGTTACGTCCTG-3′; the *Bam*HI restriction site is underlined, the ribosome binding site is in bold, and the start codon of *gusA* is italicized) and Rhi-47 (5′-ATGCGGTA CCGTTGATTCAATTGTTTGCCTCC-3′; the *Kpn*I restriction site is underlined) as a *Bam*HI–*Kpn*I fragment into pFAJ1708 and pFAJ1709. The resulting *PrnptII*–*gusA* fusions showed an expression level of approximately 2,000 Miller units in an *E. coli* background.

Fig. 3. Stability of different RK2-derived plasmids in *Rhizobium etli* CNPAF512. Strains were grown overnight in liquid TY with 1 µg of tetracycline per ml at 30°C. Subcultures were taken daily, diluted 1,000-fold, and grown in liquid TY for 10 days in the absence of tetracycline. After 0, 32, and 72 generations, samples were taken and dilutions were plated out on solid CM79 medium. After 2 days incubation at 30°C, 100 colonies per strain were picked up and replicas were tested on solid CM79 medium, with or without tetracycline. Plasmid stability (percent of plasmid-containing cells) was determined as the fraction of tetracycline-resistant colonies. Bacteroids were isolated from *Phaseolus vulgaris* cv. Limburgse after 3 weeks, as described by Michiels et al. (1998b), and plated out on solid CM79. Replica plating and determination of plasmid stability were carried out as described above. Bacteroid stability of pLAFR3 was not determined. Values are the means of at least two independent experiments; error bars denote standard deviations.

In conclusion, we described the construction of several stable RK2-derived cloning vectors that can be used for the study of genes in a wide background of gram-negative bacteria. The plasmids carry the RK2 *par* stability locus or the *spvAB* or *spvCD* postsegregational killer loci from pNGR234a of *Rhizobium* sp. NGR234. The plasmids have MCS, which are protected against read-through transcriptional activity of vector sequences by the *trpA* transcriptional terminator. *gusA*-fusion derivatives were constructed for the study of bacterial promoters. Also derivatives carrying the *nptII* promoter were made for the overexpression of genes.

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