Optimizing Gateway™ technology (Invitrogen) to construct *Rhizobium leguminosarum* deletion mutants

Lanza M*, Alborno M, Rey L, Imperial J

*Centro de Biotecnología y Genómica de Plantas, UPM-INIA and Departamento de Biotecnología, Universidad Politécnica de Madrid, Campus de Montegancedo, Carretera M-40, km 38, 28223 Pozuelo de Alarcón, Madrid, Spain. *monica.lanza@upm.es*

Summary

The study of the role of different genes in *Rhizobium leguminosarum* requires the generation of mutants by homologous recombination. In this communication we describe a novel approach to obtain deletion mutants of genes in *Rhizobium* using Gateway™ Cloning technology (Invitrogen) and a new vector (pK18-attR), both conjugative and *Rhizobium* specific, that carries the recombination tails of Gateway system. This tool is a new alternative to the classic approach based on cloning using restriction enzymes. The first step consists of designing directed oligonucleotides with specific tails for isolating recombination fragments and a resistance marker cassette to an antibiotic by PCR. The three inserts are cloned by homologous recombination in three specific vectors, in a single step. The last step consists of multisite-directed recombination of the three donor vectors to the pK18-attR destination vector. After recombination, this vector loses the *ccdB* gene, whose expression results in synthesis of a DNA gyrase that is lethal to carrier cells and thus guarantees the effectiveness in obtaining clones that carry the homologous construction to the subsequent recombination in *Rhizobium*.

Introduction

Gateway™ cloning technology uses the specific recombination of λ phage to introduce directed inserts in *E. coli* expression vectors. This strategy provides a number of advantages over traditional technology based on the use of restriction enzymes and allows incorporation of an insert for a set of expression vectors in one step. The incorporation of the insert also means the extrusion of lethal gene *cddB* after recombination. The product of the *cddB* gene is a DNA gyrase lethal to *E.coli* cells where it is expressed. This system guarantees the effectiveness of the cloning, because colonies only survive if they lose this lethal marker, and this only happens when homologous recombination has occurred and the *ccdB* gene has been replaced by the corresponding insert.

In recent years Gateway cloning technology has evolved, and new specific expression vectors have been developed for a wide variety of prokaryotic (especially *E. coli*) and eukaryotic organisms, such as yeasts and plants. Recently, the Invitrogen company has started commercializing the "multisite Gateway cloning technology" as an improvement over the previous Gateway technology, because this new system allows cloning two, three, or four inserts in a single step. This strategy is very useful to generate gene deletion mutants in organisms, where they are produced by homologous recombination from the adjacent areas of the gene to be deleted. In this study we used this method to generate deletion mutants in *Rhizobium.*

Materials and Methods

The pK18-attR expression vector was generated in this work. To construct it, the pK18mobsacB (Schafer *et al.,* 1996) conjugative vector was digested with restriction enzyme *Hind*III and the Gateway cassette, carrying the recombination-selection *ccdB* gene and a chloramphenicol resistance gene, introduced in this site. This cassette has been previously amplified by PCR and digested with *Hind*III (Figure). Vector pK18-attR can be used to generate deletion mutants in *Rhizobium leguminosarum* using the Invitrogen multisite Gateway cloning technology.

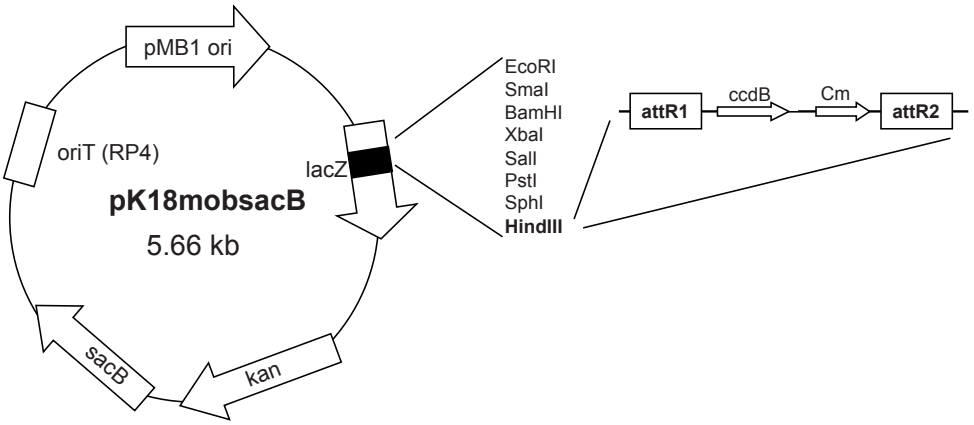

Figure. PK18-attR vector map.

Deletion mutants were generated in *Rhizobium* by conjugatively introducing the plasmid pK18-attR construct that carries the deletion construction for homologous recombination. This construct consisted of two adjacent recombination fragments of the gene to be mutated and a spectinomycin antibiotic resistance gene between those fragments. Specifically, this construction was generated as follows: First, specific primers were designed to amplify, by PCR, the three directed fragments for recombination by adding queues for the Invitrogen multiple system: tails P1 and P4 for insert 1, tails P4r and P3r for the antibiotic resistance gene, and tails P3 and P2 for the third fragment. They were all specifically recombined by BP reaction with vectors pDONR221 P1-P4, pDONR221 P3r-P4R and pDONR221 P3-P2, respectively. In this way, three independent clones, with compatible specific tails, were obtained that recombine with each other through a second recombination reaction, called LR, with the pK18-attR *Rhizobium*-specific destination vector. Both donor and target vectors endow carrier cells with resistance to the antibiotic kanamycin. Because of it, donor vectors were previously digested by the restriction enzyme *Nsi*I that breaks the of kanamycin resistance gene cassette. The subsequent selection of clones, after the LR reaction, was done with spectinomycin. Additionally, for the LR step of some vectors the pDEST22 destination vector (Invitrogen), that confers resistance to the antibiotic ampicillin but it is not conjugative to *Rhizobium*; was used. For that reason, an additional LR reaction was carried out using the pK18-attR vector as target and selecting the resulting clones with the antibiotic kanamycin.

Results and Discussion

In this paper we demonstrate an alternative strategy to generate deletion mutants of genes in *Rhizobium leguminosarum* taking advantage of Gateway technology (Invitrogen). For this purpose, a specific conjugative vector for *Rhizobium* has been generated (as described in Materials and Methods) to serve as a molecular tool for introduction of the deletion into the bacteria. The resulting mutants were confirmed by PCR and Southern blot in order to validate the method.

This system of mutant generation allows the quick mutation of several genes from an operon, because clones generated after the BP reaction are interchangeable so one of the genes can be kept and another one varied depending on whether one, two or more genes from the same operon are to be deleted. On the other hand, resistance cassettes are interchangeable so that if necessary, they could be replaced by another marker in a single LR reaction. In this way, a collection of specific directed clones is generated and these are placed in a concrete position and are interchangeable with clones of the same position. Furthermore, the sequences of the recombination tails which are generated after LR reaction are compatible with all the vectors that carry the same Gateway tails.

References

Invitrogen (2006) MultiSite Gateway® Pro Using Gateway® Technology to simultaneously clone multiple DNA fragments.

Schafer A, *et al.* (1994) *Gene* 145: 69–73.