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# A Region of the Broad-Host-Range Plasmid RK2 Causes Stable In Planta Inheritance of Plasmids in *Rhizobium meliloti* Cells Isolated from Alfalfa Root Nodules

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**We demonstrate for the first time that the broad-host-range stabilization loci from plasmid RK2 cause total retention of plasmids in cells of *Rhizobium meliloti* during symbiosis with alfalfa. Two derivatives of plasmid RK2, pRK290 and a 7.3-kb mini-RK2 plasmid, were stabilized in *R. meliloti* cells isolated from root nodules by the insertion of a 3.2-kb DNA fragment or a smaller 0.8-kb DNA fragment derived from the RK2 stabilization region.**

*Rhizobium meliloti* can establish a unique symbiotic relationship with the legume alfalfa (*Medicago sativa*) in which a plant organ, the root nodule, is developed for the maintenance of the nitrogen-fixing bacteria. Because of the agricultural importance of this symbiosis and the fundamental importance of plant-microbe interactions, *R. meliloti*, as well as other members of the family *Rhizobiaciae*, has been studied extensively with respect to genetic and physiological properties (reviewed in reference 11).

There are a variety of methods for introducing and establishing heritable factors in *R. meliloti* and related bacteria. The most widely employed plasmid vectors for this purpose are derived from broad-host-range plasmids. For example, vectors such as pRK290 (4) and pLAFR1 (7), derivatives of plasmid RK2, have been used extensively to deliver genes and complement symbiotically defective *Rhizobium* mutants in planta. However, in the case of these two vectors as well as others, a significant proportion of cells recovered from nodules generally have lost the plasmids along with their inserted genes because of the instability of the plasmid and the inability to apply antibiotic selection in planta.

Recently, even smaller derivatives of RK2, some less than 5 kb in length (14), have become available for gene cloning in the *Rhizobiaciae*. While these smaller derivatives are now more readily manipulable for gene insertions, they also tend to be even less stable than the larger RK2 derivatives. The utility of plasmid vectors for introducing genes into *R. meliloti* would be greatly enhanced if the vectors were stably maintained in the absence of antibiotic selection. Using a stabilization region of plasmid RK2 (8, 14), we constructed and investigated the properties of mini-RK2 derivatives that are stably maintained vegetatively and in planta in *R. meliloti*.

Several genetic loci that ensure a high degree of fidelity in plasmid maintenance within a bacterial culture have been identified. These loci employ diverse mechanisms to achieve plasmid stability, including resolution of plasmid multimers to monomers, the selective killing of cells which lose all copies of a plasmid, or a partitioning mechanism, in which at least one copy of a plasmid is thought to be physically segregated to each daughter cell (1, 13). While several plasmid stabilization loci that function in *Escherichia coli* have been identified, their capacity to function in heterologous bacteria is limited, probably because of the narrow host range of the plasmids from which they are derived. For our purposes a locus that would function in a broad-host-range manner was necessary. Plasmid RK2 (RP4) has been shown to encode an efficient stabilization locus, from 32.6 to 35.8 kb. This region has been shown to encode at least five genes in two operons: *parCBA* and *parDE* (8, 14, 15). These operons are divergently transcribed and autoregulated by the ParA and ParD gene products, respectively (3, 6). Within this 3.2-kb region, recent evidence indicates that the segment from 34.7 to 35.5 kb (0.8 kb, encoding the *parDE* operon) is also capable of plasmid stabilization (15). While the mechanism by which these loci achieve plasmid stability is not known, both function independently of the replicon in which they are inserted. They both have been shown to stabilize a mini-RK2 replicon in *E. coli*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, and *Azotobacter vinelandii* (14, 15). To determine whether these stabilization loci would also function in *R. meliloti*, both the 3.2- and 0.8-kb loci were tested for their capacities to stabilize mini-RK2 replicons during vegetative and in planta growth of this bacterium.

Initially, the RK2 stabilization regions were inserted into the mini-RK2 derivative pTR100 to determine whether they promoted retention of a mini-RK2 plasmid in *R. meliloti*. Figure 1 shows the overall structure of plasmids pTR100, pTR101, and pTR102, which contain no stability locus, the 0.8-kb locus, and the 3.2-kb locus, respectively. The construction of these vectors is described in the legend to Fig. 1. These plasmids were conjugally transferred into *R. meliloti* 5591 by published procedures (4). Strain Rm5591, kindly provided by Gary Ditta, is a *fix*$^+$ derivative of wild-type strain Rm102F34 which carries chromosomal pUC9 sequences and a neomycin resistance gene in the *fixLJ* region (see legend to Fig. 2).

Stability assays were conducted as described previously (14) with free-living *R. meliloti* cells carrying pTR100, pTR101, and pTR102 grown in YMB medium (17), and the results are shown in Fig. 2. pTR100 is extremely unstable in cells grown without selection, with greater than 99% loss of the plasmid after 80 generations of growth, while the derivatives bearing the 0.8- and the 3.2-kb RK2 stability regions

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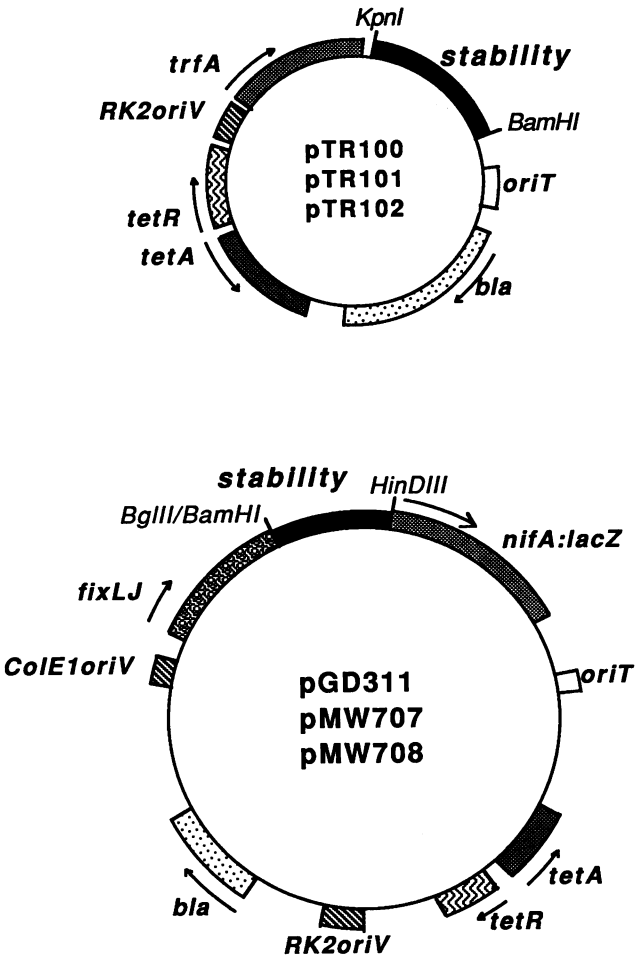


FIG. 1. Structures of relevant plasmids. pTR100 and pGD311 lack an RK2 stabilization region. pTR101 and pMW707 carry the 0.8-kb fragment, while both pTR102 and pMW708 carry the 3.2-kb segment. *trfA* refers to the RK2 replication initiation gene, and *oriV* refers to the vegetative origin of replication. *oriT* refers to the RK2 origin of transfer. Tetracycline and ampicillin resistances are indicated by *tetR*/*tetA* and *bla*, respectively. Diagrams are not drawn to scale. The estimated sizes of the plasmids in kilobase pairs are as follows: pTR100, 7.3; pTR101, 8.1; pTR102, 10.5; pGD311, 33.9; pMW707, 34.7, pMW708, 37.1. pTR100 was constructed by inserting the RK2 *tetA* and *tetR* genes from pAL4000 (9) into the *Bgl*II site of pRR10 (14), which is adjacent to *oriV*. The 0.8- and 3.2-kb RK2 stabilization loci were inserted as *Bam*HI-to-*Kpn*I fragments to generate pTR101 and pTR102, respectively. pMW707 and pMW708 were constructed from pGD311 (19). A *Bam*HI site was generated at the end of the 0.8- and 3.2-kb stability fragments by replacing the *Kpn*I site of pRR120 (15) and the *Eco*RI site of pRR71 (14) with *Bam*HI. The 0.8- and 3.2-kb regions were then isolated as *Hin*dIII-*Bam*HI fragments and inserted into pGD31, which is an intermediate construct used to create pGD311 (19). The resulting constructs, pRR144 and pMW70, respectively, were digested with *Hin*dIII and ligated with *Hin*dIII-cleaved pCHK57 (5). Cointegrates of the pGD31- and pCHK57-derived constructs were isolated as described previously (10) to generate pMW707, which carries the 0.8-kb fragment, and pMW708, which carries the 3.2-kb fragment.

are completely stable, with no loss observed over 100 generations.

*R. meliloti* cells carrying these plasmids were also inoculated onto alfalfa seedlings. Plants were grown for 4 weeks,

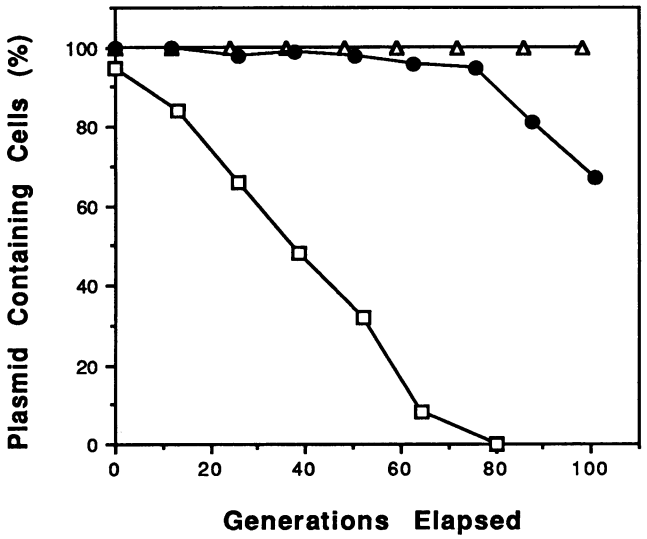


FIG. 2. Plasmid stability during vegetative growth of *R. meliloti* in the absence of selection. Open triangles, plasmids containing a stability region (including pTR101, pTR102, pMW707, and pMW708); solid circles, pGD311; open boxes, pTR100. The assays were conducted with strain Rm5591, kindly provided by Gary Ditta. This strain is a derivative of wild-type strain Rm102F34 and has pUC9 sequences, including the ampicillin resistance gene, located at the 5′ end of *fixL* and a neomycin resistance gene adjacent to the 3′ end of *fixJ*. This strain was generated by recombination, which was carried out by the marker exchange method of Ruvkin and Ausubel (16), using an intermediate broad-host-range plasmid vector (pGD55) having homologous *Rhizobium* DNA sequences flanking *fixLJ*.

after which nodules were harvested and washed sequentially with ethanol, 66% bleach, and six washes of sterile distilled water. The nodules were homogenized into crush buffer (50 mM Tris [pH 7.8], 250 mM mannitol) with a sterile glass homogenizer, and bacteria were plated onto YMB (17) with 50 μg of neomycin per ml and then grown at 30°C until individual colonies were visible. These were picked by hand onto both YMB with neomycin and YMB with neomycin and tetracycline (5 μg/ml). Plasmid stability was determined as the fraction of neomycin-resistant colonies that were also tetracycline resistant. The results shown in Table 1 indicate that pTR100 itself is extremely unstable in nodules, while the derivatives containing the 0.8- or 3.2-kb RK2 stability region are completely stable. This is the first demonstration that a

TABLE 1. Retention of stability-locus-containing plasmids in *R. meliloti* cells isolated from root nodules

| Plasmid | Stability locus | % Retained in nodules[a] |
|---|---|---|
| pTR100[b] | None | 17.7 |
| pTR101 | 0.8 kb | 100 |
| pTR102 | 3.2 kb | 100 |
| pGD311 | None | 77.7 |
| pMW707 | 0.8 kb | 100 |
| pMW708 | 3.2 kb | 100 |

[a] Results shown are the means of one experiment consisting of three replicates with 12 alfalfa plants each.

[b] pTR100, pTR101, and pTR102 were tested by using *R. meliloti* Rm5591. For pGD311, pMW707, and pMW708, strain Rm5591ΔLJ was used.

TABLE 2. Microaerobic induction of the *nifA* promoter carried on stability-locus-containing plasmids

| Plasmid[a] | Genotype | Induction[b] with: | |
|---|---|---|---|
| | | 21% $O_2$ | 1% $O_2$ |
| pGD311 | No stability locus, *fixLJ*$^+$ | 25 ± 5 | 680 ± 36 |
| pMW707 | 0.8-kb stability locus, *fixLJ*$^+$ | 21 ± 4 | 626 ± 69 |
| pMW708 | 3.2-kb stability locus, *fixLJ*$^+$ | 16 ± 6 | 671 ± 94 |

[a] The experiments were conducted with *R. meliloti* Rm5591ΔLJ, maintaining antibiotic selection for the plasmids during the course of bacterial growth.
[b] Results are in Miller units. Values shown are the means ± standard deviations of one experiment consisting of three replicates.

plasmid stability locus functions in bacteria that have grown and are maintained in root nodules.

To further test the utility of the stabilization region, its effect on maintenance of the commonly used plasmid vector pRK290 carrying symbiotically important genes was determined. The genes we chose were *fixLJ*, whose presence is essential to the establishment of a successful symbiosis. The oxygen-sensing *fixLJ* genes encode a prokaryotic two-component system which controls a symbiotic regulatory cascade. The proteins made from the *fixLJ* genes regulate many essential nitrogen fixation genes, including those for the *R. meliloti* nitrogenase, in response to oxygen availability (2, 18).

*fixLJ* sequences are expressed from the *fixL* promoter on the plasmids pGD311 (19), pMW707, and pMW708, all of which are derivatives of pRK290 and carry *nifA*::*lacZ* translational fusions and tetracycline resistance genes. pGD311 does not contain an RK2 stability locus, while pMW707 and pMW708 contain the 0.8- and 3.2-kb loci, respectively.

These constructs were introduced into *R. meliloti* 5591ΔLJ, which was also constructed by Gary Ditta and is identical to Rm5591 with the exception of a 1,323-bp internal deletion of the *fixLJ* genes on the symbiotic megaplasmid which renders this strain unable to fix nitrogen. The stabilities of pGD311, pMW707, and pMW708 were assayed in *R. meliloti* 5591ΔLJ during vegetative growth in the absence of selection as described for pTR100 and its derivatives. pGD311 is considerably more stable in the absence of selection than pTR100, and again both the 0.8- and 3.2-kb fragments carrying RK2 stability functions provided complete stability of this vector during vegetative growth (Fig. 2).

The stabilities of pMW707 and pMW708 in *R. meliloti* were also tested after the development of root nodules in the same manner as pTR100 and its derivatives. The results, shown in Table 1, demonstrate that pGD311 is more stable than pTR100 in *R. meliloti* obtained from nodules. Again, the RK2 stability regions increase the retention of this plasmid to 100%. This demonstrates that these DNA stabilization segments will function effectively with pRK290 derivatives in bacteria that have penetrated plant roots and multiplied during the formation of infection threads and mature nodules.

To test whether this stabilizing DNA segment disrupts or otherwise modifies the expression of adjacent genes, the P*nifA*:*lacZ* fusion carried on pGD311, pMW707, and pMW708 was used to assay microaerobic induction of the *nifA* promoter by FixLJ. *R. meliloti* 5591ΔLJ carrying these plasmids was subjected to microaerobic conditions, using stoppered tube assays (5), and the results are shown in Table 2. It can be seen that *nifA* expression from plasmids carrying the 0.8- or the 3.2-kb stability fragment is not altered. Additionally, the 3.2-kb stability fragment was placed upstream of the *fixJ* gene on a similar construct lacking *fixL*, and the plasmid was introduced into a strain carrying a Tn*5* insertion in *fixJ* (2). *nifA* expression from the construct containing the RK2 stability region was indistinguishable from that of an identical construct lacking the stability locus (data not shown). Therefore, the 3.2-kb stability locus can be placed upstream or downstream from other genes without affecting gene expression. Although the stability fragments have only been tested in one orientation, it is unlikely that they would alter gene expression in the other orientation, because results indicate that the promoters within the RK2 stability region are negatively autoregulated (3, 6), minimizing transcription originating from within this region.

Both the 0.8- and 3.2-kb stability regions will stabilize RK2-based plasmids in *R. meliloti* cells isolated from alfalfa root nodules. We would expect this to be the case with any plasmid capable of replicating in *R. meliloti*, because the RK2 stability regions are capable of stabilizing a replicon heterologously (8, 14, 15). Recently the 3.2-kb stabilization locus has been shown to stabilize a derivative of the cryptic *R. meliloti* plasmid pRmeGR4a in symbiotically grown cells isolated from alfalfa root nodules (12). Moreover, we expect this region to stabilize plasmids in a variety of rhizobia because of its activity in every gram-negative bacterium in which it has been tested. In addition to stabilizing plasmids in bacteria growing vegetatively and in planta, RK2 stability regions may also be useful in other situations where selection cannot be maintained, for example, when genetically altered bacteria are inoculated into the soil.

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