Research Note

Multicopy Vectors Carrying the *Klebsiella pneumoniae* *nifA* Gene Do Not Enhance the Nodulation Competitiveness of *Sinorhizobium meliloti* on Alfalfa

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It has been reported that *Sinorhizobium meliloti* strains harboring IncQ and IncP multicopy vectors containing constitutively expressed *Klebsiella pneumoniae* *nifA* exhibit an increase in nodulation competitiveness on alfalfa (J. Sanjuan and J. Olivares, Mol. Plant-Microbe Interact. 4:365-369, 1991). In our efforts to understand the mechanisms involved, in this work, we have found that the observed enhancement on nodulation competitiveness by IncQ derivatives carrying *K. pneumoniae* *nifA* was not dependent on the plasmid-borne *nifA* activity but on the sensitivity of nonresistant strains to the streptomycin carried over from growth cultures. Furthermore, it was also determined that the *nifA* of *K. pneumoniae* on an IncP vector does not have an effect on competitiveness.

Leguminous crops have been inoculated with *Rhizobium* and *Bradyrhizobium* spp. since the end of the last century with the aim to improve legume yields. However, the development of highly efficient rhizobial inoculants has been hampered by the inability of the released strains to compete for legume nodulation against the well-adapted indigenous population. This is known as “the competitiveness problem,” which has been extensively reviewed (Maier and Triplett 1996; Toro 1996). The competitive ability of rhizobial strains is affected by many environmental and genetic factors that complicate efforts to overcome this problem. Nevertheless, one strategy that could be pursued is to introduce genes that give the recipient bacteria an advantage over the indigenous population. Some genes of interest include those that produce and confer resistance to antibiotics such as trifolitoxin (Robledo et al. 1997), or those involved in improving nodulation efficiency, such as the *nfe* genes (Soto et al. 1994; Chun and Stacey 1994). Additionally, genes that give a metabolic advantage such as rhizopine synthesis and catabolism genes (*mos/moc*) (Murphy et al. 1995) or the proline dehydrogenase *putA* gene (Jiménez-Zurdo et al. 1995), could be considered. Finally, the regulatory gene *nifA* has been proposed to play a role in competitiveness in *Sinorhizobium meliloti* (Sanjuan and Olivares

1991). Sanjuan and Olivares (1991) reported that multicopy plasmids (pCK1 and pCK3) containing the *nifA* gene of *Klebsiella pneumoniae* enhance the competitive ability of *S. meliloti* strains. These authors hypothesized that this positive effect on *S. meliloti* nodulation competitiveness might be explained by the presence of *nifA*-regulated, *nfe*-analogous genes. More recently, Herrera-Cervera et al. (1997) reported that the reduced nodulation competitiveness shown by an *S. meliloti* GR4 RecA⁻ derivative strain could be enhanced by the presence of plasmid pCK3. Furthermore, it has been reported that pCK3 in *S. meliloti* has a positive effect on mycorrhiza formation (Tobar et al. 1996). The mechanism by which these multicopy vectors carrying *K. pneumoniae* *nifA* enhance *S. meliloti* competitiveness and mycorrhiza formation remains unknown. Therefore, the potential use (if any) of *K. pneumoniae* *nifA* gene as a genetic tool to enhance the competitiveness of *S. meliloti* still needs to be elucidated.

Here, in contradiction to published results (Sanjuan and Olivares 1991; Herrera-Cervera et al. 1997), we report that multicopy vectors carrying *K. pneumoniae* *nifA* do not enhance the nodulation competitiveness of *S. meliloti* on alfalfa.

Bacterial strains and plasmids used and constructed in this work are listed and described in Table 1. *Escherichia coli* and *S. meliloti* strains were routinely grown as described by Jiménez-Zurdo et al. (1995). Antibiotics were used as required at the following concentrations ($\mu\text{g ml}^{-1}$): tetracycline (10), ampicillin (200), kanamycin (50 for *E. coli* and 180 for *Sinorhizobium* strains), spectinomycin (100), streptomycin (50 for *E. coli* and 250 for *Sinorhizobium* strains), chloramphenicol (50), and gentamycin (30).

To test the effect of *K. pneumoniae* *nifA* on *S. meliloti* competitiveness, plasmid pDIL120, an analogous construction to pCK1, was made by cloning the wild-type *nifA* gene, preceded by a constitutive kanamycin resistance gene promoter, into the IncQ plasmid pMP77 (Fig. 1 and Table 1). As a control, a frameshift mutation was induced in the *nifA* gene to give pDIL119 (Fig. 1 and Table 1). The *nifA* activity produced by these constructions was determined with a plasmid carrying a P1 (*nifH* promoter)-*lacZ* fusion as a reporter in *E. coli* cells, as described by Better et al. (1985). The results indicated that the *nifA* activity exhibited by pDIL120 is similar to that of pCK1 while pDIL119 lacks this activity (data not shown).

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Competitive assays were performed with *S. meliloti* strains containing the different constructions. Alfalfa (*Medicago sativa* L.) plants were grown under axenic conditions in nitrogen-free nutrient solution as described by Olivares et al. (1980). For competition assays, 10-day-old plants (10 to 12 tubes) were inoculated with 10^5 bacteria ml^{-1} each. Prior to inoculation, rhizobia were grown to exponential phase (OD_{600} 0.4 to 0.6) in TY medium supplemented with antibiotics where appropriate for plasmid maintenance. To obtain the 1:1 ratio required in the coinoculation mixture, strains were diluted (1,000-fold) together from their respective cultures after both had reached the same optical density. To distinguish between strains occupying nodules, one of the strains of the coinoculation mixture was marked with the β -glucuronidase (GUS) gene. *S. meliloti* strains GR4 and GRM6 were marked with the construction pGUS3, while the 2011 derivative strain, M4, contains the GUS gene incorporated into its chromosome (Table 1). The plants were collected 14 to 15 days after inoculation and the roots briefly washed with water. Roots were incubated in darkness overnight at 37°C in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide; Apollo Scientific, Stockport, UK) in 50 mM sodium-phosphate buffer (pH 7.5) with sodium dodecyl sulfate 1% (wt/vol). Nodule occupancy was determined by counting blue and white nodules. As shown in Table 2, all the strains carrying IncQ derivative plasmids, including the original vectors for pCK1 and pDIL120, namely, RSF1010 and pMP77, respectively, as well as the strain carrying pDIL119 in which *nifA* had been inactivated, occupied 100% of the alfalfa root nodules. Similar results were previously reported (Sanjuan 1989; Sanjuan and Olivares 1991) with the plasmid pCK1 in different *S. meliloti* strains. However, when the bacteria were washed prior to inoculation, nodulation occupancy by both the *S. meliloti* strains carrying pDIL119 or pDIL120

decreased to about 50% (Table 2, washed samples). These results clearly indicate that the *nifA* gene on IncQ derivative plasmids is not involved in enhancing the competitiveness of *S. meliloti* strains.

In the search for a possible explanation, the cell viability of GRM6(pGUS3) and GRM6(RSF1010) was checked when coinoculated in hydroponic culture with or without alfalfa present. While GRM6 carrying RSF1010 maintains growth until a plateau is reached at about 10^7 cells ml^{-1} , the viability of GRM6 carrying pGUS3 decreased to 10^2 cells ml^{-1} in the first day after inoculation and became undetectable after 5 days. Although the presence of an alfalfa plant appeared to reduce the sensitivity of GRM6 carrying pGUS3, the initial 1:1 ratio still dropped by 1,000-fold after 5 days of inocula-

Fig. 1. Comparison of the *nifA* DNA region in pCK1, pDIL120, and pDIL119. In all three constructions, a *SalI* fragment containing the *Klebsiella pneumoniae nifA* and flanking fragments of *nifL* and *nifB* was cloned into the *XhoI* site of the kanamycin (Km) resistance gene. The indicated *KpnI* site within *nifA* was used for constructing the frameshift mutation in pDIL119 to give a truncated open reading frame ending 23 amino acids after the mutation site. Promoters are indicated by circles.

Table 1. Bacterial strains and plasmids

Strains / plasmids	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>recA endA1 gyrA96 thi-1 hsdR17 supE44 ΔlacU169(Φ80 <i>dlacZ</i>Δm15) deoR F⁻ λ</i>	Bethesda Research Laboratory
<i>Sinorhizobium meliloti</i>		
GR4	Wild-type, Nod ⁺ , Fix ⁺	This laboratory
GRM6	GR4 derivative cured of cryptic plasmid pRmeGR4b	Mercado-Blanco and Olivares 1993
2011	SU47 derivative strain, Nod ⁺ , Fix ⁺	J. Denarié
M4	2011 with <i>gus</i> gene integrated into a nonessential region between <i>alaS</i> and <i>recA</i> in the chromosome, Sm ^r	Selbitschka et al. 1995
Plasmids		
pIC20H	Cloning vector, Ap ^r	Marsh et al. 1984
pJB3KmD	Broad host range IncP cloning vector, 6052 bp, Ap ^r , Km ^r	J. M. Blatny
pMP77	Broad host range IncQ cloning vector, derivative of pKT214, <i>xyIE</i> , Cm ^r , Sm ^r	H. P. Spaink
pKT214	Broad host range IncQ cloning vector, derivative of RSF1010, Tc ^r , Cm ^r , Sm ^r	Bagdasarian et al. 1979
pRK290	Broad host range IncP cloning vector, Tc ^r	Ditta et al. 1980
RSF1010	Broad host range IncQ cloning vector, Sm ^r	Guerry et al. 1974
pGUS3	<i>nfeD</i> promoter-GUS fusion on pBI101 (Clontech), Km ^r	F. M. Garcia-Rodriguez
pCK1	RSF1010 derivative containing the <i>nifA</i> of <i>Klebsiella pneumoniae</i> , Sm ^r , Km ^r	Kennedy and Robson 1983
pCK3	pRK290 derivative containing the <i>nifA</i> of <i>K. pneumoniae</i> , Tc ^r	C. Kennedy
pWK23	Cloning vector containing <i>nifLA</i> and <i>nifBQ</i> of <i>K. pneumoniae</i> , Tc ^r	Arnold et al. 1988
pDIL15	Kanamycin cassette cloned as a <i>PstI</i> fragment from pJB3KmD into pIC20H, Ap ^r , Km ^r	This study
pDIL19	pDIL20 with disruption of the <i>nifA</i> gene by frameshift mutation created by cutting with <i>KpnI</i> and filling-in, Ap ^r	This study
pDIL20	<i>XhoI</i> fragment of pDIL15 containing most of the kanamycin resistant gene was replaced by the <i>SalI</i> fragment of pWK23 containing <i>nifA</i> , Ap ^r	This study
pDIL119	<i>HindIII</i> fragment containing <i>nifA</i> of pDIL19 cloned into pMP77, Cm ^r , Sm ^r	This study
pDIL120	<i>HindIII</i> fragment containing <i>nifA</i> of pDIL20 cloned into pMP77, Cm ^r , Sm ^r	This study

tion. On the other hand, when the bacterial cells were washed prior to coinoculation, the cell viability of both GRM6 carrying pGUS3 and GRM6 carrying RSF1010 was similarly unaffected (data not shown).

The antibiotic streptomycin is used to grow *S. meliloti* strains carrying IncQ derivative plasmids to ensure its maintenance. When the bacteria are coinoculated without washing prior to inoculation, the antibiotic streptomycin reaches a final concentration of 250 ng ml⁻¹ (1,000-fold dilution) in the hydroponic culture. We tested whether this amount of streptomycin affects the cell viability of GRM6(pGUS3) in the hydroponic culture. As shown in Figure 2, the number of viable cells of this strain decreases dramatically in the first day after inoculation although, thereafter, the surviving bacteria apparently begin to increase their number. This recovery of bacterial cell growth of the sensitive strain was not observed in the competition assays described above, which might be explained by the presence of a growing resistant competitor

strain and the consequent depletion of available carbon and nitrogen sources. Contrary to the results obtained with streptomycin, the 1,000-fold dilution of other antibiotics routinely used in our assays (tetracycline, ampicillin, kanamycin, spectinomycin, chloramphenicol, and gentamycin) showed no effect on cell viability (data not shown). Therefore, the nodule occupancy by *S. meliloti* strains carrying IncQ derivative plasmids appears to be a consequence of the sensitivity of the other strains in the coinoculation mixture to streptomycin carried over from the cultures of the *S. meliloti* strains bearing IncQ plasmid derivatives.

The effect of streptomycin carry-over on nodule occupancy should be eliminated either by washing cells prior to coinoculation or by coinoculation with both partners being resistant to this antibiotic. The latter is the case for M4, an *S. meliloti* 2011 derivative containing the GUS gene within the chromosome (Selbitschka et al. 1995), and 2011 carrying either pCK1 or RSF1010. The results presented in Table 3 with these strains confirm those obtained in Table 2 (washed samples). Once more, the strain containing the *K. pneumoniae* *nifA* (pCK1) not only failed to enhance competitiveness when compared with the strain containing RSF1010 or the control (M4 washed × 2011), but even reduced the number of nodules occupied by the carrying strain. Likewise, the comparison between washed and unwashed M4 against 2011 confirmed the effect of low concentrations of streptomycin in the results of the competitive assays.

Sanjuan and Olivares (1991) also reported that the IncP derivative plasmid pCK3 carrying *K. pneumoniae* *nifA* improved the competitive ability of *S. meliloti* strain 2011. This plasmid encodes for kanamycin resistance and therefore the effect on nodulation competitiveness cannot be attributed to the carry-over of antibiotic. We introduced plasmid pCK3 and the original vector pRK290 into strain M4 and performed competitive assays. The results shown at the bottom of Table 3 indicate that the presence of neither pCK3 nor pRK290 give significant differences in nodule occupation when compared with the wild-type strain. This implies that, as with IncQ vectors, the *nifA* of *K. pneumoniae* on an IncP vector does not have an effect on competitiveness.

From the data presented in this report we conclude that multicopy vectors carrying *K. pneumoniae* *nifA* do not enhance *S. meliloti* nodulation competitiveness on alfalfa.

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Table 2. Competitive assays with IncQ derivatives in *Sinorhizobium meliloti*

Inoculum	White nodules (%) ^a	Nodules (total no.)
GR4(pGUS3) × GR4(pCK1)	100	266
GRM6(pGUS3) × GRM6(pCK1)	100	271
GRM6(pGUS3) × GRM6(RSF1010)	100	288
GRM6(pGUS3) × GRM6(pMP77)	100	260
GRM6(pGUS3) × GRM6(pDIL119)	100	264
GRM6(pGUS3) × GRM6(pDIL120)	100	298
GRM6(pGUS3) × GRM6(pDIL119) ^b	55.3 ± 6.2	246
GRM6(pGUS3) × GRM6(pDIL120) ^b	42.9 ± 6.3	238

^a Nodules occupied by bacteria containing pGUS3 (single and double occupancy) stain blue. Error values are indicated at a 95% confidence interval

^b Washed prior to inoculation.

Fig. 2. Effect of diluted TY with or without streptomycin on viability of GRM6(pGUS3) cells in hydroponic medium (final concentration of streptomycin when present is 250 ng ml⁻¹). GRM6(pGUS3) coinoculated with only TY medium (open circles) or with TY medium containing streptomycin (black boxes). *Sinorhizobium meliloti* cell viability was analyzed during 5 days by taking 0.1-ml samples of inoculated media and plating corresponding dilutions on plates containing TY medium with kanamycin. Values represent means of three independent experiments. Error bars show corresponding standard errors. Where no bars are seen error is small.

Table 3. Competition assays with *Sinorhizobium meliloti* strain 2011

Inoculum	White nodules (%) ^a	Nodules (total no.)
M4 ^b × 2011	39.2 ± 4.1	554
M4 × 2011	1.3 ± 1.1	393
M4 × 2011(RSF1010)	26.7 ± 6.0	208
M4 × 2011(pCK1)	8.7 ± 3.4	263
M4(pRK290) ^b × 2011	47.5 ± 5.8	284
M4(pCK3) ^b × 2011	37.4 ± 5.7	273

^a Nodules occupied by bacteria containing the 2011 strain marked with *gus*, M4 (single and double occupancy), stain blue. Error values are indicated at a 95% confidence interval.

^b Washed prior to inoculation.

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