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Contribution of Cryptic Plasmids of *R. leguminosarum* bv. *trifolii* to its Phenotype

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ABSTRACT. *Plasmids in Rhizobium species are relatively large, many in number and difficult to cure. Except for the symbiotic plasmid, little is known about the functions of other cryptic plasmids. The primary objective of this investigation was to obtain plasmid cured derivatives of R. leguminosarum bv. trifolii by using a direct selection system and determine changes in the phenotypes of cured strains. Curing was achieved for one cryptic plasmid out of the four indigenous plasmids of the isolate. Two deletions were observed in the largest cryptic plasmid. Phenotypic effects observed after curing and deletions indicated that the smallest cryptic plasmid was involved in nitrogen fixation and the competitive ability of the isolate. One derivative obtained with a deletion on the largest plasmid showed a loss of competitiveness compared to the parental strain. This alteration of phenotypes suggests that these plasmids may encode genes that contribute significantly to the nitrogen fixing efficiency and saprophytic competence of Rhizobium in the soil.*

INTRODUCTION

Bacteria of genus *Rhizobium* generally contain 2-4 large plasmids (>100 kb), and in some cases, even upto 10 (Thurman *et al.*, 1985). Much of the recent research of rhizobia has been concentrated on the Sym plasmid, because of its importance in nitrogen fixation (Nuti *et al.*, 1979; Krol *et al.*, 1980). Plasmids other than pSym in rhizobia are generally termed cryptic since their functions are mostly unknown. The large number, stability and sizes of cryptic plasmids of *Rhizobium* signify that they are likely to serve some functions in the soil environment.

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In this study, an attempt was made to determine what phenotypic characters are conferred by cryptic plasmids of *R. leguminosarum* bv. *trifolii*. Nitrogen fixing efficiency and competitive ability of *Rhizobium* were studied since such characters are considered important for the survival of rhizobia in a particular environment. Survival and competitive ability of rhizobial isolates may be especially important in selecting and developing inocula which need to be genetically compatible with both host and the environment.

MATERIALS AND METHODS

The Sym plasmid of *R. leguminosarum* bv. *trifolii* isolate 16a (Karunagoda *et al.*, 1995) was identified by using the *nif* genes cloned in pBS13 [a construct of a 2.45 kb *ECo* R1 cloned fragment of the *R. leguminosarum* plasmid pRL1J1 (Hirsch, 1979), in an ampicillin resistant pUC18 vector. This fragment contains *nif* H, D and possibly K genes (Skot, pers. commu. cited by Smith, 1991)]. The pBS13 plasmid was isolated by miniplasmid preparation (Maniatis *et al.*, 1982) and restricted by *ECo* R1 and electrophoresed on an agarose gel at 100 v for 30 minutes. The *nif* gene fragment was excised from the gel and the DNA was eluted, labelled with ^{32}P and hybridized with the Southern blots (Southern, 1975) of the plasmid profile of the isolate. Following autoradiography, the Sym plasmid was identified.

Spontaneous rifampin resistant mutants of the isolate were obtained. These rifampin-resistant mutants were subjected to random Tn5B12S mutagenesis with a *nptI-sacB-sacR* cassette (confers kanamycin resistance and sucrose sensitivity) on the vector pMH1701 (Hynes *et al.*, 1989). Tn5 labelled *Rhizobium* were selected for kanamycin and rifampin resistance. Presence of *nptI-sacB-sacR* cassette in the mutants was further confirmed by hybridization of ^{32}P-labelled *nptI-sacB-sacR* cassette with the Eckhardt (1978) blots of the mutants.

The *nptI-sacB-sacR* cassette labelled bacteria were plated on TYC medium (Beringer, 1974) containing 10% sucrose and incubated at 27°C for 4 days. Colonies developing on the sucrose medium were tested for loss of the labelled plasmid by replica plating on TYC medium with and without kanamycin. For further confirmation of plasmid curing, the kanamycin-sensitive bacteria grown on TYC/sucrose plates were analyzed by Eckhardt (1978) method for the changes in the plasmid profiles.

The host dry weight was used as a measure of the nitrogen fixing efficiency of the isolates. The wild type and each derivative of cured plasmids

or with deleted plasmids of *R. leguminosarum* bv. *trifolii* isolate 16a were inoculated to *Trifolium repens* cv. Alice. Shoot dry weights were determined after drying the plants at 65°C for 42 hours for 6 weeks. The experimental design used was a randomized complete block design with 4 replicates.

Competitive ability of the derivatives was determined by inoculation of *Trifolium repens* cv. Alice seedlings with 1:1 cell ratio of the mutant and the wild type. After 6 weeks, nodules were harvested and *Rhizobium* from each nodule was isolated (Karunagoda et al., 1995). These *Rhizobium* were grown on TYC/rifampin plates to identify whether the nodules were occupied by mutants.

The relative competitiveness of *R. leguminosarum* bv. *trifolii* isolate 16a and its derivatives was assessed by Chi-square analyses of the proportions of isolate 16a and each respective derivative in the nodules. This provides information on the effect of deletions and loss of plasmids on the competitive ability of the strain.

First, the Chi-square values were calculated considering 1:1 as the expected ratio. This allows the determination of variation in the competitive abilities with respect to the wild type.

RESULTS AND DISCUSSION

Isolate 16a had 4 plasmids (Karunagoda *et al.*, 1995) and the Sym plasmid was identified as the second largest plasmid (Figure 1a).

The plasmids of the rifampin resistant mutants derived from strain 16a were able to tag with *nptI-sacB-sacR* cassette (Figure 1b). It appeared that all the plasmids were tagged with the cassette. Nevertheless, plasmid curing was achieved only for the smallest cryptic plasmid (derivative 25-27-5). Derivatives 29-27-14 and 29-27-18, were obtained with deletions on the largest plasmid (Figure 2).

There was a significant difference between the shoot dry weights of *R. legumonosarum* bv. *trifolii* 16a and its derivatives (Table 1). The smallest cryptic plasmid of the isolate was cured in 25-27-5 and the dry matter production was impaired as a consequence. Loss of this plasmid made the strain symbiotically ineffective, since its nitrogen fixing ability exhibited no significant difference from that of the uninoculated treatment, even in the presence of the Sym plasmid.

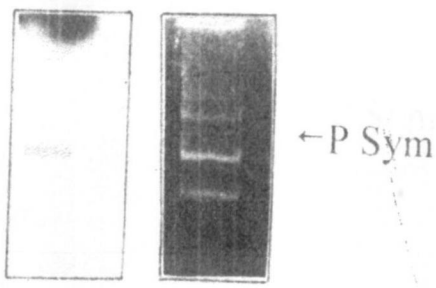

Figure 1. **Plasmid profile of *R. leguminosarum* bv. *trifolii* (left) and corresponding autoradiograph of the hybridization with *nif* probe (right.) Arrow indicates the *nif* gene labelled Sym plasmid.**

This implies that the smallest cryptic plasmid of the isolate 16a seems to have a positive effect on nitrogen fixation. It has been suggested that, in addition to the Sym plasmid, gene sequences on the smallest cryptic plasmid may be indispensable for the efficiency of the isolate.

Nitrogen fixing efficiencies of 29-27-14 and 29-27-18 did not differ significantly from that of the wild type implying that the gene sequences deleted have no significant effect on the nitrogen fixing efficiency of the isolate. No mutants produced a significantly enhanced nitrogen fixing ability when compared as the wild type. Occupancy of rhizobia in the nodules of *T. repens* cv. Alice inoculated with a mixture of 1:1 cell ratio of each derivative and wild type is given in the Table 2.

All the derivatives obtained were rifampin resistant mutants. In competition for nodule formation between 16a wild type and 16a rifampin resistant mutant inoculated in a 1:1 cell ratio, results showed that the rifampin resistant mutant was less competitive than the wild type ($X^2=5$, $P<0.05$).

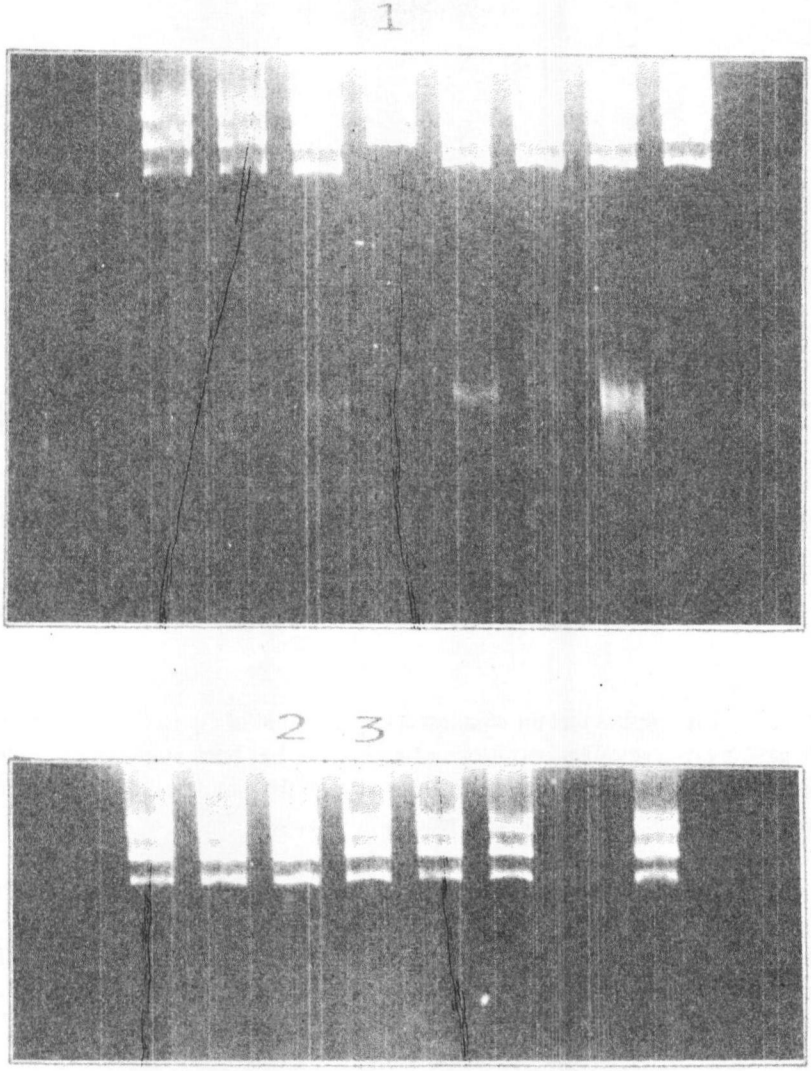

Figure 2. Changes in the plasmid profile of *R. leguminosarum* bv. *trifolii* isolate 16a after curing and deletions of plasmids
Lane 1: derivative 25-27-5, cured for the smallest plasmid
Lane 2: derivative 29-27-14, deletion on the largest plasmid
Lane 3: derivative 29-27-18, deletion on the largest plasmid

Table 1. **Response of *T. repens* cv. Alice to inoculation with *R. legumonosarum* bv. *trifolii* 16a and its derivatives.**

Derivative/isolate	Shoot dry weight (mg/plant)
25-27-5	5.85 bc
29-27-14	8.725 ab
29-27-18	8.55 ab
16 a	11.375 a
uninoculated	1.925 c

Values followed by the same letter are not significantly different at P<0.01 level.

The competitive ability of the isolate 16a had been significantly improved in the derivative 25-27-5. This derivative had been cured of the smallest cryptic plasmid and showed a higher competitive ability when compared with the rifampin resistant parent. This implies that curing of the smallest plasmid made the isolate more competitive compared to the wild type in spite of its inefficiency in nitrogen fixation.

Deletions on the largest plasmid of the isolate seem to have no effect on the competitive ability of the isolate. Derivative 29-27-14 showed significantly less competitiveness than the wild type. However, when compared with the rifampin resistant parent, it did not show any significant effect on the competitive ability. This implies that the rifampin resistance affect the competitive ability, but not the deletion. The competitive ability of the derivative 29-27-18 was not significantly different from the parental strain However, these derivatives showed a significant reduction in nitrogen fixation with compared to the rifampin resistant parent.

Rifampin-resistant mutants of *R. meliloti* were previously reported to be less competitive than their respective wild types (Lewis *et al.*, 1987) and it has been suggested that acquisition of rifampin resistance is generally associated with loss of competitiveness. This study also showed that the rifampin resistant mutant was less competitive than the wild type. However,

Table 2. **Occupancy of rhizobia in the nodules of *T. repens* cv Alice inoculated with a mixture of 1:1 cell ratio of each derivative and wild type.**

Derivative	Occupancy of rhizobia (%)		X^2_1	X^2_1
	Wild type	Derivative		
16 a/rif	75	25	0.5*	
25-27-5	45	55	0.2	9.6**
29-27-14	80	20	7.2**	0.26**
29-27-18	60	40	0.8	2.00

X^2_1 = values with the wild type (expected ratio = 1.1)
X^2_2 = values with the rif resistant mutant (expected ratio = 3:1)

* significant at 5% probability level
** significant at 1% probability level

in spite of the rifampin resistant phenotype, derivative 25-27-5 exhibited a higher competitive ability when compared with the wild type.

According to the results of this study, it would appear that there are genes on the cryptic plasmids of *R. legumonosarum* bv. *trifolii* isolate 16a which have a positive effect on nitrogen fixation as well as both positive and negative effects on the competitive ability.

The inability to cure other plasmids of *R. legumonosarum* bv. *trifolii* isolate 16a itself may give clues as to the functions earned out by those plasmids. Further experiments may be required in order to determine whether these plasmids are essential for *R. leguminosarum* bv. *trifolii* isolate 16a.

It has been postulated that cryptic plasmids of *Rhizobium* confer competitive advantage on the isolate. However, it was interesting to observe that derivative which cured the smallest plasmid (25-27-5) was more competitive than the wild type. If the Sym plasmid of this derivative could be replaced with a more efficient one, this derivative can be used as a highly

competitive inoculum in the field. However, further confirmation of the results are required.

Unfortunately, not all plasmid were successfully eliminated from the cell. It would be of interest to cure plasmids systematically from the same isolate and observe the changes in phenotypes at each step. This would provide information regarding the functions of each plasmid separately.

CONCLUSIONS

The smallest cryptic plasmid of *R. leguminosarum* bv. *trifolii* isolate 16a was able to successfully cure. Two deletions were observed in the largest cryptic plasmid. Plasmid-cured derivative has shown a reduction in its nitrogen fixing efficiency but an improvement in its competence with the parental strain.

Hence, it was suggested that the smallest cryptic plasmid of the isolate confer a phenotype which positively affects the efficiency of the isolate and negatively affects its competitive ability. The deleted gene sequences of the largest plasmid have no effect on the nitrogen fixing efficiency. However, one deletion made the strain less competitive while the other did not make any effect on the competitive ability.

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