3. White, P. R. *A handbook of plant tissue culture*, *J. Cattell, Lancaster*, 1943.
4. Murashige, T. and Skoog, F., *Physiol. Plant*, 1962, **15**, 473.
5. Schenk, R. U. and Hildebrandt, A. C., *Can. J. Bot.*, 1972, **50**, 199.

ISOLATION AND CHARACTERIZATION OF SMALL PLASMIDS FROM *RHIZOBIUM MELILOTI*

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ABSTRACT

Eight isolates of *Rhizobium meliloti* were obtained from the root nodules of *Trigonella foenum-graecum* (fenugreek). They were analysed for their plasmid content by agarose gel electrophoresis. Both large and small plasmids were observed. The presence of small plasmids (Molecular weight (M_r) less than 10×10^6) is reported here for the first time. Small plasmids were detected in at least 3 strains of *Rhizobium meliloti*. The molecular weight of pRmD04b was estimated to be 5.2×10^6. Strain Rm01 has plasmids 2 smaller than 5.2×10^6 and Rm06 has one small plasmid a little bigger than 5.2×10^6. Large plasmids were detected in Rm01, 04, 06 and 30 which were a little larger than plasmid RP4 (M_r 34×10^6). Strain Rm10 harbours 2 large plasmids only which migrate much slower than RP4 through the agarose gel.

INTRODUCTION

A number of very large plasmids have been isolated from *Rhizobium species*[1, 2]. The functional role of megaplasmids is somewhat intriguing since the total coding potential of such plasmids in certain cases may be very large. Even when it is taken into account that some or all of the *nif* and *nod* genes may be on the plasmids, the coding potential of only a fraction of the total may be accounted. In contrast to the frequent occurrence of large plasmids, small ($M_r < 10 \times 10^6$) plasmids from fast-growing Rhizobia have not been reported[1]. Such small plasmids could be useful as vectors. Here we report the isolation and characterization of small plasmids from *Rhizobium meliloti* which have been detected along with the large plasmids.

MATERIALS AND METHODS

Bacterial strains: Altogether eight strains were isolated from the root nodules of *Trigonella foenum-graecum* (fenugreek) and analysed for their plasmid content primarily by agarose gel electrophoresis. The methodology used to isolate Rhizobia was that described by Vincent[3]. The bacterial isolates were designated *R. meliloti* 01, 04 etc.

Growth media: Liquid TY medium contained 0.5% (w/v) Difco Bacto-tryptone, 0.3% (w/v) Difco Bacto-yeast extract and $CaCl_2$ (7 mM). Liquid PA medium contained 0.4% (w/v) Difco bactopeptone and 2 mM $MgSO_4$. Rhizobial strains grow somewhat poorly in PA medium but the bacterial cells produce less gummy envelope. This gives better lysis and enhanced plasmid recovery[4].

Cell lysis and cleared lysate preparation: For small and large plasmids together, the alkaline denaturation method of Hirsch *et al*[4] was followed. To isolate only small plasmids, primarily Hirt[5] method with minor modifications[6] was used. Bacterial cultures were grown to stationary phase, washed and resuspended in standard saline citrate (SSC) and lysed with sodium dodecylsulphate (SDS) at a final concentration of 1%. After lysis, NaCl (1 M final concentration) was added, gently mixed and stored at 4°C overnight. The lysates were centrifuged at 20,000 g in Sorvall RC 2B refrigerated centrifuge for 30 minutes. Lysates could be directly treated with RNAase and deprotenized with chloroform: amyl alcohol (24:1)[7].

Plasmid purification by ethidium bromide cesium chloride density-gradient centrifugation: Essentially the method of Radloff *et al*[8] was followed. Centrifugation was done in Ti 60 rotor at 40,000 RPM for 60 hr at 15°C. In most centrifugations 2 bands were observed, the lower one containing the CCC (covalently-closed circular) DNA. At times, three bands were observed with the lowest band containing the CCC DNA. The upper two bands were interpreted as has been done by Helinski[9].

Restriction cutting and agarose gel electrophoresis: Restriction analysis was carried out with enzymes from BRL and 1 unit/μg of DNA was used. Agarose gel electrophoresis was carried out according to Meyers *et al*[10] using 1% agarose for cut samples and 0.7% for plasmid resolution. The buffer used was tris acetate EDTA pH 8.

Southern hybridization: Of pRmD04b with pBR 322 probe labelled with α-^{32}P/dCTP was done according to Southern. Specific activity was 300 Ci/mmol (Amersham).

EXPERIMENTAL RESULTS

Plasmid profiles of R. meliloti strains: Electrophoresis of the cleared lysate preparation through agarose gels yielded a number of discrete bands as shown in figure 1. A gross characterization for the size of plasmids was done by comparing their mobility relative to *H. influenzae* plasmid pD7 (M_r 12×10^6) and plasmid RP4 (M_r 34×10^6). Plasmid profile and their sizes are given in table 1. Small plasmids were observed in at least three strains.

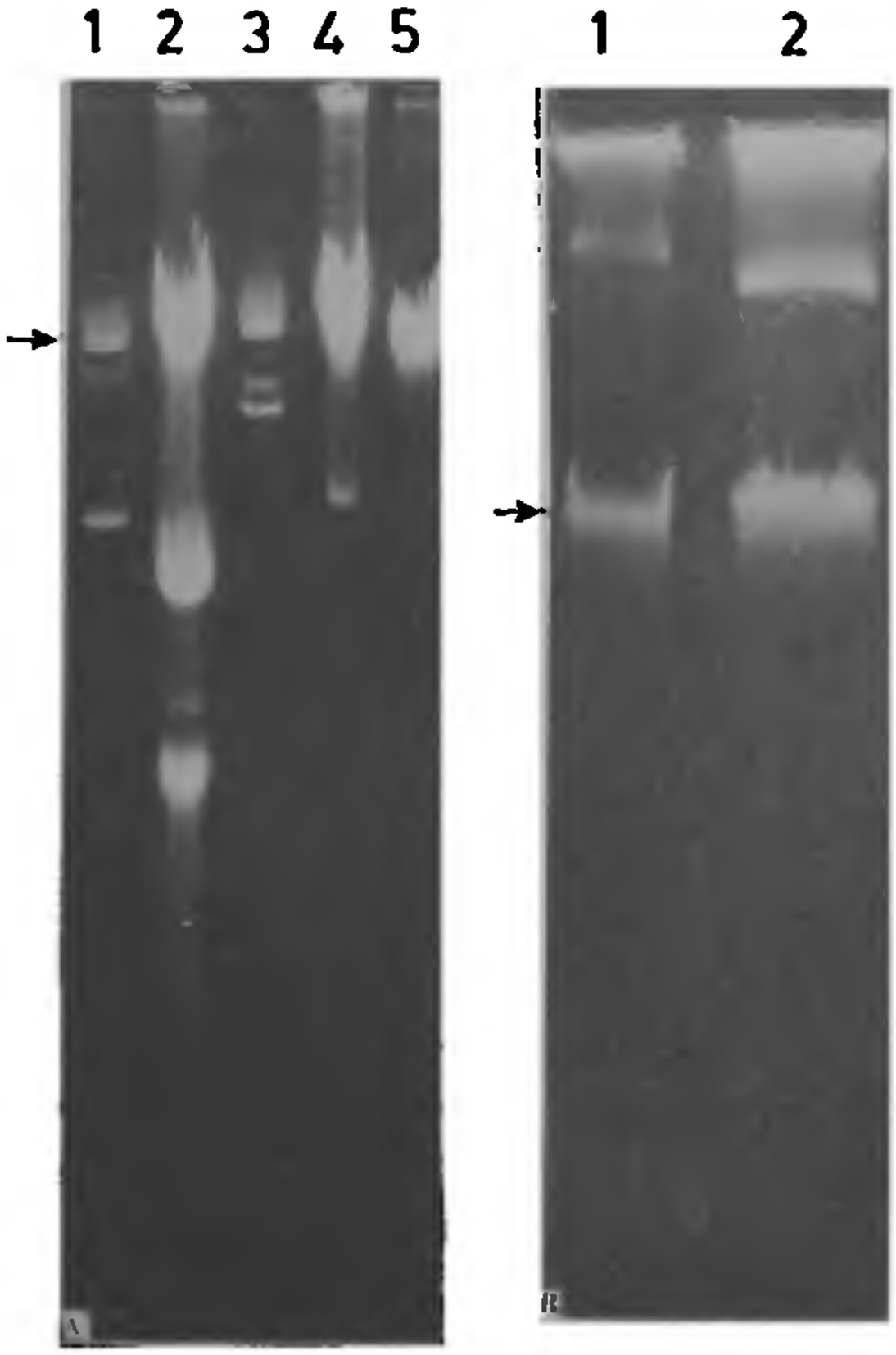

Figure 1. Agarose gel electrophoresis of plasmids from *R. meliloti* strains. A. Lane 1 pRmD04b (Hirt method) lane 2pRmD01b and pRmD01c, land 3pD7, lane 4pRmD06a, RmD06b, lane 5 pRmD04a (Hirsch method). B. Lane 1 Rm30, lane 2 RP4. The arrow indicates the position of chromosomal DNA.

Table 1 *Characterization by agarose gel electrophoresis of plasmid DNAs isolated from Rhizobium meliloti*

Strain	No. of Plasmids	Size M_r ($\times 10^6$)
Rm01	pRmD01a	> 34
	pRmD01b	< 12
	pRmD01c	< 12
Rm04	pRmD04a	> 34
	pRmD04b	$\simeq$ 5.2
Rm06	pRmD06a	> 34
	pRmD06b	< 12
Rm10	pRmD10a	$\gg$ 34
	pRmD10b	> 34
Rm12	—	—
Rm22	—	—
Rm24	—	—
Rm30	pRmD30	> 34

Molecular weight of pRmD04B: Purified pRmD04b was treated with the restriction enzyme *Bam* HI. *Bam* HI cuts it only once. The single band obtained by cutting the plasmid with *Bam* HI was compared for its mobility with phage lambda DNA standards[12] (cut with *Bam* HI and *Eco* RI separately). pRmD04b cut with *Bam* HI migrates a little slower than the 7.546 kbp DNA fragment of lambda cut with *Eco* RI and the 7.301 DNA fragment of lambda cut with *Bam* HI on the agarose gel (figure 2A), giving an estimate of M_r of about 5.2 $\times 10^6$. M_r was also estimated by the method of Meyers *et al*[10]. By this method, the M_r of plasmid pRmD04b was estimated at 5×10^6. M_r of pRmD04b was also determined by directly comparing the *Bam* HI fragment of pRmD04b with T7 DNA *Acc-I* & *Hpa-I* cut standards[12] (figure 2B).

Southern hybridization of labelled pBR322 probe with cut pRmD04b DNA: The strain from which pRmD04b was isolated is resistant to 100 μg/ml of ampicillin. In order to determine if this DNA contained any *amp*^{r} (*bla* gene) marker, ^{32}P-labelled pBR322 probe was prepared and hybridized to pRmD04b cut with *Bam* HI. No hybridization was detected (Data not shown) indicating that the plasmid

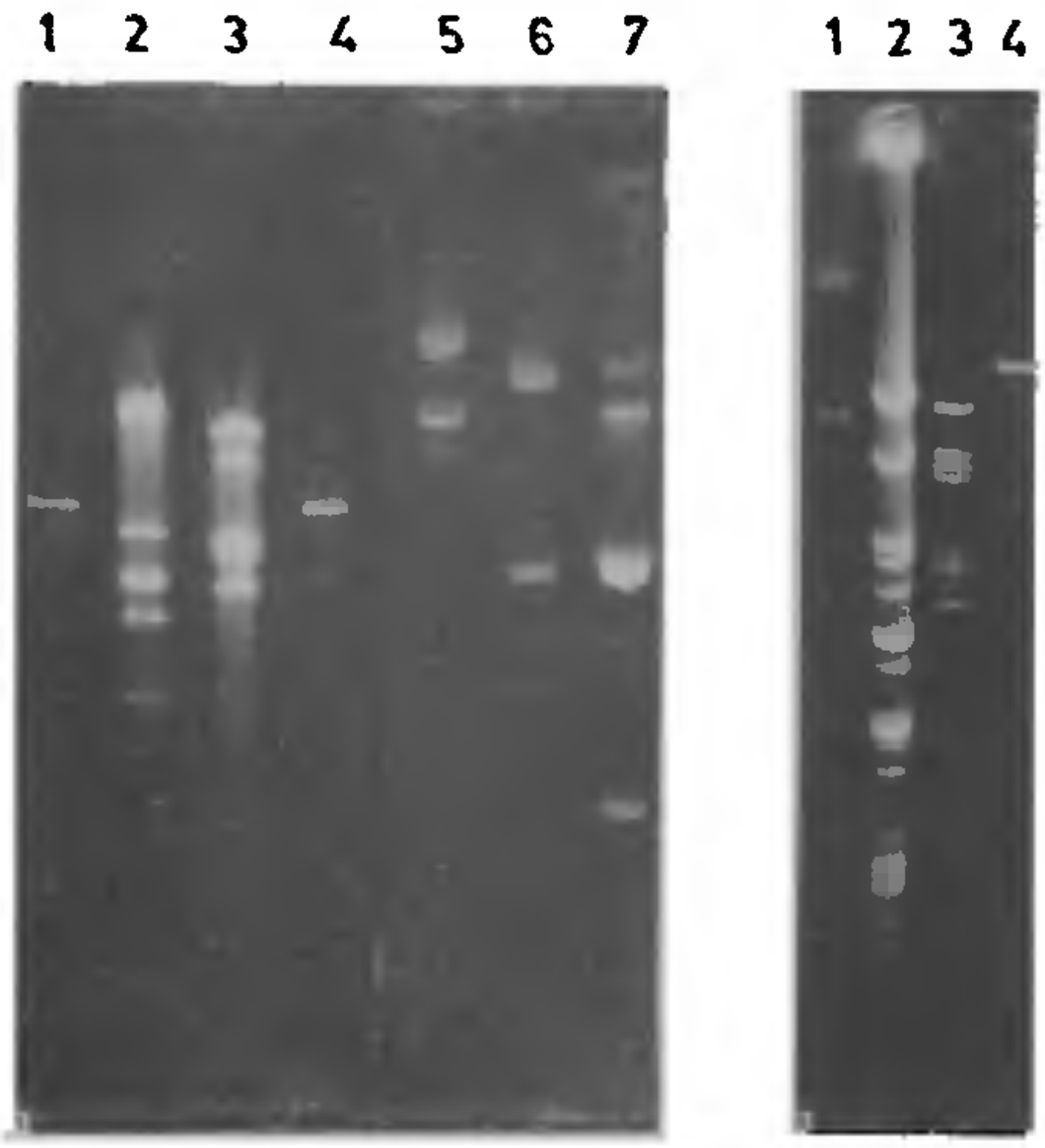

Figure 2. Agarose gel electrophoresis for determination of molecular weight of pRmD04b. **A.** Lane 1 and 4 pRmD04b cut with *Bam* HI, lane 6 pRmD04b; lane 2–phage lambda DNA cut with *Eco* RI; lane 3–phage lambda DNA cut with *Bam* HI; lane 5–pD7; lane 7–pBR322. **B.** Lane 1–pRmD04b; lane 4–pRmD04b cut with *Bam* HI; lane 2 and 3, T7 DNA digested with *Acc-I* and *Hpa-I* respectively.

likely does not contain amp^r or tet^r marker and may be cryptic.

DISCUSSION

Small plasmids from Rhizobia have not been reported thus far. Denarie *et al*[1] categorically state that a plasmid smaller than 10×10^6 M_r has not been observed. According to Nuti *et al*[2] plasmids smaller than 85 megadaltons have been observed only occasionally. In our preparations, small plasmids were observed in at least three of them. Thus these plasmids may not be that rare in the local strains. As of now it is not known if it is a multicopy plasmid. Plasmid pRmD04b could have potential for its use as a vector. However, the plasmid does not seem to contain an antibiotic resistance marker and possibly is a cryptic plasmid. It may be possible to splice cam^r transposon to pRmD04b. Cam^r is flanked on either side by a single *Bam* HI site. Such a marker would permit detection of transformants in *E. coli* with this plasmid and would in addition enable cloning this plasmid without the background of the other plasmids in the bacterial strain.

The strain Rm04 has renodulated *fenugreek*. We assume that *nod* genes are on the large plasmid and some may be even on the chromosome. The presence of transposons for antibiotic resistance genes could also use up some of the information in the megaplasmid DNA.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Sushil Kumar, IARI, New Delhi and colleagues V. P. Joshi, J. Thomas, A. A. Bhagwat and R. K. Tuli for help on several occasions. For a sample of T7 DNA molecular weight standards they are grateful to F. W. Studier, BN New York.

10 August 1983

1. Dénarié, J., Boistard, P., Casse-Delbart, F., Atherly, A. G., Berry, J. O. and Russel, P., *Int. Rev. Cytol.*, 1981, supplement **13**, 225.
2. Nuti, M. P., Lepidi, A. A., Prakash, R. K., Hoykaas, P. J. J. and Schilperoort, R. A., in *Molecular biology of plant tumors*, 1982, Academic Press, New York, p. 561.
3. Vincent, J. M., in the *Biology of nitrogen fixation*, Ed. Quispel, A. North Holland, Amsterdam. 1974, p. 7.
4. Hirsch, P. R., Van Montagu, M., Johnston, A. W. B., Brewin, N. J. and Schell, J., *J. Gen. Microbiol.* 1980, **120**, 403.
5. Hirt, B., *J. Mol. Biol.* 1967, **26**, 365.
6. Notani, N. K., *J. Biol. Sci.*, 1981, **3**, 431.
7. Marmur, J. *J. Mol. Biol.*, 1961, **3**, 208.
8. Radloff, R., Bauer, W. and Vinogard, J., *Proc. Natl. Acad. Sci. USA*, 1967, **57**, 1514.
9. Clewell, D. B. and Helinski, D. R., *Proc. Natl. Acad. Sci. USA*, 1969, **62**, 1159.
10. Meyers, J. A., Sanchez, D., Elwell, L. P. and Falkow, S., *J. Bact.*, 1976, **127**, 1529.
11. Southern, E. M., *J. Mol. Biol.*, 1975, **98**, 503.
12. McDonell, M. W. and Studier, F. W., *J. Mol. Biol.*, 1977, **110**, 119.