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Screening and Characterization of Indigenous Plasmids of *Mesorhizobium ciceri* Strains

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Twenty nine strains of *Mesorhizobium ciceri* were screened for indigenous plasmids and cured derivatives were obtained in fourteen parent strains. Based on plasmid profile, 1 to 3 plasmids were observed in all the *M. ciceri* strains and these were clubbed into 3 groups (Group 1-3). The molecular size of plasmids in all the strains ranged from 228-259 kbp. Curing of plasmid (s) was achieved with triple treatment of ethidium bromide (2 µg/ml), acridine orange (1 µg/ml) and high temperature (38 °C). Antibiotic resistance pattern of the parent strains and their cured derivatives was similar, indicating that these were true derivatives. The production of exopolysaccharide (EPS) in cured strains was less than fifty per cent of parent strains. The symbiotic effectivity of all the parent strains was higher than that of their cured derivatives.

Key words: Plasmids, *Mesorhizobium ciceri*, curing, exopolysaccharide, symbiotic effectivity, acridine orange, ethidium bromide, acetylene reducing activity.

Rhizobia are soil bacteria that are able to induce nitrogen-fixing nodules on the roots of leguminous plants and fall into 13 genera viz; *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium* (Yadav, 2008). The formation and maintenance of nitrogen-fixing nodules are the result of a complex interaction between the two partners and requires the expression and regulation of both bacterial and plant genes in a highly co-ordinated manner (Denarie *et al*., 1992; Long, 1996). Thus, rhizobia-legume symbiosis is the most efficient system which accounts for the major portion of the world's biologically fixed nitrogen, which is estimated at 70 million tones per annum (Brockwell *et al*., 1995).

Chickpea [*Cicer arietinum* (L.)] is the third most widely grown grain legume in the world (Vander *et al*., 1972) and an important rabi pulse crop of Northern India. The *Mesorhizobium ciceri* infects chickpea and forms nodules on its roots. It is now well established that plasmids exist in virtually all bacterial species including rhizobia. These plasmids are the accessory genetic elements, known as extrachromosal DNA and have the ability to self replicate (Novick, 1980). Large circular plasmids ranging in number (1-10) (Thurman *et al*., 1985) as well as in size (150-1500 kbp) (Banfalvi *et al*., 1985) are common among rhizobia. In some rhizobia (*Rhizobium, Mesorhizobium, Sinorhizobium*), most of the essential genes required for the symbiotic processes like *nod, nif, fix* etc. are located on the plasmids known as symbiotic plasmids or pSyms (Johnston *et al*., 1978; Banfalvi *et al*., 1981; Masterson *et al*.,1982; Morrison *et al*., 1983). Although plasmids have been detected in some slow growing strains of rhizobia, but these do not harbour *nif* genes which are apparently present on the chromosome (as in *Bradyrhizobium japonicum*).

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Most of the other rhizobial plasmids are not essential for the establishment of the complete symbiotic state and are called cryptic or non-symbiotic plasmids (non-pSyms). The various characteristics assigned to non-pSym plasmids include competitiveness (Bromfield *et al*., 1985), infectivity (Toro and Olivares., 1986; Zlotnikov, 1984), EPS synthesis (Carlson *et al*., 1983; Hynes *et al*., 1986), thiamine biosynthesis (Finan *et al*., 1986), dicarboxylate transport (Watson *et al*., 1988) and melanine production (Hirsch, 1979; Hirsch *et al*., 1980). The determination of these plasmid functions may be accomplished most efficiently by curing the plasmids and comparing the phenotypes of cured derivatives with that of parent strains. There is very scanty information on the plasmid profile and curing of plasmids in *M ciceri*. Very few published reports are there on this aspect (Cadahia *et al*., 1986). Keeping these observations in view, the present study was planned to prepare the plasmid profile in wild type strains of *M. ciceri*, cure the plasmids in a few strains and compare the symbiotic properties of parent and cured derivatives.

MATERIALS AND METHODS

Bacterial strains and culture media

In the present investigation, a total of 29 strains of *Mesorhizobium ciceri* were used. Out of these, 16 were isolated from root nodules of chickpea plants growing in experimental farm of CCS Haryana Agricultural University, Hisar, 8 were obtained from Deptt. of Microbiology, College of Basic Sciences & Humanities and 5 strains were procured from Division of Microbiology, IARI, New Delhi. For general culturing of strains and also for checking the purity (on YEMA congo red) of strains, yeast extract mannitol (YEM) agar medium (Vincent, 1970) was used.

Tryptone yeast extract (TY) medium (Beringer, 1974)

This medium was used for curing of plasmids.

Sloger's medium (Sloger, 1969)

This medium was used for growing chickpea plants in chillum jars for testing the symbiotic properties of *Mesorhizobium ciceri* strains.

MX-Medium

This medium was used for culturing the rhizobial strains for extraction of exopolysaccharides (EPSs) and has the following components: MoPS (morpholinepropane sulfonic acid) 50 Mm, $CaCl_2$ 1 Mm, sodium succinate 40 Mm, NH_4NO_3 7.5 mM, Na H_2PO_4 1 mM, KI 5Mm, KCl 7Mm, Na_2SO_4 1 mM, $MgSO_4$ 1 mM, $FeSO_4$ 10 µM, Na_2EDTA 10 µM, $MnSO_4$ 45 µM, $ZnSO_4$ 10 µM, H_3BO_3 50 µM,

$NaMoO_4$ 1 µM, $CuSO_4$ 1 µM, $CoCl_2$ µM, thiamine HCl 3 µM, biotin 2 µM, pyridoxine 5 µM, nicotinic acid 8 µM, pH 6.9.

Studies on plasmid profile

The method of Eckhardt (1978) as modified by Rosenberg *et al*. (1982) was used for studying the plasmid profile of parent strains.

Curing of plasmids

Plasmids were cured by a triple treatment of acridine orange (1µg/ml), ethidium bromide (2 µg/ml) and high temperature (38°C).

Estimation of exopolysaccharide (EPS)

The amount of exopolysaccharide (EPS) produced by the parent strain and the cured derivatives was estimated by using an anthrone-H_2SO_4 method modified from that described by Seifer *et al*. (1950). Exopolysaccharides were first extracted from bacterial cells and then quantified spectrophotometrically.

Symbiotic properties of the wild type and cured strains

Symbiotic characteristics of the parent and cured strains were were studied on chickpea plants grown in pots in net house under natural conditions. The seeds of chickpea were surface sterilized first in 70 % alcohol for one min and then in 0.1 % $HgCl_2$ for five min followed by 5-6 washings with sterile water. The sterilized seeds were then immersed in cell suspension (10^8-10^9 cells/ml) of each strain for 30 minutes. Five inoculated seeds were sown in sterilized sand in pots. Two seedlings were removed after some growth and three were retained for further studies. The chickpea plants were uprooted from the sand 60 days after sowing (DAS) and the following symbiotic parameters were recorded: nodule number, nodule fresh weight and dry weight, root and shoot dry weight, per cent and total shoot nitrogen.

Acetylene reducing activity

Nitrogenase activity in nodules of 60 d old plants was determined by measuring acetylene reducing activity (ARA) (Hardy *et al*. 1968).

Estimation of shoot nitrogen

Total shoot nitrogen of the plants grown under natural conditions in the net house was estimated by micro-Kjeldahl's steam distillation method (Bremner, 1960).

RESULTS AND DISCUSSION

Plasmid profile

Twenty nine different *Mesorhizobium ciceri* strains were screened for their plasmid profile. The data on plasmid profile (Table 1, Plate 1) indicate that all the strains harboured plasmids with varying number (1-3) and molecular size (228-259 kbp). Based on the number of plasmids, these strains were clubbed into 3 different groups (Table 1). In Group 1, twelve strains were placed and each of these contained only one plasmid. Group 2 contained seven strains and each harbored two plasmids. The Group 3 had ten strains, each having three plasmids. The molecular size of the plasmids (Table 1, Plate 1) also varied among the three groups. In Group 1, the molecular size ranged between 228-245 kbp; in Group 2 between 232-259 kbp and in Group 3 between 228-259 kbp. These results are in agreement with those obtained by Cadahia *et al*; 1986. The similar grouping of strains on the basis of number and size was also done by Gross *et al.* (1979), who placed 135 strains of *Rhizobium japonicum* into four groups. These strains carried combinations of two or more plasmids varying in molecular weight from 49 to 118 magadaltons. Various other species of rhizobia were reported to harbour plasmids, the number and molecular weight of plasmids, however, varied (Denarie *et al*., 1976; Casse *et al.*, 1979; Gross *et al.*, 1979; Hirsch, 1979; Zurkowski & Lorkiewicz, 1979; Brewin *et al*., 1980; Hombrecher et al., 1981;

Table 1. Number and molecular size of plasmids in *M. ciceri*

S. No.	Group	Strain	No. of plasmids	Molecular size (kbp)
1	1	MC1	1	232
2		MC2	1	235
3		MC3	1	232
4		MC4	1	228
5		MC5	1	232
6		MC6	1	235
7		MC7	1	232
8		MC13	1	242
9		MC14	1	239
10		MC15	1	242
11		MC16	1	239
12		IC59	1	245
13	2	Ca2	2	245, 235
14		Ca181	2	249, 232
15		Ca303	2	249, 232
16		Ca356	2	249, 239
17		Ca480	2	245, 235
18		Ca534	2	245, 235
19		RCD301	2	259, 245
20	3	PR2187	3	259, 249, 245
21		PR2264	3	259, 249, 245
22		PR2278	3	259, 249, 232
23		PR2301	3	259, 242, 228
24		PR2361	3	259, 245, 228
25		MC8	3	256, 242, 232
26		MC9	3	256, 242, 232
27		MC10	3	256, 239, 228
28		MC11	3	252, 245, 228
29		MC12	3	256, 242, 228

Rosenberg et al., 1982; Bender et al., 1984; Toro and Olivares, 1986; Martinez et al., 1987; Hynes *et al*.,1988; Sharma and Laxminarayanan, 1989; Brom *et al*., 1992; Hashem *et al*., 1996; Xianghong *et al*., 1997; Balchandar *et al*; 2003).

Plasmid curing

The triple combination of acridine orange (1 µg/ml), ethidium bromide (2 µg/ml) and high temperature (38°C) was used for curing different plasmids in 14 parental strains of *M. ciceri*. In Group 1, out of 12 strains (harbouring one plasmid each) only 7 strains i.e. MC 1 to MC 7 were cured of a single plasmid of molecular size 232, 235, 228, 232,235 and 234 kbp, respectively (Plate 2). In Group 2, out of 7 strains (containing 2 plasmids each), only 3 strains viz; Ca2, Ca480 and Ca534 were cured of either of the two plasmids (Plate 3). In Ca 2 and Ca 480, first plasmid of molecular size 245 kbp was absent, whereas in Ca534, second plasmid of 235 kbp was missing. In Group 3, all the 10 strains had 3 plasmids each and curing was observed in four strains only (Plate 3, 4). In MC 8, second plasmid of molecular size 242 kbp was absent, whereas in MC 9 and MC 12 first plasmid of molecular size 256 kbp was cured (Plate 3). Two cured derivatives PR2264C1 and PR2264C2 were obtained from the parental strain PR2264, whereas only one cured derivative was obtained from each of the other parental strains. In cured strain PR2264C1, second plasmid of molecular size 249 kbp was absent, whereas in PR2264C, a third plasmid of molecular size of 245 kbp was missing (Plate 4).

Characterization of parent strains and their cured derivatives

The parent strains and their cured derivatives were characterized with regard to exopolysaccharide (EPS) production and symbiotic parameters.

EPS production

The data on EPS production indicate that all the cured derivatives produced less quantity of EPS than their parent strains (Table 2). The range of EPS produced by parent strains was 37-109 µg/ml, the maximum EPS being produced by the strain PR2264 and minimum by MC6 strain. But, in cured derivatives the range of EPS produced was 9-84 µg/ml, the minimum and maximum being produced by MC6 and MC8, respectively. There was a 50 % reduction in EPS production in all the cured derivatives than their parent strains. The maximum reduction in EPS production was seen in the strain MC12 (87.3 %) and minimum in the strain Ca2 (53 %). Genes for EPS production are present on Sym plasmids as well as on the chromosome (Leigh and Walker, 1994). In the strains Ca2 and MC8, the genes required for the production of EPS may be present

Table 2. Symbiotic properties of the parent strains and their cured derivatives under pot house conditions

S. No.	Strain	EPS production (µg/ml)		
		Parent strain	Cured strain	Per cent decrease
1	MC1/MC1C	58	27	53.4
2	MC2/MC2C	70	13	81.4
3	MC3/MC3C	51	21	59.0
4	MC4/MC4C	64	23	64.0
5	MC5/MC5C	65	31	52.3
6	MC6/MC6C	37	9	76.0
7	MC7/MC7C	49	19	61.2
8	MC8/MC8C	89	84	5.6
9	MC9/MC9C	94	54	43.0
10	MC12/MC12C	87	11	87.3
11	Ca2/Ca2C	76	72	5.3
12	Ca480/Ca480C	39	18	54.0
13	Ca534/Ca534C	68	14	79.4
14	PR2264/PR2264C1	109	34	69.0
15	PR2264/PR2264C2	109	27	75.2

MC1 = Parent strain MC1C = Cured derivative of MC1

on the plasmid which was cured. It is opined that decrease in EPS production in cured strains may be due to partial deletion of the plasmid DNA region coding for EPS synthesis or due to mutation in the EPS genes.

Symbiotic properties of parent and cured strains

The data on symbiotic properties (Table 3) indicate that the host plants inoculated with parent strains showed significantly higher nodule number, nodule fresh and dry weight as compared to uninoculated control plants and plants inoculated with cured derivatives. The maximum nodule number (55 per plant), nodule fresh weight (2268 mg/plant) and nodule dry weight (297 mg/plant) were found in chickpea plants inoculated with PR2264 strain. The minimum nodule number (20/plant) and nodule fresh weight (754 mg/plant) were recorded in plants inoculated with MC5 strain, whereas minimum nodule dry weight (81.66 mg/plant) was obtained in plants inoculated with strain MC4. The maximum nodule number (18 per plant) and nodule fresh weight (1781.33 mg per plant) was found in host plants infected with cured derivatives Ca2 strain, whereas maximum nodule dry weight (216 mg/plant) was observed in plants inoculated with the strain PR2264C1. The minimum

Table 3. Symbiotic properties of the parent strains and their cured derivatives under pot house conditions

Strain	Nodule dry wt. (mg/plant)	Root dry wt. (mg/plant)	Shoot dry wt. (mg/plant)	Total shoot nitrogen (mg N/plant)	ARA activity (µ mole C_2H_2 reduced/h/plant) Parent/cured
Uninoculated	68	473	91	4.5	1.36
MC1	164	1396	1062	18.3	2.75
MC1C	92	380	944	4.0	0.97
MC2	110	1379	1043	13.9	2.72
MC2C	48	322	938	3.7	0.96
MC3	119	1087	1046	16.7	2.59
MC3C	86	374	665	1.7	0.83
MC4	82	1570	999	3.3	2.66
MC4C	16	294	949	3.3	1.07
MC5	84	1138	970	12.6	3.94
MC5C	35	248	814	2.5	1.36
MC6	171	1900	1170	21.6	3.63
MC6C	98	418	985	3.7	1.17
MC7	88	2970	978	12.7	2.92
MC7C	81	274	835	3.7	0.49
Ca2	244	1102	2080	35.9	3.96
Ca2C	206	963	941	4.7	1.36
Ca480	239	2615	1970	32.2	3.50
Ca480C	180	633	902	4.3	1.06
Ca534	287	2268	1246	26.2	3.82
Ca534C	128	534	965	4.0	1.24
MC8	237	2010	1650	31.8	4.05
MC8C	142	627	902	4.6	1.16
MC9	176	1231	1194	24.0	3.21
MC9C	119	447	835	3.5	1.36
MC12	170	2473	1176	23.5	3.30
MC12C	104	432	985	3.7	0.97
PR2264	297	2885	3847	41.5	4.05
PR2264C1	216	2888	852	4.3	1.36
PR2264C2	209	229	965	4.5	1.07
CD at 5 %	7.0	20.0	33.0	3.7	-

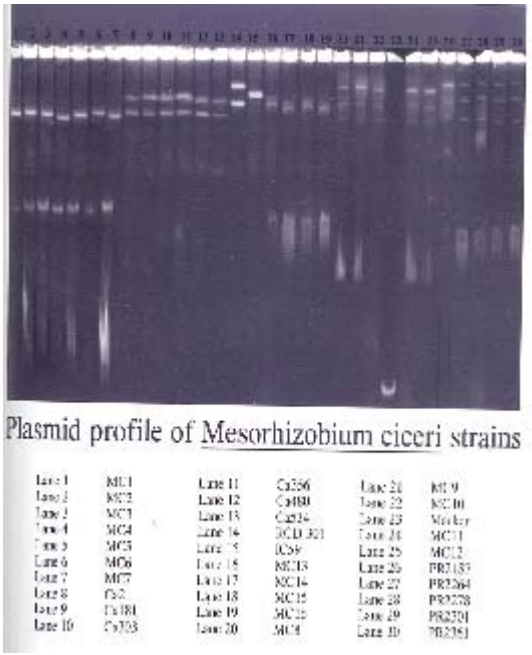

Plate 1.

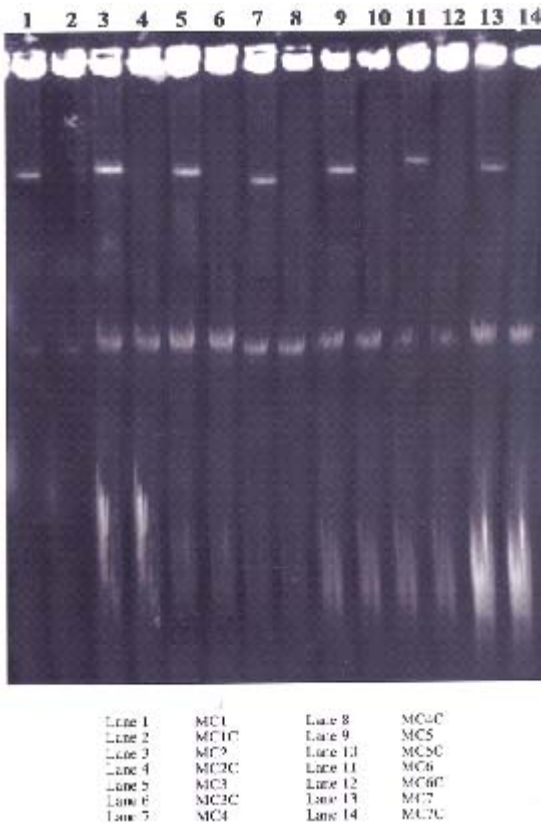

Plate 2.

nodule number (5.1/plant), as well as nodule fresh weight (377 mg/plant) and dry weight (16.1 mg/plant) were seen in plants infected with the strains MC5C and MC4C, respectively. The shoot dry weight per plant was significantly higher in chickpea plants inoculated with the parent strains. The maximum shoot dry weight in chickpea plants was induced by the strain PR2264 i.e. 3847 mg/plant and minimum by MC5 i.e. 9700 mg/plant. Among the cured derivatives, the maximum shoot dry weight of host plants was caused by MC12C strain i.e. 985.3 mg/plant and minimum by the strain MC3C i.e. 665 mg/plant. Total shoot nitrogen per plant was significantly higher in plants inoculated with all the parent strains than the plants inoculated with their cured derivatives and uninoculated control plants (Table). The maximum and minimum shoot nitrogen per plant i.e. 41.48 mg N per plant and 12.62 mg N per plant were seen in plants infected with the parent strains PR2264 and MC5, respectively. Among the cured derivatives, the maximum and minimum shoot nitrogen i.e. 4.66 mg per plant and 1.90 mg per plant were observed in host plants inoculated with the strains Ca2C and Ca3C, respectively. The comparison of parent strains and their cured derivatives is clearly discernible in the Figs. 8 & 9. A highly significant and positive correlation was observed among all the symbiotic characters viz; nodule number, nodule fresh and dry weight, shoot dry weight and total shoot nitrogen (Table). The shoot dry weight had a highly significant and positive correlation with total shoot nitrogen (p=0.696), nodule number (p=0.764), nodule fresh weight (p=0.695) and nodule dry weight (p=0.694). The total shoot nitrogen showed highly significant and positive correlation with shoot dry weight (p=0.696), nodule fresh weight (p=0.710) and nodule dry weight (p=0.696).

Acetylene reducing activity (ARA)

The observations on ARA (Table 3) indicate that the ARA in the nodules of plants inoculated with cured derivatives was 3-4 times less than that found in the nodules of plants inoculated with the parent strains. Among the parental strains, maximum ARA was observed in the nodules of chickpea plants infected with MC8 and PR2264strain, whereas minimum ARA was in the nodules of host plants inoculated with MC3 strain. Within the cured derivatives, maximum ARA

Plate 3.

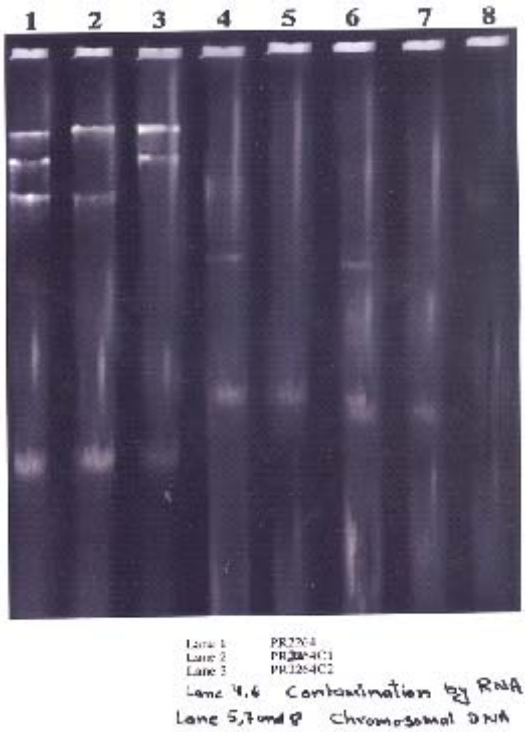

Plate 4.

was in the nodules of plants inoculated with MC5C, MC9C and Ca2C, whereas minimum ARA was seen in the nodules of plants infected with MC7C strain.

CONCLUSIONS

Based on plasmid profile studies in *M. ciceri*, one to three plasmids were observed in all the strains and their molecular size ranged from 228-259 kbp. The reduction in exopolysaccharide (EPS) production was more than fifty per cent in cured strains than their parents, whereas the symbiotic effectivity of all the parent strains was high than that of their cured derivatives.

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