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Utilization of Carbon Substrates, Electrophoretic Enzyme Patterns, and Symbiotic Performance of Plasmid-Cured Clover Rhizobia

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Plasmids in *Rhizobium* spp. are relatively large, numerous, and difficult to cure. Except for the symbiotic plasmid, little is known about their functions. The primary objective of our investigation was to obtain plasmid-cured derivatives of *Rhizobium leguminosarum* bv. trifolii by using a direct selection system and to determine changes in the phenotype of the cured strains. Three strains of rhizobia were utilized that contained three, four, and five plasmids. Phenotypic effects observed after curing of plasmids indicated that the plasmids were involved in the utilization of adonitol, arabinose, catechol, glycerol, inositol, lactose, malate, rhamnose, and sorbitol and also influenced motility, lipopolysaccharide production, and utilization of nitrate. Specific staining of 26 enzymes electrophoretically separated on starch gels indicated that superoxide dismutase, hexokinase, and carbamate kinase activities were affected by curing of plasmids. Curing of cryptic plasmids also influenced nodulation and growth of plants on nitrogen-deficient media. The alteration in the ability to utilize various substrates after curing of plasmids suggests that the plasmids may encode genes that contribute significantly to the saprophytic competence of rhizobia in soil.

Rhizobium spp. generally contain one to four large plasmids (>100 kb) in addition to the symbiotic plasmid pSym (32). Some strains may contain as many as 10 plasmids (32). Plasmids other than pSym in rhizobia are generally termed cryptic because their functions are largely unknown. The large number, stability, and sizes of cryptic plasmids in rhizobia indicate that they are likely to serve some function in the soil environment (27). Plasmids in rhizobia are difficult to cure with common curing agents (18), and functions of plasmids other than pSym have not been investigated extensively.

Genes involved in exopolysaccharide synthesis, thiamine biosynthesis (12), and dicarboxylate transport (35) are encoded by a megaplasmid in *Rhizobium meliloti*. Recent work has also shown that genes for catabolism of dulcitol, melibiose, lactose, raffinose, and β-hydroxybutyrate are carried on this megaplasmid (7, 8). Cryptic plasmids also carry genes involved in catechol catabolism (13) and in enhancement of nodulation competitiveness of *R. meliloti* (33) and *Rhizobium leguminosarum* bv. phaseoli (20).

Determination of plasmid function may be accomplished most efficiently by curing the plasmid and comparing the phenotype of the cured derivatives with that of the wild type. A method that is very useful in selectively curing strains of *R. leguminosarum* bv. viciae of plasmids was recently developed (17, 18). The method utilizes a *nptI-sacB-sacR* cassette (22) cloned into a transposon Tn*5*-Mob derivative (28). The *sac* genes make the transconjugants sensitive to sucrose; this allows for positive selection of derivatives that lose the plasmid containing the cassette and can thus grow on sucrose. We utilized this method to cure plasmids in several *R. leguminosarum* bv. trifolii strains.

In this report, we describe the effects on symbiotic activity and motility of plasmid-cured rhizobia and their ability to synthesize different enzymes, utilize various carbon compounds, and utilize nitrate as their sole nitrogen source.

MATERIALS AND METHODS

Bacterial strains and plasmids. Three strains of *R. leguminosarum* b.v. trifolii, designated W14-2, W11-9, and W8-7, were isolated from nodules of arrowleaf clover (*Trifolium vesiculosum* Savi.) plants grown in soil from east Texas.

The plasmid contents of W14-2, W11-9, and W8-7 were determined by a modified Eckhardt (11) agarose gel electrophoresis technique as described by Hynes and McGregor (17). The pSyms were identified by DNA-DNA hybridization with a biotinylated *nif* probe, pCHK12 (24). Other bacterial strains and plasmids are listed in Table 1.

Labeling of specific rhizobial plasmids with Tn*5*. Spontaneous streptomycin-resistant mutants of W14-2, W11-9, and W8-7 were obtained. These streptomycin-resistant mutants were subjected to random Tn*5*B12S mutagenesis with a *nptI-sacB-sacR* cassette on the vector pMH1701. Tn*5*-labeled rhizobia were selected for kanamycin and streptomycin resistance. To determine whether a plasmid or the chromosome was labeled, triparental crosses were made. If the chromosome was labeled, no transfer of resistance occurred. The recipient was *Agrobacterium* sp. strain UBAPF2, and the helper strain was *Escherichia coli* J-53 carrying RP4-4. *Agrobacterium* transconjugants carrying a Tn*5*-labeled rhizobial plasmid were selected for kanamycin and rifampin resistance. The identity of the labeled plasmid in *Agrobacterium* sp. was established by comparing the size of the labeled plasmid with the size of the plasmids from the donor rhizobial strain. Plasmid profiles were determined by agarose gel electrophoresis as described above.

Curing of plasmids. Tn*5*-labeled plasmids were eliminated

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TABLE 1. Bacterial strains, plasmids, and references

Strain or plasmid	Relevant characteristics	Reference
Rhizobium leguminosarum bv. viciae		
VF 39	Produces LPS I and II	17
VF 39C (plasmid cured)	Does not produce LPS I	17
Agrobacterium tumefaciens UABPF 2	Rif^r, plasmid free	19
Escherichia coli		
S17-1	Mobilizing strain for pMH1701	29
J-53	RP4-4 carrier	28
Plasmids		
pMH1701	Carries Tn*5*-Mob-*sac* derivative Tn*5*B12S	18
RP4-4	Helper plasmid for mobilizations	28
pCHK12	Used for *nifHD* gene probe	24

TABLE 2. Molecular masses of plasmids in three strains of clover rhizobia[a]

Strain	Plasmid code and size (MDa)				
	a	b	c	d	e
W14-2	150	170	260	460[b]	
W11-9	180[b]	460	660		
W8-7	80	170	200[b]	320	360

[a] Plasmid size was determined by plotting the log of the migration on the gel versus the log of the molecular mass of plasmids (160, 249, and 585 MDa) in reference strain DB1 (3).
[b] Symbiotic plasmid.

from rhizobial strains by the method of Hynes et al. (18). The *nptI-sacB-sacR*-labeled bacteria, at a concentration of 10^7 to 10^8 cells per ml, were plated on TY medium (2) containing 5% sucrose and incubated either at 28°C or at 39 to 44°C (depending on the strain) for 2 days. Plates incubated at temperatures higher than 28°C were transferred to 28°C for another 3 days. Colonies developing on the sucrose medium were tested for loss of the labeled plasmid by replica plating on TY medium with and without kanamycin. For further confirmation that the plasmid was cured, plasmid profiles of kanamycin-sensitive isolates were analyzed by agarose gel electrophoresis.

Phenotypic evaluation. Twenty-three carbon compounds were used with all rhizobial strains and cured derivatives to determine whether the ability to utilize any of these compounds was a plasmid-encoded function. The compounds included pentoses (arabinose, xylose, and ribose), hexoses (fructose, galactose, gluconate, glucose, mannose, rhamnose, and fucose), disaccharides (lactose, maltose, and sucrose), sugar alcohols (adonitol, dulcitol, mannitol, and sorbitol), polysaccharide (raffinose), a C_3 compound (glycerol), tricarboxylic acid cycle intermediates (succinate and malate), a vitamin (inositol), and the aromatic compound catechol. Carbon sources (10% [wt/vol] solutions) were neutralized to pH 7.0, filter sterilized, and added to a defined medium containing mineral salts, biotin, thiamine, pantothenic acid, and potassium nitrate (34) (but without mannitol) to provide a final concentration of 15 g of the carbon source per liter. For strains W11-9 and W8-7 and their cured derivatives, nitrate was replaced by ammonium as the nitrogen source because some derivatives did not grow well with nitrate. Inoculants for testing growth of cells on the different compounds were prepared from 48-h broth cultures grown in an orbital incubator shaker at 30°C. A defined broth medium (1) containing galactose and arabinose as carbon sources and glutamate as the nitrogen source was used. Inoculation of 5 ml of minimal medium (34) containing the different compounds was accomplished by adding 80 μl (approximately 10^8 cells) of the inoculant cultures. Cultures were grown at 30°C with shaking for 60 h, and then the optical density at 600 nm was measured. Two replicates were used for each treatment. The data were analyzed by using analysis of variance, and treatment comparisons were based on the least significant difference (0.05).

Electrophoretic enzyme patterns. Strains and derivatives were prepared for starch-gel electrophoresis, to determine the presence and mobility of enzymes by the method of Eardly et al. (10). The techniques of starch-gel electrophoresis and selective staining of enzymes were those of Selander et al. (26). The enzymes investigated included mannitol 1-phosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, glyceraldehyde-phosphate dehydrogenase (NAD), glutamate dehydrogenase (NADP), shikimic acid dehydrogenase, catalase, nucleoside phosphorylase, glutamic-oxalacetic transaminase, hexokinase, carbamate kinase, adenylate kinase, phosphoglucomutase, acid phosphatase, β-galactosidase, leucine aminopeptidase, peptidases, Phe-Leu peptidase, Leu-Gly-Gly peptidase, mannose phosphate isomerase, phosphoglucose isomerase, and superoxide dismutase. Both superoxide dismutase and glutamate dehydrogenase were stained simultaneously on the same gel, as both are oxidoreductases requiring the same staining dyes. Superoxide dismutase, however, produces a white band, and glutamate dehydrogenase produces a dark band.

Motility and symbiosis. Motility tests were performed by the method of Priefer (21), and tests of symbiotic effectiveness were evaluated by using plastic pouches (36) and crimson clover (*Trifolium incarnatum* L.) as the host plant.

LPS production. Production of lipopolysaccharide (LPS) was examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining after periodate treatment. Whole-cell preparation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and staining were performed as described by Cava et al. (6), with a few minor modifications (17).

RESULTS

Plasmid characterization. The sizes of the plasmids in each of the three strains were determined by comparing the plasmid profiles with those of previously sized plasmids in other strains (Table 2, Fig. 1). Only one pSym was present in each strain. The size of the pSym of strain W14-2 was 460 MDa. In contrast, strains W11-9 and W8-7 carried pSyms of similar sizes (180 and 200 MDa, respectively). We have used the nomenclature system of Casse et al. (5) for naming these plasmids.

Plasmid-cured strains. For strain W14-2, cured derivatives that lacked each plasmid were obtained (Fig. 1). To cure strain W14-2 of pRtrW14-2c it was necessary to incubate cells at 41°C. For strain W11-9, derivatives cured of

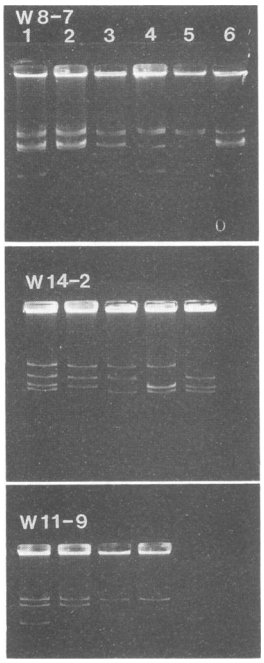

FIG. 1. Plasmid profiles of rhizobial strains W8-7, W14-2, and W11-9 in lane 1. For strain W8-7, lanes 2 through 6 contain profiles of derivatives cured of pRtrW8-7a, b, c, b and c, and e, respectively. For strain W14-2, lanes 2 through 5 contain plasmid profiles of derivatives cured of pRtrW14-2a, b, c, and d, respectively. For strain W11-9, lanes 2, 3, and 4 contain profiles of derivatives cured of pRtrW11-9a, b, and a and b, respectively.

pRtrW11-9a and pRtrW11-9b but not derivatives cured of pRtrW11-9c were obtained (Fig. 1). Incubation temperatures of 39, 41, and 44°C did not result in curing of pRtrW11-9c. For strain W8-7, derivatives cured of pRtrW8-7a and pRtrW8-7e were obtained at 28°C, but 39°C was used to cure strain W8-7 derivatives of pRtrW8-7b and pRtrW8-7c (Fig. 1). Plasmid pRtrW8-7d was not cured even at a temperature of 39, 41, or 44°C.

Phenotypes of cured derivatives. Strain W14-2 grew very poorly on ribose, gluconate, fucose, and dulcitol, strain W11-9 grew very poorly on fucose, and strain W8-7 grew very poorly on succinate. All strains grew well on the other carbon sources. The loss of some plasmids interfered with the ability of strains W14-2, W11-9, and W8-7 to utilize specific carbon compounds (Table 3). Elimination of pRtrW14-2a impaired the ability of a strain to grow on medium containing malate or lactose, whereas curing of pRtrW14-2b affected utilization of the sugar alcohol adonitol. The ability of strain W14-2 to utilize rhamnose or sorbitol was impaired by curing of pRtrW14-2c, whereas curing of the pSym (pRtrW14-2d) affected utilization of the aromatic compound catechol. In strain W11-9, only pRtrW11-9b seemed to be involved in utilization of some tested carbon compounds. Curing of this plasmid affected the ability of the strain W11-9 to utilize inositol, malate, and arabinose (Table 3). For strain W8-7, elimination of pRtrW8-7b and pRtrW8-7c impaired utilization of malate, adonitol, and glycerol (Table 3).

Motility and LPS. In addition to influencing utilization of carbon compounds, loss of some plasmids affected the motility of the strains (Table 4). Elimination of pRtrW14-2a and pRtrW8-7b affected the ability of the strains to swarm on soft agar plates. Curing of the other plasmids as well as of pRtrW11-9a and pRtrW11-9b from these strains did not affect the motility of strains. Since plasmid-encoded genes involved in LPS production have been found in several strains of *R. leguminosarum* (17) and defects in LPS production have been shown to affect motility (17, 21, 30), we examined the strains that were defective in motility for LPS production. Figure 2 shows that these strains were missing the O antigen-containing LPS I band. These mutants correspond to those described by Hynes and McGregor (17). The LPS-negative phenotype of strain W14-2 derivatives missing plasmid pRtrW14-2a was complemented by transferring the plasmid pRle336d from *R. leguminosarum* bv. viciae (17) into these strains. pRle336d restored the cured strains to a

TABLE 3. Ability of plasmid-cured derivatives of three clover rhizobial strains to utilize specific carbon sources

Strain	Carbon source[a]	Growth[b] of strain cured of plasmid:						
		a	ab	b	bc	c	d	e
W14-2	Rhamnose	+		+		−	+	
	Sorbitol	+		+		−	+	
	Adonitol	+		−		+	+	
	Malate	−		+		+	+	
	Lactose	−		+		+	+	
	Catechol	+		+		+	−	
W11-9	Inositol	+	−	−				
	Malate	+	−	−				
	Arabinose	+	−	−				
W8-7	Malate	+		−	−	+		−
	Adonitol	+		+	−	−		+
	Glycerol	+		−	−	+		+

[a] All strains and derivatives were tested on 23 carbon sources, but only results for carbon sources that provided differentiation between the wild type and a derivative are listed.
[b] +, growth not significantly different from that of the wild type; −, optical density of <10% of the wild-type optical density.

TABLE 4. Swarming of cured derivatives of clover rhizobia strains W14-2, W11-9, and W8-7 on soft agar plates[a]

Wild-type or derivative	Colony diameter (mm) after			
	30 h	48 h	72 h	Mean
W14-2	15.0	23.0	37.0	25.0
W14-2a	5.5	6.0	9.0	6.8
W14-2b	15.5	25.5	39.0	26.7
W14-2c	16.0	25.5	38.5	26.7
W14-2d	12.0	20.5	32.0	21.5
W11-9	21.0	35.0	53.0	36.3
W11-9a	20.0	33.0	53.0	35.3
W11-9b	16.0	29.0	46.0	30.3
W11-9ab	18.0	31.0	50.0	33.0
W8-7	16.0	27.0	40.0	27.7
W8-7a	15.0	26.0	39.0	26.7
W8-7b	9.0	19.0	33.0	20.3
W8-7c	17.0	28.0	41.0	28.7
W8-7bc	10.0	17.0	27.0	18.0
W8-7e	14.0	24.0	36.0	24.7

[a] TY plates (0.3% agar) were inoculated in the center with 5 μl of cells grown overnight on HP medium (4) and incubated at 28°C. The least significant difference values (0.05) for W14-2, W11-9, and W8-7 were 1.0, 1.4, and 1.1, respectively.

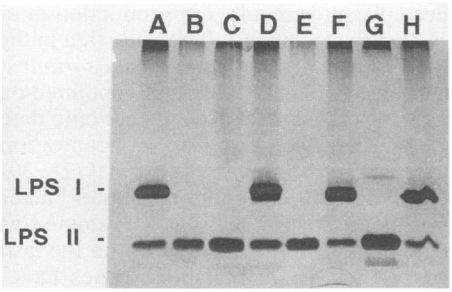

FIG. 2. Gel showing LPS production by strain W8-7 (A), strain W8-7 cured of pRtrW8-7b and pRtrW8-7c (B), strain W8-7 cured of pRtW8-7b (C), strain VF39 (D), strain VF39 cured of the plasmid coding for LPS I (E), strain W14-2 (F), strain W14-2 cured of pRtrW14-2a (G), and strain W14-2 cured of pRtrW14-2d (H).

normal colony morphology and a nonflocculating phenotype in TY broth and also restored motility. Similar complementation results were obtained when the LPS clone pDe127 (6) was used to restore pRtrW14-2a-cured strains to a wild-type LPS phenotype. Since the DNA carried on pDe127 complements the LPS, motility, and nitrogen fixation defects of all of the plasmid-cured LPS-negative strains we have studied (strain LRS39301, 336 minus pRle336d, and LRS55201 [17]; W14-2 missing pRtrW14-2a [this study]), this one locus apparently influences all of these phenotypes. As W14-2 was nitrogen fixation negative in plant tests, it was not possible to test whether complementation of the LPS phenotype affected nitrogen fixation in this strain. However, a causal relationship between these phenotypes and between the LPS and motility phenotypes has been established in other strains (6, 17, 21).

Electrophoretic mobility of enzymes. Of the 26 enzymes stained for in gels, 4 (mannitol-phosphate dehydrogenase, mannose-phosphate isomerase, acid phosphatase, and catalase) were not detected. Of the remaining 22 enzymes, the activities of 3 enzymes were influenced by curing plasmids. Superoxide dismutase (Fig. 3A) was not detected in gels after curing of pRtrW8-7e (lane o), pRtrW11-9b (lane h), pRtrW11-9a and pRtrW11-9b (lane i), and pRtrW14-2d (lane e). Hexokinase and carbamate kinase activities were present as two bands in strain W11-9 (Fig. 3B and C, lanes f and g): a strong band and a weak band. The weak band was eliminated after curing of pRtrW11-9b (Fig. 3B and C, lane h) or pRtrW11-9a and pRtrW11-9b (lane i).

Nitrate utilization. Strain W8-7 cured of pRtrW8-7e and strain W11-9 cured of pRtrW11-9b or pRtrW11-9a and pRtrW11-9b were unable to grow on any carbon source when nitrate was the source of fixed nitrogen. The addition of ammonium allowed growth of the derivatives. A test for nitrite (14) on cultures of the cured derivatives indicated the presence of nitrite. Nitrite was not detected in cultures of the wild type or in cultures of cured derivatives that could utilize nitrate.

Symbiotic characteristics. Since W14-2 was ineffective in dinitrogen fixation (Table 5), curing of plasmids could not influence this phenotype. The data indicate that curing of pRtrW14-2d resulted in loss of nodulation. Loss of pRtrW14-2a resulted in fewer nodules being formed (as compared with the number formed by the wild type) 10 and 17 days after planting. Curing of pRtrW11-9a alone or in combination with pRtrW11-9b resulted in the loss of nodulating ability (Table 5). Curing of pRtrW11-9b alone did not influence the number of nodules formed or significantly

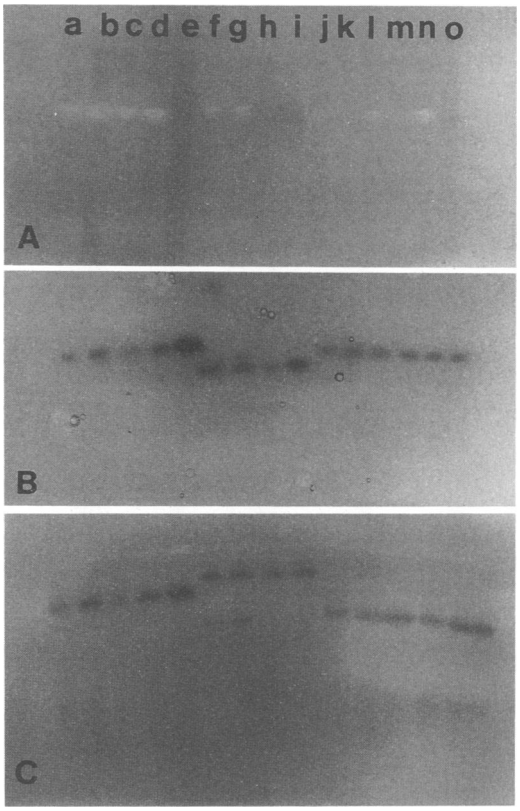

FIG. 3. Gels showing electrophoretic variation in three enzymes: superoxide dismutase (A), carbamate kinase (B), and hexokinase (C). Lanes a through e are electromorphs of strain W14-2 and derivatives cured of pRtrW14-2a, b, c, and d, respectively. Lanes f through i electromorphs of strain W11-9 and derivatives cured of pRtrW11-9a, b, and a and b, respectively. Lanes J through o are electromorphs of strain W8-7 and derivatives cured of pRtrW8-7a, b, c, b and c, and e, respectively.

reduce effectiveness of nitrogen fixation. Curing of pRtrW8-7c (pSym) resulted in the loss of nodulating ability (Table 5). Curing of plasmid pRtrW8-7b resulted in significantly fewer nodules being formed at 10 days after inoculation, but the number of nodules increased by day 18. However, there was a significant reduction in nitrogen fixation. Curing of pRtrW8-7a also reduced nitrogen fixation.

DISCUSSION

The numbers and sizes of plasmids detected in these strains were within the range of those reported in the literature for strains of *R. leguminosarum* bv. trifolii (9, 25, 38). The largest plasmid present in strain W11-9 was larger than any reported for *R. leguminosarum* bv. trifolii, but similar to some reported for *R. leguminosarum* bv. phaseoli (3) and *R. meliloti* (23). The occurrence of *nif* genes on a large plasmid such as pRtrW14-2d (460 MDa) was unusual; the sizes of pSyms reported in the literature for strains of clover rhizobia range between 130 and 200 MDa (15, 16, 38). The results for curing plasmids in the three strains of clover rhizobia demonstrated that the approach devised by Hynes et al. (18) was effective in eliminating most plasmids (Fig. 1).

It is not apparent why curing of pRtrW11-9c and pRtrW8-7d was not successful. Perhaps they were cured but

TABLE 5. Effectiveness and number of nodules formed on crimson clover by cured derivatives of clover rhizobia strains W14-2, W11-9, and W8-7 10 and 17 days after inoculation

Strain or derivative	Plant dry wt[a] (mg)	No. of nodules on day:	
		10	17
W14-2	12.3	2.4	11
W14-2a	11.3	0.5[b]	4[b]
W14-2b	14.5	1.6	8
W14-2c	12.5	2.7	11
W14-2d[c]	13.7	0.0[b]	0[b]
W11-9	61.3	1.3	10
W11-9a[c]	15.5[b]	0.0[b]	0[b]
W11-9b	44.4	1.6	10
W11-9ab	13.0[b]	0.0[b]	0[b]
W8-7	72.0	2.8	9
W8-7a	42.0[b]	2.0	8
W8-7b	27.0[b]	1.6[b]	8
W8-7c[c]	15.5[b]	0.0[b]	0[b]
W8-7bc[c]	10.0[b]	0.0[b]	0[b]
W8-7e	49.5	2.1	7
Uninoculated control	12.8	0.0	9

[a] Values are average for six plants.
[b] Significantly ($P = 0.05$) less dry matter or fewer nodules relative to the respective wild type values.
[c] Derivative cured of symbiotic plasmid.

cells died because the plasmids were essential for survival, or perhaps the plasmids are refractory to curing agents because they carry vital functions (31). These plasmids may be analogous to pRleVF39e, which was extremely difficult to cure and is absolutely required for growth of *R. leguminosarum* bv. viciae VF39 on minimal media and whose absence results in derivatives that grow at half the wild-type rate even on complete media (17).

The phenotypes expressed by plasmid-cured derivatives suggest that the ability to utilize certain carbon compounds may be plasmid encoded. All plasmids in strain W14-2 were shown to carry genes for utilization of at least one carbon compound; this was not the case for the other two strains. Some plasmids, such as pRtrW14-2a, pRtrW14-2c, pRtrW11-9b, and pRtrW8-7c, seemed to carry genes encoding the utilization of more than one carbon source. On the other hand, the loss of pRtrW11-9a and pRtrW8-7a did not affect the ability of the strains to utilize any of the carbon compounds tested. Malate utilization was a plasmid-encoded function common to all three strains studied. It should be pointed out, however, that we are unable to tell whether the lack of growth on particular compounds is the result of the absence of catabolic genes, defective transport, or other factors such as increased sensitivity to a compound as a result of membrane changes.

The presence of a plasmid carrying genes for utilization of the aromatic compound catechol in strain W14-2 may be advantageous for survival in soil. Related compounds are common in soil organic matter and are resistant to decomposition. The availability of simple carbohydrates in soil is very limited. A plasmid carrying genes for the utilization of catechol has also been detected in a *Rhizobium* strain (13).

The phenotypes of strains cured of pRtrW14-2a and pRtrW8-7b on soft agar plates suggested that these plasmids may be involved in the motility of strains W14-2 and W8-7. Loss of these plasmids resulted in production of a defective LPS that lacked the O antigen. It appears that in the majority of strains of all biovars of *R. leguminosarum* some LPS genes are plasmid encoded. It should be pointed out that the motility defects of these LPS mutants are only detectable on media such as TY with a high Ca^{2+} concentration and that the motility on minimal medium is normal (21, 30).

In previous population genetics studies of bacteria, it was generally assumed that the enzymes examined were chromosomally encoded. As we pointed out in the introduction, however, a few important metabolic genes have been detected on plasmids in *Rhizobium* spp. Our results show that curing of pRtrW11-9b resulted in the loss of a faint secondary electrophoretic band for both hexokinase and carbamate kinase. These results suggest that there may be two forms of this enzyme in strain W11-9 (Fig. 3). The two faint bands were also eliminated from W11-9 when this strain was cured of both pRtrW11-9a and pRtrW11-9b. Because the band was still present in strains cured of pRtrW11-9a, its absence in strains cured of both plasmids was probably due to curing of pRtrW11-9b.

A single strong band reflecting superoxide dismutase activity was eliminated after the curing of a single plasmid in each strain. This is the first indication that this enzyme may be encoded on a cryptic plasmid (Fig. 3). It should be noted, however, that the disappearance of an electrophoretic band as revealed by staining with a substrate is not definitive evidence that the enzyme is encoded by a plasmid; it indicates only that the activity of the enzyme was influenced.

Rhizobia are able to utilize nitrate as a their sole nitrogen source (34). This would be expected in soil organisms, since nitrate would be the primary mineral nitrogen source available unless nitrification was inhibited. Plasmids in two of the three strains of rhizobia investigated were involved in utilization of nitrate (Table 4). The presence of nitrite in the culture medium after inoculation and incubation of derivatives cured of pRtrW8-7e or pRtrW11-9b indicated that nitrate reductase was at least partially active and that the plasmids may have been involved with reduction of nitrite. Good growth of strains W8-7 and W11-9 and derivatives on ammonium-supplemented medium indicated the medium was adequate and growth was dependent on the nitrogen source. There are no published reports that plasmids in rhizobia were involved in the utilization of nitrate as an assimilatory nitrogen source.

The data in Table 5 clearly show the involvement of the pSym of each strain in nodulation. Curing of the pSym resulted in loss of nodulating ability. Curing of pRtrW14-2a, pRtrW8-7a, or pRtrW8-7b seemed to influence the nodulating ability or effectiveness in fixing nitrogen. Brink et al. (4) have also shown reduced nitrogen fixation and nodulation phenotypes of LPS mutants on clover. This is consistent with a role of pRtrW14-2a and pRtrW8-7b in LPS production. Previously, Hynes and McGregor (17) reported that plasmids (one being the plasmid carrying LPS genes) other than the pSym in *R. leguminosarum* influenced nodulation and dinitrogen fixation in peas (*Pisum sativum*) and lentils (*Lens culinaris*). Martinez-Romero and Rosenblueth (20) also reported influences of a non-pSym on competitive ability for nodulation of beans (*Phaseolus vulgaris*). Restoration of plasmids and plasmid function into our cured derivatives is required to provide definitive evidence for involvement of cryptic plasmids in symbiosis, because individual clones from the same culture may vary in nitrogen-fixing ability (37). However, we are reasonably confident that plasmids other than the pSym in strains W14-2 and W8-7

affect symbiosis because of the LPS results described and because we have also detected reiterated N *fix* genes on plasmids pRtrW14-2a and pRtrW8-7b (16a).

Overall, the results indicated that insertion of *sac* genes into the plasmids provided an effective method for selection of plasmid-cured derivatives of clover rhizobia on sucrose medium. Nevertheless, not all plasmids were successfully eliminated from the cell. Systematic elimination of plasmids from a cell provided opportunities to determine phenotypes associated with loss of plasmids. This was particularly useful for nonsymbiotic or cryptic plasmids, whose phenotypes are difficult to detect. Using this technique, we were able to demonstrate involvement of nonsymbiotic plasmids in utilization of simple sugars and dicarboxilic acids, utilization of nitrate, activity of specific enzymes, motility, and symbiotic function. Future research in our laboratory will be directed toward confirming the role of the cured plasmids by restoring them into cells and analyzing for restoration of phenotype.

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