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Megaplasmids in the Plant-Associated Bacteria *Rhizobium meliloti* and *Pseudomonas solanacearum*

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Using analytical and preparative methods, we demonstrated the presence of megaplasmids with molecular weights larger than 450×10^6 in the two plant-associated bacteria *Rhizobium meliloti* and *Pseudomonas solanacearum*. Such giant plasmids were found in 8 of 9 *P. solanacearum* strains tested and in all of the 18 *R. meliloti* strains tested.

By using agarose gel electrophoresis, high-molecular-weight plasmids have been found previously, in various strains of the *Pseudomonadaceae* and *Rhizobiaceae* families (2, 5, 6, 12, 13, 19, 20, 21). The plasmid extraction procedures used involved enrichment in supercoiled DNA and DNA concentration steps, which are likely to cause significant damage to very large covalently closed circular (CCC) DNA molecules. In two plant-associated bacterial species, the nitrogen-fixing root nodule bacterium *Rhizobium meliloti* and the plant pathogenic bacterium *Pseudomonas solanacearum*, genetic evidence suggested the presence of plasmids which could not be reproducibly detected by these extraction techniques.

A simple method of screening for plasmid DNA has been described by Eckhardt (4). This method, which involves direct lysis of bacteria on top of an agarose gel just before electrophoresis, has been used mainly in the study of low-molecular-weight plasmids such as recombinant plasmids or those present in medical isolates. Kondorosi et al. (8) devised slight modifications of this procedure, which allowed the reproducible detection of a low-mobility plasmid band in addition to the band already described in *R. meliloti* 41 (2).

In this report we demonstrate the general presence of large plasmids with molecular weights larger than 300×10^6 (megaplasmids) in *R. meliloti* and *P. solanacearum*, using a similar analytical method as well as a preparative one (16).

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To improve the reproducibility of megaplasmid detection, the Eckhardt procedure was slightly modified. Most of the modifications used for *Rhizobium* sp. were devised by Kondorosi (personal communication). Exponentially growing cells (5×10^7 cells per ml) from a liquid culture of *Rhizobium* sp. in TY medium (2) or fresh colonies of *P. solanacearum* on BG medium (1a) were washed in 0.5 ml of a 0.1% Sarkosyl solution in TE8 buffer (0.05 M Tris–0.02 M EDTA, pH 8), rinsed with TE8, and suspended in 40 μl of lysozyme mixture composed of lysozyme (7,500 U/ml), RNase I (0.3 Kunitz units per ml), 0.05% bromophenol blue, and 20% Ficoll 400 in the Tris-borate buffer (TBB) defined by Meyers et al. (12): 89 mM Tris base–2.5 mM disodium EDTA–89 mM boric acid, pH 8.2. The bacterial suspension was rapidly transferred to the slot of a vertical 0.7% agarose gel in TBB (3 mm by 140 mm by 175 mm). The cell suspension was kept at room temperature for 10 min to allow lysozyme action. Lysis was completed by adding 40 μl of a sodium dodecyl sulfate (SDS) solution in TBB containing 10% Ficoll 400. The SDS concentration used was 0.2% for *Rhizobium* sp. and 2% for *P. solanacearum*. The two layers were partly mixed together by moving a toothpick twice from side to side in each slot. One hundred microliters of overlay mixture (0.2% SDS for *Rhizobium* sp. or 2% SDS for *P. solanacearum* in TBB containing 5% Ficoll 400) was added to each slot, which was then sealed with 0.7% agarose in TBB. Electrophoresis was performed for 1 h at 8 mA and for 3 h at 40 mA. After ethidium bromide (EtBr) staining of the gel, the DNA was visualized under UV light (2).

Preparative extraction of plasmids was performed by the method of Schwinghamer (16),

with the following modifications: 200 ml of a culture of exponentially growing bacteria (5×10^8 cells per ml) in TY broth for *Rhizobium* sp. or in BG broth for *P. solanacearum* was centrifuged. The bacterial pellet was washed with 0.1% Sarkosyl in TE8 buffer and rinsed with TE8. The final cell pellet was suspended in 6 ml of 25% sucrose in TE8 and split into four fractions. To each fraction, 0.5 ml of a fresh lysozyme solution (2 mg/ml in TE8) was added immediately before adding 16 ml of TE8. After 15 to 30 min at room temperature, 1.25 ml of an aqueous solution of EtBr (10 mg/ml) was added, and lysis was completed by the addition of 2 ml of 10% Sarkosyl solution in TE8. The lysate was gently mixed, and 20.2 g of CsCl was added and dissolved slowly. Each fraction was divided between two polycarbonate tubes and centrifuged for 16 h at 35,000 rpm in a Beckman 50.2 Ti ultrarotor. After the band of linear DNA was withdrawn, the CCC DNA from all the tubes was pooled and respun in a VTi 80 ultrarotor for 4 h at 65,000 rpm to remove any contaminating chromosomal DNA. When prepared for digestion by restriction endonucleases, after removal of EtBr, the DNA from the CCC fraction was treated with phenol saturated with 20× SSC (3 M NaCl, 0.3 M sodium citrate), followed by chloroform extraction and dialysis against 10 mM Tris (pH 8).

The Eckhardt method modified for *Rhizobium* sp. was first applied to strains RCR2011 and

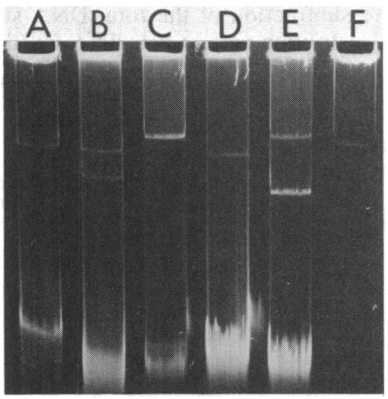

FIG. 1. Agarose gel electrophoresis of lysates from *P. solanacearum* GMI2000 (A) and K60 (F); *Agrobacterium tumefaciens* C58 (B) (the lower band is the 130 $\times 10^6$-dalton Ti plasmid, and the upper band is a cryptic pAt-C58 plasmid of 273×10^6 daltons); *R. meliloti* L5-30 (E) and its L5-30C*a*1 derivative cured of the 90×10^6-dalton pRme-L5-30*a* plasmid (C); and *Pseudomonas putida* PpS1239 harboring the 312×10^6-dalton pMG1 plasmid (D). (A) and (F), Eckhardt procedure modified for *P. solanacearum*; (B), (C), (D), and (E), Eckhardt procedure modified for *Rhizobium* sp.

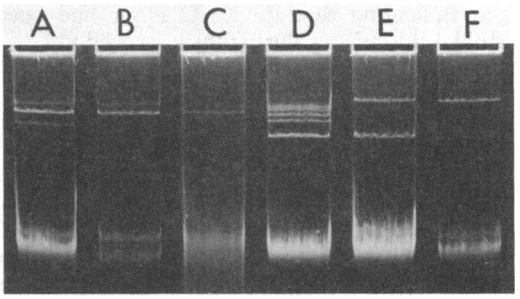

FIG. 2. Agarose gel electrophoresis of lysates from *R. trifolii* T12 (A) and its derivative which was unable to nodulate clover and was cured of pWZ2 plasmid (B); *A. tumefaciens* C58-C1 (C), carrying the reference pAt-C58 plasmid of 273×10^6 daltons; *R. leguminosarum* JB300 (D); and *R. meliloti* L5-30 (E) and A145 (F). Lysis and electrophoresis were performed by the Eckhardt procedure modified for *Rhizobium* sp.

A145, in which no plasmid could be detected by an alkaline denaturation method (2), and strain L5-30, in which a plasmid with a molecular weight of 90×10^6 (pRme-L5-30*a*) had already been described (2) and in which another CCC DNA band was occasionally detected. From the lysates of the three strains, a DNA band of low electrophoretic mobility was reproducibly seen (Fig. 1, lane E; Fig. 2, lanes E and F). These low-mobility bands were free of chromosomal DNA since when blotted from the gel onto a nitrocellulose sheet (18), they did not hybridize with a ^{32}P-labeled chromosomal *met*::Tn*5* DNA fragment from strain RCR2011 (from the pRmR5 plasmid provided by G. B. Ruvkun and F. M. Ausubel) (Fig. 3, lanes A and B). However, they did hybridize with a nonchromosomal DNA fragment from the pRmR2 plasmid (15), carrying *R. meliloti* RCR2011 *nifDH* genes (15) (Fig. 3, lanes A and B). Furthermore, the low-mobility DNA band was still present in strain L5-30C*a*1 which was cured of the pRme L5-30*a* plasmid (Fig. 1, lane C), indicating that the slowly migrating band was not a multimer of the smaller plasmid already described.

To determine whether the low-mobility bands in strains RCR2011, A145, and L5-30C*a*1 correspond to CCC DNA molecules, we tried to isolate them by a cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium density gradient centrifugation. Repeated attempts to isolate CCC DNA from strain RCR2011 by alkaline denaturation procedures were unsuccessful. On the contrary, when bacteria were lysed by the Schwinghamer procedure (16) and centrifuged in CsCl-EtBr gradients, a CCC DNA band was isolated from the three strains. This CCC DNA was submitted to agarose gel electrophoresis after EtBr removal and dialysis. Only one band that corresponded to linear DNA entered the

gel, indicating that the CCC DNA had been broken into pieces during these procedures.

The *Hin*dIII restriction pattern of CCC DNA from L5-30C*a*1 was compared with that of the smaller plasmid, pRme-L5-30*a*, isolated from wild-type strain L5-30 by an alkaline denaturation procedure (7). The two patterns were clearly different (data not shown), confirming that strain L5-30 contained, in addition to the 90 $\times$ 10^6-dalton pRme-L5-30*a* plasmid, another plasmid which was not isolated by previous procedures.

The electrophoretic mobility of the *R. meliloti* CCC DNA upper bands is clearly less than that of known large plasmids used as markers (pAt-C58 [Fig. 1, upper band, lane B] and pMG1 [Fig. 1, lane D], reported to have molecular weights of 273 $\times$ 10^6 [R. Villaroel and M. van Montagu, personal communication] and 312 $\times$ 10^6 [5], respectively). A negative logarithmic correlation has been demonstrated between electrophoretic mobility and molecular weight for CCC DNA molecules up to 180 $\times$ 10^6 (2, 12). Since extrapolations of this equation for larger molecules give underestimations of the molecular weight (2), these slowly migrating bands must represent plasmids much greater than 450 $\times$ 10^6.

Using this modified Eckhardt procedure, we looked for megaplasmids in 16 other *R. meliloti* strains of various geographical origins (2). In every strain, in addition to the smaller plasmids previously described (2), one megaplasmid of similar electrophoretic mobility was found.

To investigate whether plasmids with molecular weights greater than 450 $\times$ 10^6 were also present in other *Rhizobium* fast-growing species, we analyzed some *Rhizobium leguminosarum* and *Rhizobium trifolii* strains (Table 1) by the modified Eckhardt procedure. In *R. leguminosarum* JB300 (Fig. 2, lane D) we observed two low-mobility bands in addition to the bands already described (2, 6). With an alkaline denaturation procedure (6), these two bands were visible only in occasional preparations. Their molecular weights were clearly larger than that of pAt-C58 (273 $\times$ 10^6). *R. trifolii* T12 has been reported to carry two large plasmids (21). Again, in this strain, the use of our method revealed the presence of an additional plasmid, which was probably greater than 300 megadaltons (Fig. 2, lane A); this upper band was still present in a Nod$^-$ (nonnodulating) derivative cured of the smallest plasmid (Fig. 2, lane B).

Genetic evidence for extrachromosomal elements in the plant pathogenic bacterium *P. solanacearum* (10) had not until now been confirmed by physical data. Using the Eckhardt procedure modified for *P. solanacearum*, we observed a slowly migrating band in eight of the nine strains investigated: GMI2000, K60, 40, S210, S208, S238, and 199 (Fig. 1, lanes A and F). No CCC DNA band was detected in strain S205. Lysis of strains GMI2000 and K60 by the modified Schwinghamer procedure followed by CsCl-EtBr gradient centrifugation led to the appearance of two DNA bands. The lower one contained CCC molecules since after shearing and a second round of centrifugation, only the upper band remained. Additionally, in *P. solanacearum* strain K60, the CCC DNA was a discrete subfraction of the total DNA since its *Eco*RI restriction pattern was different (data not

TABLE 1. Bacterial strains

Bacterial species	Strain no.	Isolated from:	Geographical origin	Reference or source
A. tumefaciens	C58; C58-C1			(2)
E. coli	HB101(pRmR2)			(15)
	HB101(pRmR5)			F. M. Ausubel
P. putida	PpS1239(pMG1)			(5)
P. solanacearum	GMI2000	Tomato	Guyana	(11)
	K60	Tomato	United States	(10)
	S205	Potato	Colombia	L. Sequeira
	40	Banana	Honduras	
	S210	Plantain	Colombia	
	S208	Potato	Colombia	
	85	Tomato	Canada	(7, 17)
	S238	Potato	Australia	
	199	Tobacco	Philippines	
R. leguminosarum	JB300			(6)
R. meliloti	L5-30, 41, 311, A145, B294, Ls2A, Ve8, RCR2011, 1322, RF22, 12, V7, S14, Balsac, 102F51, 102F28, L5-30C*a*1 S26, U45			(2)
				This paper
R. trifolii	T12, T12 Nod$^-$			(21)

shown) from the *Eco*RI restriction pattern of the total DNA extracted according to Roussel and Chabbert (14).

These results demonstrate that *P. solanacearum* also contains a plasmid which could not be detected by previous techniques and which probably has a molecular weight much greater than 450×10^6. It should be noted that the modified Eckhardt procedure reproducibly gives clear CCC DNA bands of megaplasmids in *R. meliloti* (Fig. 3), whereas in *P. solanacearum*, the megaplasmid bands are less easily detected. Increasing the SDS concentration from 0.2 to 2% in the lysing and overlay mixtures improves the detection of CCC DNA in *P. solanacearum*, probably by inhibiting DNase (s), without affecting lysis of the bacteria, which appears complete with 0.2% SDS. The presence of DNase(s) could possibly explain the negative result obtained with strain S19.

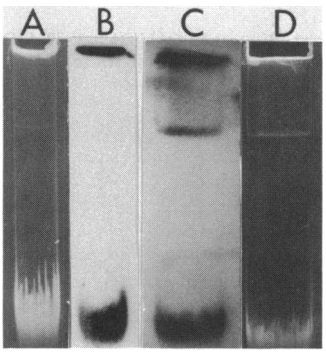

FIG. 3. Hybridization experiments. Lanes A and C show the megaplasmid band of strain RCR2011 in an agarose gel. The DNA was transferred from those gels onto nitrocellulose (18) and then was hybridized with probes consisting of cloned fragments of RCR2011 genome labeled with [^{32}P]GTP by nick translation (15). The DNA from lane A was hybridized with a chromosomal *met*::Tn*5* fragment cloned in pBR322 by G. B. Ruvkun and F. M. Ausubel (personal communication). This 10.8-kilobase fragment was obtained by *Eco*RI digestion of plasmid pRmR5, followed by purification in a sucrose gradient. The autoradiogram (lane B) revealed hybridization with only DNA trapped on the top of the gel and with linear DNA fragments. No hybridization was found with DNA transferred from the megaplasmid band. Lane D shows the autoradiogram obtained after hybridization of DNA from lane C with the fragment containing *R. meliloti nifDH* genes. This 3.9-kilobase fragment was separated from pACYC184 vector by agarose gel electrophoresis of the *Eco*RI-digested pRmR2 hybrid plasmid (15). Those genes, when marked by insertion of a transposon, were shown not to be linked to any auxotrophic marker, suggesting that *R. meliloti nifDH* genes may not be located on the chromosome (9). Furthermore, the same *nif* probe was shown to hybridize with restriction fragments from purified *R. meliloti* CCC DNA (3).

In conclusion, DNA isolation procedures that do not involve handling of DNA before the CCC DNA resolution step (by agarose gel electrophoresis or by dye-buoyant density centrifugation) allow isolation of plasmids of very high molecular weight, probably much larger than 450×10^6. Megaplasmids seem to be a rather general feature of the plant-associated bacteria *R. meliloti* and *P. solanacearum*. It has been recently shown that some of the genes controlling symbiotic properties are located on the megaplasmid of *R. meliloti* (1, 3, 8, 13a). It is logical to then ask: do those megaplasmids also control the relationship with the host plant in other *Rhizobium* species and in the plant pathogen *P. solanacearum*?

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