Only One of the Large Plasmids in *Rhizobium leguminosarum* Strain PRE is Strongly Expressed in the Endosymbiotic State

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Plasmid DNA from six strains of *Rhizobium leguminosarum* was separated by agarose gel electrophoresis and showed a large variation in the number and size of the plasmids. Blotting on to nitrocellulose and hybridization with a probe containing *nif* (nitrogen fixation) genes from *Rhizobium meliloti* demonstrated that all strains contain only one plasmid carrying *nif* genes. The molecular weights of the *nif* plasmids ranged between approximately 130×10^6 and 550×10^6 . Using DNA–DNA hybridization no homologous sequences were detected between the *nif* plasmid of strain PRE and the other plasmid present in this strain. Hybridizations with RNA from bacteroids showed that only the *nif* plasmid is strongly expressed in the endosymbiotic state. No selective amplification of plasmid DNA occurs during the transition of free-living bacteria into bacteroids.

INTRODUCTION

Bacteria of the genus *Rhizobium* are able to fix nitrogen in the root nodules of leguminous plants. Conjugation experiments have demonstrated that bacterial genes involved in the formation of root nodules are located on a large plasmid in *Rhizobium leguminosarum* (Johnston *et al.*, 1978). At least part of the structural *nif* (nitrogen fixation) genes of *R. leguminosarum* also appear to be located on a large plasmid (Nuti *et al.*, 1979). *Nif* genes and genes involved in nodule formation are located on one of the plasmids occurring in *R. meliloti* (Dénarié *et al.*, 1981). Brewin *et al.* (1980a) found that nodulating ability and hydrogenase, which enhances the efficiency of nitrogen fixation, were encoded by one of the large plasmids in *R. leguminosarum*. Hooykaas *et al.* (1981) demonstrated that in *R. trifolii*, at least a part of the structural *nif* genes, and host specificity with respect to nodulating ability, are located on one plasmid.

Since *Rhizobium* bacteria contain more than one plasmid, it has been postulated that the larger plasmids may be multimers of the smaller ones (Tichy & Lotz, 1981) or that the plasmids have otherwise homologous sequences. This would render the bacteria polyploid for certain symbiotic genes. Conversely, the plasmids may be different, perhaps carrying complementary information necessary for the establishment of a nitrogen-fixing nodule. Alternatively, only the plasmid carrying the nodulation and *nif* genes, may be important for symbiosis, the other plasmids remaining unexpressed in bacteroids. In this paper we describe experiments which support the last hypothesis.

METHODS

Bacterial strains. The six *Rhizobium leguminosarum* strains used are listed in Table 1. Strains PRE, PF2, TOM, S310a and P8 are from the Laboratory of Microbiology, Wageningen, The Netherlands. Strain 1001 originates

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from the Rothamsted collection and was obtained from Dr R. K. Prakash, Department of Biochemistry, Leiden, The Netherlands. All strains except P8 are Nod⁺ and Fix⁺ on *Pisum sativum* cv. Rondo.

Cultivation of Rhizobium bacteria and pea plants and isolation of bacteroids. These were done as described by Krol *et al.* (1980).

Plasmid isolation and agarose gel electrophoresis. These were done as described by Casse *et al.* (1978).

Isolation of plasmid DNA from agarose gels. Large plasmid DNA was isolated from a 0.6% (w/v) low-melting agarose gel (Marine Colloids, Rockland, U.S.A.). Plasmid bands were cut out, made up to 0.5 M-NaCl (from a 5 M stock solution), melted at 65 °C, extracted with phenol/cresol (phenol/*m*-cresol/hydroxyquinoline; 1000:140:0.4, by wt) (Wieslander, 1979), and the DNA precipitated with 2 vol. ethanol at -20 °C. To remove traces of agarose from the DNA the pellet was dissolved in a small volume of 0.25 M-ammonium acetate, centrifuged for 5 min at 18000 g and the DNA again precipitated from the supernatant with 2 vol. ethanol at -20 °C.

Southern blotting of large plasmid DNA. Prior to blotting intact plasmids were fragmented by incubating the gel in 0.25 M-HCl for 20 min at 20 °C (Wahl *et al.*, 1979). The gel was transferred to 0.5 M-NaOH, and blotting on to nitrocellulose filters (Sartorius, 0.1 µm pore size) was performed essentially according to Southern (1975).

Preparation of filters for hybridization. Total cellular DNA from free living bacteria and from bacteroids was isolated as described before (Krol *et al.*, 1980). After 10 min at 100 °C in 0.1 × SSC (SSC = 0.15 M-NaCl, 0.015 M-sodium citrate pH 7.0), portions containing DNA (10 µg) were made up to 6 × SSC and filtered three times through a nitrocellulose filter (Sartorius, 0.1 µm). The filters were then baked at 80 °C for 2 h.

Plasmids pSA30 and pRmR1. These were obtained from Dr F. M. Ausubel, Harvard University, Cambridge, Mass., U.S.A. Plasmid pSA30 contains the HDKY operon of *Klebsiella pneumoniae*, of which gene *H* codes for the subunits of component II, and genes *D* and *K* for the α and β subunits of component I of nitrogenase (Cannon *et al.*, 1979). Plasmid pRmR1 contains DNA from *R. meliloti* homologous to pSA30. Plasmids were isolated as described before (Krol *et al.*, 1980) and *in vitro* labelled with ³²P by nick-translation (Rigby *et al.*, 1977).

Total cellular RNA from bacteroids. This was isolated as described by Krol *et al.* (1980) and *in vitro* labelled with ³²P according to Goldbach *et al.* (1978).

Hybridizations. DNA blots were prehybridized at 63 °C for at least 6 h in 3 × SSC containing SDS (0.1%, w/v), BSA (2 µg ml⁻¹), polyvinylpyrrolidone (2 µg ml⁻¹) and Ficoll (2 µg ml⁻¹) (Denhardt, 1966), supplemented with denatured low molecular weight calf thymus DNA (50 µg ml⁻¹) and poly(A) (10 µg ml⁻¹) (for DNA-DNA hybridizations) or yeast RNA (100 µg ml⁻¹) (for DNA-RNA hybridizations). Hybridization was performed in 1 ml prehybridization buffer with *in vitro* labelled pRmR1, pSA30, *Rhizobium* large plasmid DNA, or bacteroid RNA, at 63 °C for up to 24 h. After hybridization the filters were washed three times for 2 h in 3 × SSC at 63 °C and then dried and autoradiographed using Sakura X-ray film (Konishiroku Photo Ind. Co., Japan). In the DNA-DNA filter hybridization experiments radioactivity was assayed in 8 ml Instafluor (Packard Instruments) in a liquid scintillation counter.

RESULTS

Plasmid content of strains

Six different isolates of *R. leguminosarum* were compared with regard to their plasmid content (Table 1); *R. leguminosarum* PRE is the strain commonly used in our experiments. The other strains may be distinguished from PRE by differences in their symbiotic properties such as the shape of the bacteroids (PF2 and P8), ability to nodulate *P. sativum* cv. Afghanistan (TOM), possession of a hydrogenase (S310a), or absence of nitrogen fixation (P8). Electrophoresis in 0.6% (w/v) agarose gels showed that the six isolates also differ in plasmid content (Fig. 1, lanes *a*). PRE, PF2 and 1001 each carry two plasmids, S310a three, P8 four and TOM probably also four, two of which are probably of similar size (the latter conclusion is based on the much stronger fluorescence of the middle band). The molecular weights of the plasmids in each strain were estimated by comparing their electrophoretic mobility in agarose gels with those of the two plasmids of PRE, for which molecular weights of approximately 230 × 10⁶ and 500 × 10⁶ have been determined (H. V. Tichy & W. Lotz, personal communication). The molecular weights of the different plasmids (Table 1) vary widely, from approximately 130 × 10⁶ (1001) to 600 × 10⁶ (S310a). In five strains a plasmid with a molecular weight of 500–550 × 10⁶ was found, and in four strains a plasmid with a molecular weight of 200–240 × 10⁶. No plasmids of exactly the same size were found in any of the strains.

Table 1. *Properties of the R. leguminosarum strains*

Nod⁺, Fix⁺ and Hup⁺ indicate possession of nodulation ability, nitrogen fixation and hydrogen uptake, respectively.

Strain	10 ⁻⁶ × Approx. size of plasmids	Bacteroid shape	Symbiotic properties on host plant		Remarks
			<i>Pisum sativum</i> cv. Rondo	<i>Pisum sativum</i> cv. Afghanistan	
PRE	230*, 500	Y	Nod ⁺ Fix ⁺ Hup ⁻	Nod ⁻	Broughton <i>et al.</i> (1980)
PF2	240*, 500	Spheroplasts	Nod ⁺ Fix ⁺ Hup ⁻	Nod ⁻	
TOM	180, 220, 220*, 500	Y	Nod ⁺ Fix ⁺ Hup ⁻	Nod ⁺ Fix ⁺ Hup ⁻	Broughton <i>et al.</i> (1980)
1001	130*, 550	Y	Nod ⁺ Fix ⁺	Not tested	Nuti <i>et al.</i> (1977)
S310a	400, 550*, 600	Y	Nod ⁺ Fix ⁺ Hup ⁺	Nod ⁻	Also Nod ⁺ Fix ⁻ on <i>Vicia faba</i> and <i>Lathyrus sylvestris</i>
P8	150, 200, 350, 400*	Small rods	Nod ⁺ Fix ⁻ Hup ⁻	Nod ⁻	

* Indicates plasmid carrying *nif* genes.

Fig. 1. Hybridization of *R. leguminosarum* plasmid DNA with pRmR1. Plasmid DNA from strains PRE, PF2, TOM, 1001, S310a and P8 was separated by agarose gel electrophoresis and stained with ethidium bromide. The UV-fluorescence pattern is shown in the lanes *a*. After blotting on to nitrocellulose the plasmids were hybridized with *in vitro* ³²P-labelled pRmR1 carrying *nif* genes from *R. meliloti*. The autoradiograph is shown in the lanes *b*. The arrows indicate the position of the *nif* plasmids.

Fig. 2. Comparison of the two plasmids of *R. leguminosarum* strain PRE. The two plasmids of PRE were separated by agarose gel electrophoresis and stained with ethidium bromide; the UV-fluorescence pattern is shown in lane *b*. After blotting on to nitrocellulose the plasmids were hybridized with *in vitro* ³²P-labelled pRlPa, isolated from an agarose gel (lane *a*).

Hybridization studies

To determine which of the plasmids in each strain carries *nif* genes the plasmids were blotted on to nitrocellulose filters and hybridized with *in vitro* labelled pRmR1. pRmR1 is a recombinant plasmid containing a 3.9 kb insert with at least a part of the structural *nif* genes of *R. meliloti* (Ruvkun & Ausubel, 1980). The autoradiograph (Fig. 1, lanes *b*) shows that in each isolate only one plasmid hybridized with pRmR1, suggesting that only one of the plasmids carries *nif* genes. There is a large variation in size between the plasmids carrying *nif* genes: in 1001 the *nif* plasmid is nearly half the size of the *nif* plasmid of PRE, while in S310a it is more than twice as large. Also, strain P8 appeared to have a *nif* plasmid, although it is Fix⁻ on all hosts tested.

Comparison of pRlPa and pRlPb

The two plasmids detected in strain PRE were compared in more detail. The *nif* plasmid of PRE (referred to as pRlPa), was isolated from an agarose gel, labelled *in vitro* with ³²P and used for hybridization with the two separated PRE plasmids blotted on to nitrocellulose. The autoradiograph, shown in Fig. 2, shows that pRlPa only annealed to itself, but not to the larger plasmid (referred to as pRlPb). This indicates the absence of appreciable homology between

Fig. 3

Fig. 4

Fig. 3. Autoradiographs showing hybridization of isolated plasmids of *R. leguminosarum* strain PRE with RNA from bacterioids. Plasmids pRIPa and pRIPb (see Fig. 2) were isolated from an agarose gel and digested with *EcoRI* (lanes *b* and *c*). The fragments were separated by agarose gel electrophoresis, blotted on to nitrocellulose and hybridized with *in vitro* ^{32}P -labelled RNA from bacterioids. The autoradiographs are shown in lanes *b* and *c*. For comparison total DNA from free-living bacteria was digested with *EcoRI* and after separation by agarose gel electrophoresis, hybridized with *in vitro* ^{32}P -labelled ribosomal RNA from free living bacteria (lane *a*). The arrow at the left indicates the position of the fragment of chromosomal DNA strongly hybridizing with ribosomal RNA. The arrows at the right indicate the position of phage λ DNA *HindIII* fragments used as molecular weight markers.

Fig. 4. Hybridization of plasmid DNA of *R. leguminosarum* strain PRE with RNA from bacterioids. The two plasmids of PRE were separated by agarose gel electrophoresis and stained with ethidium bromide. The UV-fluorescence pattern is shown in lane *b*. After blotting on to nitrocellulose the plasmids were hybridized with *in vitro* ^{32}P -labelled RNA from endosymbiotic bacterioids. The autoradiograph is shown in lane *a*.

the two plasmids. The hybridization of pRIPb with a blot of the separated plasmids was not done, because pRIPb is contaminated with pRIPa. This is due to breaking of supercoiled plasmids, causing tailing towards the origin. Therefore a preparation of pRIPb will always show some hybridization with pRIPa.

Table 2. Hybridization of pSA30 with total DNA from *R. leguminosarum* PRE bacteria and bacteroids

Total DNA from bacteria and bacteroids was blotted on to nitrocellulose filters (10 µg DNA per filter), prehybridized with calf thymus DNA and hybridized with *in vitro* labelled pSA30 (specific activity 7×10^6 d.p.m. µg⁻¹) in $3 \times$ SSC at 63 °C for the time indicated. After washing in $3 \times$ SSC the filters were counted in a liquid scintillation counter. The figures are corrected for background.

DNA on filter	Amount of pSA30 hybridized to DNA on filters [d.p.m. (µg DNA) ⁻¹]		
	Time of hybridization		
	7 h	14 h	24 h
Total bacterial DNA	43.2	99.0	140.9
Total bacteroid DNA	42.7	84.8	132.8

Transcription of plasmids

To examine if the extensive transcription of plasmid-borne genes which we found previously (Krol *et al.*, 1980) is from a single plasmid or from both, the two plasmids of PRE were isolated from an agarose gel. The amount of pRIPb obtained from a gel is less than the amount of pRIPa: in this experiment approximately 0.5 µg pRIPb and 1 µg pRIPa were isolated. *Eco*RI digests of the plasmids were separated in a 0.7% (w/v) agarose gel, blotted on to nitrocellulose filters and hybridized with *in vitro* ³²P-labelled RNA from bacteroids. The autoradiograph is shown in Fig. 3, lane *c*. Many fragments of pRIPa (the *nif* plasmid) hybridize, the pattern being in agreement with that obtained earlier by hybridization of bacteroid RNA with plasmid DNA isolated by CsCl–ethidium bromide centrifugation (Krol *et al.*, 1980). In lane *b*, which shows the *Eco*RI fragments from pRIPb, the weaker hybridization is probably due to contamination of pRIPb with pRIPa as indicated above, since the pattern of bands in lane *b* corresponds exactly to that of the most strongly hybridizing bands in lane *c*. The more weakly hybridizing bands in lane *c* are not, or hardly, visible in *b*. An exception is the band a little below the position corresponding to 2.8×10^6 which is more intense in lane *b* relative to the other bands than in lane *c*. In lane *a* of Fig. 3 the hybridization of *Eco*RI-digested total bacterial DNA with purified bacterial ribosomal RNA is shown. It can be seen that ribosomal RNA hybridized strongly to a fragment with a molecular weight of approximately 2.8×10^6 , presumably the location of the ribosomal RNA gene on the chromosome. In the *Eco*RI pattern of pRIPa and pRIPb a fragment of similar size also hybridizes strongly with the ribosomal RNA, indicating that the isolated plasmids are contaminated with chromosomal DNA. Therefore the hybridization observed in lane *b* of Fig. 3 must be attributed to contamination of pRIPb with pRIPa and chromosomal DNA. No specific hybridization of bacteroid RNA with pRIPb is detectable, suggesting that the large plasmid is not expressed in root nodules. This conclusion was confirmed by an experiment in which plasmids were separated in an agarose gel, blotted on to nitrocellulose and hybridized with ³²P-labelled bacteroid RNA. The autoradiograph, shown in Fig. 4, exhibits hybridization only with pRIPa.

No selective amplification of *nif* plasmid DNA

The use of cloned *nif* genes makes it possible to examine whether a small amplification of the *nif* plasmid might occur during the differentiation of bacteria into bacteroids. Therefore total DNA from bacteria and bacteroids was isolated, blotted on to nitrocellulose filters and hybridized with *in vitro* ³²P-labelled pSA30. The results (Table 2) show that the rates of hybridization of pSA30 with the two DNAs are equal. This indicates that there is no selective amplification of *nif* plasmid DNA.

DISCUSSION

Rhizobium strains have been shown to contain several plasmids varying in size (Tichy & Lotz, 1981). If a CsCl-ethidium bromide gradient is used for the isolation of large plasmids from *Rhizobium* (Casse *et al.*, 1978; Spitzbarth *et al.*, 1979; Nuti *et al.*, 1979; Krol *et al.*, 1980; Dénarié *et al.*, 1981), a mixture of the plasmids present is obtained, in which the larger plasmids will be under-represented, if they occur at all, because they are more sensitive to shearing forces (Dénarié *et al.*, 1981). To study the different plasmids separately we therefore used agarose gel electrophoresis, which makes it possible to detect the presence and the relative amount of the different plasmids by ethidium bromide staining (Fig. 1). The separated plasmids can be blotted on to a nitrocellulose filter, but it is necessary to reduce the size of the DNA before blotting by incubation of the gel in 0.25 M-HCl (Wahl *et al.*, 1979). Such blots make it possible to localize sequences on each of the plasmids by annealing with DNA or RNA probes. Using pRmR1 as a probe we have shown that different strains of *R. leguminosarum*, varying in plasmid content, contain only a single *nif* plasmid. The *nif* plasmids differ remarkably in molecular weight: from 130×10^6 in strain 1001, to 550×10^6 in strain S310a. There is no correlation between the size of the *nif* plasmid and the phenotype of the symbiosis: PRE and 1001 have similar symbiotic properties, but differ by approximately 100×10^6 in the molecular weight of their *nif* plasmids. Conversely, PRE, PF2 and TOM differ in symbiotic properties, but have a *nif* plasmid of approximately the same size. Strain S310a has a very large *nif* plasmid (550×10^6) and also possesses genes for hydrogen uptake (*hup* genes). However, Brewin *et al.* (1980a) located genes involved in the Hup⁺ phenotype on a 190×10^6 plasmid, so that Hup⁺ is not necessarily correlated with a larger size of *nif* plasmid. Strain P8 is Fix⁻, yet possesses a *nif* plasmid with a molecular weight of 400×10^6 ; hence it is unlikely that a large deletion is involved. This strain reduces acetylene at a very low rate (the maximal rate for P8 is less than 0.1% of the maximal rate for PRE) and only for a very short period after nodule formation. It also fails to maintain leghaemoglobin synthesis (T. Bisseling, unpublished results). Apparently P8 is defective not in a *nif* gene but in some other gene involved in establishing effective symbiosis. The significance of the variation in size of the *nif* plasmids from different strains of *R. leguminosarum* therefore remains a mystery as does the function of the other plasmids present. For strain PRE we have demonstrated that there is no detectable homology between the two plasmids, which would have rendered PRE diploid for certain genes. Brewin *et al.* (1980b) found a similar plasmid pattern for strain TOM to that presented here, only their size estimates were smaller than ours. By plasmid transfer experiments they demonstrated that one of the intermediate-sized TOM plasmids carries genes for nodulation and fixation. By blotting of the plasmids and hybridization with pSA30 it was shown that this plasmid also contains nitrogenase genes (Hombrecher *et al.*, 1981). Four other plasmids of different strains of *R. leguminosarum* or *R. phaseoli* showed the same linkage of *nif* genes and nodulating ability. Also in *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981) and in *R. trifolii* (Hooykaas *et al.*, 1981) nodulation genes and nitrogenase genes appear to be located on a single plasmid.

Of the two plasmids of *R. leguminosarum* strain PRE, only the *nif* plasmid is strongly expressed in bacteroids, suggesting that only the *nif* plasmid carries information for the formation of effective nodules. It should be recognized that genes which are only expressed during the early stages of root infection and nodule formation, and also genes showing reduced expression, have probably not been detected in the experiments presented here. Our experiments with strain PRE do not exclude the possibility that with one of the other strains of *R. leguminosarum* or with one of the other *Rhizobium* species, other plasmids, besides the *nif* plasmid, are involved in symbiotic nitrogen fixation. Our results suggest, however, that the information for nodule formation is located on a single plasmid and that, in addition to this plasmid, a variable number of cryptic plasmids may occur in *Rhizobium*.

One reason for the location of symbiotic genes on a single plasmid might be the possibility

of amplification of this plasmid, resulting in the bacteroids being polyploid for symbiotic genes. During the development of *Rhizobium* into mature bacteroids the total DNA content per cell increases five- to sevenfold (Bisseling *et al.*, 1977). Reynders *et al.* (1975) demonstrated that amplification of a part of the DNA during the development of bacteroids does not occur, but since they used reannealing kinetics, a two- or threefold amplification of a small portion of the DNA, e.g. a plasmid, would have remained undetected. Using cloned *nif* genes it was possible to demonstrate that the copy numbers of *nif* genes in bacteria and bacteroids are identical, so that there is no amplification of *nif* plasmids. Thus the strong expression of genes on the *nif* plasmid is apparently not caused by amplification of *nif* plasmid DNA.

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