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Instability of Cryptic Plasmids Affects the Symbiotic Effectivity of *Rhizobium leguminosarum* bv. *viceae* Strains

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A total of fifty-two strains of *Rhizobium leguminosarum* biovar *viceae* were newly isolated from effective root nodules of faba bean plants (*Vicia faba* 'Herz Freya'). When tested with *Pisum sativum* 'Poneka', 20 of the 52 strains showed a significantly reduced ability to fix nitrogen. Such strains were classified as Fix' for this host. Fully effective nodules developed at a frequency of about 2% on pea plants infected with Fix' strains. Segregant strains isolated from such nodules were stably Nod^+Fix^+ with *P. sativum* and *V. faba* and usually had an altered plasmid profile (e.g., deletions of different sizes in a cryptic plasmid). Conjugal transfer of a mobilizable derivative of the genetically unstable cryptic plasmid pRleF41b of Fix' strain *R. leguminosarum* bv. *viceae* F41 into the Fix^+ strain P13 resulted in the co-transfer of the Fix' phenotype. A gene library of the Fix' wild type strain F41 was constructed. Overlapping cosmid clones carrying the wild type DNA sequences from the genetically unstable region of plasmid pRleF41b have been identified. Several lines of experimental evidence suggest that determinants for a host-dependent reduction of nitrogen fixation (*hrf*) are located on a 13-kb *Bam*HI fragment of this 210-kb plasmid.

Additional keywords: host-range, plasmid rearrangements, reiterated sequences, symbiotic nitrogen fixation.

Soil bacteria of the genus *Rhizobium* are able to fix atmospheric nitrogen in symbiosis with leguminous plants (Vincent 1980). The symbiotic interaction is highly specific and a given rhizobial strain nodulates only a restricted number of legumes. Strains of *R. leguminosarum* bv. *viceae* Jordan, for example, normally nodulate species of the plant generae *Pisum*, *Vicia*, *Lathyrus*, and *Lens*, which constitute the pea cross-inoculation group. Depending on the bacterium-host combination, however, there are considerable differences in the effectiveness of such symbiotic associations. Variation in the symbiotic effectiveness was observed when a particular *R. l.* bv. *viceae* strain nodulated plants from different genera of the pea cross-inoculation group (Van den Berg 1977), or different species within a given genus (Van Brussel *et al.* 1982), or even different varieties of one species (Mytton *et al.* 1977; Hobbs and Mahon 1982).

In *Rhizobium meliloti* Dangeard and in the three biovars of *Rhizobium leguminosarum* (*viceae*, *trifolii* Jordan, and *phaseoli* Jordan) most of the genetic determinants for the formation of root nodules (*nod* genes) and nitrogen fixation (*nif* and *fix*) genes are located on large symbiotic plasmids (pSym; reviewed by Prakash and Atherly 1986). Most strains of these *Rhizobium* species, however, contain several large plasmids in addition to their pSym. Very little is known about the functions encoded by the cryptic rhizobial plasmids. In some cases, it has been demonstrated that such plasmids carry genes that are essential for symbiotic nitrogen fixation. Genes that are required for the formation of effective root nodules have been located on a second megaplasmid of *R. meliloti* (Finan *et al.* 1986; Hynes *et al.* 1986). In a strain of *R. l.* bv. *viceae*, genes that were necessary for the formation of nitrogen-fixing nodules have been located on two cryptic plasmids distinct from the pSym of the strain (Hynes and McGregor 1990).

In *R. l.* bv. *viceae*, some plasmids have been shown to code for bacteriocin production (Hirsch 1979) or melanin production (Hynes *et al.* 1988). Some cryptic plasmids were shown to be self-transmissible (Johnston *et al.* 1982) and could be allocated into different incompatibility groups (O'Connell *et al.* 1987). It has been reported that cryptic plasmids may also negatively influence the symbiotic effectivity of a given rhizobial strain. DeJong *et al.* (1981) have reported that the presence of the bacteriocinogenic plasmid pRL3JI in strain *R. l.* bv. *viceae* 300 decreased the effectivity of this strain. For the species *Rhizobium loti*, it has been demonstrated that a strain of *R. loti* Janis *et al*, heat-cured of a large *Lotus pedunculatus* Cav. than its parent strain (Pankhurst *et al.* 1986).

The results presented in this study indicate that functions encoded by cryptic plasmids of *R. l.* bv. *viceae* wild type strains may exert a host-dependent influence on the symbiotic effectivity of these rhizobia.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Strains of *Rhizobium* were grown at 28° C on tryptone-yeast extract (TY) medium (Beringer 1974). *Escherichia coli* strains were grown at 37° C on PA medium (antibiotic medium no. 3; Oxoid Ltd, Basingstoke, England) or Luria-Bertani medium (Miller 1972). The final concentrations of antibiotics were: 40 mg of rifampicin, 100 mg of streptomycin, and 100 mg of

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kanamycin per liter of medium for *Rhizobium*; 20 mg of chloramphenicol, 20 mg of kanamycin, 30 mg of tetracyclin, and 200 mg of ampicillin per liter of medium were used for *E. coli*.

Isolation of *R. l.* bv. *viceae* strains. Fifty-two wild type strains of *R. l.* bv. *viceae* have been isolated from the root nodules of 10 *Vicia faba* 'Herz Freya' plants harvested from a large faba bean field of the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau near Freising in Bavaria, Germany. Only one rhizobial strain per nodule has been isolated (for isolation procedure, see Tichy and Lotz 1981).

Plant test for *R. l.* bv. *viceae* strains. Seeds were surface-sterilized with 0.1% (w/v) mercuric chloride solution. After extensive rinsing with sterile water, the seeds were germinated for three days on agar plates. They were then transferred to 250-ml Erlenmeyer flasks containing vermiculite and 100 ml of nitrogen-free Jensen solution (Jensen 1942). An overnight culture of *Rhizobium* (0.1 ml) was used as inoculum. Plants were grown for 21 days in a growth chamber (day/night temperatures, 20°/16° C [peas], 19°/14° C [faba beans]; light intensity, 6 klx, 16-hr photoperiod; relative humidity, day/night, 60/75%).

Nitrogenase and uptake hydrogenase assays. For the nitrogenase assay, excised roots were placed in 120-ml glass tubes, which were sealed with rubber caps. Ten percent of the atmosphere was replaced with acetylene, and samples were taken after 30 min for ethylene determination by a gas chromatograph (model 5570A, Hewlett-Packard, Avondale, PA). Gas-phase samples were injected into a 1.5-m × 3-mm column of Poropak N with a N_2 carrier and an H_2 flame ionization detector at 150° C. The assay used for uptake hydrogenase (Hup) activity has been described (Tichy and Lotz 1985).

Bacterial matings. For transfer of mobilizable plasmids between strains of *Rhizobium*, donor and recipient cells were grown to log-phase in liquid culture. The rhizobia were mixed with log-phase cells of *E. coli* strain HB101 carrying the helper plasmid pRK2013 (at a ratio of 2:2:1). Matings were carried out on nitrocellulose filters (0.2 μm pore size) on TY plates at 28° C for 16 hr.

Transformation of *E. coli* cells. Competent *E. coli* cells were prepared and transformed with plasmid DNA according to the method of van Die *et al.* (1983).

Agarose gel electrophoresis of plasmids and restriction fragments. The plasmid content of bacterial strains was analyzed by agarose gel electrophoresis with the Eckhardt

Table 1. Bacterial strains and plasmids used in this study

Bacteria	Relevant characteristics	Origin
Escherichia coli		
HB101	*pro*, *leu*, *thi*, *lac*Y, *end*A, *rec*A *hsd*R, *hsd*M, Sm$^{r a}$	Boyer and Roulland-Dussoix 1969
S17-1	*thi*, *pro*, *rec*A, *hsd*R, *hsd*M, RP4-2-Tc::Mu-Km::Tn*7*, Tp$^{r a}$, Sm^{r}	Simon et al. 1983
BHB2688	N205, *rec*A, (λimm434, *cI*ts, b2, *red*3, *Eam*4, *Sam*7)	Hohn 1979
BHB2690	N205, *rec*A, (λimm434, *cI*ts, b2, *red*3, *Dam*15, *Sam*7)	Hohn 1979
Rhizobium leguminosarum bv. *viceae*		
F28	Nod^{+}Fix^{+} phenotype with *Vicia faba and Pisum sativum*	Field isolate from BLBP[b]
F41	Nod^{+}Fix^{+} phenotype with *V. faba*; Nod^{+}Fix′ phenotype with *P. sativum*	Field isolate from BLBP
SF41	Sm^{r} spontaneous mutant of F41	This study
RF41	Rf$^{r a}$ spontaneous mutant of F41	This study
F41-1	Segregant of F41; Fix^{+} with *P. sativum*; loss of pRleF41b	This study
SF411	Integration of plasmid pIBS13::Tn*5* into pRleF41b of strain SF41	This study
P13	Rf^{r} spontaneous mutant of strain PRE Nod^{+}Fix^{+} phenotype with *P. sativum*	Selbitschka and Lotz 1984
P131	Derivative of P13, containing the pRleF41b hybrid plasmid of strain SF411; Nod^{+}Fix′ phenotype with *P. sativum*	This study
P131-1	Segregant of P131; Fix^{+} with *P. sativum*	This study
P131-2	Segregant of P131; Fix^{+} with *P. sativum*	This study
pRK2013	Km^{r}, helper plasmid	Figurski and Helinski 1979
pMMB33	Km$^{r}_{a}$ cos, IncQ	Frey *et al.* 1983
pACYC184	Cm$^{r a}$, Tc$^{r a}$	Chang and Cohen 1978
pSUP202	Cm^{r}, Tc^{r}, Ap$^{r a}$, mob	Simon *et al.* 1983
pGB5	*nif*HD from *Rhizobium leguminosarum* PRE in pSUP201	Schetgens *et al.* 1984
pHF5	*nod*-specific DNA from *R. leguminosarum* B10 in pACYC184	Fees *et al.* 1985
pRlF69	pRleF41b-homologous DNA in pMMB33	This study
pRlF245	pRleF41b DNA in pMMB33	This study
pIBS6	3.4-kbp *Eco*RI fragment of pRlF69 in pACYC184	This study
pIBS7	13-kbp *Bam*HI fragment of pRlF245 in pACYC184	This study
pIBS13	13-kbp *Bam*HI fragment of pRlF245 in pSUP202	This study
pIBS13::Tn*5*	Tn*5*-carrying derivative of pIBS13	This study

[a]Ap^{r}, ampicillin resistant; Cm^{r}, chloramphenicol resistant; Km^{r}, kanamycin resistant; Rf^{r}, rifampicin resistant; Sm^{r}, streptomycin resistant; Tp^{r}, trimethoprim resistant.
[b]Bayerische Landesanstalt für BodenKultur und Pflanzenbau, Freising.

gel technique (Eckhardt 1978). Sizes of plasmids were estimated from their electrophoretic mobility using the plasmids of *R. meliloti* MVII/1 (Burkhardt and Burkhardt 1984) as standards. DNA fragments were separated on 0.7 agarose gels in TA buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA; pH 7.8) at 80 V.

DNA isolation procedures. Large scale plasmid DNA preparations were made by the Brij/DOC lysis (Hardies *et al.* 1979). Plasmid DNA from *Rhizobium* was isolated following the procedure described by Hansen and Olsen (1978). Small-scale plasmid DNA preparations were performed according to Holmes and Quigley (1981). Total DNA of *R. l.* bv. *viceae* for use in cosmid cloning experiments as well as for use in hybridization experiments was prepared according to Gärtner *et al.* (1988).

Construction of *R. l.* bv. *viceae* F41 gene library. Total DNA from *R. l.* bv. *viceae* strain F41 was partially digested with *Sau*3AI and size-fractionated by sucrose gradient centrifugation (Maniatis *et al.* 1982). The size of DNA restriction fragments present in each fraction was determined by electrophoresis in 0.5% agarose gels, and fractions containing DNA fragments of 27–35 kb in size were pooled and used for cloning. Vector "arms" from cosmid pMMB33 were prepared (Frey *et al.* 1983; method B) and ligated with size-fractionated *R. l.* bv. *viceae* F41 DNA. Preparation of packaging extracts from *E. coli* strains BHB2688 and BHB2690 as well as in vitro packaging of aliquots of the ligation product was done as described by Maniatis *et al.* (1982).

Hybridization procedures. Plasmid DNA or purified DNA fragments were labeled with α-[^{32}P]-dATP (3,000 Ci/mmol; Amersham Buchler, Braunschweig, Germany) by nick translation, following the procedure of Rigby *et al.* (1977). Eckhardt gels of *R. l.* bv. *viceae* plasmids, as well as restriction fragments separated in agarose gels, were transferred to nitrocellulose filters (BA 85; Schleicher & Schuell, Dassel, Germany) by the method of Southern (1975). For dot-blot hybridization experiments, plasmid DNA was dotted onto nylon membranes (Biodyne A; Pall, Dreieich, Germany) following the supplied protocols. The DNA hybridization of labeled probes to blots and nylon membranes was carried out for 15 hr at 43° C in 5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's solution (20 mg ficoll 400.000, 20 mg polyvinyl-pyrrolidone, 20 mg BSA per milliliter), 0.1% sodium pyrophosphate, 50 g of denatured calf thymus DNA per milliliter. After hybridization, filters were washed consecutively in the following solutions: three times with 2× SSC, 0.1% sodium dodecyl sulfate (SDS), 20 mM phosphate buffer for 10 min; two times with 1× SSC, 0.1% SDS, 20 mM phosphate buffer for 15 min; and once for 30 min with 0.2× SSC, 0.1% SDS, 20 mM phosphate buffer at 68° C. The dried blots and nylon membranes were autoradiographed at −70° C on X-Omat AR film (Eastman Kodak Co., Rochester, NY) with intensifying screens.

DNA probes used for identification of pSym. Plasmids pGB5 and pHF5 were used as *nif*- and *nod*-specific DNA probes, respectively. Plasmid pGB5 carries the nitrogenase structural genes *nif*HD from *R. l.* bv. *viceae* PRE (Schetgens *et al.* 1984). In plasmid pHF5, a 2.6-kb *Bam*HI fragment of *nod*-specific DNA from *R. l.* bv. *viceae* strain B10 has been cloned into the vector pACYC184 (Fees *et al.* 1985).

RESULTS

Plasmid pattern and host-dependent Fix phenotype of newly isolated *R. l.* bv. *viceae* strains. Fifty-two wild type strains of *R. l.* bv. *viceae* were isolated from individual nodules of faba bean plants (*V. faba* 'Herz Freya'). Analysis of plasmid content revealed that the field isolates were very heterogeneous: three to eight plasmids of different sizes (ranging from 20 kb to more than 1,000 kb) were detected by agarose gel electrophoresis in each strain. The pSym carrying nodulation (*nod*) and nitrogen fixation (*nif*) genes was identified in each strain by Southern hybridization experiments. Only one plasmid per strain hybridized to the *nif*- and *nod*-specific probes (plasmids pGB5 and pHF5, respectively; data not shown)

In nodulation assays, all strains formed effective root nodules on their original host *V. faba* (phenotype Nod$^+$ Fix$^+$). None of the strains expressed an uptake hydrogenase activity (phenotype Hup$^-$). The acetylene-reducing activity of two representative strains, (strains F28 and F41) is shown in Table 2. When tested on pea plants (*P. sativum* 'Poneka') 20 of the 52 isolates showed a significantly reduced ability to fix nitrogen. In this case, numerous small root nodules were formed that showed only a slight pink colour indicating a low content of leghemoglobin. The acetylene-reducing activity of strains F28 and F41 with *P. sativum* is compared in Table 2. The amount of acetylene reduced by strain F41 was only about 5% of that of the Fix$^+$ strain F28. Strains showing reduced nitrogen fixation effectivity for *P. sativum* were classified as Fix′. Figure 1 shows the root nodules of a pea plant induced by Fix′ strain F41.

To investigate the host range of the Fix′ phenotype, strain F41 was tested with other host plants of the pea cross-inoculation group (*Lathyrus sativus* L., *Lens culinaris*

Table 2. Acetylene reduction activity of wild type strains *Rhizobium leguminosarum* bv. *viciae* F28, F41, P13, and their derivatives nodulating different host plants of the pea cross-inoculation group

R. leguminosarum bv. *viciae*	Host plant	C_2H_4 production μmoles/h/plant[a]	Fix-phenotype
F28	*Vicia faba*	4.50 ± 0.84	Fix$^+$
	Pisum sativum	4.94 ± 1.06	Fix$^+$
F41	*V. faba*	4.66 ± 1.07	Fix$^+$
	P. sativum	0.18 ± 0.12	Fix′
	Lathyrus sativus	0.25 ± 0.30	Fix′
	Vicia angustifolia	0.13 ± 0.21	Fix′
	Lens culinaris	0.80 ± 0.29	Fix$^+$
F41-1	*V. faba*	3.12 ± 1.02	Fix$^+$
	P. sativum	4.25 ± 0.70	Fix$^+$
	L. sativus	3.70 ± 0.85	Fix$^+$
	V. angustifolia	0.85 ± 0.49	Fix$^+$
	L. culinaris	0.64 ± 0.27	Fix$^+$
P13	*P. sativum*	4.39 ± 0.39	Fix$^+$
	V. faba	2.27 ± 0.83	Fix$^+$
P131	*P. sativum*	0.05 ± 0.04	Fix′
	V. faba	2.61 ± 1.02	Fix$^+$
P131-1	*P. sativum*	2.96 ± 0.89	Fix$^+$
P131-2	*P. sativum*	1.53 ± 0.34	Fix$^+$

[a]Values are means of five replicates ± standard error.

Medik, and *Vicia angustifolia* L.). As with *P. sativum*, strain F41 was poorly effective on sweet pea and vetch but showed a Fix^+ phenotype with lentils (Table 2).

About 2% of the nodules that were induced by Fix′ strains on pea roots were large and fully effective (Fig. 1; large arrow). To exclude the possibility of contaminations in the rhizobial cultures, spontaneous rifampicin-resistant derivatives were isolated from some of the Fix′ strains. In all cases tested, the selective marker was retained in reisolates from the fully effective pea root nodules that occasionally appeared.

Nodulation assays showed that the large, effective nodules were formed by stable Fix^+ segregants of the respective wild type strains. As shown in Table 2, segregant strain F41-1 (derivative of strain F41) expressed a normal Fix^+ phenotype with *P. sativum*. It also exhibited a Fix^+ phenotype with the original host *V. faba* as well as with *L. sativus*, *V. angustifolia*, and *L. culinaris*. In contrast, reisolates from small pea root nodules again induced the formation of numerous small and few large nodules on the roots of *P. sativum*.

The plasmid pattern of Fix^+ segregants of different wild type *R. l.* bv. *viceae* strains showing a Fix′ phenotype with *P. sativum* has been analyzed by Eckhardt gel electrophoresis. Surprisingly, most segregants showed an altered plasmid pattern. The plasmid profiles of 51 independently isolated Fix^+ segregants of the Fix′ strain F41 have been grouped into four classes: 27% of the Fix^+ segregants investigated showed no changes in their plasmid profile (class I). Deletions smaller than 5 kb remained undetected under our experimental conditions with the Eckhardt gel technique; 21% had deletions ranging from 5 to 50 kb in the cryptic plasmid pRleF41b of strain F41 (class II); 45% of the segregants had lost pRleF41b (class III); in 6% of the segregants plasmid rearrangements affecting pRleF41b had apparently occured (class IV). Examples of segregant plasmid profiles are shown in Figure 2. The plasmid profiles of representative Fix^+ segregants of each class remained stable over nodulation passages with either *P. sativum* or *V. faba* as host plant.

Cloning of DNA from pRleF41b affected by genetic instability. By using the cosmid pMMB33, a gene library of Fix′ strain F41 has been established. The resulting gene bank composed 1,000 selected clones, the insert DNA averaging 30 kb. For the identification of cosmids carrying pRleF41b DNA, total plasmid DNA was extracted from strain F41, [^{32}P]-labeled, and hybridized to dot blots of cosmid DNA prepared from 600 individual clones. In this way, 93 recombinant cosmids have been identified showing sequence homology to the plasmid preparation from strain F41 (data not shown).

To identify cosmids carrying cloned DNA from the genetically unstable region of plasmid pRleF41b, total plasmid DNA from the Fix^+ segregant strain F41-3 was prepared, [^{32}P]-labeled, and hybridized to dot-blots of the 93 cosmids. Strain F41-3 harbours a derivative of plasmid pRleF41b, which has a deletion of about 50 kb (pRleF41-3b; see also Fig. 2A, lane 5), and it was assumed that cosmids carrying wild type DNA that corresponded to that area deleted in pRleF41-3b would not hybridize to the probe. Accordingly,

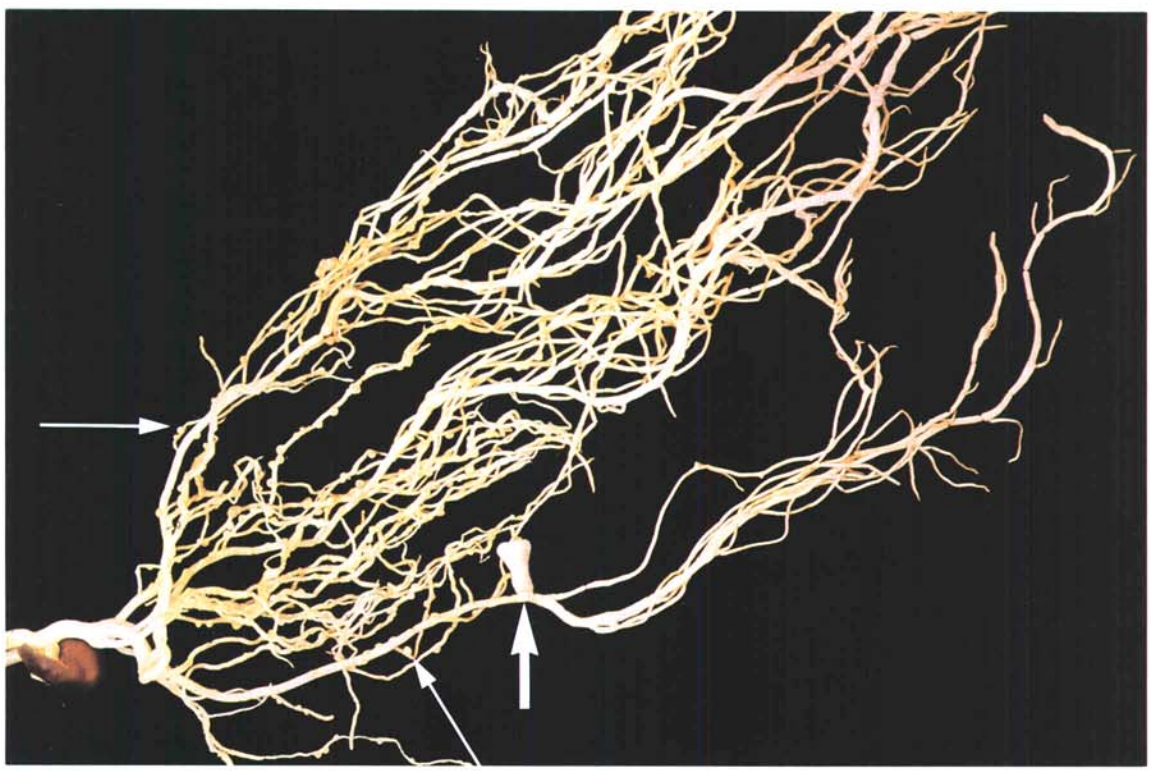

Fig. 1. Root system of *Pisum sativum* 'Poneka' (after 4 wk of cultivation) carrying nodules induced by *Rhizobium leguminosarum* bv. *viceae* F41. In addition to the numerous small nodules (small arrows) induced by the Fix′ wild type bacteria, a nodule of normal size (large arrow) has been induced by a Fix^+ segregant of strain F41.

one cosmid (pRlF69), which did not hybridize to plasmid DNA of strain F41-3, was identified. Hybridization of radioactively labeled pRlF69 DNA to Southern blots of Eckhardt gels of strains F41 and F41-3 confirmed this assumption. Cosmid pRlF69 hybridized to plasmid pRleF41b from strain F41 only and not to its deletion derivative in strain F41-3 (Fig. 3).

This result indicated that the DNA contained in cosmid pRlF69 corresponded to the sequence deleted in pRleF41-3b. However, analysis of this cosmid revealed that it contained a large insert of apparently chromosomal DNA, and that only a part of the insert DNA was homologous to pRleF41b. Later experiments revealed that sequences homologous to pRleF41b were present in more than one copy in the genome of strain F41. This finding explained how the cloned DNA of pRlF69 could be chromosomal. The pRleF41b homologous sequence was localized on a 3.4-kb *Eco*RI fragment of cosmid pRlF69. This was done by hybridizing [^{32}P]-labeled plasmid DNA of strain F41 to a Southern blot of *Eco*RI-digested pRlF69 DNA (data not shown) The 3.4-kb *Eco*RI fragment was subsequently cloned into the vector pACYC184 resulting in plasmid pIBS6. Thus, we had constructed a probe that was homologous to a particular region of the unstable plasmid pRleF41b.

To identify additional cosmids carrying large inserts spanning the approximately 50-kb deletion in plasmid pRleF41-3b, pIBS6 DNA was radioactively labeled and hybridized to dot blots of the 93 recombinant cosmids showing homology to the plasmid DNA of strain F41. In this way, seven cosmids that hybridized to pIBS6 were identified. In a subsequent experiment, DNA from two of the seven cosmids (pRlF224 and pRlF245) as well as plasmid DNA from the wild type strain F41 and from its Fix$^+$ segregant F41-3 was *Bam*HI-digested and electrophoretically separated. The Southern blots were hybridized to [^{32}P]-labeled DNA of the corresponding cosmid. The autoradiograms indicated that the cosmids pRlF224 and pRlF245 had a 13-kb fragment each of which was present in plasmid pRleF41b but had no corresponding fragment in the segregant plasmid pRleF41-3b (data not shown).

Mapping of pRleF41b DNA affected by genetic instability. A *Bam*HI restriction map of a region of plasmid pRleF41b affected by the deletion in pRleF41-3b has been established (Fig. 4). This was achieved in two ways. The arrangement of *Bam*HI fragments within cosmids pRlF224 and pR1F245 was established by analysis of partial *Bam*HI digests of the respective cosmids. The length of the contiguous *Bam*HI fragments that were partially present in both cosmids was calculated from hybridization patterns of *Bam*HI-digested pRleF41b DNA with cosmids pRlF224 and pRlF245 as hybridization probes.

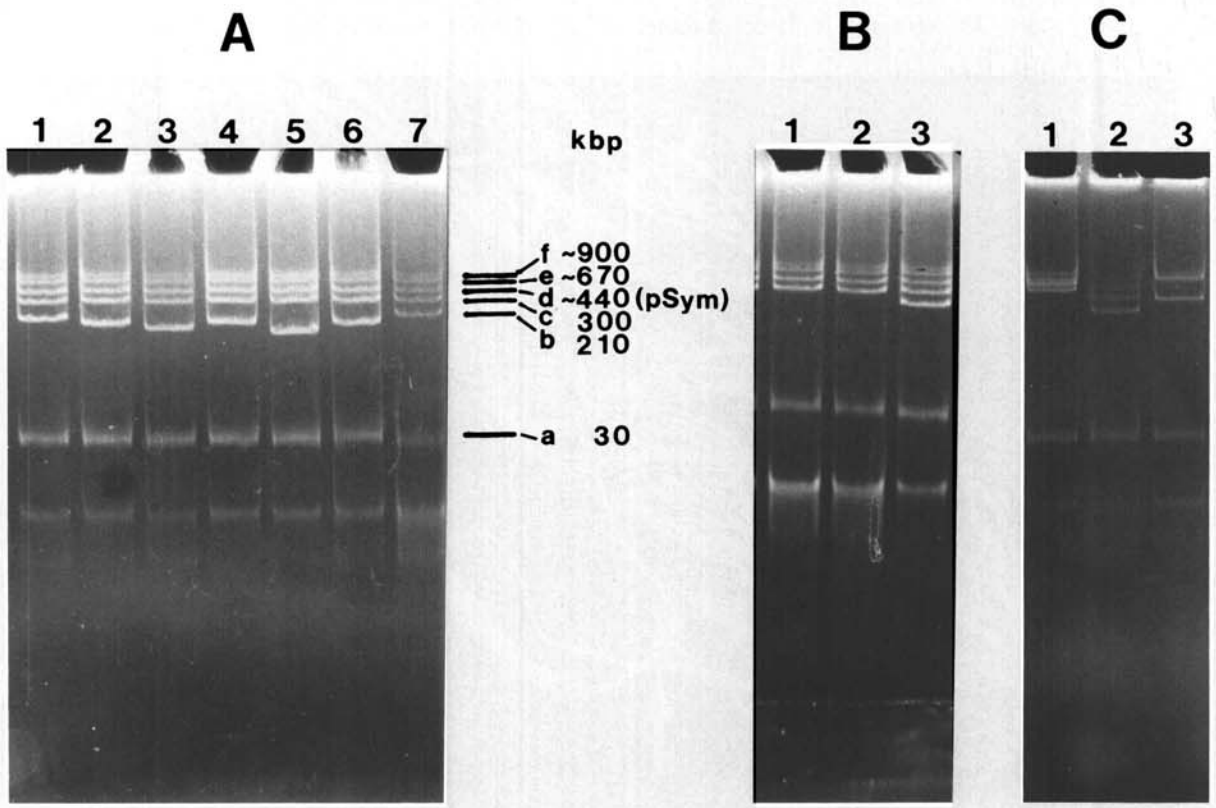

Fig. 2. Plasmid profile of the Fix' wild type strain *Rhizobium leguminosarum* bv. *viceae* F41 and of its Fix$^+$ segregants. **A,** plasmid profile of strain F41 (lane 7) and its class II Fix$^+$ segregants with a deletion in plasmid pRleF41b: RF41-2 (lane 1), RF41-3 (lane 2), RF41-4 (lane 3), F41-2 (lane 4), F41-3 (lane 5), and F41-4 (lane 6). **B,** plasmid profile of strain F41 (lane 3) and its class III Fix$^+$ segregants (loss of pRleF41b): F41-1 (lane 1) and RF41-1 (lane 2). **C,** plasmid profile of strain F41 (lane 2) and two class IV Fix$^+$ segregants (with apparent rearrangements of plasmid DNA): RF41-10 (lane 1) and RF41-11 (lane 3).

We have asked whether the 13-kb *Bam*HI fragment of pRleF41b was generally affected by the genetic instability leading to Fix$^+$ segregants of the Fix' strain F41. To test this possibility, the 13-kb *Bam*HI fragment (subcloned from cosmid pRlF245 into pACYC184 to give pIBS7) has been probed against the *Bam*HI-digested total DNA of strain F41 and its Fix$^+$ segregants. Four independently isolated Fix$^+$ segregants of wild type strain F41 (F41-1, F41-2, F41-3, and F41-4) and four Fix$^+$ segregants (RF41-1, RF41-2, RF41-3, and RF41-4) from the rifampicin-resistant spontaneous F41 mutant RF41 have been analyzed. Previous experiments had shown that six of these Fix$^+$ segregants tested had deletions in their respective pRleF41b plasmid (class II segregants; see also Fig. 2A). The values (kb) of the deletions are as follow: segregant RF41-2 (5 kb), RF41-3 (24 kb), RF41-4 (44 kb), F41-2 (24 kb), F41-3 (50 kb), and F41-4 (29 kb). Segregant strains RF41-1 and F41-1 had lost the genetically unstable plasmid pRleF41b (class III segregants; see Fig. 2B). The experiment also included *Bam*HI-digested plasmid DNA from wild type strain F41, as well as from its derivatives F41-3 and RF41-2.

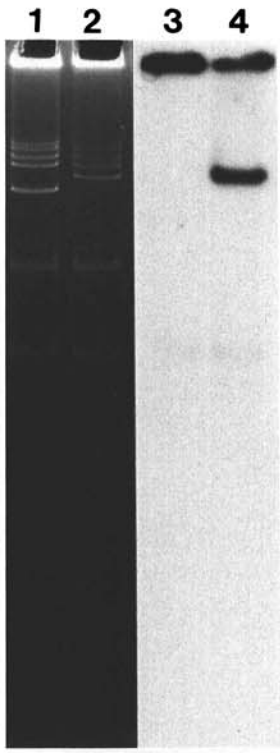

Fig. 3. Southern blot analysis of plasmids of the Fix' wild type strain *Rhizobium leguminosarum* bv. *viceae* F41 (lanes 2 and 4) and of its Fix$^+$ segregant F41-3 (lanes 1 and 3). Cosmid pRlF69, carrying DNA homologous to the unstable region of the cryptic plasmid pRleF41b has been [^{32}P]-labeled and used as a hybridization probe. The agarose gel (lanes 1 and 2) and the correspondiing autoradiography (lanes 3 and 4) are shown.

Positive hybridization signals that comigrated with the 13-kb insert of the probe were detected in the plasmid preparation and the total DNA prepared from wild type strain F41 (Fig. 5, lanes 12 and 13). In strain F41, the 13-kb probe insert showed homology to four additional DNA bands of sizes 1.8, 5.1, 6.8, and 9.5 kb. The hybridizing bands are absent in the case of Fix$^+$ segregants RF41-1 and F41-1 (lanes 10 and 11), which have lost plasmid pRleF41b. Therefore, the mentioned hybridizing DNA bands of strain F41 must have originated from plasmid pRleF41b. In addition, the 13-kb *Bam*HI fragment appeared to be homologous with chromosomal DNA of strain F41. Strains RF41-1 and F41-1 each showed two bands homologous to the probe (9.9 and 18.8 kb, respectively), whereas no homology was detected between the probe and the other plasmids of strain F41.

In the case of the Fix$^+$ segregant strains RF41-3 (Fig. 5, lane 4), RF41-4 (lane 5), and F41-4 (lane 9), the probe was found not to hybridize to a 13-kb *Bam*HI fragment (or a deletion derivative of this fragment). This indicates that the 13-kb *Bam*HI fragment has been deleted completely

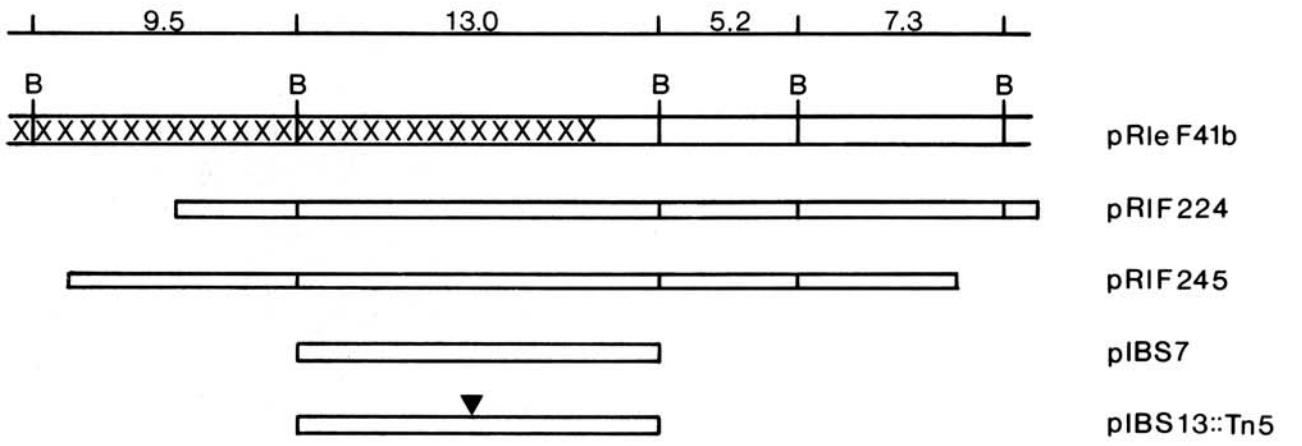

Fig. 4. *Bam*HI restriction map of the 35-kb fragment from the unstable region of plasmid pRleF41b of *Rhizobium leguminosarum* bv. *viceae* F41. The hatched region indicates DNA sequences deleted in plasmid pRleF41-3b of the Fix$^+$ segregant F41-3. The *Sau*3AI inserts of cosmids pRlF224 and pRlF245 (31.8 kb and 32 kb, respectively) are shown. The 3.4-kb *Eco*RI insert of plasmid pIBS6 that was used as a probe for the identification of cosmids pRlF224 and pRlF245 shows homology to the 13-kb *Bam*HI fragment. Plasmid pIBS7 (13-kb *Bam*HI fragment in pACYC184) served as a probe in the hybridization experiments shown in Figure 5. Plasmid pIBS13::Tn*5* (13-kb *Bam*HI fragment, carrying Tn*5*, in pSUP202) has been used to construct a mobilizable derivative of plasmid pRleF41b. The location of the Tn*5* insertion on the 13-kb fragment is indicated by a triangle. The size of all *Bam*HI fragments is given in kilobase pairs (kbp).

from the plasmid DNA of these strains. In strain F41-2 (lane 6), which also lacked the 13-kb fragment, a very faint, additional fragment of approximately 0.5 kb had hybridized to the probe, indicating that the 13-kb fragment was also almost completely deleted from the unstable plasmid of this strain. A hybridizing band of 13 kb was also missing in the *Bam*HI-digested total DNA as well as the plasmid DNA of segregant RF41-2. Instead, a 17-kb fragment had hybridized to the probe (Fig. 5, lanes 2 and 3). This new fragment could result from a 5-kb deletion

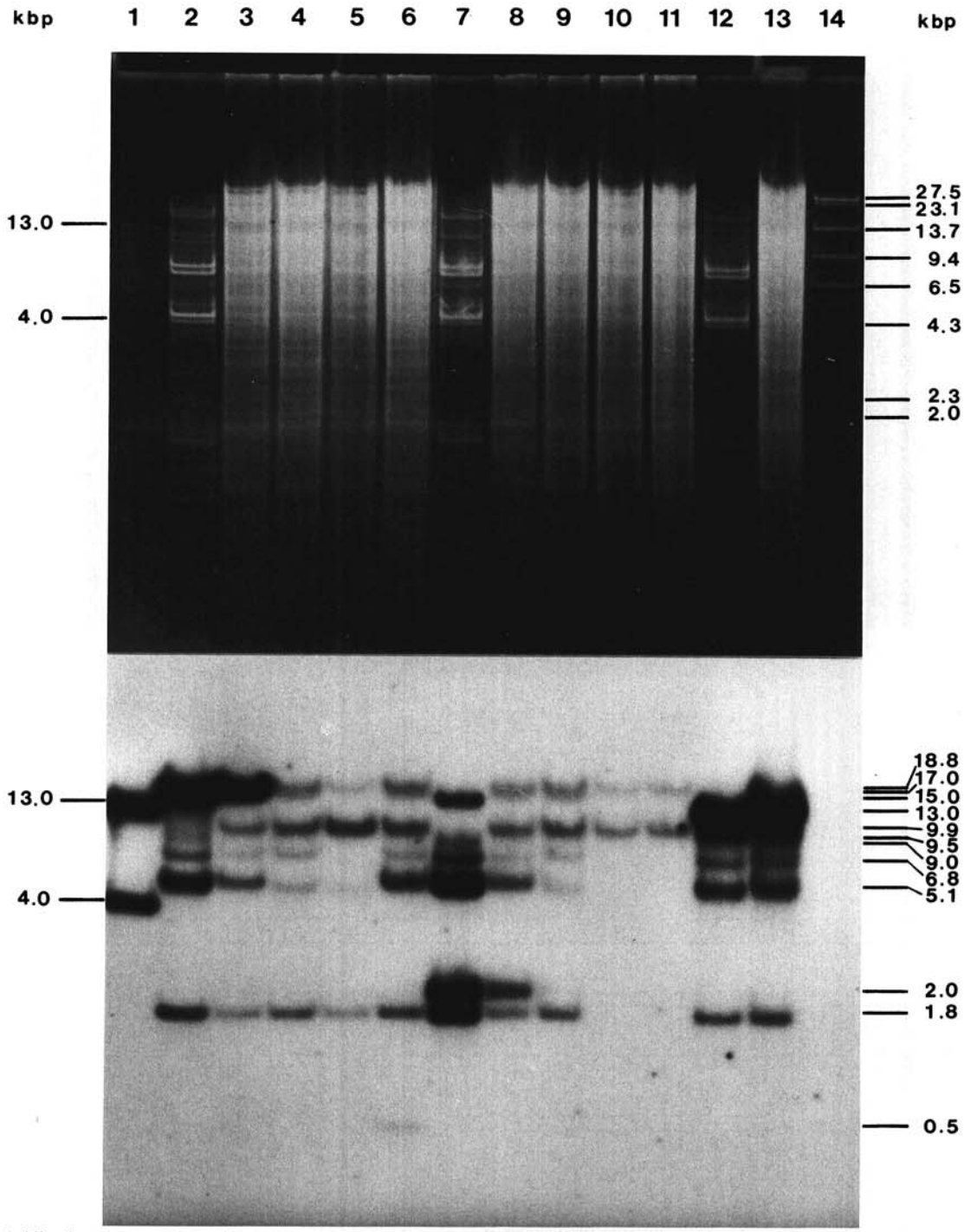

Fig. 5. DNA hybridization pattern of the Fix' wild type strain *Rhizobium leguminosarum* bv. *viceae* F41 and of the corresponding Fix$^+$ segregants. The agarose gel and the corresponding autoradiogram are shown. Plasmid pIBS7 (13-kb *Bam*HI fragment in pACYC184) was [^{32}P]-labeled and hybridized to Southern blots of *Bam*HI-digested plasmid DNA (lanes 1, 2, 7, and 12) or total DNA (lanes 3–6, 9–11, and 13). Lane 1, pIBS7; lane 2, RF41-2; lane 3, RF41-2; lane 4, RF41-3; lane 5, RF41-4; lane 6, F41-2; lane 7, F41-3; lane 8, F41-3; lane 9, F41-4; lane 10, RF41-1; lane 11, F41-1; lane 12, F41; lane 13, F41; lane 14, lambda/*Hin*dIII and pMMB33/*Bam*HI. The size of the lambda marker fragments and of the hybridizing fragments is given in kilobase pairs (kbp). For plasmid profiles of the Fix$^+$ segregants used in this experiment, see Figure 2.

affecting contiguous sequences of the linked 13- and 9.5-kb *Bam*HI fragments (see Fig. 4).

Segregant strain F41-3 lacked the 13-kb *Bam*HI hybridizing fragment as well as the 9.5-kb fragment that shows homology to the 13-kb fragment (Fig. 5, lane 7). Instead, a strongly hybridizing, new band of 2.0 kb in size (Fig. 5, lanes 7 and 8) and two further bands of a medium (15 kb) and a very weak signal strength (9.0 kb) hybridized to the probe (lane 7). From the intensity of the signals, we assumed that the 15.0-kb as well as the 9.0-kb hybridizing fragments seen in lane 7 originated from the 9.5-kb fragment by rearrangement events, whereas the 2.0-kb fragment was a deletion derivative of the 13-kb fragment. The fact that the 15.0-kb as well as the 9.0-kb hybridizing fragments were not visible in lane 8 (total DNA of strain F41-3) is very probably attributable to the partial homology of the 13-kb fragment with these new bands resulting in a signal that is not detectable in the presence of an excess of nonspecific DNA fragments.

In summary, the results presented in Figure 5 show that the 13-kb fragment has been affected by genetic instability in all of the Fix^+ segregants tested.

The Fix′ phenotype is co-transferred with plasmid pRleF41b. Preliminary tests have indicated that the indigenous plasmids of *R. l.* bv. *viceae* F41 are not self-transmissible. Because our results strongly indicated that the postulated *hrf* determinants were located on plasmid pRleF41b, we asked whether the genetic information present on this plasmid caused the Fix′ phenotype or whether additional genetic information was required, delivered *in trans* by the genome of strain F41 to result in the Fix′ phenotype. Therefore, we constructed a mobilizable derivative of plasmid pRleF41b. For this purpose, the 13-kb *Bam*HI fragment of pIBS7 was cloned in the mobilizable plasmid pSUP202 resulting in plasmid pIBS13. A Tn*5*-carrying derivative of plasmid pIBS13 (pIBS13::Tn*5*) has been isolated following the method described by Klipp and Pühler (1984). The Tn*5* insertion site in pIBS13::Tn*5* has been mapped (Fig. 4).

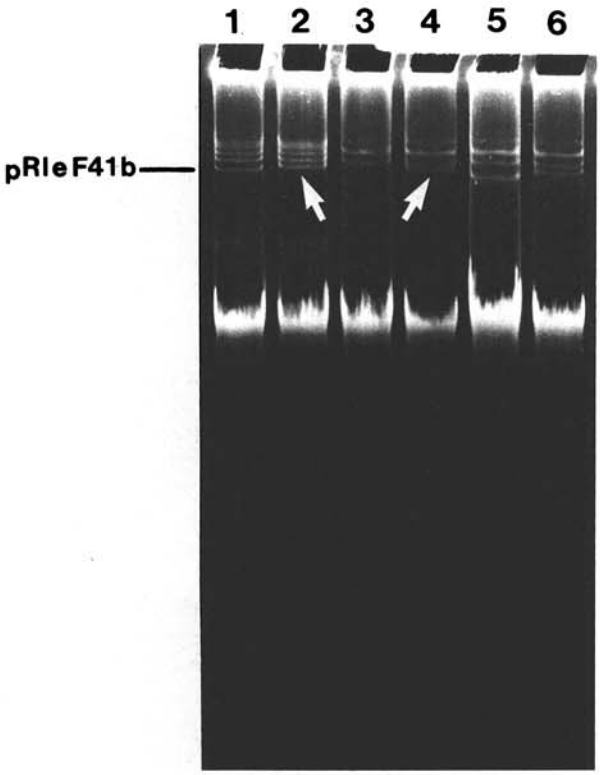

Fig. 6. Plasmid pattern of Fix′ strains F41 (lane 1), SF411, and P131 (the latter two harboring a mobilizable derivative of pRleF42b [lanes 2 and 4, arrows]), of Fix^+ strain *Rhizobium leguminosarum* bv. *viceae*, P13 (lane 3) and of the Fix^+ segregants P131-1 (lane 5) and P131-2 (lane 6) of transconjugant strain P131.

With the help of the mobilizing strain *E. coli* S17-1 plasmid pIBS13::Tn*5* was transferred into *R. l.* bv. *viceae* SF41, a spontaneous streptomycin-resistant mutant of wild type strain F41. Kanamycin-resistant (Km^r) transconjugants arose at a frequency of about 8.5×10^{-3}. The integration of plasmid pIBS13::Tn*5* into the cryptic plasmid pRleF41b via homologous recombination was verified in Southern hybridization experiments (data not shown). In a triparental cross, the mobilizable co-integrate plasmid of strain SF411 (Fig. 6, lane 2) was transferred via the helper plasmid pRK2013 into Fix^+ *R. l.* bv. *viceae* strain P13, which offered a heterologous genetic background. The Tn*5*-mediated Km^r served as the selection marker. The frequency of Km^r transfer was approximately 3×10^{-8}. Eckhardt gel analysis of transconjugant strain P131 confirmed that the plasmid had been transferred to the recipient strain P13 (Fig. 6, lanes 3 and 4).

Faba bean and pea plants were inoculated with strain P131. Whereas the presence of the transferred plasmid in this strain did not significantly affect the symbiotic nitrogen fixation with *V. faba*, it led to a one hundred-fold decrease in symbiotic nitrogen fixation with *P. sativum* (Table 2). Pea plants infected with strain P131 showed the same characteristics (i.e., numerous small nodules and symptoms of nitrogen starvation) as plants inoculated with the original Fix′ strain F41. Moreover, strain P131 induced the formation of fully effective root nodules at a frequency of 2–5%. The plasmid profiles of two reisolates (strains P131-1 and P131-2) from such nodules revealed an alteration in their plasmid pattern: the transferred plasmid had a deletion of approximately 50 kb in strain P131-1 and of approximately 20 kb in strain P131-2 (Fig. 6, lanes 5 and 6). With *P. sativum*, the segregant strains P131-1 and P131-2 reduced significantly more acetylene than strain P131 but less than parent strain *R. l.* bv. *viceae* P13 (Table 2). These results show that the presence of the transferred co-integrate plasmid in strain P131 resulted in a Fix′ phenotype with *P. sativum*.

DISCUSSION

This study shows that genetic information of indigenous plasmids distinct from the symbiotic plasmid of *R. l.* bv. *viceae* wild type strains may reduce the efficiency of symbiotic nitrogen fixation with *P. sativum*, but not with *V. faba*. We propose that such sequences can contribute to the variation in symbiotic efficiency of *R. l.* bv. *viceae* wild type strains observed with different hosts of the pea cross-inoculation group. We postulate as a working hypothesis that these sequences carry "*hrf*" determinants (for host-

dependent reduction of nitrogen fixation).

Preliminary light microscopic observations show that the Fix′ pea root nodules induced by wild type strain *R. l.* bv. *viceae* F41 and the Fix$^+$ nodules induced by segregant strain F41-1 after 4 wk of plant cultivation both showed infection threads and infected plant cells containing bacteriods. In Fix′ nodules sections of uninfected plant cells accumulating starch granules could be observed.

Most of the spontaneous Fix$^+$ segregants examined had an altered plasmid profile, showing either a loss of or a deletion in one of their indigenous plasmids. In the case of *R. l.* bv. *viceae* F41, which has been examined by us in more detail, the postulated *hrf* determinants are very probably located on plasmid pRleF41b. We have demonstrated that the 13-kb *Bam*HI restriction fragment of pRleF41b is directly affected by the genetic instability in all of the Fix$^+$ segregants examined (e.g., in the Fix$^+$ segregant RF41-2, which has a deletion of approximately 5 kb in this plasmid, Fig. 2A, lane 1; Fig. 5, lanes 2 and 3).

Sequences homologous to the 13-kb *Bam*HI fragment were found in all of the 20 Fix′ wild type strains examined; the probe hybridized to the respective unstable plasmid (but not to pSym) of these strains (our unpublished results).

In summary, these results indicate that at least part of the postulated *hrf* determinants are located on the 13-kb *Bam*HI fragment.

With all of the 20 Fix′ *R. l.* bv. *viceae* wild type strains spontaneous loss of *hrf* determinants occured at a high frequency. The observed plasmid rearrangements of *R. l.* bv. *viceae* strains that we report here resemble those described for *R. l. bv. phaseoli.* Plasmid-borne rearrangements in a strain of *R. l.* bv. *phaseoli*, which resulted in either sequence amplification, deletion, co-integrate formation, or loss of plasmids have been described by Brom *et al.* (1991). However, a host-dependent negative influence of cryptic plasmids on symbiotic nitrogen fixation of *R. l.* bv. *phaseoli* strains has not been reported by these authors.

Reiterated sequences have been found in strains of various *Rhizobium* species (Flores *et al.* 1987 and references therein), and in some cases it has been shown that these sequences are involved in the generation of rearrangements (Kaluza *et al.* 1985; Hahn and Hennecke 1987). We have shown that the 13-kb *Bam*HI fragment exhibits sequence homology to additional regions of plasmid pRleF41b (*Bam*HI fragments of 1.8, 5.1, 6.8, and 9.5 kb; see Fig 5., lanes 12 and 13). In some of the 20 Fix′ wild type strains, the 13-kb probe hybridized not only to the unstable plasmid but also to a second cryptic plasmid. Co-integrates originating from both of these plasmids have been observed in some Fix$^+$ segregants of these strains. Some of the cosmid clones of the F41 gene library, which hybridized to two of the indigenous plasmids of strain F41, also hybridized to its chromosomal DNA. Homologous recombination between reiterated DNA sequences could therefore account for the observed plasmid rearrangements as well as for the instability of cosmid pRlF245 that was observed after it was introduced into segregant strain F41-1 for complementation analyses (our unpublished results). Alternatively, the plasmid rearrangements could be due to the transposition of insertion sequence elements because an insertion sequence-like element has been detected in *R. l.* bv. *viceae* F7 (Simon *et al.* 1991), one of the 20 Fix′ strains from which Fix$^+$ segregants with an altered plasmid pattern have been isolated (our unpublished results).

Changes in the symbiotic properties of *R. l.* bv. *trifolii* and bv. *phaseoli* strains, which were correlated with plasmid rearrangements, have been reported by several authors (Beynon *et al.* 1980; Djordjevic *et al.* 1982; Christensen and Schubert 1983; Wang *et al.* 1986). In these cases, however, the strains under investigation contained the *R. l.* bv. *viceae* pSym pJB5JI, which had been introduced in addition to the indigenous pSym. It was suggested that the different host-range genes are functionally incompatible (Johnston *et al.* 1978; Beynon *et al.* 1980). Although our hybridization results rule out the presence of two pSym in the Fix′ strains, our results do not conclusively exclude the possibility that the proposed *hrf* determinants may represent single host-range genes of a different *Rhizobium* species.

To our knowledge, a negative effect of rhizobial wild type cryptic plasmids on the symbiotic nitrogen fixation has only been reported for *R. loti.* Pankhurst *et al.* (1986) have shown that heat-curing of the (only) indigenous plasmid (pRlo2037) of *R. loti* strain NZP2037 increased the symbiotic nitrogen fixation with *Lotus pedunculatus* (but not with *L. tenuis*). The enhanced fixation of the plasmid-cured derivative was associated with an increased number of (normally effective) nodules per *L. pedunculatus* host plant. The occurence of spontaneous plasmid segregants showing an increase in symbiotic nitrogen fixation has not been described in this system.

The presence of the co-integrate plasmid in Fix$^+$ strain P13 resulted in a reduced symbiotic effectiveness of the transconjugant strain P131 with *P. sativum* but not with *V. faba.* This effect is therefore attributable to DNA sequences present on the mobilizable derivative of plasmid pRleF41b. This postulation is further supported by the occurrence of Fix$^+$ segregants of strain P131 that have lost DNA sequences present on the transferred co-integrate plasmid. This finding clearly demonstrates that the genetic information present on pRleF41b alone is sufficient to result in the Fix$^+$ phenotype, and that the *hrf* determinants are able to function in a heterologous genetic background.

The *hrf* determinants on pRleF41b of *R. l.* bv. *viceae* F41 will now be analyzed by site-directed cassette mutagenesis (Kokotek and Lotz 1989). What is the effect of *hrf*-specific functions on the root nodule development of *P. sativum*? In our nodulation assays with *P. sativum* (controlled conditions, only one rhizobial strain per host plant), the Fix′ wild type strains of *R. l.* bv. *viceae* induced many small root nodules. In contrast, under natural conditions in the soil (host-rhizosphere) the Fix′ strains may be out-competed for nodule formation by Fix$^+$ strains. Most of the rhizobial strains used in research today have originally been isolated from natural populations in the soil via root nodule formation, using a given host as "trap plant." With this strategy, only the most competitive strains may have been isolated for a given plant genotype. Consequently, rhizobial strains carrying incompatible (or subcompatible) genetic information for a given host plant may have been overlooked or underestimated. Such "incom-

patible" genetic information (e.g., carried on cryptic plasmids) may be essential for competitive saprophytic growth of rhizobial strains in the soil.

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