Distribution of *repC* plasmid-replication sequences among plasmids and isolates of *Rhizobium leguminosarum* bv. *viciae* from field populations

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The distribution of four classes of related plasmid replication genes (*repC*) within three field populations of *Rhizobium leguminosarum* in France, Germany and the UK was investigated using RFLP, PCR-RFLP and plasmid profile analysis. The results suggest that the four *repC* classes are compatible: when two or more different *repC* sequences are present in a strain they are usually associated with different plasmids. Furthermore, classical incompatibility studies in which a Tn5-labelled plasmid with a group IV *repC* sequence was transferred into field isolates by conjugation demonstrated that group IV sequences are incompatible with each other, but compatible with the other *repC* groups. This supports the idea that the different *repC* groups represent different incompatibility groups. The same field isolates were also screened for chromosomal (*plac12*) and symbiotic gene (*nodD–F* region) variation. Comparison of these and the plasmid data suggest that plasmid transfer does occur within field populations of *R. leguminosarum* but that certain plasmid–chromosome combinations are favoured.

Keywords: *Rhizobium leguminosarum* bv. *viciae*, plasmids, *repC*, compatibility, natural populations

INTRODUCTION

The soil bacterium *Rhizobium leguminosarum* forms nitrogen-fixing nodules on the roots of several temperate leguminous plants. Most of the genes required for symbiotic function are carried on a large plasmid, the symbiotic plasmid or pSym (Johnston *et al.*, 1978; Prakash *et al.*, 1981). According to the host-specificity genes present on this plasmid the species has been divided into three biovars, bv. *phaseoli*, *trifolii* and *viciae* (Jordan, 1984). Most *R. leguminosarum* strains also carry between one and seven additional plasmids varying in size from 40 to 900 kb. These plasmids are usually referred to as cryptic, i.e. of no known function, although several studies have demonstrated that they have physiological roles in free-living and/or symbiotic *R. leguminosarum*: for exopolysaccharide (Skorupska *et al.*, 1991) and melanin (Hynes *et al.*, 1988) production,

carbon source utilization (Baldani *et al.*, 1992) and symbiotic characteristics such as level of nitrogen fixation (Thurman *et al.*, 1985; Hynes & McGregor, 1990; Selbitschka & Lotz, 1991). The plasmid component of the genome is usually stably maintained through successive generations, although occasional loss of part or all of a plasmid may occur during laboratory culturing and/or storage. Self-transmissible pSym and cryptic plasmids have been described (Johnston *et al.*, 1978; Brewin *et al.*, 1980); plasmid transfers are frequently associated with plasmid losses or rearrangements (Djordjevic, 1982; Soberon-Chavez, 1991; Brom *et al.*, 1991; Geniaux & Amarger, 1993). Moreover, studies of *R. leguminosarum* field populations provide indirect evidence that pSym transfer occurs in the environment (Young & Wexler, 1988; Laguerre *et al.*, 1992). The high plasticity of the plasmid component of the genome observed in laboratory studies is, therefore, likely to represent a natural phenomenon.

The aim of the current survey was to investigate this natural level of plasmid plasticity, with the longer-term

Abbreviation: ERIC, enterobacterial repetitive intergenic consensus.

Table 1. *R. leguminosarum* bv. *viciae* strains and plasmids used

Strain/plasmid	Characteristics*	Reference/source
<i>R. leguminosarum</i> bv. <i>viciae</i>		
BA5, BA7, BA9, BA11, BA12, BA19, BB2, BB4, BB12, BB18, BB19, BB20, BC1, BC11, BD3, BD12, BD13, BD15, SA1, SA2, SA6, SA10, SA20, SB19, SC1, SC3, SC8, SC12, SC14, SD1, SD2, SD11, SD13, SD19, SD20	Field isolates from Bretennières, France	INRA, Dijon
FIII-12, FIII-36, FIII-37, FIII-50, FIII-61, FIII-70, FIII-71, FIII-72	Field isolates from Schlüterhof, Germany	W. Lotz, Erlangen, Germany
RES-1, RES-2, RES-3, RES-4, RES-5, RES-6, RES-7, RES-8, RES-9, RES-10	Field isolates from Harpenden, UK	P. R. Hirsch, Rothamsted, UK
P262SpSt	Field isolate which had spontaneously lost its pSym; Sm ^r Sp ^r	Geniaux & Amarger (1993)
248, 306, 309	Field isolates	Hirsch <i>et al.</i> (1980)
1062/pRL1JI, 1062/pRL3JI, 1062/pRL4JI	Transconjugant strains	Hirsch <i>et al.</i> (1980)
RSM2004	248 with pRL1JI:: <i>cx</i> d; Km ^r	Hirsch & Spokes (1994)
Plasmids		
plac12	26.2 kb fragment containing the <i>lac</i> region of <i>R. l.</i> bv. <i>viciae</i> B155 cloned into pLAFR1	Young & Wexler (1988)
pIJ1246	6.6 kb <i>Eco</i> RI fragment carrying <i>nod</i> <i>EFDABC</i> genes of pRL1JI of <i>R. l.</i> bv. <i>viciae</i> 248 cloned into pSUP202; Ap ^r Tc ^r	Downie <i>et al.</i> (1985)
pGEM-T	Ap ^r , <i>lacZ</i>	Promega
pCT0370I	750 bp of <i>repC</i> group I of plasmid pBA7b of <i>R. l.</i> bv. <i>viciae</i> strain BA7 cloned into pGEM-T; Ap ^r	Turner <i>et al.</i> (1996)
pCT0370II	750 bp of <i>repC</i> group II of plasmid pBA11b of <i>R. l.</i> bv. <i>viciae</i> strain BA11 cloned into pGEM-T; Ap ^r	Turner <i>et al.</i> (1996)
pCT0370III	750 bp of <i>repC</i> group III of plasmid pSD13a of <i>R. l.</i> bv. <i>viciae</i> strain SD13 cloned into pGEM-T; Ap ^r	Turner <i>et al.</i> (1996)
pCT0370IV	750 bp of <i>repC</i> group IV of plasmid pSD11a of <i>R. l.</i> bv. <i>viciae</i> SD11 strain cloned into pT7-Blue-T vector; Ap ^r	Turner <i>et al.</i> (1996)

* *R. l.*, *Rhizobium leguminosarum*; Ap^r, Km^r, Sm^r, Sp^r, Tc^r, resistance to ampicillin, kanamycin, streptomycin, spectinomycin and tetracycline, respectively.

aim of assessing the potential for and frequency of plasmid transfer within *R. leguminosarum* field populations. Previously such studies have been restricted due to an absence of markers for the majority of plasmids; indeed they were largely confined to pSym. We have recently described homologues of the plasmid replication gene *repC* and determined their distribution in three field populations of *R. leguminosarum* bv. *viciae* (Turner *et al.*, 1996). As part of this work PCR primers were designed that specifically amplify sequences belonging to each of the four major groups (I–IV) of *repC* identified. In this study these primers and their products have been used to assess the distribution of such sequences within three *R. leguminosarum* populations, their distribution within the plasmid component of the

genome and the relationship of *repC* sequence variation to plasmid compatibility.

METHODS

Bacterial strains, plasmids and culture conditions. The strains used in this study are listed in Table 1. They were isolates of *R. leguminosarum* bv. *viciae* recovered from root nodules of *Pisum sativum* grown at three field sites: Bretennières in France, Schlüterhof in Germany and Harpenden in the UK. Diversity within the French and German populations had been analysed previously by determining plasmid profile patterns for French isolates and ERIC-PCR fingerprints for German isolates (L. Rigottier-Gois & W. Lotz, unpublished data). From the 136 isolates sampled in Bretennières, 35 isolates belonging to 18 distinct plasmid profiles were chosen. The

eight isolates from Germany represented each of the distinct ERIC-PCR fingerprints identified within the 72 isolates analysed. The ten isolates from the UK were selected randomly from within the population of Harpenden. The plasmids used in this study are listed in Table 1. pCTO370I–IV each contain a 750 bp product corresponding to one of the four *repC* groups (I–IV respectively) defined by Turner *et al.* (1996). *R. leguminosarum* strains were maintained on Bergersen's agar medium (Bergersen, 1961) supplemented with 0.02% yeast extract and were cultivated in TY (Beringer, 1974) at 28 °C for DNA preparations. *Escherichia coli* DH5 α derivatives containing the pCTO370I–IV plasmids were grown at 37 °C in LB (Sambrook *et al.*, 1989) supplemented with ampicillin (100 μ g ml⁻¹).

Conjugation experiments. Patch matings and transconjugant screening for symbiotic proficiency were done as described by Geniaux & Amarger (1993). Filter matings were carried out by depositing overnight TY cultures (1 ml) of donor and recipient onto 24 mm diameter cellulose nitrate membrane filters (Millipore, pore size 0.45 μ m, type HA) and by incubating bacteria side up for 12 h at 28 °C. The filters were mixed in 10 ml MilliQ sterile water, and after dilution the cell suspension was spread on appropriately amended TY plates (Sm, streptomycin 200 μ g ml⁻¹; Km, kanamycin 50 μ g ml⁻¹). Transconjugants were purified and checked for the loss of indigenous *repC* group III or group IV plasmids by probing blots of Eckhardt gels with pCTO370III or pCTO370IV.

DNA methods. Plasmid profiles were obtained by agarose gel electrophoresis as described by Eckhardt (1978) and modified by Wheatcroft *et al.* (1990). Plasmid DNA for probes was isolated from 15 h *E. coli* cultures using the QIAGEN Plasmid Midi Kit, according to the manufacturer's instructions. DNA purification and restriction with *Eco*RI, electrophoresis, blotting, probe labelling and hybridization were performed as described by Laguerre *et al.* (1992), except that the membrane used was Nytran-N (Schleicher & Schuell) and that chemiluminescence detection of digoxigenin-labelled DNA (Boehringer Mannheim) was used. The blots were hybridized successively with two probes following the protocol supplied by the manufacturer. Conditions for PCR amplifications using RSM2004-specific primers (5' TCA AAC CAG GTA AGG CGA CG 3' and 5' ATT GAA TTC ACG TCA CTA AG 3') were the same as those described for group-specific amplification by Turner *et al.* (1996). The products were sequenced using the Dye Terminator Cycle Sequencing kit (Perkin Elmer) and an ABI 377 automated sequencer, according to the manufacturer's instructions. RES-6 group II PCR products were cloned into pGEM-T prior to sequencing, which was carried out using the plasmid as template and primers SP6 and T7, according to the manufacturer's instructions.

Products for restriction analysis of the group I and group II *repC* sequences were obtained by PCR amplification using the general primers RC1/RC3 and the conditions described by Turner *et al.* (1996). When group III- and IV-specific primers were used the conditions for PCR were: 1.5 mM MgCl₂, 20 μ M dNTPs, 0.1 μ M of each primer and 1 U *Taq* polymerase (Appligene) per 100 μ l reaction, and the amplification programme was: 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 59 °C (group III) or 58 °C (group IV) for 1 min, 72 °C for 2 min; the last cycle was followed by a final extension step of 3 min. The PCR products (8 μ l) were digested with 10 U restriction endonucleases in 10 μ l reaction volumes according to the manufacturer's recommendations. The following restriction endonucleases were used: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Msp*I, *Nde*II and *Rsa*I (Appligene). Similarities be-

tween the restriction profiles of pairs of *repC* sequences were estimated from the proportion of shared restriction fragments to the total number of restriction fragments identified in all strains examined by using a simple matching coefficient (Sokal & Michener, 1958). A dendrogram was constructed from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973). *Hae*III analysis of PCR-amplified *nodD*–*F* regions was performed according to Laguerre *et al.* (1996).

RESULTS AND DISCUSSION

Background genetic diversity of the isolates

Table 2 summarizes the results obtained when chromosomal diversity, symbiotic gene diversity and plasmid content were examined in 53 field isolates of *R. leguminosarum*. Chromosomal diversity was investigated by probing Southern blots of *Eco*RI-restricted genomic DNA with *plac*12 (Laguerre *et al.*, 1992). Six classes were identified (a, i, n, o, p, q). Variation within the symbiotic genes of the isolates was investigated by RFLP analysis of PCR-amplified *nodD*–*F* gene fragments (Laguerre *et al.*, 1996): eight distinct *Hae*III restriction patterns were obtained (A, B, C, D, G, J, M, N).

The four most common *nodD*–*F* patterns (A, D, G, C) were each found associated with several different chromosomal backgrounds, for example *nodD*–*F* pattern A was associated with chromosomal backgrounds a3, i1, i5, i6, o and p. Similar patterns have been observed previously (Schofield *et al.*, 1987; Young & Wexler, 1988; Demezas *et al.*, 1991; Laguerre *et al.*, 1992), and these have been used to infer that pSym exchange does occur within field populations. When combined, the chromosomal and *nodD*–*F* data define 24 different chromosomal/*nodD*–*F* associations. Some of these associations were common to strains from more than one site. For example the associations between *plac*12 type i3 and *nod*-type J or between *plac*12 type i5 and *nod*-type D are found in both French and German isolates, BB4 and FIII-50, BD13 and FIII-36 (Table 2). Such findings suggest that certain associations of *R. leguminosarum* host and symbiotic plasmids are particularly stable and have been maintained independently within the distinct populations.

The plasmid profile of each isolate was assessed initially using Eckhardt gel electrophoresis (Eckhardt, 1978). A typical gel is shown in Fig. 1(a). Plasmid profiles were obtained for 51 of the 53 isolates; unambiguous plasmid patterns could not be resolved for isolates FIII-12 and RES-1. The 51 isolates each contained between two and seven distinct plasmid bands and there were 31 different plasmid profiles (Table 2, Fig. 2). Prolonged laboratory culture resulted in the loss of a plasmid from a minority of isolates. This was the case for some isolates with profiles 7 or 15 which after laboratory cultures showed modified profiles 7' and 15' (Fig. 2). Isolates SC14, BA9 (modified profile shown, 7') and FIII-71 (unmodified profile shown, 7) all lost their third smallest plasmid band and isolates SC8 and BD15 (modified profiles

Table 2. Chromosomal diversity, symbiotic gene diversity, plasmid content and diversity of *repC* hybridizing sequences in field isolates of *R. leguminosarum* bv. *viciae*

Strains	<i>plac12</i> type	<i>nod</i> type	Plasmid type*	Size (kb) of <i>EcoRI</i> restriction fragment hybridizing with:			
				<i>repCI</i>	<i>repCII</i>	<i>repCIII</i>	<i>repCIV</i>
BA7, BB19, BD12, SA2	i4	D	1	7	14, 9.8	1.2	8
SD1	i5	A	2	4.4	—	1.2	8
BD13	i5	D	3	4.4	14, 9.8	1.2	4.5
BA12, SC1, SB19	i4	D	4	14	—	1.2	8.6
BB2	i1	D	5	—	—	1.2	8
BA11, BA19, BC11, SD19	i3	D	6	—	14, 9.8	1.2	8
BB20, SA6, FIII-71	i3	D	7	8.2	14, 9.8	1.2	8
BA9, SC14	i3	D	7'	8.2	—	1.2	8
BB4	i3	J	8	—	—	1.2	8
SA1	i1	A	9	—	—	1.2	8
BB12	i1	G	10	—	—	1.2	—
BB18	i1	G	11	—	—	1.2	8
SD13	i1	A	12	—	—	1.2	8
SD11	i6	A	13	—	—	1.2	4.5
SC12	i5	G	14	—	—	1.2	—
BA5, SD2	i4	D	15	—	14, 9.8	1.2	4.5
BD15, SC8	i4	D	15'	—	—	1.2	4.5
SA20, RES-3	a3	G	16	—	—	—	—
SC3	a3	A	17	—	—	—	11
BC1, SA10, SD20	a1	D	18	4.4	14, 9.8	—	8.6
BD3	a1	D	18	12	14, 9.8	—	8.6
FIII-12	o	A	no	—	—	1.5	8
FIII-70	p	A	19	7	14	1.2	8
FIII-72	i2	D	20	7	14	1.2	8
FIII-36, FIII-61	i5	D	21	14	—	1.2	8.6
FIII-50	i3	J	22	—	14, 9.8	1.2	8
FIII-37	a3	M	23	7	14	—	8
RES-1	q	C	no	—	—	1.2	—
RES-9	q	G	24	—	—	1.2	—
RES-2	n1	B	25	—	—	1.2	—
RES-4	n1	C	26	—	—	1.2	—
RES-7	n2	C	27	—	—	2.8	—
RES-8	n1	C	28	—	—	1.2	—
RES-5	a3	D	29	5.8	—	—	8.6
RES-6	a3	N	30	5.8	14, 7.7	—	—
RES-10	a3	D	31	—	—	—	8

no, Not obtained.

* 7' and 15' correspond to modified profiles 7 and 15 observed after prolonged laboratory culture of isolates.

shown, 15') lost their smallest plasmid bands. Plasmid loss from isolate SC14 was observed independently in both the Dijon and York laboratories; the other plasmid losses were only documented at Dijon (BA9, SC8, BD15) or York (FIII-71). In all instances this corresponded to loss of a group II *repC* sequence in PCR analyses.

Several different isolates from the same field site shared identical plasmid profiles, for example French strains BA7, BB19, BD12 and SA2 or German isolates FIII-36 and FIII-61. More occasionally isolates from different

field sites shared the same plasmid profile: the German isolate FIII-71 shared plasmid profile 7 with French isolates SA6 and BB20.

Of the three methods used to assess genetic diversity, plasmid profiles revealed the greatest level of variation between isolates. Strains with identical (e.g. BA7, BB19, BD12, SA2) or very similar plasmid profiles (e.g. profiles 6 and 7) share *plac12* and *nodD-F* patterns, but isolates with identical *plac12* and *nodD-F* patterns do not always share the same plasmid profile. For example

Fig. 1. (a) Agarose gel electrophoresis of plasmids of *Rhizobium* isolates. (b) Southern blot of the same gel hybridized to the *repCIII* probe. Lanes: 1, BA7; 2, SD1; 3, BD13; 4, BA12; 5, BB2; 6, BA11; 7, SA6; 8, SC14; 9, BB4; 10, SA1; 11, BB12; 12, BB18; 13, SD13; 14, SD11; 15, BA5; 16, SC12; 17, BA5.

isolates with *plac12* pattern i4 and *nodD-F* pattern D have a number of different plasmid profiles (profiles 1, 4, 15, 15') (Table 2). These strains must be closely related in their chromosome and symbiotic gene organization but have apparently lost or gained plasmids and/or undergone plasmid rearrangements, again supporting a high level of plasticity within the plasmid component of *R. leguminosarum* field populations. On the basis of this characterization of *plac12* patterns, *nodD-F* patterns and plasmid profiles, the 53 isolates can be grouped into 35 different genotypes (Table 2).

Plasmid distribution and diversity: *repC* amplification

Turner *et al.* (1996) used PCR to isolate and partially sequence plasmid replication (*repC*) sequences from 22 *R. leguminosarum* field isolates (some of which were included in the present study). The results of our earlier study suggest that there are at least four distinct but related groups of *repC* sequence (I–IV). The partial sequence data obtained for the 22 PCR-amplified *repC* sequences showed that the divergence between sequences clustered in the same group was less than 3% for groups I and II but was as much as 15% for groups III and IV. To see if these levels were representative of the divergence found in a greater sample of isolates, RFLP analysis of *repC* PCR products of the 37 French isolates was undertaken; the results are summarized in Fig. 3.

When *repC* sequences belonging to several different groups were present within one isolate, the use of the general RC1 and RC3 primer sequences led to amplification of *repC* group I and II sequences, where these were present either singly or jointly. There were no restriction polymorphisms associated with either the group I or the group II amplification products, which is consistent with the low level of sequence variation found in these groups (Turner *et al.*, 1996). Amplification of the *repC* group III sequences using the general primer pair only occurred if both group I and II sequences were absent, and amplification of group IV sequences was restricted to isolates BB18 and SD11. This reinforces the conclusion of Turner *et al.* (1996) that some *repC* sequences may match the general primers more closely, and be amplified more efficiently than others.

The group III- and IV-specific PCR primers were used to amplify these sequences for RFLP analysis. All of the 32 group IV amplification products had identical restriction profiles to SD11, except BB18 (Fig. 3). The group III amplification products were much more polymorphic. Five different restriction profiles were obtained. Three of these patterns correspond to distinct sequences within the group III cluster of the phylogenetic trees of Turner *et al.* (1996). Furthermore, the restriction fragment variation (Fig. 3) is in accordance with the observations of these authors, since the group III PCR-RFLP results imply a greater level of sequence variation than do the group I and II results.

Plasmid distribution and diversity: *repC* hybridization

Southern blots of *EcoRI*-digested total genomic DNAs from all 53 field isolates used for the *plac12* analysis were probed sequentially with the four *repC* probes. Each probe hybridized to some but not all of the 53 isolates. All isolates except two hybridized with at least one of the probes, and ten isolates hybridized with all four probes. Isolates with the same genetic background (*plac12*, *nodD-F* and plasmid profile) always hybridized to the same combination of *repC* probes. The different hybridization patterns obtained with each probe are summarized in Table 2 and illustrated in Fig. 4. At least two different hybridization patterns were obtained with each probe. The group III hybridization patterns were the least polymorphic, all isolates except two hybridizing with a fragment of 1.2 kb; and the group I hybridization patterns were the most polymorphic, six fragments being recognized ranging in size from 4.4 to 14 kb (Fig. 4).

The group I and III *repC* probe sequences have *EcoRI* sites at positions 682 and 570 respectively, and these are conserved in the equivalent amplification products from the field isolates (not shown). An *EcoRI* digest of genomic DNA would, therefore, be predicted to generate two hybridizing fragments, but only a single fragment was observed in each case. There is a conserved *EcoRI* site in the reverse primer RC3 (Turner *et al.*, 1996); if this were conserved in the group I and III sequences then fragments of approximately 70 and 180 bp respectively

Fig. 2. Schematic representation of the different plasmid profiles listed in Table 2 and of the hybridization patterns obtained with each of the five probes tested: *nod* probe pIJ1246 (←), *repCI* probe (□—), *repCII* probe (→), *repCIII* probe (—▽—), *repCIV* probe (—◇—). —, Band corresponding to possible plasmid doublet. 7' and 15' represent modified profiles 7 and 15 observed after prolonged laboratory culture of isolates.

would be generated after *EcoRI* digestion. These fragments would have run off the gel and would not have been detected in the hybridization studies.

The conserved internal *EcoRI* sites of the group I and group III amplified sequences mean that the polymorphisms seen must be associated with sequences upstream of *repC*. If *repAB* sequences lie immediately upstream of *repC*, as they do in the six fully sequenced replicators (Nishiguchi *et al.*, 1987; Tabata *et al.*, 1989; Turner & Young, 1995; Freiberg *et al.*, 1997; Ramirez-Romero *et al.*, 1997; Bartosik *et al.*, 1995; GenBank accession number U60522), the polymorphic sites seen in sequences upstream of *repA*, since the hybridizing fragments are all 4.4 kb or larger, while those of group III would lie within *repA* or *repB*. The significantly lower frequency at which polymorphisms were detected with the group III probe compared with the group I

probe may reflect sequence constraints imposed by the presence of upstream *repAB* coding sequences. This apparent association of the group III *repC* with similar *repAB* sequences may imply that the partition and replication systems have co-evolved. PCR-based studies to look at the variation between *repAB* sequences amongst and between plasmids of known *repC* groups are under way to address this question.

The restriction fragment hybridizing to the group IV *repC* probe varied in size from 4.5 to 11 kb, but the polymorphic sites cannot be located from this information because there is no internal *EcoRI* site in the group IV PCR products. The *EcoRI* restriction polymorphisms detected with the group I, III and IV probes again suggest that plasmid transfer has occurred. Identical hybridization patterns were found in a range of different chromosomal backgrounds, and in strains from different field sites.

Fig. 3. UPGMA (unweighted pair group method with arithmetic mean) dendrogram showing the relationships between *repC* sequences revealed by PCR-RFLP analysis with eight endonucleases. *repC* sequence of pRmeGR4a from *Sinorhizobium meliloti* (Mercado-Blanco & Olivares, 1993) was included by inferring its RFLP from the sequence available in the GenBank database (accession number X69105).

Fig. 4. *EcoRI*-digested total DNA from *R. leguminosarum* bv. *viciae* field isolates hybridized with: (a) *repCI* probe, lanes 1 to 6, strains BA7, BD13, BA12, SA6, BD3, RES-5; (b) *repCII* probe, lane 1, strain BA11; (c) *repCIII* probe, lanes 1 and 2, strains SD13 and FIII-12; (d) *repCIV* probe, lanes 1 to 4, strains BA7, SD11, SC3, BC1.

The group II probe hybridized with two fragments (14 kb and 9.8 or 7.7 kb) in all but three of the 23 positive strains, suggesting that two copies of this sequence were present in the majority of strains. RFLP analysis of group II PCR products showed that *EcoRI* does not cut the target sequence of any of the amplification products. Furthermore, the probe hybridized with two bands after total DNA was digested with *Bam*HI, which also does not cut the probe sequence, strongly suggesting that there really are two copies of the group II *repC* sequence. Only three field isolates, FIII-70, FIII-72 and FIII-37, have a single band hybridizing with the group II sequence (14 kb). Again it is not possible to say whether the polymorphisms are associated with variation within the replicator sequences or not.

Localization of the *repC* sequences on different plasmids

A more detailed investigation of the plasmid profiles was achieved by Southern analysis of Eckhardt gels with the four *repC* probes, and with a *nodEFDABC* probe to identify the symbiotic plasmid of each isolate (Downie *et al.*, 1985). Fig. 1(b) shows typical hybridization results and Fig. 2 shows a schematic representation of the plasmid profiles and the hybridization patterns obtained with each of the five probes for all 51 isolates. The distribution of *repC* groups using this screen was the same as that described following probing of the *EcoRI*-digested genomic DNAs (Table 2). At least one *repC* sequence was present in each of the 51 isolates for which plasmid profiles were obtained, except isolates SA20 and RES-3. In all cases the *repC* sequences were shown to be plasmid-associated. When *repC* sequences belonging to different groups were present in an isolate they were usually associated with different plasmids. In general each group-specific *repC* probe hybridized with only one plasmid in each isolate (see below).

There are eight profiles in Fig. 2 (profiles 1, 7 strain BB20, 9, 18, 19, 20, 22, 23) that have a single plasmid band highlighted by two different *repC* probes. One explanation for this is that each dual-hybridizing band represents a doublet of co-migrating but distinct plasmids, as documented for the smallest plasmid band of *R. leguminosarum* strain 3841 (Hirsch *et al.*, 1980). This may be the case for some of the plasmid bands, for example the third-smallest band of SA1 (Fig. 1, lane 10) is more intense than the other plasmid bands of this isolate and may represent a doublet. This and other possible plasmid doublets are indicated as a thick line in Fig. 2. Where there is no obvious increase in band intensity it seems more likely that these bands represent dual-replicon plasmids (second-smallest band in profile 1, 20, 23; third-smallest band in profile 7 strain BB20). Plasmids carrying multiple, compatible replication systems have been described for other plasmid systems (Lane & Gardener, 1979; Danbara *et al.*, 1980). Furthermore, cointegrate, dual-replicon plasmids that form and are stable in *Agrobacterium* are known (Hooykaas *et al.*, 1980; Ooms *et al.*, 1982). These are now known to form between mini-replicons carrying different *repC*

genes (Paul Hooykaas, personal communication), and the *Paracoccus versutus* plasmid pTAV1 (Bartosik *et al.*, 1995) has two functional replicator regions (GenBank accession numbers U42228 and U60522), both of which have *repC* genes related to those of *R. leguminosarum*.

Genetic background and plasmid stability

It is evident from Table 2 that plasmid groups and *nod* types are not distributed randomly across the different chromosomal classes. For example, the group III probe hybridized with plasmids in strains of all chromosome types, except those belonging to class a, regardless of their *nodD-F* pattern and their country of origin. This might suggest that group III plasmids cannot be transferred into the class a background, or that they cannot be maintained in it. To investigate this further, patch matings were made between BB18, which contains a group III pSym (Fig. 2), and strain P262SpSt, which has an a1-type chromosome and has been cured of its pSym (Table 1). Transconjugants resistant to spectinomycin and streptomycin were screened for symbiotic proficiency in plant nodulation trials (Geniaux & Amarger, 1993). Transfer resulting in symbiotic proficiency was observed and group III *repC* sequences could be detected in antibiotic-resistant bacteria isolated from nodules by group-specific PCR. These sequences were rapidly and completely lost when bacteria isolated from the nodules were grown on laboratory media, the equivalent of non-selective conditions. These bacteria had also lost their ability to nodulate peas. Laguerre *et al.* (1992) noted that strains belonging to chromosomal *plac12* type a carry two large (~450 and 800–900 kb) plasmids that have proven impossible to cure. These plasmids might influence the capacity of these strains to accommodate *repC* group III plasmids. They do not have *repC* genes that are closely related to group III, but it is conceivable that they express incompatibility through some other essential maintenance function such as partition. The possibility that *repA* and *repB* gene products are involved in incompatibility is being investigated.

Do the different *repC* groups correspond to incompatibility classes?

Turner *et al.* (1996) proposed that the distinct *repC* groups identified in *R. leguminosarum* field isolates might correspond to different plasmid incompatibility classes. Plasmids are compatible if they can be co-maintained by a host cell under non-selective conditions. The large size and cryptic nature of many of the plasmids carried by *R. leguminosarum* field isolates makes classical plasmid incompatibility studies difficult to undertake. However, the plasmid source of the partial *repC* sequence cloned from strain RSM2004 has been identified. The RSM2004 *repC* sequence clusters with group IV sequences, but is not amplified by the group IV-specific primer set (Turner *et al.*, 1996). PCR primers were designed to amplify the RSM2004 *repC* sequence and these amplified a single product from RSM2004 and from a 1062/pRL1JI transconjugant (gift of P. Hirsch,

Rothamsted, UK), but not from the parental 1062 strain. Thus the RSM2004 partial *repC* sequence belongs to plasmid pRL1JI. Plasmid pRL1JI has been shown to be incompatible with plasmids pRL3JI and pRL4JI identified in strains 306 and 309 respectively (Brewin *et al.*, 1982). PCR analysis of strains 306 and 309, and 1062/pRL3JI and 1062/pRL4JI transconjugants using the RSM 2004-specific primer set generated a single PCR product with all four strains. Sequencing of the PCR products obtained from strains RSM2004, 306 and 309 revealed that all three were identical to the published sequence for the RSM2004 clone (EMBL accession number Z69844).

The finding that the incompatible plasmids pRL1JI, pRL3JI and pRL4JI have identical partial *repC* sequences is consistent with the hypothesis that *repC* is an incompatibility determinant or is tightly linked to an incompatibility determinant. To assess the extent of incompatibility within and between *repC* groups Sm^r field isolates were selected and then used in conjugation studies with RSM2004/pRL1JI::Tn5. Group-specific PCR analysis of two Km^rSm^r transconjugants of both SA6 and BB18 suggest that acquisition of pRL1JI::Tn5 is associated with loss of the indigenous group IV sequence. Eckhardt analysis of these strains (not shown) indicated that the plasmid band corresponding to the indigenous group IV plasmid has been lost, although acquisition of a discrete band corresponding in size to pRL1JI::Tn5 was not always observed and plasmid rearrangements were also seen. As mentioned in the Introduction, such rearrangements are often associated with plasmid transfers.

The *repC* plasmid hybridization patterns for strains BB20 and RES-6, plasmid profiles 7 and 30 respectively, are unusual, because each contains two different plasmids that hybridize with the same probe (the group I and II probes respectively). In the case of BB20 one also hybridized with the group II probe. This plasmid band did not appear to be a doublet and could represent a cointegrate plasmid in which the group I *repC* is not active/dominant and does not contribute to incompatibility. Neither of the two group II plasmids of RES-6 hybridized with any other *repC* probe. The PCR-RFLP data collected for the French isolates suggest that when two *repC* group II sequences are present in these strains the RFLP pattern is identical for both copies. PCR-RFLP analysis (*Hinf*I, *Hae*III, *Pst*I and *Taq*I) of the product obtained from strain RES-6 using the group II primers gave a complex restriction pattern in which the sum of the restriction fragment bands was approximately twice that expected (~1.1 kb). The pattern appeared to be a composite of two different restriction patterns: that of the BA11 clone (group II) and that of the sequence previously cloned from RES-6 (data not shown). The RES-6 group II amplification product was cloned into pGEM-T (Promega) and clones with restriction patterns corresponding to each of these were selected, and sequenced. The sequence data support the RFLP data: one sequence was identical to the full-length sequence cloned from BA11, and the second was distinct and the

same as the RES-6 clone (EMBL accession number Z69866).

This RES-6 sequence shows approximately 15% divergence from the other sequences that have been collected into group II, and it is possible that this is sufficient to make this replicon compatible with other group II sequences. Work is under way to address this question. An alternative explanation is that they are not compatible, but that one or both of the plasmids found carry a second functional replication system and it is this that enables their coexistence. The majority (158 out of 271) of all the bands identified in the plasmid profiles do not hybridize with any of the four *repC* probes used in this study, indicating that there must be a number of additional plasmid replication systems that are not detected by our probes. Even if such a replicator had an homologous *repC*, we might not have detected it because of the high sequence divergence found among *repC* genes: neither of the replicators found on pTAV1, for example, would be predicted to hybridize with, or be amplified by any of the probes used in this study under the conditions used.

The data presented in this paper generally support our hypothesis (Turner *et al.*, 1996) that the *repC* sequence groups correspond to plasmid incompatibility groups. The apparent exceptions have possible explanations that are amenable to testing, and further study will reveal more about the evolutionary dynamics of this complex family of replicons.

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