Diversity of *repC* plasmid-replication sequences in *Rhizobium leguminosarum*

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Homologues of the plasmid replicator gene *repC* were detected and characterized in a sample of *Rhizobium leguminosarum* strains. Conserved PCR primers were designed from published sequences of *repC*; they amplified a fragment of about 750 bp from 39 out of 41 strains tested, and also from several *Sinorhizobium* strains, including *S. meliloti*. Restriction endonuclease digestion showed that the PCR product from individual strains, though uniform in size, was often heterogeneous in sequence. PCR products from 24 field isolates of *R. leguminosarum* from France, Germany and the UK were cloned and partially sequenced from both ends. Phylogenies constructed from the 5' and 3' ends (200 bp each) were largely congruent and demonstrated four clearly defined groups plus several unique strains. Published *Agrobacterium repC* sequences fall within the phylogeny of *R. leguminosarum* sequences, though not within any of the four groups. Specific pairs of PCR primers were designed for each of the four groups; 29 out of 41 *R. leguminosarum* strains gave a PCR product of the expected size with more than one group-specific primer pair. We hypothesize that the sequence groups correspond to incompatibility groups of *Rhizobium* plasmids.

Keywords: *Rhizobium leguminosarum* plasmids, *repC*, compatibility, plasmid probes

INTRODUCTION

Large, low-copy-number plasmids are common in the *Rhizobiaceae*. These plasmids (> 100 kb in size) often encode characteristics that are important taxonomic markers, e.g. the ability of agrobacteria to induce plant tumours and the ability of rhizobia to enter into nitrogen-fixing symbioses with legumes. The plasmids in this group of bacteria appear to be related, since plasmids isolated from agrobacteria have been shown to replicate in rhizobia and vice versa, and incompatibility between replicons isolated from the different genera has been noted (Hynes *et al.*, 1985; O'Connell *et al.*, 1987; Mozo *et al.*, 1990). Incompatible plasmids are not maintained within the same host cell under non-selective conditions; this is thought to be the result of shared replication, replication control and/or partition functions (Novick, 1987).

A single plasmid is sufficient to confer symbiotic proficiency in *Rhizobium leguminosarum* strains (Martínez *et al.*, 1987). However, the symbiotic plasmid (pSym) is generally only a minor component of the extra-chromosomal DNA of an *R. leguminosarum* strain. The majority of strains contain between one and six additional, distinct cryptic plasmids. A minority of non-pSym plasmids affect symbiotic proficiency, although the cause of these phenotypes is not fully understood (Hynes & McGregor, 1990; Velázquez *et al.*, 1995). The plasmid complement of *R. leguminosarum* strains is stable under laboratory and field conditions, implying that the plasmids contribute advantageously to cell maintenance and/or replication. Some *R. leguminosarum* plasmids are apparently important for cell survival since they cannot be completely cured (Hynes *et al.*, 1989), or their removal results in a strain with significantly impaired growth rates (Hynes & McGregor, 1990). The genetics that underpin the stable maintenance of multi-replicon genomes in *R. leguminosarum* have not been studied in detail. Plasmid transfer studies have revealed cryptic plasmids that are incompatible with some symbiotic plasmids (Brewin *et al.*, 1982) and symbiotic plasmids that are compatible with other pSyms (Geniaux

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The EMBL database accession numbers for the *repC* sequences in this paper are Z69840–Z69879.

Table 1. Bacterial strains used

Strains	Characteristics	Reference/source
<i>Escherichia coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>d lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
<i>Agrobacterium tumefaciens</i>	/pTiB6S3; C58 chromosomal background Rif ₂₀	J. Escuerdo, Leiden, The Netherlands
<i>Rhizobium leguminosarum</i> 3841	Sm ^R derivative of strain of 300/pRL8JI	Johnston & Beringer (1975)
RSM2004	Inoculant strain at UK release site (1987)	Hirsch & Spokes (1994)
SC8, BA5, BA7, BA12, BA11, SC14, SA1, BC1, SA10, BB18, SD13, SC12 and SD11	Field isolates from Brettennières, France	
FI11-12, -20, -36, -37, -38, -49, -50, -51, -52, -60, -61, -68, -69, -70, -71 and -72	Field isolates from Schlüterhof, Germany	W. Lotz, Erlangen, Germany
RES-1 to RES-10	Field isolates from Harpenden, UK	P. Hirsch, Rothamsted, UK
<i>Sinorhizobium</i> strains		
<i>S. meliloti</i> A12, 14, 18, 19, 111, 212–216, 218, 219, 312–316, 318 and 319	Field isolates from Granada, Spain	N. Toro, Granada, Spain
<i>S. teranga</i> ORS 1009	Type strain	de Lajudie <i>et al.</i> (1994)
<i>S. saheli</i> ORS 609	Type strain	de Lajudie <i>et al.</i> (1994)

& Amarger, 1993). These observations are not unexpected since the only genes that are always associated with a plasmid belonging to one compatibility grouping are those involved in plasmid maintenance (Couturier *et al.*, 1988).

We have recently described a replicator region isolated from the indigenous *R. leguminosarum* cryptic plasmid pRL8JI (Turner & Young, 1995). This replicator region shows significant organizational and sequence similarity to two replicator regions isolated previously from the *Agrobacterium* plasmids pRiA4b (Nishiguchi *et al.*, 1987) and pTiB6S3 (Tabata *et al.*, 1989). These three replicator regions are each predicted to comprise three genes, *repA*, *B*, and *C*, with a conserved intergenic sequence (*igs*) located between the *repB* and *C* genes. The *repC* component codes for an essential replication function, whereas *repA* and *B* code for ancillary, stabilizing functions (Tabata *et al.*, 1989). The predicted translation products of *repA* and *B* show amino acid sequence similarities to the partition proteins of the *Escherichia coli* phage P1 and plasmid RK2 (Williams & Thomas, 1992).

The two cloned *Agrobacterium* plasmid replicator regions are known to be compatible (Tabata *et al.*, 1989), even though they share significant amino acid sequence identity (≈ 40 – 55 %) across each of the predicted RepA, B and C peptides. A similar level of sequence identity exists between these two replicator regions and the pRL8JI replicator. A fourth replicator region isolated from a *Sinorhizobium meliloti* strain has also been described. This replicator region, from pRmeGR4a (Mercado-Blanco & Olivares, 1993), contains a *repC* homologue but neither

repA nor *repB* homologues. The predicted pRmeGR4a RepC has limited (≈ 23 %) amino acid identity to the three other RepC proteins. The *repC* genes and their predicted peptide products show little similarity to any other DNA or protein sequences currently in the databases. This is in keeping with the observations of Kobayashi & Bailey (1994), who investigated plasmid diversity in bacteria isolated from plant phyllospheres using the hybridized probes of Couturier *et al.* (1988). These probes, primarily designed to detect plasmids belonging to different incompatibility groups in clinical isolates, hybridized to few plasmids isolated from an agricultural environment.

An historical bias towards understanding the plasmid biology of clinically important bacteria has meant that plasmid systems in other bacteria have been overlooked. The potential use of microbes in agriculture, both in biological control and crop improvement, has stimulated interest in the plasmid biology of soil bacteria. This interest stems largely from the fact that plasmids are potential sources of horizontal gene transfer within soil populations. Gene transfer into, or out of, a genetically engineered microbe in the environment has serious implications for the use or design of such organisms.

A long-term aim of the present study is to assess the potential for, and frequency of, horizontal plasmid transfer within field populations of *R. leguminosarum*. The first requirement for such a study is a set of plasmid-specific probes that are representative of the plasmids present in soil populations. These can then be used to screen for plasmid transfer into tagged release strains. The presence of a unique family of plasmid replication

sequences (*repC*) in each of four cloned *Rhizobiaceae* replicator regions suggested that this gene might be a good target sequence for the design of PCR probes.

METHODS

Bacterial strains, plasmids and growth conditions. All bacterial strains used are listed in Table 1. *R. leguminosarum* field isolates represented the dominant genotypes present in each field site (W. Lotz & P. Hirsch, personal communications).

E. coli DH5 α was grown at 37 °C in LB and on L agar (Sambrook *et al.*, 1989). When selection was required ampicillin was added to a final concentration of 100 μ g ml⁻¹. *R. leguminosarum* and *Sinorhizobium* strains were grown at 28 °C in TY (Beringer, 1974); this medium was solidified with 1% (w/v) agar as required. Plasmids pGEM-T (Promega) or pT<blue-T-vector (Novagen) were used to clone PCR products.

General methods. Standard methods were used for the preparation and transformation of *E. coli* competent cells and for DNA manipulations and analyses (Sambrook *et al.*, 1989). Plasmid DNA for sequencing and restriction analyses was prepared from 1.5 ml of an overnight, 37 °C *E. coli* culture using Wizard Mini-Prep Columns (Promega) or the Quiagen plasmid midi kit according to the manufacturers' instructions. Restriction endonucleases, *Taq* polymerase and T4 DNA ligase from Promega were used according to the manufacturer's instructions. PCR products were cleaned using Wizard PCR-Cleanup columns (Promega) prior to cloning.

Double-stranded plasmid DNAs were denatured with alkali prior to sequencing by the dideoxynucleotide chain-termination method using either [α -³²S]thio-dATP (NEN-Du Pont), modified T7 polymerase (Sequenase; US Biochemical) and T7 and SP6 forward and reverse primers (Promega), or [α -³²P]-dATP (Isotopchim), Pharmacia¹⁷ Sequencing Kit and 18-2 and 18-1 forward and reverse primers (Novagen).

PCR was performed on whole cells or on DNA purified according to the method of Laguerre *et al.* (1992), using the *Taq* polymerase from Promega supplied with MgCl₂-free 10 $\times$ buffer. Conditions for amplification with the general primer set were: 2 mM MgCl₂, 200 μ M dNTPs, 40 pmol each primer and 0.5 U *Taq* per 50 μ l reaction. The amplification cycle used was: 97 °C for 2 min, followed by 35 cycles of: 48 °C for 40 s, 72 °C for 2 min and 93 °C for 40 s; the last cycle was followed by an additional annealing step and a final extension step of 10 min. Conditions for group-specific PCR amplifications were: 1.5 mM MgCl₂, 200 μ M dNTPs, 40 pmol of each primer and 0.5 U *Taq* per 50 μ l reaction. Thermal cycling (25 cycles) was as above except that the annealing step was at 58 °C.

RESULTS AND DISCUSSION

Most *R. leguminosarum* strains have *repC*-like sequences

Peptide sequence comparisons of the pTiB6S3, pRiA4b and pRL8JI *RepC*s identified regions of highly conserved amino acid residues (Fig. 1a). Investigation and com-

Fig. 1. (a) *RepC* peptide sequence alignments. Two highly conserved regions that were investigated as possible general primer sites are indicated in bold in the consensus sequence. Arrows above the pRL8JI sequence indicate the position and orientation of PCR primers: primers RC1 and RC3 are shown as solid arrows and group-specific primers as dashed arrows. (b) Nucleotide sequence alignments at the conserved amino acid sequences and primer sequences of RC1 and RC3.

Table 2. PCR amplification using the general *repC* primer set (RC1/3) or group-specific primer sets

Strains	Primer sets used in PCR amplifications*					Sequence group of cloned PCR product
	RC1/3	Group I	Group II	Group III	Group IV	
<i>R. leguminosarum</i>						
SC8	+	—	—	+	+	III
BA5	+	—	—	+	+	III
BA7	+	+	+	+	+	I
BA12	+	+	—	+	+	I
BA11	+	—	+	+	+	II
SC14	+	+	+	+	+	II
SA1	+	—	—	+	+	III
BC1	+	+	+	—	+	I
SA10	+	+	+	—	+	
BB18	+	—	—	+	+	IV
SD13	+	—	—	+	+	III
SC12	+	—	—	+	—	
SD11	+	—	—	+	+	IV
FIII-72	+	+	+	+	+	
FIII-69	+	+	+	+	—	
FIII-71	+	+	+	+	+	II
FIII-68	+	+	+	—	+	II
FIII-61	+	+	—	+	+	
FIII-60	+	+	—	+	—	I
FIII-51	+	+	+	+	—	
FIII-52	+	+	+	+	+	
FIII-50	+	—	+	+	+	
FIII-49	+	—	+	+	+	
FIII-36	+	+	—	+	+	I
FIII-38	+	+	—	+	—	
FIII-12	+	—	—	—	+	
FIII-20	+	—	—	—	—	unique
FIII-70	+	+	+	+	+	I
FIII-37	+	+	+	—	+	
RES-1	+	—	—	+	—	unique
RES-2	—	—	—	+	—	
RES-3	—	—	—	—	—	
RES-4	+	—	—	+	—	II
RES-5	+	+	+	—	+	unique
RES-6	+	+	+	—	—	II
RES-7	+	—	—	+	—	III
RES-8	+	—	—	+	—	III
RES-9	+	—	—	+	—	
RES-10	+	—	—	—	+	
3841	+	—	—	—	—	
RSM2004	+	—	—	+	—	
<i>S. teranga</i>	+	—	—	—	ND	
<i>S. sabeli</i>	+	—	—	—	ND	
<i>S. meliloti</i> A14	+	—	—	—	ND	
A111	+	—	—	—	ND	
A212	+	—	—	—	ND	
A312	+	—	—	—	ND	

+, PCR product of expected size; —, no amplification. ND, Not done.

Fig. 2. *Pst*I restriction fragments of *repC* PCR products amplified by the general primers RC1 and RC3. The PCR product amplified from *R. leguminosarum* strain FIII-71 is shown uncut (lane 1) and cut with *Pst*I (lane 2). Restriction patterns of products amplified from cloned *repC* sequences corresponding to groups I (BA12), II (BA11), III (SC8) and IV (RSM2004), are shown in lanes 3–6 respectively. The PCR product obtained from *S. saheli* is shown cut with *Pst*I (lane 7) and uncut (lane 8).

parison of the corresponding DNA sequences identified conserved nucleotide sequences that might serve as suitable PCR primer sites. The two regions chosen as primer sites and the sequences of the resultant forward (RC1) and reverse (RC3) primers are shown in Fig. 1. PCR amplification using these primers with *R. leguminosarum* 3841, from which the pRL8JI replicator region was originally cloned, and *Agrobacterium tumefaciens*/pTiB6S3 generated products of the expected size (≈ 750 bp) (data not shown).

PCR amplifications undertaken using the RC1/3 primer set with 39 *R. leguminosarum* field isolates from three field sites in Germany (16 strains), France (13 strains) and the UK (10 strains) gave amplification products with 37 of the 39 strains screened (Table 2). Although the PCR annealing temperature used (48 °C) is relatively low, only products of the expected size were seen following agarose gel electrophoresis (Fig. 2, lane 1), suggesting that the primers were amplifying specific sequences and not priming randomly. In addition, amplification using this primer set was obtained with *R. leguminosarum* strain RSM2004, which has selectable markers to enable it to be easily

Fig. 3. Phylogenetic relationships of partial *repC* sequences obtained from *R. leguminosarum* field isolates. (a) Relationships derived from the 200 bp immediately following the RC1 primer site of the cloned DNAs. (b) Relationships derived from the 200 bp immediately preceding the RC3 primer site. Neighbour-joining trees (Saitou & Nei, 1987) were rooted using pRmeGR4a as the outgroup, and the percentage of bootstrap trials supporting a branch is shown when this exceeded 75% and the branch length exceeded 1%. Four major groups of sequences, each amplified by group-specific primers, are indicated by roman numerals and by the shaded areas. Homologous published *repC* sequences are also included: pRmeGR4a from *S. meliloti* (Mercado-Blanco & Olivares, 1993), pRiA4b from *Agrobacterium rhizogenes* (Nishiguchi et al., 1987), pTiB6S3 from *A. tumefaciens* (Tabata et al., 1989) and pRL8JI from *R. leguminosarum* (Turner & Young, 1995).

Group/ strain designation	Forward primer site		Reverse primer site	
	85	104	642	660
pRneGR4a	TCGGAACCGT	GACGCGTGCC	GCGGGCAACG	GCTCTTCTGG
pRiA4b	TCAAACAAGG	CAAGACAGCG	CTTGCTGATG	TAGAGTCAAT
BB18	TTAAGCCAGG	CAAGACGGCG	CTCAGTGATG	TGAATTC AAT
SD11	TTAAGCCGGG	CAAGACAGCG	CTCAGTGATG	TGAATTC AAT
RSM2004	TCAAACCAGG	TAAGCGAGCG	CTTAGTGACG	TGAATTC AAT
RES-1	TCAAGCCCGG	CAAATCGGTC	ATGACAGACG	TCACAGAAAT
group I	TCAAGCCGGG	AAAATCGGTC	ATGGGAGACG	TAGCGGGCGT
RES-5	TCGAACCTGG	AATTACCGCG	ATTGAGGACC	TGGCGATTGG
group III	TCAAGCCGGA	TCAGTCGGTC	ACAGCGCAGC	TTGAACTGTT
PIII-20	TCGACCCGGC	CAATCTATCG	GCCGAGCAGG	TCGCCACCGC
pTib6S	TGGCGCCTGA	CGCTACCGCA	AGACAGCTTG	TGSAGGAGAT
group II	ATGTCCTCGA	CACCTCGGTA	TTACAAGACA	TCGCAACGAG
pRL8JI	CAATAGCGGC	TGGTCGGGTC	CTCCAGGACC	TGATTTGAG

Fig. 5. Sequence alignments of the target sites for group-specific forward and reverse primers. The numbers above each column refer to the nucleotide position in the pRL8JI *repC* coding sequence. Group IV is represented by strains BB18 and SD11; the group IV primers were made with mixed nucleotides at the sites shown in bold.

from all these sequences that the site of such a recombination event would be hard to identify.

The four *repC* sequence groups are widespread in *R. leguminosarum* field populations

Sequence comparison of all 27 pairs of partial sequences enabled the design of group-specific PCR primers to amplify *repC* sequences corresponding to each of the four main groups (I–IV) identified from phylogenetic analysis (Fig. 5). To facilitate the longer-term aims of this research (an assessment of plasmid transfer in soil populations of *R. leguminosarum*) one requirement for specific primers is that they should not amplify *repC* sequences present in tagged release strains. Since RSM2004 is such a strain, the group IV primers were designed so that they would not amplify the *repC* sequence isolated from this strain. All group-specific primer sequences correspond to the same two 20 bp regions of the *repC* partial sequences (dashed arrows in Fig. 1). All the cloned sequences were amplified only by the corresponding group-specific primer pair, except that the RSM2004 cloned sequence was not amplified by any of them.

Table 2 summarizes results from PCR amplifications using these primer sets on the 39 *R. leguminosarum* field isolates. Amplification products were often obtained for more than one primer set from one strain, e.g. BA7 gave a positive result with all four primer sets. These results agree with the restriction enzyme analyses, which strongly suggested that multiple *repC*-like sequences are present in a substantial proportion of the *R. leguminosarum* field isolates screened.

Homologous *repC* sequences were not universally amplified by the general primer pair. For example, RES-2 did

not generate a PCR product with the general primer set, but did with the group III-specific primer set (Table 2). This presumably reflects sequence divergence at the general primer sites in this strain, since group III sequences were amplified and cloned from other strains (e.g. SC8 and RES-8). Group III and IV sequences were only cloned from strains that did not contain group I or group II sequences, suggesting that the group I and II *repC* sequences may match the general primers more closely than do the group III and IV sequences, and are consequently amplified more efficiently. This interpretation is supported by the relative intensities of the bands in Fig. 2: the restriction fragments corresponding to groups I and II are the strongest and those corresponding to groups III + IV restriction fragments are relatively weak.

No amplification products were obtained from strain RES-3 using any of the primer sets. This does not, of course, mean that the plasmids of this strain do not have *repC* genes. In view of the high level of sequence diversity that we have documented, it is very likely that in this and other strains there are *repC* genes that have diverged too much to amplify with any of the limited number of primers that we have used so far.

Sinorhizobium strains also have *repC*-like sequences

PCR using the RC1/3 primer set was used to assess whether related sequences are present in other species of rhizobia, including a number of *S. meliloti* strains isolated from a field site in Spain and type strains of *S. sabeli* and *S. teranga*, originally isolated from Senegal. Amplification products were generated in 18 out of 21 *S. meliloti* strains and with both the *S. sabeli* and *S. teranga* strains. Again restriction enzyme analysis suggested that the PCR products represented heterogeneous mixtures of related sequences in many of the strains tested (Fig. 2, lane 7). However, when six *Sinorhizobium* strains were tested with the group I, II or III primer sets they did not generate amplification products. Thus the *repC* sequences present in *Sinorhizobium* strains appear to belong to groups other than I, II or III, which were the dominant *repC* classes amplified and cloned from *R. leguminosarum* strains.

Wider implications

The genetic diversity of the *repC* sequences from *R. leguminosarum* is so great that some sequences are as different from each other as they are from the published sequences from *Agrobacterium* plasmids. This, together with the finding of related sequences in *Sinorhizobium*, suggest that the range of *repC* sequences that we have described is not specific to *R. leguminosarum*, but may be characteristic of the whole branch of the alpha proteobacteria that includes these genera. In view of the high level of sequence divergence, it is possible that different *repC* groups were already present in the common ancestor of this branch. However, it is also likely that considerable plasmid transfer has occurred subsequently, since conjugative plasmid transfer between these genera has been demonstrated experimentally (Martínez *et al.*, 1987), and

certain *Rhizobium* and *Agrobacterium* plasmids are incompatible with each other and therefore presumably closely related (Hynes *et al.*, 1985; O'Connell *et al.*, 1987; Mozo *et al.*, 1990). We are currently surveying a wide range of related species to ascertain the distribution of the *repC* groups that we have already identified.

We think it is likely that the *repC* groups correspond to plasmid incompatibility groups, though there is as yet no direct evidence for this interpretation. In the *Agrobacterium* plasmids, the *repC* gene encodes an essential replication function (Tabata *et al.*, 1989) so it could plausibly be a determinant of incompatibility. The plasmids pTiB6S3 and pRiA4b are compatible, and the divergence between their *repC* sequences is comparable to that among the four groups that we have identified, suggesting that this level of divergence is sufficient (and may perhaps be necessary) to allow compatibility. Further studies are clearly needed to examine the possible role of *repC* in plasmid incompatibility and to assess the functional significance of the sequence diversity. In addition, the *repA* and *repB* genes are adjacent to *repC* in the *Agrobacterium* plasmids and in pRL8JI, though as yet we have no information for the other *R. leguminosarum* plasmids. The *repA* and *repB* genes have distant homologues in the partition functions of IncP plasmids and phage P1 (Williams & Thomas, 1992), and are also likely to affect compatibility. If *repC* is, or is closely linked to, the major determinant of plasmid incompatibility, then the sequences and primers that we have developed may form the basis for a general scheme for typing plasmids in this group of the alpha proteobacteria.

The primers will also be useful tools for studies of plasmid transfer in the field, which is of interest in the context of risk assessment for the release of genetically modified bacteria. One approach that we are pursuing is to monitor the acquisition of plasmids from the indigenous population by a marked release strain that initially lacks some or all of the *repC* sequences that our primers recognize. More generally, a better understanding of the relationships, origins, host ranges and other properties of indigenous bacterial plasmids will have wide-ranging implications for the development of safe and effective microbial biotechnology.

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