Characterization of the RepFVII Replicon of the Haemolytic Plasmid pSU233: Nucleotide Sequence of an *incFVII* Determinant

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We have isolated a replication region (designated RepFVII) from the IncFVII plasmid, pSU233. Hybridization experiments showed that RepFVII has high homology to the RepFIII replicon of the plasmid pSU316 and, therefore, that pSU233 is another member of the RepFIIA family. We have also located the incompatibility gene (*incFVII*) from RepFVII. The analysis of its sequence revealed the same organization described for several other replicons belonging to the same family.

INTRODUCTION

Bacterial plasmids able to synthesize an F-like pilus have been grouped within the 'F-complex' (Bradley *et al.*, 1980; Willets & Skurray, 1980). Initial results indicated that there were no relationships among the replication regions of IncF plasmids belonging to different incompatibility groups (Sharp *et al.*, 1973). However, Bergquist *et al.* (1986) showed that three replicons previously described for IncFI plasmids, namely RepFIA, RepFIB and RepFIC, are widely distributed among IncF plasmids belonging to six incompatibility groups (IncFI to IncFVI). The RepFIIA replicon of IncFII plasmids is homologous to RepFIC and plasmids homologous to RepFIC/RepFIIA have been classified as belonging to the RepFIIA family (Saadi *et al.*, 1987).

During the study of the incompatibility properties of haemolytic plasmids, de la Cruz *et al.* (1979) reported the existence of a plasmid able to synthesize an F-like pilus and compatible with all previously known IncF plasmids. This plasmid, named pSU233, was classified as IncFVII.

In this paper we report the isolation of a replicon, RepFVII, from pSU233. RepFVII shows homology with RepFIII, a member of the RepFIIA family (Bergquist *et al.*, 1986; J. Lopez and others, unpublished), and thus belongs to this family. In addition, a fragment encoding the IncFVII incompatibility has been located and its nucleotide sequence determined. A comparison with several other IncF plasmids incompatibility genes is presented.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* derivative DH5 α (F⁻ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 (lacZYA-argF)U169 ϕ 80dlacZ M15*; Bethesda Research Laboratories) was the bacterial strain used for the experiments described in this work. The plasmid vectors used were pGEM-3Z (Promega Biotec) and pHGS399 (Takeshita *et al.*, 1987). pSU3040 is a plasmid containing the RepFIII replicon and has been described previously (Rodríguez *et al.*, 1983). pSU221 is a Tn802 insertion derivative of the IncFVII haemolytic plasmid pSU233 and was described by de la Cruz *et al.* (1979). pSU502 is an IncFVI plasmid also described by de la Cruz *et al.* (1979). The plasmids pSU3201, pSU3202, pSU3203 and pSU3204 were constructed during this work (see Results).

DNA manipulations. Plasmid DNA was isolated after caesium chloride/ethidium bromide density gradient centrifugation of lysates obtained by the alkaline procedure described by Ish-Horowicz & Burke (1981). The purification of restriction fragments from either agarose or acrylamide gels was done as described by Maniatis *et*

al. (1982). The conditions of digestion, ligation and transformation have been previously reported (Andres *et al.*, 1987). When intramolecular ligations were required, the DNA concentrations were calculated as suggested by Perbal (1984).

The transfer of DNA fragments to nitrocellulose filters and subsequent hybridization were basically done according to Meinkoth & Wahl (1984). The hybridizations were carried out at 65 °C in 6 × SSC, 1 × Denhardt's reagent, 100 µg denatured herring sperm DNA ml⁻¹ and 0.1% SDS. (1 × SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate; 1 × Denhardt's reagent is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone.) Before exposure the filters were washed at a final stringency of 0.2 × SSC.

Incompatibility tests. Competent cells harbouring the resident plasmid were transformed and selected only for the incoming plasmid. Three transformant colonies were resuspended in L-broth and, after appropriate dilutions, plated onto L-agar plates without selection. The loss of any of the plasmids was tested by transferring 50 colonies from each plate to L-agar plates containing the appropriate antibiotics. To rule out any recombinational event, DNA preparations from ten individual colonies were analysed by agarose gel electrophoresis.

DNA sequence determinations. The *Sau3A* fragment containing the *inc* gene was cloned within the *Bam*HI site of the vector pGEM-3Z (Promega Biotec) and sequenced by the enzymic chain termination method of Sanger adapted for double-stranded DNA (Korneluk *et al.*, 1985).

RESULTS AND DISCUSSION

Definition of RepFVII, the minimal IncFVII replicon

pSU221 is a Tn3 insertion derivative of the IncFVII haemolytic plasmid pSU233 (de la Cruz *et al.*, 1979). To test whether IncFVII plasmids have any homology to the plasmids belonging to the RepFIIA family, we digested pSU221 with the endonuclease *Eco*RI and probed the digest with a 2.9 kb *Bgl*II-*Xba*I fragment containing part of the minimal replicon of the IncFIII plasmid pSU316 (Rodriguez *et al.*, 1983). The hybridization experiment showed a region of strong homology in an *Eco*RI fragment 15 kb in size. No other fragments showed any homology (data not shown). This is the fragment known to contain the inserted Tn3 (de la Cruz *et al.*, 1979). If the homology corresponds to a functional replication region, the recircularization of the 15 kb fragment would result in an ampicillin-resistant miniplasmid. Thus, the *Eco*RI digestion was ligated in conditions favouring the recircularization of the fragments and the mixture was used to transform competent *E. coli* cells selecting for ampicillin resistance. As expected, a 15 kb miniplasmid was obtained. This miniplasmid was termed pSU3201. Its map is shown in Fig. 1.

The homology between pSU3201 and RepFIII, the replicon of the IncFIII plasmid pSU316, is located in two *Pst*I fragments, 4.2 and 1.1 kb in size respectively (Fig. 2, A). These *Pst*I fragments are contiguous. Part of the 4.2 kb fragment corresponds to the *bla* gene of the Tn802 transposon. In order to confirm that this fragment is a functional replication region, we constructed the miniplasmid pSU3202 by intramolecular ligation of the *Bgl*II-*Bam*HI fragment shown in Fig. 1.

Isolation and sequencing of an IncFVII determinant

When comparing the nucleotide sequences of four replicons belonging to the RepFIIA family, Saadi *et al.* (1987) defined alternating regions of homology (HRs) and nonhomology (NHRs). One of the regions of conserved homology (HRII in Saadi *et al.*, 1987) corresponds to the incompatibility gene *inc*. In order to locate this gene in the RepFVII replicon, we digested pSU3201 with several restriction endonucleases; the digests were blotted onto nitrocellulose filters and probed with a 0.5 kb *Pst*I fragment which contains the incompatibility gene of the plasmid pSU316 (Rodriguez *et al.*, 1983). The results shown in Fig. 2 (B to E) indicate that the minimal fragment of pSU3201 that showed homology with the *incFIII* gene is a *Bgl*II-*Sa*I fragment of 0.84 kb. The restriction map of this fragment is identical to the same region of R100, an IncFII plasmid also belonging to the RepFIIA family (Fig. 1). The 0.84 kb fragment was cloned in pHGS399 to produce the plasmid pSU3204. As shown in Table 1, pSU3204 is strongly incompatible with its parent plasmids (i.e. pSU221, pSU3201 and pSU3202) but compatible with the plasmids pSU3040 and pSU502 belonging respectively to the IncFIII and IncFVI incompatibility groups.

Fig. 1. Physical map of the plasmids constructed during this work. Both pSU3201 and pSU3202 are autonomously-replicating miniplasmids obtained by deletions of pSU221. pSU3203 is a pBR322 clone containing a 1.1 kb *Pst*I fragment from pSU3202. The comparison of the *Pst*I fragment of pSU3203 with the same region of R100 (Rosen *et al.*, 1980) is also shown. Restriction sites are: P, *Pst*I; L, *Sal*I; E, *Eco*RI; B, *Bgl*II; BH, *Bam*HI; S, *Sau*3A.

Fig. 2. Southern blot hybridizations of pSU3201. Digests were transferred to nitrocellulose filters as described in Methods. The arrows at the sides indicate the fragments showing homology. The size of these fragments is also indicated. The different parts of the figure correspond to different gels. A and A', pSU3201 digested with *Pst*I and hybridized with a 2.9 kb fragment of the RepFIII replicon of pSU316 (Rodriguez *et al.*, 1983). B and B', Double digeston *Pst*I-*Bgl*II of pSU3201 hybridized with a 0.5 kb probe containing the *incFIII* gene (Rodriguez *et al.*, 1983). The same probe was used for hybridizing with the following digestions of pSU3201: C and C', *Sal*I-*Bgl*II; D and D', *Pst*I; E and E', *Bam*HI-*Bgl*II; F and F', *Sal*I-*Pst*I.

R1	GATCGTTTAAAGGAATTTTGTGGCTGGCCACGCCGTAAGGT
R100	
P307	A.....
pINV	A.....T.....
pSU316	A.....A.....
pSU221	
R1	GGCAAGGAACTGGTTCTGATGTGGATTACAGGAGCCAGA
R100	
P307	...G.....
pINV	...G.....G.....
pSU316	...G.....
pSU221	...G.....
R1	AAAGCAAAAACCCCGATAATCTTCTTCAACTTTGGCGAAT
R100	T.....G.....
P307	A.....A..T..G.....CG
pINV	T.....G.....
pSU316	...TG.....T.....G.....
pSU221	...TG.....G.....C
R1	ACGAAAAGATTACCGGGGCCAC T TAAACCGTATAGCC
R100	
P307	.G..G.....T.....
pINV	.AG.....
pSU316	GA.....T.....TAA.AA G.....T.C.....A
pSU221	.T.....AT..AA.....
R1	AACAATTCAGCTATGCGGGAGTATAGTTATATGCCCGGA
R100	
P307	
pINV	...CA.....
pSU316	.G.GG..GCT.....
pSU221	
R1	AAAGTTCANG
R100	
P307	A.....AC.....
pINV	
pSU316	A.....T.....
pSU221	A.....
R1	TTGTAAGTGCAGGATGGTGTGACT
R100	
P307	G.....
pINV	
	ACA.....

Fig. 3. Comparison of the nucleotide sequences of the *Sau3A* fragment containing the *inc* gene of several *IncF* plasmids. The gene is transcribed leftwards. Boxes labelled -35 and -10 represent respectively the -35 and -10 consensus promoter regions (Rosenberg & Court, 1979). The coding sequence of the *IncRNA* of plasmid R1 is indicated by the large box. Arrows above the sequence represent the stems of the two loops of the *IncRNA* which are indicated by the small boxes between the arrows (Gerhart *et al.*, 1986). Sequences were taken from Rosen *et al.* (1980; R1 and R100), Saadi *et al.* (1987; P307), López *et al.* (unpublished; pSU316) and Silva *et al.* (1988; pINV).

Comparison of the incompatibility genes of several *IncF* plasmids

The incompatibility genes of all the *IncF* plasmids so far mapped are contained in a 262 bp *Sau3A* fragment. There is an analogous fragment within the 0.84 kb fragment of pSU221 which expresses the *IncFVII* phenotype. The nucleotide sequence of this *Sau3A* fragment of pSU3201 was determined and is shown in Fig. 3.

Table 1. *Incompatibility properties of pSU3204*

Plasmid pSU3204 was always used as the donor plasmid. At least 150 colonies of each pair were assayed for the presence of plasmid.

Resident plasmid	Percentage plasmid loss	Plasmid lost	Incompatibility
pSU221	100	pSU221	+
pSU3201	100	pSU3201	+
pSU3202	100	pSU3202	+
pSU3040	0	none	—
pSU502	0	none	—

The *inc* gene of replicons belonging to the RepFIIA family encodes a small RNA molecule. This RNA is expressed constitutively from its own promoter (Rosen *et al.*, 1981; Stougaard *et al.*, 1981). The existence of this promoter seems to be essential for the controlled replication of RepFIIA-family replicons. As expected, the consensus sequences of the -35 and -10 regions of the promoter are conserved in the nucleotide sequence of pSU221 as well (Fig. 3).

The study of the secondary structure of the IncRNA of the IncFII plasmid R1 revealed the existence of two loops, namely loop I and loop II. Loop II is presumably involved in the recognition of the target RNA. In fact many copy number mutations have been mapped in positions that would alter loop II structure (Giskov & Molin, 1984; Gerhart *et al.*, 1986). pSU221 presents three mutations in the region coding for the stem of loop II. These mutations are good candidates for the change of incompatibility group of the plasmid pSU221.

In conclusion, the RepFVII replicon of pSU221 is another member of the RepFIIA family of replicons. The *incFVII* gene shows very few changes with respect to other RepFIIA-family plasmids. Our results, together with other studies on the evolution of RepFIIA-family plasmids (for review see Saadi *et al.*, 1987), indicate that the divergence to incompatibility groups is a very recent event. Such a rapid divergence to different incompatibility phenotypes suggests that incompatibility is probably an undesirable side-effect of the replication control mechanisms. Another interesting effect of this rapid evolution is that the conservation of most of the sequences in the replication region would allow exchange of genes among different replicons. As suggested by Saadi *et al.* (1987), this shuffling of replication genes would probably increase the 'survival value' of IncF plasmids.

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