Isolation and evolutionary analysis of a RepFVIB replicon of the plasmid pSU212

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We have isolated at least two different replication regions from pSU401, a Tn802 insertion derivative of the IncFVI plasmid pSU212. One of the replication regions (RepFVIB) is highly homologous to the RepFIIA replicon of IncFII plasmids, and thus belongs to the RepFIIA family. We have also cloned the incompatibility determinant (*incFVI*) and the copy number control gene (*cop*) from RepFVIB and determined their nucleotide sequences. The analysis of the sequences supports the idea of a modular evolution of RepFIIA plasmids.

Introduction

The 'F-complex' includes all the plasmids able to synthesize an F-like pilus (Bradley, 1980; Willetts & Skurray, 1980). Although initial hybridization results indicated that, apart from the genes involved in conjugation, there was no homology among IncF plasmids belonging to different incompatibility groups, both detailed hybridization studies (Bergquist *et al.*, 1986) and nucleotide sequence data (Ryder *et al.*, 1982; Saadi *et al.*, 1987; López *et al.*, 1989*a, b*) have shown that the basic replicons of IncF plasmids are very similar. Three different types of replicons have been identified, namely RepFIA, RepFIB and RepFIC. This last one was extensively characterized first in IncFII plasmids and is therefore also called RepFIIA. All the replicons homologous to it have been grouped within the RepFIIA family (Saadi *et al.*, 1987).

The classification of IncFVI plasmids has been always controversial. Zabala *et al.* (1983) isolated and characterized a miniplasmid from the IncFVI plasmid pSU502. This miniplasmid had no homology with other plasmids belonging to the IncF supergroup. More recent studies by Bergquist *et al.* (1986) indicated that pSU104 and pGL611a, another two IncFVI plasmids studied, had homology with the RepFIC (RepFIIA) probe. In order to further characterize the region of IncFVI plasmids having homology with the RepFIIA replicon, we have isolated a miniplasmid (pSU3251) from pSU401, a

Tn802 insertion derivative of the IncFVI plasmid pSU212 (de la Cruz *et al.*, 1979). The replicon of pSU3251 (RepFVIB) has all the characteristics of a RepFIIA family replicon. The study of its homology with some other replicons belonging to the RepFIIA family is described, and the evolution of RepFIIA replicons is discussed.

Methods

Bacterial strains and plasmids. The *Escherichia coli* derivative DH5 α [*F⁻ endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ (lacZYA-argF)-U169, ϕ 80dlacZ M15; Bethesda Research Laboratories] and the minicell-producing strain DS410 (*sup⁺ lac⁺ minA minB rpsL*) (Dougan & Sherratt, 1977) were the bacterial strains used.*

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). pSU3201 is a miniplasmid derived from pSU233 and its construction has been reported previously (López *et al.*, 1989*b*). pSU401 is a Tn802 insertion derivative of the haemolytic plasmid pSU212 (Monti-Bragadin *et al.*, 1975). It belongs to the IncFVI incompatibility group (de la Cruz *et al.*, 1979). Plasmids pSU3251 and pSU3252 were constructed during this work and will be described below.

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 reported previously (Andrés *et al.*, 1987). When intramolecular ligations were required, the DNA concentrations were calculated as suggested by Perbal (1984).

Abbreviation: ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession number X55896.

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, 0.1% SDS. Before exposure the filters were washed at a final stringency of 0.2× SSC. (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.)

Incompatibility tests. Competent cells harbouring the resident plasmid were transformed and selected only for the incoming plasmid. Four 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 10 individual colonies were analysed by agarose gel electrophoresis.

Analysis of plasmid-encoded proteins. Minicells from stationary-phase L-broth cultures of strain DS410 containing plasmids were prepared as described by Andrés *et al.* (1979). Plasmid-encoded proteins were labelled with 25 µCi (925 kBq) of [¹⁴C]protein hydrolysate (55 mCi mg-atom⁻¹; Amersham) and analysed by electrophoresis through 15% (w/v) polyacrylamide gels.

DNA nucleotide sequence determinations. The fragments containing either the incompatibility determinant or the *cop* gene of the RepFIIA region of pSU401 were cloned in the plasmid vector pGEM-3Z (Promega Biotec). Deletions produced by the exonuclease III method (Henikoff, 1984) were sequenced by the enzymic chain-termination method of Sanger adapted for double-stranded DNA (Korneluk *et al.*, 1985). The sequences of both strands were assembled and analysed using the Microgenie program package (Queen & Korn, 1984).

Results and Discussion

Isolation of the RepFVIB replication region

The isolation of a replication region from an IncFVI plasmid was previously described by Zabala *et al.* (1983). They reported that the replication region isolated from an IncFVI plasmid (a region which we will refer to as RepFVIA) has no homology with several IncF plasmids belonging to different incompatibility groups. Thus, RepFVIA is probably unique to IncFVI plasmids. Most IncFVI plasmids, however, have a region of homology with the RepFIC replicon of IncF plasmids (also known as RepFIIA) (Bergquist *et al.*, 1986). All these results raised the question whether the homology corresponds to a functional replication region. To answer this question, we constructed miniplasmids from pSU401, a Tn802 insertion derivative of the haemolytic plasmid pSU212. We digested pSU401 DNA with *Eco*RI. A 1:3 dilution of the digestion mixture was religated and used to transform competent *E. coli* DH5α cells, selecting for resistance to ampicillin. The analysis of the transformants revealed the presence of at least three different miniplasmids. One of them, pSU3251, hybridized with the 0.5 kb *Pst*I fragment containing the *incFIII* gene of pSU316 (Rodríguez *et al.*, 1983), a plasmid belonging to

Fig. 1. Restriction map of the RepFVIB miniplasmid pSU3251 obtained from pSU401. The *Bgl*II-*Sal*I fragment homologous to the *incFIII* gene as well as the same region of plasmids R100 and pSU233 are also shown. Restriction sites are: P, *Pst*I; E, *Eco*RI; B, *Bgl*II; L, *Sal*I; H, *Hind*III.

the RepFIIA family. Subsequent analysis by restriction and hybridization allowed us to locate the homology in a 0.8 kb *Bgl*II-*Sal*I fragment (see the map in Fig. 1). The restriction map of this region is very similar to the corresponding region of the plasmid R100, one of the prototypes of the plasmids belonging to the RepFIIA family. Thus, this region has the physical characteristics of a RepFIIA replicon. To distinguish it from that described by Zabala *et al.* (1983), we will refer to it as RepFVIB.

Isolation of an IncFVI incompatibility determinant

It could be argued that the fact that pSU3251 has homology with the *incFIII* gene does not prove that RepFVIB is a functional replicon. To test this, we used an indirect approach. We first cloned the region that, according to both the restriction map and the hybridization experiments, should contain the incompatibility determinant. The cloning was done in the Cm^R vector pHSG399. The plasmid pSU3252, containing the 0.8 kb *Bgl*II-*Sal*I fragment, was assayed for incompatibility against either the parental plasmid or the miniplasmid pSU3251. The results, shown in Table 1, indicated that (i) the 0.8 kb fragment contains an incompatibility determinant; (ii) this incompatibility determinant is able to suppress the replication of the miniplasmid pSU3251, which strongly suggests that pSU3251 is replicated from the RepFVIB replicon; and (iii) the fragment does not

Table 1. Incompatibility of the clone pSU3252

pSU3252 was always used as the donor plasmid. Two hundred colonies of each pair were assayed for the presence of the plasmids.

Resident plasmid	Percentage of plasmid loss	Plasmid lost	Inc
pSU221 (IncFVII)	0	None	—
pSU401 (IncFVI)	0	None	—
pSU3040 (IncFIII)	0	None	—
pSU3251 (IncFVI)	100	pSU3251	+
pSU3201 (IncFVII)	18	pSU3201	—*

* The percentage of incompatibility does not increase at 10 generations. Thus, we consider the plasmids as compatible. For details see Methods and de la Cruz *et al.* (1979).

inhibit the replication of the parental plasmid pSU401. This last result was consistent with that of Zabala *et al.* (1983) and indicated the presence of more than one functional replicon in pSU401. In addition, pSU3252 showed partial incompatibility towards the IncFVII miniplasmid pSU3201. The reason for this partial incompatibility is unknown.

The putative *cop* gene from pSU3251 expresses a 9.5 kDa polypeptide

The replication of RepFIIA replicons is controlled by at least two plasmid-encoded inhibitors. One of them is a polypeptide encoded by the *copB* gene. This gene is located in a fragment adjacent to the incompatibility determinant. By analogy with R100, the 0.8 kb *Bgl*II-*Sal*I fragment should also contain the *copB* gene. We cloned the fragment in pGEM-3Z. The recombinant plasmid was expressed in a minicell system. The analysis of the proteins synthesized (Fig. 2) indicated that this fragment codes for a protein of approximately 9.5 kDa, similar to the proteins described for other replicons belonging to the RepFIIA family (Molin *et al.*, 1981; Andrés *et al.*, 1987 and references therein).

Analysis of the nucleotide sequence of the copy number control region of pSU3251

The structure of the replicons belonging to the RepFIIA family has been established based on nucleotide sequence data. They basically consist of an origin of replication (*oriV*), whose location in R1 has been reported by Masai *et al.* (1983). The frequency of initiation of replication at *oriV* is regulated by the availability of a 33 kDa *cis*-acting protein, the product of the gene *repA*. The *repA* gene is transcribed from two

Fig. 2. Expression of the *cop* gene in a minicell system. pSU3251-containing minicells were prepared and labelled as described in Methods. The proteins were analysed in a 15% polyacrylamide gel and compared with those produced by minicells containing only the cloning vector without insert and with the protein produced by a plasmid containing the *cop* gene of the IncFVII plasmid pSU233. Lanes: A, molecular mass markers; B, pGEM-3Z; C, pSU3251; D, a plasmid containing the *cop* gene of the IncFVII plasmid pSU233 cloned in pHSG399 (Takeshita *et al.*, 1987). Bla and LacZ represent respectively the β -lactamase and LacZ peptide produced by pGEM-3Z. Cat is the chloramphenicol acetyltransferase produced by pHSG399.

promoters, *copBp* and *repAp*. The control of the levels of the RepA protein is exerted by at least two other genes present in the replicon, namely *copB* and *copA*. The product of *copB* acts at the -35 region of the *repA* promoter (Riise & Molin, 1986), which is normally repressed. On the other hand, *copA* encodes an antisense RNA which interacts with the *repA* gene transcripts. This interaction results in lower levels of the RepA protein (see Nordström *et al.*, 1988, for a review).

We have already mentioned that RepFVIB belongs to the RepFIIA family. To compare RepFVIB with other RepFIIA replicons, we determined the nucleotide sequence of the whole 0.8 kb fragment which contains both *copA* and *copB* genes (Fig. 3). Among all the plasmids belonging to the RepFIIA family whose sequence has been reported, RepFVI was most similar to

Fig. 3. Nucleotide sequence of the copy number control region of pSU212. The -35 and -10 RNA polymerase recognition sites (Rosenberg & Court, 1979) and Shine-Dalgarno (SD) sequences (Shine & Dalgarno, 1979) are boxed. Large boxes labelled cop, repA and 7K are the coding sequences of Cop, part of RepA and the putative 7 kDa proteins respectively. The incompatibility determinant (inc) is underlined. HRI, HRII, NHRI and NHRII represent the homology/non-homology regions described by Saadi *et al.* (1987).

R100. The similarity starts with the *copB* promoter at positions 22 and 46 (-35 and -10 regions respectively), which is identical to the same region of R100. The Shine-Dalgarno consensus sequence of *copB* is also conserved

(position 80). Within the coding sequence of the *copB* gene, there is only one nucleotide change with respect to R100 (C by T at position 281) and this does not result in an amino acid substitution.

Rosen *et al.* (1980) described the existence of another two ORFs, 3K and 7K respectively, in the replication region of the IncFII plasmid R100. The 7K ORF seems to be implicated in the regulation of plasmid replication. The current hypothesis suggests that the translation of the 7K ORF prevents secondary structure formation by ('irons') the RepA mRNA and therefore interferes with the binding of CopA to CopT, which is known to be initiated by interactions between the single-stranded loops at the top of complementary hairpins in the two RNAs. This inhibition of binding would result in an enhanced expression of the RepA protein (Wagner *et al.*, 1987).

The detailed analysis of the sequence of pSU3251 revealed the existence of a premature stop codon (TAA) at position 500. Thus, the translation of the '7K' ORF of pSU316 would produce a truncated polypeptide of about 2 kDa. A premature stop codon in the 7K ORF was also described for the RepFIIIA replicon of pSU316 (López *et al.*, 1989a). In spite of the presence of the stop codon, in both RepFIIIA and RepFVIB the '7K' ORF would still overlap the *copA* gene. Thus, our data are not contradictory with the hypothesis of 'ironing'. Moreover, the presence of the stop codon supports the idea that the protein itself is not necessary for the regulation as suggested by Wagner *et al.* (1987).

Evolution of RepFIIA replicons

Saadi *et al.* (1987) were the first to report that the homology among the members of the RepFIIA family is not evenly distributed along the replicon, but clustered in regions which they defined as homology regions (HR) separated by non-homologous regions (NHR). As they also noted, these modules contain both a gene and the target of the product of that gene. Thus, although defined in terms of homology, the modules also have a functional meaning. The first one, which includes part of the *copB* gene and its target, the -35 region of the *repA* promoter, would correspond to the NHRI region (module *copB/-35*). The second module of homology (HRII) includes the region coding for the antisense RNA, i.e. the incompatibility determinant, and its overlapping target *copT* (module *copA/copT*). NHRII, according to Saadi *et al.* (1987), includes *repA* and its target *oriV* (module *repA/oriV*).

In order to better understand the evolution of the plasmids belonging to the RepFIIA family, we have defined several alleles of the modules that constitute the basic replicon. All the modules belonging to the same allele have more than 95% nucleotide sequence similarity. The classification is summarized in Table 2. The alleles shown here correspond to functional alleles as

Table 2. Summary of plasmid-specific alleles of the modules of RepFIIA replicons

Plasmid	Module*		
	<i>copB/-35</i>	<i>copA/copT</i>	<i>repA/oriV</i>
P307 (IncFI)	I	I	I
F (IncFI)	I	I	I†
R1 (IncFII)	II	II	II
R100 (IncFII)	III	II	II
pSU316 (IncFIII)	II	III	II
pSU233 (IncFVII)	IV	VII	II‡
pSU212 (IncFVI)	III	VI	II‡

* The modules included in the same allele class have more than 95% homology.

† The RepFIIA replicon of F is interrupted by the insertion of Tn1000.

‡ The classification is based on restriction analysis and preliminary sequence data.

suggested by both incompatibility and complementation studies.

The first interesting conclusion from the classification shown in Table 2 is that there are only two *repA/oriV* alleles. One of them, the allele I, seems to be exclusive to IncFI plasmids (F and P307). IncFI plasmids contain specific alleles for all the modules considered (Table 2). Thus, IncFI plasmids are clearly different from the rest of the plasmids listed and probably constitute a subfamily within the RepFIIA family.

The large number of alleles of the *copA/copT* module is noteworthy. The different alleles are, however, very similar. It is known that a single base-pair change in the incompatibility determinant is enough to alter the incompatibility behaviour of a plasmid (Lacatena & Cesareni, 1981; Tomizawa & Itoh, 1981). Thus, it is very likely that replicons within the RepFIIA family are rapidly evolving by creating new *copA/copT* alleles.

There are four alleles of the *copB/-35* module. One of them is exclusive to IncFI plasmids. The other alleles were described for the IncFII plasmids R1 and R100 and the IncFVII plasmid pSU233. As both R1 and R100 share the incompatibility determinant, the initial idea was that there had been a divergence in their *copB* genes. Later results from López *et al.* (1989a) indicated that there is no link between *copA/copT* and the *copB/-35* module. For instance, pSU316 and R1 were shown to belong to different incompatibility groups, that is, they have different *copA/copT* alleles, and have the same *copB/-35* allele. In this paper we have demonstrated that this phenomenon is rather frequent. pSU212 shares the *copB/-35* module with R100 but is much more related to pSU233 in *copA/copT* (Fig. 4).

These results strongly support the idea of modular evolution of RepA replicons, as already proposed by

Fig. 4. Comparison of the nucleotide sequence of the *Sau3A* fragment containing the *inc* gene of pSU3251 with those of the same region of other RepFIIA replicons. Boxes labelled -35 and -10 represent respectively the -35 and -10 consensus promoter sequences (Rosenberg & Court, 1979). The sequence in the large box corresponds to the IncRNA of plasmid R1. The arrows above the sequence represent the stems of the two loops of the IncRNA which are indicated by small boxes between arrows (Wagner & Nordström, 1986). Sequences were taken from Stougaard *et al.* (1981; R1), Ohtsubo *et al.* (1986; R100), Saadi *et al.* (1987; P307), López *et al.* (1989a, pSU316) and López *et al.* (1989b, pSU221).

Saadi *et al.* (1987). The process might be similar to the exchange of exons in eukaryotic chromosomes and by analogy we have called it 'gene shuffling' (López *et al.*, 1989a). The way in which it occurs is still unknown, although homologous recombination carried out by a RecBC-like system is a very likely possibility.

In summary, the evolution of RepFIIA replicons by

module-exchange ('gene shuffling') allows a very rapid divergence of replicons by giving them the possibility of recruiting control genes from other regions of the chromosome. The clear advantage of this type of modular recombination is that both the product and its target evolve at the same time, thus avoiding misfunctions of the control system during the evolutionary process.

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