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

JAVIER LÓPEZ, DOLORES DELGADO, ISABEL ANDRÉS, JOSÉ M. ORTIZ and JOSÉ C. RODRÍGUEZ*

Departamento de Biología Molecular, Universidad de Cantabria, Polígono de Cazofía s/n, 39011 Santander, Spain

(Received 4 October 1990; revised 8 January 1991; accepted 6 February 1991)

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 IncFI 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., 1989a, 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 mini-plasmid from the IncFVI plasmid pSU502. This mini-plasmid 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 mini-plasmid (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 DH5α [F- endA1 hsdRI7 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)-U169, 80lacZ 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 Biotech) and pHG5399 (Takeshita et al., 1987). pSU3040 is a plasmid containing the RepFIII replicon and has been described previously (Rodriguez 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 mini-plasmid derived from pSU233 and its construction has been reported previously (López et al., 1989b), pSU3401 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).
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 André et al. (1979). Plasmid-encoded proteins were either the incompatibility determinant or the cop gene of the RepFIIA family. Subsequent analysis by restriction map and hybridization allowed us to locate the homology in a region of pSU401. The BglII-SaI fragment homologous to the incFIII gene as well as the same region of plasmids R100 and pSU233 are also shown. Restriction sites are: P, PstI; E, EcoRI; B, BglII; L, SalI; H, HindIII.

**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 EcoRI. 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 Psrl fragment containing the incFIII gene of pSU316 (Rodriguez et al., 1983), a plasmid belonging to the RepFIIA family. Subsequent analysis by restriction and hybridization allowed us to locate the homology in a 0.8 kb BglII–SalI 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 CmR vector pHSG399. The plasmid pSU3265, containing the 0.8 kb BglII–SalI 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

<table>
<thead>
<tr>
<th>Resident plasmid</th>
<th>Percentage of plasmid loss</th>
<th>Plasmid lost</th>
<th>Inc</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSU221 (IncFVII)</td>
<td>0</td>
<td>None</td>
<td>−</td>
</tr>
<tr>
<td>pSU401 (IncFVII)</td>
<td>0</td>
<td>None</td>
<td>−</td>
</tr>
<tr>
<td>pSU3040 (IncFIII)</td>
<td>0</td>
<td>None</td>
<td>−</td>
</tr>
<tr>
<td>pSU325 (IncFVI)</td>
<td>100</td>
<td>pSU3251</td>
<td>+</td>
</tr>
<tr>
<td>pSU3201 (IncFVII)</td>
<td>18</td>
<td>pSU3201</td>
<td>−</td>
</tr>
</tbody>
</table>

* 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 BglII-SalI 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 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 Nordstrom 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 RepFIIIA replicons

Saadi et al. (1987) were the first to report that the homology among the members of the RepFIIIA 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 RepFIIIA 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 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 RepFIIIA 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 RepFIIIA 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
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 RepFIA 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.

This work was supported by grant 0007/85 from the Comision Asesora de Investigacion Cientifica y Tecnica (CAICYT). J. L. is a predoctoral recipient of a grant from the Spanish Ministry of Education (Plan de Formacion del Personal Investigador).

References


