Incompatibility Properties of the IncFIII/FIV Haemolytic Plasmid pSU316 when Integrated in the *Escherichia coli* Chromosome

By JESÚS NAVAS, FERNANDO DE LA CRUZ*, JOSÉ C. RODRIGUEZ, ISABEL ANDRÉS, GABRIEL PULGAR AND JOSÉ M. ORTIZ

Departamento de Bioquímica, Facultad de Medicina, Polígono de Cazoña s/n, Santander, Spain

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The haemolytic plasmid pSU316 is incompatible with members of the IncFIII and IncFIV incompatibility groups. Plasmid pSU307 (pSU316 *hlyC*::Tn5) was inserted by integrative suppression into the chromosome of JW112, a temperature-sensitive *dnaA* mutant of *Escherichia coli*. The incompatibility properties of this strain (SU51) were studied and it was found that: (1) plasmid pSU306 (pSU316 *hlyA*::Tn802) was rapidly lost from strain SU51 both at 30 °C and 42 °C; (2) the IncFIII plasmid pSU397 (ColB-K98::Tn802) was lost from strain SU51 at 42 °C but not at 30 °C; and (3) the IncFIV plasmid R124 was stably maintained in strain SU51 at both temperatures. Revertants of pSU307 to the autonomous state could be obtained from SU51. These revertants exerted incompatibility towards the prototype plasmids pSU306, pSU397 and R124 in the same way as pSU307 itself. Thus, strain SU51 provided a suitable method for distinguishing the three different incompatibility determinants of plasmid pSU316.

INTRODUCTION

Plasmid incompatibility is the phenotypic expression of the inability of two plasmids to be stably maintained in the same bacterial cell line. Plasmid incompatibility has been used as a criterion for plasmid classification, particularly in epidemiological studies (Datta, 1979; Sasakawa *et al.*, 1980). Also, incompatibility is a manifestation of the mechanisms of control of plasmid replication and partitioning (Lane, 1981; Nordström *et al.*, 1980; Seelke *et al.*, 1982; Timmis *et al.*, 1981) and is often used as a tool for analysing these processes.

The α -haemolytic plasmid pSU316 is particularly interesting because it is incompatible with representatives of both the IncFIII (represented by ColB-K98) and IncFIV (represented by R124) groups (de la Cruz *et al.*, 1979). The incompatibility status of these two groups is controversial because their members have been reported to show incompatibility reactions against members of other F incompatibility groups (Gasson & Willetts, 1975; Säsärman *et al.*, 1980). Furthermore, hybridization experiments showed that plasmid pSU316 had a similar degree of homology to ColB-K98 and R124 as it did to the compatible F-like plasmids R386 (IncFI) and R1 (IncFII) (de la Cruz *et al.*, 1980a).

This work describes a genetic characterization of the incompatibility determinants of plasmid pSU316. Plasmid pSU316 was inserted in the chromosome of a *dnaA*-ts mutant of *Escherichia coli* by integrative suppression (Nishimura *et al.*, 1971) and the incompatibility properties of the plasmid studied in the integrated state. In this way, it has been possible to distinguish between incompatibility reactions specific for IncFIII, IncFIV and the presumptive replicon-specific incompatibility of plasmid pSU316.

METHODS

Bacterial strains and plasmids. The strains and plasmids used are described in Tables 1 and 2, respectively.

Genetic experiments. The techniques used for bacterial growth, conjugation and incompatibility testing have been described previously (de la Cruz *et al.*, 1979). Experiments designed to detect the switch-off of replication of

Abbreviations: Cb, carbenicillin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin.

Table 1. *Escherichia coli* K12 strains used

Strain	Relevant genotype	Source/Reference
EC185	F ⁻ prototroph <i>gyrA</i>	Authors' laboratory collection
DF110	F ⁻ <i>lac pro thi rpsL</i>	Authors' laboratory collection
W3110 Rif ^r	F ⁻ prototroph <i>rpoB</i>	Authors' laboratory collection
J53-1	F ⁻ <i>pro met gyrA</i>	Authors' laboratory collection
JW112	F ⁻ <i>lac rpsL dnaA508</i>	Wechsler & Gross (1971)
UB5201	F ⁻ <i>pro met gyrA recA56</i>	Bennett & Richmond (1976)
UB1780	F ⁻ <i>pro met gyrA recA56 bla</i> (Tn802)	Bennett & Richmond (1976)
SU51	JW112::pSU307	This work

Table 2. *Plasmids used*

Plasmids	Properties/Origin	Relevant phenotype	Size (kb)	Reference
pSU316	Wild-type Hly plasmid	Hly ⁺ Tra ⁺ IncFIII/FIV	77	de la Cruz <i>et al.</i> (1979)
pSU306	pSU316 (<i>hlyA</i> ::Tn802)	Ap ^r /Cb ^r Tra ⁺ IncFIII/FIV	82	de la Cruz <i>et al.</i> (1979) and this work
pSU307	pSU316 (<i>hlyC</i> ::Tn5)	Km ^r Tra ⁺ IncFIII/IV	82	This work
ColB-K98	Wild-type Col plasmid	Cba ⁺ Tra ⁺ IncFIII	70	Frydman & Meynell (1969)
pSU397	ColB-K98::Tn802	Ap ^r /Cb ^r Cba ⁺ Tra ⁺ IncFIII	75	This work
R124	Wild-type R plasmid	Tc ^r Tra ⁺ IncFIV	124	Hedges & Datta (1972)
pSU307/8	Plasmid revertant from SU51	Km ^r Tra ⁺ IncFIII/IV	71	This work

the integratively suppressed strain SU51 and plasmid-containing derivatives were carried out as described by Molin & Nordström (1980). Basically, strains growing exponentially in Penassay broth (AM3; Difco) at 30 °C were diluted into pre-warmed (42 °C) medium, and growth was followed spectrophotometrically in a Spectronic 20 colorimeter (Bausch & Lomb) at 600 nm.

Genetic labelling of plasmids with transposons. Plasmids were labelled with transposons Tn802 and Tn5 by the following tri-parental mating technique. Strain UB1780 (Bennett & Richmond, 1976) carrying Tn802, and strain ECS605 carrying Tn5 (A. Pühler, personal communication) were mixed with strains carrying the plasmid to be labelled and the final recipient, DF110 (streptomycin-resistant; Sm^r), and incubated at 37 °C for about 18 h. Transconjugants were selected on streptomycin (300 µg ml⁻¹; Compañía Española de Penicilina y Antibióticos) and either carbenicillin (Cb; 500 µg ml⁻¹; Beechams) for pSU316::Tn802 and ColB-K98::Tn802 or kanamycin (Km; 50 µg ml⁻¹; Laboratorios Lefa S.A.) for pSU316::Tn5.

A total of 48 Sm^r Km^r colonies were isolated from the EC185(pSU316) × ECS605 × DF110 mating; 20 were Hly⁻. One derivative was chosen because it showed a high transfer frequency (4 × 10⁻⁴ Km^r transconjugants per donor cell in 1 h crosses). The physical structure of the pSU316::Tn5 plasmid present (called pSU307) was determined by restriction endonuclease analysis (Fig. 1). A ColB-K98::Tn802 plasmid (called pSU397) was constructed in a similar fashion.

Integrative suppression of the dnaA-ts508 mutation. Plasmid pSU307 was transferred to strain JW112 (Sm^r) by conjugation at 30 °C. Transconjugant Km^r Sm^r colonies were isolated, grown at 30 °C, and plated on Penassay (Antibiotic Medium no. 3; Difco) agar plates at 30 °C and 42 °C. The efficiency of plating at 42 °C in relation to that at 30 °C was about 10⁻⁵. Individual colonies that grew at 42 °C were purified and checked for Km^r and for the presence of plasmids by a screening technique (Kitts *et al.*, 1982). One strain (SU51) was chosen for further study because the following tests indicated that it carried pSU307 integrated in its chromosome. (1) No plasmid band was detectable when DNA from cleared lysates of SU51 was analysed by agarose gel electrophoresis. (2) The Km^r determinant was not transferred at a detectable frequency [$< 10^{-8}$ compared to 3 × 10⁻³ Km^r transconjugants per recipient in JW112(pSU307) control matings]. (3) A ³²P-labelled RNA probe obtained by transcription of pSU307 DNA *in vitro* (de la Cruz *et al.*, 1980b) was tested for hybridization with EcoRI-cleaved DNA prepared from SU51 by the method of Southern (1975). The hybridization pattern corresponded to the EcoRI bands of pSU307 itself (data not shown). (4) Plasmid pSU306 is stably maintained in strain JW112, but not in strain SU51 (Table 3). (5) Revertants of SU51 carrying pSU307 in the autonomous state were obtained from SU51 after prolonged growth at 30 °C (see below).

Isolation of revertants of SU51. In order to obtain revertants of SU51 with pSU307 in the autonomous state, the strain was grown for many generations at 30 °C by repeated subculture in fresh medium. This culture was mixed with strain W3110 Rif^r. After mating for 6 h at 30 °C, Km^r Rif^r transconjugants were isolated at a frequency of about 3 × 10⁻⁶ per donor cell.

Biophysical and biochemical techniques. The method of Kitts *et al.* (1982) was used to check for the presence of plasmids and measure their size. Plasmid DNA was purified from Triton X-100 cleared lysates by precipitation with polyethylene glycol, followed by caesium chloride/ethidium bromide density-gradient centrifugation as described previously (de la Cruz *et al.*, 1980*b*). The methods used for cleavage of DNA with restriction enzymes, for agarose gel electrophoresis, preparation of ^{32}P -labelled RNA from plasmid DNA *in vitro*, hybridization of labelled RNA with DNA on nitrocellulose filters and autoradiography have all been described previously (de la Cruz *et al.*, 1979, 1980*b*).

RESULTS

Genetic labelling of plasmids

Plasmids pSU316 and ColB-K98 can be identified readily because they produce α -haemolysin and colicin, respectively, but these markers cannot be selected. In order to achieve positive and mutually distinguishable selection, the plasmids were labelled by transposon insertion. Thus, pSU316 was labelled with Tn5, to produce plasmid pSU307 (Fig. 1). In addition, pSU306 (pSU316 : Tn802) was already available (de la Cruz *et al.*, 1979; Fig. 1). As can be seen in Fig. 1, no detectable deletion or any other rearrangement accompanied the insertions in pSU306 and pSU307.

Plasmid ColB-K98 is about 70 kb in length. The insertion of Tn802 that produced plasmid pSU397 caused an increase in size of 5 kb.

Incompatibility properties of SU51

Strain SU51 was constructed as described in Methods. In order to analyse the incompatibility properties of plasmid pSU307 in the integrated state, derivatives of SU51 were constructed that contained plasmid pSU306, pSU397 or R124. The inheritance of these plasmids was analysed (Table 3). Plasmid pSU306 was very unstable in strain SU51 at both 30 °C and 42 °C. On the other hand, plasmid R124 was completely stable at both temperatures. Plasmid pSU397 was stable at 30 °C but was lost at high rates after growth at 42 °C. The incompatibility results obtained with pSU307 in the autonomous state are also included in Table 3 for comparison.

It has been described that plasmids which consist of multiple replication regions can be stably co-maintained in strains containing an incompatible plasmid integrated in the chromosome (Molin & Nordström, 1980). In these cases, what is affected is the growth of the strain itself; this phenomenon is known as switch-off of replication. Thus, we tested whether derivatives of strain SU51 containing either R124 on pSU397 had their growth impaired by the presence of these plasmids. It was shown that, after a shift to 42 °C, strain SU51(R124) had a doubling time of 58 min, almost identical to that of strain SU51 itself (57 min). However, strain SU51(pSU397) grew at 42 °C with a doubling time of 76 min. It can be assumed from these results that pSU397, but not R124, causes switch-off of replication of strain SU51.

Three conclusions can be drawn from these results. First, the incompatibility of integrated pSU307 towards R124 is not expressed. Second, the incompatibility of integrated pSU307 towards pSU397 is expressed at 42 °C, but not at 30 °C. Finally, the incompatibility against pSU306, the isogenic plasmid, is expressed at both temperatures.

Incompatibility properties of autonomous plasmid derivatives obtained from strain SU51

In order to confirm that strain SU51 still contained intact all the incompatibility determinants present in the original plasmid pSU307, revertants of pSU307 to the autonomous state were isolated from SU51. Six independent revertants of strain SU51 carrying pSU307 in the autonomous state were analysed for their plasmid content and structure. All six strains contained a single plasmid. Analysis of the electrophoretic patterns produced by digestion of these plasmid DNA molecules with endonuclease *Bgl*II showed that these revertants are similar to plasmid pSU307 but have suffered genetic rearrangements during the integration/excision process (Fig. 2). Three types of revertants were obtained. Although their structures have not been investigated in detail, inspection of their restriction patterns suggested the type of genetic rearrangements that had occurred [see Bukhari & Kamp (1980) for detailed explanations]. Type I revertants have several extra fragments and are probably the result of the acquisition of

Fig. 1. Restriction map of the *hly* region of plasmids pSU316, pSU306 (pSU316 *hlyC*::Tn802) and pSU307 (pSU316 *hlyC*::Tn5). The restriction map of pSU316 is known (de la Cruz *et al.*, 1980*b* and unpublished). The sites of insertion of Tn802 and Tn5 were mapped after single and double digestions with the relevant enzymes (data not shown). The position of the *hly* genes has been taken from Goebel & Hedgpeth (1982). The restriction enzymes used were *Eco*RI (▲), *Hind*III (△), *Bam*HI (◆) and *Bgl*II (■). B4/B3 indicates the *Bgl*II site that cleaves between fragments *Bgl*II-3 (11.5 kb) and *Bgl*II-4 (8.3 kb) of pSU316. The relative sizes of the sequences corresponding to the insertions of the transposons have been scaled down (1:2).

Table 3. Incompatibility properties of strain SU51 and derivatives

Plasmid	Percentage incompatibility against:					
	SU51*		Revertants†		pSU307‡ (<i>t</i> = 0)	
	<i>t</i> = 0§	<i>t</i> = 20	<i>t</i> = 0	<i>t</i> = 20	Resident	Donor
pSU306 { 30 °C	32	100	98	100	100	100
{ 42 °C	100	—				
R124 { 30 °C	0	0	42	86	43	6
{ 42 °C	0	0				
pSU397 { 30 °C	0	0	2	14	4	40
{ 42 °C	0	100				

* Derivatives of SU51 containing plasmids pSU306, pSU397 or R124 were constructed by transferring those plasmids to SU51 by conjugation either at 30 °C or 42 °C (depending on the temperature at which the incompatibility was to be studied) and selecting for Sm^r and a marker on the plasmid. The figures are the percentages of cells that have lost the marker of the donor plasmid after growth in drug-free medium for 0 or 20 generations (*t*). In the cases in which the donor plasmid seemed to be stably inherited, its presence was demonstrated by the plasmid screening technique.

† As above, except that strain UB5201 Nal^r (pSU307/8) was the recipient. The donor plasmids were present in strain DF110. The incompatibility was studied at 37 °C.

‡ Doubles were constructed by conjugating derivatives of strain DF110 containing plasmids pSU306, pSU397 or R124 with W3110(pSU307). The incompatibility was studied at 37 °C. The results of crosses performed in both directions (i.e. with pSU307 being either the resident or the donor plasmid) have been recorded. In all cases the resident plasmid was lost except in (||) in which the donor plasmid (pSU307) was preferentially lost.

§ Plating at *t* = 0 involves resuspending in broth the original colonies that were obtained on the selective plates, plating on drug-free plates and replica plating these new colonies on to drug-containing plates. Thus, it is assumed that the loss of the donor plasmid occurs during growth on the non-selective plates.

additional DNA sequences from an adjacent region of the bacterial chromosome. Type II revertants, which lack two fragments and have acquired two new fragments instead, could be the result of inversion or substitution. Type III revertants probably arose after deletion of a segment of pSU307 DNA containing regions of DNA from the pSU307 fragments *Bgl*II-3 (11.5 kb) and *Bgl*II-5 (4.5 kb). All the three plasmid types contain the 7.2 kb fragment *Bgl*II-4 of pSU307, which is known to contain the replication region of the plasmid (unpublished results).

Fig. 2. *Bgl*II restriction endonuclease cleavage patterns of plasmid revertants from strain SU51. Plasmid DNA was obtained as described in Methods and digested with 2 units of endonuclease *Bgl*II for 1 h at 37 °C. The products of such digestions were run in 1% (w/v) agarose gels as described previously (de la Cruz *et al.*, 1979). The sizes (in kb) of the *Bgl*II fragments of pSU307 are indicated on the left-hand side of the Figure. λ DNA digested with *Hind*III (track 1) provided fragment size markers. All the other samples were digested with *Bgl*II: track 2, pSU307; tracks 3–10, DNA obtained from individual Km^r transconjugant colonies obtained after the mating SU51 $\times$ W3110 Rif^r . Three types of restriction patterns (I, II and III) were obtained.

All these rearrangements involve DNA regions from the site of the Tn5 insertion downstream from the *hly* genes (see Fig. 1).

One of the type II derivatives (called pSU307/8) was chosen for the incompatibility tests. It was transferred to the *recA* strain UB5201 at a frequency of 3×10^{-4} Km^r transconjugants ml^{-1} after a 90 min mating at 37 °C. Three transconjugant colonies were used to construct double-plasmid strains carrying pSU307/8 along with either pSU306, pSU397 or R124. The incompatibility tests (Table 3) show that pSU307/8 is incompatible with both pSU397 and R124. Thus, SU51 must harbour the *IncFIII* and *IncFIV* incompatibility determinants although they are not expressed normally. The incompatibility between R124 and pSU307 is unusual because it results in highly asymmetric segregation. Thus, pSU307/8 was transferred to strain UB5201(R124) and the incompatibility between these plasmids was studied. It was demonstrated that pSU307/8 (the donor) was lost in 9% ($t = 0$ generations) and 27% ($t = 20$ generations) of the colonies, so that it maintains the asymmetric incompatibility behaviour of pSU307.

DISCUSSION

F-like plasmids of *Enterobacteriaceae* have been classified into six incompatibility groups (Datta, 1979; de la Cruz *et al.*, 1980a). The incompatibility determinants of two of them, the *IncFI* and *IncFII* groups, have been studied in depth (Lane, 1981; Timmis *et al.*, 1981), but the others have been hardly studied at all. We are interested in *IncF* determinants because comparison of plasmid replication control regions may shed light on the evolutionary relationships among the F-like plasmids.

When plasmid pSU307 was integrated in the *E. coli* chromosome, at least three kinds of incompatibility determinants were distinguished. The first one, active only against isogenic plasmids such as pSU306, is shown by incompatibility at the permissive temperature. A second, called *IncFIII* is only active against *IncFIII* plasmids at the restrictive temperature. The third, the *IncFIV* incompatibility of pSU307, is only active when both plasmids are autonomous. The dissection of these incompatibility determinants at the molecular level is now being undertaken in our laboratory and results (unpublished) suggest that these three kinds of incompatibility correspond to different DNA sequences present in plasmid pSU307.

In the case of SU51(pSU397), plasmid pSU397 is stable at 30 °C, but is eliminated at the restrictive temperature, where the integrated plasmid presumably directs replication of the bacterial chromosome. At the permissive temperature, the bacterial origin, and thus the chromosomal machinery for the control of replication, is presumably used (Chandler *et al.*, 1977). Assuming this to be the case for SU51, it is tempting to speculate that the reason for this incompatibility is that pSU307 and the IncFIII plasmids share a site necessary for replication and/or partition, but do not share a diffusible product (i.e. a repressor) acting as a copy-number control gene product. The repressor will only work on pSU316 derivatives and, therefore, will produce incompatibility against pSU306, but not against pSU397, at the permissive temperature.

The situation found between the integrated pSU307 and R124 is similar to that found where F is unilaterally displaced by IncHI plasmids from strains containing F in the autonomous but not in the integrated state (Smith *et al.*, 1973). In order to interpret the interactions between pSU316 and R124, the following facts have to be taken into account: (1) R124 retains its incompatibility properties when integrated in the bacterial chromosome (Sasakawa *et al.*, 1980); (2) R124 is stably maintained in strain SU51 both at the permissive and the non-permissive temperatures (this work); (3) R124 is compatible with ColB-K98 (Hedges & Datta, 1972); (4) when strains are constructed containing R124 and pSU316, both in the autonomous state, pSU316 is selectively eliminated, irrespective of the direction of the cross (de la Cruz *et al.*, 1979); and (5) R124 does not switch-off the replication of SU51 at 42 °C (this work).

Plasmids may show a complex set of incompatibility reactions for two reasons: firstly, different mechanisms (e.g. control of replication and partitioning) can lead to the expression of incompatibility (as discussed by Novick & Hoppensteadt, 1978). Secondly, plasmids can carry more than one set of replication/partition systems brought together by the evolutionary history of the element (possibly due to plasmid fusions in the past). The results presented in this work shed some light on the plasmid interactions underlying the expression of the incompatibility reactions shown by plasmid pSU316. In summary, when integrated in the bacterial chromosome, plasmid pSU316 expresses a *trans*-acting function (such as a copy-number control gene product) which causes elimination of isogenic derivatives, but this *trans*-acting function neither eliminates IncFIII nor IncFIV plasmids. Elimination of IncFIII plasmids is most probably brought about by competition of *cis*-acting genes (i.e. sites) necessary for initiation of replication or partitioning as discussed above. This interaction is mutual since pSU397 causes switch-off of SU51 replication. Thus, plasmid pSU316 and IncFIII plasmids share only part of their incompatibility determinants. This phenomenon is very well documented for plasmids belonging to the IncFI group (Bergquist *et al.*, 1982). R124 presents a unilateral incompatibility towards pSU316 which is suppressed when pSU316 is integrated in the bacterial chromosome. This behaviour could be explained if R124 contained two sites controlling initiation of replication and/or partitioning, either of which could be used in order to assure stable inheritance of the plasmid. One of them could interact with plasmid pSU316 when in the autonomous state and thus produce unilateral incompatibility. When integrated in the bacterial chromosome, however, plasmid pSU316 could use the chromosome partition system and therefore the incompatibility against R124 would be suppressed. However, the existing data cannot rule out alternative explanations.

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