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Identification and Expression of a Copy Number Control Gene in the IncFIII Hemolytic Plasmid pSU316

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A DNA fragment carrying both the IncFIII determinant and a copy number control gene of the hemolytic plasmid pSU316 has been cloned in pBR322. Deletion derivatives of the hybrid plasmid generated by *Bal* 31 digestion, which no longer exhibit the IncFIII phenotype, fall into two complementation groups when tested against a pSU316 miniplasmid derivative. Type 1 mutants exhibit the copy number control (Cop$^+$) phenotype whereas type 2 mutants do not. Restriction analysis of type 1 and type 2 mutants allowed us to locate the *cop* gene of pSU316 in a 700-base-pair fragment adjacent to the IncFIII determinant. Plasmid expression in a minicell system suggests that the product of the *cop* gene of pSU316 could be a 13,000-dalton protein.

The replication of tightly controlled plasmids, namely IncFI and IncFII, has been extensively characterized (12, 17, 21). At least three plasmid-borne functions (incompatibility, copy number control, and a function needed for the initiation of new rounds of replication) have been found to be essential for control of the replication of these plasmids. Although it is assumed that other conjugative plasmids may be similarly controlled, little is known about the mechanisms operating in plasmids belonging to other incompatibility groups.

The hemolytic plasmid pSU316 has recently been described and characterized (1). It is unusual with respect to its incompatibility properties because it expresses incompatibility toward both IncFIII and IncFIV plasmids (9). Two different genetic incompatibility determinants appear to be responsible for this effect (16, 18).

We have previously reported the construction of two pSU316 derivatives, pSU3025 and pSU3027, containing essential functions for replication (18). A copy number control gene present in pSU3027 appears to be lacking in pSU3025, since the latter is in a very high copy number (340 copies per genome). To characterize this gene, we have cloned a DNA fragment from pSU3027 that is able to reduce the number of copies of pSU3025. Expression of the hybrid plasmid containing the copy number control gene (*cop*) in a minicell system showed the presence of a 13,000-dalton protein that might be the product of the *cop* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work were the *Escherichia coli* derivatives C600 (F$^-$ *fhuA21 dra-1*) (3), UB5021 (*pro met recA56 gyrA*) (5), and DS410 (*sup*$^+$ *lac*$^+$ *minA minB rpsL*) (10). The plasmids used in this work and their relevant properties are listed in Table 1.

LB medium was prepared as described by Bertani (6). Solid medium was prepared by adding 1.2% agar to the liquid medium. Antibiotics were added to the following concentrations: carbenicillin, 500 μg/ml; and kanamycin sulfate, 50 μg/ml.

Isolation of plasmid DNA. Plasmid DNA preparations were obtained after cesium chloride-ethidium bromide density gradient centrifugation of cleared lysates (7) or by the alkaline lysis procedure described by Ish-Horowicz and Burke (11).

DNA cleavage, ligation, and *Bal* 31 deletions. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs and used as recommended by the supplier. Deletions with the nuclease *Bal* 31 were carried out as described by Maniatis et al. (13).

If restriction endonuclease-generated fragments were subsequently to be ligated, the solutions were heat pulsed (75°C for 15 min) to inactivate the restriction endonuclease. After 60 min on ice, ATP, dithiothreitol, and T4 DNA ligase were added to final concentrations of 100 mM, 10 mM, and 5 U/ml, respectively, and the solutions were incubated at 14°C for 16 h.

Transformation. Transformations were carried out by the method of Cohen et al. (8). Transformed cells were incubated at 37°C for 90 min to allow the expression of drug

TABLE 1. Plasmids used in this work

Plasmid	Relevant properties	Reference
pSU307	IncFIII-IncFIV, Cop$^+$, Km^r	16
pSU316	IncFIII-IncFIV, Cop$^+$, Hly$^+$	9
pSU3027	(pSU316 *Eco*RI fragments E1 + E9) IncFIII-IncFIV, Cop$^+$, Hly$^-$, Ap^r	18
pSU3025	(pSU316 *Bgl*II fragment B4) IncFIII, Cop$^-$, Ap^r	18
pSU3040	(Km^r, Ap^s derivative of pSU3025) IncFIII, Cop$^-$	This work
pSU3034	(*Hpa*I deletion derivative) pBR322-pSU316 *Sal*GI fragment S1, IncFIII, Cop$^+$, Ap^r	This work
pSU3039	(*Hin*dIII-*Hpa*I deletion derivative of pSU3040) IncFIII, Cop$^+$, Ap^r	This work
pSU3042	(*Bal* 31 deletion derivative of pSU3039) IncFIII$^-$, Cop$^+$, Ap^r	This work
pSU3043	(*Bal* 31 deletion derivative of pSU3039) IncFIII$^-$, Cop$^-$, Ap^r	This work

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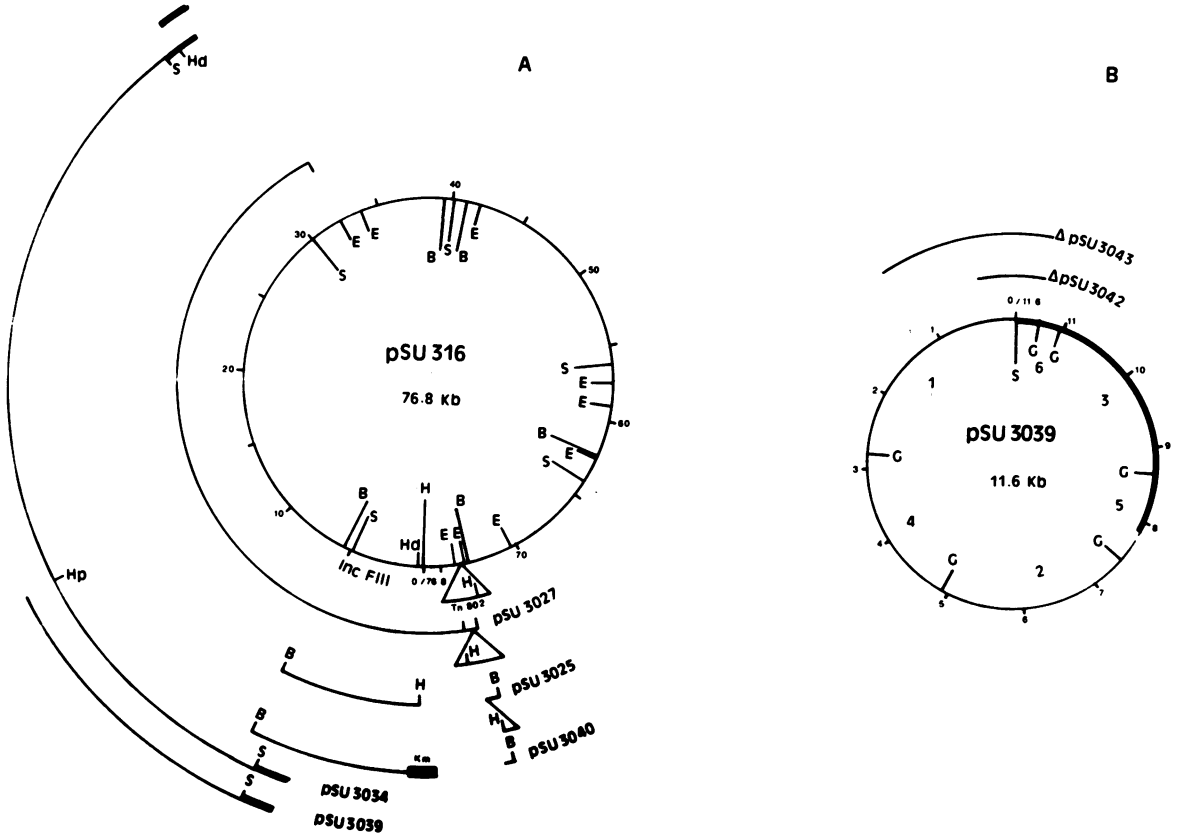

FIG. 1. (A) Restriction map of plasmid pSU316 and of several derivatives used in this work. The possition of the Tn*802* insertion is shown in the map. Plasmids pSU3025, pSU3027, and pSU3040 are mini-plasmids containing the replication region of pSU316. pSU3025 and pSU3027 carry the Ap^r gene of Tn*802*, and pSU3040 carries the Km^r determinant of Tn*5*. Plasmid pSU3034 is a hybrid plasmid containing the *Sal*I fragment of pSU316 S-1 cloned in pBR322. Plasmid pSU3039 is a *Hin*dIII-*Hpa*I deletion derivative of pSU3034. Abbreviations: H, *Bam*HI; E, *Eco*RI; B, *Bgl*II; S, *Sal*GI; Hd, *Hin*dIII; Hp, HpaI; X, *Xba*I. The black box in the pSU3040 map represents the Km^r gene from Tn*5*, and heavy lines in both the pSU3034 and pSU3039 maps represent pBR322 DNA. (B) Schematic representation of the IncFIII Cop$^+$ hybrid plasmid pSU3039 and of the extent of the deletions present in the *Bal* 31 deletion derivatives pSU3042 (Cop$^+$) and pSU3043 (Cop$^-$). The thick line represents the cloning vector pBR322. Abbreviations: S, *Sal*I; G, *Bgl*I.

resistance and then plated on L-agar plates containing the appropriate antibiotic at the above concentrations.

Agarose gel electrophoresis. Analytical agarose gel electrophoresis was performed in either vertical or horizontal slab gels at agarose concentrations between 0.7 and 1.2%, as previously described (2).

Determination of variation in plasmid copy number. Essentially, the procedure of Tomizawa (22) was followed, but plasmid-digested DNA instead of intact plasmid DNA was used. Heterotransformant bacteria carrying chimeric and test plasmid were grown overnight in 10 ml of L broth at 37°C, and plasmid DNA was extracted by the method of Ish-Horowicz and Burke (11). Aliquots were digested with the appropriate restriction endonucleases, and DNA fragments were separated in 1.2% agarose gels. The fragments were stained in the gels with ethidium bromide and photographed under UV light. The negatives were scanned in a Vernon densitometer, and the areas under the peaks formed by fragments of each plasmid were determined.

Analysis of plasmid-encoded proteins. Minicells from stationary-phase LB cultures of strain DS410 containing the indicated plasmids were prepared as previously described (2). Plasmid-encoded proteins were labeled with 25 μCi of ^{14}C protein hydrolysate (55 mCi/mg-atom; Amersham Corp.) and analyzed by electrophoresis through 15 to 25% polyacrylamide gels containing sodium dodecyl sulfate followed by autoradiography.

Incompatibility tests. Incompatibility tests were done essentially as described previously (18). Transformants were selected only for the incoming plasmids. At least three individual colonies from each transformation plate were grown in L broth for 20 generations (t = 20) and plated onto L-agar plates without selection. One hundred individual colonies from each plate were transferred with a toothpick

TABLE 2. Loss of the IncFIII phenotype of pSU3039 as a function of the time of the *Bal* 31 reaction

Time of reaction (min)	No. of Ap^r colonies	No. of Ap^r Km^r colonies	% of IncFIII$^-$ colonies
0	800	0	0
2	175	14	8
3	115	12	10
4	97	38	39
5	100	55	55
6	28	17	61
7	31	23	74
8	37	29	79

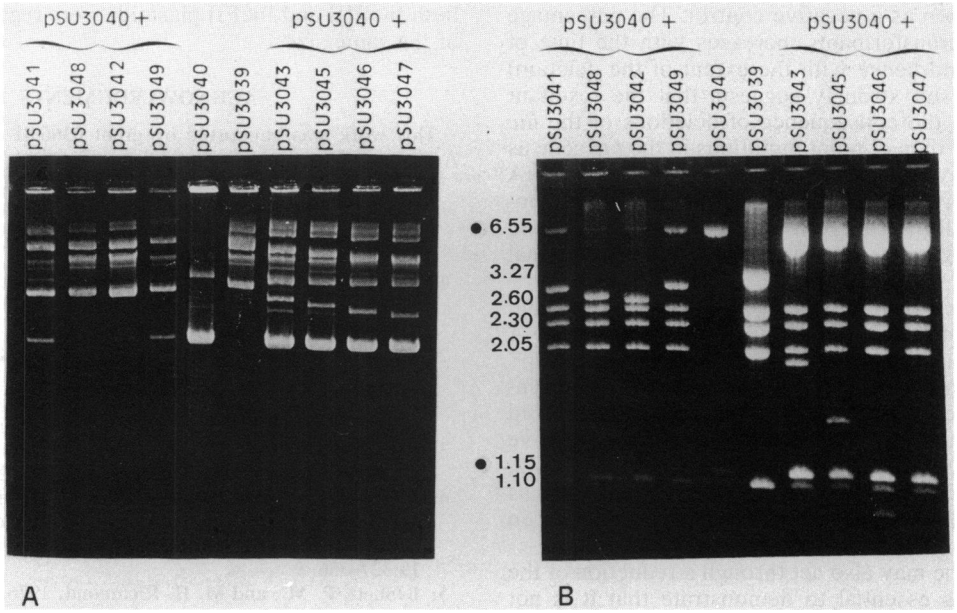

FIG. 2. Identification of Cop$^+$ and Cop$^-$ hybrid clones. (A) Agarose gel electrophoresis of undigested plasmid DNA obtained from overnight cultures of heterotransformant colonies. Samples were run on 0.7% agarose gels. (B) *Bgl*I digestion of 10 μl of the same plasmid DNAs, run on 1% agarose gels. Bands marked with a black dot correspond to pSU3040 digestion.

onto L-agar plates containing the appropriate antibiotics. To rule out any recombinational event in which no loss of any resistance phenotype was observed, DNA preparations from 10 colonies were analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

Cloning of a DNA fragment from pSU316 carrying the copy control gene. To clone a DNA fragment carrying the *cop* gene of pSU316, we used the miniplasmid system composed of pSU3027 and pSU3025. The longest pSU316 *Sal*I-generated fragment (25.0 kilobases [kb]), which overlaps 600 base pairs to the left end of pSU3025 (Fig. 1A), was inserted into the cloning vector pBR322, giving rise to plasmid pSU3034 (29.3 kb). This hybrid plasmid should also contain the IncFIII determinant of pSU316 (18). It is, however, unsuitable for complementation tests with pSU3025 or its derivatives, because the presence of IncFIII determinants in both plasmids does not allow stable maintenance. To circumvent this problem, several endonuclease-generated deletions were produced.

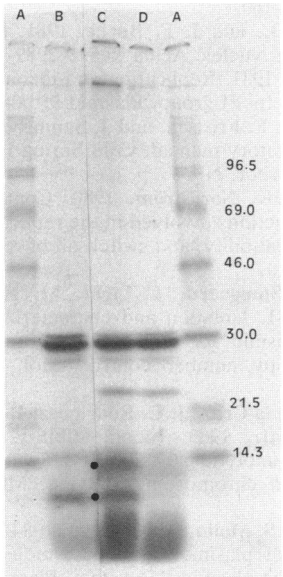

FIG. 3. Plasmid-encoded proteins from minicell-producing strain DS410. The dots indicate proteins absent from the Cop$^-$ plasmid preparation. Lanes: A, molecular weight standards; B, pBR322; C, pSU3042; (Cop$^+$); D, pSU3043 (Cop$^-$).

A *Hin*dIII-*Hpa*I double digestion of pSU3034 plasmid DNA allowed deletion of the 17.0-kb DNA segment located between the *Hin*dIII site of the cloning vector and the *Hpa*I site proximal to the right end of the cloned fragment (Fig. 1A). The linear DNA generated in this digestion was used to transform *E. coli* C600 competent cells. The resulting plasmid, pSU3039 (11.7 kb), possesses a unique *Sal*I site located within the DNA sequence where the IncFIII determinant has been shown to lie. Further deletion of this determinant was achieved by *Bal* 31 nuclease digestion of pSU3039 previously linearized with *Sal*I endonuclease.

Complementation of the number of copies of pSU3025 by deletion derivatives of pSU3039. The miniplasmid pSU3025, which, like pSU3027, is derived from pSU316 but is maintained in high copy number, was considered a good system with which to assay the complementation of pSU3039 deletion derivatives. To make it appropriate for this purpose, we replaced its ampicillin resistance determinant by the kanamycin resistance gene present in Tn*5* (4). The resulting miniplasmid, pSU3040, contains all the replication region of pSU3025 and the Km^r determinant of Tn*5* (Fig. 1A).

The pSU3039 *Bal* 31-digested DNA (see above) was ligated and used to transform *E. coli* UB5201 competent cells carrying plasmid pSU3040. The IncFIII determinant of pSU3039 should not allow plasmid pSU3040 to be stably inherited, and only chimeric molecules in which the *inc* determinant has been deleted would produce stable heteroplasmid transformants. pSU3039 DNA was also used

in the transformation as a negative control. The percentage of heteroplasmid transformants increases with the time of *Bal* 31 reaction (and hence with the extent of the deletion) (Table 2). This result strongly suggests that the resultant Inc$^-$ phenotype is the consequence of deletions of the *inc* determinant rather than of point mutations in the *inc* gene as a result of selective pressure. The analysis of the DNA content of the heteroplasmid cells by agarose gel electrophoresis revealed in all the cases the presence of both plasmid species, thus ruling out the possibility of a recombinational event.

A *trans*-acting *cop* gene carried by the hybrid plasmid should decrease the amount of pSU3040 plasmid DNA present in the cells and therefore permit Cop$^+$ and Cop$^-$ hybrid plasmids to be distinguished among the transformants. Of 120 clones analyzed, 9 (7.5%) showed a marked decrease in the amount of pSU3040 DNA. A representative example is shown in Fig. 2. Plasmids pSU3041, pSU3042, pSU3048, and pSU3049 cause the Cop$^+$ phenotype, whereas pSU3043, pSU3045, pSU3046, and pSU3047 lack the *cop* gene.

Since the *inc* gene may also act through a reduction of the copy number, it is essential to demonstrate that it is not expressed in the deletion derivatives. To do that, we carried out several incompatibility tests, whose results showed that (i) all the pairs mentioned above are stable and (ii) in the pairs formed by the deletion derivatives and pSU307, no loss of any of the plasmids was observed at $t = 20$ (see Materials and Methods).

pSU307 is a derivative of pSU316 with Tn*5* inserted within the *hly* determinant (16). It is in the same copy number as pSU316 (i.e., three or four copies per cell) (18). Therefore, any effect of the *inc* gene should result in the loss of the plasmid. Because such loss has not been observed, we concluded that the *inc* gene is not expressed at detectable levels in the deletion derivatives. This result strongly suggests that the reduction in the pSU3040 copy number is due solely to the expression of the *cop* gene.

Digestion of the DNA of the deletion derivatives with the endonuclease *Bgl*I showed that pSU3042 is the plasmid still expressing the Cop$^+$ phenotype in which the largest amount of DNA has been deleted and pSU3043 is the plasmid expressing the Cop$^-$ phenotype with the smallest deletion. The extent of the deletions in both pSU3042 and pSU3043, as well as the *Bgl*I map of pSU3039, is shown in Fig. 1B. The region of pSU3042 deleted in pSU3043 (ca. 700 base pairs) must contain the *cop* determinant, at least in part.

Cop$^+$ and Cop$^-$ plasmids. In a minicell system, plasmid pSU3042 (Cop$^+$) expresses two proteins, of 13,000 and 9,000 daltons, which are not expressed by plasmid pSU3043 (Cop$^-$) (Fig. 3). One of these, the 13,000-dalton protein, is the most likely product of the *cop* gene for two reasons. First, the 9,000-dalton protein is also expressed by pBR322. The absence of the latter from the pSU3043 profile could be explained in several different ways. For instance, deletions in pBR322 could affect its production. Alternatively, the 9,000-dalton protein could be produced in pBR322 under the influence of the Tet promoter, and insertions within the *Sal*I site would inhibit its production, providing that no other promoter is inserted. Second, the size of the putative Cop protein agrees with the values reported by other authors for the Cop protein in IncFII plasmids (15). Our results (I. Andrés and J. C. Rodriguez, unpublished data) indicate that there is a great deal of homology between the replication regions of IncFII and IncFIII plasmids (14, 18–20), and therefore it would not be surprising if the copy numbers of both IncFIII and IncFII plasmids were regulated by proteins of the same size.

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