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Properties and incompatibility behavior of miniplasmids derived from the bireplicon plasmid pCG86

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Summary. Many plasmids belonging to the F incompatibility groups contain more than one basic replicon. The chimeric plasmid pCG86 is an example of such a multireplicon plasmid. The two basic replicons of pCG86, RepFIIA/FIC and RepFIB have been cloned and re-ligated, the copy numbers of the clones have been determined, and the incompatibility behavior of plasmids containing the ligated replicons and the individual replicons has been studied. The bireplicon plasmids are not expected to be incompatible as recipients with monoreplicon RepFIB or RepFIIA/RepFIC plasmids, since when one replicon is challenged by an incoming replicon, the other should be able to handle the plasmid's replication. In our studies, we found that challenge with either monoreplicon plasmid resulted in incompatibility. This incompatibility was increased in bireplicon plasmids in which RepFIB was duplicated. We conclude that in the bireplicon plasmids, challenging the replication control of one replicon by an incompatible plasmid can interfere with the replication originating from the second replicon.

Key words: Replicon interactions – Bireplicon plasmids

Introduction

During recent years it has been shown that many plasmids belonging to IncF incompatibility groups possess more than one basic replicon (Bergquist et al. 1982; for review see Couturier et al. 1988). Three basic replicons have been characterized in these plasmids and were found to be present in various combinations (Bergquist et al. 1986). In plasmids belonging to incompatbility group FI (IncFI) the three basic replicons have been named RepFIA, RepFIB and RepFIC (Bergquist et al. 1986). There is no homology between these replicons. RepFIC is similar to the replicon RepFIIA present in IncFII plasmids, although plasmids containing RepFIIA are compatible with plasmids containing RepFIC (Saadi et al. 1987).

We have previously characterized the conjugative chimeric R/Ent plasmid pCG86 (Mazaitis et al. 1981). This 117 kb plasmid contains two basic replicons, RepFIB and a hybrid replicon, RepFIIA/FIC (Picken et al. 1984). Both replicons have been cloned and characterized by restriction endonuclease mapping and partial sequencing. We were interested in studying possible interactions *in cis* between the two replicons and to this end we constructed bireplicon plasmids containing RepFIB and RepFIIA/FIC, and analyzed their incompatibility behavior. We also used another bireplicon plasmid, P307, to construct some of the clones described in the present study. P307 contains the replicons RepFIB and RepFIC (Picken et al. 1984; Saadi et al. 1987). RepFIB of P307 appears to be identical with RepFIB of pCG86. RepFIC, as mentioned above, is very similar to other IncFII basic replicons.

In the present paper we describe interactions between RepFIB and RepFIIA/FIC that are not present when the replicons are introduced separately *in trans* and that cannot be explained on the basis of their copy number control mechanisms. A monoreplicon plasmid with RepFIB has a copy number of one, a monoreplicon plasmid with RepFIIA/FIC has a copy number of three, and a bireplicon plasmid with both replicons has a copy number of three, as expected. RepFIB is presumably shut off in bireplicon plasmids. Either replicon is potentially functional and should be able to take over plasmid replication when the other one is inhibited. Yet we found that challenge with either a RepFIIA/FIC plasmid or a RepFIB plasmid resulted in incompatibility. Moreover, the presence of the bireplicon plasmid interfered with the establishment of a RepFIB monoreplicon plasmid by transformation. Our results indicate that under some circumstances there are interactions *in cis* between the two replicons.

Materials and methods

Bacterial strains and plasmids. The following F$^-$ *Escherichia coli* strains were used as hosts for the plasmids: C600; *thi, thr, leu*; SC201; *polA214, his, arg*A, *met*B, *leu, lac*Y, *dra/din, streptomycin, thy, polA*, temperature sensitive. The plasmids and the clones derived from them are described in Table 1.

Tests for incompatibility. The incoming plasmid was transferred by transformation to the strain with the resident plasmid, with selection for the incoming plasmid only. The incompatibility experiments presented were carried out with clones lacking the *tra* genes to prevent subsequent rounds of mating on the selective plate. The offspring were purified

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Table 1. Description of plasmids used in this work

Plasmid number	Type[a]	Phenotype[b]	Replicons and replication genes present	Reference
pCG86	no	Tc^r, Sp-Sm^r, Su^r, Hg^r, LT^+, ST^+, Tra^+	RepFIIA/FIC, RepFIB	Mazaitis et al. 1981
pDXRR3	c	Ap^r, Tc^s	copA or IncRNA of R100 and replication genes of pBR322	Womble et al. 1984
3780 (Tn^5 insertion in EntP307)	no	Km^r, LT^+, ST^+, Tra^+	RepFIB, RepFIC	R. Maas, unpublished
KLF1 (F *thr*$^+$ *leu*$^+$)	no	Thr^+, Leu^+, Tra^+	RepFIA (miniF), RepFIB, RepFIC (incomplete), all from F	Pfister et al. 1976
PRM133	c	Sp^r (Ω)	RepFIIA/FIC, RepFIB	This work
pRM136	c	Sp^r (Ω)	RepFIIA/FIC, 2 × RepFIB	This work
pRM3937	c	Sp^r (Ω)	RepFIIA/FIC	This work
pRM3947	c	Ap^r	Base pairs 0-836 of RepFIIA/FIC and replication genes of pUC19	This work
pRM3994	c	FIB Cop Inc^+, Sp^r (Ω)	RepFIB	This work
pRR933	c	Cm^r	RepFIIA (mini-R100)	Miki et al. 1980
pSS3928	c	Sp^r (Ω)	RepFIB (from P307)	This work
pSS3945	c	Sp^r (Ω)	RepFIC	Saadi et al. 1987
pWM5	c	Tc^r	RepFIIA/FIC	Picken et al. 1985
pWM104	c	Km^r	RepFIB (from P307)	Picken et al. 1985
pWM111	c	Km^r	RepFIB (from pCG86)	Picken et al. 1985
pWM112	c	Km^r	RepFIIA/FIC	Picken et al. 1985
pWM113	c	Ap^r, Tc^r	RepFIC and replication genes of pBR325	Saul et al. 1989
pWM114	c	Ap^r, Tc^r	RepFIB (from pCG86) and replication genes of pBR325	This work
pWM2981	no	Sp-Sm^r, Su^r, Hg^r, LT^+, ST^+, Tra^+	RepFIIA/FIC, RepFIB	Mazaitis et al. 1981
pWM2982	no	Tc^r, Sp-Sm^r, Su^r, Hg^r, LT^+, ST^+, Tra^+	RepFIB	Mazaitis et al. 1981

[a] no, naturally occurring; c, constructed
[b] LT, heat labile enterotoxin; ST, heat stable enterotoxin; Tra, transfer; Tc, tetracycline; Sp, spectinomycin; Sm, streptomycin; Su, sulphonamide; Hg, mercury; Km, kanamycin

once by streaking to single colonies on the same selective medium. At least 40 colonies were then tested by replica plating for unselected markers present in the resident plasmid. Thus, in the described incompatibility test, one is testing for the percentage segregation of the resident plasmid after one round of growth or approximately 25 cell generations. Controls were carried out to correct for spontaneous loss of the resident plasmid.

Incompatibility measured by segregation from the heteroplasmid state as a function of time. Donor DNA was introduced by transformation, and the cells were suspended in TYE (tryptone yeast extract, Difco) medium. Expression of the newly introduced DNA was allowed for 90–120 min at 37° C and suitable cell dilutions were plated on media selective for the incoming plasmid only. The transformed cell suspension was kept overnight in the refrigerator and was used to inoculate flasks in which the cells were grown under selective conditions for the donor plasmid DNA. Samples were plated after given periods of growth on media selective for the donor. Loss of the resident plasmid was measured by replica plating, using 50–100 colonies. Note that the first plating differs from the preceding "Tests for incompatibility" in that it omits one round of growth on agar plates.

Preparation of DNA for transformation and of probe fragments. Plasmid DNA was prepared by cesium chloride gradient centrifugation of the cleared bacterial lysates. Probe DNA was prepared by restriction of the appropriate plasmid DNA and separation of the fragments by agarose gel electrophoresis (1% low melting temperature agarose or 1% agarose). The band was either extracted from low melting temperature agarose (Maniatis et al. 1982) or electrobound onto NA-45 DEAE membrane (Schleicher and Schuell) and eluted with buffered 1 M NaCl as described by the manufacturer.

The replicon probes. RepFIB: This probe is a 4.3 kb *Eco*RI fragment obtained from pCG86, capable of autonomous replication (Picken et al. 1984). For convenience of preparation it was subcloned into pBR325 (pWM114). The RepFIB fragment has been sequenced (Saul et al. 1989). RepFIIA/FIC: The hybrid replicon FIIA/FIC was subcloned into pUC19 as a fragment extending from base pair (bp) 1 to bp 836. This fragment codes for *copB*, *copA* and part of the *RepA1* gene.

Labeling of the probes. The fragments were labeled with ^{32}P by nick-translation with radioactive dCTP (New England Nuclear, specific activity 3000 Ci/mmol) to a specific activity of ca. 5×10^7 cpm/μg of probe (Rigby et al. 1977).

Hybridization and washing. The hybridizations were done in 50% formamide/5 × SSC at 37° C. In general, an approximately ten-fold molar excess of probe over complementary immobilized DNA was used. The washings were done as described previously (Maas 1983).

Determination of copy number. The strains were grown in tryptone-yeast extract medium (TYE) with the appropriate

drug for plasmid selection to an OD of 0.25 as measured in a model 401A Lumetron colorimeter (580 filter in place). The viable counts for each strain at this optical density were determined at least in duplicate with a spiral plater (Spiral Systems Instruments). They were of the order of 10^8 cells/ml. Aliquots (3 ml) were centrifuged and resuspended in 1 ml cold 0.5 N NaOH, 1.5 N NaCl. The suspensions were placed in a boiling water bath for 3 min for lysis of the cells and denaturation of the DNA, and cooled in watery ice. The lysates were neutralized with 1 ml of cold 1 M TRIS, pH 7, 2 M NaCl, and kept on ice. For each strain, aliquots of 5, 10, 25, 50, 75 and 100 µl were filtered onto GeneScreenPlus (NEN) membranes, using a Schleicher and Schuell filtering manifold. To insure that all aliquots were filtered at comparable rates (and gave uniformly sized circles), the aliquots were made up to a constant volume of 100 µl with plasmid-free lysed cells, such as cells of strain C600. Sets of immobilized DNA were hybridized with an appropriate replicon probe. One set of aliquots always belonged to a strain for which the copy number is known. For example, F, copy number of one to two, has one gene copy of RepFIB. The hybridized membranes were then exposed to Kodak X-Omat AR5 film with one intensifying screen. Spots of equal intensity could easily and reproducibly be matched and the number of replicon copies per cell could be determined by correcting for the aliquot volumes and viable counts of each matched pair (always with reference to a standard: F for RepFIB and pCG86 for RepFIIA/FIC). Plasmid pCG86 could be used as a standard after it was matched to F (RepFIB probe). We found that this procedure gave reproducible results and was feasible for large numbers of strains (two dozen strains could be handled in the course of a day). The method is well suited for determining the copy number of plasmids with identical replicons in common, as the extent of hybridization for any one probe has to be the same for any matched set. The matching could be done even more accurately using the BRL Hybri-Slot™ manifold (which became available subsequently). When the latter was used, 1 ml rather than 3 ml of cell suspension at OD 0.25 was centrifuged, followed by lysis and neutralization as before (final volume, 2 ml).

Alternatively, when plasmids carried the same gene for spectinomycin resistance, the relative numbers of gene copies were determined by comparing the levels of spectinomycin resistance on solid media (Maas and Davis 1950) as well as by hybridization to a spectinomycin probe (2.0 kb fragment of pHP45, see below).

Copy number adjustment experiments. The chimeric plasmid pWM114 was transformed into the *polA* ts strain SC201 (see Bacterial strain and plasmids). The miniplasmid pSS3928 was also transformed into SC201. pSS3928 (RepFIB) is *polA* independent in its replication (Gardner et al. 1985), whereas the ColE1 derivative pBR325 is *polA* dependent. Thus at 41° C (non-permissive temperature) the above chimeric plasmid replicates under RepFIB control. In order to minimize cell death, these strains were grown in 2 × YT (Miller 1972) supplemented with 0.5% glucose and 20 µg/ml thymine. When the cells reached an OD at 580 nm of 0.05 in a model 401A Lumetron (5×10^7 cells/ml) they were diluted with an equal volume of fresh medium. This was repeated for as many doublings as were followed in each experiment. Four milliliter samples were taken before each dilution (i.e., at each doubling) and the copy number was determined for each sample as described in "Determination of copy number" using the BRL Hybri-Slot.

For each experiment the strains were grown overnight with aeration at 30° C. Each strain was diluted 1:20 into each of two growth flasks containing fresh medium (2 × YT, 0.5% glucose, thymine, selective drug). One set of growth flasks was aerated by rotary agitation in a water bath at 30° C, and the other similarly at 41° C.

The 30°–41° C shift involved a lag and an inoculum with a certain number of dead cells, and what we refer to in the Results section as the "first" division is strictly speaking later than the first division (see Table 3).

Construction of chimeric plasmids analogous to pCG86. These were constructed by ligating a 4.3 kb *Eco*RI fragment

Table 2. Copy numbers of plasmids used in this work

Plasmid number	Replicons	Method Hybridization with			Level of Sp^r
		FIB	FIIA/FIC	Sp	
pWM2981 (no)	RepFIIA/FIC, RepFIB	3	3		
pWM2982 (no)	RepFIB	1			
pWM5 (c)	RepFIIA/FIC		3		
pWM112 (c)	RepFIIA/FIC		3		
pRM3937 (c)	RepFIIA/FIC		2.5		2
pWM104 (c)	RepFIB	1			
KLF1 (F) (no)	RepFIA, RepFIB, RepFIC incomplete	1.5[a]			
pSS3928 (c)	RepFIB	1			1
pRM133 (c)	RepFIIA/FIC, RepFIB	3	3	6	1.5
pRM136 (c)	RepFIIA/FIC, RepFIB duplicated	6	3	6	
pRM3994 (c)	RepFIB				8

All determinations were carried out in tryptone yeast extract (TYE) at 37° C. Plasmid pRM136 carries a RepFIB duplication but its copy number remains at three. Note that the measured copy number is three when the probe used is homologous to a non-duplicated gene or set of genes (FIIA/FIC for pRM136)

[a] Used as standard, copy number defined as 1.5. KLF1 is an F′ plasmid carrying the chromosomal markers *thr*$^+$ *leu*$^+$

(self-replicating) coding for RepFIB, a 7.2 kb *Eco*RI fragment (self-replicating) coding for RepFIIA/FIC, and the drug resistance marker (omega fragment of plasmid pHP45) coding for spectinomycin/streptomycin resistance (Prentki and Krisch 1984). Omega was used because it has translational and transcriptional terminators at both ends. Transformants were selected on spectinomycin containing medium (50 μg/ml) and those that possibly carried bireplicon plasmids were identified by colony hybridization with each of the two replicon probes, and by restriction analysis.

Results

Copy numbers of replicons and their control

In this section we report the copy number of the two basic replicons of pCG86, RepFIB and RepFIIA/FIC. We also describe adjustment following deviation from the normal copy number of RepFIB. It should be noted that, although several types of copy number control mechanisms have been described, two seem to be prevalent. One is partly dependent on the inhibition of translation of a replication activator protein by a homologous countertranscript. It has been analyzed in R100 (Womble et al. 1984) and is also present in RepFIIA/FIC (this laboratory, unpublished results). The other involves, in part, the binding to DNA repeat segments (iterons) of a rate limiting activator protein, a mechanism that has been demonstrated for the replicons RepFIA of F (Tsutsui and Matsubara 1981; Abeles 1986) and R of P1. The mechanism of control for RepFIB appears to be the second, by analogy to RepFIA (see copy number adjustment results below).

In Table 2 we report the relative copy numbers in logarithmically growing cells of the replicons RepFIB (1) and RepFIIA/FIC (2.5–3), as well as those of bireplicon plasmids, using plasmid KLF1 as a standard, the copy number of which is defined as 1.5. The copy number of naturally occurring bireplicon plasmids, as well as the in vitro constructed ones, is the same as that of the higher copy number of the two replicons (copy number is 3 for all bireplicon plasmids containing RepFIIA/FIC). It should be emphasized at this point that our copy number determinations involved the matching of spots of equal intensity and surface area for any one pair of plasmids, with a final reference to plasmid F. The six dilutions for each sample provided an adequate range for the matching of intensities. The copy number calculation was made by taking into account the dilution for that particular spot, and the viable count of the cell culture used in the experiment. In the middle range two-fold differences in dilution were easily discernible in the autoradiograms (Fig. 1). All copy number determinations were carried out at least twice, and were reproducible. Some examples are shown in Fig. 1 with the replicon probes RepFIB and RepFIIA/FIC, and with the spectinomycin resistance probe.

The rate of copy number adjustment (return to normal copy number after elevation, for example) reflects the mechanism of copy number control. This is discussed in detail by Nordström and co-workers (Nordström et al. 1984). They describe two types of adjustment, a gradual hyperbolic function, characteristic of countertranscript RNA control, and a step function, characteristic of control by DNA repeats. Our results of copy number adjustment experiments, performed as described in Materials and methods, are shown in Table 3 and Fig. 2. We were interested in the copy number adjustment behavior of RepFIB since nothing is known about its mechanism of replication control. The pBR325-FIB clone used in the experiment has a copy number of eight, which is elevated with respect to the normal RepFIB copy number of one because the plasmid replicates under the control of pBR325. Since pBR replication is *polA* dependent and the cointegrate plasmid is in this case in a *polA* ts (temperature sensitive) host, a temperature shift as carried out in the experiment forces the cointegrate plasmid to replicate under RepFIB control, the latter being *polA* independent. The adjustment of copy number from eight to one is indicative of the replication control of RepFIB. The expected values shown in Table 3

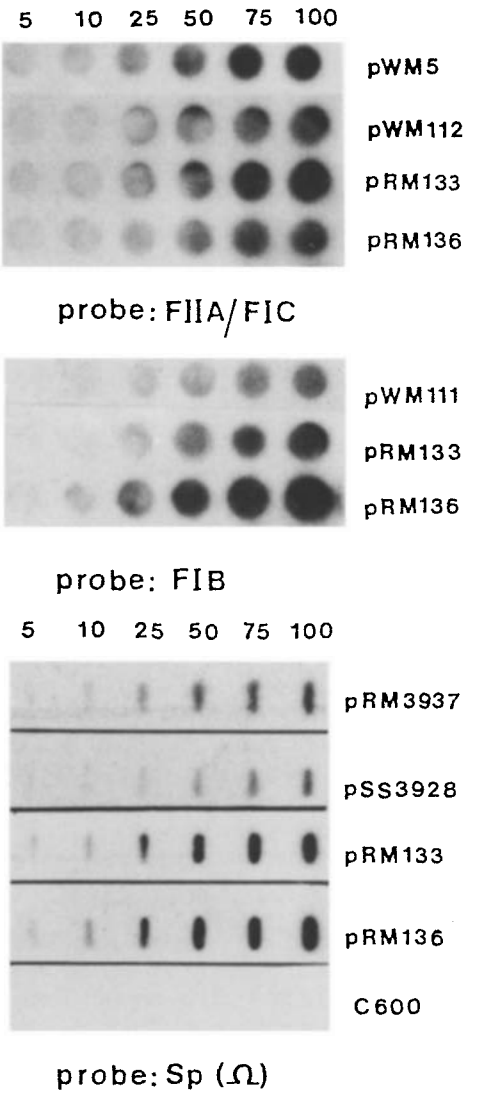

Fig. 1. Copy number determinations of a few constructed plasmids by hybridization with replicon probes or a drug resistance probe. pWM5, pWM112 and pRM3937 are mini RepFIIA/FIC plasmids while pWM111 and pSS3928 are mini RepFIB plasmids. The multiorigin plasmids were: pRM133; RepFIIA/FIC + RepFIB + 2 × Ω, and pRM136; RepFIIA/FIC + 2 × RepFIB + 2 × Ω. The numbers at the top of the panels represent the volumes in microliters of lysed and neutralized plasmid-containing cells. The spot intensities were matched on the autoradiogram with the best exposure. The sets shown for each probe come from one "pot" of probe and one autoradiogram, although not necessarily one membrane

Table 3. The copy number adjustment of RepFIB from an elevated value

Generations[a]	Copy number of pWM114 (pBR325-RepFIB)		
	Observed	Expected step	Expected hyperbolic
0.0	8.0[b]	8.0	8.0
1.3	3.0	3.3	3.8
2.3	1.3	1.6	2.4
3.3	1.0	1.0	1.7
4.3	1.0	1.0	1.4

[a] Doubling time, 45 min
[b] Identical numbers were obtained in two separate experiments

for a step-function type of control were calculated on the basis of the assumption that RepFIB replication is arrested when the copy number is greater than one. Therefore, since there is no plasmid DNA synthesis, each time the cells double, the copy number is halved, until the copy number is reduced to one. The expected values for a hyperbolic type of control, such as seen in IncFII plasmids, were obtained from Table 4 in Womble and Rownd (1986). Where the control is of the hyperbolic type, plasmid DNA synthesis continues during a "down" adjustment and is controlled by the concentration of available negative regulator. The results shown in Table 3 suggest that RepFIB adjusts to a lower copy number by a step-function shut-off.

Incompatibility of the component replicons of pCG86

The incompatibility properties of the replicons RepFIIA/FIC and RepFIB are unequivocal when the single replicons are tested against each other (Table 4). They are fully compatible *in trans*. The hybrid replicon RepFIIA/FIC of pCG86 is incompatible with itself, with R1 and with R100 (the latter two not shown). The replicon RepFIB is incompatible with itself, whether derived from pCG86 or P307

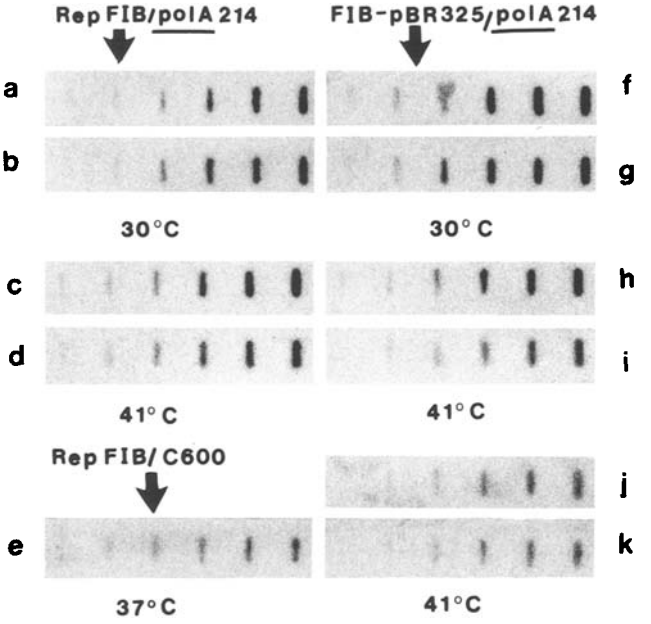

Fig. 2a–k. Copy number determination for copy number adjustment of RepFIB-pBR325 as a result of the switch-off of pBR325 replication functions in a *polA* temperature sensitive (T^s) host. Samples (4 ml) containing 5×10^7 cells/ml were taken at each point. The samples for panels f and g were diluted two-fold because of the high copy number. The volumes of lysed and neutralized cells used for DNA immobilization in each panel were 5, 10, 25, 50, 75 and 100 µl. The hybridization was with a RepFIB probe. Control panels a, b, c, d and e were as follows: a, mini RepFIB plasmid pSS3928 in a *polA* T^s host after two doublings at 30° C; b, pSS3928 in a *polA* T^s host after four doublings at 30° C; c, pSS3928 in a *polA* T^s host after two doublings at 41° C; d, pSS3928 in a *polA* T^s host after four doublings at 41° C; e, pSS3928 in C600 at 37° C. The remaining panels are for the actual temperature shift of RepFIB-pBR325 in the same *polA* T^s host. Panel f, two doublings at 30° C; g, four doublings at 30° C; h, one "doubling" (1.3 generations) at 41° C; i, two doublings at 41° C; j, three doublings at 41° C; k, four doublings at 41° C

Table 4. Incompatibility of some representative plasmids

Incoming	Percentage loss of indicated resident plasmid					
	FIIA/FIC pRM3937	FIB pSS3928	FIB pWM111	Copy mutant FIB pRM3994	FIIA/FIC FIB pRM133	FIIA/FIC 2 × FIB pRM136
1. pWM112 (FIIA/FIC)	100	0	–	–	36	100
2. pRM3947 (pUC-IncRNA)	100	–	–	–	0	65
3. pWM111 (FIB)	0	100	–	94	100[b]	100[b]
4. pWM114 (pBR-FIB)	7	100	–	99	25	55
5. pWM113 (pBR-FIC)[a]	7	0	–	–	–	–
6. pRM3994 (copy mutant-FIB)	–	–	62	–	–	–
7. 0 DNA	5	2.5	2.5	0	0	17.5

Incompatibility is defined as percent displacement of the resident plasmid after one round of growth on solid media (ca. 25 generations) with selection for the incoming plasmid only. In the experiments described this was based on a minimum of 40 colonies (where no more than 10 came from one transformant) which were tested for loss of the resident plasmid by replica plating

[a] Note that RepFIC and RepFIIA/FIC are compatible (Saadi et al. 1987)
[b] Very poor frequency of transformation
Typical standard deviations (pRM133 as resident): line 7 (0 DNA), $\sigma_n = 0$, and line 1 (FIIA/FIC challenge), $\sigma_n = 2.8$

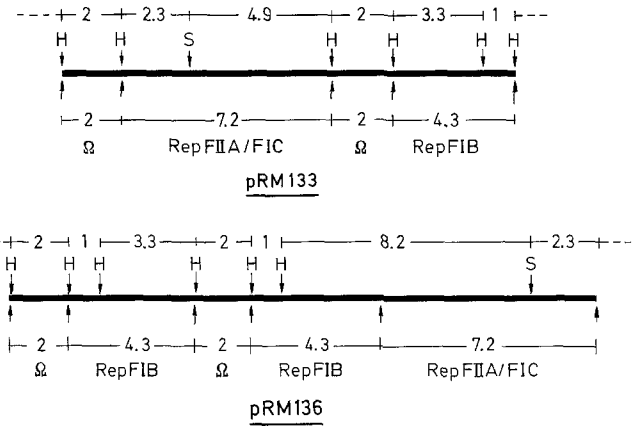

Fig. 3. Schematic representation of linearized structures of the plasmid cointegrates pRM133 and RM136. *Lower arrows* indicate *Eco*RI sites used in cloning. *Upper arrows* indicate restriction sites used to analyze the position and orientation of the cloned fragements. The numbers are distances in kb obtained experimentally and by computerized restriction analysis of the replicon DNA sequences. S, *Sal*I; H, *Hin*dIII

Table 5. Restriction analysis of pRM133 and pRM136

	Size of restriction fragments in kb obtained by digestion with	
	*Sal*I	*Sal*I + *Hin*dIII
pRM133	One fragment between 21 and 9.4	4.9
		3.3
		2.3
		2.0[a]
		1.0
pRM136	One fragment between 21 and 9.4, larger than linear pRM133	ca. 8
		3.3
		2.3
		2.0[a]
		1.0

The restriction digests were electrophoresed in 0.8% agarose, using TRIS-borate buffer, pH 8, together with two sets of molecular weight standards (Boehringer Mannheim)

[a] In both cases this band was more intense than the one immediately preceding it

(see Introduction) or with F (results with F not shown). In the RepFIIA-RepFIC type of replicons, i.e., where copy number control is mediated in part by a countertranscript, incompatibility is expressed by the incoming subcloned IncRNA region provided that the RNA product reacts with its target (Womble et al. 1984). Thus, the subcloned IncRNA region of R100 and of pCG86 each express incompatibility toward the cloned replicon RepFIIA/FIC of pCG86 (data shown in part, Table 4).

The in vitro constructed analogs of pCG86

The in vitro constructions were carried out as described in Materials and methods. Of 1120 transformants, 57 gave positive hybridization with both a RepFIB and a RepFIIA/FIC probe. Twenty were eliminated because they contained more than one plasmid, as demonstrated by gel electrophoresis (Kado and Liu 1981). Of the remainder, two prototypes were chosen for further study (pRM133 and pRM136) on the basis of their incompatibility behavior and the fact that upon digestion with *Eco*RI they yielded the three fragments used in the original ligation (FIB, FIIA/FIC and omega). Structural differences between the two selected plasmids are demonstrated in Table 2 and Fig. 3, where it can be seen that pRM133 is similar to pCG86, in that it contains one RepFIB replicon and one RepFIIA/FIC replicon. Plasmid pRM136, on the other hand, contains a duplication of RepFIB. Restriction with *Eco*RI, *Sal*I (one site in the FIIA/FIC fragment) and *Hin*dIII (one site in the FIB fragment) is consistent with the proposed duplication for the following reasons. The duplication (documented by probing) was already obvious from the intensities of the ethidium bromide stained bands of the *Eco*RI digests. *Sal*I digestion linearized pRM133 (15.5 kb) and pRM136 (19.8 kb). These results and those obtained by double digestion with *Sal*I and *Hin*dIII are summarized in Table 5. The orientation of the replicon fragments deduced from the restriction results is shown in Fig. 3.

Incompatibility of in vitro constructed analogs of pCG86, when used as resident plasmids

We would expect a bireplicon plasmid containing RepFIIA/FIC and RepRIB, such as pRM133, to replicate under RepFIIA/FIC control. When this bireplicon cointegrate is challenged in an incompatibility test by an incoming RepFIIA/FIC plasmid, we would expect RepFIB to take over the replication of the bireplicon cointegrate as the plasmid copy number drops during the incompatibility challenge. If the cointegrate is challenged by an incoming repFIB plasmid we would expect the cointegrate to continue replicating under RepFIIA/FIC control.

In Table 4 we see that pRM133 is displaced by both a mini-RepFIIA/FIC plasmid and by a RepFIB cointegrate plasmid. Therefore, the rescue by the unchallenged replicon in the resident plasmid is not quite complete. With incoming RepFIIA/FIC there is no rescue when RepFIB is duplicated (Table 4, line 1). This could be due to malfunctioning of the duplicated replicon. The same interference with rescue by RepFIIA/FIC when RepFIB is duplicated is observed when the subcloned IncRNA gene of pCG86 is used as the incoming plasmid (Table 4, line 2). Although the copy number of the IncRNA subclone is much higher than that of the self-cloned replicon pWM112, the effects on rescue are not as pronounced for both pRM133 and pRM136. We have no simple explanation for the difference in effectiveness between the autonomous RepFIIA/FIC replicon and its subcloned IncRNA gene, other than that a functioning origin could compete for the rate limiting initiator protein. This idea is supported by the experimental fact that an incoming RepFIIA/FIC origin region cloned in a high copy number vector slightly destabilizes a resident RepFIIA/FIC plasmid (incompatibility of 5%–10% as defined in Materials and methods, data not shown).

When a pBR325-RepFIB cointegrate plasmid is used as the incoming plasmid (Table 4, line 4), although replication is presumably controlled by the RepFIIA/FIC component of pRM133 and pRM136, respectively, we see incompatibility. Since we do not think that the RepFIB origins of pRM133 and pRM136 are being utilized, we propose that the observed incompatibility is due to interference with replication from the RepFIIA/FIC origin. A possible interpretation for our findings is that the incoming RepFIB in-

Table 6. The establishment of the heteroplasmid state with RepFIB plasmids in strains containing bireplicon plasmids with a RepFIB component

	Incoming plasmid DNA		
	pWM111 (RepFIB)[a]	pWM111 (RepFIB)	pWM112 (RepFIIA/FIC)[a]
Resident plasmid	pRM133 (RepFIB, RepFIIA/FIC)	pSS3928 (RepFIB)	pRM133 (RepFIB, RepFIIA/FIC)
No. transformants per ml	40–140	5000	ca 10000
No. heteroplasmid strains after 90–120 min	0%–13%[b]	68%	94%–100%

[a] A summary of three separate experiments. Range of values indicates upper and lower limit
[b] All surviving "heteroplasmids" that were analyzed by agarose gel electrophoresis were actually cointegrates

When pBR325-RepFIB (copy no. is eight) was used as a resident, the inhibition of transformation observed when a RepFIB plasmid was introduced by transformation was practically identical. When pSS3928 (RepFIB) and pWM5 (RepFIIA/FIC) were independent resident plasmids (heteroplasmid recipient) RepFIB donor DNA gave normal frequencies of transformation as well as the rapid segregation of the resident RepFIB characteristic for this replicon

teracts with the resident RepFIB component(s) to create a steric hindrance for the replication originating from RepFIIA/FIC.

It should be noted that when autonomously cloned RepFIB is introduced into cells containing the bireplicons pRM133 and pRM136 (Table 4, line 3), the frequency of transformation is very low. In elaboration of this finding, Table 6 illustrates that it is the heteroplasmid state that does not seem to be established (Table 6, column 1). RepFIB, however, has no trouble establishing a temporary heteroplasmid state in cells containing a RepFIB plasmid (no other replicon) with a different drug marker (Table 6, middle column). In contrast to RepFIB, the last column in Table 6 shows that RepFIIA/FIC can establish the temporary heteroplasmid state in cells containing the bireplicon pRM133. Austin and co-workers (Austin et al. 1982) also found that they could not isolate heteroplasmid cells containing P1 incompatibility functions subcloned in high copy number vectors and autonomous P1. Thus, P1 as well as other plasmids to be mentioned in the Discussion, appear to behave like RepFIB in this respect. We believe that ligation of RepFIB to another functioning replicon of higher copy number so that the pertinent RepFIB functions are switched off, creates a barrier or interference to an incoming plasmid that must utilize its RepFIB replicon. There is always the possibility that the failure to establish a heteroplasmid state is caused by the higher copy number of RepFIB when it is a component of a cointegrate plasmid, rather than by RepFIB being switched off. To this end, identical experiments were performed to detect the temporary heteroplasmid state using a RepFIB copy mutant (pRM3994, copy number is eight) as resident plasmid (data not shown). The copy number mutant behaved like normal RepFIB in that there was no inhibition of transformation. Yet pRM3994 is incompatible in both directions with normal RepFIB (Table 4, lines 3, 4, 6). Therefore, only switched-off RepFIBs show the phenomenon of prevention of establishment of an incoming obligate RepFIB plasmid. This will also be considered in the Discussion.

Discussion

We have investigated the incompatibility properties of bireplicon plasmids containing the basic replicons RepFIIA/FIC and RepFIB, present in the chimeric R/Ent plasmid pCG86. The bireplicons we constructed were much smaller than the 117 kb pCG86 and contained only the two basic replicons, together with a marker gene for drug resistance. The copy numbers of the bireplicon plasmids and their cloned component replicons were determined, as well as the rate of adjustment to a normal copy number from an elevated value of one of them (RepFIB). The purpose of the study was to see if the incompatibility behavior of these bireplicon plasmids could be explained on the basis of what we know about copy numbers and their controls in RepFIIA/FIC and RepFIB. On this basis we expected to see no incompatibility when a resident bireplicon plasmid is challenged by a monoreplicon plasmid containing either RepFIIA/FIC or RepFIB.

We did, however, observe some incompatibility when the RepFIB-RepFIIA/FIC cointegrate plasmids were challenged with either RepFIB or RepFIIA/FIC plasmids. This incompatibility was reproducible (at least 80 colonies tested, no more than 10 originating from one transformant) and the values were above control values (Table 4, line 7, same number of colonies tested).

On the basis of our copy number determinations (three for RepFIIA/FIC, one for RepFIB and three for the cointegrate plasmids) we expected RepFIB to be switched off in the cointegrate plasmids and thus not to be a target for incompatibility expressed by incoming RepFIB. We therefore postulate that the observed RepFIB incompatibility is due to the inhibition of replication originating from RepFIIA/FIC, possibly by introducing a steric block into the progress of the replication fork, for the reason that inhibition is accentuated in the cointegrate containing a duplication of RepFIB (Table 4, line 4).

The incompatibility observed on challenge of the resident bireplicon pRM133 with a RepFIIA/FIC plasmid (Table 4, line 1) is also accentuated in pRM136 (Table 4, lines 1, 2). This finding is also suggestive of a steric effect produced by a switched-off RepFIB, which is enhanced by the presence of two RepFIBs in tandem, although one of the latter is flanked by the genetic element omega with its transcriptional and translational stops at both ends (Fig. 3). It has been proposed that *incA* can restrain replication by causing steric hindrance to the origin function in P1 (Pal and Chattoraj 1988). These workers have shown that purified RepA protein can bind to two sites simulta-

neously and cause DNA looping. Such a mechanism could explain our results with the RepFIB duplications.

Another finding in the present work is that when RepFIB in the resident plasmid is ligated to a replicon of copy number higher than one, we cannot introduce into a cell containing it a plasmid replicating solely under RepFIB control. We obtained identical results using pBR325-RepFIB as the resident plasmid (data not shown), and similar results were obtained when both the resident and incoming plasmids were conjugative (the experiments were carried out by mating, with almost no offspring, data not shown). A similar observation has been made for RepFIA (D. Womble, personal communication) and for RK2 transformation of RK2-ColE1 plasmid containing cells (Figurski et al. 1979). A possible mechanism could be that switched-off RepFIB prevents the start of replication of incoming RepFIB. If, on the other hand, this were a case of inhibition of the incoming plasmid replication by a *trans*-acting diffusible inhibitor, then we should be able to see a normal transformation frequency by simply selecting for the incoming RepFIB. If it were incompatibility due to competition for an autoregulated or constitutive initiator, then the incoming RepFIB should make more initiator (autoregulated control) or its own initiator (constitutive control). It seems that a complex of resident RepFIB (part of a cointegrate) and its inhibitor acts as a repressor of initiation of the incoming RepFIB plasmid. A. Abeles and S. Austin have proposed such a model for the replication control of P1 (personal communication) where the cloned *incA* repeats in a high copy number vector prevent replication at a P1 origin *in trans*. In cases where we introduce RepFIB ligated to another replicon, the transformation frequency is normal because the replication of the incoming plasmid can initiate at an origin other than the RepFIB origin. In this latter case we see expression of RepFIB incompatibility. We conclude from our findings that strong inhibition of transformation is not a general measure of incompatibility as previously proposed (Timmis et al. 1975), since it is confined to a particular form of replication control (like that of RepFIA or P1, for instance) and to restricted conditions.

The complete DNA sequence of RepFIB derived from P307 has been determined (Saul et al. 1989) and the organization of its genetic components appears to be like that of P1 and RepFIA (an open reading frame coding for a Rep protein flanked by repeat sequences). The copy mutant pRM3994 differs from normal RepFIB by one amino acid in the Rep protein (P. Bergquist, personal communication) and it is possible that this protein is not sensitive to autoregulation. The copy mutant can however express incompatibility toward RepFIB, and yet as a resident when it is providing an eightfold dose of repeats it fails to inhibit the establishment of an incoming RepFIB. We feel that the above finding supports the requirement of a switched-off RepFIB for the inhibition of transformation by RepFIB.

It has been proposed that the copy number of chimeric plasmids will not be less than that of the component plasmid with the higher copy number (Pritchard and Grover 1981). This argument has been shown to be valid in many cases, and indeed pCG86 and its analogous constructs have a copy number of three, reflecting that of the higher copy number component RepFIIA/FIC.

Our experiments on the adjustment of copy number have shown that RepFIB exhibits a step-function shut-off when it adjusts from an elevated copy number to its normal one. In view of its subsequently derived structure, we can assume that RepFIB, which bears no homology with RepFIA or P1, controls its replication by similar mechanisms.

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