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Killing of *Escherichia coli* cells modulated by components of the stability system ParD of plasmid R1

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Summary. The proteins P10 and P12 have been shown to be gene products of a new stability system, ParD, of plasmid R1. It is now shown that an R1 miniplasmid, pAB112, carrying a *trans*-complementable amber mutation in the gene of the P10 protein, is lethal for the host in the absence of suppression. This lethal effect is suppressed in a *sup*F background and also by deletions in pAB112 that affect the gene of the P12 protein. These data indicate that the P12 protein has a lethal effect on the host and that this effect is neutralized by the P10 protein. The possibility that the stabilization conferred by the ParD system could be due to a counterselection, mediated by P12, of cells that lose the plasmid at cell division, is discussed.

Key words: Plasmid R1 – Stability functions – ParD system – Cell killing functions – *kis* and *kid* loci

Introduction

The resistance factor R1 is a plasmid of enterobacteria (Meynell and Datta 1967) that in spite of its low copy number, is very stably inherited (Nordström and Aagaard-Hansen 1984; Nordström et al. 1984). This stable inheritance reflects not only the efficiency of the copy number control system of the plasmid, but also the activity of additional stability functions that are located away from the basic replicon of the plasmid (Nordström et al. 1980) and that correspond to two different stability systems called ParA and ParB (Gerdes et al. 1985). The ParB system acts by counterselecting the cells that lose the plasmid at cell division (Gerdes et al. 1986), while the ParA system is probably required for an even distribution of the plasmid copies in the same process (Gerdes and Molin 1986). Systems involved in non-random distribution of the plasmids at cell division have also been found in other low copy number plasmids, such as F (Ogura and Hiraga 1983), P1 (Austin and Abeles 1983) and the *inc*FII plasmid, NR1 (Miki et al. 1980); and a system that results in the death of the segregants was described for the first time in the sex factor F (Ogura and Hiraga 1983; Jaffé et al. 1985). In addition to ParA and ParB, R1 has a stability system that is located in the proximity of the basic replicon and that has been called ParD (Bravo et al. 1987). Two genes, *p10* and *p12*, that code for proteins of approximately 9.3 and 12 kDa (P10 and P12, respectively), are components of the ParD system. In this communication we provide evidence that an amber mutation in *p10* is lethal for the host in the absence of suppression and that this killing effect is abolished by deletions that remove the 3′ end of *p12*. This killing phenotype is discussed in the light of the stabilization mediated by ParD. We propose new denominations for the genes of the P10 and P12 proteins, *kis* (*s*uppressor of *k*illing) and *kid* (*k*illing *d*eterminant), respectively.

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Material and methods

Bacterial strains and plasmids. All the bacterial strains were *Escherichia coli* K12. The wild-type strain, W3110 (Bachmann 1972), the *sup*F(ts) strain, OV2 (Donachie et al. 1976) and the *sup*F strain, KSu3 (obtained from R. Hayward) were used or tested as host for the different plasmids shown in Fig. 1. A *min*B derivative of OV2 (this work) was used as a minicell producing strain carrying a thermosensitive *sup*F allele; the *min*B allele was introduced by cotransduction with a tetracycline resistance marker (source strain B1688 from R. D'Ari). BMH71-18 (Gronenborn 1976) was used in experiments involving vectors M13mp8 or M13mp9 (Messing and Vieira 1982). pET80 and pAB1120 are *cop*B derivatives of the R1 miniplasmid pKN1562 and of the amber mutant described here, respectively, and they were constructed by deleting in vitro the *Bgl*II fragment to remove the promoter and part of the *cop*B gene (Riise et al. 1982). The relevant features of other plasmids used in this work are shown in Fig. 1.

Growth media. The bacteria were grown in LB medium (Lennox 1955) except the minicell producing strains that were grown in M9-based media (Bravo et al. 1987). Kanamycin or ampicillin were added as required at final concentrations of 25 μg/ml and tetracycline at a concentration of 10 μg/ml. The media were solidified, as required, with bacto agar (1.5%). H medium (Amersham M13 cloning and sequencing manual) was used for the propagation of M13-based recombinants. The growth of cultures in liquid medium was followed by the absorbance at 450 nm, which was measured in a Gilford spectrophotometer.

Hydroxylamine mutagenesis of plasmid DNA. The basic protocol used (Humphreys et al. 1976), as well as the modifications introduced (Bravo et al. 1987), have been described.

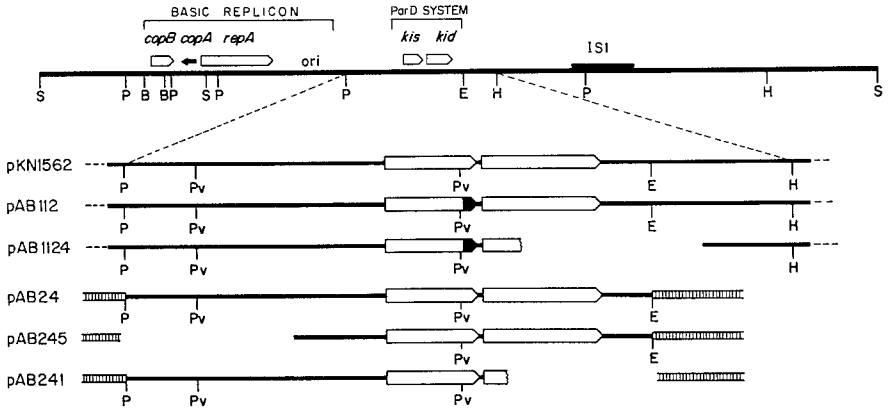

Fig. 1. Physical map of plasmids used in this work. Only features relevant to the information presented in this study are shown. ⊏⊐ open reading frames (the region of *kis* located after the amber mutation described in this paper is marked in *black*); ⟶ RNA CopA; ▬ R1 sequences; ▬ IS*1* sequence; ▥ pBR322 sequences. The fragment that includes the ParD system is enlarged; deletions of the R1 sequence are indicated by an *internal discontinuity*. The relevant informaiton on pKN1562 (Molin and Nordström 1980), pAB24, pAB241 and pAB245 (Bravo et al. 1987) has been described but is repeated here for easier comprehension. S, *Sal* I; P, *Pst*I; B, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II (the targets of *Pvu*II are detailed only in the enlarged region)

DNA preparations, enzymatic manipulations and transformation with DNA. Mini-preparations of plasmid DNA were done as described (Maniatis et al. 1982). Alternatively, the DNA was obtained from clear lysates (Clewell and Helinski 1969), after purification in CsCl-ethidium bromide gradients (Stougaard and Molin 1981). Single-strand and replicative forms of M13mp recombinants were prepared as described by Amersham (M13 cloning and sequencing manual). Restriction endonucleases, T4 DNA ligase or nuclease Bal31 were used according to the specifications of the suppliers (Bethesda Research Laboratories, Boehringer Mannheim or New England Biolabs). Electrophoresis of DNA in agarose gels was done as described (Maniatis et al. 1982). Competent cells were prepared and transformed as described (Cohen et al. 1972; Messing 1983).

DNA sequencing. The enzymatic method of Sanger (Sanger et al. 1977) was followed, with the modifications described by Amersham (M13 cloning and sequencing manual). Appropriate recombinants derived from the vectors M13mp8 or M13mp9, were used to sequence the relevant region of the *kis* gene of pAB112 and the borders of the deletion that affects the *kid* gene of pAB1124.

Analysis of proteins expressed in minicells. The minicells were prepared from cultures of OV2 *min*B or from this strain containing plasmids pET80 or pAB1120 (copy number derivatives of pKN1562 and pAB112, respectively, this work). Labelling with ^{35}S-methionine of the proteins synthesized in them, was done at 30° C or 42° C for 45 min: suspensions of approximately 2×10^9 minicells in a volume of 200 µl, were incubated with 8 µCi of ^{35}S-methionine (1400 Ci/mmol). Before adding the label, the minicell suspensions were incubated for 10 min at 30° C or 42° C, respectively. The basic protocol for the purification of minicells and the analysis of proteins synthesized in them have been described (Frazer and Curtiss 1975). The proteins were separated by electrophoresis in SDS-urea-polyacrylamide gels according to the protocols adapted by Bethesda Research Laboratories, and the labelled proteins were identified by autoradiography.

Results

Isolation of the pAB112 mutant

DNA of the R1 ParD$^+$ miniplasmid, pKN1562, conferring kanamycin resistance to the cells, was mutagenized in vitro with hydroxylamine and introduced by transformation in cells of the *sup*F(ts) strain, OV2. The transformants were selected at 30° C and in LBT (LB plus 20 µg/ml thymine) medium containing kanamycin. Subsequently, the transformants were propagated at 30° C and 42° C on LBT (LB plus 20 µg/ml thymine) plates without antibiotic and then tested for growth at 30° C in the presence of kanamycin. Transformants that have lost the plasmid during the propagation in non-selective medium, and with it their resistance to kanamycin, were found with a frequency of 0.8%. Some of them (10%–20%), contained plasmids that were lost preferentially when the propagation in the non-selective medium was done at 42° C. Among these transformants, the pAB112 mutant, which is indistinguishable from the parental miniplasmid pKN1562 by restriction analysis (see Fig. 1), was chosen for further characterization. OV2 cells transformed with pAB112 DNA gave kanamycin resistant clones that had the same phenotype as the initial transformant, showing that the mutation was located in the plasmid.

pAB112 contains an amber mutation that affects kis, *one of the components of the stability system ParD*

The unstable maintenance of pAB112 in the culture could be due to a failure in plasmid replication at 42° C. However, preliminary analysis (data not shown) indicated that the mutation had not affected either *cop*A, the gene that controls replication of R1, or *rep*A, the gene coding for a protein which is specifically required for replication of R1. Instead, the mutation of pAB112 was located within the

Table 1. *Trans*-complementation of the thermosensitive phenotype of pAB112

Plasmid in OV2[a]	Viability on LBT-kanamycin plates[b]	
	30° C	42° C
pKN1562	1.6×10^8	1.5×10^8
pAB112	0.8×10^8	$< 1.0 \times 10^5$
pAB112+pAB24	1.3×10^8	1.4×10^8
pAB112+pAB241	0.7×10^8	0.8×10^8
pAB112+pAB245	1.3×10^8	1.2×10^8
pAB112+pBR322	1.0×10^8	$< 1.0 \times 10^5$

[a] R1 miniplasmid determines resistance to kanamycin and pBR322 recombinants resistance to tetracycline

[b] The viability indicates the number of viable cells in a colony. To determine this value, a colony selected at 30° C on an LBT (LB plus 20 μg/ml thymine) plate containing 25 μg/ml kanamycin (LBT-Km_{25}) was resuspended in 0.9% NaCl, diluted 10^4- and 10^5-fold in the same medium and 0.1 ml of these dilutions was spread on LBT-Km_{25} plates that were incubated at 30° C or 42° C. After 14 h incubation, the number of kanamycin resistant colonies was scored, and the viability determined. Minute colonies appeared in the plates corresponding to cells harbouring pAB112 or pAB112 and pBR322, when the plates were incubated at 42° C for periods longer than 18 h

1.449 kbp *Pst* I–*Eco*RI fragment that is adjacent to the origin of replication of R1 and that contains the stability system ParD. Indeed, a pBR322 recombinant, pAB24, carrying the corresponding wild-type fragment, was able to *trans*-complement the thermosensitive phenotype of the pAB112 mutant (see Fig. 1, Table 1). Reiteration of the complementation analysis with the recombinants pAB245 and pAB241 (see Fig. 1, Table 1), strongly suggested that the mutation maps inside *kis*, one of the genes of the ParD system.

The *kis* gene of pAB112 was sequenced with chain terminating inhibitors (Sanger et al. 1977). This analysis indicated that the mutation was a GC-AT transition located at position 3156 (coordinates of Rosen et al. 1980), that changes codon 74 of the *kis* gene into an amber termination codon (Fig. 2). The mutation was denominated *kis*74.

The sequence analysis predicts (see Fig. 2), that the mutation should result in a truncated P10 protein of 8 kDa, 12 amino acids shorter than the 9.3 kDa wild-type P10 protein. To test this prediction, the mobilities of the P10 proteins corresponding to the parental and mutant miniplasmids, were compared under suppressing and non-suppressing conditions using the minicell system. A *min*B derivative of the *sup*F(ts) strain OV2 was used in the analysis as the source of the minicells and to facilitate detection of the P10 proteins, the *copB* derivatives of pKN1562 and pAB112 plasmids called, respectively, pET80 and pAB1120, were used (see Material and methods).

The proteins synthesized at 30° C or at 42° C by minicells containing the *cop*B derivatives, were labelled with ^{35}S-methionine, fractionated by electrophoresis, and analyzed by autoradiography as indicated in Materials and methods. Consistent with the prediction based on the sequence analysis, minicells containing pAB1120 synthesized at 42° C a truncated polypeptide with a relative mobility in the gels close to 8.0 kDa, and at 30° C a polypeptide that moved into the 9 kDa region (Fig. 3; tracks 5, 6). Minicells containing pET80 synthesized, both at 30° C and 42° C, a P10 protein with a relative mobility in the gels of 9 kDa (Fig. 3; tracks 3, 4), a value close to the one deduced from the sequence. Notice that in all cases, the P12 protein (the product of *kid*), that has a relative mobility in the gels close to 10.5 kDa, is synthesized efficiently.

The kis*74 mutation is lethal for the cell in the absence of suppression*

The instability of pAB112 in OV2 when the cells are grown at 42° C could be due to the combined effect of the counterselection of cells containing pAB112 in the absence of suppression and the takeover of the culture by plasmid-free cells already present at 30° C. To evaluate this possibility, the viability of cultures of OV2 containing either pAB112 or its *cop*B derivative, pAB1120, was determined at different times after shifting the temperature from 30° C to 42° C; the total number of viable cells and kanamycin resistant cells was determined. Controls were carried out with cultures containing either pKN1562 or its *cop*B derivative, pET80. The results of these experiments are shown in Fig. 4.

A comparison of the increase of kanamycin resistant cells indicates that the cells carrying either pAB112 (Fig. 4A) or its copy mutant derivative pAB1120 (Fig. 4B), stop dividing approximately 100 min after the shift to 42° C and then they die. The killing effect is more clearly shown in cells containing the pAB1120 plasmid: 150 min after these cells stop dividing, their viability falls more than 100 times. We also observed that the size of the colonies formed at 30° C by the kanamycin resistant cells that remained viable, was smaller than the ones of the control

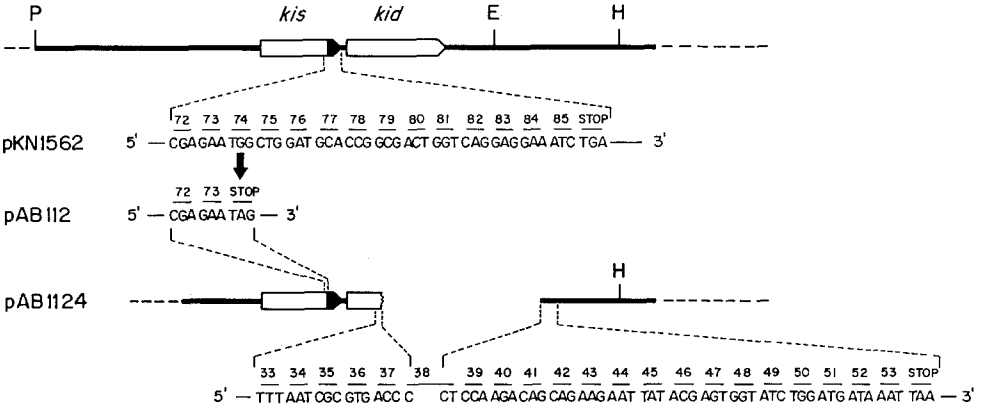

Fig. 2. Sequence information on the mutation in *kis* carried by pAB112 and on the deletion in *kid* of pAB1124. The specific base change and codon altered by the mutation and the sequences joined by the deletion are indicated. ▬ R1 sequences; ▭ open reading frames corresponding to *kis* and *kid*. The region of *kis* located after the amber mutation is marked in *black*

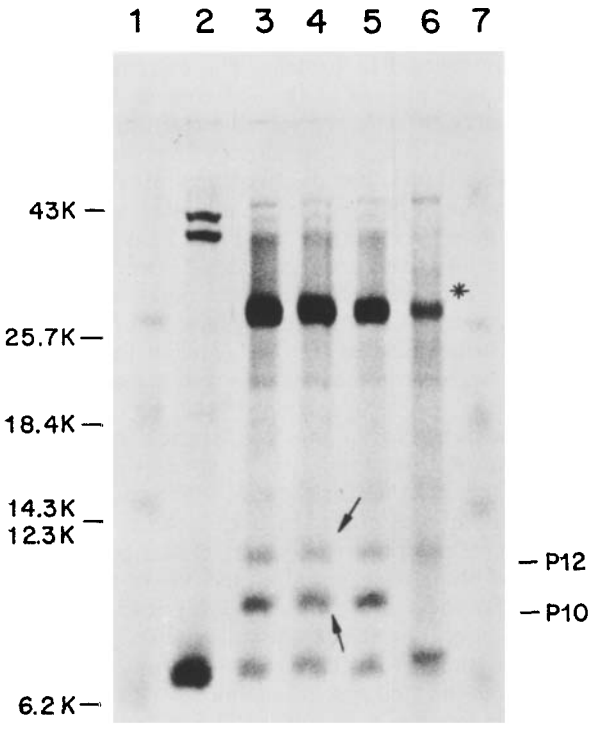

Fig. 3. Autoradiograms of ^{35}S-labelled proteins synthesized in minicells. Proteins synthesized in minicells at 30° C or 42° C, were labelled with ^{35}S-methionine for 45 min after a preincubation period of 10 min at the same temperature. The proteins were separated by electrophoresis in SDS-urea-polyacrylamide (15%) gels. Tracks 1, 7, molecular weight standards; 2, proteins snythesized by plasmid-free minicells; 3, 4, proteins synthesized by minicells containing pET80 at 30° C or 42° C, respectively; 5, 6, proteins synthesized by minicells containing pAB1120 at 30° C or 42° C, respectively. Tracks 2–6 contain similar amounts of proteins. The diffuse band just above the front in track 6 is probably the product of the truncated P10 protein. *, product of the kanamycin resistance gene. The intensity of this band compared to the bands corresponding to Kis (amber fragment) and Kid is clearly reduced in track 6

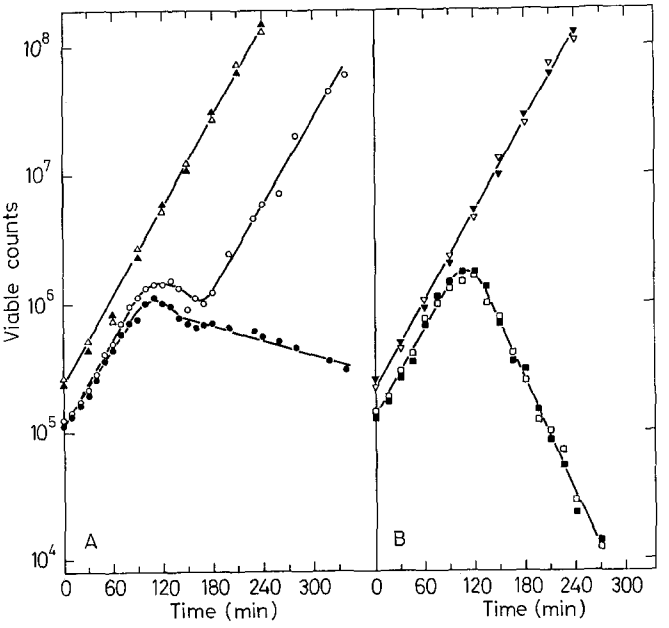

Fig. 4A, B. Viability of cultures of OV2 containing pKN1562, pAB112, pET80 or pAB1120 at different times after shifting the temperature from 30° C to 42° C. Appropriate dilutions of the cultures were plated on LBT (LB plus 20 µg/ml thymine) medium with or without kanamycin (25 µg/ml); the plates were incubated at 30° C and the colonies appearing overnight were counted. **A** Viable cells corresponding to a culture of OV2/pKN1562 (△, ▲) or to a culture of OV2/pAB112 (○, ●). Total viable cells (△, ○) and kanamycin resistant cells (▲, ●). **B** Viable cells corresponding to a culture of OV2/pET80 (▽, ▼) or to a culture of OV2/pAB1120 (□, ■). Total viable cells (▽, □) and kanamycin resistant cells (▼, ■)

Table 2. Efficiency of transformation of different plasmids in suppressing or non-suppressing hosts

Plasmid	Total number of transformants					
	OV2		KSu3		W3110	
	30° C	42° C	30° C	42° C	30° C	42° C
pKN1562	69	65	118	150	200	125
pAB112	56	0	150	110	0	0
pAB1124	180	162	193	240	120	144

A minipreparation of 20 ng plasmid DNA was used in each transformation. The transformation mixtures were heat shocked for 5 min at 30° C, incubated for 1 h in LBT (LB plus 20 µg/ml thymine) at 30° C and then plated on LBT medium supplemente with kanamycin. The plates were incubated either at 30° C or 42° C overnight, and the total number of colonies that appeared was scored. OV2 is a *sup*F(ts) strain, KSu3 a *sup*F strain, and W3100 a wild-type strain

culture (data not shown). In addition, the longer the cells stayed at 42° C, the smaller was the size of these colonies. This indicated that the physiology of the cells containing these plasmids that survive at 42° C was affected. In fact, an examination of these cells in the phase-contrast microscope, showed that they formed small filaments (data not shown).

In cultures of OV2 containing pAB112, the total number of viable cells recovered, with an exponential increase 180 min after the temperature shift up due to the growth of plasmid free cells (Fig. 4A). As in this culture the fraction of plasmid free cells before the shift to 42° C was approximately 9%, and the generation time of OV2 at 42° C is 30 min, the recovery observed reflects basically the takeover of the culture by this initial population. Note that in cultures of OV2 containing plasmid pAB1120 and growing at 30° C, the percentage of plasmid free cells is extremely low (<than 0.1%); consequently, a takeover by plasmid-free cells was not observed in these cultures after the shift to 42° C.

We conclude that the presence of the amber mutation described here is lethal to the non-supressing host and that the unstable phenotype of the pAB112 mutant in OV2 at 42° C is the indirect consequence of this effect.

If the presence of the amber mutation of pAB112 is lethal to the host, the frequency of transformation of pAB112 in a non-suppressing strain, W3110, should be drastically reduced. The results presented in Table 2 indicated that this was indeed the case. Note that an efficiency

of transformation similar to the one obtained with the parental plasmid, pKN1562, was obtained with pAB112, when the host contained a functional SupF tRNA (OV2 at 30° C but not at 42° C, or KSu3 at both temperatures).

Suppression of the killing phenotype of pAB112 by genetic inactivation of the kid *gene*

As previous results indicated that the phenotype associated with a missense mutation in *kis* could be suppressed by deletions that inactivated the *kid* gene (Bravo et al. 1987), we tested if this was also the case for the lethal effect associated with the amber mutation in *kis*. For this purpose, the 3′ end of *kid* was deleted in vitro using Bal31. The resulting plasmid, pAB1124 (see Figs. 1, 2), that was rescued at 30° C in OV2 by transformation, was not lethal for this host at 42° C. In addition, pAB1124 transformed with similar efficiency (Table 2) a non-suppressing host (W3110, or OV2 at 42° C) or a *sup*F one (OV2 at 30° C or KSu3 at 30° C or 42° C). Spontaneous derivatives of pAB112 carrying deletions that allowed the OV2 host to grow at 42° C, were also isolated. In all cases the *kid* gene was affected by the deletions (data not shown).

These results indicate that genetic inactivation of the *kid* gene of pAB112 suppresses the lethal effect mediated by *kis*74. This in turn suggests that the product of *kis* antagonizes the killing effect mediated directly or indirectly by the product of *kid*.

Discussion

In this paper we describe the isolation and characterization of an amber mutation affecting *kis*, one of the genes of the ParD stability system of R1 that is close to the origin of replication (Bravo et al. 1987). The analysis presented here indicates that the P10 (Kis) protein antagonizes a killing effect mediated by the P12 (Kid) protein, the other component of the ParD system.

How do these results fit with the ones that indicate that ParD is a stability system? We propose that ParD, like the Ccd system of F (Ogura and Hiraga 1983; Jaffé et al. 1985) and the ParB system of R1 (Gerdes et al. 1986), stabilizes the plasmid by interfering with the growth or viability of the cells that lose the plasmid at cell division and that this interference is the result of the action of the P12 protein. This model implies that the activity of the P10 protein decays faster than the activity of the P12 protein in the segregants. The low efficiency of the wild-type ParD system in stabilizing R1 miniplasmids (at least in hosts and conditions so far tested, Bravo et al. 1987), could be due to a slow decay of the wild-type P10 protein. This interpretation is in agreement with the fact that, after the shift to 42° C, the cells of OV2 containing pAB112 grow and divide actively for three generations before they start dying.

The model predicts that an alteration of the activity and/or stability of the P10 protein could lead to an interference with growth or viability of the host and in particular cases could enhance the counterselection of segregants mediated by the P12 protein. Data obtained with an R1 miniplasmid, pAB17, that carries a missense mutation in the *kis* gene (Bravo et al. 1987), support this prediction. This mutant can interfere with growth and viability of the host in particular conditions (rich medium or high temperature) and is very stably maintained in cell populations. As both effects can be suppressed by insertions or deletions that inactivate *kid*, it seems clear that they are the consequence of a non-antagonized P12 protein. The extreme stability of pAB17 can be explained as the consequence of an efficient counterselection of the segregants due to amplification in them of the levels of non-neutralized P12 protein.

We have also found that the frequency of transformation of a recombinant that carries the inhibitor of R1 replication, *cop*A, in cells containing the pAB17 mutant, is reduced by at least 4 orders of magnitude (A. Bravo, unpublished results). The above hypothesis explains this result as the consequence of the efficient displacement of the mutant from the cells mediated by *cop*A, and of the efficient killing of the segregants mediated by the mutated ParD system.

The parallelism between the ParD system of R1 and the Ccd system of F is striking. Both systems code for a component which is lethal for the host and another which is an antagonist of this effect. In both systems the genes of these components are probably part of an operon, with the gene of the antagonist preceding the one of the killing component. Like the amber mutation in *kis* described here, an amber mutation, *ham*22, in the antagonist of the Ccd system is also lethal for the host (Karoui et al. 1983). The ParD system, as the Ccd system, stabilizes the plasmid in the absence fo the RecA functions and in both systems the cells affected by the killing functions form small filaments (to be published). However, the sequence analysis shows clear differences between the corresponding components of the two systems. The conservation of functions and genetic organization in the two systems, in spite of the differences found at the sequence level, underlines the relevance of their role in plasmid maintenance during evolution. A careful comparison of the hydropathy curves of the antagonists and killing components of the two systems indicates that, although the profiles of the killing components are clearly different, the NH_2 and COOH regions of the antagonists are similar (to be published). It may be significant that the two mutations described by our group affecting the antagonist of the ParD system and leading to different phenotypes, map precisely in these regions.

R1 contains at least three stability systems, ParA, ParB and ParD, two of which, ParB and ParD code for cell killing functions. Under the experimental conditions evaluated so far, the ParA or ParB systems are approximately 100 times more efficient than the ParD system. However, the exact conservation fo the ParD system in the *inc*FII replicon, R100, the proximity of this system to the basic replicon of R1 and R100 and the fact that a mutated ParD system can be at least 10 times more efficient than the ParA or ParB systems (Bravo et al. 1987), argues in favour of the significance of ParD in nature.

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Note added in proof

After submission of this paper, Tsuchimoto et al. (J Bacteriol 170:1461–1466, 1988) reported a stable maintenance system, Pem, of R100 which was found to be identical to ParD of R1 (Bravo et al. Mol Gen Genet 210:101–110, 1987).

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