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Stability of ColE1-like and pBR322-like plasmids in *Escherichia coli*

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Summary

The average copy number, the level of ampicillin resistance conferred by one plasmid, and the degree of plasmid multimerization were determined for several ColE1-like and pBR322-like plasmids. From the results obtained, the variance of the units of partition corresponding to each plasmid studied was calculated. Experimentally determined plasmid stability was compared with that calculated using the variance of the units of partition and the ratio between the generation times of plasmid-free and of plasmid-carrying cells, assuming that the units of partition are distributed randomly between daughter cells.

Stability of the pBR322-like plasmids present mainly as monomers in the bacterial host was consistent with random partitioning, whereas pBR322-like plasmids, present mainly as dimers, and the ColE1-like plasmid showed greater stability than that predicted with random partitioning at cell division.

Introduction

Plasmids are extrachromosomal DNA replicons present in many bacteria in a defined number of copies per cell. For some plasmids, this number may be as low as one to six, whereas for others it may be as high as 100. Many low-copy and multi-copy plasmids are stably maintained in a bacterial population after many generations of growth under non-selective conditions; however, most of the multi-copy plasmids constructed *in vitro* and used as cloning vectors are unstable and are lost from cultures at frequencies ranging from 10^{-2} to 10^{-5} per cell per generation (Summers and Sherratt, 1984).

Five main mechanisms for ensuring plasmid stability have been proposed: (i) a precise replication control (Davison, 1984; Lacatena *et al.*, 1984); (ii) an active partition mechanism mediated by the attachment of a plasmid-specific DNA sequence to a cellular partition system (Miki *et al.*, 1980; Gustafsson *et al.*, 1983); (iii) site-specific recombination of multimers to provide an adequate number of units for partitioning (Austin *et al.*, 1981; Sherratt *et al.*, 1986); (iv) post-segregational killing, which prevents the appearance of plasmid-free cells in a growing culture (Jaffe *et al.*, 1985; Gerdes *et al.*, 1986); and (iv) temporal inhibition of cell division if the plasmid replication process does not provide a sufficient number of plasmid copies per cell cycle (Ogura and Hiraga, 1983a; Miki *et al.*, 1984). Different plasmids display distinct strategies to ensure an accurate partition and therefore stability. The low copy-number plasmids, F, R1 and prophage P1, and the intermediate copy-number plasmid, pSC101, in addition to other strategies to ensure their stability, code for functions involved in active partition (Meacock and Cohen, 1980; Nordström *et al.*, 1980; Austin and Abeles, 1983; Gustafsson *et al.*, 1983; Ogura and Hiraga, 1983b). However, there are no reports of active partition mechanisms for high copy-number plasmids. The stability of plasmids is a complex phenomenon; however, as has been suggested by Summers and Sherratt (1984), bacterial cells carrying high copy-number plasmids with no active partition mechanism can generate plasmid-free cells if the number of units of partition in some cells close to division is relatively low.

The present study was designed to experimentally test the hypothesis that partition of high copy-number plasmids is at random. For this purpose, a bacterial population was considered to be formed by various cell sub-populations carrying different numbers of units of partition and therefore showing different probabilities for generation of plasmid-free cells. It was also considered that in a bacterial population growing without selective pressure, the variance of units of partition remains constant after reaching an equilibrium. If this hypothesis is correct, the stability of these plasmids should be a function of the variance of the number of units of partition in cells near division.

Plasmids having different transcriptional patterns as well as *rop*$^+$ and *rop*$^-$ plasmids were selected. The plasmid transcriptional pattern can modify the degree of plasmid multimerization and, therefore, plasmid stability (Bujard *et al.*, 1985). The *rop* gene encodes for a protein that represses plasmid replication (Lacatena *et al.*, 1984) and probably determines a broader plasmid copy-number variance (Summers and Sherratt, 1985).

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Plasmid copy number, plasmid multimerization, and single-cell resistance to ampicillin were determined in strain C600 carrying each of the plasmids tested. From the results obtained, the variance of the units of partition was calculated. Plasmid stability was experimentally determined and compared with that calculated using the experimentally obtained values for variance of the units of partition.

Results

Average plasmid copy number and level of ampicillin resistance conferred by one plasmid

The average plasmid copy number per cell determined by a method which yields an accurate plasmid to chromosome ratio (Womble *et al.*, 1977) was 11.5 for ColE1 (average of two independent experiments) and 11 for pT7 (average of three experiments) (Table 1). The copy numbers for plasmids pBR322, pIO1, pBR327 and pBR329 were determined by fluorescence densitometry (Projan *et al.*, 1983) using plasmid pT7 as control. With this method, the average copy number for plasmid pBR322 was 61; for pIO1, 37; for pBR327, 74; and for pBR329, 20 (Table 1). The copy number for plasmid pBR327 (*rop*$^-$) was, as expected, higher than that for pBR322 (*rop*$^+$) (Lacatena *et al.*, 1984). However, the copy number for plasmids pIO1 and pBR329 which carry the *rep* region of pBR322 and pBR327, respectively, was lower than the number expected. The main difference between pBR322 and pBR327 and the derivative plasmids pIO1 and pBR329 is the insertion in the *Eco*RI site of the former plasmids of a fragment of 877 bp carrying the gene *cat* (*Experimental procedures*; Covarrubias and Bolivar, 1982).

Since there is a linear correlation between resistance to ampicillin and gene dosage (Uhlin and Nordström, 1977), the level of ampicillin resistance conferred by one plasmid was calculated from the distribution of single-cell resistance to different concentrations of ampicillin (Fig. 1). From these data, the average level of ampicillin resistance in the bacterial population was calculated. The average level of resistance to ampicillin and the average plasmid copy number were used to determine the resistance level to ampicillin conferred by one plasmid (Table 1). Plasmid pT7 conferred a higher resistance to ampicillin than that conferred by the other plasmids tested. This higher resistance level is probably due to the presence in pT7 of a stronger *bla* promoter or to a higher degree of transcriptional readthrough into this gene from distal promoters. The ampicillin resistance conferred by one copy of the derivative plasmids pIO1 or pBR329 was lower than that conferred by the original plasmids pBR322 or pBR327, respectively. As mentioned before, the former plasmids contained an insertion of 877 bp in the unique *Eco*RI site present in both pBR322 and pBR327. This insert separates the anti-tet promoter (Covarrubias and Bolivar, 1982) from the *bla* gene; therefore, it is possible that in pIO1 and pBR329 the number of transcripts containing the *bla* gene is lower than that in pBR322 and pBR327.

Table 1. Plasmid copy number and level of ampicillin resistance conferred by one plasmid to strain C600.

Plasmid	Average plasmid copy number/cell[a]	Average ampicillin resistance ($\mu g\ ml^{-1}$)[b]	Ampicillin resistance ($\mu g\ ml^{-1}$)/copy[c]
pT7	11	2300	210
pBR322	61	7400	120
pIO1	37	1600	43
pBR327	74	6700	90
pBR329	20	900	45

a. The copy numbers were estimated by determination of CCC DNA (pT7) and by fluorescence densitometry (pBR322, pIO1, pBR327, and pBR329).
b. Calculated from histograms, as shown in Fig. 2. The histograms were obtained from experiments represented as in Fig. 1.
c. Ratio between the average plasmid copy number/cell and average ampicillin resistance ($\mu g\ ml^{-1}$).

Plasmid multimerization

The degree of plasmid multimerization for the plasmids tested is shown in Table 2. Multimerization of plasmid pIO1 was calculated by both electron microscopy and densitometry, as described in the *Experimental procedures*. The results obtained with these two methods were similar; therefore plasmid multimerization for the other plasmids was determined using only the second method. The degrees of multimerization for ColE1 and pT7 were similar. The relatively high percentage of dimers presented by these two plasmids was unexpected. ColE1 encodes a determinant, *cer*, which converts multimers to monomers (Summers and Sherratt, 1984); pT7 carries the resolution system encoded by Tn*1* (Arthur and Sherratt, 1979) in addition to the *cer* determinant. The prevalent molecular form of plasmids pBR322 and pBR327 was the dimer; meanwhile pIO1 and pBR329 were present mainly as monomers.

Distribution of plasmid copy number in the bacterial population at different times

The distribution of plasmid copy number in cells growing exponentially under non-selective pressure at different times was studied. These distributions were determined in a cell culture started with cells from Luria plates containing a relatively high concentration of Tc ($50\ \mu g\ ml^{-1}$). The average copy number at time zero was, as expected, relatively high (Fig. 2). However, after approximately 50

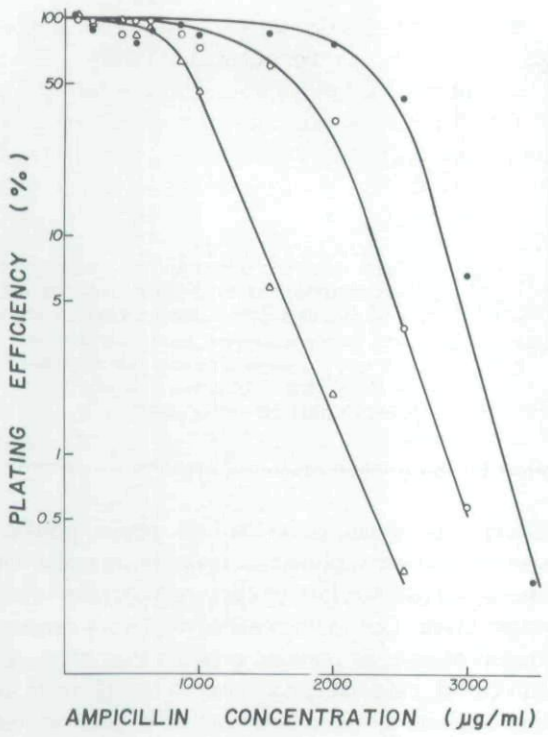

Fig. 1. Plating efficiency of cells carrying different ColE1-like plasmids. Cultures were grown as described in the assay for plasmid stability (*Experimental procedures*) and appropriate dilutions were spread onto plates containing various concentrations of ampicillin. Colonies were counted after incubation for 18–20 h at 37°C. (●) pT7; (○) pIO1; (△) pBR329.

generations and up to 100 generations of growth, both the average plasmid copy number and the relative distribution of plasmid copy number in cells carrying plasmids were similar (Fig. 2). The percentages of cells without plasmids after 50 and 100 generations of growth were 4 and 40, respectively. These results showed that the variance of plasmid copy number in the subpopulation of cells containing plasmids remained constant after reaching a condition of equilibrium; meanwhile, the number of plasmid-free cells increased with time.

Table 2. Plasmid multimerization in strain C600.

Plasmid	Percentage of Molecules[a]		
	Monomers	Dimers	Tetramers
ColE1	83.8	16.2	0.0
pT7	85.7	14.3	0.0
pBR322	0.4	92.6	7.0
pIO1	91.5	8.5	0.0
pBR327	0.0	97.8	2.2
pBR329	89.8	10.2	0.0

a. Calculated from electron microscopy photographs (pIO1) and by fluorescence densitometry (ColE1, pT7, pBR322, pIO1, pBR327, and pBR329).

Variance of the units of partition

The variance of the units of partition corresponding to plasmids pT7, pBR322, pIO1, pBR327, and pBR329 was calculated from the distribution of single-cell resistance to ampicillin. This distribution was determined in cells near to cell division (*Experimental procedures*). The percentages of cells resistant to different concentration ranges of ampicillin were calculated from the distribution of single-cell resistance to ampicillin represented in histograms. The different concentrations of ampicillin were expressed as plasmid copy-number equivalents (Table 1) and the plasmid copy number was then expressed as units of partition calculated from the values of plasmid multimerization (Table 2), assuming that the degree of plasmid multimerization is the same for all cells. The histograms representing the variance of units of partition for plasmids pT7, pBR322, pIO1, pBR327, and pBR329 are shown in Fig. 3. These histograms show the distribution of cells carrying from one to 15 units of partition. The variance of the units of partition was different for each of the plasmids tested. The bacterial population of cells carrying plasmids pT7, pBR322 or pBR327 showed a higher cell fraction with fewer than 10 units of partition than that of cells carrying pIO1 or pBR329 (Fig. 3).

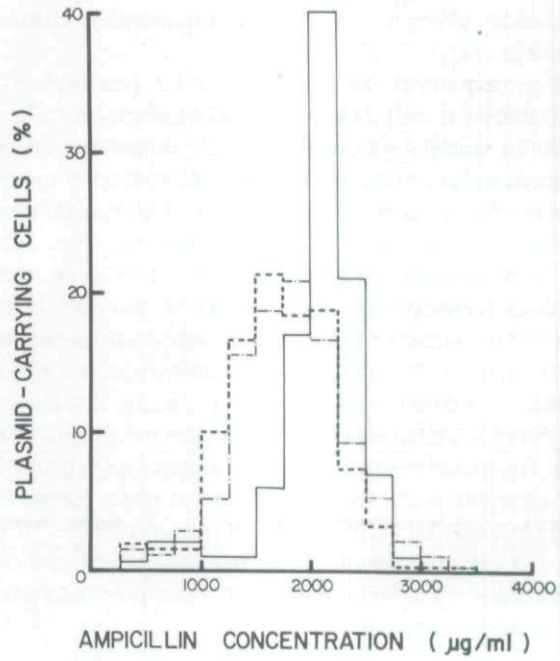

Fig. 2. Distribution of cells carrying plasmid pIO1 resistant to various concentrations of ampicillin. Cells pre-grown in Luria medium containing tetracycline (50 μg ml^{-1}) were grown in Luria broth as in the assay for plasmid stability (*Experimental procedures*). Appropriate dilutions were spread onto plates containing different concentrations of ampicillin after 0 (–), 53 (---), or 109 (--) generations of growth. Colonies were counted after incubation for 18–20 h at 37°C.

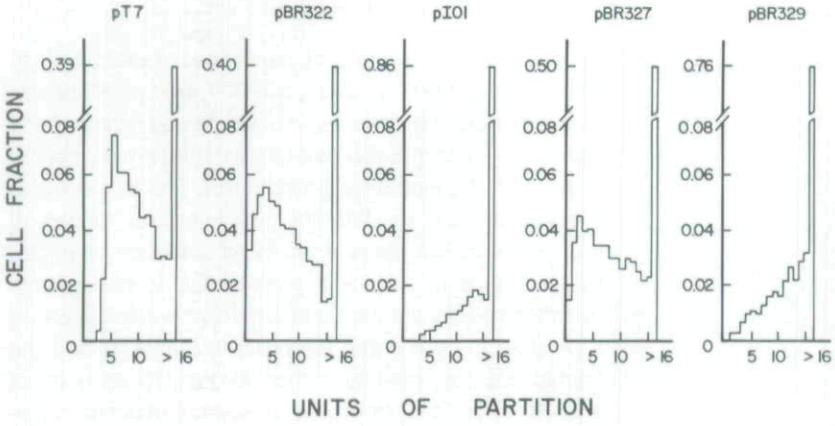

Fig. 3. Variance of units of partition. This variance was calculated from the distribution of single-cell resistance to ampicillin, expressed as plasmid copy-number equivalent, in cells near division and from the values of plasmid multimerization (Table 2). Strain C600 carries plasmid pT7, pBR322, pIO1, pBR327 or pBR329.

Experimental and predicted plasmid stability

Plasmid stability was determined in cell cultures started with cells grown on Luria plates or on Luria plates containing different concentrations of ampicillin or tetracycline. Similar slopes were found in plots of percentage of resistant cells versus time, regardless of the different lag periods required to detect plasmid-free cells in those cell cultures. As expected, the lag period was shorter for cell cultures started with cells from Luria plates, or from Luria plates containing a low antibiotic concentration, than for cell cultures started with cells from Luria plates containing a high antibiotic concentration (data not shown).

The kinetics of plasmid stability for plasmids pT7, pBR322, pIO1, pBR327, and pBR329 are shown in Fig. 4. Plasmid stability was experimentally determined in cell populations in which the variance of plasmid copy number had reached a condition of equilibrium (Fig. 2). Equation 5 (*Appendix*) was used to calculate plasmid stability using the experimentally determined values for variance of the units of partition (Fig. 3) and factor R, the ratio of the generation times of plasmid-free and plasmid-carrying cells (Table 3). Plasmid pT7 was stable under the experimental conditions used; however, using Equation 5 (*Appendix*), a loss rate was predicted for this plasmid (Fig. 4A). The experimental and the calculated loss rate values for plasmids pIO1 and pBR329 are in good agreement (Figs 4C and 4E); for plasmids pBR322 and pBR327 (Figs 4B and 4D), however, the experimentally determined loss rates were significantly lower than those predicted using Equation 5 (*Appendix*).

Discussion

Stability of high copy-number plasmids depends mainly on efficient mechanisms of copy number control and of recombination to maintain the plasmid molecules as monomers. For these plasmids, no active partition mechanism has been reported. In this work we tested the hypothesis that partition for high copy-number plasmids is a random event. Our mathematical model for random distribution of units of partition predicts that only subpopulations of cells carrying fewer than 12 units of partition generate a measurable number of plasmid-free cells after 100 generations of growth.

Five different plasmids were selected for this study: pT7 (an Ap^R Col^- derivative of ColE1); pBR322 *rop*$^+$ and its derivative pBR327 *rop*$^-$; pIO1 *rop*$^+$ (carrying the replication region of pBR322 but the resistance genes of pBR329) and its derivative pBR329 *rop*$^-$.

This study shows that the variance of units of partition of cell cultures started with cells pre-grown under high selective pressure and grown without selective pressure, remained constant after reaching an equilibrium (Fig. 2). The lag period needed to reach the equilibrium depends on the variance presented by the cells with which the culture is started. Plasmid-free cells were detected after the variance of units of partition reached this equilibrium.

The variance of units of partition for plasmid pT7 showed that a high fraction of cells (0.48) near division carry fewer than 12 units (Fig. 3). This variance is mainly determined by the presence of a relatively high percentage

Table 3. Ratio (*R*) of the generation times of strain C600 and C600 carrying different plasmids.

Plasmid	*R*[a]
–	1.000 ± 0.115
ColE1	0.967 ± 0.119
pT7	0.995 ± 0.091
pBR322	1.010 ± 0.193
pIO1	1.030 ± 0.102
pBR327	1.048 ± 0.192
pBR329	1.019 ± 0.113

a. Data obtained from an average of 10 independent determinations.

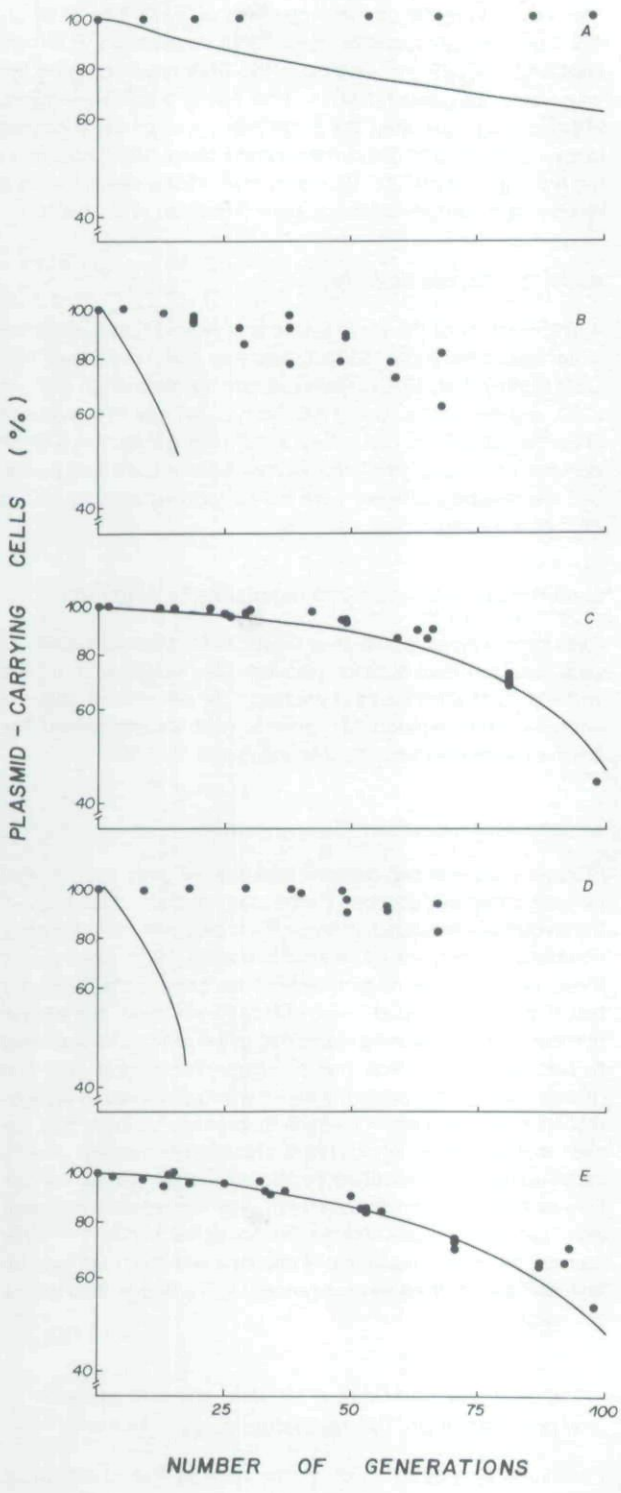

Fig. 4. Plasmid stability. Plasmid stability was experimentally determined (*Experimental procedures*) in cell cultures started with cells that had reached a condition of equilibrium (Fig. 2). Experimental values for plasmid stability are represented by solid circles (●). Plasmid stability calculated using Equation 5 (*Appendix*) and the experimentally determined values for variance of units of partition (Fig. 3) and for factor *R* (Table 3) is represented by a continuous line. Strain C600 carrying plasmid: A, pT7; B, pBR322; C, pIO1; D, pBR327; or E, pBR329.

of pT7 molecules as dimers (Table 2). Plasmid ColE1 exhibited a similar degree of dimerization (Table 2). These data were unexpected since plasmids ColE1 and pT7 carry the *cer* locus for site-specific recombination to ensure the monomerization of oligomeric molecules (Summers and Sherratt, 1984). In addition, pT7 carries the gene *tnpR* and the internal resolution site (*res*) corresponding to Tn*3* (Arthur and Sherratt, 1979) for the resolution of cointegrate molecules or cointegrate-like molecules (dimers). The relatively high percentage of ColE1 and pT7 dimers probably means that the host cell used (C600) is partially deficient in the recombination activities needed to recombine dimers or that the transcriptional patterns of these plasmids interfere with intraplasmidic recombination (Bujard *et al.*, 1985), despite the presence in ColE1 and pT7 of *cer* and of the resolution system of Tn*3* in pT7.

Plasmid pT7, which carries an inactivated gene for production of colicin, showed stable inheritance in a growing cell population; however, a random partition model predicts unstable behaviour for this plasmid (Fig. 4). This suggests that pT7 must have an active partition mechanism to ensure stability. This active mechanism could be the specific plasmid-nucleoid interaction described by Kline *et al.* (1976).

Plasmids pBR322 and pBR327 were present in cells mainly as dimers (Table 2) and, as expected (Lacatena *et al.*, 1984; Summers and Sherratt, 1985), the *rop*$^-$ plasmid exhibited a higher average copy number and slightly broader variance than those exhibited by the *rop*$^+$ plasmid (Table 1, Fig. 3). Plasmids pIO1 and pBR329, which were present in cells mainly as monomers (Table 2), displayed a lower copy number and a different pattern of variance relative to those presented by plasmids pBR322 and pBR327 (Table 1, Fig. 3). These differences could be partially explained by the insertion of the *cat* gene, in plasmids pIO1 and pBR329, which separates the anti-tet promoter from the *bla* gene and from the *ori* region (Covarrubias and Bolivar, 1982). It is possible that the number of transcripts containing the *bla* gene or transcripts reaching the *ori* region is lower in these plasmids than in pBR322 or pBR327. This could explain the lower plasmid copy number and lower ampicillin resistance per gene copy shown by plasmids pIO1 and pBR329 relative to those shown by pBR322 or pBR327 (Table 1). The insertion of the *cat* gene could also explain the monomerization of plasmids pIO1 and pBR329. It has been reported that the insertion of transcriptional elements outside the replication region can interfere with replication and recombination of ColE1-derived plasmids (Bujard *et al.*, 1985). Therefore, the monomerization of plasmids pIO1 and pBR329 could be explained by a modification of the transcriptional pattern resulting from the insertion of the *cat* gene in these plasmids. Plasmids pIO1 *rop*$^+$ and

pBR329 *rop*$^-$, besides being present in cells mainly as monomers (Table 2), showed a relative copy number and variance that do not correspond to that expected on the basis of their *rop* genotype. Plasmid pBR329 *rop*$^-$ presented a lower average copy number and a less broad variance than those presented by plasmid pIO1 *rop*$^+$ (Table 1, Fig. 3). We have no explanation for these minor differences in plasmid copy number and variance between pIO1 and pBR329.

Plasmid stability experimentally determined or calculated for the pBR322- and pBR327-derived plasmids (pIO1 and pBR329, respectively) which are present in cells mainly as monomers was similar (Fig. 4). On the other hand, the plasmid stability determined for plasmids pBR322 and pBR327 present in cells mainly as dimers, was different from that calculated for these plasmids, assuming a random distribution of units of partition (Fig. 4). These plasmids showed significantly greater stability than that predicted by a model of random partition. The stability of plasmids present mainly as dimers may be partially explained if dimers are present mainly in cells carrying a high copy number of plasmid molecules; monomers, meanwhile, are present mainly in cells carrying a low copy number of plasmids. A higher degree of plasmid dimerization in cells carring a high copy number of plasmids can be explained by a saturation-like process in these cells of the multimers' recombination systems. The histograms representing the variance of units of partition (Fig. 3) were obtained using the values of plasmid multimerization (Table 2) and assuming a uniform distribution of dimers in the bacterial population.

The data presented in this work suggest that plasmid pT7 has an active partition mechanism to ensure stability, and show that a random partition model, in which plasmid stability depends on the variance of the units of partition in cells near division, explains the behaviour of pBR322-like plasmids present in cells mainly as monomers. Stability of plasmids present mainly as dimers or with a complex pattern of multimerization probably also depends on the variance of units of partition. For these plasmids, a non-uniform distribution of dimers or of different multimers in the bacterial population must be considered.

Experimental procedures

Bacterial strain and plasmids

Escherichia coli K12 strain C600 *thr*$^-$, *leu*$^-$, *thi*$^-$, *lac*$^-$ was used in this work. The plasmids used were ColE1, pT7, pBR322, pBR327, pIO1, and pBR329. Plasmid pT7 represented a control stable plasmid. This plasmid is a derivative of ColE1::Tn*1* (pDS1109) (Dougan and Sherratt, 1977) that carries Tn*1* inserted in the *col* gene and a deletion of one of the inverted repeats of Tn*1* and of a portion of the *tnpA* gene (see below). Plasmids pIO1 and pBR329 are derived from the unstable plasmids pBR322 (Bolivar *et al.*, 1977) and pBR327 (Soberón *et al.*, 1980), respectively. Plasmids pIO1 and pBR329 are identical in the DNA region carrying the drug resistance genes *tet*, *bla*, and *cat*, but differ in the *rep* region. Plasmid pIO1 contained the same *rep* region as pBR322 (see below), and pBR329 (Covarrubias and Bolivar, 1982) contained the *rep* region of pBR327 (Soberón *et al.*, 1980), which carries a deletion that comprises the *rop* gene (Lacatena *et al.*, 1984).

Assay of plasmid stability

A single colony of the strain tested was picked from a selective plate, inoculated into a 125 ml Erlenmeyer flask containing 10 ml Luria broth (Miller, 1972), incubated with agitation (210 r.p.m.) at 37°C, and the culture diluted into fresh Luria broth at intervals to keep the cell density below 2–3 $\times$ 10^8 cells per ml. From these cultures, samples were diluted and spread onto Luria agar plates, and the resulting colonies were tested for resistance to 200 μg ml^{-1} of ampicillin.

Determination of single-cell resistance to ampicillin

Cells from cultures grown as in the assay for plasmid stability or cells collected from sucrose gradients (see below) were diluted and spread on Luria plates containing different concentrations of ampicillin and prepared 12 h prior to each determination. The colonies were counted after 24 h incubation at 37°C.

Determination of plasmid copy number

Plasmid copy-number determinations were performed in cell cultures grown as described for the assay of plasmid stability. As the level of cell resistance to ampicillin is proportional to the copy number of the *bla* gene (Uhlin and Nordström, 1977), an aliquot of these cultures was diluted, spread on Luria plates and the resulting colonies tested for resistance to ampicillin. The relative number of cells containing plasmids (Ap^R) in the culture was used to calculate the average plasmid copy number per cell. The relative copy-number determinations were performed using fluorescence densitometry of ethidium bromide-stained gels, as described by Projan *et al.* (1983), and by dye-buoyant density centrifugation, as described by Womble *et al.* (1977). For the fluorescence densitometric method, clear lysates were prepared from cell suspensions containing an equal number of cells carrying the control plasmid pT7 and cells with the plasmid to be tested. Plasmid DNAs were linearized by *Eco*RI digestion before gel electrophoresis.

Isolation of plasmid DNA, restriction enzyme analysis, and purification of DNA restriction fragments

Plasmid DNA was isolated by the alkaline lysis method, as described by Maniatis *et al.* (1982). Purified plasmid DNA was digested with restriction enzymes according to the reaction conditions specified by the supplier, and the fragments were separated by electrophoresis in 0.8% agarose slab gels or in 7.5% polyacrylamide gels in 50 mM Tris/2 mM EDTA/20 mM sodium acetate/1.8 mM NaCl (pH 8.0). DNA fragments were purified from the gels by electroelution or by gel fusion when low melting-point agarose was used (Maniatis *et al.*, 1982).

Construction of plasmids pT7 and pIO1

Plasmid ColE1::Tn*1* (Dougan and Sherratt, 1977) was partially digested with *Pst*I and ligated with T4 DNA ligase (Maniatis *et al.*, 1982). The ligation mixture was used to transform strain C600 and ampicillin resistance transformants were selected. These transformants were screened for plasmids which presented a deletion of the *Pst*I fragment D, which contains one of the inverted repeats of Tn*1* and a portion of the *tnpR*; thus the resulting deleted Tn*1* cannot transpose. A plasmid presenting that deletion was named pT7. To construct plasmid pIO1, plasmid pBR322 (Bolivar *et al.*, 1977) was digested with *Pst*I and *Bam*HI and the largest fragment containing the origin of replication and the 3′ end of genes *bla* and *tet* was purified; plasmid pBR329 (Covarrubias and Bolivar, 1982) was digested with *Pst*I, *Bam*HI, and *Ava*I and the largest of the fragments containing the chloramphenicol resistance gene and the 5′ end of the genes *bla* and *tet* was purified. These fragments were ligated with T4 DNA ligase, and the ligation mixture was used to transform strain C600. Transformants resistant to chloramphenicol, ampicillin and tetracycline were selected. A plasmid carrying the selected markers and showing the expected restriction pattern was named pIO1.

Separation of cell subpopulations in sucrose gradients

Bacterial cultures were grown to exponential phase as in the assay for plasmid stability. Aliquots (7.5 ml) of these cultures were washed with BSG (0.85% NaCl; 0.03% KH_2PO_4; 0.06% Na_2HPO_4; 0.001% gelatin; Roozen *et al.*, 1971), resuspended in 1 ml of BSG and centrifuged at 4000 r.p.m. in a Sorvall HB-4 rotor for 7 min at 4°C in a 28 ml sucrose gradient prepared by freezing and slowly thawing a 30% sucrose solution containing 100 mM NaCl, 5 mM EDTA, and 10 mM Tris (pH 7.5). The lower third of the broad cell band obtained was collected, washed in BSG, and re-run in another sucrose gradient as before. Cells from the lower half of the cell band were collected. These cells represented mainly large cells close to division. In the experimental conditions used, cell aggregates formed a pellet. Separation of cells was performed under conditions of sterility.

Determination of plasmid multimerization

The degree of plasmid multimerization was determined by two different methods in cell cultures grown in the conditions described for plasmid stability. (i) Plasmid DNA molecules isolated by the alkaline lysis method were visualized in agarose slab gels (Maniatis *et al.*, 1982). The different molecular forms were identified using as control plasmid DNA molecules linearized or relaxed by *Eco*RI or by DNAse I digestions, respectively. Fluorescence densitometry was used to calculate the percentage of ColE1, pT7, pBR322, pIO1, pBR327, and pBR329 monomer and oligomer molecules (Projan *et al.*, 1983). (ii) Purified plasmid pIO1 DNA molecules were mounted by the cytochrome *c* spreading method as described by Davis *et al.* (1971) and visualized by electron microscopy. A total of 508 molecules were visualized to determine the degree of plasmid multimerization.

Appendix

Plasmid stability

The mathematical analysis of plasmid stability assumes that the partition of the plasmids between daughter cells is at random.

For a cell containing u plasmid units of partition, the probability (P) of generating a plasmid-free cell in one cell division is given by the following equation:

$$P = 2\frac{1}{2^u} \tag{1}$$

and the fraction of plasmid-free cells (T) in a bacterial population is:

$$T = \frac{P}{2} = \frac{1}{2^u} \tag{2}$$

Since a bacterial population consists of different subpopulations of cells containing different numbers of units of partition and, therefore, different probabilities of generating plasmid-free cells, the total fraction (F) of plasmid-free cells in a bacterial population at equilibrium is:

$$F = \sum_{i=1}^{m} T_i S_i \tag{3}$$

where, m is the number of subpopulations in the bacterial population, T_i is the fraction of plasmid-free cells in the subpopulation i and S_i is the fraction of subpopulation i in the bacterial population.

Equation 3 defines the total fraction of plasmid-free cells generated in one generation of growth; therefore, to calculate the kinetics of plasmid stability, the fraction of plasmid-free cells generated in the previous generation has to be considered. The fraction of plasmid-free cells in generation n is defined by F_n:

$$F_n = (1-F_{n-1})F + F_{n-1} \tag{4}$$

where F_n is the fraction of plasmid-free cells generated by cells carrying the plasmid in the generation n and F_{n-1} represents the fraction in the previous generation and F is the constant corresponding to equation (3).

The slope in plots of percentage of resistant cells versus time is modified if the generation time of cells containing plasmids and of plasmid-free cells is different. Therefore, the ratio (R) between the generation times of plasmid-free cells and of plasmid-carrying cells is considered in the following equation:

$$\Phi_n = [1-(F_{n-1})R]F + F_{n-1}R \tag{5}$$

where Φ_n is the corrected total fraction of plasmid-free cells in a bacterial population after n generations of growth.

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