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Effects of Genes Exerting Growth Inhibition and Plasmid Stability on Plasmid Maintenance

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Plasmid stabilization mediated by the *parA*$^+$ and *parB*$^+$ genes of the R1 plasmid and the *ccd*$^+$ and *sop*$^+$ genes of the F plasmid was tested on a mini-R1 plasmid and a pBR322 plasmid derivative. The mini-R1 plasmid is thought to be unstably inherited owing to a low copy number and to random segregation of the plasmid at cell division, whereas cells harboring the pBR322 derivative used in this work are lost through competition with plasmid-free cells, mainly as a result of the shorter generation time of cells without plasmids. The pBR322 derivative carries a fusion between part of the *atp* operon of *Escherichia coli* and the bacteriophage lambda p_R promoter, and the *c*I857 repressor gene. The insertion of *sop*$^+$ from the F plasmid or *parB*$^+$ from the R1 plasmid reduced the loss frequency by a factor of 10^3 for the pBR322 derivative and by at least a factor of 10^2 for the mini-R1 plasmid. Insertion of *parA*$^+$ from the R1 plasmid decreased the loss frequency of the pBR322 derivative by a factor of 10 and that of the mini-R1 plasmid by a factor of 50. When *ccd*$^+$ from the F plasmid was inserted, the loss frequency of the pBR322 derivative was decreased by a factor of 10, but it had only a marginal effect on the stability of the mini-R1 plasmid. In no case was any significant structural instability of the plasmids observed.

The decreasing frequency of plasmid-harboring cells in a culture growing in the absence of selective pressure for the maintenance of the plasmid is caused mainly by segregational loss at cell division. The maintenance of the plasmid may be further impeded by a difference in the growth rates of cells with and without plasmids. To reduce the inhibition of growth, which is often caused by strong gene expression from in vitro-constructed recombinant DNA molecules, two approaches have been used. One is to use inducible promoters in front of the inserted gene, and the other is to use conditional runaway replication vectors (9). In both cases it is important to reduce the frequency of plasmid-free segregants in the culture, since these may outgrow the plasmid-harboring cells within a short time after induction. The use of an expression vector with a constitutive promoter further emphasizes the importance of the segregational stability of the vector, since the growth advantage of the plasmid-free segregants prevails through the entire cultivation period.

We have attempted to improve the stability of a low-copy-number plasmid which is unstable primarily owing to random loss at cell division and of a high-copy-number plasmid whose instability is strongly enhanced by a reduced growth rate of plasmid-harboring cells compared with that of plasmid-free segregants. Stabilization was attempted through insertion of *sop*$^+$ (14) and *ccd*$^+$ (13) from the F plasmid and *parA*$^+$ (4) and *parB*$^+$ (5) from the R1 plasmid. *sop*$^+$ and *parB*$^+$ were both found to be particularly efficient in stabilizing the two types of plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strain MC1000 (2) was used throughout. The plasmids and their relevant genotypes are listed in Table 1. The vector plasmids are also shown in Fig. 1.

Media and growth conditions. LB medium (1) was used for all genetic experiments. For the stability experiments cells were grown for at least 10 generations in AB medium (3) supplemented with thiamine (1 mg/liter), glucose (2 g/liter), leucine (25 mg/liter), and antibiotics (50 mg/liter), harvested by centrifugation to remove the antibiotics, and suspended in the appropriate growth medium without antibiotics. The cultures were kept in a steady state of growth by being continuously diluted in fresh medium, with the optical density at 450 nm never allowed to exceed 0.2. Each dilution was made to contain an appropriate number of cells (more than 10,000), both with and without plasmid. At appropriate time points the cultures were assayed by replica plating for the frequency of plasmid-harboring cells.

Measurements of growth. Growth was measured by monitoring the optical density at 450 nm. Generation times were determined graphically, and differences in generation times down to 1% were detectable. The generation time of cells harboring plasmids was used to calculate the number of generations used in the experimental plots.

Genetic techniques. Standard genetic experiments were performed by the methods of Maniatis et al. (10).

Plasmid stability tests. Plasmid pBOE106 is a derivative of pBR322, which carries the gene encoding kanamycin resistance from the transposon Tn*5* (8), the gene for the temperature-sensitive *c*I857 repressor, and the p_R promoter from bacteriophage lambda. The p_R promoter is fused to part of the *atp* operon from *E. coli* encoding the a and c subunits (the *Hin*dIII-*Nar*I fragment [12]). Hence, expression of the a and c subunits is under temperature regulation. At high temperature (40°C), overexpression of the a subunit leads to decreased protein synthesis as a result of a collapse of the membrane potential (17, 18), and at this temperature cells harboring the plasmid die within 2 h.

The construction of this plasmid served two purposes. First it enabled us to detect very low levels of plasmid-free cells. This was obtained by determining the viable counts at 40°C, when only cells without plasmid form colonies, and a concomitant determination of viable counts at 30°C, when all cells form colonies. When plating cells at 40°C from a culture of MC1000 harboring pBOE106, we found that none of the

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TABLE 1. Plasmids and their relevant genotypes

Plasmid	Replicon	Size (kb)	Antibiotic resistance	Stability locus	Reference
pBOE106	pBR322	9	Ap^r Km^r	None	This work
pBOE113	pBR322	11	Ap^r Km^r	*parA*	This work
pBOE115	pBR322	11	Ap^r Km^r	*parB*	This work
pBOE122	pBR322	11	Ap^r Km^r	*ccd*	This work
pBOE124	pBR322	11	Ap^r Km^r	*par*	This work
pBOE135	pBR322	12	Ap^r Km^r	*sop*	This work
pOU82	mini-R1	12	Ap^r	None	9
pKG46	mini-R1	14	Ap^r	*parA*	4
pOU95[a]	mini-R1	13	Ap^r	*parB*	5
pBOE147	mini-R1	16	Ap^r Km^r	*ccd*	This work
pBOE148	mini-R1	18	Ap^r Km^r	*sop*	This work

[a] Plasmid pOU95 is identical to pPR95 (5).

surviving clones were resistant to ampicillin or kanamycin. This confirmed that the temperature-resistant cells had arisen from segregational loss of the plasmid. This property of plasmid pBOE106 made it an attractive test plasmid for assessing the effect of stabilization functions. Second, the plasmid-mediated growth inhibition could be modulated by cultivating the cells at different intermediate temperatures.

An alternative test plasmid for low-level instability is pOU82, which is a vector derived from the low-copy-number plasmid R1. Insertion of the p_R promoter and the *c*I857 allele of the λ repressor gene upstream of the replication genes caused the replication rate to become temperature dependent in such a way that at 30°C the plasmid copy number is 1 to 2 per chromosome, whereas at 42°C (induction of p_R transcription) the copy number increases to such high values that the cells eventually die (runaway replication [9]).

Under conditions when the fraction of plasmid-carrying cells was in the range 0.01 to 0.99, the actual ratio of plasmid-harboring cells was determined by replica plating single-cell colonies onto nonselective and selective media at 30°C.

Construction of pBOE106 derivatives. Plasmid pBOE113 was made by inserting the *parA*$^+$-carrying *Eco*RI fragment of pKG46 in the *Eco*RI site of pBOE106. pBOE115 was made by a series of steps, resulting in the insertion of the *parB*$^+$-carrying *Eco*RI fragment of pOU95 between the *Sal*I sites. Plasmid pBOE122 carries the *Bam*HI-*Xho*I (*ccd*$^+$) fragment from the F plasmid inserted between the *Bam*HI and *Sal*I sites, and pBOE124 carries the *Eco*RI-*Ava*I fragment of pPM31 (the *par*$^+$ fragment of pSC101 [11]) inserted between the *Sal*I sites. Plasmid pBOE135 was constructed in two steps. First the *Xho*I-*Sal*I fragment of the F plasmid containing the *sop*$^+$ genes and the replication functions was inserted between the *Sal*I sites of pBOE106. The replication functions of the F plasmid were subsequently removed by an *Eco*RV-*Sma*I deletion in the F DNA to yield pBOE135.

Construction of pOU82 derivatives. Plasmids pKG46 (4) and pOU95 (5) carry the *parA*$^+$ and *parB*$^+$ fragments of R1, respectively, inserted in the *Eco*RI site of pOU82. Plasmid pBOE147 carries the *Bam*HI-*Xho*I fragment containing the *ccd*$^+$ function of the F factor inserted between the *Bam*HI and *Eco*RI sites of pOU82, together with the gene from Tn*5* encoding kanamycin resistance (8). Plasmid pBOE148 carries the *Eco*RV-*Sal*I fragment containing the *sop*$^+$ genes of the F plasmid, together with the gene encoding kanamycin resistance inserted between the *Bam*HI and *Eco*RI sites of pOU82. The *Eco*RI site was filled out with Klenow polymerase.

RESULTS

Maintenance of pBOE106 at different levels of growth inhibition. In the experiment described below we attempted to demonstrate that plasmid pBOE106 (described in detail in Materials and Methods) is lost with variable frequency, dependent on the rate of transcription into the *atpB* gene, and that this difference in loss rate is caused mainly by differential levels of growth inhibition of plasmid-carrying cells. At intermediate temperatures (33 to 37°C), expression of the a subunit is not sufficient to kill the host cells but results in severe growth inhibition, and even at low temperature (30°C), the plasmid causes approximately 8% inhibition of growth of the host cells (estimated from Fig. 2).

The results of growth experiments with MC1000 harboring pBOE106 cultivated at different temperatures are shown in Fig. 2. The same preculture, grown at 30°C in the presence of 50 mg of kanamycin per liter, was used for inoculation to ensure the same ratio of plasmid-free cells in all subcultures at time zero. It is evident that the apparent rate of plasmid loss is higher at the high temperatures, at which growth inhibition due to overexpression of the a subunit is more severe.

The maintenance of a plasmid can be simulated on a computer by recursive use of the following formulas, where each recursion corresponds to one generation time of the plasmid-harboring cells: $N = N \exp[(\ln 2)T_p/T] + 2fN_p$, and $N_p = 2N_p(1 - f)$, where N is the number of cells without a plasmid, N_p is the number of cells with a plasmid, T is the

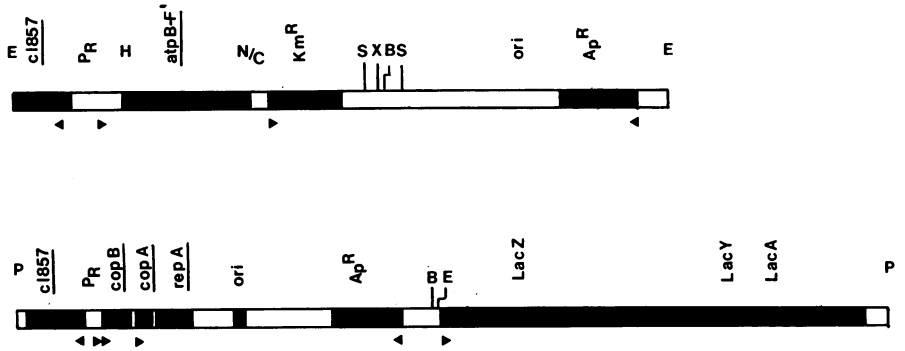

FIG. 1. Plasmid pBOE106 (top) and the mini-R1 plasmid pOU82 (bottom). The relevant endonuclease sites are indicated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nar*I; S, *Sal*I; X, *Xho*I. The arrowheads indicate location and direction of transcription from known promoters.

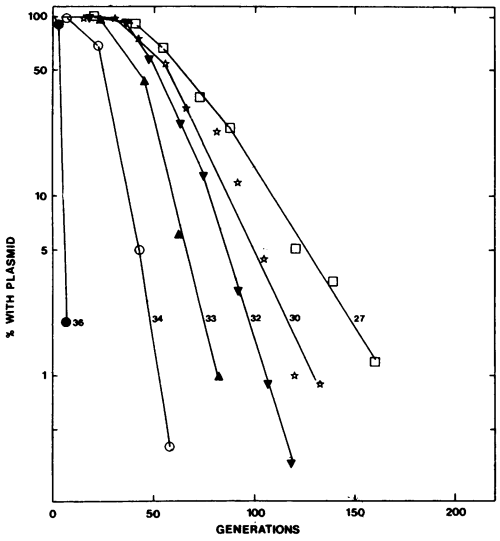

FIG. 2. Maintenance of pBOE106 in MC1000 grown in glucose minimal medium at the following temperatures: 27°C (□), 30°C (☆), 32°C (▼), 33°C (▲), 34°C (○), and 36°C (●).

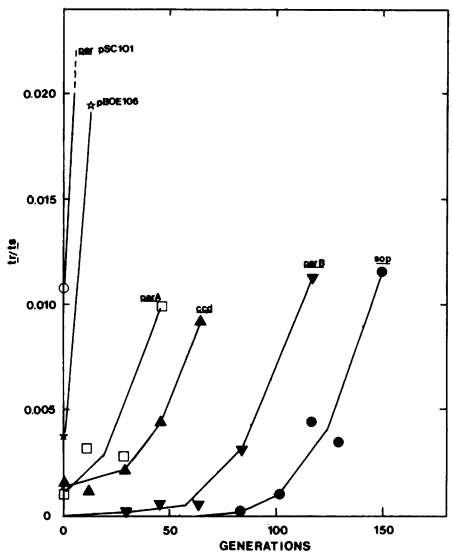

FIG. 4. Ratio of temperature-resistant and temperature-sensitive cells in cultures of MC1000 harboring pBOE106 (☆), pBOE113 (□), pBOE115 (▼), pBOE122 (▲), pBOE124 (○), or pBOE135 (●) grown in glucose minimal medium at 30°C.

generation time of cells without a plasmid, T_p is the generation time of cells with a plasmid, and f is the loss frequency per cell per generation of the plasmid. The outcome of such simulations where the loss frequency f arbitrarily was set to 0.001 and where different ratios of generation times with and without plasmid were tested is shown in Fig. 3. The pronounced similarity between the maintenance of pBOE106 at different temperatures and the computer simulation suggests that the major reason for the different kinetics of loss of pBOE106 at the different temperatures is the temperature-dependent expression of the a subunit of the *atp* operon, which affects the cellular growth rate.

We realize that the above conclusion is based on the assumptions that (i) N_p/N is initially the same in all subcultures, and (ii) f does not vary significantly among the different subcultures. However, we think that these assumptions are justified since (i) the subcultures were simply aliquots of a preculture grown with kanamycin at 30°C, and (ii) there is no reason to assume that the plasmid copy numbers should be changed by very small increases in the transcription rate from p_R (from 30 to 36°C the increase in the transcription rate is less than a factor of 3 [unpublished data and reference 9]). Moreover, the p_R promoter is well separated from the replication region of pBR322 (Fig. 1). Therefore, if copy numbers are essentially the same in all subcultures, the loss frequency f should also be constant.

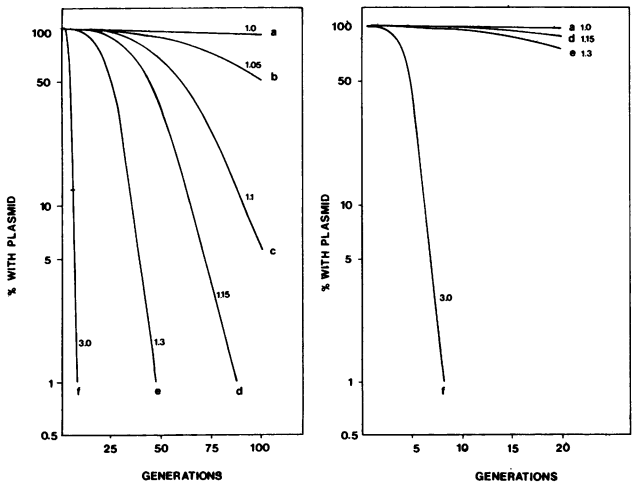

FIG. 3. Percentage of cells harboring plasmid versus generations from a computer simulation. The loss frequency is set to 0.001, and the following ratios between generation times of cells with and without plasmid (T_p/T) were used: 1 (a), 1.05 (b), 1.1 (c), 1.15 (d), 1.3 (e), and 3 (f).

Stabilization of plasmids with stability functions from different low-copy-number plasmids. Stabilization of pBOE106 was attempted by the insertion of either *ccd*$^+$ or *sop*$^+$ from the F plasmid, *parA*$^+$ or *parB*$^+$ from the R1 plasmid, or *par*$^+$ from pSC101. Figure 4 shows the ratio of temperature-resistant (plasmid-free) cells to the number of cells in cultures harboring pBOE106 carrying each of the different stabilization regions when grown at 30°C. Assuming that the temperature-resistant cells are cells without plasmids and that the plating efficiency is unaffected by the temperature, maximal estimates of the loss frequencies can be read as the

TABLE 2. Maximal estimates of loss frequencies of the stabilized plasmids

Plasmid	Replicon	Stability locus	Loss frequency
pBOE106	pBR322	None	5×10^{-4}
pBOE133	pBR322	*parA*	5×10^{-5}
pBOE115	pBR322	*parB*	5×10^{-7}
pBOE122	pBR322	*ccd*	5×10^{-5}
pBOE124	pBR322	*par*	$>5 \times 10^{-4}$
pBOE135	pBR322	*sop*	$<5 \times 10^{-7}$
pOU82	mini-R1	None	5×10^{-2}
pKG46	mini-R1	*parA*	1×10^{-3}
pOU95	mini-R1	*parB*	$<1 \times 10^{-4}$
pBOE147	mini-R1	*ccd*	1×10^{-2}
pBOE148	mini-R1	*sop*	1×10^{-4}

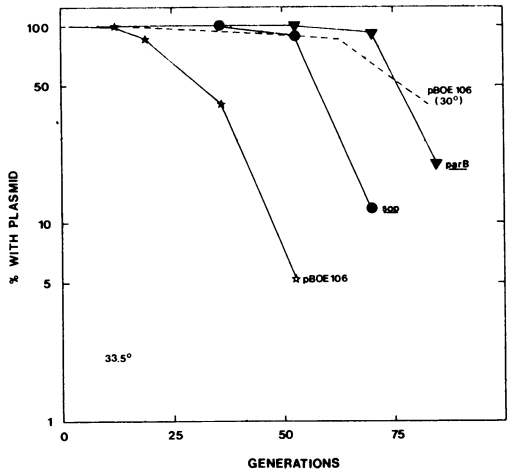

FIG. 5. The percentage of cells with plasmid in cultures of MC1000 harboring pBOE106 (☆), pBOE115 (▼), or pBOE135 (●) grown in glucose minimal medium at 33.5°C. – – – –, Maintenance of MC1000 harboring pBOE106 grown in the same medium at 30°C.

initial slopes in the curves of Fig. 4 (values presented in Table 2). All cultures were kept growing exponentially until plasmid-free segregants were detected by replica plating. The plasmids stabilized with the *parB*$^+$ locus or the *sop*$^+$ genes were very stably maintained, the *parA*$^+$ locus and the *ccd*$^+$ genes significantly improved the maintenance of the plasmid, and the *par*$^+$ locus of pSC101 was without effect.

A stability test experiment with MC1000 harboring pBOE106, pBOE115 (*parB*$^+$), or pBOE135 (*sop*$^+$) was performed at 33.5°C, at which the plasmids mediate 20 to 25% inhibition of growth. The results are shown in Fig. 5. This experiment demonstrated that the fidelity of plasmid maintenance can be increased by use of stability functions from low-copy-number plasmids even when the plasmid mediates a relatively high level of growth inhibition. The *parB*$^+$ locus was more effective under these conditions than the *sop*$^+$ genes, in contrast to the experiment performed at 30°C (approximately 8% inhibition of growth). There were no detectable differences in the growth rates of the cells harboring the different plasmids when grown in medium selective for the plasmids (data not shown); therefore the difference in the kinetics of loss of the plasmids is probably due only to differences in the loss frequencies of the plasmids.

The low-copy-number plasmid pOU82, a mini-R1 derivative, is mainly unstably inherited owing to segregational loss of the plasmid at cell division; i.e., a plasmid-encoded inhibition of growth was never detectable (data not shown). The effect of the stabilization functions on the maintenance of pOU82 is shown in Fig. 6 and Table 2. As was the case with pBOE106, insertion of the *parB*$^+$ locus or the *sop*$^+$ genes in the mini-R1 plasmid resulted in a high fidelity of plasmid maintenance. The maintenance of the mini-R1 plasmid carrying the *parA*$^+$ locus was greatly improved, while the *ccd*$^+$ locus was without significant effect. The *par*$^+$ locus of pSC101 was not tested on the mini-R1 plasmid.

Each stability experiment was terminated by analysis of several plasmid-containing clones for structural changes in the plasmid and for the presence of cointegrates (data not shown). All the plasmids used in this work were found to be structurally stable, and fewer than 1% of the pBR322-derived plasmids were dimerized. Dimers and multimers were never detected for the low-copy-number plasmids.

DISCUSSION

Little is known at present about the molecular mechanisms involved in plasmid stabilization. However, this work demonstrates the usefulness of naturally occurring stabilization functions in the stabilization of unstable plasmids in *E. coli*.

An important word of caution should be added. Although we have not been able to detect any significant copy number effects on our test plasmids after insertion of the different stabilizing DNA fragments (data not shown), it cannot be excluded that the stabilizing activities exerted by the different inserted regions are influenced somewhat by flanking sequences in the plasmids. Such effects may make accurate stability factors conditional, but we strongly believe that the similarity we observed in the data presented for two very different plasmid vectors makes it reasonable to argue that some natural stabilization functions are more efficient than others.

Neglecting the rare occurrence of cells which at the point of division harbor the plasmid in only one copy, segregational instability of plasmids can be overcome by an efficient partitioning function. The *sop*$^+$ genes of the F plasmid have been proposed to encode a partitioning function analogous to the centromers of eucaryotic chromosomes (14). The very efficient stabilization of the pBR322 derivative and the mini-R1 derivative shown here to be mediated by the *sop*$^+$ gene and the fact that *sop*$^+$ can stabilize minichromosomes (14) and pSC101 derivatives (unpublished results) support this idea. The proposed mode of action of the *ccd*$^+$ locus of the F plasmid is that of inhibiting host cell division when the copy number of the plasmid is too low for a partitioning function to be active, i.e., when the copy number of the plasmid is 1 per cell at the time of division (13). It therefore was surprising that the *ccd*$^+$ locus was more efficient in stabilization of the high-copy-number plasmid (the pBR322 derivative) than of the low-copy-number plasmid (the mini-R1 derivative). However, new results about the *ccd*$^+$ locus indicate that *ccd*$^+$ acts by killing the plasmid-free segregants (7).

Recently a model for the mechanisms of the *parB*$^+$ locus of the R1 plasmid was proposed (5). For this model it is argued that one of the *parB*$^+$ gene products is able to kill plasmid-free segregants with a very high efficiency. In accordance with this model, *parB*$^+$ is able to stabilize any replicon in *E. coli* ranging from pBR322 to minichromosomes. If *parB*$^+$ from the R1 plasmid and *ccd*$^+$ from the F plasmid exhibit the same type of activity, it remains to be

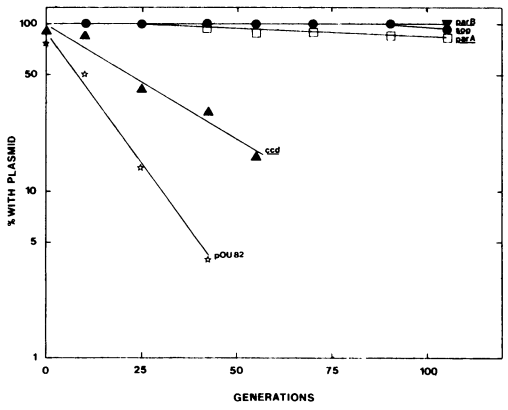

FIG. 6. Percentage of cells with plasmid in cultures of MC1000 harboring pOU82 (☆), pKG46 (□), pOU95 (▼), pBOE147 (▲), or pBOE148 (●) grown in glucose minimal medium at 30°C.

explained why *parB*$^+$ is very efficient in stabilization of virtually all types of plasmids, whereas *ccd*$^+$ is much less efficient in stabilizing both pBR322 derivatives and mini-R1 derivatives.

The *parA*$^+$ gene from the R1 plasmid has been proposed to be a true partitioning function (4). If so, the results presented here indicate that it is not as efficient, even on R1-derived plasmids, as the *sop*$^+$ genes from the F plasmid. It works poorly on pBR322 derivatives and has little, if any, effect on p15A replicons (4).

The *par*$^+$ locus from pSC101 did not show any stabilizing effect on plasmid pBOE106. It has previously been reported that the *par*$^+$ locus from plasmid pSC101 could to some extent improve the maintenance of a pBR322 derivative (15), and it has been shown to work efficiently on p15 A replicons (11), but it is without effect on the maintenance of minichromosomes (6). The explanation of these conflicting results may have to await an understanding of the molecular mechanism behind the *par*$^+$ stabilization.

The temperature-dependent loss rate of pBOE106 (and the computer simulation) demonstrates the importance of the plasmid-mediated growth inhibition on the maintenance of otherwise stable high-copy-number plasmids. We believe that the poor maintenance of pBOE106 in MC1000 is mainly, if not solely, a consequence of the level of expression of the a subunit of the *atp* operon. Accordingly, we found that pBR322 was stably maintained for more than 100 generations under similar growth conditions. By analogy, most maintenance problems of pBR322 derivatives carrying foreign genes are probably caused by growth inhibition exerted by the desired gene product.

The results presented in this paper also indicate a general method for increasing the stability of plasmids in *E. coli*, use of the naturally occurring plasmid stabilization genes. The results obtained with the *sop*$^+$ locus from the F plasmid and the *parB*$^+$ locus from the R1 plasmid indicate that these functions are particularly effective in stabilization of vectors used for production purposes. However, growth conditions and strain backgrounds used in industrial fermentations may influence the efficiency of the stabilization functions.

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