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Mini-P1 Plasmid Partitioning: Excess ParB Protein Destabilizes Plasmids Containing the Centromere *parS*

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The partition system of the unit-copy plasmid P1 consists of two proteins, the *parA* and *parB* gene products, and a *cis*-acting site, *parS*. Production of high levels of the P1 ParB protein, from an external promoter on a high-copy-number vector, inhibits the propagation of λ–mini-P1 prophages and destabilizes other P1-derived plasmids. The interference by ParB protein depends on the *parS* site, or centromere, of the P1 partition region; plasmids lacking *parS* are unaffected. The defect is more severe than the defect due to mutations that simply eliminate *par* function. In the presence of excess ParB protein, plasmids carrying *parS* are more unstable than would be predicted from a random distribution at cell division. The destabilization is a segregation defect, as the copy number of *parS*-bearing plasmids is not decreased under these conditions. Thus, it appears that ParB protein binds to *parS*; if too much protein is present, it sequesters such plasmids so they cannot be properly, or even randomly, partitioned. This suggests that under normal conditions, ParB protein recognizes and binds to *parS* and may be the protein responsible for pairing plasmids during the process of partitioning at cell division.

To assure their stability, unit-copy-number plasmids, such as the prophage of bacteriophage P1, must actively partition newly replicated copies to daughter cells at cell division. High-copy-number plasmids, such as pBR322 (about 20 copies per host chromosome), usually partition by random segregation; statistically, the chance of a newborn cell not receiving any plasmid is extremely low (21), and these plasmids are stable under normal conditions. However, in the absence of an active partition system, random distribution of low-copy-number plasmids results in their rapid loss from a growing population of cells (3, 5, 21), as long as the growth of plasmidless segregants is not inhibited. The P1 genome contains a genetically and physically defined partition region, *par*, separate from the region required for DNA replication and its control (3). P1 *par* can be used to stabilize other replicons, such as certain unstable F derivatives (2) or pBR322 derivatives whose copy number is reduced in *polA* hosts (1). The region contains two *trans*-acting genes, *parA* and *parB*, and a *cis*-acting site, *parS*. It has been proposed that *parS* is a procaryotic analog of a centromere, that one or both of the P1 Par proteins recognize and bind to this site, and that this complex attaches to the cellular partition apparatus during cell division (3).

The P1 prophage, a 90-kilobase plasmid, contains many genes that are expressed in the lysogen but are not required for replication or partition (26). To avoid this complexity, P1 derivatives were constructed containing essentially only the replication and *par* regions in an integration-deficient lambda vector. Such λ–mini-P1 chimeras, for example, λ–P1:5R (25), grow lysogenically as P1 plasmids, with a copy number similar to that of intact P1 (one to two per host chromosome), and are thus dependent on *par* for stability (5, 25). The partition system is very efficient; in a *recA* host (in which plasmid dimerization is avoided), λ–mini-P1 is lost at a frequency of 0.01% per generation (7).

Mutations in, or deletions of, *par* result in very unstable plasmids, although such plasmids can be maintained in the presence of selection (3, 5). In addition, par^+ plasmids can be destabilized by plasmids carrying *parS*, and by high-copy-number plasmids producing ParA protein (1). The latter result indicates that excess partition protein can also inhibit proper partitioning. This study shows that the remaining partition component, ParB protein, also blocks normal partitioning when it is in excess; however, the destabilization is much greater than that obtained by either complete inactivation of the partition system (by mutation) or interference with its function (by *parS*-mediated destabilization).

MATERIALS AND METHODS

Strains. *Escherichia coli* DH5 [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1*) was used for all plasmid experiments, and *E. coli* YMC (*supF58*) (11) was used for λ propagation and the genetic crosses described below.

Media and antibiotics. All experiments were performed in LB medium (Quality Biological Ltd.) and on LB plates (GIBCO Diagnostics). The antibiotics and concentrations were: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin, 25 μg/ml.

Plasmids and plasmid constructions. The P1 partition region (*par*) and plasmids used in this study are diagrammed in Fig. 1. The *par* plasmids pALA270 and pALA207 (1) were used as the sources of P1 DNA for the subcloning described here. Restriction enzymes and enzymes used for cloning were purchased from Bethesda Research Laboratories, Inc., Boehringer Mannheim Biochemicals, New England BioLabs, Inc., and Pharmacia and used according to the supplied directions of Maniatis et al. (18). Eight-base-pair (bp) phosphorylated *SalI* and *XhoI* linker molecules (New England BioLabs) were attached to DNA ends according to Maniatis et al. (18), and 10-bp nonphosphorylated *BamHI* linkers (Boehringer Mannheim) were attached as described below.

The *trp* promoter vector, pRPG48, contains the *trp* operator and promoter (*trp-PO*) from *Serratia marcescens* on a 94-bp fragment inserted between the *EcoRI* and *BamHI* sites of pBR327 (9); the direction of transcription is from *EcoRI* to *BamHI*. The P1 *parB* gene from *BglII* to *DraI* (pBEF104) or

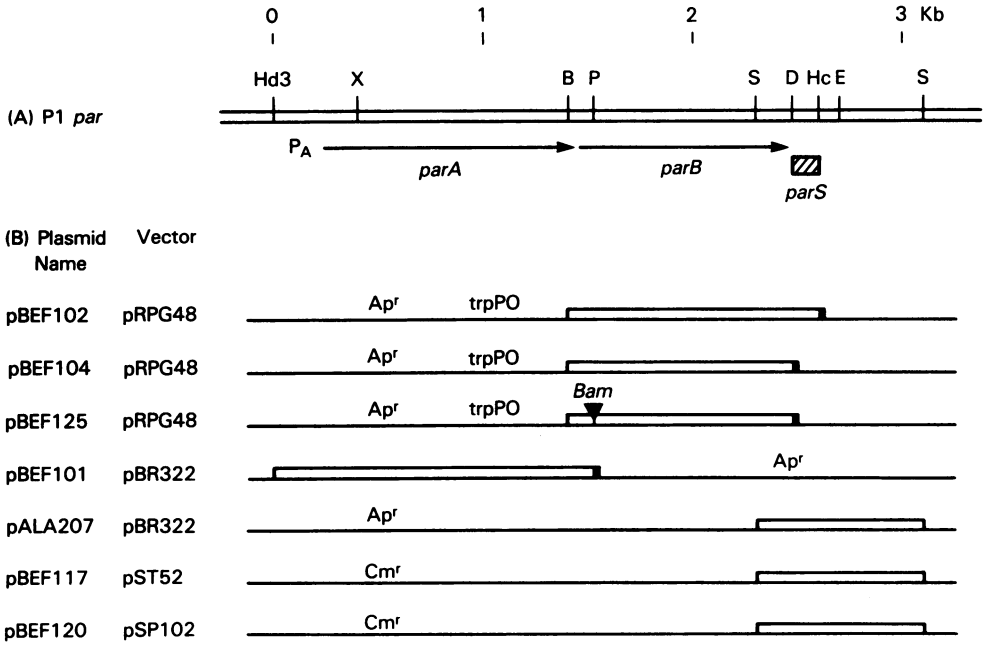

FIG. 1. Plasmids of the P1 partition region (*par*) used in this study. (A) Map of the entire *par* region. The position of the relevant restriction sites, the *parA* and *parB* coding regions, the promoter upstream of *parA* (P_A), and the boundaries of *parS* were determined by Abeles et al. (1). Only the *Hin*cII and *Sau*3AI restriction sites involved in the subcloning are shown; all other sites are unique within the region. Abbreviations: B, *Bgl*II; D, *Dra*I (*Aha*III); E, *Eco*RV; Hc, *Hin*cII; Hd3, *Hin*dIII; P, *Pvu*I; S, *Sau*3AI; X, *Xho*I. (B) Plasmid maps show the vector DNA as a thin line, and the P1 inserts as open boxes directly below the corresponding region on the map in part A. All plasmids were constructed for this analysis (see Materials and Methods), except for pALA207 which is described elsewhere (1). The *trp* promoter and operator (*trpPO*) from *S. marcescens* and the position of the *Bam*HI linker inserted into the *parB* gene in pBEF125 (*Bam*) are indicated. The position of *Sal*I linker DNA (■) is also indicated. Kb, 1,000 bp.

from *Bgl*II to *Hin*cII (pBEF102) was cloned into the *Bam*HI and *Sal*I sites, respectively, of pRPG48, after attachment of synthetic *Sal*I linker DNA to the downstream sites (Fig. 1). The *Bam*HI insertion mutant, pBEF125, was constructed as follows. A plasmid containing *parA* and *parB* in pBR327 (pBEF119) was partially digested with *Pvu*I, producing full-length fragments cut within either *parB* or the β-lactamase gene. The DNA ends were repaired by T4 DNA polymerase and ligated to 10-bp nonphosphorylated *Bam*HI linkers. In this way, only one linker was attached to the 5′ end of each fragment. Full-length linear molecules were separated from uncut circular molecules, smaller fragments, and free linkers by gel electrophoresis in low-melting-point agarose, and cut out from the gel. The DNA (in agarose) was diluted fivefold in a solution of 10 mM Tris (pH 8) and 1 mM EDTA, heated to 70°C for 10 min (producing 10-bp cohesive ends), allowed to reanneal slowly (1 to 2 h at 20°C), and used to transform DH5. All ampicillin-resistant (Ap^r) transformants (pBEF119::*Bam*) contained a *Bam*HI site replacing the *Pvu*I site within *parB* (Fig. 1), resulting in a net 8-bp insertion 85 bp downstream from the start codon. The *Bgl*II-*Dra*I (now *Sal*I) fragment was cloned into pRPG48 as described above. The plasmid pRPG18, a gift from R. P. Gunsalus (University of California at Los Angeles), contains *E. coli trpR* in pACYC184 (13).

The other vectors used were pBR322 (8), pST52, a high-copy-number chloramphenicol-resistant (Cm^r) plasmid derived from RSF1030 and compatible with pBR322 derivatives (24), and pSP102, a P1–*ori* miniplasmid (23). The *parA* gene, from *Hin*dIII to *Pvu*I (Fig. 1), was cloned into the *Hin*dIII-*Sal*I region of pBR322, after attachment of *Sal*I linker DNA to the downstream end, to yield pBEF101. *parS*, on a 794-bp *Sau*3AI fragment from pALA207, was inserted into the *Bgl*II site of pST52 to give pBEF117 and into the *Bam*HI site of pSP102 to give pBEF120 (Fig. 1).

Construction of λ–mini-P1 Km^r derivatives. λ *c*I857–P1:5R-3 (25) was the parent phage for all constructions. The kanamycin resistance gene from Tn*903* was purchased as a cassette (*kan*) on the plasmid pUC-4K (Pharmacia).

(i) λ*kan*–P1:5R. The *kan* cassette was inserted between the *Sal*I sites of λ–P1:5R (in the right arm of λ), and the DNA was packaged in vitro with commercial extracts (Boehringer Mannheim). After infection of YMC at 32°C, individual plaques were tested for kanamycin-resistant (Km^r) lysogens and used to prepare phage stocks. The position and orientation of the *kan* cassette was confirmed by restriction mapping analysis of the resulting phage DNA. In the λ*kan*–P1:5R used here, *kan* is transcribed from right to left on the standard phage map (25).

(ii) λ–P1:5R *par* mutants. Plasmid *par* mutations were constructed with the pUC-4K *kan* cassette and then crossed into Km^s λ–P1:5R. Plasmids were chosen to contain approximately 400 to 1,400 bp of P1 DNA on either side of the *kan* insertion, to ensure that crossovers on both sides of *kan* would be about equally likely. The *kan* cassette, cut with *Sal*I, was inserted into the *Xho*I site in *parA* of pBEF101 (Fig. 1); and cut with *Bam*HI, into the *Bam*HI (*Pvu*I) site of *parB*::*Bam* (of pBEF119::*Bam* above). The *parS* mutation was a deletion between the *Dra*I and *Eco*RV sites, which were changed to *Sal*I and *Xho*I, respectively, and the *kan* cassette was inserted between them. All *par* mutant plasmids were thus both Ap^r and Km^r.

λ–P1:5R phage was grown on YMC containing the above plasmids at 37°C, and the resulting phage lysates were used to infect YMC, this time selecting for Km^r lysogens at 32°C. Of the Km^r lysogens, 2 to 10% were also Ap^s, indicating that

TABLE 1. Lysogenization by λ–mini-P1 of the cells containing subcloned *par* plasmids

Resident plasmid	No. of cells/ml		Ratio of lysogens to survivors
	Survivors (Ap^r)	Lysogens (Ap^r Km^r)	
pRPG48 (*trp-PO* vector)	2×10^7	2×10^7	1
pBEF104 (*trp-PO parB*)	2×10^7	7×10^3	0.0003
pBEF101 (*parA*)	3×10^7	3×10^7	1
pALA207 (*parS*)	3×10^7	3×10^7	1
pBEF125 (*trp-PO parB::Bam*)	4×10^7	4×10^7	1
pBEF102 (*trp-PO parB parS*)	2×10^7	2×10^7	1

the *par* mutation, but not the entire plasmid, had been crossed into the phage. Km^r Ap^s lysogens were colony purified and then heat induced to make phage stocks. The structures of the resulting mutant phages were confirmed by restriction mapping.

Lysogenization. Fifty microliters of overnight DH5 (*recA*) cultures containing the *par* plasmid(s) (grown in LB plus maltose plus the appropriate antibiotic) was infected with the desired λ–mini-P1 derivative at a multiplicity of 5 to 10, allowed to adsorb for 15 min at room temperature, added to 1 ml of medium nonselective for the prophage, and grown at 32°C for 60 to 90 min before plating on selective (with kanamycin) and nonselective (without kanamycin) plates at 32°C. Under these conditions, 20 to 30% of infected cells survived the infection; the ratio of lysogens to survivors was identical when colonies from nonselective plates were later tested for kanamycin resistance.

Transformations. Competent cells were prepared by $CaCl_2$ treatment (10) and left on ice for 18 to 24 h before transformation. Cells (0.2 ml) were incubated with the indicated amount of DNA for 50 min at 0°C, heat shocked for 2 min at 42°C (34°C for temperature-sensitive cells), left at room temperature for 5 min, mixed with 0.5 ml of nonselective medium, and grown for 1 h at 37°C (or 32°C) before plating on selective and nonselective plates.

Copy-number measurements. The copy numbers of the plasmids used in this study were determined relative to pSP10, a pBR322 derivative (23), by a method modified from that of Tomizawa (28). Bacteria were grown in LB medium with the appropriate antibiotic(s) at 37°C to log phase (A_{600} of approximately 0.6). A portion of a DH5 culture containing the plasmid(s) of interest, corresponding to 8.3 ml of a culture with A_{600} of exactly 0.6, was mixed with an equivalent amount of DH5(pSP10). Total plasmid DNA was isolated by alkaline lysis (18), linearized with restriction enzymes, and separated by agarose gel electrophoresis. The DNA species were quantitated by densitometry either of autoradiograms of Southern blots (18) or of photographic negatives of the ethidium bromide-stained gels. All copy-number measurements of the plasmids in this study are reported relative to pBR322 or to each other. Cultures of highly unstable plasmids usually contained some (2 to 25%) plasmidless segregants (even with selection), determined by plating cells on nonselective plates and then transferring the cells (with toothpicks) to selective plates. Copy-number measurements are reported for the drug-resistant portion of the population.

RESULTS

Inhibition of the growth of λ–mini-P1 by high levels of P1 ParB protein. The P1 *par* region has been completely sequenced (1) (Fig. 1A). Both *parA* and *parB* are presumably transcribed from the promoter upstream from *parA*, which is autoregulated by one or both proteins (4). An additional putative promoter for *parB* was found by sequence analysis at the end of the *parA* reading frame, but the amount of ParB protein made from it is unknown. To express high levels of *parB* in the absence of *parA*, the *parB* gene was subcloned behind the *trp* promoter (Fig. 1). The vector, pRPG48, contained the *trp* operator and promoter (but not the attenuator) from *S. marcescens* (in pBR327 [9]), which are recognized and repressed by the *E. coli trp* repressor (19). The copy number of pRPG48 was about 20% higher than that of pBR322 (see Materials and Methods). The *parB* gene was subcloned with (pBEF102) and without (pBEF104) *parS*, which is immediately downstream from *parB* (Fig. 1). A *parB* mutation, *parB::Bam*, was constructed by insertion of a *Bam*HI linker early in the gene. The P1 test plasmid used in these experiments was the lambda–mini-P1 hybrid prophage, λ–P1:5R, into which the kanamycin resistance gene from Tn*903* was inserted to use drug resistance for lysogen selection.

E. coli recA (DH5) cells containing the recombinant (Ap^r) plasmids were infected with λ*kan*–mini-P1, and the survivors were plated onto selective (containing ampicillin and kanamycin) and nonselective (containing ampicillin) plates to determine the lysogenization frequency. All plasmid-containing strains were found to lysogenize normally, except for cells containing the *parB* plasmid pBEF104 (Table 1); it appeared that λ–mini-P1 could not be maintained in cells making excess ParB protein, even under selection. Because the number of survivors of the λ infection was similar in all strains, it appeared that the cells containing this *parB* plasmid had been infected and lysogeny established long enough to repress the λ lytic functions, suggesting an inhibition of λ–mini-P1 maintenance rather than just establishment. This was confirmed by reversing the order of lysogenization and transformation: λ–mini-P1 lysogens were transformed with normal frequency by the *parB* plasmid when only the incoming plasmid was selected, but selection for both plasmid and the resident prophage resulted in no transformants (Table 2). Thus, the *parB* plasmid was able to displace a resident λ–mini-P1. The inhibitory effect of *parB* differed from the effect of the other two partition elements, *parA* and *parS* (Fig. 1). The *parA* plasmid pBEF101 contains the entire *parA* gene, including its own promoter, and is almost identical to the *parA* plasmid described and used

TABLE 2. Displacement of resident λ–mini-P1 by the *parB* plasmid pBEF104

Transforming plasmid[a]	No. of transformants/cell per fmol of plasmid	
	Selecting for incoming plasmid only (Ap^r)	Selecting for incoming plasmid and resident λ–mini-P1 (Ap^r Km^r)
pRPG48 (vector)	2.1×10^{-5}	2.1×10^{-5}
pBEF104 (*trp-PO parB*)	2.7×10^{-5}	$< 10^{-8}$

[a] Competent λ*kan*–mini-P1 lysogens were transformed with 25 ng of plasmid DNA (see Materials and Methods).

TABLE 3. Inhibition by excess ParB protein suppressed by excess *parS* or a repressor of *parB* expression

Resident plasmids		No. of cells/ml		Ratio of lysogens to survivors
Ap^r	Cm^r	Survivors (Ap^r Cm^r)	Lysogens (Ap^r Cm^r Km^r)	
pRPG48 (vector)	pST52 (vector)	6×10^7	6×10^7	1
pRPG48 (vector)	pBEF117 (*parS*)	5×10^7	5×10^7	1
pBEF104 (*trp-PO parB*)	pST52 (vector)	4×10^7	3×10^3	$\leq 10^{-4}$
pBEF104 (*trp-PO parB*)	pBEF117 (*parS*)	2×10^7	2×10^7	1
pBEF104 (*trp-PO parB*)	pRPG18 (*trpR*)[a]	5×10^7	5×10^7	1

[a] The plasmid pRPG18 contains the *E. coli trpR* gene, encoding the *trp* repressor, in pACYC184 (13).

previously by Abeles et al. (1). The plasmid pALA207 contains a 794-bp *Sau*3AI fragment covering *parS* (1). Neither plasmid blocked lysogenization by λ–mini-P1 (Table 1), although the prophage was subsequently unstable once selection was removed ([1] and data not shown).

Several lines of evidence demonstrate that the ParB protein, transcribed from the *trp* promoter, is responsible for this inhibitory effect. First, a strain containing a cloned *parB* insertion mutant (pBEF125 [Fig. 1]) was lysogenized normally by λ–mini-P1 (Table 1), and the prophage in this strain was stable in the absence of selection (data not shown). Second, of the rare λ–mini-P1 lysogens that grew in the presence of excess ParB protein [in DH5(pBEF104) in Table 1], 90% were very small colonies that would not grow when restreaked on plates containing kanamycin. The remaining 10%, when cured of the prophage, were relysogenized by new λ–mini-P1 at a normal frequency; the *parB* plasmids in these strains had acquired an insertion within the *parB* coding sequence (data not shown). Finally, the presence of excess *trp* repressor, supplied by a second plasmid compatible with the *parB* plasmid, suppressed the inhibition of lysogenization (Table 3). None of the above conditions reduced the average copy numbers of any of the *parB*-containing plasmids. In addition, the amount of ParB protein made by these plasmids had no measurable effect on the growth rate of the host cells (data not shown).

The inhibitory effect of the *parB* plasmid pBEF104 was specific for plasmids bearing the P1 partition region. λ–mini-F hybrid plasmids, which contain the replication and partition regions of the sex factor F (2), lysogenized cells with the *parB* plasmid pBEF104 normally (data not shown). This result also confirmed that λ infection was not blocked by excess ParB protein.

***parS* required for the inhibitory effect.** The presence of *parS* on the same plasmid expressing ParB protein blocked the effect of excess ParB protein on λ–mini-P1 lysogenization. Cells containing the *parB parS* plasmid pBEF102 were lysogenized by λ–mini-P1 at a normal frequency; the presence of *parS* suppressed the inhibition of λ–mini-P1 maintenance (Table 1). Austin and Abeles (3) have proposed that one or both of the P1 Par proteins bind to *parS* to form a complex recognized by the cellular partition apparatus. Therefore, the *parS* suppression of the inhibition by ParB protein could be due to titration of excess protein by the site. Alternatively, *parS* may reduce the amount of ParB protein made from the plasmid by retroregulation (a *cis* effect). However, *parS* in *trans* to *parB*, on a second compatible high-copy-number plasmid (pBEF117 [Fig. 1]) also suppressed the *parB* effects and allowed λ–mini-P1 to lysogenize normally (Table 3). Therefore, titration of the ParB protein, the distribution of the protein among many rather than few *parS* sites, most likely accounts for the behavior of the *parB parS* plasmid. These results suggested that ParB protein binds to *parS*, and lead to the prediction that *parS* on the λ–mini-P1 would be necessary for the inhibitory effect by ParB protein. Two sets of experiments were performed to test this idea.

First, I tested mutations within P1 *par* to determine which components on the λ–mini-P1 prophage were required for *parB* inhibition. Mutations were constructed in vitro on plasmids, by using a drug cassette *kan* (the kanamycin resistance gene from Tn*903*), and crossed into λ–mini-P1 phage. The *parA* and *parB* mutations were *kan* insertions early in the reading frame of each gene. The *parS* mutation was a 225-bp *par* deletion (from the *Dra*I to *Eco*RV sites of the partition region in Fig. 1), replaced by the *kan* cassette. The resulting λ–mini-P1 prophages (in the absence of any other plasmid) were unstable, as expected for *par* mutants. After overnight growth in nonselective medium, over 95% of the cells had lost the prophage for each mutant.

Cells containing either the *parB* plasmid pBEF104 or the vector pRPG48 were lysogenized by the λ–mini-P1 *par* mutants. As predicted, lysogeny of λ–mini-P1Δ*parS* was not inhibited by excess ParB protein (Table 4). λ–mini-P1 *parA* and λ–mini-P1 *parB* mutants, on the other hand, could not escape the inhibition. Therefore, production of ParA and ParB proteins from the infecting λ–mini-P1 were not required but the *parS* site was required for the effect. This

TABLE 4. Lysogenization by λ–mini-P1 *par* mutants

Phage	Position of *kan*[a]	Ratio of lysogens to survivors[b] in DH5 with:	
		pRPG48 (vector)	pBEF104 (*trp-PO parB*)
λ*kan*–P1:5R	Lambda	0.8	6×10^{-5}
λ–P1:5R *parA*::*kan*	*Xho*I insertion	0.8	2×10^{-5}
λ–P1:5R *parB*::*kan*	*Pvu*I insertion	0.9	8×10^{-5}
λ–P1:5R Δ*parS kan*	*Dra*I-*Eco*RV substitution	0.9	0.8

[a] See Fig. 1 for the position of relevant restriction sites. Construction of the *par* mutants is described in Materials and Methods.

[b] Experiments were performed exactly as described for Tables 1 and 3; only the final lysogenization ratio is reported here, but the absolute numbers were similar to those from Tables 1 and 3.

experiment also shows that λ–mini-P1 *par* mutants showed no inherent defect in lysogeny; the mutants could be maintained with selection. Thus, the instability imposed on λ–mini-P1 by excess ParB protein was greater than if the plasmid were simply deficient in ParB protein.

Second, the effect of excess ParB protein was tested on plasmids containing only *parS*. The P1 miniplasmid, pSP102, which contains the P1 replication region but not *par*, has a copy number of eight per host chromosome, higher than that of λ–mini-P1 due to deletion of the regulatory region *incA* (23). *parS* cloned into pSP102 (pBEF120; Fig. 1) could not be transformed into cells containing the *parB* plasmid pBEF 104, whereas transformation by pSP102 was unaffected (Table 5). Cells without ParB protein were transformed equally well by both plasmids. On the other hand, the high-copy-number *parS* plasmid pBEF117 (derived from pST52, with a copy number similar to pBR322) could be transformed into cells producing ParB protein, although the transformation frequency was about 10-fold lower than that into cells without ParB protein (Table 5). This result suggests that the inhibitory effect of excess ParB protein can be overcome if the protein is distributed among a sufficient number of *parS* binding sites (as in the titration effect in Table 3).

Destabilization of *parS* plasmids by excess ParB protein. Once transformed into cells producing excess ParB protein, the high-copy-number *parS* plasmid pBEF117 was unstable in the absence of selection. After 100 generations of nonselective growth, 85% of the cells had lost the plasmid (Fig. 2A). In the absence of ParB protein, this *parS* plasmid, which normally partitions by random segregation, was stable. In addition, the other high-copy-number *parS* derivative, pBEF102, which supplies its own ParB protein (Fig. 1), was also slightly unstable, whereas the corresponding plasmid without *parS*, pBEF104, was by itself very stable (Fig. 2B). By comparison, the plasmid pSP108, another P1-*ori*-derived replicon (23) with a copy number about one-fourth that of pBR322 (see Materials and Methods) was more stable than either *parS* plasmid under these conditions (Fig. 2B). In rich growth medium, the plasmid copy number per cell is usually severalfold higher than the copy number per host chromosome, because each rapidly growing cell contains several host chromosomes (27). Under such growth conditions, the low-copy-number plasmid pSP108, which lacks *par*, was only slightly unstable; 2 to 3% of the cells had lost this plasmid after 100 generations. The instability of high-copy-number *parS* plasmids in the presence of excess ParB protein indicated a decrease in the number of partitionable units per cell, at least below that of a plasmid such as pSP108 (Fig. 2). However, the copy number of *parS* plasmids did not decrease in the presence of excess ParB protein; in fact, there was a small but measurable increase. The copy number of pBEF117 (from Fig. 2A) in the presence of ParB protein supplied by pBEF104, relative to the copy number in the presence of the vector pRPG48, was 1.5 ± 0.2. Similarly, the copy number of the *parB parS* plasmid pBEF102 (from Fig. 2B) relative to the *parB* plasmid pBEF104 was 1.3 ± 0.1. Plasmids isolated from cells containing excess ParB protein showed no evidence of dimerization or catenation (data not shown). These observations rule out an actual decrease in plasmid copy number, and thus excess ParB protein does not cause a replication defect. The apparent decrease suggested that the plasmids were sequestered by ParB protein before or during cell division, so that the number of partitionable units per cell was lower than the number of plasmids per cell. Thus, random segregation of individual plasmids was actively prevented. Plasmid seques-

TABLE 5. Effect of excess ParB protein on transformation by *parS* plasmids

Expt	Transforming plasmid[a]	No. of transformants/cell per fmol of plasmid in DH5 with:	
		pRPG48 (vector)	pBEF104 (*trp-PO parB*)
1	pSP102 (vector)	1×10^{-4}	1×10^{-4}
	pBEF120 (*parS*)	1×10^{-4}	$<10^{-7}$
2	pST52 (vector)	6×10^{-4}	8×10^{-4}
	pBEF117 (*parS*)	5×10^{-4}	6×10^{-5}

[a] Under the growth conditions used for transformation, the average copy number of pBEF120 was about one-half that of pBEF117 (Materials and Methods). Experiments 1 and 2 were done with different preparations of competent cells. Ten nanograms of each plasmid was used experiment 1; 20 ng was used in experiment 2.

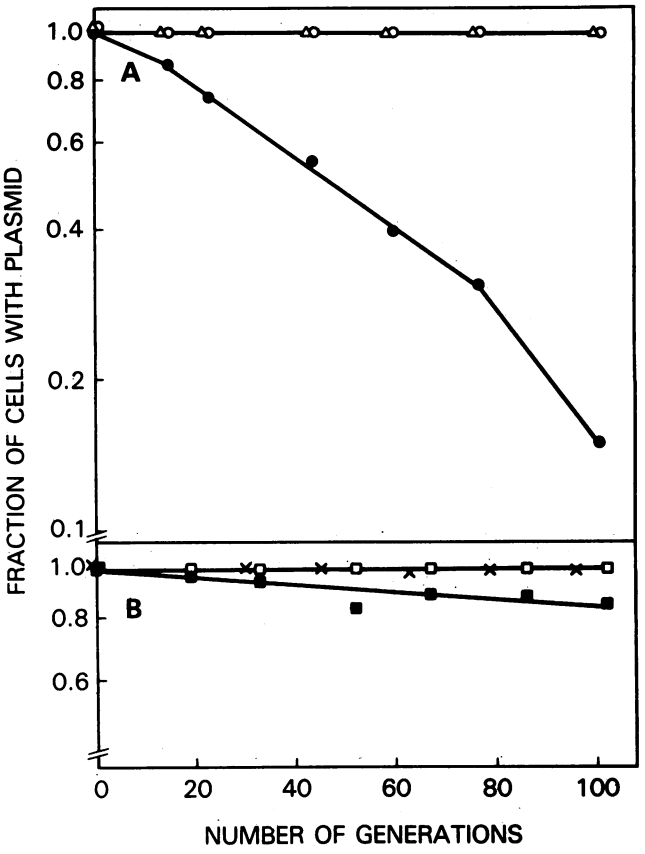

FIG. 2. Stability of *parS* plasmids. (A) Stability of the *parS* plasmid pBEF117 (Cm^r) in *trans* to *parB* on pBEF104 (●), to the vector pRPG48 (○), or to the *parB*::*Bam* mutant on pBEF125 (△) at 37°C. Cells containing both plasmids (grown in the presence of ampicillin and chloramphenicol) were diluted into medium nonselective for pBEF117 (with ampicillin only), and plated onto nonselective plates at the indicated number of generations (calculated from the viable cell count). Individual colonies were tested (by transfer with toothpicks) for chloramphenicol resistance. At least 100 colonies were tested for each culture at every time point. (B) Stability of pBEF102 (*parB parS*) (■), pBEF104 (*parB*) (□), and pSP108 (×). Experiments were performed as above, except that selective medium included ampicillin for pBEF102 and pBEF104, or chloramphenicol for pSP108, and nonselective medium had no antibiotics.

tration explains the inability of low-copy-number *parS* plasmids, such as λ–mini-P1, to grow under these conditions. Presumably λ–mini-P1 is stringently controlled at such a low copy number that all plasmids are sequestered to one of the daughter cells at cell division; consequently, colonies could not grow out on plates selective for the prophage. A transient accumulation of plasmids in some daughter cells would be expected from a segregation defect. This might explain why the copy number of the high-copy-number *parS*-bearing plasmids appeared slightly higher in the presence of ParB protein than in its absence, if after asymmetric segregation, normal copy numbers were restored to the daughter cell with few copies (by replication) more rapidly than to the cell with more copies (by subsequent cell divisions). In any case, a segregation defect caused by high levels of expression of ParB protein seems reasonable, as *parB* is a partition, not a replication, gene (3). The possible nature of this defect and its implications for normal P1 partitioning are discussed below.

DISCUSSION

The P1 ParA and ParB proteins are required for normal partitioning of the prophage at cell division. Also essential is a *cis*-acting site on the partitioned plasmid called *parS*. I have shown here that excess ParB protein interferes with the proper segregation of P1 derivatives. Plasmids producing high levels of ParB protein destabilized *parS*-containing plasmids. When the plasmid with *parS* was of high copy number, both the *parB*- and *parS*-bearing plasmids could be maintained in the presence of selection, but once the selection was removed, the *parS* plasmid was lost from the cell population. When the *parS* plasmid was of low copy number, the destabilization was so strong that cells containing both plasmids could not grow, even under selection. The effect could be weakened by increasing the number of *parS* sites in the same cell (Tables 1 and 3). The behavior of *parS*-bearing plasmids under these conditions suggested that ParB protein was binding to itself, as well as to *parS*, causing the plasmids to segregate as aggregates or clumps. By decreasing the number of partitionable units, plasmids became more unstable than would be predicted from a random distribution of individual plasmids at cell division.

The interference by ParB protein required only *parS* DNA and supports the model, proposed by Austin and Abeles (3), that one (or both) partition proteins bind to *parS* (the centromere) and pair plasmids before cell division. My results suggest that in vivo, ParB protein can bind to itself and to *parS*, so that under normal conditions, ParB protein may be the proposed pairing protein. According to the model, the Par protein-*parS* complex (which may or may not contain ParA protein) attaches to the cellular partition apparatus, and at the time of septation, the complex dissociates so that the members of each plasmid pair go into different daughter cells. Presumably, overproduction of the pairing protein, in the absence of enough of the effector of dissociation, unbalances the system and is responsible for the destabilization of P1 derivatives observed here.

Although the *parS* region is the only DNA sequence required for ParB protein-mediated instability, the precise limits required have not been defined. The instability of the *parB parS* plasmid (pBEF102) compared with the *parB* plasmid (pBEF104) (Fig. 2B) indicates that the 112-bp region between *Dra*I and *Hin*cII is necessary for ParB protein binding, because the two plasmids differ only in the presence of this sequence (Fig. 1). The sequence includes the minimal 49-bp *parS* region described by Friedman et al. (12). Whether sequences to the left of *Dra*I are also required, such as those required to produce the incompatibility phenotype of *incB* (12), remains to be determined. The plasmid containing a 794-bp fragment covering *parS*, pBEF117, was more unstable than pBEF102 (Fig. 2). This observation may indicate that a sequence to the right of the 112-bp region interacts with ParB protein to increase the partition interference; however, other possibilities are equally likely. For example, the copy number of pBEF102 was slightly higher than that of pBEF117, and thus pBEF102 may more easily have escaped the sequestering effects of ParB protein.

Although the defect in λ–mini-P1 lysogenization by excess ParB protein was alleviated by supplying extra *parS* sites (in *cis* or *trans*; Tables 1 and 3), normal partition was not completely restored and the prophages in such strains were unstable (over 70% of cells lost λ–mini-P1 after 20 generations in nonselective medium). Because excess *parS* sites alone greatly destabilize λ–mini-P1 (1), it is difficult to determine whether prophage instability in strains containing both excess *parB* and *parS* is due to one or both components. Nevertheless, it is clear that *parS* suppresses the sequestration of λ–mini-P1 by excess ParB protein sufficiently to allow lysogenization, possibly by nucleating more clumps of plasmid DNA and allowing a more random segregation of prophages.

Because *parA* is not required for *parB* destabilization (Table 4), ParA protein is not necessary for ParB protein to bind *parS* plasmids, but one cannot make any conclusions about the role of ParA protein during normal partitioning. It may regulate the production of ParB protein, be responsible for proper attachment of the ParB protein-*parS* complex to the cellular partition proteins (or membrane), be the dissociation effector discussed above, or any combination of such functions, all of which could explain the destabilizing effects of excess ParA protein (1).

The phenomenon of plasmid sequestration, preventing random distribution of individual molecules, has been observed for another plasmid system, pSC101 (29). Certain derivatives deleted for the centromerelike site appear to segregate as groups, not individuals, suggesting that such plasmids are not counted as separate molecules in the absence of the *par* locus (29). On the other hand, the sequestering effects of ParB protein described here are dependent on the presence of the centromere (Table 4 and Fig. 2B), not on its absence. Presumably both sequestration phenomena reflect different ways of interfering with the normal partition process.

The partition functions of mini-F are very similar to those of mini-P1 in genetic organization, although almost totally nonhomologous in sequence (4, 20). Two plasmid-encoded proteins, the products of *sopA* and *sopB*, and a *cis*-acting site, *sopC* (or *incD*) are required for stable maintenance (6, 22). The SopB protein has been shown to bind to *sopC* (14), the centromere of mini-F (15, 17). Thus, the similarity between F and P1 has been extended to the binding of ParB protein to the centromere of P1. *sopB* in high copy number destabilizes mini-F, although the effect is weak, since both mini-F and the *sopB* plasmids can coexist in the same cell in the presence of selection (20, 22). Recently, Kusukawa et al. (16) have shown that this destabilization is due to overproduction of the SopB protein, suggesting that in mini-F, as well as mini-P1, the levels of SopB or ParB proteins, relative to other partition proteins and sites, must be carefully controlled to ensure proper segregation. Because P1 and F have evolved, or maintained, remarkably similar partition

regions (although with different specificities), it seems likely that both use similar mechanisms to segregate daughter plasmids (3, 22). The nature of the interaction of the Par proteins with the centromere and with the host is still a mystery in both systems, and its characterization is the next step in determining the mechanism which accomplishes equipartition of daughter plasmids to newborn cells.

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LITERATURE CITED

1. **Abeles, A. L., S. A. Friedman, and S. J. Austin.** 1985. Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organization of the P1 partition region. J. Mol. Biol. **185**:261–272.
2. **Austin, S., and A. Abeles.** 1983. Partition of unit-copy miniplasmid to daughter cells. I. P1 and F miniplasmids contain discrete, interchangeable sequences sufficient to promote equipartition. J. Mol. Biol. **169**:353–372.
3. **Austin, S., and A. Abeles.** 1983. Partition of unit-copy miniplasmids to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and a centromere-like site at which it acts. J. Mol. Biol. **169**:373–387.
4. **Austin, S., and A. Abeles.** 1985. The partition functions of P1, P7, and F miniplasmids, p. 215–226. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
5. **Austin, S., F. Hart, A. Abeles, and N. Sternberg.** 1982. Genetic and physical map of a P1 miniplasmid. J. Bacteriol. **152**:63–71.
6. **Austin, S., and A. Wierzbicki.** 1983. Two mini-F-encoded proteins are essential for equipartition. Plasmid **10**:73–81.
7. **Austin, S., M. Ziese, and N. Sternberg.** 1981. A novel role for site-specific recombination in maintenance of bacterial replicons. Cell **25**:729–736.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
9. **Cecchini, G., C. R. Thompson, B. A. C. Ackrell, D. J. Westenberg, N. Dean, and R. P. Gunsalus.** 1986. Oxidation of reduced menaquinone by the fumarate reductase complex in *Escherichia coli* requires the hydrophobic FrdD peptide. Proc. Natl. Acad. Sci. USA **83**:8898–8902.
10. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 140. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Dennert, G., and V. Henning.** 1968. Tyrosine-incorporating amber suppressor in *Escherichia coli*. J. Mol. Biol. **33**:322–329.
12. **Friedman, S., K. Martin, and S. Austin.** 1986. The partition system of the P1 plasmid, p. 285–295. *In* Banbury Report no. 24. Antibiotic resistance genes: ecology, transfer, and expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Gunsalus, R. P., G. Zurawski, and C. Yanofsky.** 1979. Structural and functional analysis of cloned deoxyribonucleic acid containing the *trpR-thr* region of the *Escherichia coli* chromosome. J. Bacteriol. **140**:106–113.
14. **Hayakawa, Y., T. Murotsu, and K. Matsubara.** 1985. Mini-F protein that binds to a unique region for partition of mini-F plasmid DNA. J. Bacteriol. **163**:349–354.
15. **Helsberg, M., and R. Eichenlaub.** 1986. Twelve 43-base-pair repeats map in a *cis*-acting region essential for partition of plasmid mini-F. J. Bacteriol. **165**:1043–1045.
16. **Kusukawa, J., H. Mori, A. Kondo, and S. Hiraga.** 1987. Partitioning of the F plasmid: overproduction of an essential protein for partition inhibits plasmid maintenance. Mol. Gen. Genet. **208**:365–372.
17. **Lane, D., R. Rothenbuehler, A. Merrillat, and C. Aiken.** 1987. Analysis of the F plasmid centromere. Mol. Gen. Genet. **207**: 406–412.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Miozzari, G. F., and C. Yanofsky.** 1978. The regulatory region of the *trp* operon of *Serratia marcescens*. Nature (London) **276**: 684–689.
20. **Mori, H., A. Kondo, A. Ohshima, T. Ogura, and S. Hiraga.** 1986. Structure and function of the F plasmid genes essential for partitioning. J. Mol. Biol. **192**:1–15.
21. **Nordström, K., S. Molin, and H. Aagaard-Hanson.** 1980. Partitioning of plasmid R1 in *E. coli*. I. Kinetics of loss of plasmid derivatives deleted of the par region. Plasmid **4**:215–227.
22. **Ogura, T., and S. Hiraga.** 1983. Partition mechanism of F plasmid: two plasmid gene-encoded products and a *cis*-acting region are involved in partition. Cell **32**:351–360.
23. **Pal, S. K., R. J. Mason, and D. K. Chattoraj.** 1986. P1 plasmid replication. Role of initiator titration in copy number control. J. Mol. Biol. **192**:275–285.
24. **Som, T., and J. Tomizawa.** 1982. Origin of replication of *Escherichia coli* plasmid RSF1030. Mol. Gen. Genet. **187**:375–383.
25. **Sternberg, N., and S. Austin.** 1983. Isolation and characterization of P1 minireplicons, λ-P1:5R and λ-P1:5L. J. Bacteriol. **153**:800–812.
26. **Sternberg, N., and R. Hoess.** 1983. The molecular genetics of bacteriophage P1. Annu. Rev. Genet. **17**:123–154.
27. **Swack, J. A., S. K. Pal, R. J. Mason, A. L. Abeles, and D. K. Chattoraj.** 1987. P1 plasmid replication: measurement of initiator protein concentration in vivo. J. Bacteriol. **169**:3737–3742.
28. **Tomizawa, J.** 1985. Control of colEI plasmid replication: initial interaction of RNA I and the primer transcript is reversible. Cell **40**:527–535.
29. **Tucker, W. T., C. A. Miller, and S. N. Cohen.** 1984. Structural and functional analysis of the *par* region of the pSC101 plasmid. Cell **38**:191–201.