Characterization of the Stable Maintenance Properties of the *par* Region of Broad-Host-Range Plasmid RK2

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A 3.2-kb fragment encoding five genes, *parCBA/DE*, in two divergently transcribed operons promotes stable maintenance of the replicon of the broad-host-range plasmid RK2 in a vector-independent manner in *Escherichia coli*. The *parDE* operon has been shown to contribute to stabilization through the postsegregational killing of plasmid-free daughter cells, while the *parCBA* operon encodes a resolvase, ParA, that mediates the resolution of plasmid multimers through site-specific recombination. To date, evidence indicates that multimer resolution alone does not play a significant role in RK2 stable maintenance by the *parCBA* operon in *E. coli*. It has been proposed, instead, that the *parCBA* region encodes an additional stability mechanism, a partition system, that ensures that each daughter cell receives a plasmid copy at cell division. However, studies carried out to date have not directly determined the plasmid stabilization activity of the *parCBA* operon alone. An assessment was made of the relative contributions of postsegregational killing (*parDE*) and the putative partitioning system (*parCBA*) to the stabilization of mini-RK2 replicons in *E. coli*. Mini-RK2 replicons carrying either the entire 3.2-kb (*parCBA/DE*) fragment or the 2.3-kb *parCBA* region alone were found to be stably maintained in two *E. coli* strains tested. The stabilization found is not due to resolution of multimers. The stabilizing effectiveness of *parCBA* was substantially reduced when the plasmid copy number was lowered, as in the case of *E. coli* cells carrying a temperature-sensitive mini-RK2 replicon grown at a nonpermissive temperature. The presence of the entire 3.2-kb region effectively stabilized the replicon, however, under both low- and high-copy-number conditions. In those instances of decreased plasmid copy number, the postsegregational killing activity, encoded by *parDE*, either as part of the 3.2-kb fragment or alone played the major role in the stabilization of mini-RK2 replicons within the growing bacterial population. Our findings indicate that the *parCBA* operon functions to stabilize by a mechanism other than cell killing and resolution of plasmid multimers, while the *parDE* operon functions solely to stabilize plasmids by cell killing. The relative contribution of each system to stabilization depends on plasmid copy number and the particular *E. coli* host.

The loss rate of randomly segregating plasmid copies upon cell division is predicted to increase as plasmid copy number decreases. Most low-copy-number plasmids, however, have been found to be highly stable under nonselective growth conditions, suggesting that these plasmids encode genetic systems other than replication control that ensure their stable maintenance within a growing population.

Several mechanisms of plasmid stabilization have been described. It has been shown that copy up mutations in the *Bacillus subtilis* plasmid pTA1060 and the *Escherichia coli* plasmid pSC101 result in an increase in plasmid copy number that increases plasmid stability (7, 31). However, the metabolic burden associated with the maintenance of a high plasmid copy number may in turn result in a selective advantage for plasmid-free cells that arise within the population. For this reason, this mechanism of stabilization is not likely to be widespread among naturally occurring plasmids. Another mechanism, the selective killing of plasmid-free segregants, has been shown to effectively stabilize plasmids. Several such plasmid systems, each varying with respect to the mode of cell killing, have been identified. Examples include the production of extracellular colicin toxins by the Col family of plasmids (38) and intracellular toxins encoded by the *ccd* locus of plasmid F, the *hok/sok* and *kis/kid* loci of plasmid R1, and the *phd/doc* locus of plasmid P1 (5, 6, 16, 17, 22, 28, 32, 43). The intramolecular resolution

of plasmid multimers, which increases the number of unit copies available for distribution to daughter cells, is another mechanism that can contribute to plasmid stability. Examples of extrachromosomal elements encoding multimer resolution systems (*mrs*) include the prophage form of bacteriophage P1, plasmids F, pR46, and pSDL2, and the ColE1 plasmid family (2, 4, 11, 25, 27, 48). However, for relatively low copy number plasmids such as RK2, an *mrs* cannot account for the observed plasmid stability since the maximum number of single units available for segregation is well below that required to ensure a low plasmid loss rate during random segregation of plasmid molecules upon cell division (40). Several plasmid-encoded stabilization systems, consisting of a *cis*-acting site and *trans*-acting protein(s), also have been described. It has been proposed that these systems provide stable maintenance by an active process of distribution of plasmid copies to daughter cells. This type of stabilization system, designated partitioning, has been shown for plasmids F, NR1/R1, and pTAR and the plasmid form of the bacteriophages P1 and P7 (1, 15, 16, 23, 29, 35, 49).

The 60-kb, antibiotic-resistant, IncP1α plasmid RK2 (identical to plasmids RP1, RP4, R18, and R68 [8]) is stably maintained at a low copy number (five to eight copies per chromosome [51]). A stabilization locus encoded within a 6.8-kb *Pst*I fragment of this broad-host-range plasmid has been previously described (44). A region within this fragment termed RK2 *par*, located at approximately kb 32.6 to 35.8 on the RK2 map (37), has been shown to stabilize plasmids in a broad-host-range,

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
TG1	F′ *lacI*q *proA*+*B*+ *lacZ*ΔM15 *sup*0	9
MV10Δlac	*thr-1 leu-6 lacY1 thi-1 tonA21 supE44 ΔtrpE5 Δlac* linked to Tn*10*	46
Plasmids		
pRR10	Mini-RK2, *bla oriT lacZ*α	40
pRR10ts97	pRR10 with *trfA* ts97	40
pBS7-0.7	pRR10 + 0.7-kb RK2 *par* region (kb 35.0 to 35.7)	This work
pRR15	Mini-R6K, *npt lacZ*α	40
pCE25-2.3	pRR10 + 2.3-kb RK2 *par* region (kb 32.8 to 35.1)	This work
pRR46	pRR15 + *parDE* promoter, *parD*, and 1/2 of *parE* (to the *Esp*I site)	42
pRR41-3.2	pRR10ts97 + 3.2-kb RK2 *par* region (kb 32.8 to 36.0)	40
pBS41-2.3	pRR10ts97 + 2.3-kb RK2 *par* region (kb 32.8 to 35.1)	This work
pRR41-0.7	pRR10ts97 + 0.7-kb RK2 *par* region (kb 35.0 to 35.7)	40
pRR54	pRR10 + 3.2-kb RK2 *par* region (kb 32.8 to 36.0)	40

vector-independent manner (18, 40, 44). The RK2 *par* region encodes five genes in two operons (*parCBA* and *parDE*) transcribed by divergent, autoregulated promoters (18, 40). The *parCBA* operon contains a system for plasmid multimer resolution including a *cis*-acting site that is required for resolving multimers and is located between the two *par* promoters (10, 12). The *parA* gene, essential for multimer resolution activity, encodes a resolvase protein that exhibits considerable homology to the Tn*3* transposon resolvase family (18). ParB exhibits endonuclease activity (19), but little else is known regarding the role of ParB or the *parC* gene product in promoting RK2 stability. Studies carried out to date have not assessed the plasmid stabilization activity of the *parCBA* operon alone.

The *parDE* operon is carried on a 0.7-kb fragment of the RK2 3.2-kb *par* region and has been shown to promote plasmid maintenance through postsegregational cell killing under conditions such that the copy number of the plasmid on which the fragment is carried becomes too low or the plasmid is lost (41, 42). In the absence of ParD, ParE exhibits bacterial toxicity which is associated with cell filamentation, whereas the presence of the ParD protein, provided either in *cis* or in *trans*, neutralizes the growth-inhibiting or filamentation activity of the ParE protein (42). Thus, the two proteins ParD and ParE appear to function as a toxin-antitoxin killing system analogous to the *kis/kid* and *ccd* mechanisms previously described (5, 6, 22, 32, 33). Another growth-inhibitory locus on RK2, *psa* (postsegregation arrest), has been recently identified by Jovanovic et al. (24). Although the exact location of the *psa* locus on RK2 is unknown, it has been shown to arrest the growth of plasmid-free segregants independently of the *parDE* system and without the concomitant filament formation. Recent findings by Sia et al. (46) indicate that conjugal transfer plays a role in stable maintenance of RK2 under conditions that favor plasmid transfer to plasmidless segregants (i.e., solid surfaces).

In this study, we have assessed the relative contributions of several possible mechanisms to the stabilization of mini-RK2 replicons carrying the *parCBA* and *parDE* operons separately and together. Our findings indicate that the *parCBA* operon functions to stabilize by a mechanism other than cell killing and resolution of plasmid multimers, while the *parDE* operon functions to stabilize plasmids by a cell-killing mechanism. The relative contributions of these two stabilization mechanisms depend on the plasmid copy number and the particular *E. coli* host. When both operons are present, the *parDE* operon promotes plasmid stabilization by killing those cells that lose the plasmid by either a failure of replication control or possibly partitioning errors.

MATERIALS AND METHODS

Materials. Restriction endonucleases, the Klenow fragment of *E. coli* DNA polymerase, T4 DNA polymerase, and T4 DNA ligase were obtained from commercial suppliers and were used as described in the manufacturers' specifications. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). [α-32P]dATP (3,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.).

Bacterial strains and plasmid construction. The bacteria and plasmids used in this study are listed in Table 1. The *E. coli* strains were grown in Lennox L broth (34) (Gibco Scientific, Grand Island, N.Y.) at the temperatures noted. Antibiotics were added as necessary to final concentrations of 200 μg/ml for penicillin and 50 μg/ml for kanamycin. Restriction endonuclease digestions, fill-in reactions of 5′ ends by the Klenow fragment, removal of 3′ ends by T4 DNA polymerase, DNA ligation, agarose gel electrophoresis, and *E. coli* transformation of plasmid DNA have been previously described (30). Plasmid DNA was isolated by alkaline lysis according to the method of Birnboim and Doly (3).

Plasmid stabilization assays. Two types of stabilization assays were used, depending on the number of generations of growth of the test bacterium and plasmid. Short-term assays were used for analyzing constructs based on the vector pRR10ts97 in *E. coli* cells grown at 33.5°C (40). Similar but longer-term assays were used for analyzing constructs based on the vector pRR10 (which exhibits a lower rate of loss than pRR10ts97 derivatives) in *E. coli* cells grown at 30°C. Overnight liquid cultures of *E. coli* containing the plasmids of interest were obtained under antibiotic selection. For both types of assays, an aliquot of cells was diluted 10⁴- to 10⁶-fold into prewarmed LB broth and grown under selection to mid-log phase at the appropriate assay temperature. The remaining stationary-phase cells were used to isolate plasmid DNA to verify the presence and integrity of the DNA. Throughout the entire stabilization assay, antibiotic selection was maintained on the R6K derivatives in experiments in which ParD was provided in *trans*. The mid-log cells were then diluted 10⁶-fold into prewarmed LB broth containing kanamycin, to maintain the R6K derivative, pRR15 or pRR46, and an aliquot of the culture was plated onto prewarmed LB agar containing kanamycin. The diluted cultures were then grown to mid-log to late log phase and serially transferred to fresh LB medium containing kanamycin, and dilutions were plated onto LB agar containing kanamycin. Short-term assays required only one serial transfer; long-term assays required that log-phase growth be extended for approximately 75 generations because of lower plasmid loss rates. All plates were incubated at 30°C, and the percentage of cells maintaining the specific RK2 plasmid derivative was determined by replica plating at least 200 colonies from cells plated (LB with kanamycin) at the various sampling intervals onto selective medium (LB with penicillin). Percent plasmid loss per generation was calculated as previously described (41) and averaged over at least three trials.

Plasmid copy number determination. Relative plasmid copy number was determined essentially according to a modification of the method described by Projan et al. (39). The relative numbers of copies of pRR10ts97 and pRR10 present in mid-log-phase *E. coli* cells grown at 30, 33.5, and 37°C for pRR10 and 30 and 33.5°C for pRR10ts97 were determined. Cell cultures were grown in LB broth at each assay temperature with antibiotic selection and moderate shaking for at least five generations until the optical density at 600 nm was 0.65 to 0.70. The percentage of cells containing the mini-RK2 plasmids at each assay temperature was determined by plating dilution aliquots of each culture on LB agar with and without antibiotic. In all experiments, the percentage of cells containing the plasmid was >99%.

An aliquot of stationary-phase *E. coli*(pRR54) cells was added to each culture to provide an external standard for monitoring DNA recovery. Plasmid DNA was recovered by the alkaline lysis procedure and cleaved by *Hin*dIII, which yields a single band for each plasmid. Restriction-digested DNA was run on a 0.8% agarose gel and stained with ethidium bromide. Gels were photographed on a
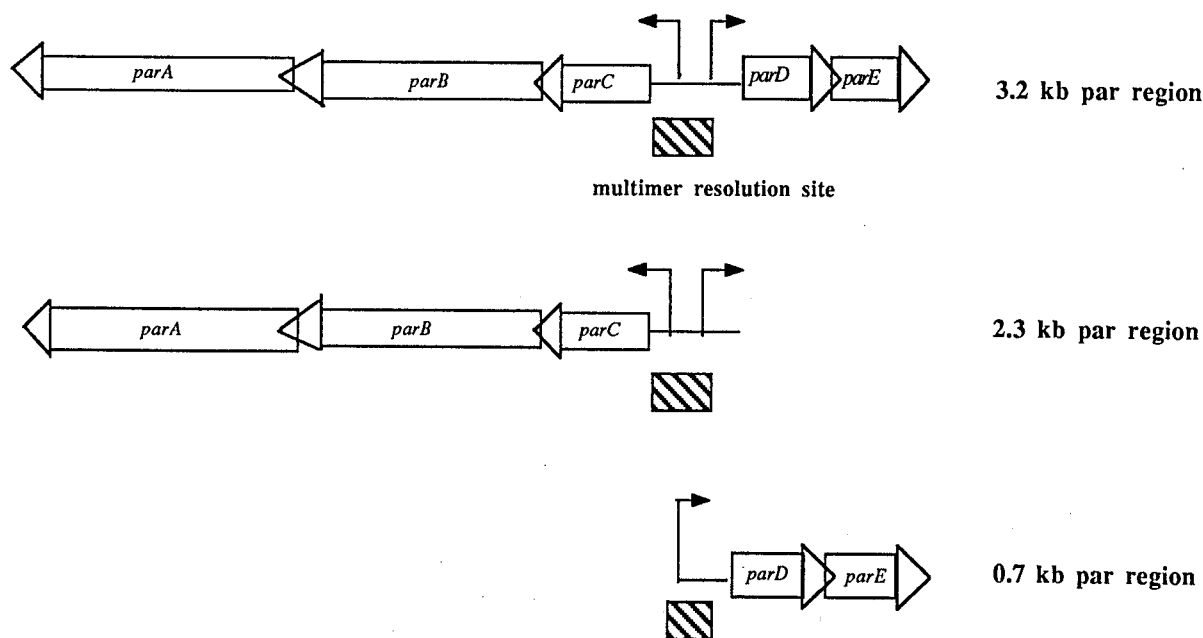
FIG. 1. Genetic structure of the 3.2-kb RK2 minimal stabilization region. The minimal region for complete stabilization is located between coordinates 32.8 and 35.9 on plasmid RK2 (18, 40, 42). The arrows represent the locations and directions of the promoters for the *parCBA* and *parDE* operons. Each hatched box represents the location of the resolvase site. The 3.2- and 2.3-kb regions contain a complete resolvase site. The 0.7-kb region carries only a partial resolvase site.

UV transilluminator with Polaroid 55 (instant positive-negative) film. Negatives were scanned on an LKB UltraScan XL model 2222 laser densitometer (LKB Instruments, Inc., Rockville, Md.). Densitometric analyses of peaks were conducted with Ambis Imaging software (Ambis, San Diego, Calif.).

Analysis of plasmid multimers. The extent of multimerization of plasmids pRR10ts97 and pRR10 with and without the 2.3-kb *parCBA* region was determined by initially isolating plasmid DNA by alkaline lysis and then Southern blotting according to the method of Roberts and Helinski (41). Essentially, 5 ml of cell culture was grown overnight at 30 and 33.5°C, the plasmid DNA was isolated, and approximately equal amounts of DNA were electrophoresed on a 0.8% agarose gel. Standards for the positions of the various forms of plasmid DNA (covalently closed circular, open circular, and linear) were obtained by P1 endonuclease digestions of cesium chloride-purified pRR10 DNA according to the method of Hwang and Kornberg (21) but omitting sodium hydroxide from the stop reaction.

The probe chosen for hybridization with the plasmid DNA was the 169-bp *Dra*I-to-*Bam*HI fragment of *oriV* isolated from pTJS65 (45). This fragment was chosen because it contains a portion of the AT- and GC-rich regions of the *oriV* region of RK2 (bp 533 to 696) but does not contain any of the inverted repeat sequences (47). The probe was labeled by nick translation and hybridized to filters as previously described (30). The labeled filters were autoradiographed with Kodak X-Omat AR 50 X-ray film at −70°C with an intensifying screen.

Enumeration of cell filaments. The morphological properties of cells undergoing loss of plasmids pRR10 and pRR10ts97 with and without the various RK2 *par* regions were examined. Aliquots of cells were removed immediately upon inoculation in LB broth and at periodic intervals during growth at 30, 33.5, and 37°C and spread onto microscope slides. The samples were then negatively stained with 2% Congo red (Sigma), washed with acid alcohol (3 ml of concentrated HCl added to 100 ml of 95% alcohol), and air dried. The lengths of at least 200 cells from each time point were estimated, and the percentage of filamented cells was calculated by dividing the number of filaments by the total number of cells counted. Although log-phase *E. coli* cells were used as controls for maximum normal cell length (i.e., greater than two single cell lengths was considered to represent a filament), filament formation generally was underestimated by counting only those cells with a minimum of three single cell lengths.

RESULTS

Relative contribution of cell killing to mini-RK2 plasmid stability. Several studies have focused on determining the mechanism(s) by which the 3.2-kb *parCBA/DE* region functions to ensure stable plasmid maintenance of RK2 and its derivatives. Previously, the 3.2-kb *par* region has been shown to encode a site-specific recombination system which resolves plasmid multimers (13, 18, 40). However, from studies using mini-RK2 derivatives, it was concluded that dimer resolution contributes little, if at all, to RK2 stability because of the lack of detectable levels of multimer formation even in the absence of a functional *mrs* and the continued plasmid instability of mini-RK2 plasmids which lacked the *parCBA* operon but were constructed to contain another resolution system (13, 18, 41). It has been further proposed that the *parCBA* operon encodes a plasmid partitioning system that contributes to the overall stabilization activity of the 3.2-kb region. Recently Roberts et al. (42) reported the presence of a postsegregational inhibitory system on a 0.8-kb fragment of the 3.2-kb *par* region which contained the *parDE* operon. This inhibitory system arrested and killed plasmid-free segregants that arose during cell division. The present study was undertaken to evaluate the relative contributions of this postsegregational cell-killing system and the putative partition system to overall mini-RK2 plasmid maintenance.

To determine whether a stabilization system in addition to the postsegregational killing system was encoded by the 3.2-kb RK2 *par* operon, initially we constructed *E. coli* strains that carried either the complete 3.2-kb *parCBA/DE* system, the 2.3-kb *parCBA* operon, or a 0.7-kb *parDE* operon on the temperature-sensitive RK2 minireplicon pRR10ts97 (Fig. 1). The pRR10ts97 vector is a replication temperature-sensitive derivative of pRR10 as a result of a mutation in the RK2 *trfA*-encoded replication initiation protein (19a). This mutant RK2 replicon has been used extensively in stability assays because its reduced plasmid copy number facilitates more rapid loss of the minireplicon compared with its wild-type parental plasmid under nonselective growth conditions (40). In the present study, for some of the strains carrying the various RK2 *par* regions, the cell-killing activity of the toxic protein ParE was suppressed by constitutively supplying excess antitoxin protein ParD from a compatible, coresident plasmid. In these cases, antibiotic selection was maintained throughout the assays on either the compatible mini-R6K plasmid (pRR46) carrying the antitoxin

TABLE 2. Stabilization of a temperature-sensitive RK2 minireplicon by RK2 *par* regions in *E. coli* strains grown at the semipermissive temperature of 33.5°C

Plasmid	Stabilization region	Loss rate (% per generation)[a] in strain:			
		TG1[b]	TG1 (*parD*)[c]	MV10Δlac[b]	MV10Δlac (*parD*)[c]
pRR10ts97	None	14.23	15.30	3.67	4.92
pRR41-3.2	*parCBA/parDE*	0.01	14.84	0.02	2.03
pBS41-2.3	*parCBA*	5.01	5.43	4.55	6.51
pRR41-0.7	*parDE*	0.10	5.93	0.00	2.65

[a] Average number of generations is 25 in LB medium, as described in Materials and Methods.
[b] pRR15 in *trans*.
[c] pRR46 in *trans* carrying *parD*.

gene *parD* or the mini-R6K control plasmid, pRR15, which lacks *parD* (41, 42). Using the coresident plasmid system to suppress the cell-killing activity encoded by the *parDE* operon allows detection of additional stabilization functions encoded by the *parCBA* operon.

After 25 generations of nonselective growth (for the mini-RK2 plasmids) at 33.5°C, a semipermissive replication temperature for the *trfA* ts97 allele, the *E. coli* strains TG1 and MV10Δlac exhibited little loss of the minireplicon (pRR10 ts97) carrying either the 3.2-kb or the 0.7-kb *par* region when pRR15 was present in *trans* (>99% of the plasmid retained; Table 2). When ParD was supplied by the coresident plasmid pRR46, however, significant loss rates of pRR10ts97, carrying either the 3.2-kb or the 0.7-kb *par* region, were observed in both *E. coli* strains (Table 2). Although the 2.3-kb *parCBA* region showed no stabilization activity in MV10Δlac at 33.5°C (Table 2), there did appear to be an intermediate level of stabilization activity in *E. coli* TG1 compared with the loss rate of the vector alone. Moreover, the presence of *parD* on pRR46 did not affect this partial stabilization in *E. coli* TG1.

Since the *parCBA* operon provided some degree of stabilization in one of the *E. coli* hosts tested, we questioned whether the use of the temperature-sensitive minireplicon at a semipermissive temperature negatively influenced any stabilizing activity encoded by the *parCBA* operon. Consequently, similar plasmid stability assays were conducted at the permissive replication temperature of 30°C with the same pRR10ts97 derivatives. At this temperature, the *parCBA* region was found to promote efficient plasmid stabilization in both *E. coli* strains when pRR15 was the coresident plasmid (>99% of the plasmid retained; Table 3). The *parCBA/DE* and *parDE* regions also exhibited similar high levels of stabilization at the lower temperature (>99% of the plasmid retained; Table 3). Different stabilization results were obtained with cells grown at 30 and at 33.5°C when the strains contained the coresident plasmid pRR46 which provided the ParD protein. Only those *E. coli* strains carrying the 0.7-kb *par* region, in which the killing

function should be suppressed, exhibited plasmid loss at 30°C. Strains carrying either the 2.3-kb or the 3.2-kb *par* region in the presence of the pRR46 coresident plasmid exhibited little, if any, instability, although presumably the cell-killing mechanism in the case of plasmids carrying the 3.2-kb region was suppressed by the ParD antitoxin protein (>99% of the plasmid retained; Table 3).

These results indicate that a stabilization mechanism other than postsegregational killing contained on the 3.2-kb (*par CBA/DE*) fragment and on the 2.3-kb (*parCBA*) fragment was active in *E. coli* strains carrying the temperature-sensitive plasmid and grown at 30°C. The substantial increase in plasmid pRR10ts97 loss rates (ranging from 6- to 50-fold) at 33.5°C was considered to be due to a decrease in plasmid copy number of pRR10ts97 in the *E. coli* strains grown at this semipermissive replication temperature.

Stabilization of a wild-type mini-RK2 replicon by the *parCBA* operon. To further explore whether the observed differences in the stabilizing capabilities of the 2.3-kb *par* region were the result of a change in plasmid copy number or also involved an effect of higher temperature on the stabilization mechanism, stability assays were carried out with a wild-type mini-RK2 replicon (pRR10). The loss per generation observed for pRR10 ranged from 0.08 to 0.19% in either the absence or the presence of *parD* supplied by a compatible coresident plasmid (Table 4). The insertion of either the *parCBA/DE* or *parDE* region into pRR10 resulted in complete stabilization of this RK2 minireplicon in *E. coli* TG1 grown at the three temperatures (Table 4). Moreover, the 2.3-kb *parCBA* region also effectively stabilized pRR10 in TG1 regardless of the growth temperature (Table 4). When *parD* was supplied by pRR46 to suppress the *parDE*-associated cell-killing activity, only cells carrying the 0.7-kb *par* region exhibited a loss rate comparable to that of cells carrying the control pRR10 plasmid without a *par* insert at the various temperatures (Table 4). In contrast to the previous stability results for cells carrying pRR10ts97 and grown at 33.5°C, suppression of the cell-killing function did not

TABLE 3. Stabilization of a temperature-sensitive RK2 minireplicon by RK2 *par* regions in *E. coli* strains grown at the permissive temperature of 30°C

Plasmid	Stabilization region	Loss rate (% per generation)[a] in strain:			
		TG1[b]	TG1 (*parD*)[c]	MV10Δlac[b]	MV10Δlac (*parD*)[c]
pRR10ts97	None	0.43	0.30	0.48	0.74
pRR41-3.2	*parCBA/parDE*	0.00	0.01	0.00	0.03
pBS41-2.3	*parCBA*	0.06	0.03	0.07	0.00
pRR41-0.7	*parDE*	0.04	0.48	0.00	0.56

[a] Average number of generations is 80 in LB medium, as described in Materials and Methods.
[b] pRR15 in *trans*.
[c] pRR46 in *trans* carrying *parD*.

TABLE 4. Stabilization of a wild-type RK2 minireplicon at
different temperatures by RK2 *par* regions in *E. coli* TG1

Plasmid	Stabilization region	Loss rate (% per generation)[a] at:					
		30°C		33.5°C		37°C	
		−ParD[b]	+ParD[c]	−ParD	+ParD	−ParD	+ParD
pRR10	None	0.13	0.10	0.12	0.19	0.08	0.12
pRR54	*parCBA/DE*	0.00	0.00	0.00	0.02	0.00	0.00
pCE25-2.3	*parCBA*	0.00	0.00	0.00	0.00	0.00	0.00
pBS7-0.7	*parDE*	0.00	0.13	0.00	0.10	0.01	0.17

[a] Average number of generations is 80 in LB medium, as described in Materials and Methods.
[b] pRR15 in *trans*.
[c] pRR46 in *trans* carrying *parD*.

result in the destabilization of the plasmid carrying the 3.2-kb *parCBA/DE* region (Table 4). Similarly, the *parCBA* region also continued to stabilize pRR10 when ParD was provided by the coresident plasmid pRR46 (Table 4). These results indicate that the lowered stabilization activity seen when the 2.3-kb *parCBA* region was inserted into the ts97 RK2 derivative and the cells were grown at 33.5°C is likely due to the lowered plasmid copy number rather than any direct temperature effect on the stabilization mechanism.

Determination of relative plasmid copy number. Although the 2.3-kb *parCBA* fragment was ineffective or only partially effective in stabilizing pRR10ts97 in either *E. coli* MV10Δlac or TG1, respectively, grown at 33.5°C, the 2.3-kb region provided complete stabilization when either strain was grown at 30°C or when the 2.3-kb region was carried by the wild-type mini-RK2 replicon, pRR10 (Table 4). To determine a value or range for the number of copies of either pRR10 or pRR10ts97 possibly necessary for the *parCBA* region to provide efficient stabilization, relative copy number measurements were carried out. The copy number of pRR10ts97 was estimated to be 50% lower in *E. coli* TG1 cells grown at the semipermissive temperature of 33.5°C compared with the copy number in cells grown at 30°C (Table 5). In contrast, significant differences were not observed in the copy number of the wild-type pRR10 vector in TG1 cells grown at 30, 33.5, and 37°C (Table 5). These findings suggest that the temperature effect on the stabilizing activity of the 2.3-kb *parCBA* region is due to the lower plasmid copy number of pRR10ts97 in cells grown at the semipermissive temperature. The rate of loss of the plasmids without the *parCBA* and/or *parDE* region indicates that pRR10 is present at a copy number of approximately 11 per dividing cell [based on the equation $P = 2(0.5^n)$] at all temperatures assayed, while pRR10ts97 is present at a copy number ranging from a low of approximately four to six per dividing cell at 33.5°C to up to eight to nine per dividing cell at 30°C. While these copy numbers are theoretical estimates based on plasmid loss rates, they are consistent with the relative differences seen

TABLE 5. Relative copy numbers of wild-type and temperature-
sensitive RK2 minireplicons in *E. coli* TG1 at different temperatures

Plasmid	Avg relative copy no.[a]		
	30°C	33.5°C	37°C
pRR10	0.93 ± 0.07	1.02 ± 0.05	1.0[b]
pRR10ts97	0.82 ± 0.03	0.47 ± 0.06	ND[c]

[a] Values represent averages of at least three assays ± standard deviations.
[b] Copy number relative to pRR54 as described in Materials and Methods.
[c] ND, not determined.

in the copy number estimates summarized in Table 5. Clonal analyses of pRR10ts97 and pRR10 containing the various DNA fragments of the *par* region indicated that the copy numbers of these two plasmids were similar either with or without the *par* DNA inserts (data not shown).

Filament formation associated with the *par* regions. Recent findings by Jovanovic et al. (24) and Roberts et al. (42) have demonstrated that the loss of the mini-RK2 or intact RK2 plasmid carrying the *parDE* region from *E. coli* results in the formation of filamentous cells. Examples of other plasmid-encoded systems that induce filament formation upon plasmid loss include the *ccd* locus of plasmid F, *pem* of plasmid R100, and *parD* of plasmid R1 (6, 22, 52). These observations were extended to evaluate filamentation levels associated with the *parCBA/DE*, *parCBA*, and *parDE* operons when the *parDE* killing activity was suppressed by providing ParD from a coresident plasmid.

When *E. coli* TG1 was grown at 30°C in LB broth, a higher percentage (1.5- to 4.0-fold) of filamentation was observed with cells carrying plasmid pRR10ts97 with the 0.7-kb *parDE* region than with cells containing plasmid pRR10ts97 carrying either the 3.2-kb or the 2.3-kb *par* region (Fig. 2A). When ParD was provided by the coresident plasmid pRR46 to suppress plasmid-free cell-killing activity, the frequency of filament formation observed with cells carrying a *parDE* plasmid did not differ greatly from that of cells carrying either the *parCBA/DE* or *parCBA* region (Fig. 2A). However, *E. coli* TG1 cells grown at 33.5°C and carrying plasmid pRR10ts97 containing a *parDE* or *parCBA/DE* insert exhibited a higher percentage (>10-fold) of filamentation than cells carrying the 2.3-kb *parCBA* region (Fig. 2B). When the extent of filament formation was determined for cells containing plasmid pRR10 carrying either the *parCBA/DE*, *parCBA*, or *parDE* region insert, only cells carrying the *parDE* operon produced a relatively high level of filaments (Fig. 2C). When ParD was provided by a coresident plasmid, little filamentation was observed in cells containing pRR10 with the 0.7-kb *par* region (Fig. 2C). The results of the filamentation assays indicate that the *parDE* operon, when in the context of the entire 3.2-kb *parCBA/DE* operon, plays a greater role in plasmid stabilization when the *parCBA* operon is less effective or ineffective at plasmid stabilization as in the case of *E. coli* cells carrying the pRR10ts97 plasmid at 33.5°C.

Analysis of the 2.3-kb *parCBA* plasmid constructs for multimer formation. Previous studies by Roberts and Helinski (41) and Gerlitz et al. (18) have shown that the multimer resolution activity encoded by the *parCBA/DE* region appears to contribute little, if at all, to stabilization of mini-RK2 replicons. Moreover, Gerlitz et al. (18) reported that plasmid constructs containing deletions in *parB* and *parC* yet retaining an intact *mrs* were unstable. However, since the majority of these studies were conducted with *E. coli* strains grown at 37°C and carrying plasmids with an insert of either the *parCBA/DE* or *parDE* region, it was necessary to determine the multimeric state of plasmids pRR10ts97 and pRR10 containing the 2.3-kb *parCBA* region in *E. coli* TG1 strains grown at 30 and 33.5°C, respectively, the experimental conditions of this study.

Plasmid DNA was isolated from the various cultures and examined by Southern blotting (Fig. 3). A relatively small percentage of multimers existed in strains carrying either pRR10ts97 or pRR10 and grown at either 30 or 33.5°C, respectively (Fig. 3, lanes 1 to 6, 9, and 10). In addition, the relatively low level of multimers did not significantly increase or decrease with respect to temperature. Moreover, plasmids containing the *parCBA* operon alone appeared to be largely monomeric at both 30 and 33.5°C regardless of the minirepli-
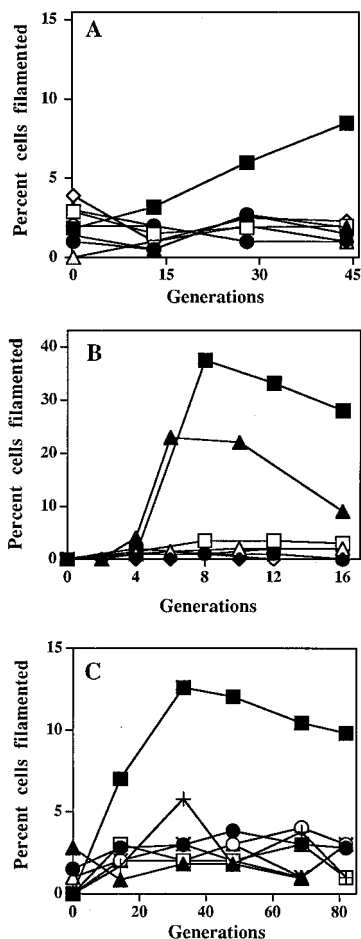
FIG. 2. Extent of cell filamentation with *E. coli* TG1 cells carrying the various RK2 stabilization regions on either pRR10ts97 grown at 30°C (A) and 33.5°C (B) or pRR10 at 37°C (C) in the absence (pRR15) or presence (pRR46) of ParD. The percentage of filamentation in cells carrying pRR15 along with pRR10 (+), pRR10ts97 (◆), or their derivatives carrying the stabilization region *parCBA/DE* (▲), *parCBA* (●), or *parDE* (■) or cells carrying pRR46 along with pRR10 (×), pRR10ts97 (◇), or their derivatives *parCBA/DE* (△), *parCBA* (○), or *parDE* (□) was estimated by counting only cells that appeared to be greater in size than three cell lengths.
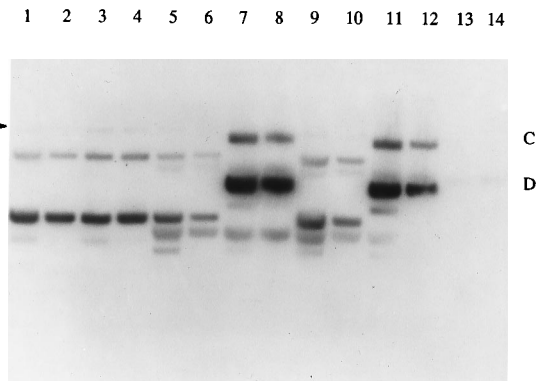

FIG. 3. Analysis of mini-RK2 plasmid multimerization. The effects of temperature and either the presence or absence of the 2.3-kb *parCBA* region on the multimeric state of the wild-type mini-RK2 plasmid pRR10 and the temperature-sensitive plasmid pRR10ts97 were examined by isolation of plasmid DNA from *E. coli* TG1 cells and detecting plasmid forms by Southern blotting as described in Materials and Methods. The various lanes represent plasmid DNA from *E. coli* TG1 cells carrying the following plasmids: lanes 1 and 2, plasmid pRR10ts97 at 30 and 33.5°C, respectively; lanes 3 and 4, plasmid pRR10 at 30 and 33.5°C, respectively; lanes 5 and 6, plasmids pRR10ts97 and pRR15 at 30 and 33.5°C, respectively, lanes 7 and 8, plasmid pRR10ts97 carrying the 2.3-kb *parCBA* stabilization region and pRR15 at 30 and 33.5°C, respectively; lanes 9 and 10, plasmids pRR10 and pRR15 at 30 and 33.5°C respectively; lanes 11 and 12, plasmid pRR10 carrying the 2.3-kb *parCBA* stabilization region and pRR15 at 30 and 33.5°C, respectively; lanes 13 and 14, plasmid pRR15 at 30 and 33.5°C, respectively. Band positions: A, open circular monomer pRR10ts97 and pRR10 (lanes 1 to 6, 9, and 10); B, supercoiled monomer pRR10ts97 and pRR10 (lanes 1 to 6, 9, and 10); C, open circular monomer pRR10ts97 (2.3 kb) and pRR10 (2.3 kb) (lanes 7, 8, 11, and 12); D, supercoiled monomer pRR10ts97 (2.3 kb) and pRR10 (2.3 kb) (lanes 7, 8, 11, and 12). The weak bands below position B in the cultures grown at 30°C are likely pRR10ts97 or pRR10 with a different super-helical density or deleted forms of these plasmids. The arrowhead indicates the expected position of supercoiled dimers of pRR10ts97 and pRR10.

con tested (Fig. 3, lanes 7, 8, 11, and 12). It should also be noted that Roberts and Helinski (41) previously demonstrated that the ColE1 *cer* site can functionally substitute for the RK2 *mrs* and yet was unable to provide effective stabilization of pRR10ts97 in various *E. coli* strains. Therefore, while the resolution of plasmid dimers does contribute to plasmid stabilization in certain plasmid systems (e.g., ColE1 [26]), the role of *mrs* in stabilizing RK2 minireplicons does not appear to be significant under the growth conditions used in this study.

DISCUSSION

An RK2 region which lies between coordinates 32.6 and 35.8 on the RK2 map encodes five genes (*parC, parB, parA, parD,* and *parE*) on two divergently transcribed operons. This region has been shown to promote complete plasmid stabilization in a vector-independent manner (18, 40, 44). In this study, we have attempted to separate the various components of this stabilization system and characterize their relative contributions to mini-RK2 plasmid maintenance.

Results of stabilization assays conducted at 30°C with *E. coli* TG1 and MV10Δlac carrying pRR10ts97 indicated that the *parCBA* operon was indeed capable of promoting plasmid maintenance to approximately the same extent as the *parDE* and the combined *parCBA/DE* operons. However, when the antitoxin gene *parD* was carried on a coresident, compatible plasmid (i.e., pRR15), mini-RK2 derivatives carrying only the *parDE* operon were lost at a high rate, presumably because of the ParD-mediated suppression of cell killing. In contrast, minireplicons containing either the *parCBA/DE* or *parCBA* operon were stably maintained in the presence or absence of the coresident plasmid carrying the *parD* gene, suggesting that although cell killing is an important plasmid-stabilizing mechanism, another mechanism present on the 3.2-kb *par* region is functioning to provide complete plasmid stabilization.

When stabilization assays were carried out at a semipermissive temperature for replication of the ts97 allele, however, we found that the *parCBA* operon alone is not effective in stabilizing the RK2 minireplicon. In contrast, the *parCBA/DE* or the *parDE* operon alone provided effective plasmid-stabilizing activities under these conditions. When ParD was provided by a coresident plasmid, the *parCBA/DE* and *parDE* operons failed to stabilize the plasmid. These results indicate that depending on the particular growth conditions used, cell killing of plasmid-free segregants or a second stabilization mechanism, possibly partitioning, is predominantly responsible for plasmid maintenance.

Stabilization analyses conducted with the wild-type pRR10 minireplicon revealed that all three of the *par* DNA fragments (*parCBA/DE, parCBA,* and *parDE*) used in this study were capable of effectively promoting plasmid maintenance. When

ParD was provided by a coresident plasmid, stabilization activity was suppressed only in the case of plasmids carrying the *parDE* operon insert. Thus, in the case of *E. coli* cells carrying pRR10, although postsegregational cell killing was suppressed when the antitoxin protein ParD was provided by plasmid pRR46, the *parCBA* operon provided a substantial level of plasmid stabilization activity. Together, the results obtained with the minireplicons of RK2 carrying either the wild-type *trfA* gene or the ts97 allele of *trfA* indicate that changes in plasmid copy number can affect the relative contribution of postsegregational cell killing and possibly partitioning to plasmid stabilization. At a relatively low plasmid copy number, the *parCBA*-encoded partitioning mechanism appears to be less efficient, thereby resulting in an increased number of plasmidless cells produced upon cell division. Under these conditions, postsegregational cell killing provides an effective backup mechanism to maintain a plasmid in a growing population of cells. The failure of the *parCBA* operon to provide effective partitioning of pRR10ts97 at the lower copy number, as in the case of cells grown at the semipermissive temperature, may indicate a requirement for a particular threshold copy number (i.e., greater than four to six copies per dividing cell) for efficient stabilization.

Previously published results have indicated that the toxin ParE inhibits the growth of newly formed daughter cells that have lost a plasmid carrying a *parDE* operon (42). Furthermore, this growth inhibition was shown to be associated with cell filament formation and cell killing. We have observed a higher percentage of filamentation with cells carrying the 0.7-kb *parDE* region than with cells containing either the 3.2-kb *parCBA/DE* or 2.3-kb *parCBA* operon. In contrast, when ParD is provided by a coresident plasmid, cell-killing activity is suppressed and the percentage of filamentation observed with cells carrying plasmids with a 0.7-kb insert does not differ greatly from that of cells carrying either the 3.2-kb or the 2.3-kb region. These results are consistent with the idea that the killing mechanism encoded by *parDE* functions as a secondary or backup system when plasmid copy number becomes sufficiently low that the stability function encoded by *parCBA* is no longer effective. In contrast to the findings reported by Roberts and Helinski (41), we observed efficient stabilization of the mini-RK2 replicons with the 0.7-kb *parDE* region regardless of either the growth conditions or the *E. coli* host background tested. This discrepancy between the activity of the 0.8-kb fragment used in the previously published study (41) and that of the 0.7-kb fragment used in this study may be due to a decrease in expression of the *parE* toxin gene as a result of the presence of the *parCBA* promoter on the 0.8-kb fragment.

Although the *parCBA* operon encodes a system for plasmid multimer resolution, previous studies have indicated that this multimer resolution activity alone is unlikely to account for the observed stabilization (13). A possible alternative mechanism for the observed stabilization provided by the *parCBA* operon is active partitioning. A partition system has been proposed for several plasmids, including plasmids F (20, 35, 36), R1/NR1 (23, 49, 50), and P1 (1). In general, most of the partition systems studied to date are contained on a single operon with a *cis*-acting site which acts as an incompatibility determinant and two genes encoding partition proteins capable of functioning either in *cis* or in *trans*. While an in *cis* site that also acts as an incompatibility determinant has not been identified to date in the case of the RK2 *par* region, Sia et al. (46) have reported incompatibility between two compatible plasmids both carrying the *parCBA/DE* region, supporting the hypothesis that a partitioning function is encoded by the RK2 *par* region. In addition, because the partitioning process may be sensitive to

levels of either one or all of the *par* proteins, autoregulation is another important feature common to many plasmid partitioning systems (14, 23). Previous studies have shown that the *parCBA* promoter of RK2 is autorepressed by ParA (10, 12).

Our findings support a model whereby a second stability function, possibly partitioning encoded by the *parCBA* operon, acts as the predominant plasmid stabilization system under conditions of normal growth and plasmid copy number in at least certain *E. coli* strains. When the plasmid copy number decreases, as in the experimental case of cells carrying a temperature-sensitive replicon growing at a semipermissive temperature or perhaps in a different bacterial host, the secondary or backup system of postsegregational cell killing encoded by the *parDE* operon prevents the survival of plasmid-free daughter segregants. It is reasonable to propose that the presence of both stabilization mechanisms encoded by plasmid RK2 ensures the stable maintenance of this plasmid in *E. coli* and other gram-negative bacterial hosts. A recently published study by Sia et al. (46) employing specific deletions of the various regions of the 3.2-kb stabilization region of a relatively intact plasmid RK2 is consistent with different relative contributions of the two mechanisms to the stabilization of plasmid RK2 in different bacterial hosts.

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