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# Contribution of Different Segments of the *par* Region to Stable Maintenance of the Broad-Host-Range Plasmid RK2

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**A 3.2-kb region of the broad-host-range plasmid RK2 has been shown to encode a highly efficient plasmid maintenance system that functions in a vector-independent manner. This region, designated *par*, consists of two divergently arranged operons: *parCBA* and *parDE*. The 0.7-kb *parDE* operon promotes plasmid stability by a postsegregational killing mechanism that ensures that plasmid-free daughter cells do not survive after cell division. The 2.3-kb *parCBA* operon encodes a site-specific resolvase protein (ParA) and its multimer resolution site (*res*) and two proteins (ParB and ParC) whose functions are as yet unknown. It has been proposed that the *parCBA* operon encodes a plasmid partitioning system (M. Gerlitz, O. Hrabak, and H. Schwabb, J. Bacteriol. 172:6194–6203, 1990; R. C. Roberts, R. Burioni, and D. R. Helinski, J. Bacteriol. 172:6204–6216, 1990). To further define the role of this region in promoting the stable maintenance of plasmid RK2, the *parCBA* and *parDE* operons separately and the intact (*parCBA*/*DE*) *par* region (3.2 kb) were reintroduced into an RK2 plasmid deleted for *par* and assayed for plasmid stability in two *Escherichia coli* strains (MC1061K and MV10Δlac). The intact 3.2-kb region provided the highest degree of stability in the two strains tested. The ability of the *parCBA* or *parDE* region alone to promote stable maintenance in the *E. coli* strains was dependent on the particular strain and the growth temperature. Furthermore, the insertion of the ColE1 *cer* site into the RK2 plasmid deleted for the *par* region failed to stabilize the plasmid in the MC1061K strain, indicating that the multimer resolution activity encoded by *parCBA* is not by itself responsible for the stabilization activity observed for this operon. To examine the relative contributions of postsegregational cell killing and a possible partitioning function encoded by the intact 3.2-kb *par* region, stability assays were carried out with ParD provided in *trans* by a compatible (R6K) minireplicon to prevent postsegregational killing. In *E. coli* MV10Δlac, postsegregational killing appeared to be the predominant mechanism for stabilization since the presence of ParD substantially reduced the stability of plasmids carrying either the 3.2- or 0.7-kb region. However, in the case of *E. coli* MC1061K, the presence of ParD in *trans* did not result in a significant loss of stabilization by the 3.2-kb region, indicating that the putative partitioning function was largely responsible for RK2 maintenance. To examine the basis for the apparent differences in postsegregational killing between the two *E. coli* strains, transformation assays were carried out to determine the relative sensitivities of the strains to the ParE toxin protein. Consistent with the relatively small contribution of the postsegregational killing to plasmid stabilization in MC1061K, we found that this strain was substantially more resistant to killing by ParE in comparison to *E. coli* MV10Δlac. A transfer-deficient mutant of the *par*-deleted plasmid was constructed for the stable maintenance studies. This plasmid was found to be lost from *E. coli* MV10Δlac at a rate three times greater than the rate for the transfer-proficient plasmid, suggesting that conjugation can also play a significant role in the maintenance of plasmid RK2.**

A number of plasmid-encoded systems which enable plasmids to be stably maintained during exponential growth in the absence of selection have been described. At least four basic mechanisms for ensuring plasmid stable maintenance have been established: copy number control of replication, an active partition system which consists of an in *cis* site and *trans*-acting proteins, site-specific resolution of plasmid multimers to maximize the number of plasmid monomers for partitioning, and postsegregational killing which prevents plasmid-free daughter cells from arising during cell division (10, 13, 23).

The broad-host-range plasmid RK2 (60 kb) is maintained at the relatively low copy number of five to eight copies/chromosome in *Escherichia coli* (38) and is stably maintained in a wide range of gram-negative bacteria (6, 29, 31, 34, 38). RK2 contains a 3.2-kb region designated *par* which has been shown to provide stable maintenance of RK2 and mini-RK2 replicons (8, 29, 31, 32, 34, 35). The *par* region contains five genes in two divergent operons (8, 29, 35). The first operon, contained within a 2.3-kb fragment, consists of three genes, *parC*, *parB*, and *parA*. The second operon includes two genes, *parD* and *parE*. The ParD and ParE proteins function as an antitoxin and a toxin, respectively, similar in activity to the previously described postsegregational killing systems of the F (*ccd*), R1 (*kis*/*kid*), and P1 (*phd*/*doc*) plasmids (2, 12, 14, 19, 21). ParD is known to combine with and neutralize the toxic effects of the ParE protein (15).

Although a clear understanding of the mode of stabilization provided by the *parCBA* operon of RK2 has not been established, the *parA* gene has been shown to encode a resolvase protein which in conjunction with its multimer resolution (*res*) site is active for resolving plasmid multimers (7, 8, 29). The *res* site is found within a 100-bp region between the two promoters $P_{parCBA}$ and $P_{parDE}$. Earlier work using mini-RK2 replicons indicated that multimer resolution activity specified by the *parCBA* operon by itself does not account for the level of stabilization observed (29, 31, 35). ParB has been shown to be

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TABLE 1. *E. coli* strains and plasmids used

| Strain or plasmid | Genotype and relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| MV10Δlac | *thr-1 leuB6 lacY-1 thi-1 tonA21 supE44 rfvD1* Δ*trpE5* λ$^-$ Δ(*argF-lac*) *deoC1*::Tn*10* | 34 |
| MC1061K | F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL*(Str$^r$) *hsdR2* $r_K^-$ $m_K^+$ *mcrA mcrB1* Km$^r$ | 27 |
| DH5α | F *endA1 hsdR17 supE44 thi1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [Φ80*dlac*Δ(*lacZ*)*M15*] | 16 |
| HB101 | F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20*(Str$^r$) *xyl-5 mtl-1 recA13* | 1 |
| C2110Nal$^r$ | *polA his* rha spontaneous nalidixic acid-resistant mutant | 16 |
| MV10ΔlacNal$^r$ | Spontaneous nalidixic acid-resistant mutant of MV10Δlac | 34 |
| Plasmids | | |
| pRK2526 | RK2 *tetA*::*lacZYA* | 34 |
| pRK21382 | RK2526 Δ*parCBA*/*DE*, Sp$^r$ | 34 |
| pRR71-H | pUC19 derivative containing *parCBA*/*DE* on a *Hin*dIII fragment | This work |
| pRR120-0.7H | pBluescript II SK(+) containing *parDE* on a *Hin*dIII fragment | This work |
| pRK21382-3.2 | pRK21382 containing *parCBA*/*DE* | This work |
| pRK21382-0.7 | pRK21382 containing *parDE* | This work |
| pVW8703 | pUC8Cm containing *traFG* | G. Waters |
| pCE8703 | pVW8703 containing a deletion-substitution within *traG* | This work |
| pCE60 | *traG* mutant, derivative of pRK2526 | This work |
| pCE61 | *traG* mutant, derivative of pRK21382 | This work |
| pCE61-3.2 | *traG* mutant, derivative of pRK21382-3.2, Km$^s$ | This work |
| pCE61-0.7 | *traG* mutant, derivative of pRK21382-0.7, Km$^s$ | This work |
| pBS51 | pUC19 derivative containing *parCBA* on a *Hin*dIII fragment | 35 |
| pCE61-2.3 | pCE61 containing *parCBA*, Km$^s$ | This work |
| pRR46 | Mini-R6K with *parD* inserted into the polylinker region | 35 |
| pCE46 | Cm$^r$ derivative of pRR46 | This work |
| pCE15 | pCE46 deleted for *parD* | This work |
| pMM40 | ColE1 derivative with inducible *tac* promoter | 17 |
| pAS4 | pMM40 containing *parE* with an ATG start and expressed from the *tac* promoter | 15 |
| pAS6 | pMM40 containing *parE* with a TTG start and expressed from the *tac* promoter | 15 |
| pKS490 | pUC8 containing the *cer* site from ColE1 | 36 |
| pCE61-*cer* | pCE61 containing the *cer* fragment from pKS490 | This work |
| pCE490 | pUC8 containing the ColE1 *cer* site from pCE61-*cer* | This work |

an endonuclease, but its contribution to the stable maintenance provided by the *parCBA* operon is not known. It has been proposed that the ParA, ParB, and ParC proteins make up an active partition complex (8, 29, 31, 35).

Initially it was shown that the entire 3.2-kb region carrying either the *parCBA* and *parDE* operons or the *parDE* operon alone provided effective stabilization of an RK2 minireplicon in a variety of gram-negative bacteria (29, 31). In a later study carried out only in *E. coli*, the *parCBA* operon was isolated on a 2.3-kb fragment and shown to be effective in stabilizing a mini-RK2 plasmid (35). The relative contributions of the *parCBA* and *parDE* operons to the highly effective stabilization activity of the whole 3.2-kb region were determined in assays using a mini-RK2 plasmid and found to be dependent on the particular *E. coli* host and the temperature of growth. Recently, deletions were carried out on an intact RK2 plasmid carrying *lacZ* (34) to remove either the entire 3.2-kb region or each operon separately. The various RK2 deletions were then tested for stable maintenance in *E. coli* and five other gram-negative bacteria. Sia et al. (34) found that the relative importance of *parCBA* and *parDE* varied depending on the host bacterium.

In the present study, the stabilization activities of the entire *parCBA*/*DE* region and the *parCBA* and *parDE* operons alone were determined by reinserting these operons, isolated on DNA fragments of lengths of 3.2, 2.3, and 0.7 kb, respectively, into the intact RK2 plasmid deleted for the *par* region. The activities of these operons individually and together were determined in two different *E. coli* hosts that differed in sensitivity to the ParE toxin protein, which is responsible for postsegregational killing by the *parDE* operon. In addition, the stabilizing activity of the intact *parCBA*/*DE* region was determined in the different *E. coli* hosts under conditions where the postsegregational killing activity was prevented by providing the ParD protein in *trans* by using a second compatible plasmid carrying the *parD* gene. This has allowed an assessment of the relative contributions in different *E. coli* hosts of the *parCBA* stabilization system and postsegregational killing (*parDE*) to plasmid RK2 maintenance when both systems are present on the plasmid.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase, T4 DNA ligase, linkers, and shrimp alkaline phosphatase were obtained from commercial suppliers and used as recommended by the manufacturers. 5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal) was purchased from Gold Biotechnology, Inc. (St. Louis, Mo.). [α-$^{32}$P]dATP (3,000 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Isopropyl-β-D-thiogalactoside (IPTG) and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

**Strains and media.** The bacteria and their sources are listed in Table 1. *E. coli* strains were grown in LB medium (22) (GIBCO BRL Scientific, Grand Island, N.Y.). MacConkey agar (Difco Laboratories, Detroit, Mich.) was used for growth of *E. coli* MV10Δlac as noted. Antibiotics for *E. coli* selection were added to final concentrations of 250 μg/ml for penicillin, 50 μg/ml for kanamycin, 50 μg/ml for spectinomycin, 25 μg/ml for chloramphenicol, and 10 μg/ml for nalidixic acid.

**Plasmid construction.** All DNA manipulations such as restriction enzyme digestion, filling in of 5′ overhangs with Klenow fragment, shrimp alkaline phosphatase treatment, DNA ligation, agarose gel electrophoresis, and transformation of *E. coli* have been previously described (20). Plasmid DNA was isolated by either the boiling lysis (11) or the alkaline lysis (20) procedure.

Plasmid pRK21382 (Fig. 1) is an RK2 derivative that has been deleted for the entire 3.2-kb *par* region (34). This plasmid was kindly provided by E. Sia and D.

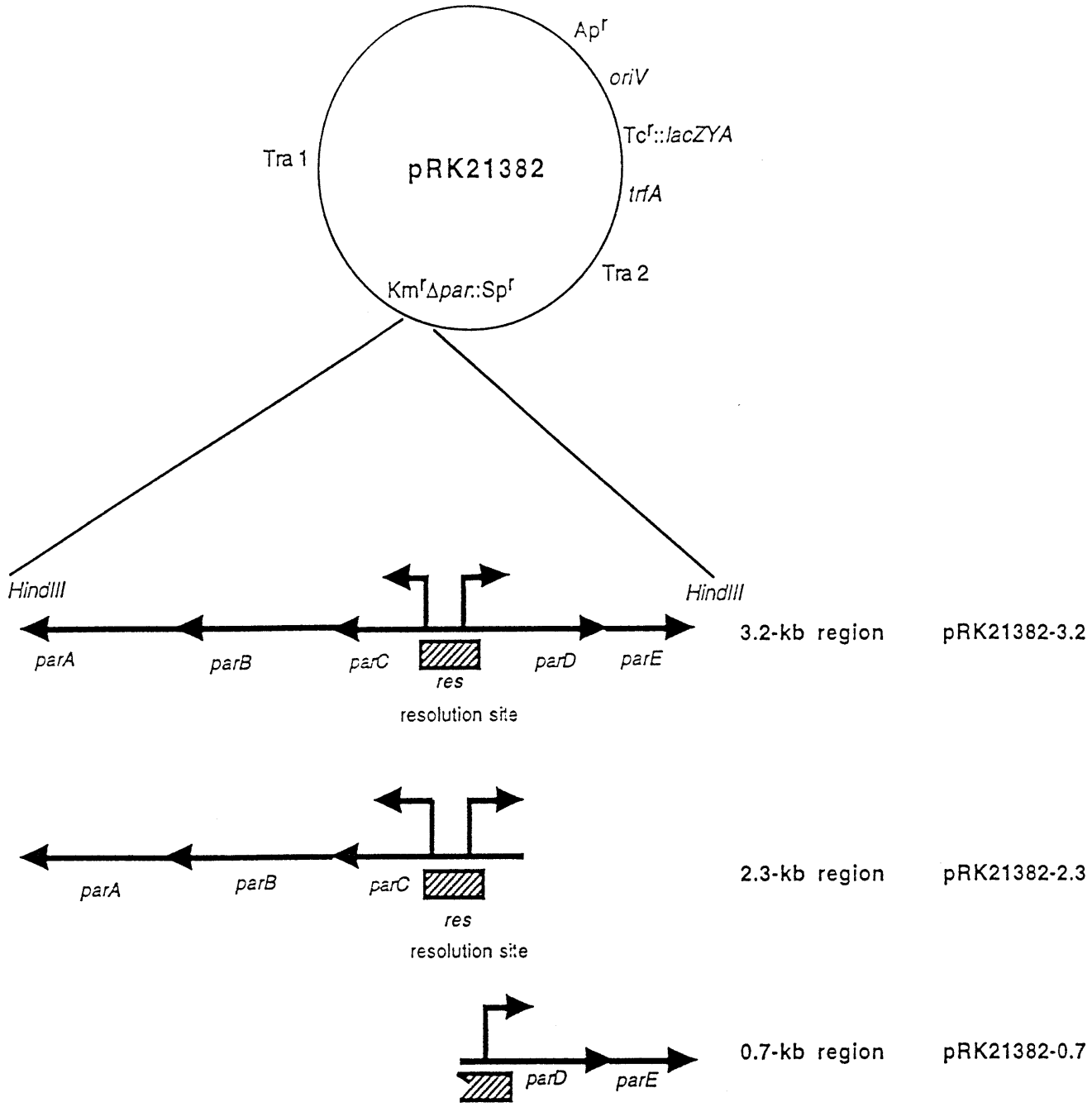


FIG. 1. RK2 carrying different *par* regions. The relative positions of the kanamycin resistance gene ($Km^r$), conjugal transfer regions (Tra1 and Tra2), ampicillin resistance gene ($Ap^r$), origin of replication (*oriV*), replication initiation protein gene (*trfA*), *lacZYA* insertion, and region of insertion of the *par*-containing fragments are indicated. The insertion site of the three *Hin*dIII fragments containing the *par* regions is at the *Hin*dIII sequence within the kanamycin resistance gene, and these insertions result in an inactivation of this gene. The orientation of the insert in each case is not known.

Figurski. pRK21382 carries the *lac* operon which was inserted into the tetracycline resistance gene. Plasmid pRK21382-3.2 is a derivative of plasmid pRK21382 that contains the *parCBA* and *parDE* operons on a 3.2-kb *Hin*dIII fragment. For its construction, plasmid pRR71, a pUC19 derivative which contains the 3.2-kb *par* region of RK2 in the polylinker region (27a), was modified by insertion of *Hin*dIII linkers into the unique polylinker *Eco*RI site of pRR71, which was blunt ended by using *E. coli* DNA polymerase Klenow fragment to generate plasmid pRR71-H. The 3.2-kb *par* region was removed from pRR71-H as a *Hin*dIII fragment and inserted into pRK21382 to generate pRK21382-3.2. The orientation of the 3.2-kb fragment carrying *parCBA/DE* in pRK21382-3.2 was not determined. Plasmid pRK21382-0.7 was constructed by insertion of the *parDE* operon from plasmid pRR120-0.7H into pRK21382. Plasmid pRR120-0.7H is a derivative of plasmid pRR120 [pBluescript II SK(+) derivative containing the *parDE* operon in the polylinker region] (28). The *Xho*I site in the polylinker of pRR120 was filled in with *E. coli* Klenow enzyme, and a *Hin*dIII linker was added. The *parDE* region was isolated as an approximately 0.7-kb *Hin*dIII fragment and inserted into the *Hin*dIII-cleaved pRK21382. Orientation of the 0.7-kb fragment containing the *parDE* region was not determined in pRK21382-0.7.

A conjugally transfer-deficient RK2 plasmid was generated by using plasmid pCE8703, a derivative of plasmid pVW8703. pVW8703 (38a) is a pUC8-Cm vector that contains the *traFG* genes of RK2 cloned into the *Hin*dIII and *Eco*RI

sites of the polylinker. The unique *Not*I site in the *traG* gene of pVW8703 was cleaved, and *E. coli* DNA polymerase Klenow fragment was used to fill in the 5′ overhang. A *Sma*I linker with a β-turn proline codon (TCCCCCGGGGGA) was inserted into the blunt-ended site, resulting in pCE8703, which is $TraG^-$ as a result of the insertion. Replacement of the *traG* gene in the RK2 derivatives pRK21382, pRK21382-3.2, pRK21382-0.7, and pRK2526 with the defective *traG* gene was carried out in vivo as described by Coles et al. (5). The *traG* mutant derivatives with the various *par* regions were designated pCE61, pCE61-3.2, pCE61-0.7, and pCE60, respectively. Plasmid pCE61-2.3 was constructed by insertion of the *parCBA* operon, isolated from pBS51 (35) as a *Hin*dIII fragment, into the *Hin*dIII site of plasmid pCE61. To confirm the presence of the correct RK2 *par* fragments in the conjugal transfer-deficient plasmids, Southern blot analysis was carried out with specific *par* DNA sequences as probes, but the orientation of the fragment was not determined (20).

Plasmid pCE46 was constructed by using plasmid pRR46 (30), a mini-R6K derivative containing the *parD* gene. The chloramphenicol resistance gene was isolated from pBR325 (26) on a 2.0-kb *Hin*dIII and *Hin*cII fragment. This 2.0-kb fragment was blunt ended and then inserted into *Sma*I-cleaved pRR46. The control plasmid, pCE15, was constructed by deleting the *parD* gene from pCE46 by digestion with *Bam*HI and *Kpn*I, blunting the ends, and religation.

For the construction of pCE61-*cer* plasmid pKS490 (33), a pUC8 derivative containing the ColE1 *cer* fragment was digested with *Sma*I, followed by the addition of *Hin*dIII-phosphorylated linkers to yield pKS490H. The *cer* site was then isolated as a *Hin*dIII fragment and inserted into the *Hin*dIII site of pCE61. Positive clones of pCE61-*cer* were then screened by Southern blotting using the *cer* site sequence as a probe. To test the functionality of the *cer* site in pCE61-*cer*, the *cer* site was reisolated from pCE61-*cer* and reinserted into pUC8 to generate plasmid pCE490. Dimers pCE490 and pUC8 were isolated from agarose gels and transformed into *E. coli* HB101 and MC1061K. Plasmid DNA from transformants containing pCE490 and pUC8 was isolated by boiling lysis and run on a 0.85% agarose gel to determine the proportion of dimers to monomers.

**Determination of transfer frequency.** Conjugation experiments were performed on solid media as previously described (39), using *E. coli* MV10Δlac carrying the RK2 plasmid derivatives as donors and MV10ΔlacNal$^r$ (spontaneous nalidixic acid-resistant mutant of MV10Δlac) as the recipient. Cultures were grown to mid- to late log phase, mixed at a donor-to-recipient ratio of 1:4, and vacuum filtered through 0.05-μm-pore-size filters, followed by incubation of the filters on LB plates for 1 h at 37°C. Filters were subsequently placed into 1 ml of 150 mM NaCl and vortexed. Serial dilutions were plated onto selective media to determine the exact number of recipients, donors, and exconjugants. Plates were incubated at 37°C for 16 to 20 h. Frequencies were expressed as the number of exconjugants per recipient cell.

**Plasmid stabilization assays.** Stabilization assays have been previously described (31). Briefly, overnight liquid cultures of *E. coli* containing the various RK2 *traG* derivatives were grown under antibiotic selection at either 30 or 37°C. An aliquot of each culture was diluted $10^4$- to $10^6$-fold in prewarmed LB broth and grown under selection to mid-log phase at either 30 or 37°C. The remaining aliquot of each overnight culture was used to isolate plasmid DNA to confirm the presence of the particular plasmid. At time zero, cells were diluted into LB broth and maintained without selection for 50 to 200 generations of log-phase growth. Aliquots of cells were plated onto either LB agar containing X-Gal (40 μg/ml) or MacConkey agar, depending on the *E. coli* strain and plasmid, to determine the percentage of cells maintaining the plasmid. For the pCE61-*cer* studies, aliquots of cells were plated onto LB agar, and the resulting colonies were tested for antibiotic resistance by replica plating onto LB agar with and without antibiotics in order to determine the portion of cells that had retained the plasmid. Plates were incubated at 37°C for 16 to 20 h. The percent plasmid loss per generation was calculated by using the following formula: $(1 - \sqrt[n]{F_f/F_i}) \times 100$, where $n$ is the number of generations elapsed, $F_i$ is the fraction of cells containing the plasmid at the initial time point, and $F_f$ is the fraction of cells containing the plasmid at the final time point.

**Analysis of plasmid stability with ParD in *trans*.** Assays were carried out as described above except that antibiotic selection was maintained continuously on the mini-R6K derivative carrying *parD* in *trans*. Aliquots of cells were then plated onto MacConkey or LB agar containing X-Gal (40 μg/ml), with antibiotic selection for the R6K derivatives to assess RK2 plasmid stability.

**Toxicity of ParE.** Transformations were carried with 0.25 to 1 μg of plasmid DNA. pMM40 is a ColE1-derived plasmid that contains an inducible *tac* promoter (17). Plasmids pAS6 and pAS4 are derivatives of pMM40 containing the ParE gene with ATG and TTG translational starts, respectively (15). Various dilutions of the transformation mixtures were made in LB liquid and plated on LB agar containing penicillin and with or without 1 mM IPTG and incubated at 37°C for approximately 16 h. The number of transformants per microgram of DNA was determined for each transformation assay.

## RESULTS

**Contribution of conjugal transfer to RK2 stability.** Numerous studies have demonstrated that the broad-host-range plasmid RK2 is capable of high-frequency conjugal transfer among many diverse gram-negative bacteria (9). To determine the relative contribution of conjugal transfer to RK2 stable maintenance, the essential transfer gene *traG*, located in the Tra1 region of RK2 (39), was inactivated by insertional mutagenesis in plasmids pRK2526 (intact RK2 with a *lacZYA* insert in the tetracycline resistance gene), pRK21382 (a derivative of RK2526 with the *par* region deleted), and the *par*-containing derivatives of pRK21382 by homologous recombination with a pUC8 plasmid carrying an insertional mutation in *traG*. The *traG*-defective plasmid construct of the *par*-deleted pRK21382 is designated pCE61. The transfer frequencies of the various RK2 derivatives were determined in *E. coli* MV10Δlac grown on solid LB medium.

TABLE 2. Contribution of conjugal transfer to RK2 maintenance[a]

| Plasmid | Stabilization region | TraG | Transfer frequency | Plasmid loss rate (%/generation)[b] |
|---|---|---|---|---|
| pRK21382 | None | + | $3.4 \times 10^{-1}$ | 1.10 |
| pCE61 | None | − | $<1.0 \times 10^{-6}$ | 3.63 |
| pRK21382-3.2 | *parCBA/DE* | + | $3.6 \times 10^{-1}$ | 0.00 |
| pCE61-3.2 | *parCBA/DE* | − | $<1.0 \times 10^{-6}$ | 0.00 |
| pRK21382-0.7 | *parDE* | + | $1.5 \times 10^{-2}$ | 0.01 |
| pCE61-0.7 | *parDE* | − | $<1.0 \times 10^{-7}$ | 0.15 |

[a] Results reported for transfer frequencies, and plasmid losses per generation are averages from at least two experiments. *E. coli* MV10Δlac served as both donor and recipient for conjugal transfer measurements carried out at 37°C.

[b] Stability assays were carried out at 37°C in LB broth for approximately 100 generations.

Results of the conjugal transfer assays indicated that the mutation in *traG* resulted in an approximately $10^5$ decrease in transfer frequency in comparison to the $traG^+$ plasmid (Table 2). In addition, plasmid loss rates were approximately threefold higher in cells containing pCE61 (*traG par*) than in cells containing pRK21382 ($traG^+$ *par*) (Table 2). By using pCE61 and its derivatives, plasmid-containing cells can be distinguished from plasmid-free cells by the presence of blue versus white colonies that develop after plating on either X-Gal medium or the presence of red versus white colonies that appear on MacConkey agar. A reduction in the ability of the *par*-deleted plasmid to be maintained is also observed when the *traG* mutation is present in the plasmid carrying the *parDE* operon. In the case of the RK2 derivative carrying both *par* operons, there was no effect of reducing conjugal transfer on plasmid stability since both the $traG^+$ and the *traG* derivatives were stably maintained. These results suggest that conjugal transfer reduces the loss rate of the RK2 plasmid when its stabilization mechanisms are not fully functional but that conjugal transfer by itself is not sufficient to compensate for all of the plasmid loss when the entire 3.2-kb *par* region is not present. Sia et al. (34) have obtained similar results in which the essential *traJ* gene of RK2 was mutated and the consequence of this reduction in conjugal transfer was loss rates which were significantly greater in the case of the RK2 derivative deleted for the *par* region.

**Contribution of the *parCBA* and *parDE* operons to RK2 plasmid maintenance.** Several studies have focused on determining the mechanism by which the 3.2-kb *parCBA/DE* region functions to ensure stable plasmid maintenance of RK2 and mini-RK2 replicon derivatives (29, 31, 35). The 0.7-kb *parDE* operon within the 3.2-kb *par* region encodes a postsegregational killing system that either prevents or at least lowers the frequency of production of plasmid-free segregants during cell division (15, 30). The 2.3-kb *parCBA* operon has been shown to encode a site-specific recombination system which resolves plasmid multimers and two proteins with unknown functions

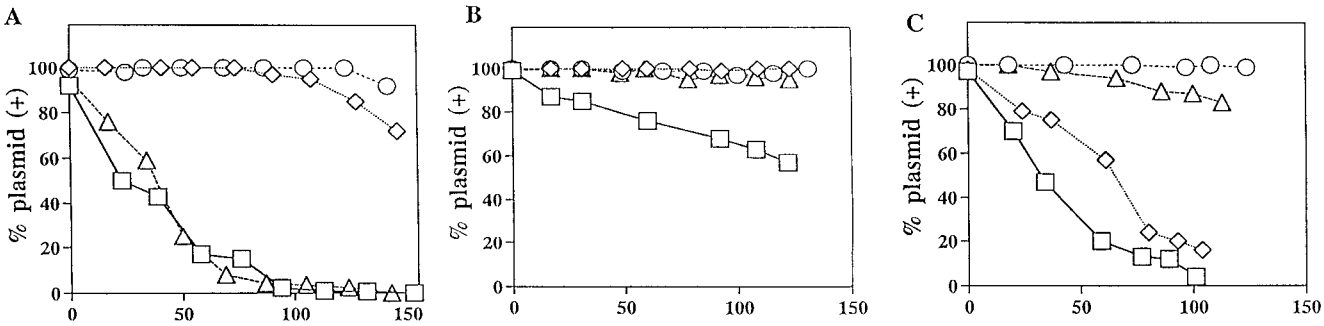


FIG. 2. Stabilization of RK2 by different *par* regions in two *E. coli* strains. (A) Stability in *E. coli* MV10Δlac at 37°C; (B) stability in *E. coli* MV10Δlac at 30°C in LB; (C) stability of *E. coli* MC1061K at 37°C in LB. Plasmids used were pCE61 (□), pCE61-3.2 (○), pCE61-2.3 (△), and pCE61-0.7 (◇).

(7, 8, 29). However, several studies using RK2 minireplicons have concluded that multimer resolution alone is not responsible for stabilization of the minireplicons by the *par* region since the ColE1 *cer* site does not prevent loss of the plasmid in the absence of the *parCBA* operon, nor were detectable levels of multimers observed in these mini-RK2 plasmid stabilization studies (29, 31, 35). In this study, we used an intact RK2 plasmid defective in conjugal transfer to assess the roles of the *parCBA* and *parDE* operons in RK2 plasmid maintenance instead of the minireplicons of RK2 that were used in the earlier studies.

The *traG* derivative of RK2 (pCE61) containing either the 3.2-kb *parCBA/DE*, the 2.3-kb *parCBA*, or the 0.7-kb *parDE* region was transferred into and established in two *E. coli* strains (MV10Δlac and MC1061K). After 100 generations of nonselective growth at 37°C in LB broth, *E. coli* MV10Δlac exhibited little loss of whole RK2 carrying either the *parCBA/DE* or the *parDE* region (Fig. 2A). In contrast, the 2.3-kb *parCBA* region did not provide a high level of stability in *E. coli* MV10Δlac (Fig. 2A). Since Sobecky et al. (35) recently reported that the *parCBA*-mediated stabilization with mini-RK2 replicons as the test system may show some dependency on plasmid copy number or temperature conditions of cell growth, the intact RK2 stabilization assay was also carried out in *E. coli* MV10Δlac at 30°C. The earlier study with minireplicons (35) found that the plasmid copy number of a temperature-sensitive minireplicon at 30°C was approximately twice that at 33.5°C. As shown in Fig. 2B, the 2.3-kb *parCBA* region exhibited a high degree of stabilization activity in *E. coli* MV10Δlac at 30°C. In addition, either *parCBA/DE* or *parDE* alone effectively stabilized the plasmid at 30°C. In the second *E. coli* strain tested, MC1061K, it was the *parDE* region alone which was unable to provide efficient stabilizing activity of intact RK2 when grown at 37°C in LB broth (Fig. 2C). The presence of either *parCBA/DE* or *parCBA* provided greater than 95% plasmid retention for 100 generations in *E. coli* MC1061K (Fig. 2C). Sia et al. (34) similarly found reduced effectiveness of the *parCBA* operon in *E. coli* MV10Δlac grown at 37°C.

**Sensitivity of *E. coli* strains MV10Δlac and MC1061K to the *parE* protein.** It has been previously shown that the *parE* gene encodes a toxic protein which is responsible for the killing of plasmid-free segregants (15, 30). The ParD protein complexes with the ParE protein and acts as an antitoxin to the toxic effects of ParE. Presumably, when a plasmid carrying the *parDE* operon is lost from a cell, ParD is no longer present in sufficient concentrations to prevent the killing action of the ParE protein and the plasmidless cell dies. Kinetics studies using minireplicons of RK2 carrying the *parDE* operon demonstrated a correlation between loss of viability of cells and loss of plasmid-containing cells with time in a growing culture (30). It was previously found that certain *E. coli* strains were more sensitive to the ParE toxin than others (15). The possibility was considered that the substantial difference in effectiveness of the *parDE* operon in stabilizing intact RK2 in the *E. coli* strains MC1061K and MV10Δlac may be due to greater sensitivity of the MV10Δlac strain to the ParE protein. To test this possibility, MC1061K and MV10Δlac were transformed with two different plasmids, pAS4 and pAS6, each containing *parE* under the control of the inducible promoter *tac* (15). Plasmid pAS6 contains the *parE* gene and its native and weaker translational start signal (TTG). Plasmid pAS4 contains the *parE* gene with the stronger translational start signal (ATG). Previous work has shown that plasmid pAS4 produces substantially higher levels of the ParE protein than plasmid pAS6 (15). As shown in Table 3, IPTG induction of either pAS6 or plasmid pAS4 for ParE production resulted in no transformants of *E. coli* MV10Δlac, whereas many pAS6 transformants were obtained with strain MC1061K in the absence of induction. The results suggest that the apparent greater resistance of MC1061K to the ParE protein is the reason for the lesser effectiveness of the *parDE* operon to stabilize the RK2 plasmid in MC1061K than in MV10Δlac.

**Effect of ParD in *trans* on the stability of RK2 plasmids carrying various *par* regions.** The stabilization of plasmids containing the 0.7-kb *parDE* operon is dependent on the ability of ParE to kill plasmid-free segregants that may arise in the population due to either replication or partition errors. Supplying ParD in *trans* allows plasmid-free segregants to survive through the neutralization of ParE toxicity (15, 35). To examine the contribution of the *parDE* operon to the overall stabilization seen for the 3.2-kb region in MV10Δlac and MC1061K, ParD was supplied in *trans* by a coresident plasmid (pCE46) that was maintained by antibiotic selection. As shown

TABLE 3. Sensitivity of *E. coli* strains to ParE[a]

| Plasmid | No. of transformants obtained | | | |
|---|---|---|---|---|
| | *E. coli* MC1061K | | *E. coli* MV10Δlac | |
| | Uninduced | Induced | Uninduced | Induced |
| pMM40 | $2.4 \times 10^5$ | $2.8 \times 10^5$ | $9.0 \times 10^4$ | $1.9 \times 10^5$ |
| pAS6 | $3.4 \times 10^5$ | $3.8 \times 10^5$ | $2.8 \times 10^4$ | 0.0 |
| pAS4 | $4.2 \times 10^5$ | 0.0 | $1.4 \times 10^5$ | 0.0 |

[a] Transformation mixtures were plated on LB agar containing penicillin (250 μg/ml) with and without IPTG (1 mM).

TABLE 4. Stability of RK2 plasmids when ParD is provided in *trans*

| Plasmid | Stabilization region | Loss rate (%/generation)[a] | | | |
|---|---|---|---|---|---|
| | | MV10Δlac | MV10Δlac (ParD)[b] | MC1061K | MC1061K (ParD)[b] |
| pCE61 | None | 5.55 | 4.96 | 3.15 | 2.45 |
| pCE61-3.2 | *parCBA/DE* | 0.00 | 3.02 | 0.16 | 0.17 |
| pCE61-0.7 | *parDE* | 0.60 | 3.20 | 1.58 | 2.23 |

[a] Number of generations ranged from 100 to 200 in LB medium at 37°C.
[b] ParD is provided by plasmid pCE46.

in Table 4, in the case of MV10Δlac, providing ParD in *trans* substantially reduced the stabilization of RK2 by either the 3.2-kb region or the *parDE* operon alone, suggesting that the stabilization activity of the 3.2-kb region at 37°C is largely due to postsegregational killing encoded by the *parDE* operon (Table 4). This finding is consistent with the relative ineffectiveness of the *parCBA* region alone in stabilizing RK2 in this host strain at 37°C (Fig. 2A). In contrast, the stabilization activity of the 3.2-kb region in MC1061K is not affected by the presence of ParD in *trans*, which is consistent with the overall effectiveness of the *parCBA* operon alone in stabilizing the plasmid (Fig. 2C) and the relative insensitivity of MC1061K to the toxic activity of the ParE protein (Table 3). These results, in contrast to those obtained with *E. coli* MV10Δlac, indicate that the stabilization provided by the *parCBA/DE* operons in *E. coli* MC1061K is largely due to the activity of the *parCBA* operon.

**Multimer resolution and its role in stabilization.** Several plasmid systems have been shown to be stabilized by a functional multimer resolution system (4, 18, 25). The ParA protein encoded by the *parCBA* region of RK2 and its *res* site constitute a highly effective system for resolving multimers and are therefore capable of stabilizing plasmids particularly under conditions of high levels of multimer formation and/or low copy number. However, previous studies with mini-RK2 replicons indicated that the stabilization activity of the *parCBA* operon is unlikely to be due in large part to the multimer resolution system within this operon (29, 31, 35). To determine if that was the case for the intact RK2 plasmid, the *cer* site of ColE1 was inserted into the RK2 *par*-deleted plasmid pCE61. As shown in Table 5, whereas the *parCBA* region was highly effective in stabilizing the pCE61 plasmid (0.03% loss per generation of pCE61-2.3 in MC1061K), the presence of the *cer* site produced only a modest increase in stabilization of the plasmid. To prove the functionality of the *cer* site insert in pCE61, the fragment containing the *cer* site was removed from pCE61-*cer* and inserted into a derivative of plasmid pUC8 (pCE490), which was then tested for its ability to monomerize pCE490 dimers in several *E. coli* strains. It was found that the *cer* site was completely functional in resolving dimers of the pUC8 derivative (data not shown). Thus, in the case of the intact plasmid RK2, a functional multimer resolution system cannot replace the activity of the *parCBA* operon in providing stabilization of the RK2 plasmid.

TABLE 5. Determination of stabilization of RK2 by the ColE1 *cer* site in *E. coli* MC1061K

| Plasmid | Stabilization region | Loss rate (%/generation)[a] |
|---|---|---|
| pCE61 | None | 3.36 |
| pCE61-2.3 | *parCBA* | 0.03 |
| pCE61-*cer* | ColE1 *cer* | 2.11 |

[a] Calculated for 150 generations in LB medium at 37°C.

## DISCUSSION

Most of the work to date characterizing the *parCBA/DE* stabilization region of RK2 has been carried out with mini-replicons of RK2. An exception is a recent study using an RK2 plasmid that is intact except for the insertion of a DNA fragment carrying *lacZYA* into the tetracycline resistance gene (34). The importance of examining the role of the entire *par* region as well as the separate *parCBA* and *parDE* operons in providing maintenance of the RK2 replicon in the context of the many other genes and sequences of the intact plasmid led to the present study. This study examines the effects on stable maintenance of reinsertion of these *par* regions into the RK2 *par*-deleted plasmid in the presence and absence of the ParD protein which was provided in *trans* by a second compatible plasmid. Two *E. coli* hosts that display substantial differences in the ability to maintain RK2 carrying either the *parCBA* or the *parDE* operon alone were used. In addition, the ability of the ColE1 *cer* multimer resolution system to replace the *parCBA* operon in stabilizing intact RK2 was examined. The restoration of stabilization activity by the reinsertion of either the intact or parts of the *par* region complements and extends the earlier study using various *par* deletions and allows us to examine in future studies the effect of various point mutations and deletions in the *par* region on stabilization of the intact RK2 plasmid.

For this study, a *traG* mutation was introduced into the *par*-deleted RK2 plasmid to prevent conjugal transfer of the plasmid and therefore minimize the possibility that conjugal transfer from RK2-containing cells to cells that have lost the plasmid would occur and thus mask any instability due to the absence of all or parts of the *par* region. In the course of working with this *traG* mutant of the *par*-deleted plasmid, similar evidence supporting a role of conjugal transfer in plasmid maintenance was obtained by Sia et al. (34).

Stability measurements on constructs of the RK2 plasmid carrying either the entire 3.2-kb *parCBA/DE* region or the *parCBA* and *parDE* operons alone in *E. coli* MV10Δlac and MC1061K clearly demonstrated major strain differences in the activities of the two operons when the cells were grown at 37°C. While the entire 3.2-kb *parCBA/DE* region stably maintained RK2 in both strains, the *parDE* operon was relatively ineffective in *E. coli* MC1061K whereas the *parCBA* operon poorly stabilized RK2 in *E. coli* MV10Δlac at 37°C. At 30°C, however, the *parCBA* operon was quite effective in stabilization of RK2 in *E. coli* MV10Δlac. Sobecky et al. (35) have observed a higher copy number of a mini-RK2 temperature-sensitive replicon at 30°C than at 33.5°C; therefore, we examined the possibility that the intact RK2 plasmid also displayed a higher copy number at a lower temperature. Indeed, the copy number of *par*-deleted RK2 in *E. coli* MV10Δlac at 30°C was found to be 50% greater than when the strain was grown at 37°C (data not shown). This lower copy number at 37°C may at least partly explain the ineffectiveness of the *parCBA* operon to stabilize in *E. coli* MV10Δlac particularly if there is a threshold of plasmid copy number for *parCBA* effectiveness. Alternatively, the function or expression of the *parCBA* operon may show some temperature sensitivity in *E. coli* MV10Δlac or the lower growth rate of this strain at 30°C may affect the activity of the *parCBA* stabilizing system. Plasmid stabilization studies that have been carried out with *Bacillus stearothermophilus*, *E. coli*, and *Rhodococcus equi* have led to the proposal that growth rate,

temperature, and bacterial strains can have an effect on plasmid maintenance (3, 24, 37).

It is clear that *E. coli* MC1061K is significantly less sensitive to ParE killing than *E. coli* MV10Δlac. This is the likely explanation for the relative failure of the *parDE* operon to stabilize RK2 in MC1061K. MV10Δlac, however, exhibits a high level of sensitivity to ParE killing, which likely accounts for the effectiveness of stabilization at 37°C by the *parDE* postsegregational killing system in this particular strain. The basis for this difference in ParE sensitivity exhibited by these two *E. coli* strains is unknown. Sia et al. (34) using the RK2 plasmid with specific deletions of the *par* region similarly found a high level of effectiveness of stabilization at 37°C by the *parDE* region in MV10Δlac and relatively ineffective stable maintenance by the *parCBA* region. However, Sia et al. (34) did not examine other *E. coli* strains. In both this earlier work and the present study, the intact 3.2-kb *parCBA/DE* region is highly effective in stabilizing RK2 in *E. coli* MV10Δlac.

Because of the ability of the ParD protein to complex with and inactivate the ParE protein, it is possible to assess the contribution of the postsegregational killing mechanism to the level of stabilization observed with the intact 3.2-kb *parCBA/DE* region. This was done by establishing in both *E. coli* strains tested (MV10Δlac and MC1061K) an R6K plasmid minireplicon (pCE46) that was compatible with the RK2 replicon and expresses ParD constitutively. Under these conditions, any loss of an RK2 plasmid carrying the *parDE* operon would not result in cell killing by ParE because of the presence of ParD expressed from pCE46 which was stably maintained by antibiotic selection. With this approach, it is clear that the *parDE* operon functions as a postsegregational killing mechanism even in the context of the intact 3.2-kb *parCBA/DE* region and that this killing mechanism plays a major role in the overall stabilization provided by the *parCBA/DE* region in MV10Δlac and a minor role, if any, in MC1061K. Again, the results are consistent with the relative tolerance levels of the two strains to ParE.

Finally, the contribution of multimer resolution in RK2 stabilization by *parCBA/DE* or *parCBA* alone was examined. Previously, it has been shown that the ColE1 *cer* multimer resolution system consisting of the ColE1 plasmid *cer* site and host genes did not increase the stable maintenance of a mini-RK2 replicon. However, the effect of the *cer* multimer resolution system on stabilization of intact RK2 had not been tested previously. It was particularly important to test the effect of *cer* on the stability of the intact RK2 plasmid despite the previous results with the mini-RK2 replicon since it has been shown for the multimer resolution systems *psi* and *loxP* that their ability to replace the *dif* system is dependent on their location in the chromosome (19a). It is clear from the results of the present study that at least in *E. coli* MC1061K, the *cer* system provides at best only a moderate level of stabilization of RK2 under conditions where the *parCBA* region is highly effective. It was further shown that the same *cer* sequence that was inserted into RK2 is functional in resolving a pUC8 plasmid derivative in the same MC1061K strain. It is also worth noting that RK2 naturally carries a functional Tn*1* transposon with an active ParA-like multimer resolution system (4a). These results support a mechanism for stabilization by the *parCBA* region other than multimer resolution of plasmid dimers or higher multimeric forms. Preliminary results with deletions within the *parCBA* region indicate that the *parA* gene is absolutely critical for the stabilization seen with this operon whereas the *parB* and/or *parC* genes in certain *E. coli* strains enhance stabilization by *parA* (6a).

Consistent with the earlier observations of Sia et al. (34), we have found in this study that the 3.2-kb *par* region is highly effective in stabilization of a relatively intact RK2 plasmid in different strains of gram-negative bacteria grown under different conditions. Although the results of both studies indicate that the 3.2-kb *parCBA/DE* is highly effective in many of the host tested, there may be other regions of RK2 necessary for stable maintenance of the plasmid under certain growth conditions or in different hosts. For example, in addition to the *par* region, the *kilE* gene has been shown to be important for RK2 stable maintenance in *Pseudomonas aeruginosa* (40). Moreover, it is clear that the relative contributions of the *parCBA* and *parDE* operons to RK2 stabilization vary depending on the organism, temperature of growth, and possibly copy number. It is perhaps surprising that even within the same bacterial species significant differences can be found in different strains with regard to the effectiveness of the *parCBA* and *parDE* operons in providing stable maintenance of the RK2 plasmid. There is little question, however, that the combined 3.2-kb *parCBA/DE* region has evolved into a highly effective and broad-host-range stabilization system. A major question is the mechanism of stabilization by the intact *par* system beyond postsegregational killing encoded by *parDE*. Of particular interest is the possibility that *parCBA* alone or in conjunction with *parDE* form a complex nucleoprotein structure that ensures plasmid distribution to each daughter cell during cell division by a partitioning-like mechanism.

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