

Role of the *parCBA* Operon of the Broad-Host-Range Plasmid RK2 in Stable Plasmid Maintenance

CARLA L. EASTER,¹† HELMUT SCHWAB,² AND DONALD R. HELINSKI^{1*}

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0322,¹ and Institut fuer Biotechnologie, Technische Universitaet Graz, A-8010 Graz, Austria²

Received 1 June 1998/Accepted 9 September 1998

The *par* region of the stably maintained broad-host-range plasmid RK2 is organized as two divergent operons, *parCBA* and *parDE*, and a *cis*-acting site. *parDE* encodes a postsegregational killing system, and *parCBA* encodes a resolvase (ParA), a nuclease (ParB), and a protein of unknown function (ParC). The present study was undertaken to further delineate the role of the *parCBA* region in the stable maintenance of RK2 by first introducing precise deletions in the three genes and then assessing the abilities of the different constructs to stabilize RK2 in three strains of *Escherichia coli* and two strains of *Pseudomonas aeruginosa*. The intact *parCBA* operon was effective in stabilizing a conjugation-defective RK2 derivative in *E. coli* MC1061K and RR1 but was relatively ineffective in *E. coli* MV10Δlac. In the two strains in which the *parCBA* operon was effective, deletions in *parB*, *parC*, or both *parB* and *parC* caused an approximately twofold reduction in the stabilizing ability of the operon, while a deletion in the *parA* gene resulted in a much greater loss of *parCBA* activity. For *P. aeruginosa* PAO1161Rif^r, the *parCBA* operon provided little if any plasmid stability, but for *P. aeruginosa* PAC452Rif^r, the RK2 plasmid was stabilized to a substantial extent by *parCBA*. With this latter strain, *parA* and *res* alone were sufficient for stabilization. The *cer* resolvase system of plasmid ColE1 and the *loxP*/Cre system of plasmid P1 were tested in comparison with the *parCBA* operon. We found that, not unlike what was previously observed with MC1061K, *cer* failed to stabilize the RK2 plasmid with *par* deletions in strain MV10Δlac, but this multimer resolution system was effective in stabilizing the plasmid in strain RR1. The *loxP*/Cre system, on the other hand, was very effective in stabilizing the plasmid in all three *E. coli* strains. These observations indicate that the *parA* gene, along with its *res* site, exhibits a significant level of plasmid stabilization in the absence of the *parC* and *parB* genes but that in at least one *E. coli* strain, all three genes are required for maximum stabilization. It cannot be determined from these results whether or not the stabilization effects seen with *parCBA* or the *cer* and *loxP*/Cre systems are strictly due to a reduction in the level of RK2 dimers and an increase in the number of plasmid monomer units or if these systems play a role in a more complex process of plasmid stabilization that requires as an essential step the resolution of plasmid dimers.

Plasmids use a variety of mechanisms to maintain themselves within bacterial populations. Plasmid-encoded systems for stable maintenance include copy number control, active partitioning systems, multimer resolution, and postsegregational killing (18). RK2, identical to RP4, is a 60-kb broad-host-range plasmid that exists in *Escherichia coli* at five to eight copies/chromosome (40). It is a member of the IncPα family of plasmids and can be stably maintained in a wide range of gram-negative bacteria (12, 33, 37, 38, 40). A stability region of RK2 designated *par* is comprised of five genes in two divergently oriented autoregulated operons, *parCBA* and *parDE* (15, 33, 38). The coding frames in each of the two operons are translationally coupled. *parDE* encodes a postsegregational killing system and comprises a 0.7-kb region. The ParD protein acts as an antitoxin to neutralize the toxic effects of the ParE protein (22). This system has a function similar to that of the postsegregational killing systems of plasmids F (*ccd*), R1 (*kis/kid*), R100 (*pemI/pemK*), and P1 (*phd/doc*) in ensuring that

plasmid-free cells that may arise after cell division do not survive (7, 14, 20, 21, 24, 25, 28).

The *parCBA* operon comprises a 2.3-kb region and encodes three proteins, ParA, ParB, and ParC. The ParA protein, along with its *res* site, located between promoters P_{*parCBA*} and P_{*parDE*}, functions as a site-specific recombination system homologous to the Tn3 family of resolvases (13). *parB* encodes a nuclease with homology to a *Staphylococcus* nuclease (9, 17a). The function of ParC has yet to be determined. The role of these three proteins in stabilizing RK2, other than multimer resolution by ParA, is not known. It has been proposed that these three proteins together make up or contribute to an active partitioning complex (15, 17a, 33, 37, 38).

Recently, it was shown that the *parCBA* region is capable of stabilizing both minireplicons of RK2 and the intact RK2 plasmid in *E. coli* and several other gram-negative bacteria (12c, 37). These studies involved the use of an RK2 plasmid with deletions of the *par* operons (37) and reinsertion of the intact *parCBA* and *parDE* operons together or separately into the intact RK2 plasmid with *par* deletions (12c). It was found that the *parCBA* region could stabilize RK2 but that the level of stability provided was dependent on the strain and the growth conditions under which the assays were carried out. In order to further characterize the *parCBA* operon and its role in the stabilization of RK2, precise deletions which maintained the translational coupling arrays were made in each of the three

* Corresponding author. Mailing address: Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0322. Phone: (619) 534-3638. Fax: (619) 534-0559. E-mail: helinski@biomail.ucsd.edu.

† Present address: Department of Molecular Microbiology, School of Medicine, Washington University, St. Louis, MO 63110-1093.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype and other relevant characteristics	Source or reference
Strains		
<i>Escherichia coli</i>		
MV10	<i>thr-1 leuB6 lacY1 thi-1 tonA21 supE44 rfbD1 ΔtrpE5 λ⁻</i>	37
MV10Δlac	MV10 Δ(<i>argF-lac</i>) <i>deoC1::Tn10</i> (Tet ^r)	37
MC1061K	F ⁻ <i>araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac) X74 rpsL</i> (Strs ^r) <i>hsdR2 r_K m_K⁺ mcrA mcrB1 Km^r</i>	32
RR1	F ⁻ Δ(<i>gpt-proA</i>)62 <i>leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20</i> (Str ^r) <i>xyl-5 ml-1</i>	27
DH5 α	F ⁻ <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZY-argF)U169 deoR [80dlacΔ(lacZ)M15]</i>	23
DPB635	Wild type	6
DBP636	DPB635 <i>topA66</i>	6
<i>Pseudomonas aeruginosa</i>		
PAO1161Rif	Spontaneous Nal ^r Rif ^r derivative of PAO1161	5a
PAC452Rif	Spontaneous Rif ^r derivative of PAC452	31
Plasmids		
pCE61	Derivative of RK21382 with <i>parCBA</i> and <i>parDE</i> deletions	12a
pCE61-2.3	pCE61 containing <i>parCBA</i>	12a
pCE61-91	pCE61 containing <i>parA</i>	This work
pCE61-92	pCE61 containing <i>parAB</i>	This work
pCE61-94	pCE61 containing <i>parAC</i>	This work
pCE61-95	pCE61 containing <i>parBC</i>	This work
pVW8703	pUC8Cm containing <i>traFG</i>	42
pAL4000	RSF1010 derivative with promoterless luciferase gene	16
pTD10	pAL4000 with <i>parCBA</i> promoter	12
pEKA28	IncQ (RSF1010) pMHL3 derivative	36b
pEKA30	Derivative of pEKA28 containing Cre	36b
pEKA28G	Gentamicin-resistant derivative of pEKA28	This work
pEKA30G	Gentamicin-resistant derivative of pEKA30	This work

structural genes, and the resulting deletion derivatives were cloned into RK2 with *par* deletions. The stability of these various constructs in three strains of *E. coli* and two strains of *Pseudomonas aeruginosa* was then determined. Furthermore, the ability of two multimer resolution systems, *cer* of the ColE1 plasmid and *loxP*/Cre of the P1 plasmid, to replace the *parCBA* operon for plasmid stabilization was assessed. It is clear from the results obtained that *parA*, along with its *res* site, can in some circumstances provide a significant level of plasmid stabilization in the absence of *parB* and *parC* in both *E. coli* and *P. aeruginosa*. In addition, the *loxP*/Cre multimer resolution system provides a substantial level of stability in the absence of the *parCBA* operon in all three *E. coli* strains. It is not clear from the results whether these different resolution systems act solely to increase the number of plasmid monomer units for segregation or whether they play a role in a more complex process of stabilization of plasmid RK2 during cell growth and division.

MATERIALS AND METHODS

Materials. Restriction endonucleases, the 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. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Strains and media. The bacteria used and their sources are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium (29) (GIBCO BRL Scientific, Grand Island, N.Y., or Sigma). Antibiotics used for plasmid selection with *E. coli* 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, 10 μg/ml for tetracycline, 15 μg/ml for gentamicin, and 10 μg/ml for nalidixic acid. *P. aeruginosa* strains were grown in nutrient broth (NB) (Difco Laboratories, Detroit, Mich.). Antibiotics used for plasmid selection with *P. aeruginosa* were added to final concentrations of 500 μg/ml for spectinomycin, 100 μg/ml for carbenicillin, and 75 μg/ml for rifampin.

Plasmid constructions. All DNA manipulations, such as restriction enzyme digestion, filling in of 5' overhangs with the Klenow fragment, shrimp alkaline phosphatase treatment, DNA ligation, agarose gel electrophoresis, and transformation of *E. coli*, have been described elsewhere (27). Plasmid DNA was isolated by either the boiling lysis (19) or the alkaline lysis (27) procedure. Construction of the pCE61 and pCE61-2.3 plasmids has been described elsewhere (12c). Several deletion constructs in which different *Par* proteins were truncated while retaining translational coupling were constructed. A derivative of pCE61 carrying the *parCBA* operon with deletions in both *parC* and *parB* was constructed as follows. An *SphI*-*BamHI* fragment carrying the *parA* gene was isolated from plasmid pGMA7 (15) and ligated to pUC18 digested with *SphI* and *BamHI*, resulting in plasmid pUCGMA7. The *BamHI* fragment from pGMA41 (15) containing the promoter *P_{parCBA}* and the *parD* gene was isolated and ligated to *BamHI*-digested pUCGMA7. The resulting plasmid, containing the *BamHI* fragment from pGMA41 in the correct orientation, was designated pCESH201. In order to delete the *parD* gene, pCESH201 was digested with *AflIII* and filled in, and then *HindIII* linkers were added. The resulting plasmid, pCE91, was digested with *HindIII*, and the *HindIII* fragment carrying *parA* plus the deletions in *parB* and *parC* (bp 1023 to 1701) (15) was ligated to *HindIII*-digested pCE61. The resulting plasmid was designated pCE61-91.

Construction of a plasmid carrying *parCBA* with a deletion in the *parB* gene (bp 1018 to 1380) (15) was carried out by first isolating a *BamHI*-*SphI* fragment containing *parA* and part of the *parB* gene from pGMA45 (15) and ligating that fragment to pUC18 digested with *BamHI* and *SphI* to produce plasmid pUCGMA45. pUCGMA45 was digested with *BamHI* and ligated with the *BamHI* fragment from pGMA45 (15) which contained the *parC* and *parD* genes. The orientation of the *BamHI* fragment was checked by clonal analysis, and the clone with the correct orientation was designated pCESH202. pCESH202 was digested with *AflIII*, filled in, and linked to *HindIII* to remove *parD*. The resulting plasmid, designated pCE92, was used for the cloning of pCE61-92. pCE61-92 was produced by insertion of the *HindIII* fragment from pCE92 carrying the *parCBA* region with a deletion in *parB* into *HindIII*-digested pCE61.

Construction of the *parC* deletion derivative (bp 1549 to 1701) (15) was carried out in the following manner. First, a plasmid carrying a truncated *parC* was constructed by insertion of a *SalI*-*RsaI* fragment from pGMA30 (15) containing portions of *parB* and *parC* into *SalI*-*EcoRV*-digested pBSkII (cloning vector from Stratagene). The resulting plasmid, pBS-Sal/Rsa, was digested with *EcoRI*, and the unique site was filled in to produce plasmid pBS-Sal/RsaE*. Plasmid pUCGMA16 was constructed by insertion of an *SphI*-*BamHI* fragment from

pGMA16 (15) containing the *parCBA* operon into pUC18 digested with *SphI* and *BamHI*. The *Sall-BamHI* fragment from pBS-Sal/RsaE* was ligated with *Sall-BamHI*-digested pUCGMA16. The resulting plasmid was designated pUCGMA16R. The *BamHI* fragment from pGMA41 was isolated and ligated to *BamHI*-digested pUCGMA16R. The resulting plasmid, pCESH204, was digested with *AflII*, filled in, and linked with *HindIII* to remove *parD* and to add a *HindIII* site. The resulting *HindIII*-linked plasmid was designated pCE94 and was used for the subsequent insertion of the *parCBA* operon with a *parC* deletion into the intact RK2 plasmid pCE61. The *HindIII* fragment from pCE94 was isolated and inserted into *HindIII*-digested pCE61. The resulting plasmid was named pCE61-94.

In order to construct a *parCBA* operon with deletions of segments of the *parA* gene (bp 692 to 940) (15), several intermediate plasmids were constructed. An *SphI-BamHI* fragment from plasmid pGMA49 (15) carrying *parB* and a portion of *parA* was isolated and inserted into pUC18 digested with *SphI* and *BamHI*. The resulting plasmid was designated pUCGMA49. Plasmid pGEM5-*parB** was used for the next step. To construct pGEM5-*parB**, pGEM5-*parB*, which carries the *parB* gene (36a), was digested with *SacII* and *AvaI*, and oligonucleotides containing a *BamHI* site and *SacII* and *AvaI* ends (GGATCCCCGACTTCACCGAGTTGCAGGTCGGCGGGATCGCGC) (CGGATCCCCGCGACCTGCAACCTGGTGAAGTCGGGATCCGCC) were inserted into the compatible ends. The resulting plasmid, pGEM5-*parB**, was digested with *BamHI* and *SacI* and inserted into the *BamHI-SacI* sites of pUCGMA49 to produce plasmid pUCGMA49A⁻. A *Sall-EcoRI* fragment from plasmid pGMA27SB (this work), which is a pUC18 derivative carrying the *parB*, *parC*, and *parD* genes on the *Sall-BamHI* fragment from pGMA27 (15), was isolated and inserted into *Sall-EcoRI*-digested pUCGMA49A⁻. We designated the resulting plasmid pCESH205. pCESH205 was digested with *AflIII*, filled in, and linked with *HindIII* to remove *parD* and to add a *HindIII* site (pCE95). The *HindIII* fragment carrying the *par* region with the deletion in *parA* was isolated and inserted into pCE61 digested with *HindIII*. The resulting construct was designated pCE61-95. The pCESH series of pUC18 derivatives was checked for the integrity of the downstream genes by an in vitro assay for ParB nuclease activity (17a). The integrity of the deletion derivatives of the RK2 plasmid was examined by an in vivo assay for ParA resolvase activity (33).

Plasmid pEKA28 (36c) is a tetracycline-resistant derivative of plasmid pMHL3 (a derivative of plasmid RSF1010) and was constructed by inserting the tetracycline resistance gene into the unique *HindIII* site of pMHL3. pEKA30 (5) is a derivative of pEKA28 which contains the *cre* gene of P1 inserted into the unique *EcoRI* site of pEKA28. pEKA28 and pEKA30 were kindly provided by E. Sia and D. Figurski. Gentamicin-resistant plasmids were constructed by insertion of the gentamicin resistance gene carried on a *HindIII* fragment from plasmid pUC19-G/S (41). Plasmid pUC19-G/S is a derivative of pUC19 which contains the gentamicin resistance gene (36) cloned into the multiple cloning region of pUC19. Plasmids pEKA28G and pEKA30G, gentamicin-resistant derivatives of plasmids pEKA28 and pEKA30, respectively (5), were constructed by digestion with *HindIII* and insertion of the gentamicin resistance gene carried on a *HindIII* fragment from pUC19-G/S.

Conjugal plasmid transfer. Plasmid pCE61 and its derivatives were established in *P. aeruginosa* PAC452Rif^r and PAO1161Rif^r by conjugal mating with *E. coli* DH5 α carrying both the specific pCE61 derivative and pVW8703 (pUC8-Cm derivative and source of the TraG protein) (42). pCE61 and its derivatives carry a mutation in *traG* and are therefore unable to transfer unless TraG is provided in *trans*. Exconjugants were selected with NB agar containing rifampin, spectinomycin, and carbenicillin.

Plasmid stabilization assays. Stabilization assays were previously described (34). Overnight liquid cultures of *E. coli* or *P. aeruginosa* containing the various RK2 derivatives were grown under antibiotic selection at 30 or 37°C. A portion of each culture was diluted 10⁻⁴ to 10⁻⁶-fold in prewarmed LB broth for *E. coli* or NB for *P. aeruginosa* and grown under antibiotic selection to the mid-log phase at 30 or 37°C. The remaining portion of each overnight culture was used to isolate plasmid DNA to confirm the presence and integrity of the particular plasmid. At time zero, cells were diluted into antibiotic-free LB broth or NB and maintained without selection for 50 to 200 generations of log-phase growth. Portions of the cell cultures were plated onto antibiotic-free LB agar for *E. coli* strains or antibiotic-free NB agar for *P. aeruginosa* strains and then replica picked onto LB or NB agar with and without the appropriate antibiotic(s) to determine the percentage of cells maintaining the plasmid. The percent plasmid loss per generation was calculated as described previously (12c).

Luciferase assay. *E. coli* MV10 (the tetracycline-sensitive parent of MV10 Δ lac) and RR1 carrying the various RK2 plasmids were transformed with plasmids pAL4000 and pTD10 (12, 16). Strains were grown to the mid-log phase at 30°C, and luciferase assays were carried out with a luciferase assay system from Promega (product no. E1500) and following manufacturer recommendations. Activity was measured with a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, Calif.).

Analysis of plasmids carrying the *loxP/Cre* system of P1. The ability of the P1 multimer resolution system *loxP/Cre* to stabilize pCE61 and its derivatives was tested as follows. Plasmid pCE61 contains the *loxP* site of P1 (5, 37). pCE61 was established in *E. coli* RR1 along with the RSF1010 plasmids pEKA28 (vector) and pEKA30 (vector with Cre protein expressed from its native promoter). pEKA28G and pEKA30G (constructed as described above) were established in

E. coli MV10 Δ lac and MC1061K along with pCE61. *E. coli* MV10 Δ lac and MC1061K containing plasmids pCE61-2.3 and pCE61-91, carrying the *parCBA* and *parA* genes, respectively, along with either pEKA28 or pEKA28G were also constructed. Stability assays were carried out as described above with LB medium at 30°C, except that selection was maintained on the pEKA28 and pEKA30 plasmids or their gentamicin-resistant derivatives. The stability of the pCE61 plasmid and its derivatives in the presence of the pEKA28 and pEKA30 plasmids or their gentamicin-resistant derivatives was assessed by replica plating onto LB agar plates containing spectinomycin and calculating the percentage of cells retaining the various pCE61 plasmids.

RESULTS

Effects of deletions within the three structural genes of the *parCBA* operon on RK2 maintenance in three *E. coli* strains. Previous studies that used the intact RK2 plasmid examined the contributions to stability of the intact *par* region and the *parCBA* and *parDE* operons separately (12c, 37). These earlier studies showed that the intact *par* region provided a high level of stability to RK2 under all growth conditions and in all strains tested. The abilities of the two individual *par* operons to stabilize RK2 were dependent on the strain and growth conditions. To determine the requirement of each of the three genes within the *parCBA* operon for the stabilization of RK2 maintenance by this operon, deletion derivatives of the *parCBA* operon were constructed and inserted into plasmid pCE61 (Fig. 1). Since the three genes in the *parCBA* operon are translationally coupled (15), the deletions were constructed in a manner that maintained the translational reading frame of each gene containing a deletion. Plasmid pCE61 itself is a derivative of RK2 with a deletion of the entire *par* region and containing defective *traG*, so that it is incapable of conjugal transfer. The stability of pCE61 with the various inserts was tested in three *E. coli* strains, MC1061K, RR1, and MV10 Δ lac. Previously, it was shown that the intact *parCBA* operon by itself was very effective in stabilizing pCE61 in MC1061K but relatively ineffective in MV10 Δ lac. When various deletion derivatives of the *parCBA* operon were tested in MC1061K, there was a substantial reduction of stabilization as a result of a deletion in *parC* or *parB* and a total loss of stabilization with a *parA* deletion (Table 2). For *E. coli* RR1, the intact *parCBA* operon by itself, as in the case of MC1061K, provided a high level of stable maintenance of plasmid pCE61. In this strain, however, a deletion within the *parC* or *parB* gene had relatively little effect on the stabilization activity of the *parCBA* operon (Table 2). However, deletions in both the *parB* and the *parC* genes significantly reduced the effectiveness of the *parCBA* operon. Finally, the stability of pCE61 carrying intact *parCBA* and deletion derivatives of *parCBA* was tested with *E. coli* MV10 Δ lac. As shown before (37), the *parCBA* operon was found to be relatively ineffective in stabilizing pCE61 in this *E. coli* strain. A deletion in *parC*, *parB*, or both had no effect on the low level of stabilization seen with the *parCBA* operon, and a deletion in *parA* resulted in a complete loss of *parCBA* stabilization activity. Thus, in two strains in which the *parCBA* operon was effective, MC1061K and RR1, deletions in *parB*, *parC*, or both somewhat reduced (approximately twofold) the stabilization activity of the operon, while a deletion in *parA* resulted in a much greater loss of stabilization activity.

Contributions of the *parCBA* region to stability in two strains of *P. aeruginosa*. Since RK2 is a broad-host-range plasmid with the ability to be stably maintained in a wide range of gram-negative bacteria, we decided to test the effect of deletions in both the *parB* and the *parC* genes on *parCBA* activity in two strains of *P. aeruginosa*, PAC452Rif^r and PAO1161Rif^r. pCE61 and its derivatives were established in each strain, and the stability of the plasmid was assessed over 75 to 100 generations. For strain PAC452Rif^r, the *parCBA* operon or a *parC parB*

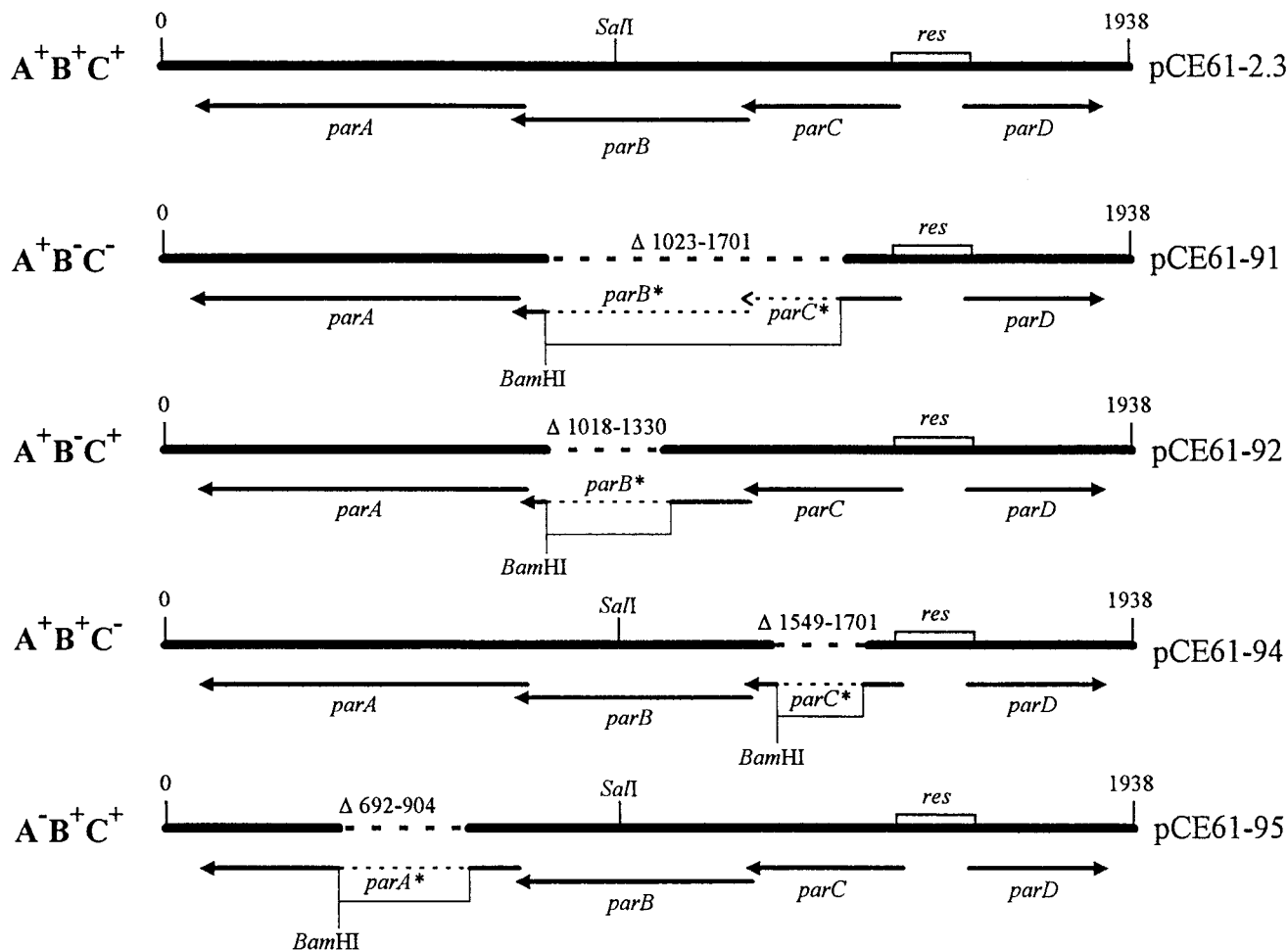


FIG. 1. RK2 derivatives carrying different deletions in the *par* region. The site of insertion of the fragments carrying deletions in the *par* region was at the *Hind*III sequence within the kanamycin resistance gene, as described in Materials and Methods. Insertions at this site result in inactivation of the kanamycin resistance gene. The orientation of the insert in each case is not known. The positions of the various deletions have been described by Gerlitz et al. (15).

parA⁺ deletion derivative provided a significant level of stabilization (Fig. 2A). After 100 generations, greater than 75% of the cells retained the plasmid carrying *parC*⁺ *parB*⁺ *parA*⁺ (pCE61-2.3) or *parC* *parB* *parA*⁺ (pCE61-91). For strain PAO1161Rif^r, neither *parCBA* nor *parC* *parB* *parA*⁺ was able to provide a substantial level of stabilization (Fig. 2B). Less than 25% of the cells contained the plasmid after approximately 75 generations of growth. It is of interest that in both of these strains, the intact *par* region (*parCBA* and *parDE*) functioned very well in stabilizing the pCE61 plasmid in that

greater than 95% of the cells retained the plasmid after 100 generations of growth (data not shown).

Assessing ParA levels in vivo for the various deletion constructs. Various constructs that contained a deletion in either *parB* or *parC* or both were designed to minimize the effects of the deletions on the downstream expression of ParA levels. This fact is of importance to ensure that any effect of a deletion in the upstream *parC* and *parB* genes is not the result of a change in the amount of ParA provided by the translationally coupled operon. To test the activity and relative amounts of

TABLE 2. Stability of RK2 plasmids carrying deletion derivatives in three *E. coli* strains

Strain	Rate of loss (% per generation) ^a of:					
	pCE61 (<i>parCBA</i>)	pCE91-2.3 (<i>parC</i> ⁺ <i>B</i> ⁺ <i>A</i> ⁺)	pCE61-91 (<i>parCBA</i> ⁺)	pCE61-92 (<i>parC</i> ⁺ <i>BA</i> ⁺)	pCE61-94 (<i>parCB</i> ⁺ <i>A</i> ⁺)	pCE61-95 (<i>parC</i> ⁺ <i>B</i> ⁺ <i>A</i>)
MC1061K	0.65	0.04	0.27	0.23	0.22	0.57
RR1	0.52	0.07	0.19	0.03	0.04	0.35
MV10 Δ lac	0.85	0.45	0.33	0.35	0.33	0.88

^a Stability assays were carried out at 30°C for an average of 50 to 100 generations. The results are from a single experiment; however, essentially similar results were obtained when the experiment was repeated two more times.

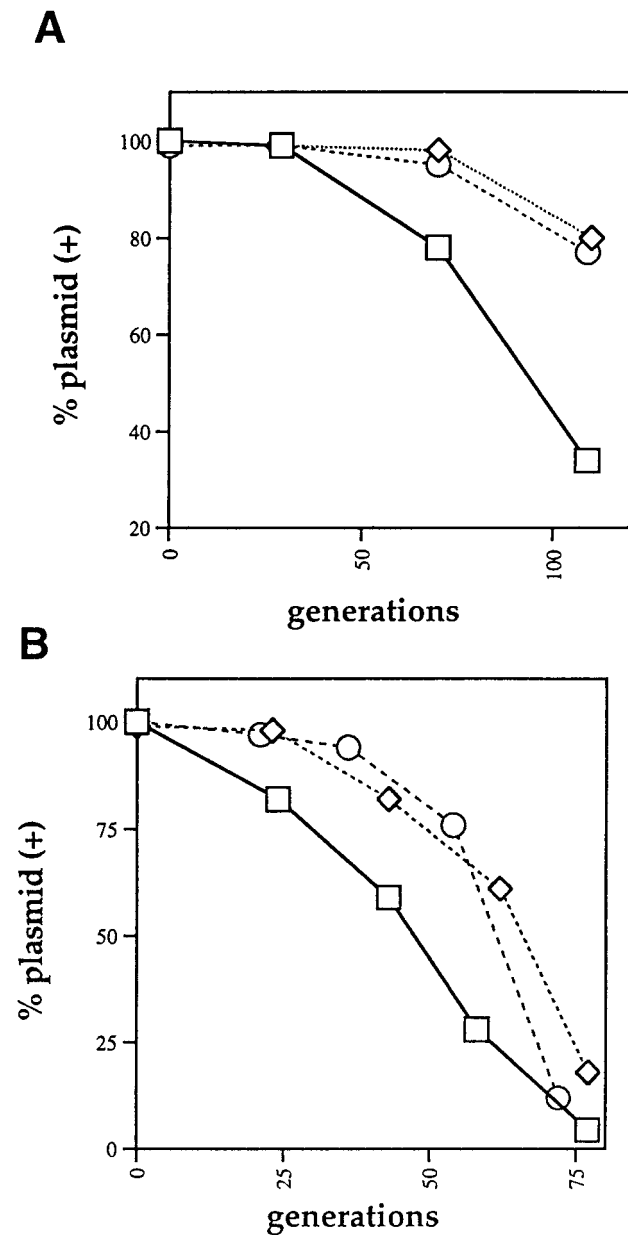


FIG. 2. Stabilization of RK2 by different *par* regions in two strains of *P. aeruginosa*. (A) Stability in *P. aeruginosa* PAC452Rif⁺ at 37°C in NB. (B) Stability in *P. aeruginosa* PAO1161Rif⁺ at 37°C in NB. Symbols: □, pCE61; ◇, pCE61-2.3 (*parC*⁺*B*⁺*A*⁺); ○, pCE61-91 (*parCBA*⁺).

ParA produced by the various deletion derivatives, we used a luciferase assay. Plasmid pTD10 is an RSF1010 derivative that carries the luciferase gene fused to the *parCBA* promoter (12, 16). It has been shown that the amount of luciferase activity expressed from the promoter of this fusion construct is a function of the level in vivo of the ParA protein. The ParA protein represses the activity of this promoter by binding to the operator region (12). pTD10 was established in *E. coli* MV10 and RR1 along with various pCE61 plasmid derivatives (*E. coli* MV10Δlac is tetracycline resistant; therefore, its tetracycline-sensitive parent, MV10, was used). As shown in Table 3, the level of ParA produced by the intact *parCBA* operon substantially reduced the level of expression of luciferase in both *E.*

TABLE 3. Use of a *parCBA* promoter and a luciferase fusion construct to measure ParA levels provided by various deletion constructs

Plasmids	Stabilization region	Luciferase units ^a of <i>E. coli</i>	
		RR1	MV10
pCE61 + pAL4000	None	251	131
pCE61 + pTD10	None	2,024	1,443
pCE61-2.3 + pTD10	<i>parCBA</i>	619	383
pCE61-91 + pTD10	<i>parA</i>	652	422
pCE61-92 + pTD10	<i>parB</i>	491	473
pCE61-94 + pTD10	<i>parCA</i>	164	950
pCE61-95 + pTD10	<i>parCB</i>	2,092	2,693

^a Luciferase units were calculated as (reading 1 + reading 2)/200(optical density at 600 nm).

coli RR1 and MV10 (compare pCE61 and pCE61-2.3). Constructs having a deletion in *parC* (pCE61-94), *parB* (pCE61-92), or both *parB* and *parC* (pCE61-91) similarly resulted in a substantial reduction of luciferase expression by plasmid pTD10, indicating that these deletion constructs produced levels of ParA comparable to that produced by the intact *parCBA* construct. Not surprisingly, the construct with a deletion in *parA* failed to repress luciferase expression. Based upon the results obtained with these two *E. coli* strains, it appears that the functional *parC* and *parB* genes are directly responsible for the loss of the stabilization activity of the *parCBA* operon for plasmid RK2 in strain RR1, as deletions in the upstream genes do not result in a reduction of ParA levels.

Stability of the plasmid RK2 in a *topA* *E. coli* strain. Previous studies with minireplicons of the pSC101, F, and P1 plasmids with deletions of a stabilization region revealed increased levels of plasmid stability when the plasmids were carried in an *E. coli topA* mutant (4, 6, 10, 11). It has been proposed that the increased negative superhelicity of plasmid DNA as a result of a deficiency in the type I topoisomerase (TopA) enzyme in a *topA* mutant results in improved distribution of the plasmid to daughter cells during cell division (4, 6, 10, 11). It was previously demonstrated that ParA binds to the *res* site at three distinguishable positions (13). To assess whether or not the increased stabilization of RK2 by the *parCBA* operon, including its *res* site, in *E. coli* is due to a superhelical change in the DNA as a result of binding of the ParA protein and/or the ParB and ParC proteins to the plasmid, the effect of a *topA* mutation on RK2 stabilization was tested. The *par* deletion plasmid pCE61 was established in the *E. coli topA* strain DPB636 and the isogenic *topA*⁺ parent DPB635, and the rates of plasmid loss were compared. There was no increase in the stability of the pCE61 plasmid in strain DPB636 (0.32% loss per generation in pDB635 versus 0.46% loss in DB636). Since, unlike the case with pSC101, F, and P1, the presumed increase in the negative superhelicity of RK2 in the *topA* mutant did not increase the stability of the plasmid, it is unlikely that the stabilization seen with *parA* and its *res* site is due to a change in RK2 superhelicity as a result of binding of the ParA protein and/or the other Par proteins.

Resolvase activity contributes to RK2 stabilization. The resolvase activity of ParA suggests that the stabilization of RK2 observed when ParA and its *res* site are inserted into pCE61 is due to the resolution of dimers and/or higher-multimer forms of the plasmid. It was previously observed that the *cer* multimer resolution system, when inserted into pCE61, did not increase the stability of the plasmid in *E. coli* MC1061K (12c). To explore further the role of resolvase activity in plasmid stabi-

TABLE 4. Effect of insertion of the multimer resolution systems of plasmids P1 and ColE1 on the stable maintenance of RK2 with deletions of the *par* region

Plasmid(s)	Resolvase system	Rate of loss (% per generation) ^a with strain		
		MC1061K	MV10Δlac	RR1 ^b
pCE61 + pEKA28G	None	1.50	0.45	2.71
pCE61 + pEKA30G	<i>loxP</i> /Cre	0.13	0.02	0.14
pCE61 <i>cer</i>	<i>cer</i>	1.24	0.83	0.05

^a The number of generations ranged from 75 to 90 in LB medium at 30°C. Similar results were obtained when the experiment was repeated.

^b Plasmids pEKA28 and pEKA30 were used *in trans* in this strain instead of plasmids pEKA28G and pEKA30G.

lization, we took advantage of the fact that plasmid pCE61 was constructed with a vector excision system which necessitated replacing the *par* region with a spectinomycin resistance gene cassette and the *loxP* site of prophage P1 (5). Thus, by supplying the Cre recombinase protein in *trans* from a compatible plasmid, it was possible to determine if multimer resolution via this system was able to stabilize pCE61. pCE61 was established in *E. coli* RR1, MC1061K, and MV10Δlac along with plasmid pEKA30G, which is a derivative of the compatible plasmid RSF1010 containing the *cre* gene. In addition, pCE61 and the RSF1010 vector without the *cre* gene were established in all three strains as a control. In all three strains, the rate of loss of pCE61 was higher than previously observed for this plasmid in the absence of the RSF1010 vector (Tables 1 and 4). This finding may have been due to a low level of incompatibility between the two plasmids. Nevertheless, it is clear that the *loxP*/Cre system provided a high level of stability for pCE61 in all three *E. coli* strains (Table 4). Because of this surprising result with the *loxP*/Cre system and in view of the earlier observation that the *cer* system did not stabilize pCE61 in MC1061K, the effect of inserting the *cer* system into RK2 was reinvestigated with MC1061K and examined with *E. coli* MV10Δlac and RR1. As shown in Table 4, the *cer* system once again failed to stabilize pCE61 in MC1061K; however, this multimer resolution system was effective in stabilizing the plasmid in RR1. As in the case of MC1061K, insertion of the *cer* site into RK2 did not improve its stability in MV10Δlac. That the *cer* system was capable of resolving multimers in all three strains was shown in an earlier study (12c).

DISCUSSION

The *par* region of plasmid RK2, consisting of two divergently oriented operons, *parCBA* and *parDE*, clearly plays a major role in the stabilization of this broad-host-range plasmid in a growing population of bacteria. While the mechanism of stabilization by *parDE* is well documented, the role of the *parCBA* operon in stabilizing this plasmid is still not fully understood. In addition, although the stabilization seen with the complete *par* region can be accounted for largely by the summation of the activities of the *parCBA* and *parDE* operons acting individually, we cannot rule out the possibility that the two operons act synergistically in providing virtually complete stabilization when the *par* region is intact.

In an attempt to obtain some insight into the mechanism by which *parCBA* stabilizes RK2, a series of precise deletions were constructed to specifically remove each of the three genes in the operon. Since the three genes are translationally coupled, care was exercised to maintain the reading frames in each of the various deletions. Results from the stability assays were

somewhat equivocal with respect to the necessity of the *parB* and *parC* genes. In the two *E. coli* strains in which the *parCBA* operon is effective in stabilizing RK2, MC1061K and RR1, the inactivation of *parB*, *parC*, or both *parB* and *parC* caused an approximately twofold reduction in the activity of the operon. However, a deletion in the *parA* gene caused a much greater loss of *parCBA* activity. In *P. aeruginosa* strains, in which *parCBA* shows a high level of stabilization activity, the inactivation of *parB* and *parC* did not diminish the activity observed. These results indicate a key role for the ParA- and *res* site-specific recombination system in the stabilization activity that is observed for the *parCBA* operon. However, when the level of ParA produced by the deletion constructs was approximated in two of the *E. coli* strains by determining the level of repression of the *par* promoter by ParA, it appeared that the reduced stabilization activity seen when *parB*, *parC*, or both genes were inactivated was not due to an effect of the deletions on ParA production.

These various observations do not, of course, provide any insight into the role of *parB* and *parC* in the stabilization seen for the intact *parCBA* operon in *E. coli* strains. If the stabilization activity of the *parCBA* operon is due solely to site-specific recombination, ParB and ParC may be accessory proteins that enhance the activity of ParA acting on its *res* site in resolving dimers and/or higher-multimer forms. The biochemical product of ParA acting on *res* sites in a dimeric plasmid is a catenate of two monomers (1–3). Additional enzymatic steps are required to separate the monomers. ParB, a calcium-dependent nuclease (17a), and ParC conceivably could play a role in resolving these catenates. Furthermore, differences in the requirements for *parB* and *parC* for full activity of the *parCBA* operon in different hosts may reflect differences in the abilities of different strains to compensate for the loss of *parB* and/or *parC* by providing host proteins with comparable activities. Alternatively, ParB and ParC could play an adaptive role in directing the *parA-res* system to an appropriate site in the bacterium where the multimer resolution system can facilitate segregation of the plasmid to daughter cells. The translational coupling of the three genes in the *parCBA* operon argues, albeit weakly, for the formation of a specific complex by the three proteins. If this were the case, then, of course, other proteins and even other multimer resolution systems might supplant the components of the *parCBA* system in a host-dependent manner in facilitating partitioning of the plasmid during cell division.

The high level of effectiveness of the *loxP*/Cre system in stabilizing plasmid RK2 was surprising in view of the failure in earlier studies of the *cer* multimer resolution system to stabilize the plasmid in one *E. coli* strain tested (12c). In addition, a significant level of RK2 dimers was not observed in the *E. coli* strains used in this study despite an effort to detect higher forms of RK2 with a procedure which involves direct lysis in agarose gels and which detects high-molecular-weight forms of supercoiled DNA (unpublished observations). Similar results have been reported for pBR322 derivatives containing intact or deletion RP4 *par* fragments (38). It has been argued, however, that even very low amounts of the dimeric form of a plasmid under certain circumstances can substantially increase the rate of loss of the plasmid (39). It is also worth pointing out that the plasmid RK2 derivative, pCE61, used in this study carries a functional TnI transposon that includes an active resolvase system (8, 12b, 17, 30). Grinter et al. (17) showed that deletion of the TnI transposon in RK2 had little effect on the stability of the plasmid in comparison with deletion of the *par* region. The TnI system obviously cannot compensate for the loss of the *parA-res* system in providing for stabilization of the RK2

plasmid. Again, this idea may not be surprising, since even the highly effective ColE1 plasmid *cer* system was found, unlike the *loxP*/Cre multimer resolution system, to be active in only one of three *E. coli* strains in substituting for the loss of the *parCBA* operon. This finding suggests that the actual level of resolvase activity in a particular strain, the nucleotide sequence context, or perhaps the topological position of the multimer resolution site within the supercoiled plasmid DNA influences the effectiveness of the system in functioning as a resolvase system for stabilizing the plasmid. Studies involving the *dif* locus of the *E. coli* chromosome, the site of multimer resolution of the chromosome within the terminus region, demonstrated a position requirement for the functionality of this sequence (26). In addition, the Dif phenotype of an *E. coli* strain caused by deletion of the *dif* locus could be suppressed only by replacement with a functional multimer resolution site such as *psi* (pSC101) or *loxP* (and Cre) in the same terminus region and not at another site on the chromosome (26). Finally, it is worth emphasizing that studies on the effectiveness of the various multimer resolution systems and the contribution of the *parB* and *parC* genes to the effectiveness of the *parCBA* operon in stabilizing the broad-host-range plasmid RK2 have been limited to relatively few bacterial hosts. A more substantial role for all three genes in stabilizing the RK2 plasmid may be revealed by extension of the analysis to a more diverse set of bacteria.

A much larger question is whether or not the juxtapositioning of the *parCBA* and *parDE* operons in the RK2 plasmid is fortuitous or whether it reflects an interaction between the five proteins and their DNA in providing virtually complete stabilization of the plasmid in a wide range of bacterial hosts. One study carried out with whole RK2 and *E. coli* MV10Δlac failed to show enhancement of *parCBA* stabilization activity on RK2 when the *parDE* products were provided in *trans* from a compatible plasmid (unpublished observations). Again, it will be of interest to extend the analysis to other bacteria to determine if the two operons act synergistically. Finally, all five proteins from these two operons have been purified (13, 17a, 21a, 22, 35) and are now available for determining if the proteins from the two operons interact with each other at the biochemical level. These additional approaches should provide some clarification of whether or not the *parCBA* products or multimer resolution systems such as *cer* and *loxP*/Cre stabilize RK2 only by increasing monomer units (or decreasing the number of potentially destabilizing dimeric forms of the plasmid) or if these systems play a role in a complex plasmid stabilization mechanism that requires as one essential step the resolution of dimeric forms of the plasmid. The view of a more complex function of the RK2 *par* region is supported by the recent finding that the resolution of dimer chromosomes at the *dif* locus of *E. coli* is coupled to cell division (38a). In this hypothetical stabilization system, *parDE* may work in concert with the *parCBA* products in providing for the stable maintenance of RK2 in a wide range of bacterial hosts.

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