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Different Relative Importances of the *par* Operons and the Effect of Conjugal Transfer on the Maintenance of Intact Promiscuous Plasmid RK2

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The *par* region of the broad-host-range, IncPα plasmid RK2 has been implicated as a stability determinant by its ability to enhance the maintenance of mini-RK2 plasmids or heterologous replicons in a growing population of host cells. The region consists of two operons: *parCBA*, which encodes a multimer resolution system, and *parDE*, which specifies a postsegregational response mechanism that is toxic to plasmidless segregants. To assess the importance of this region to the stable maintenance of the complete RK2 plasmid in different hosts, we used the vector-mediated excision (VEX) deletion system to specifically remove the entire *par* region or each operon separately from an otherwise intact RK2 plasmid carrying a *lacZ* marker. The *par* region was found to be important to stable maintenance of RK2*lac* (pRK2526) in *Escherichia coli* and five other gram-negative hosts (*Agrobacterium tumefaciens*, *Azotobacter vinelandii*, *Acinetobacter calcoaceticus*, *Caulobacter crescentus*, and *Pseudomonas aeruginosa*). However, the relative importance of the *parCBA* and *parDE* operons varied from host to host. Deletion of *parDE* had no effect on the maintenance of pRK2526 in *A. calcoaceticus*, but it severely reduced pRK2526 maintenance in *A. vinelandii* and resulted in significant instability in the other hosts. Deletion of *parCBA* did not alter pRK2526 stability in *E. coli*, *A. tumefaciens*, or *A. vinelandii* but severely reduced plasmid maintenance in *A. calcoaceticus* and *P. aeruginosa*. In the latter two hosts and *C. crescentus*, the Δ*parCBA* mutant caused a notable reduction in growth rate in the absence of selection for the plasmid, indicating that instability resulting from the absence of *parCBA* may trigger the postsegregational response mediated by *parDE*. We also examined the effect of the conjugal transfer system on RK2 maintenance in *E. coli*. Transfer-defective *traJ* and *traG* mutants of pRK2526 were stably maintained in rapidly growing broth cultures. On solid medium, which should be optimal for IncP-mediated conjugation, colonies from cells containing the pRK2526 *tra* mutants displayed significant numbers of white (Lac$^-$) sectors on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates, whereas sectors appeared rarely in colonies from *tra*$^+$ plasmid-containing cells. Both the *traJ* and *traG* mutations further reduced the maintenance of the already unstable Δ*par* derivative. Thus, these experiments with defined mutations in an intact RK2 plasmid have revealed (i) that the *par* region allows RK2 to adapt to the different requirements for stable maintenance in various hosts and (ii) that conjugal transfer can contribute to the maintenance of RK2 in a growing population, particularly under conditions that are favorable to RK2 transfer.

The extraordinary success of plasmids in nature relies largely on plasmid-specified functions that promote their dissemination and ensure their persistence in growing populations of bacteria. The highly promiscuous, self-transmissible plasmids of incompatibility group P (IncPα and IncPβ) are particularly remarkable not only for their conjugal host range, which includes gram-negative bacteria, gram-positive bacteria, and yeasts, but also for their stable maintenance as autonomously replicating elements in a wide range of gram-negative bacterial hosts (36, 57). We are studying the IncPα plasmid RK2 (22, 36) to identify and understand the functions responsible for successful inheritance of IncP plasmids by diverse bacteria.

RK2 is a 60,099-bp self-transmissible plasmid isolated from an antibiotic-resistant strain of *Klebsiella aerogenes* cultured from a burn wound (22, 36). The replication of RK2 depends on an origin of unidirectional replication (*oriV*) (30, 56) and a replication initiator gene (*trfA*), which codes for two related polypeptides that bind to repeated sequences (iterons) in the *oriV* region (25, 37, 38, 54). Precise deletion of *trfA* from RK2 results in the failure of RK2 to replicate in at least nine gram-negative hosts (4), and minimal RK2 replicons composed essentially of *oriV* and *trfA* are capable of replication in several gram-negative hosts (46, 47). Therefore, a single plasmid gene is both necessary and sufficient for initiation of RK2 replication at *oriV* in a variety of bacterial hosts.

One of the striking properties of RK2 is its exquisitely stable maintenance in different bacterial species during unselected growth (47). This property clearly involves more than the *trfA* and *oriV* replication determinants because mini-RK2 plasmids are notably unstable (44, 46, 47). The exact mechanisms by which RK2 accomplishes the difficult task of stable maintenance in diverse bacterial populations are not yet known. One region likely to be important is the 3.3-kb *par* region, which is able to stabilize mini-RK2 plasmids and other replicons in *Escherichia coli*, *Pseudomonas aeruginosa*, *Azotobacter vinelandii*, and *Agrobacterium tumefaciens* (40). The *par* region contains five genes (*parABCDE*) transcribed in two divergent,

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autoregulated operons (7, 9, 15, 41). The *parCBA* operon expresses a site-specific recombinase (ParA) (10, 18, 40) that also functions as a repressor to autoregulate transcription of the operon (7, 9). ParA alone can resolve multimeric plasmid forms by interacting with a specific sequence located in the *parC* promoter region (10). However, the resolvase activity of ParA is insufficient to explain the stabilization phenotype conferred by the *parCBA* operon (15, 40), and it has been proposed that ParA is part of an active partition apparatus that also involves the ParB and ParC proteins (15). The *parDE* operon is transcribed from a promoter that is negatively regulated by the ParD protein (7, 9). Recently, it has been demonstrated that the *parDE* operon specifies a postsegregational mechanism that is able to stabilize mini-RK2 plasmids by inhibiting the growth of plasmidless segregants (23, 43). In this system, ParE is a toxic protein whose activity is controlled by ParD (43).

Other RK2 loci have also been proposed to be involved in plasmid maintenance. The four operons of the *kilA*, *kilC*, and *kilE* determinants are nonessential for replication or conjugal transfer (13, 36). Because they are conserved on IncPα plasmids and coregulated with replication and transfer functions (31, 48, 50, 58), it has been suggested that these determinants are involved in RK2 maintenance or broad host range (13, 36). The IncC and KorB proteins from the *korA* operon of RK2 have been hypothesized to be partition functions because they exhibit sequence similarity to the SopA and SopB partition proteins of F plasmid, including a nucleotide binding motif in IncC that is conserved in other partition proteins (32). Recently, experiments with RK2 have indicated the existence of a second postsegregational inhibitory determinant (*psa*) that may function in plasmid maintenance (23). Thus, while the RK2 *par* region is capable of decreasing the rate of appearance of plasmidless segregants when cloned onto unstable RK2 miniplasmids and heterologous replicons, other maintenance functions may also be operating in the intact RK2 plasmid. In this study, we determined the significance of the *par* genes for the overall stability of RK2. We constructed specific deletions that removed the entire *par* region, the *parCBA* operon, or the *parDE* operon in an essentially wild-type RK2 plasmid to assess the importance of the *par* operons in the stable maintenance of RK2 in a variety of gram-negative bacterial species. In addition, we examined the contribution of the conjugal transfer system to RK2 maintenance in the presence and absence of *par*.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. The *E. coli* K-12 strains used were the following: MV10 (*thr-1 leuB6 lacY1 thi-1 tonA21 supE44 rfbD1* Δ*trpE5* λ$^-$); EKA340 [MV10 Δ(*argF-lac*) *deoC1*::Tn*10*]; a *recA* derivative, EKA340.2 (EKA340 *deoC1*::Tn*10*Δ*tet recA1 srl*::Tn*10*), made by P1 transduction of *recA1 srl*::Tn*10* from PG1118 (17) into a tetracycline-sensitive (Tc^s) derivative (28) of EKA340; EKA83, a spontaneous rifampin-resistant (Rif^r) mutant of MV10; EKA133 [MV10 Rif^r (λ*cre*$^+$)] (4); EKA516, a spontaneous nalidixic acid-resistant (Nal^r) mutant of EKA340; EKA539 [EKA340 (λDKC266 P1 *repA*$^+$)]; DF4063 (MV10 Nal^r) (4); SM10 [*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21 recA1* (Muc) Ω(RP4-2-Tc::Mu)] (53); BR2943 [*hsdR17 thi-1 relA1 supE44 endA1 gyrA96 recA1* (λDKC266 P1 *repA*$^+$)] (4); EKA260 [JMB9.1 (λDKC266 P1 *repA*$^+$)] (4); and AS11 [*thr leu ara thi gal lacY tonA malA xyl mtl minA minB* (λ R6K *pir*$^+$)] (49). Non-*E. coli* species were *Acinetobacter calcoaceticus* BD413 (47), *Caulobacter crescentus* CB15N C500 (47), *A. vinelandii* UW (47), *A. tumefaciens* A136 (47), and *P. aeruginosa* PAC452 (39).

The RK2 *tetA*::*lac* plasmid pRK2526 contains the *E. coli lac* operon (*lacZYA*) with the *lacL8 uv5* CAP-independent promoter (52) and the 5′ end of *lacZ* from the *lacZ*α portion of pUC19 (63) inserted at the *Sal*I site in the *tetA* gene of RK2 (16). Construction of pRK2526Δ*par* (pRK21382), pRK2526Δ*parCBA* (pRK21522), and pRK2526Δ*parDE* (pRK21526) by the vector-mediated excision (VEX) deletion system (4) is illustrated in Fig. 1A. Cloning of the RK2 *par* region was achieved by treating the *par*$^+$-pVEX double cointegrate RK2 plasmid, pRK21372, with Cre recombinase in vitro (Fig. 1B). Resolution by Cre at the *loxP* sites flanking the *par* region yields two products: (i) a derivative of RK2 deleted for *par* and marked with the gene for spectinomycin resistance (Sp^r) and (ii) the excised circular DNA containing the *par* region, the chloramphenicol resistance (Cm^r) marker, and the P1 and R6K origins of replication from the pVEX vectors (4). The reaction products were used to transform the RepA$^+$ strain EKA260 with selection for Cm^r to select for the *par*-containing plasmid, pRK21549 (Fig. 1B). Colonies were screened for sensitivity to tetracycline, spectinomycin, penicillin, and kanamycin, to verify the absence of the RK2 derivative. Plasmid pEKA12 is a Δ*par* derivative of pRK21549 constructed by digestion with *Xba*I, whose cleavage sites flank the *par* region in pRK21549. Plasmids from Cm^r transformants of EKA260 were screened for loss of the *par* fragment. Plasmid pVW52X is a derivative of pVWDG23110, which carries the Tra1 region of RP4 (61), with an *Xba*I linker insertion in the *traJ* gene (60). Plasmid pRK21553 is a derivative of pVW52X that contains a trimethoprim resistance (Tp^r)-encoding *Xba*I fragment from pEKA3 (51) inserted at the linker. The *traJ*::Tp^r allele was transferred to RK2 by homologous recombination. RK2 was conjugally transferred to a strain containing pRK21533; individual Pn^r Tp^r transconjugants were patched onto a Luria-broth (LB) plate, incubated overnight, and then replica plated to a lawn of EKA83 on an LB-kanamycin-rifampin plate to screen for failure to transfer. The structure of the transfer-defective plasmid RK2*traJ*::Tp (pRK21556) was confirmed by restriction analysis. To construct *traJ*::Tp mutants of pRK2526 and pRK2526Δ*par*, EKA340 strains containing pRK2526 or pRK2526Δ*par* in addition to pRK21556 (RK2*traJ*::Tp) were streaked onto M9–CAA–trimethoprim–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates (for pRK2526) and M9–CAA–trimethoprim–spectinomycin–X-Gal plates (for pRK2526Δ*par*). Blue colonies were isolated and tested for the presence of a transfer-defective plasmid, as described above. The structures of plasmids pRK21558 (pRK2526 *traJ*::Tp^r) and pRK21575 (pRK2526Δ*par traJ*::Tp^r), isolated from colonies that did not conjugate, were confirmed by restriction analysis. Plasmid pVW8703 is a pUC8Cm derivative that carries the *traF* and *traG* genes (60). The construction of the *traG* mutants of pRK2526 (pCE60) and pRK2526Δ*par* (pCE61) is described elsewhere (8).

Media. LB or M9-CAA medium (29) was used for *E. coli*, *A. calcoaceticus*, and *P. aeruginosa*; PYE was used for *C. crescentus* (47); Burk's modified minimal or C medium was used for *A. vinelandii* (47); and yeast extract-peptone was used for *A. tumefaciens* (47). For *E. coli*, antibiotics were used at the indicated concentrations (micrograms per milliliter): chloramphenicol, 50; kanamycin, 50; nalidixic acid, 20; penicillin, 150; spectinomycin, 50; trimethoprim, 50; and rifampin, 100. To select for the presence of RK2, kanamycin was used at 30 μg/ml for *A. calcoaceticus*, at 500 μg/ml for *P. aeruginosa*, and at 10 μg/ml for *C. crescentus*. Nalidixic acid was used at 30 μg/ml for *C. crescentus* and 20 μg/ml for *A. tumefaciens*. X-Gal was used at 40 μg/ml to distinguish plasmid-containing from plasmidless cells and at 80 μg/ml to quantitate sectors in colonies.

DNA procedures. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, *E. coli* DNA polymerase I Klenow fragment, and Cre recombinase were purchased from commercial suppliers and used as recommended. Purification of plasmid DNA (24), agarose and polyacrylamide gel electrophoresis (29), transformation of *E. coli* (6), and Southern blot hybridization (55) have been described elsewhere.

Conjugation. Broth cultures of donor and recipient strains were grown overnight under selection for resident plasmids. *E. coli* and *P. aeruginosa* were grown at 37°C; all other strains were grown at 30°C. Cells were washed in LB or M9, and 100-μl aliquots of donor and recipient strains were mixed on an LB plate and incubated for 4 h at the appropriate temperature. Controls consisted of mock matings containing donor alone and recipient alone. The mating mixtures were scraped from the plate with a toothpick and resuspended in 1 ml of LB or M9. Serial dilutions were plated on appropriate selective media. Mating mixtures consisting of *E. coli* donors and non-*E. coli* recipients were prepared similarly, except that the cells were mixed on the nonselective solid medium appropriate for the recipient strain and the mixtures were incubated for 12 h at the appropriate temperature before plating on selective media.

Plasmid stability assays. Bacterial strains were grown overnight at 37°C in broth with selection for the plasmid. *E. coli* and *P. aeruginosa* were grown at 37°C; all other strains were grown at 30°C. The cultures were then diluted 10^6-fold (except *C. crescentus*, which was diluted 10^4-fold) into medium lacking antibiotics, grown to stationary phase, and then diluted 10^6-fold. The cycle was repeated until the cultures were grown for 80 to 200 generations. At the time of each dilution, strains containing derivatives of *lacZ*$^+$ pRK2526 were plated on agar containing X-Gal to determine cell titers and to assay Lac$^+$ (blue) and Lac$^-$ (white) colonies. For stability assays with *lacZ*$^+$ RK2 derivatives, cells were plated on nonselective medium at the time of each dilution, and 100 colonies were picked onto selective and nonselective plates. To test for genetic complementation of pRK2526Δ*par*, strains were grown overnight with selection for both plasmids and then diluted into medium containing chloramphenicol to select for the complementing plasmid (pRK21549 or pEKA12) only. At various times, cells were plated on medium containing chloramphenicol and X-Gal to screen for the presence of pRK2526Δ*par*. For all stability assays, the formula used to calculate the average percent loss per generation is the following:

$$\left(1 - \sqrt[n]{\frac{F_f}{F_i}}\right) \times 100$$

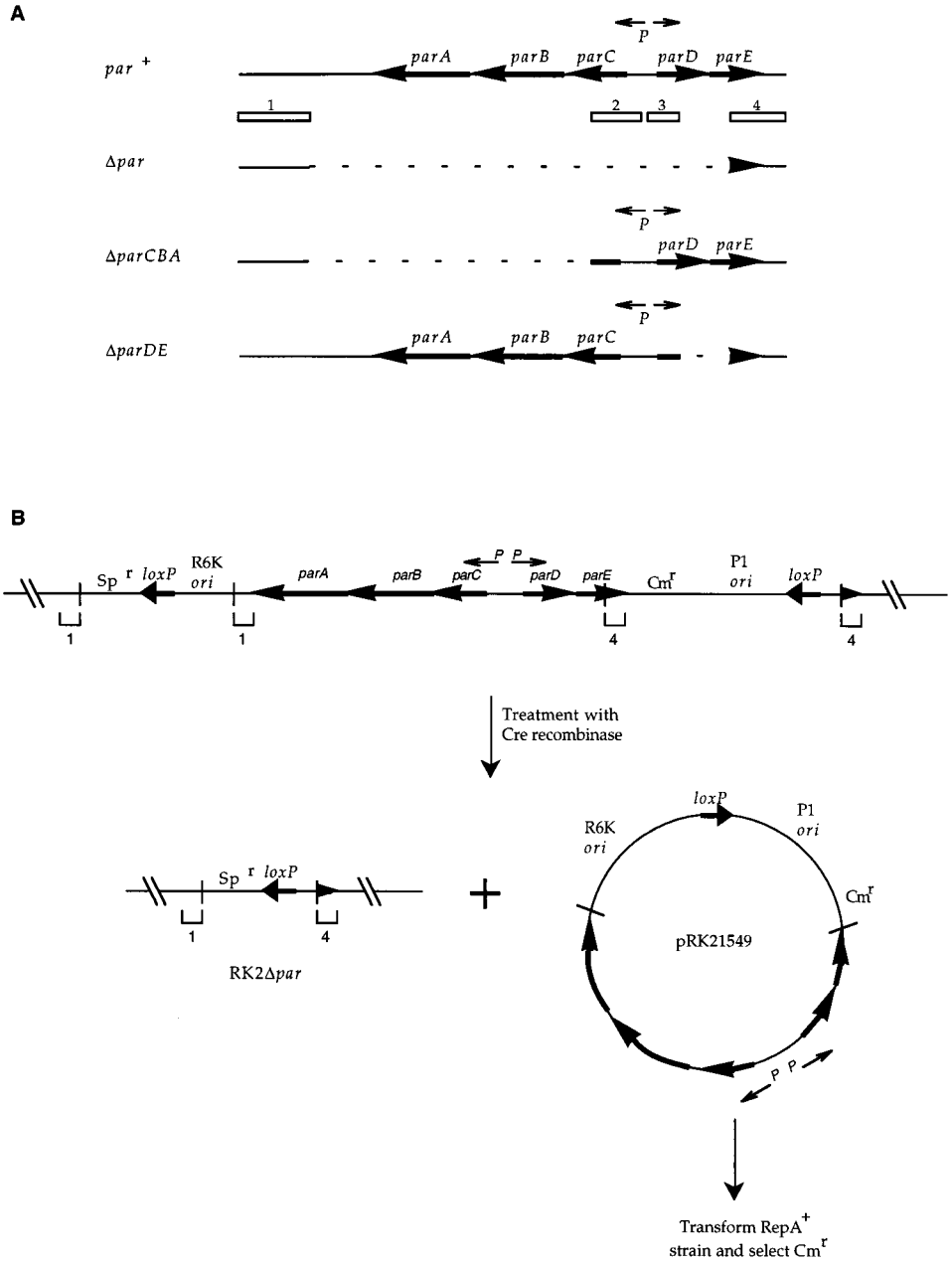

FIG. 1. Deletion and cloning of the RK2 *par* region. (A) Physical and genetic maps of *par* deletion mutants of pRK2526. The wild-type par^+ region of pRK2526 is shown at the top. The regions missing (dashed lines) in the Δ*par* deletion-substitution mutants are shown below. P indicates the region containing the divergent promoters for the *parCBA* and *parDE* operons; the small horizontal arrows indicate the direction of transcription. Bold arrows on the map indicate the *par* structural genes. Deletion-substitution mutants of pRK2526 were constructed in vivo by the VEX method as previously described (4). The open boxes labeled 1, 2, 3, and 4 show the fragments cloned into the pVEX vectors for recombination with RK2. Fragment 1 is a 373-bp fragment (nucleotides [nt] 32789 to 33161 of RK2 [36], 2 is a 319-bp fragment (nt 35020 to 34702), 3 is a 171-bp fragment (nt 35080 to 35250), and 4 is a 288-bp fragment (nt 35480 to 35767). Fragments 1 and 4 were used to delete the entire region (Δ*par*) essentially as shown below in panel B except that Cre resolution was done in EKA133. Likewise, 1 and 2 were used to delete the *parCBA* operon (Δ*parCBA*) and 3 and 4 were used to delete the *parDE* operon (Δ*parDE*). The VEX vectors (4) used for cloning the fragments were the following: fragment 1, pVEX2172; fragment 2, pVEX1261; fragment 3, pVEX2172; and fragment 4, pVEX1262. In each case, the region deleted (dotted lines) was replaced with a 2.1-kb fragment containing an Sp^r determinant flanked by transcriptional and translational terminators (4). (B) Cloning of the *par* region by VEX recombination in vitro. The double cointegrate (pRK21372) arising from recombination with pVEX vectors containing fragments 1 and 4 is shown on the top line. Resolution of the double cointegrate was done with Cre recombinase in vitro. The excised plasmid, pRK21549, was obtained by transformation of a P1 $RepA^+$ strain, as described in Materials and Methods.

where n is the number of generations, F_i is the fraction of plasmid-containing cells at the initial time point, and F_f is the fraction of plasmid-containing cells at the final time point (40). This formula assumes no difference in the growth rate of plasmid-containing and plasmid-cured cells.

RESULTS

Stability of RK2 and RK2 *tetA*::*lac* (pRK2526). To facilitate the assay for stable maintenance of RK2 mutants, we used a *lacZ*$^+$ derivative of RK2 (pRK2526), which has the *E. coli lacZYA* operon inserted within the tetracycline resistance *tetA* gene. The *lac* operon is expressed from the CAP-independent *lacL8 uv5* mutant promoter (52) to permit expression in different bacterial hosts. The presence of pRK2526 in *E. coli* (Δ*lacIZYA*), *P. aeruginosa*, *C. crescentus*, *A. vinelandii*, *A. calcoaceticus*, and *A. tumefaciens* resulted in the formation of blue colonies on medium containing X-Gal, indicating expression of β-galactosidase in all strains. To determine if the presence and/or expression of the *lac* operon affect pRK2526 maintenance in the host, we compared the stability of RK2 and that of pRK2526 in *E. coli* and *P. aeruginosa* during growth in the absence of selection. Both plasmids were lost at a rate of less than 0.2% per generation for 200 generations in both *E. coli* and *P. aeruginosa*. Thus, pRK2526, like RK2, is stably maintained in these hosts. Comparable stability was observed for plasmid pRK2526 in the other hosts (see below).

Unstable maintenance of pRK2526Δ*par* in *E. coli*. To determine the contribution of *par* to stable maintenance, the region was deleted from pRK2526 with the VEX system for making precise deletion-substitution mutations in large genomes (4). Small fragments from the ends of the *par* region (Fig. 1) were cloned into the pVEX vectors, and the resulting plasmids were inserted into pRK2526 by homologous recombination to produce a double cointegrate with directly repeated *loxP* sequences flanking the *par* region. Site-specific recombination at the *loxP* sites by Cre recombinase resulted in deletion of the *par* region and insertion of a spectinomycin resistance determinant flanked by transcriptional terminators. The 2.3-kb deletion in pRK2526Δ*par* (pRK21382), which removed *parA*, *parB*, *parC*, *parD*, and the 5′-end coding region of *parE* (Fig. 1), was confirmed by Southern hybridization (data not shown).

E. coli MV10 Δ*lac* (EKA340) strains containing pRK2526 or pRK2526Δ*par* (pRK21382) were plated without antibiotic selection on X-Gal medium. The pRK2526-containing strain gave rise to colonies that were uniformly blue as expected, while nearly all the colonies of the pRK2526Δ*par*-containing strain showed white sectors (Fig. 2). Cells cultured from the white sectors lacked all plasmid markers and showed no evidence of plasmid DNA as determined by gel electrophoresis (data not shown). Sectored colonies are expected from cells that produce plasmidless segregants at a significant frequency. Thus, this phenotype indicated that the absence of the *par* region reduces the stability of pRK2526 in *E. coli*. The strains were grown without selection in broth and plated on X-Gal medium at various times to determine the proportion of plasmidless cells (white colonies). The *par*$^+$ plasmid pRK2526 was stably maintained for 125 generations as expected, whereas the Δ*par* mutant pRK21382 was lost at a faster rate (Fig. 3). A crude approximation of the difference in stability was obtained by calculating the average rate of loss of the plasmid from the population over the duration of the experiment. The *par*$^+$ plasmid, pRK2526, was lost at 0.2% per generation, whereas the Δ*par* plasmid, pRK21382, was lost at a rate of 3.4% per generation. Similar results were obtained with RK2 and a Δ*par* derivative of RK2 (data not shown). Thus, the *par* region is important for the stable maintenance of RK2 in *E. coli*.

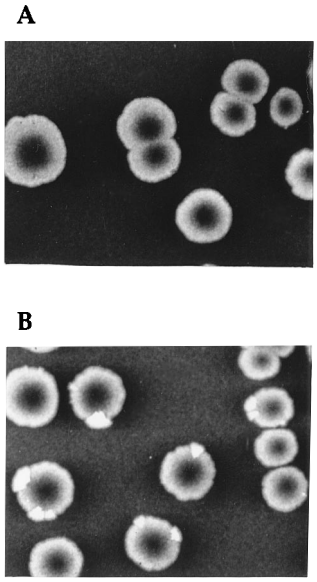

FIG. 2. Sectored colonies from pRK2526Δ*par*-containing *E. coli* cells. Strains were grown overnight in LB medium containing kanamycin to select for the presence of the plasmid. Cultures were diluted and plated in the absence of selection on LB X-Gal plates. Panel A shows colonies from strain EKA340 containing the pRK2526 (*par*$^+$) control plasmid; panel B shows the EKA340 strain containing the pRK2526Δ*par* mutant (pRK21382).

Characterization of pRK2526Δ*par* instability. Although pRK2526Δ*par* was highly unstable in *E. coli*, it was never completely lost from the population. After 80 generations of nonselective growth, the plasmid remained in approximately 4% of the cells in the culture for at least the next 45 generations (Fig. 3). Plasmid-containing colonies obtained from cells after 125 generations displayed phenotypes that differed from that of the original strain. When plated on nonselective plates containing X-Gal, these isolates formed colonies with significantly fewer sectors than did the parent strain, indicating the presence of plasmid or host suppressor mutations that alleviated the instability of pRK2526Δ*par*. These potentially interesting variants are being studied.

To test whether the instability of pRK2526Δ*par* is caused by altered plasmid copy number, we determined the MICs of ampicillin for isogenic strains containing RK2, RK2Δ*par* (pRK21373), pRK2526, and pRK2526Δ*par* (pRK21382). Because resistance to ampicillin increases linearly with increasing copies of the β-lactamase gene (34), it is useful for comparing the relative copy numbers of plasmids that carry this marker. We found no differences in the concentrations of ampicillin required to inhibit the growth of strains containing the Δ*par* and *par*$^+$ plasmids (data not shown). Therefore, the Δ*par* mutation does not appear to cause instability by altering plasmid copy number.

One of the known functions of the *par* region is the multimer resolution activity of ParA (10, 18, 40). If the instability of pRK2526Δ*par* results solely from the inability to resolve multimers, then preventing multimer formation by abolishing homologous recombination should suppress the instability (2). The pRK2526 and pRK2526Δ*par* plasmids were transferred into an isogenic Δ*lac recA1* mutant strain (EKA340.2) and plated on nonselective medium containing X-Gal. Sectors appeared in the colonies of the pRK2526Δ*par*-containing strain,

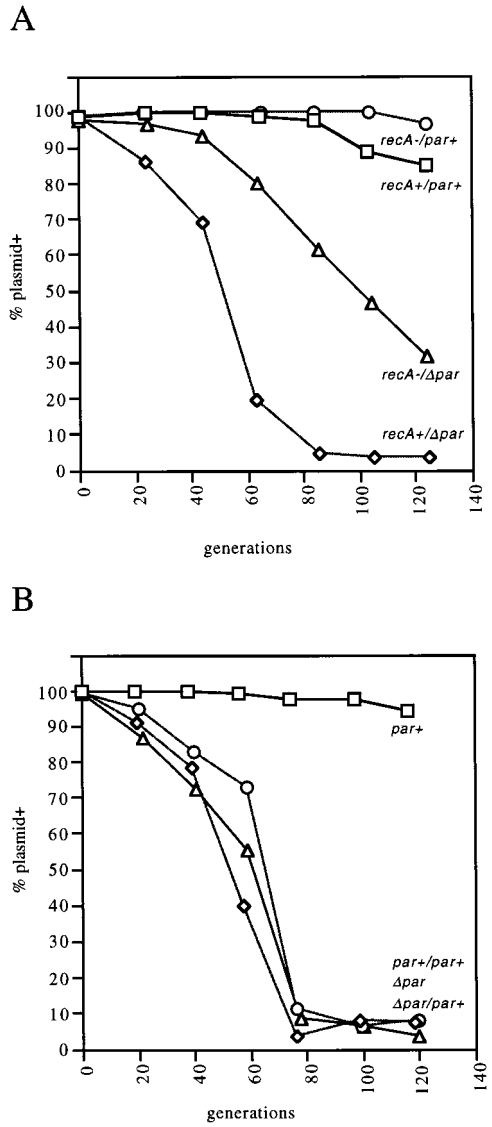

FIG. 3. Properties of pRK2526Δ*par* in *E. coli*. (A) Stability of pRK2526Δ*par* (pRK21382) in *recA*$^+$ and *recA* mutant *E. coli* hosts. EKA340 (*recA*$^+$) and EKA340.2 (*recA* mutant) strains containing pRK2526 and pRK21382 were grown in the absence of selection for 120 generations, as described in Materials and Methods. The fraction of cells containing plasmid was determined by plating cells on LB X-Gal medium approximately every 20 generations. □, EKA340 (pRK2526); ◇, EKA340(pRK21382); ○, EKA340.2(pRK2526); △, EKA340.2 (pRK21382). (B) Effect of *par* in *trans*. EKA539 strains containing the cloned *par* region (pRK21549) or the vector (pEKA12) and pRK2526 or pRK21382 were grown in the absence of selection for the RK2 derivative for 120 generations, as described in Materials and Methods. The fraction of cells containing pRK2526 or pRK21382 was determined as described in Materials and Methods. /*par*+ indicates the presence of *par* in *trans*. □, EKA539(pRK2526, pEKA12); ◇, EKA539 (pRK21382, pEKA12); ○, EKA539(pRK2526, pRK21549); △, EKA539 (pRK21382, pRK21549).

indicating that the Δ*par* plasmid is still unstable under conditions in which multimers do not form at any significant frequency. Broth assays confirmed the instability, although they did reveal that pRK2526Δ*par* was somewhat more stable in the *recA* mutant host than in the *recA*$^+$ host (Fig. 3).

We tested the ability of the *par* region in *trans* to complement the instability of pRK2526Δ*par*. For at least 120 generations, the unstable phenotype of pRK2526Δ*par* was unaffected by the presence of the cloned *par* region (pRK21549) or the vector alone (pEKA12) (Fig. 3). Thus, the Δ*par* mutation cannot be complemented in *trans*. While the cloned *par* region in *trans* had no effect on the stability of the pRK2526Δ*par* plasmid, it significantly destabilized the *par*$^+$ plasmid pRK2526 (Fig. 3). This result shows that the *par* region causes incompatibility with RK2 and that sensitivity to *par*-mediated incompatibility requires the presence of at least some portion of the *par* region on the target plasmid.

Stability of Δ*parCBA* and Δ*parDE* mutants of pRK2526 in *E. coli*. To determine the relative contributions of the divergent *parCBA* and *parDE* operons of the *par* region, we used VEX to construct pRK2526 deletion mutants that lack the individual *par* operons (Fig. 1). As with Δ*par*, these deletions were marked by insertion of the transcriptionally isolated spectinomycin resistance gene. *E. coli* strains containing pRK2526 Δ*parCBA* (pRK21522) and pRK2526Δ*parDE* (pRK21526) were plated on nonselective medium containing X-Gal. Strains containing pRK2526 (*par*$^+$) or pRK2526Δ*parCBA* showed no evidence of plasmid loss, while the pRK2526Δ*parDE*-containing strain gave rise to sectored colonies (data not shown). Stability assays in broth confirmed that the deletion of *parCBA* did not reduce pRK2526 maintenance in *E. coli*, while the deletion of *parDE* caused a considerable loss of stability (Fig. 4).

Stability of Δ*par*, Δ*parCBA*, and Δ*parDE* mutants of pRK2526 in different gram-negative bacterial hosts. The maintenance of the Δ*par*, Δ*parCBA*, and Δ*parDE* mutants of pRK2526 was examined in five other gram-negative hosts of RK2 (Fig. 4). For all species tested, deletion of the entire *par* region from pRK2526 resulted in a significant increase in the number of plasmidless segregants in a growing culture. However, plasmids deleted for the individual *par* operons demonstrated that the relative contributions of these operons vary from host to host.

As in *E. coli*, the *parCBA* deletion had no obvious effect on plasmid stability in *A. tumefaciens* and *A. vinelandii*. This indicates either that *parCBA* does not function in these hosts or that there is a redundant function that maps outside the *par* region. In contrast, the *parCBA* deletion produced striking effects on plasmid maintenance in *A. calcoaceticus* and *P. aeruginosa* and a detectable effect in *C. crescentus*. In these strains, pRK2526Δ*parCBA* was very unstable and the colonies were markedly smaller than those arising from plasmid-free and pRK2526-containing cells, even in the absence of selection for the plasmid. This phenotype indicates that the absence of the *parCBA* operon caused pRK2526Δ*parCBA* to become toxic to these hosts. The apparent high rate of plasmid loss in these strains is likely to be exaggerated by the differences in the growth rates of plasmid-containing and plasmid-free cultures, since plasmid-free segregants should rapidly outgrow the plasmid-containing cells in the culture. Mutants lacking the entire *par* region did not confer the slow growth phenotype, indicating a possible involvement of *parDE* in this phenotype.

The Δ*parDE* mutation affected the stability of pRK2526 in all hosts except *A. calcoaceticus*, in which the plasmid was as stable as the wild-type *par*$^+$ plasmid. The greatest instability occurred in *A. vinelandii*, for which approximately 90% of the culture was plasmid free by 40 generations.

Effect of conjugal transfer on the maintenance of pRK2526. We considered the possibility that conjugal transfer might enhance RK2 maintenance in a clonal population by allowing the reintroduction of RK2 into plasmidless segregants. Studies have indicated that IncP plasmid-containing donor cells are best able to conjugate on solid surfaces, likely because of the properties of the sex pilus expressed by IncP plasmids (5). We therefore expected that the contribution of conjugal transfer to

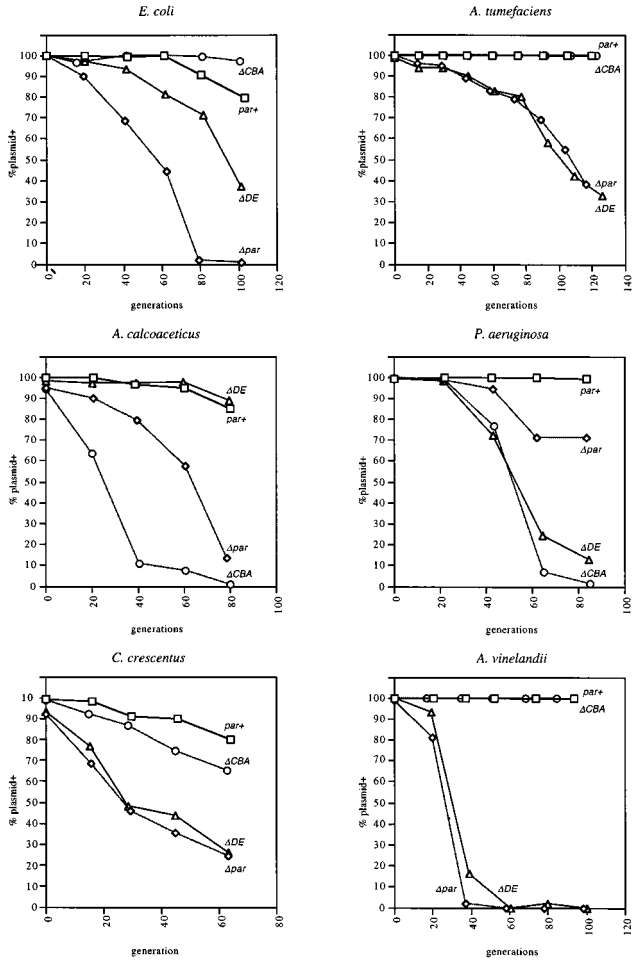

FIG. 4. Stability of pRK2526Δ*par*, Δ*parCBA*, and Δ*parDE* mutants in different gram-negative hosts. Strains of *E. coli*, *A. tumefaciens*, *A. calcoaceticus*, *P. aeruginosa*, *C. crescentus*, and *A. vinelandii* containing pRK2526 (*par*$^+$) (□), pRK21382 (Δ*par*) (◇), pRK21522 (Δ*parCBA*) (○), and pRK21526 (Δ*parDE*) (△) were grown without selection for the plasmid for 60 to 120 generations as described in Materials and Methods. The percentage of plasmid-containing cells was determined as described in Materials and Methods.

plasmid maintenance would be negligible in these experiments, since the bacteria were grown in rapidly shaking broth cultures and plated immediately upon removal of the cultures from the shaker. Nevertheless, significant levels of plasmid transfer were detected in an experiment in which equal numbers of RK2-containing donors and plasmidless recipients were deliberately mixed and grown together under similar conditions (62). Therefore, we constructed and tested transfer-defective derivatives of pRK2526 to assess the role of conjugation in the maintenance of RK2 in broth culture. Mutant alleles of *traJ* and *traG* were moved into pRK2526 by homologous recombination to produce plasmids pRK21558 and pCE60, respectively. Both plasmids are completely defective in conjugal transfer (data not shown), but they were maintained as stably (<0.2% loss per generation) in *E. coli* during 200 generations of unselected growth as was the *tra*$^+$ parental plasmid pRK2526 (data not shown). Thus, elimination of the conjugal transfer system from pRK2526 did not significantly increase the occurrence of plasmidless segregants in a broth culture.

We observed different resulted when plasmid maintenance was assayed in cells growing on solid medium, conditions that

TABLE 1. Effect of the *tra* and *par* mutations on maintenance of pRK2526 derivatives on solid medium

Plasmid(s)[a]	Genotype	No. of sectors[b]	No. of colonies	No. of sectors/colony
pRK2526	*traJ*$^+$ *par*$^+$	3	128	0.02
pRK21558	*traJ par*$^+$	22	221	0.1
pRK21382	*traJ*$^+$ Δ*par*	97	226	0.4
pRK21575	*traJ* Δ*par*	191	230	0.8
pCE60 plus pUC8Cm	*traG par*$^+$	72	214	0.3
pCE60 plus pVW8703	*traG par*$^+$ *traG*$^+$ *traF*$^+$	8	321	0.02
pCE61 plus pUC8Cm	*traG* Δ*par*	228	120	1.9
pCE61 plus pVW8703	*traG* Δ*par* *traG*$^+$ *traF*$^+$	52	111	0.5

[a] EKA340 was the host for all plasmids.
[b] For each strain, several plates containing fewer than 50 colonies each were examined with a dissecting microscope. Sectors were assayed after 2 days of growth at 37°C.

are favorable to RK2-mediated conjugation (5). Colonies from *E. coli* cells containing the pRK2526 *traJ* mutant and the pRK2526 *traG* mutant displayed white sectors on X-Gal medium at a significant frequency (Table 1). Both *traJ* and *traG* mutations also further reduced the maintenance of the pRK2526Δ*par* plasmid. The sectors in colonies from the *tra* mutant strains (both *par*$^+$ and Δ*par*) arose earlier than those from the *tra*$^+$ Δ*par* strain (data not shown). In the case of the *traG* mutants, it was possible to test for complementation; the presence of *traG* in *trans* reduced the sectoring frequency. These results indicate that the contribution of conjugal transfer to plasmid maintenance can be significant when the cells are growing under conditions that are favorable to conjugation.

DISCUSSION

High-molecular-weight, low-copy-number plasmids commonly contain several distinct loci to enhance their maintenance in an expanding population of host cells (33). The genetic analysis of plasmid stability functions has relied on stabilization phenotypes arising from the attachment of the stability determinants to an unstable miniplasmid or heterologous replicon. However, assessment of the significance of a particular determinant for the overall stability of the parental plasmid requires the construction of null mutations in an otherwise wild-type plasmid. This approach is particularly useful for the study of stability determinants of broad-host-range plasmids like RK2 because the behavior of miniplasmids or heterologous replicons used as test vectors varies considerably from host to host (45, 47).

In this study, we used the VEX system (4) to construct mutants of a *lacZ*$^+$ derivative of RK2 (pRK2526) in which the entire *par* region, the *parCBA* operon, or the *parDE* operon was specifically deleted. The behavior of these mutants was examined in *E. coli* and five other gram-negative hosts to determine the importance of the *par* region and the individual *par* operons to the stable maintenance of RK2 in these different hosts. We found that the absence of the entire *par* region, encompassing both the *parCBA* and *parDE* operons, significantly reduced the stability of RK2 in all hosts tested (*E. coli*, *A. calcoaceticus*, *A. vinelandii*, *A. tumefaciens*, *C. crescentus*, and *P. aeruginosa*). However, pRK2526 mutants lacking only the *parCBA* operon or the *parDE* operon displayed strikingly different phenotypes in the different hosts. Thus, while the *par*

region is important for stable maintenance in all hosts, the relative importance of the functions encoded by the *parCBA* and *parDE* operons varies from host to host.

The copy number of RK2 in *E. coli* has been measured as 5 to 7 per bacterial chromosome (57) and 15 per exponentially growing cell (11). At a copy number of 7, random distribution of plasmids in the cell should allow a calculated loss rate of 6×10^{-5} per generation (3). While the wild-type RK2 and pRK2526 plasmids are remarkably stable in most hosts, the Δ*par* mutations led to a high rate of loss without any significant reduction in copy number, suggesting that the pRK2526Δ*par* plasmids are not randomly distributed in the cell. It is possible that one of the functions of *par* is to randomize the RK2 plasmids in the cell so that every daughter cell has a high probability of acquiring at least one copy. Alternatively, copies of RK2 may form complexes naturally, and the function of *par* may be to ensure proper separation of plasmid molecules from the complexes and subsequent segregation to each of the daughter cells.

The *parDE* operon has recently been shown to specify a postsegregational inhibitory system that arrests or kills plasmidless segregants (23, 43), and the *parCBA* operon expresses a site-specific recombinase (ParA) capable of resolving plasmid multimers (10, 18, 40). However, several lines of evidence indicate that resolution by ParA does not account for the stabilizing effect of the *parCBA* operon. In studies with a miniplasmid, use of a different resolution system in place of the *parCBA* operon did not enhance plasmid stability even though multimers were resolved (41). In this study, the Δ*parCBA* mutation reduced the stability of pRK2526 in three hosts despite the presence of another resolvase (TnpR) from Tn*1* (36), which should compensate for any loss of resolution by ParA. Finally, a *recA* host failed to suppress the instability of pRK2526Δ*par*. Thus, the *parCBA* operon very likely specifies a stability mechanism that does more than resolve plasmid multimers. Gerlitz et al. (15) have suggested that the ParC, ParB, and ParA proteins are the components of a partition apparatus that helps to segregate plasmids to both daughter cells at cell division.

The inability of the *par* region to complement the Δ*par* mutation in *trans* is consistent with all the proposed functions for the *par* region. (i) ParA-catalyzed multimer resolution requires a *cis*-acting site that is located in the *parC* promoter region (10, 40). (ii) An active partition system similar to those of the P1 (1) and F (21) plasmids would also require a specific sequence on the plasmid. No such element has been identified for RK2, but it is reasonable to expect that, if it exists, it lies within the *par* region because other partitioning systems have *cis*-acting sequence elements in the regions that encode the partition proteins (33). (iii) Finally, proper functioning of the *parDE* postsegregational response system requires that the *parD* gene, whose product controls the toxic ParE protein (43), be present only on RK2. We also found that the *par* region in *trans* is incompatible with the par^+ plasmid pRK2526. This is consistent with the presence of either an active partition mechanism (by analogy to plasmids F and P1 [21, 33]) or the postsegregational response system, whose effectiveness should be lost when the *parD* regulator is provided in *trans.*

Loss of the *parDE* operon significantly reduced the stability of pRK2526 in all hosts except *A. calcoaceticus*. Thus, the postsegregational inhibitory system specified by this operon is an important factor in RK2 maintenance in a wide variety of hosts. These results extend the findings of Roberts and Helinski (41) that a small DNA segment containing the *parDE* operon can stabilize a mini-RK2 plasmid in *P. aeruginosa*, *A. vinelandii*, *A. tumefaciens*, and, under certain conditions, *E. coli*. The degree to which pRK2526 stability was affected by the Δ*parDE* mutation varied greatly in the different hosts. The rate of loss of plasmids pRK2526Δ*parDE* and pRK2526Δ*par* from *A. vinelandii* is so severe that the mutants are essentially defective in host range. Thus, the *parDE* operon appears to be critical for stable maintenance in this host.

While the Δ*parDE* mutation had no effect on plasmid stability in *A. calcoaceticus*, it is nevertheless likely that ParD and ParE still function in this host. Deletion of the *parCBA* operon resulted in slowly growing colonies and rapid plasmid loss. A similar phenotype was also observed for the *parCBA* mutant in *P. aeruginosa*, except that plasmid stability was affected by the Δ*parDE* mutation. In *C. crescentus*, the Δ*parCBA* mutant caused a slow growth phenotype with little or no effect on plasmid stability. In all cases, the slow growth phenotype was relieved by the further deletion of *parDE*. One explanation for these results is that the Δ*parCBA* mutation causes the plasmid to become unstable in these hosts and triggers the *parDE* postsegregational response mechanism, which then inhibits the growth of plasmidless segregants. This model predicts that *parD* in *trans* will suppress the slow growth phenotypes. Why would the Δ*parDE* mutation fail to reduce the stability of pRK2526 if the *parDE* postsegregational response system is functional in this host? If *parCBA* (and possibly other factors) is sufficient to confer a high degree of stability in *A. calcoaceticus*, the *parDE* system becomes unnecessary. In *P. aeruginosa* and *C. crescentus*, the Δ*parDE* mutation confers some detectable instability, suggesting not only that *parDE* is functional but also that it contributes significantly to the maintenance of RK2 in these hosts.

An alternative model to explain the Δ*parCBA* phenotypes in *A. calcoaceticus*, *P. aeruginosa*, and *C. crescentus* is that unregulated expression of the *parC* remnant, encoding the N-terminal 78 amino acids of the 86-amino-acid protein (Fig. 1), is toxic in these hosts. Because ParA is responsible for autoregulation of transcription from the *parC* promoter (7, 9), the absence of *parA* in the Δ*parCBA* mutant would allow increased transcription from the *parC* promoter. The transcriptional terminators in the Sp^r determinant (12) should prevent transcription from extending beyond the *par* region, but the *parC* remnant will be expressed. In hosts in which the slow growth phenotype is not observed, the truncated ParC polypeptide may be unstable or poorly expressed, or it may fail to interact with its target. This model can be distinguished from the previous model by the prediction that the slow growth phenotype will be suppressed by *parA* in *trans*. Roberts and Helinski have shown that a region containing the N-terminal 63 amino acids of ParC could have a destabilizing effect on miniplasmids unless the *parCBA* operon was present in *trans*; this would suggest that overexpression of portions of ParC could affect the growth of *E. coli* (42). If true, then host mutations that suppress the ParC toxicity might identify a gene whose product interacts with ParC.

The highly promiscuous conjugal transfer system of RK2 is clearly important for the spread of RK2 to new bacterial populations (19, 20, 59). Because conjugal transfer has the potential to reintroduce RK2 into plasmid-free daughter cells, we also explored the possibility that it is a factor in the maintenance of RK2 within a bacterial population. Transfer-defective (Tra^-) derivatives of RK2 were constructed by introducing mutations in *traJ*, which encodes a well-defined function that binds to the origin of conjugal transfer (14, 35, 64), and *traG*, which specifies a protein thought to link the *oriT*-relaxase complex to the conjugation pore (26, 27). The results obtained with these Tra^- mutants demonstrated that loss of conjugal transfer does not detectably alter RK2 maintenance in *E. coli* growing

in broth culture. This finding is not particularly surprising because conjugal transfer of RK2 occurs best on solid surfaces. In contrast, the *traJ* and *traG* mutations resulted in obvious plasmid loss within colonies growing on solid medium, as evidenced by the formation of plasmid-free sectors. This result has two implications: (i) the stability functions of RK2, including *par*, are not completely adequate to maintain RK2 in all cells of a clonal population growing on solid, rich medium; and (ii) the RK2 conjugal transfer system is able to compensate for plasmid loss that occurs under these conditions. An alternative explanation, that the *tra* mutations destabilize RK2 in cells grown on solid medium, is less attractive in light of the similar phenotypes exhibited by two different *tra* mutants. We conclude that the conjugal transfer system of RK2 can be a significant factor in reducing the proportion of plasmidless segregants in a growing population when growth conditions are favorable to conjugation.

In summary, we have introduced specific mutations into an otherwise intact RK2 plasmid. Our results have demonstrated that the *par* region is important for stable RK2 maintenance in all hosts tested and that conjugal transfer can also be a significant factor in the maintenance of RK2 in a growing population. The different relative importance of the *parCBA* and *parDE* operons in the different hosts once again underscores the varying requirements for the stable maintenance of RK2 in diverse gram-negative hosts and further indicates that RK2 is able to adapt by relying on stability functions best suited to a particular host.

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