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# The *kilE* Locus of Promiscuous IncPα Plasmid RK2 Is Required for Stable Maintenance in *Pseudomonas aeruginosa*

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**Eight coordinately regulated operons constitute the *kor* regulon of the IncPα plasmid RK2. Three operons specify functions required for replication initiation, conjugative transfer, and control of gene expression. The functions of the other operons, including those of the four coregulated operons that compose the *kilA*, *kilC*, and *kilE* loci, have not been determined. Here, we present the first evidence that a *kil* determinant is involved in IncP plasmid maintenance. Elevation of KorC levels specifically to reduce the expression of the KorC-regulated *kilC* and *kilE* operons severely affected the maintenance of both the IncPα plasmid RK2*lac* and the IncPβ plasmid R751 in *Pseudomonas aeruginosa* but had little effect on plasmid maintenance in *Escherichia coli*. Precise deletion of the two *kilE* operons from RK2*lac* was achieved with the VEX mutagenesis system for large genomes. The resulting plasmid showed significant loss of stability in *P. aeruginosa* only. The defect could be complemented by reintroduction of *kilE* at a different position on the plasmid. The instability of the RK2*lac* Δ*kilE* mutant did not result from a reduction in average plasmid copy number, reduced expression of *kilC*, decreased conjugative transfer, or loss of the *korE* regulator. We found that both the *par* and *kilE* loci are required for full stability of RK2*lac* in *P. aeruginosa* and that the *par* and *kilE* functions act independently. These results demonstrate a critical role for the *kilE* locus in the stable inheritance of RK2 in *P. aeruginosa*.**

The highly promiscuous plasmids of incompatibility group P (IncP) have gained considerable attention as agents for gene exchange and evolution in bacteria. They are able to promote conjugation between remarkably diverse organisms, including gram-negative and gram-positive bacteria and several species of yeast (9, 21, 22, 39, 64). In addition, they can persist as autonomously replicating elements in a wide array of gram-negative bacteria (59). The replicative promiscuity poses particularly formidable challenges for IncP plasmids. How do these plasmids achieve appropriate gene expression and effect functional interactions with host components in widely different genera of bacteria? Studies to understand the genetic basis for these remarkable abilities have focused on the identical IncPα plasmids RK2, RP1, RP4, and R68 and on the related but distinct IncPβ plasmid R751 (39, 62).

RK2 is a 60,099-bp, self-transmissible IncPα plasmid originally isolated from an antibiotic-resistant *Klebsiella aerogenes* strain cultured from a burn wound (24, 39). The promiscuous conjugation system of RK2 involves at least nineteen genes in mating pair formation and DNA processing (20, 39). The replication of RK2 depends on a single plasmid gene, *trfA*, which is both necessary (4) and sufficient (44, 45) for the initiation of vegetative replication at the origin of replication (*oriV*) in all hosts tested (12, 36, 54, 55). However, *trfA* and *oriV* are not adequate to impart the remarkable stability that RK2 exhibits in its various bacterial hosts (44–46). Two plasmid maintenance functions are specified by the *par* locus (17, 41). A postsegregational arrest mechanism that inhibits or kills plasmidless segregants emerging after cell division is encoded by the *parDE* operon (27, 42). A multimer resolution system and a possible active partition system are expressed by the adjacent *parCBA* operon (11, 17, 19, 41). The complete *par* locus is an important determinant of RK2 stability in all hosts tested, although the relative importance of *parDE* and *parCBA* varies from host to host (50). Another RK2 stability function may be specified by the cotranscribed *incC* (35, 61) and *korB* genes (13, 31, 57). The IncC polypeptide sequence has some similarity to known active partition proteins of other plasmids (37). KorB is a sequence-specific DNA binding protein that acts as a transcriptional repressor at several RK2 promoters and binds to sites of unknown function located outside promoter regions (6, 39, 48, 53). By analogy to the well-characterized systems of plasmids F and P1 (2, 23), IncC and KorB have been proposed to form an active partition system (37).

An intriguing and unique feature of IncP plasmids is the regulatory network known as the *kor* regulon (13, 14, 39). On RK2 the *kor* regulon coordinately controls the expression of eight plasmid operons. The *kor* regulon genes include the essential replication initiator gene, *trfA*; the conjugation genes of the Tra2 operon; the *korA*, *korB*, *korC*, *korF*, and *korG* regulatory determinants; the putative stability gene, *incC*; and several operons with genes of unknown function. The existence of the *kor* regulon was revealed by the presence of multiple host-lethal *kil* determinants (*kilA*, *kilB*, *kilC*, and *kilE*) whose toxic expression is controlled by the "*kil* override" determinants *korA*, *korB*, and *korC* (13, 30). The *kilB* locus was found to code for conjugal transfer genes in the Tra2 operon (38, 63). Despite the detailed molecular analysis of the *kilA*, *kilC*, and *kilE* loci, little is known about their functions. The *kilA* locus consists of an operon of three genes (*klaABC*) conserved on IncPα plasmids but missing from IncPβ plasmids (18, 60, 65). The presence of all three genes results in the inhibition of conjugal transfer of a coresident IncW plasmid (15, 18). In addition, mutations that confer resistance to tellurite map to this operon, and all three genes are required for tellurite resistance (18, 56, 65). The relationship of these disparate phenotypes to the actual function of *kilA* is not clear. The *kilC* locus is part of a KorA- and KorC-regulated operon of three genes (*klcA*, *klcB*, and *korC*) located near the origin of replication on both

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TABLE 1. Plasmids

| Plasmid | Phenotype | Relevant genotype | Description | Reference or source |
|---|---|---|---|---|
| pJAK13 | $Sp^r$ | *tacp* | Expression vector with IncQ replicon | 32 |
| pRK2526 | $Ap^r$ $Km^r$ $Tc^s$ $Lac^+$ | *tetA*::*lacZ*$^+$ *Y*$^+$ *A*$^+$ | RK2 with the *lac* operon inserted in *tetA* | 50 |
| pRK21382 | $Ap^r$ $Km^r$ $Tc^s$ $Lac^+$ $Sp^r$ | *tetA*::*lacZ*$^+$ *Y*$^+$ *A*$^+$ Δ*par1* | pRK2526 with *par* deleted and replaced by an $Sp^r$ marker | 50 |
| pRK21595 | $Ap^r$ $Km^r$ $Tc^s$ $Lac^+$ $Cm^r$ | *tetA*::*lacZ*$^+$ *Y*$^+$ *A*$^+$ Δ*kilE1* | pRK2526 with *kilE* deleted and replaced by a $Cm^r$ marker | This study |
| pRK21724 | $Ap^r$ $Lac^+$ | ϕ(*kleAp-lacZ*$^+$) | RK2 *kleA* promoter fused to *lacZ* on a mini-F replicon | 67 |
| pRK21770 | $Ap^r$ $Km^r$ $Tc^s$ $Lac^+$ $Sp^r$ $Cm^r$ | *tetA*::*lacZ*$^+$ *Y*$^+$ *A*$^+$ Δ*par1* Δ*kilE1* | pRK2526 with both *par* and *kilE* deleted and replaced by $Sp^r$ and $Cm^r$ markers, respectively | This study |
| pRK21918 | $Sp^r$ | ϕ(*tacp-korC*$^+$) | pJAK13 with *korC* downstream of *tacp* | This study |
| pRK21921 | $Ap^r$ $Tc^s$ $Lac^+$ $Cm^r$ $Km^s$ | *tetA*::*lacZ*$^+$ *Y*$^+$ *A*$^+$ Δ*kilE1 aphA*::*kilE*$^+$ | pRK21595 with the entire *kilE* region inserted in *aphA* | This study |
| R751 | $Tp^r$ | *kilC*$^+$ *kilE*$^+$ | IncPβ | 26 |
| R772 | $Km^r$ | *kilC*$^+$ *kilE*$^{+a}$ | IncPβ | 7 |

[a] Has homology to the *kilC* and *kilE* regions of RK2, as detected by hybridization (52).

IncPα and IncPβ plasmids (33, 60). KorC has been identified as a transcriptional repressor (13, 29, 58). The KlcA sequence has similarity to that of the ArdB antirestriction protein of IncN plasmid pKM101, and a segment of the predicted KlcB protein sequence is similar to sequences in the KorA and TrbA repressor proteins (25, 33). However, the functions of the KlcA and KlcB proteins remain to be determined. The *kilE* locus of RK2 consists of two operons regulated by KorA and KorC (30, 58). The *kleA* operon contains two genes (*kleAB*), and the *kleC* operon has four (*kleCDEF*) (30). The sequences of the KleA and KleC polypeptides are related, suggesting a gene duplication (30, 58). This idea is supported by the finding that the *kilE* locus of the IncPβ plasmid R751 has a single *kilE* operon composed of only four RK2-related genes (*kleA*, *kleB*, *kleE*, and *kleF*) plus an unrelated putative fifth gene, *kleG* (60).

We have suggested that the regulated *kil* loci are likely to be important for RK2 and have speculated that they code for functions needed to maintain RK2 in different hosts (13, 14, 18). The potential significance of the *kilA*, *kilC*, and *kilE* loci was strengthened by the finding that they are coregulated with genes for replication and conjugation. Here, we report the first direct evidence for the involvement of a *kil* determinant in plasmid maintenance. We show that the *kilE* locus is required for the stable inheritance of RK2 in *Pseudomonas aeruginosa.*

## MATERIALS AND METHODS

**Bacteria and plasmids.** The strains used were *Escherichia coli* EKA340 [*thr-1 leuB6 thi-1 tonA21 supE44 rfbD1* Δ*trpE5* Δ(*argF-lac*) *deoC1*::Tn*10*] (50), *E. coli* DF4063 (*thr-1 leuB6 thi-1 tonA21 supE44 rfbD1* Δ*trpE5 gyrA*) (50), *P. aeruginosa* PAC452 (40), and PAC452Rif, a spontaneous rifampin-resistant mutant of PAC452 (this study).

Plasmids are described in Table 1. Plasmid pRK21918 was constructed by cloning an *Eco*RI-*Hin*dIII fragment containing *korC* (RK2 coordinates, 4400 to 4854) into *Eco*RI- and *Hin*dIII-digested pJAK13 (32), a derivative of pMMB67 (5) with a streptomycin resistance determinant inserted into the β-lactamase gene. This cloning places the *korC* gene downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tacp* promoter (1). Plasmid pRK21595 (pRK2526Δ*kilE*) was constructed by using the VEX system for making precise deletions in large genomes as described previously (4). Flanking DNA fragments used to make the deletion had RK2 coordinates 4427 to 4673 and 2365 to 2607. The fragments were cloned into the pVEX vectors pVEX1211 and pVEX2211 (4), respectively, for recombination with pRK2526. The deletion resulted in replacement of the *kilE* locus with a 1.6-kb chloramphenicol resistance determinant and a single *loxP* site. Plasmid pRK21921 was constructed by first inserting a fragment containing the RK2 *kilE* region (RK2 coordinates, 2365 to 4612) into the *Hin*dIII site of pRK21891, a P15A replicon carrying a large region of RK2 (coordinates, 12991 to 60099). Insertion of the *kilE* fragment into pRK21891 disrupted the RK2 gene for kanamycin resistance, *aphA*. The *kilE* insertion was oriented such that transcription of the *kilE* operons was directed toward the transcriptional terminator downstream of the *aphA* gene. The resulting plasmid, pRK21919 ($Tc^r$ $Km^s$), was introduced into a strain containing pRK2526Δ*kilE* ($Tc^s$ $Km^r$). A $Tc^r$ $Km^r$ cointegrate was isolated by conjugation and resolved by homologous recombination to produce pRK21921 ($Tc^s$ $Km^s$), in which the *aphA* gene of pRK2526Δ*kilE* is replaced by *aphA*::*kilE*. Plasmid pRK21770 (pRK2526Δ*par*Δ*kilE*) was similarly constructed by homologous recombination between RK2Δ*par* (pRK21373), which is $Tc^r$ and has the *par* region replaced by a spectinomycin resistance marker, and RK2*lac* Δ*kilE* (pRK21595), which is $Tc^s$ and has the *kilE* region replaced by a chloramphenicol resistance marker.

**Media.** Growth of all strains was on either Luria-Bertani (LB) or M9-CAA media (43). M9-CAA medium was supplemented with 50 μg of tryptophan per ml for growth of *E. coli* EKA340. Antibiotics were added to the following concentrations (micrograms per milliliter) for *E. coli*: chloramphenicol, 50; kanamycin, 50; nalidixic acid, 20; penicillin, 150; rifampin, 100; spectinomycin, 50; tetracycline, 15; and trimethoprim, 50. For *P. aeruginosa*, the following concentrations (micrograms per milliliter) were used: carbenicillin, 500; kanamycin, 500; rifampin, 100; streptomycin, 100 (broth) or 400 (agar); and trimethoprim, 500. To detect $Lac^+$ colonies, solid medium contained 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. One millimolar IPTG was used for induction of *tacp-korC* on pRK21918.

**DNA procedures.** DNA manipulations with restriction endonucleases, T4 DNA ligase, and *E. coli* DNA polymerase I Klenow fragment were done according to the manufacturers' recommendations. The preparation of plasmid DNA was done by a standard alkaline lysis protocol (3) or column purification (Qiagen Inc., Chatsworth, Calif.). The purification of DNA fragments was done by the crush-and-soak method (34) or by β-agarase treatment (FMC Bioproducts, Rockland, Maine). Agarose and polyacrylamide gel electrophoresis (43) and transformation of *E. coli* (8) have been described elsewhere.

**Plasmid stability assays.** Strains were grown overnight at 37°C in broth with selection for resident plasmids. The cultures were then diluted $10^5$- to $10^6$-fold into nonselective media, grown to stationary phase, and then diluted as described above into fresh nonselective media. This cycle was repeated until the strains were grown for 60 to 160 generations. At the time of each dilution, the cultures were plated on LB X-Gal medium to determine cell titers and to assay for $Lac^+$ and $Lac^-$ colonies, indicating the presence or absence of the RK2*lacZ* derivatives. For the assays in which pRK21918 was maintained in *trans* to pRK2526, R751, or R772, strains were grown overnight with selection for both plasmids and then diluted into medium containing selection for pRK21918 only. At the time of dilution, cells were plated on LB X-Gal medium for pRK2526 (as described above) or on LB medium for R751 or R772. To score for the presence of R751 or R772, individual colonies were picked to selective medium appropriate for each plasmid. Plots of stability assays contain the average values of at least two experiments.

**Plasmid copy number.** The copy numbers of pRK2526 and pRK21595 in *E. coli* and *P. aeruginosa* hosts were determined essentially as described previously (10) with the following modifications. Cells were grown for at least five generations in LB broth with selection and then harvested, washed in ice-cold M9 salts (43)–0.1% $NaN_3$, and kept on ice until being filtered onto a nylon membrane (Amersham Hybond-N) with a 96-well manifold. Cell number was determined directly by microscopy with a hemocytometer. Serial fivefold dilutions of cells were loaded onto the membrane. The DNA hybridization probe was a 272-bp RK2 *oriT* fragment that was labeled by random priming with digoxigenin-conjugated dUTP (Boehringer Mannheim). The probe was applied to filters as

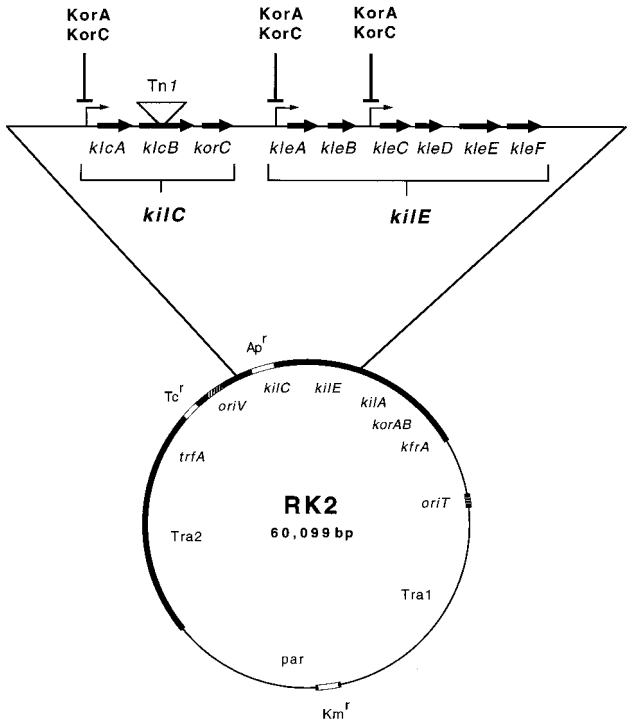


FIG. 1. The KorC-regulated genes of plasmid RK2. The *kilC* and *kilE* regions (39) have been enlarged relative to the circular map of RK2. On the circular map, the solid blocks represent the locations of *kor* regulon genes. The open blocks represent the locations of antibiotic resistance genes. The stippled blocks indicate the origin of vegetative replication (*oriV*) and the origin of transfer (*oriT*). On the enlarged region, the large arrows represent open reading frames. The thin angled arrows indicate the KorA- and KorC-regulated promoters of the *kilC*, *kleAB*, and *kleCDEF* operons. The presence of the Tn*1* insertion within the *klcB* open reading frame is indicated.

described previously in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (43)–50% formamide–0.1% *N*-lauroylsarcosine–0.02% sodium dodecyl sulfate–2% Boehringer Mannheim blocking reagent. After hybridization, filters were washed in both 2× and 0.1× SSC solutions containing 0.5% sodium dodecyl sulfate. Detection of digoxigenin-labeled probe was done as described by the supplier (Boehringer Mannheim). Exposed films were scanned with a densitometer (Molecular Dynamics Model 300A), and the peak areas were quantified with the Imagequant program (Molecular Dynamics). The amount of DNA in unknown samples was determined by comparison to dilutions of a plasmid DNA standard prepared as described previously (10). *E. coli* SM10 (51) was used as a chromosomal DNA standard, as it contains an insertion of RP4 (equivalent to RK2) in its chromosome. To determine the average number of plasmid copies per chromosome, the amounts of DNA per cell detected in the pRK2526 and pRK21595 samples were divided by the amounts detected in SM10 samples.

**Conjugative transfer assays.** Broth matings were done under stability assay conditions. Overnight cultures of donors and recipients were mixed 1:1 at approximately $10^3$ cells per ml in fresh LB medium. The cultures were grown to saturation at 37°C with shaking, serially diluted, and plated on appropriate selective media for transconjugants. Filter matings were done as previously described (50) for one h at 37°C with a donor-to-recipient ratio of 1:10. The *E. coli* donor host was EKA340 and the recipient was DF4063. Transconjugants were selected on LB-Km-Nal plates. The *P. aeruginosa* donor host was PAC452 and the recipient was PAC452Rif. Transconjugants were selected on LB-Km-Rif plates.

TABLE 2. Effect of *tacp-korC* on the expression of a *kleAp-lacZ* reporter

| Coresident plasmid[a] | Description | IPTG | β-Galactosidase activity (Miller units) | Fold reduction |
|---|---|---|---|---|
| pJAK13 | *tacp* vector | None | 1576.8 | —[b] |
| | | 1 mM | 1332.6 | — |
| pRK21918 | *tacp-korC* | None | 55.6 | 28 |
| | | 1 mM | 4.6 | 289 |

[a] The *kleAp-lacZ* reporter was present on plasmid pRK21724.
[b] —, not applicable (control).

## RESULTS

**Involvement of KorC-regulated genes in the stable maintenance of RK2.** KorC is a small, basic, DNA binding protein that represses transcription of the *kilC* operon and the two operons of the *kilE* locus (Fig. 1). The KorC target is a 17-bp palindromic sequence that overlaps the −10 sequence in all three promoters (39). No other full or half sites occur on RK2 (28, 39). Thus, it was possible specifically to test the importance of the KorC-regulated operons by artificially elevating KorC levels in the cell to reduce their expression.

To control the level of KorC, we placed the *korC* gene

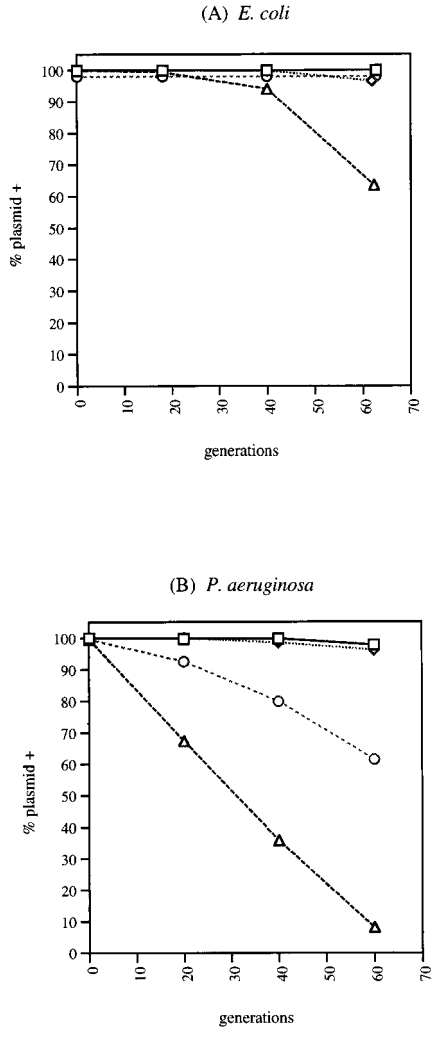


FIG. 2. Effect of elevated KorC levels on the maintenance of RK2*lac* in *E. coli* and *P. aeruginosa* hosts. Strains of *E. coli* EKA340 (A) and *P. aeruginosa* PAC452 (B) containing RK2*lac* (pRK2526) and either pJAK13 (vector) or pRK21918 (*tacp-korC*$^+$) were grown for 60 generations with selection for pJAK13 or pRK21918 only, as described in Materials and Methods. The strains were grown both in the presence and absence of IPTG. The percentage of RK2*lac*-containing cells for each strain was assayed on X-Gal plates. □, pRK2526, pJAK13, no IPTG; ◇, pRK2526, pJAK13, +IPTG; ○, pRK2526, pRK21918, no IPTG; △, pRK2526, pRK21918, +IPTG.

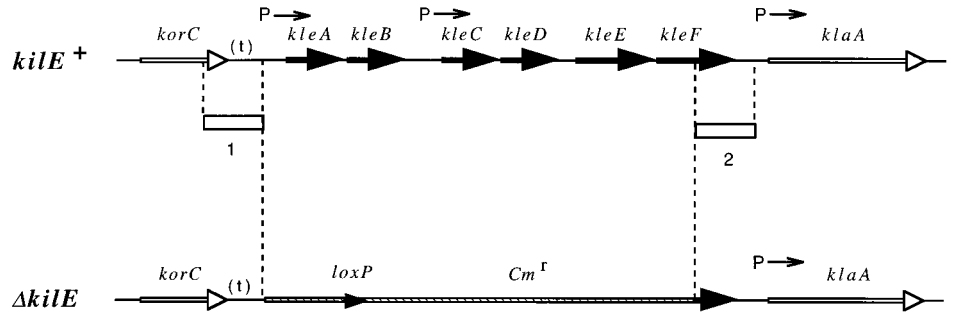


FIG. 3. VEX-mediated deletion of the *kilE* region from RK2*lac*. The *kilE* regions in both RK2*lac* (pRK2526) and RK2*lac* Δ*kilE* (pRK21595) are depicted. P indicates the location of a promoter, and small horizontal arrows indicate the direction of transcription. (t) indicates a putative transcriptional terminator. The *kilE* structural genes are indicated by bold arrows. Open arrows show the genes immediately flanking the *kilE* region. Deletion of the *kilE* region was performed with VEX (4) as described in Materials and Methods. Open boxes 1 and 2 indicate the fragments cloned into the pVEX vectors for recombination with pRK2526. The deletion resulted in the replacement of *kilE* with a 1.6-kb chloramphenicol resistance determinant and a single *loxP* site.

downstream of the inducible *tacp* promoter on a broad-host-range IncQ vector. Functional expression of *korC* from this plasmid (pRK21918) in *E. coli* was monitored by using a *lacZ* reporter fused to the *kleA* promoter on a low-copy plasmid (Table 2). Even uninduced, *korC* expression was sufficient to reduce expression of the reporter by a factor of 28 relative to the control, indicating significant expression of *korC* from pRK21918. Induction by IPTG led to further repression of the *kleA* promoter as expected. The *tacp* promoter has been shown to be similarly expressed and regulated in *P. aeruginosa* (16).

We tested the effect of elevated KorC levels on the maintenance of RK2 in both *E. coli* and *P. aeruginosa*. To facilitate detection of plasmidless segregants, we used the RK2*lac* plasmid pRK2526, an otherwise wild-type RK2 plasmid that contains a *lacZYA* insertion in the gene for tetracycline resistance, *tetA*. We have shown previously that RK2*lac* is stably maintained in *E. coli* and *P. aeruginosa* under nonselective growth conditions (50). The presence of the *tacp-korC* plasmid pRK21918 had no discernable effect on the stability of RK2*lac* in *E. coli* under uninduced conditions and caused only modest instability in this host when *korC* expression was induced with IPTG (Fig. 2). In contrast, *tacp-korC* significantly destabilized RK2*lac* in *P. aeruginosa* under uninduced conditions, and IPTG induction of *korC* caused an even greater rate of plasmid loss. Thus, reduced expression of the *kilC* and/or *kilE* operons leads to a substantial loss of RK2*lac* stability in *P. aeruginosa*.

***kilE* is required for stable maintenance in *P. aeruginosa*.** We specifically tested the importance of the *kilE* region for the maintenance of RK2. Both promoters and the six genes of the *kilE* region were deleted from RK2*lac* and replaced with a chloramphenicol resistance marker by using the VEX system (4) (Fig. 3). The resulting RK2*lac* Δ*kilE* plasmid (pRK21595) showed only a slight loss of stability in *E. coli* (Fig. 4A). In contrast, RK2*lac* Δ*kilE* was clearly unstable in *P. aeruginosa* (Fig. 4B). After 140 generations of nonselective growth, less than 10% of *P. aeruginosa* host cells carried RK2*lac* Δ*kilE*, whereas >99.9% of the cells retained RK2*lac* under the same conditions. When the data were plotted as the log percent plasmidless cells versus the number of generations of nonselective growth (Fig. 4C), it was readily apparent that plasmidless segregants lacking RK2*lac* Δ*kilE* appeared at the first time point following removal of selection. Furthermore, the kinetics of loss do not appear to reflect an enrichment for plasmidless segregants over the course of the experiment, since the growth rates of plasmidless or cured *P. aeruginosa* strains and the RK2*lac*- or RK2*lac* Δ*kilE*-containing strains were indistinguishable (data not shown).

The reduced stability of RK2*lac* Δ*kilE* is not caused by a reduction in average copy number. Hybridization analysis of both *E. coli* and *P. aeruginosa* strains revealed that the number of plasmid copies per chromosome for RK2*lac* Δ*kilE* was not significantly different from that for RK2*lac* in either host (Table 3).

To test if altered plasmid structure caused by the deletion-substitution mutation of RK2*lac* Δ*kilE* was responsible for the unstable phenotype, we determined if ectopic reinsertion of the *kilE* region could restore stability to RK2*lac* Δ*kilE* in *P. aeruginosa*. The entire, intact *kilE* region was inserted into the kanamycin resistance gene of RK2*lac* Δ*kilE* to make pRK21921. Stability assays with pRK21921 for *P. aeruginosa* demonstrated that the reintroduction of the *kilE* region almost completely restored stability (Fig. 5). This result proves that the *kilE* region is required for stable maintenance in *P. aeruginosa*.

Our previous work has shown that the conjugative transfer system of RK2 can contribute to plasmid maintenance in a growing population of *E. coli* under some conditions (50). In reconstruction experiments in which equal numbers of differentially marked plasmid-containing and plasmidless cells were mixed, we were able to detect transfer of RK2*lac* *kilE*$^+$ and RK2*lac* Δ*kilE* in *P. aeruginosa* during the course of the stability assay used here (data not shown). However, the frequency of transfer of RK2*lac* *kilE*$^+$ (0.2 transconjugants per donor) was not significantly different from that of RK2*lac* Δ*kilE* (0.4 transconjugants per donor) over one cycle (approximately 20 generations). In addition, filter matings demonstrated that RK2*lac* *kilE*$^+$ and RK2*lac* Δ*kilE* were indistinguishable in conjugative transfer in both *E. coli* (4.5 and 4.0 transconjugants per

TABLE 3. Copy numbers of RK2*lac* and RK2*lac* Δ*kilE* in *E. coli* and *P. aeruginosa*

| Host | Plasmid | Description | Copies per chromosome[a] |
|---|---|---|---|
| *E. coli* EKA340 | pRK2526 | *kilE*$^+$ | 9.6 ± 2.2 |
| *E. coli* EKA340 | pRK21595 | Δ*kilE* | 11.1 ± 0.3 |
| *P. aeruginosa* PAC452 | pRK2526 | *kilE*$^+$ | 11.5 ± 1.2 |
| *P. aeruginosa* PAC452 | pRK21595 | Δ*kilE* | 10.6 ± 0.8 |

[a] Determined by DNA-DNA hybridization as described in Materials and Methods.

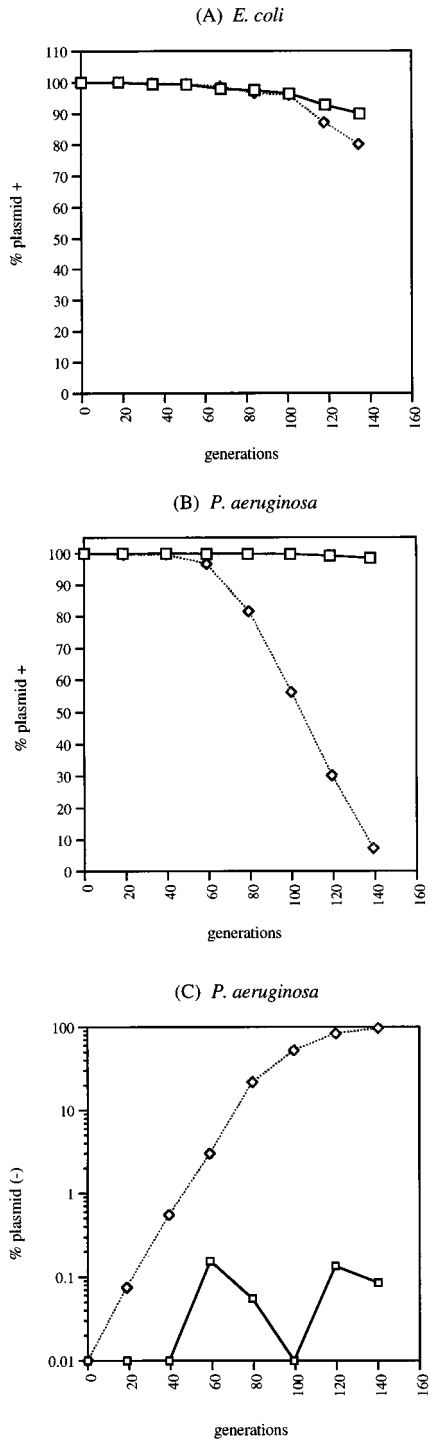


FIG. 4. Effect of the Δ*kilE* mutation on the maintenance of RK2*lac*. Strains of *E. coli* EKA340 (A) and *P. aeruginosa* PAC452 (B) containing RK2*lac* (pRK2526) (□) or RK2*lac* Δ*kilE* (pRK21595) (◇) were grown for 140 generations without plasmid selection as described in Materials and Methods. The percentage of plasmid-containing ($Lac^+$) cells in each culture was monitored on X-Gal plates. Panel C shows the same data as panel B plotted as the percent plasmidless cells on a log scale versus the number of generations of nonselective growth.

donor, respectively) and *P. aeruginosa* (6.4 and 8.2 transconjugants per donor, respectively). These results indicate that the effect of the Δ*kilE* mutation on RK2 plasmid maintenance in *P. aeruginosa* does not arise from decreased conjugative transfer.

We considered the possibility that the instability of RK2*lac* Δ*kilE* in *P. aeruginosa* is caused by the absence of two of the three KorC boxes on RK2. Because *korC* is constitutively expressed from the upstream *bla* promoter of transposon Tn*1* (29), the loss of two KorC binding sites may allow more KorC to be available for binding to the remaining KorC operator in the *kilC* promoter. The resulting reduction of *kilC* expression might conceivably be responsible for the instability of RK2*lac* Δ*kilE* in *P. aeruginosa*. This model was ruled out by showing that the unstable phenotype of RK2*lac* Δ*kilE* was not suppressed by providing additional KorC operators in *trans* on a multicopy plasmid (data not shown).

We also tested the possibility that RK2*lac* Δ*kilE* instability results from the loss of *korE*, a regulatory determinant that maps within the *kilE* region (39, 47). *korE* is not expressed as part of the *kleA* or *kleC* operons nor is it regulated by KorC. *korE* enhances the repressive activity of the RK2 KorA protein at both the *trfA* and *kilA* promoters (47, 68). This activity does not correspond to an open reading frame, and it is possible that *korE* expresses a regulatory RNA (28). The absence of such a factor could alter RK2 gene expression in *P. aeruginosa* and negatively affect plasmid maintenance. However, providing *korE* in *trans* on a multicopy plasmid did not restore stability to RK2*lac* Δ*kilE* in *P. aeruginosa* (data not shown).

Thus, the ability of RK2*lac* to be stably maintained in *P. aeruginosa* is lost by elevation of KorC levels in the cell and by removal of the *kilE* region. Taken together, these results indicate that expression of one or more genes from the *kilE* region is important for stable inheritance of RK2*lac* in *P. aeruginosa*.

**The *par* and *kilE* loci contribute independently to the stability of RK2 in *P. aeruginosa*.** Our earlier studies revealed that deletion of the *par* region from RK2*lac* results in a significant loss of stability in both *P. aeruginosa* and *E. coli* (50). To test if *par* and *kilE* act independently, we constructed a derivative of RK2*lac* in which both the *kilE* and *par* regions are deleted (pRK21770) and compared its stability with those of both single mutants.

The stability of the Δ*par*Δ*kilE* double mutant in *E. coli* was found to be similar to that of the RK2*lac* Δ*par* single mutant (pRK21382) (Fig. 6A). This result was expected because the Δ*kilE* mutation has only a slight effect on RK2*lac* maintenance in *E. coli*. For *P. aeruginosa*, the Δ*par*Δ*kilE* double mutant is considerably more unstable than both the RK2*lac* Δ*par* and RK2*lac* Δ*kilE* single mutants (Fig. 6B). This result shows that the *par* and *kilE* regions specify stability functions that act independently of each other to stabilize RK2*lac* in *P. aeruginosa*.

**Involvement of the KorC-regulated genes in the stable maintenance of IncPβ plasmids.** IncPβ plasmid R751 also contains KorC-regulated *kilC* and *kilE* operons that are related to those of IncPα plasmid RK2 (60). To investigate the possibility that KorC-regulated genes also play a role in the maintenance of IncPβ plasmids, we examined the effect of elevated KorC levels on the stable maintenance of R751 in *E. coli* and *P. aeruginosa* (Fig. 7). As we observed for RK2, the stability of R751 was affected only slightly by KorC in *E. coli*, and only when *korC* expression was induced by IPTG. However, there was a substantial loss of stability of R751 in *P. aeruginosa* under both uninduced and induced conditions of *korC* expression. We obtained nearly identical results with another IncPβ plasmid, R772 (data not shown). These results demonstrate clearly that

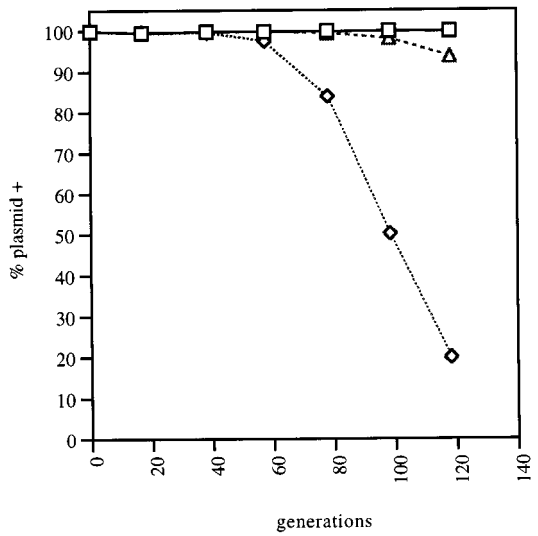


FIG. 5. Complementation of the Δ*kilE* mutation in *cis*. The maintenance of plasmids RK2*lac* (pRK2526) (□), RK2*lac* Δ*kilE* (pRK21595) (◇), and RK2*lac* Δ*kilE aphA*::*kilE*$^+$ (pRK21921) (△) was monitored for 120 generations in *P. aeruginosa* PAC452 as described in the legend for Fig. 3. The results are plotted as the percentages of plasmid-containing cells, as assayed on X-Gal plates.

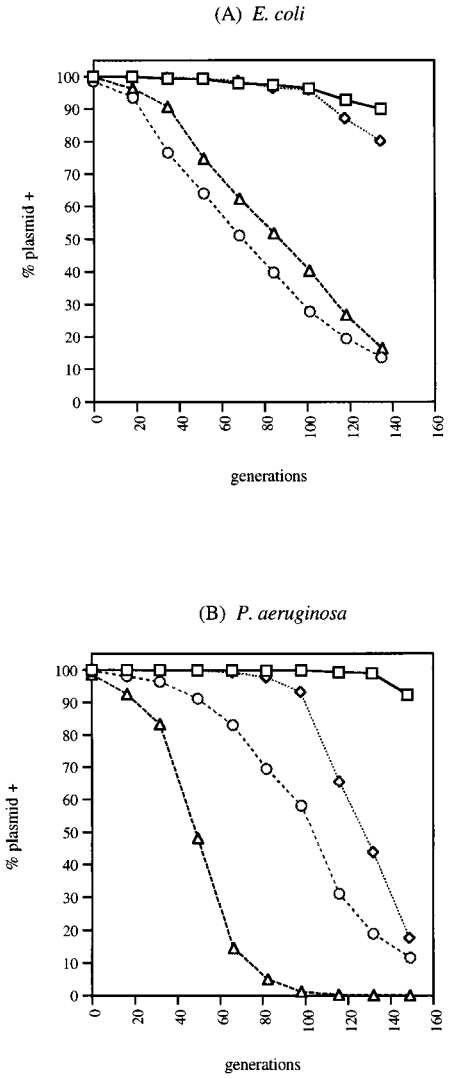


FIG. 6. Effect of the Δ*par*Δ*kilE* double mutation on the maintenance of RK2*lac*. Strains of *E. coli* EKA340 (A) and *P. aeruginosa* PAC452 (B) containing either RK2*lac* (pRK2526) (□), RK2*lac* Δ*par* (pRK21382) (○), RK2*lac* Δ*kilE* (pRK21595) (◇), or RK2*lac* Δ*par*Δ*kilE* (pRK21770) (△) were grown for 140 generations without selection, as described in Materials and Methods. The percentages of plasmid-containing cells were assayed on X-Gal plates.

KorC-regulated genes are also critical for stable maintenance of IncPβ plasmids in *P. aeruginosa*.

## DISCUSSION

The *kor* regulon is a unique feature of the broad-host-range IncP plasmids. For IncPα plasmid RK2, eight operons are controlled by combinations of the repressors KorA, KorB, and KorC (39). Except for those of the *trfA* operon (replication initiation), the Tra2 operon (conjugative transfer), and the *korA* operon (regulation), the functions of the operons have not been identified. Four operons, encompassing twelve genes, constitute the toxic *kilA*, *kilC*, and *kilE* determinants, whose unregulated expression is lethal to *E. coli* host cells (13, 18, 30, 33). The results of this study support our hypothesis that these determinants are involved in the stable maintenance of RK2 (13, 18). We found (1) that reducing *kilC* and *kilE* expression by elevating KorC in the cell caused a severe loss of stability of both IncPα and IncPβ plasmids in *P. aeruginosa* and (2) that deletion of *kilE* from RK2 results in a plasmid with significantly reduced stability in *P. aeruginosa*. The results show that at least one gene from the *kilE* region is required for stable maintenance of RK2 in *P. aeruginosa*.

Our previous studies (50) have demonstrated that stable maintenance of intact RK2 in *P. aeruginosa* requires the *par* region, which contains two divergently transcribed operons that are not regulated as part of the *kor* regulon. The *parCBA* operon encodes a multimer resolution system and is also thought to function as an active partition system (11, 17, 19, 41). The *parDE* operon specifies a postsegregational arrest mechanism which kills or inhibits the growth of plasmidless segregants (27, 42). Deletions of *par* and its individual operons have demonstrated the importance of this region to the stable maintenance of RK2 in at least six different gram-negative bacterial hosts (50). In this work we have shown that *par* and *kilE* are both required for full stability of RK2 in *P. aeruginosa*. We considered the possibility that *kilE* and one of the *par* determinants act in the same branch of a plasmid stabilization pathway. However, the Δ*par*Δ*kilE* double mutant is substantially more unstable than either single mutant in this host. Thus, the *kilE* and *par* functions act independently. This result is consistent with studies showing that the *par* region alone is able to confer increased stability on mini-RK2 plasmids in *P. aeruginosa* (41).

The Δ*kilE* mutation produced a slight, but reproducible, effect on the stability of RK2 in *E. coli*. In contrast, *par* is critical to stable maintenance in *E. coli*. Thus, *par* alone or *par* and another determinant must stabilize RK2 in *E. coli*. It is possible that a second determinant, if it exists, is encoded by the *kilC* operon because elevated *korC* expression, which reduces *kilC* expression, had a noticeable effect on RK2 stability in *E. coli*. Other determinants could also be involved, and there is evidence for a possible involvement of *incC* (37) and the *kilA* operon (49) in the stable maintenance of RK2 in *E. coli*.

The IncPβ plasmid R751 also contains *kilC* and *kilE* loci. Like those on RK2, the *kilC* and *kilE* promoters are regulated

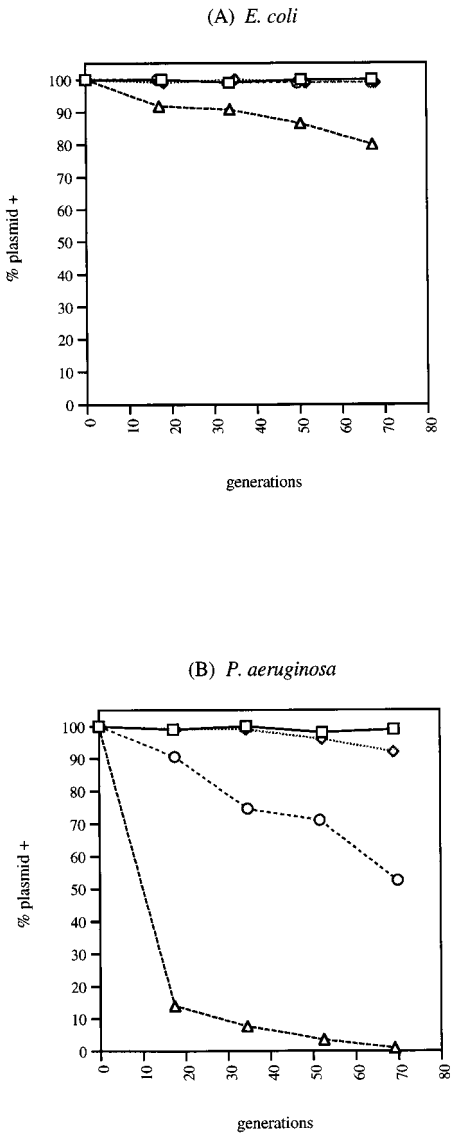


FIG. 7. Effect of elevated KorC levels on the maintenance of IncPβ plasmid R751 in *E. coli* and *P. aeruginosa*. Strains of *E. coli* EKA340 (A) and *P. aeruginosa* PAC452Rif (B) containing R751 and either pJAK13 (vector) or pRK21918 (*tacp-korC*$^+$) were grown for 70 generations with selection for pJAK13 or pRK21918 only, as described in Materials and Methods. Strains were grown both in the presence and absence of IPTG. The percentage of R751-containing cells for each strain was assayed as described in Materials and Methods. □, R751, pJAK13, no IPTG; ◇, R751, pJAK13, +IPTG; ○, R751, pRK21918, no IPTG; △, R751, pRK21918, +IPTG.

by *korA* and *korC*, and they are predicted to be of comparable strengths (28). In addition, the KorA and KorC operators are nearly identical to those of RK2, and they are located in precisely the same positions within the promoters (39, 60). However, relative to that of RK2, the stability of R751 was much more severely affected by elevated KorC levels in *P. aeruginosa*. Plasmid R751 does not contain the *par* region present on RK2 (39). It is possible that R751 maintenance in *P. aeruginosa* is more dependent on expression of genes from the *korC*-regulated *kilC* and *kilE* operons than is that of RK2. In addition, R751 enjoys full stability in *P. aeruginosa* in the absence of the *par* locus, whereas the analogous Δ*par* mutant of RK2 is unstable. Thus, the *kilC* and/or *kilE* operons of R751 may be more effective in promoting stability in *P. aeruginosa* than the *kilC* and/or *kilE* determinants of RK2. Although the *kilC* and *kilE* operons of R751 are closely related to those of RK2, there are notable differences. The *kilE* locus of R751 contains a single operon of five genes (60). The *kleABEF* genes are closely related to those of RK2, but a putative fifth gene of the operon, *kleG*, has no homolog in RK2. The presence of the *kleG* gene or the subtle sequence differences exhibited by the other *kle* gene products might be responsible for the greater effectiveness of the R751 *kilE* determinant in *P. aeruginosa*. Alternatively, variations in the *kilC* operon might account for the difference. The *kilC* operon of R751 is intact (60), whereas the *kilC* operon of RK2 is interrupted by a transposon Tn*1* insertion in *klcB*, the second gene of the operon (33). *kilC* may be involved in the stability of IncP plasmids in *P. aeruginosa*, since elevated KorC levels had more of an effect on RK2*lac* stability than did the deletion of *kilE*. Furthermore, we have found that elevated KorC levels further destabilize the RK2*lac* Δ*kilE* mutant (66). We are continuing to investigate the role of the *kilC* region in the maintenance of IncP plasmids.

Is the *kilE* stabilization function specific for *P. aeruginosa*? Both the elevated-KorC-level experiments and the phenotype of the Δ*kilE* mutant demonstrate substantial loss of stability in *P. aeruginosa* but only a slight loss of stability in *E. coli*. Such a host-specific phenotype could be explained if one or more of the *kilE* gene products exhibit a high degree of specificity for a component of the *P. aeruginosa* host. In *E. coli*, the relevant target may be missing or sufficiently different from that of *P. aeruginosa* such that a functional interaction does not occur in *E. coli*. In this case, other stability functions would take over for RK2 in *E. coli*. An alternative explanation is that *kilE* functions well in both hosts and that another stability function compensates for the absence of *kilE* in *E. coli* but not in *P. aeruginosa*. Thus, RK2 shows host-specific differences with respect to its stability functions, but whether host specificity is a property of *kilE* is not yet known. To distinguish between these models, we are currently attempting to control the toxicity of *kilE* to allow us to test if it is sufficient to stabilize an unstable miniplasmid in *P. aeruginosa* and *E. coli*. We were surprised to find that *P. aeruginosa* appears to be more sensitive to the toxicity of unregulated *kilE* expression than is *E. coli* (66). The correlation of the toxicity and stabilization phenotypes in *P. aeruginosa* and *E. coli* is consistent with the host specificity model for *kilE* function.

We do not yet know how *kilE* stabilizes RK2 in *P. aeruginosa*. Although *kilE* could encode functions analogous to known plasmid stabilization systems, such as partition or postsegregational lethality, it is also possible that *kilE* enhances RK2 maintenance in a different way. For example, the rate of loss of the RK2*lac* Δ*kilE* mutant, which has an average copy number of at least 20 per dividing cell, is greater than that predicted for a random distribution of plasmids to daughter cells (2). This raises the possibility that *kilE* could act to make plasmids available for partition, perhaps by preventing sequestration or aggregation of plasmids at the time of cell division. A *kilE*-encoded protein might also enhance stability by affecting the regulation of an RK2 gene located outside the locus, in which case the *kilE* region would be insufficient to stabilize a heterologous replicon.

The development of the VEX system to permit precision mutagenesis in an otherwise wild-type RK2 has allowed us to determine which loci are important to the replication and stable maintenance of RK2 in its various hosts (4, 50). This approach is particularly useful because the integrated regulatory structure of RK2 complicates the analysis of individual segments of the plasmid out of the context of the *kor* regulon.

Our studies so far have revealed that the replication initiator gene, *trfA*, is essential in all hosts tested (4). In contrast, the stabilization functions encoded by the *parCBA* and *parDE* operons and the *kilE* locus are important in some but not all hosts. Thus, one strategy for the broad host range exhibited by the IncP plasmids is the combination of a universally functional replicon with host-specific stability functions. The property of host specificity may illustrate a requirement for plasmid products to interact with host cell components to promote plasmid maintenance. We are intrigued by the possibility that the functions encoded by the *kilA*, *kilC*, and *kilE* operons may reveal additional insights into plasmid-host interactions and the poorly understood process of DNA inheritance in bacteria.

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## REFERENCES

1. **Amann, E., J. Brosius, and M. Ptashne.** 1983. Vector bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*. Gene **25:**167–178.
2. **Austin, S., and K. Nordström.** 1990. Partition-mediated incompatibility of bacterial plasmids. Cell **60:**351–354.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
4. **Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski.** 1993. Precise deletions in large prokaryotic genomes by vector-mediated excision (VEX): the *trfA* gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. **230:**174–185.
5. **Bagdasarian, M., R. Lurz, B. Ruckert, F. C. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors and host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237–247.
6. **Balzer, D., G. Ziegelin, W. Pansegrau, V. Kruft, and E. Lanka.** 1992. KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in regions essential for conjugative plasmid transfer. Nucleic Acids Res. **20:**1851–1858.
7. **Coetzee, J. N., G. Lecatsas, W. F. Coetzee, and R. W. Hedges.** 1979. Properties of R plasmid R772 and the corresponding pilus-specific phage PR772. J. Gen. Microbiol. **110:**263–273.
8. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
9. **Datta, N., and R. Hedges.** 1972. Host ranges of R factors. J. Gen. Microbiol. **70:**453–460.
10. **Durland, R. H., and D. R. Helinski.** 1990. Replication of the broad-host-range plasmid RK2: direct measurement of intracellular concentrations of the essential TrfA replication proteins and their effect on plasmid copy number. J. Bacteriol. **172:**3849–3858.
11. **Eberl, L., C. S. Kristensen, M. Givskov, E. Grohmann, M. Gerlitz, and H. Schwab.** 1994. Analysis of the multimer resolution system encoded by the *parCBA* operon of broad-host-range plasmid RP4. Mol. Microbiol. **12:**131–141.
12. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
13. **Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton.** 1982. Broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. Proc. Natl. Acad. Sci. USA **79:**1935–1939.
14. **Figurski, D. H., C. Young, H. C. Schreiner, R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and T. F. D'Amico.** 1985. Genetic interactions of broad host-range plasmid RK2: evidence for a complex replication regulon, p. 227–241. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corporation, New York, N.Y.
15. **Fong, S. T., and V. A. Stanisich.** 1989. Location and characterization of two functions on RP1 that inhibit the fertility of the IncW plasmid R388. J. Gen. Microbiol. **135:**499–502.
16. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
17. **Gerlitz, M., O. Hrabak, and H. Schwab.** 1990. Partitioning of broad-host-range plasmid RP4 is a complex system involving site-specific recombination. J. Bacteriol. **172:**6194–6203.
18. **Goncharoff, P., S. Saadi, C.-H. Chang, L. H. Saltman, and D. H. Figurski.** 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. J. Bacteriol. **173:**3463–3477.
19. **Grinter, N. J., G. Brewster, and P. T. Barth.** 1989. Two mechanisms for the stable inheritance of plasmid RP4. Plasmid **22:**203–214.
20. **Guiney, D. G.** 1993. Broad host-range conjugation and mobilizable plasmids in gram-negative bacteria, p. 75–103. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Publishing, New York, N.Y.
21. **Hayman, G. T., and P. L. Bolen.** 1993. Movement of shuttle plasmids from *Escherichia coli* into yeasts other than *Saccharomyces cerevisiae* using *trans*-kingdom conjugation. Plasmid **30:**251–257.
22. **Heinemann, J. A., and G. Sprague.** 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature **340:**205–209.
23. **Hiraga, S.** 1992. Chromosome and plasmid partition in *Escherichia coli*. Annu. Rev. Biochem. **61:**283–306.
24. **Ingram, L. C., M. H. Richmond, and R. B. Sykes.** 1973. Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of *Pseudomonas aeruginosa* strains isolated from burns. Antimicrob. Agents Chemother. **3:**279–288.
25. **Jagura-Burdzy, G., F. Khanim, C. A. Smith, and C. M. Thomas.** 1992. Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. Nucleic Acids Res. **20:**3939–3944.
26. **Jobanputra, R. S., and N. Datta.** 1974. Trimethoprim R factors in enterobacteria from clinical specimens. J. Med. Microbiol. **7:**169–177.
27. **Jovanovic, O. S., E. K. Ayres, and D. H. Figurski.** 1994. Host-inhibitory functions encoded by promiscuous plasmids: transient arrest of *Escherichia coli* segregants that fail to inherit plasmid RK2. J. Mol. Biol. **237:**52–64.
28. **Jovanovic, O. S., and D. H. Figurski.** Unpublished results.
29. **Kornacki, J. A., R. S. Burlage, and D. H. Figurski.** 1990. The *kil-kor* regulon of broad-host-range plasmid RK2: nucleotide sequence, polypeptide product, and expression of regulatory gene *korC*. J. Bacteriol. **172:**3040–3050.
30. **Kornacki, J. A., C.-H. Chang, and D. H. Figurski.** 1993. The *kil-kor* regulon of promiscuous plasmid RK2: structure, products, and regulation of two operons that constitute the *kilE* locus. J. Bacteriol. **175:**5078–5090.
31. **Kornacki, J. A., P. J. Balderes, and D. H. Figurski.** 1987. Nucleotide sequence of *korB*, a replication control gene of broad host-range plasmid RK2. J. Mol. Biol. **198:**211–222.
32. **Kornacki, J. A.** Unpublished results.
33. **Larsen, M. H., and D. H. Figurski.** 1994. Structure, expression, and regulation of the *kilC* operon of promiscuous IncPα plasmids. J. Bacteriol. **176:**5022–5032.
34. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
35. **Meyer, R., and M. Hinds.** 1982. Multiple mechanisms for expression of incompatibility by broad-host-range plasmid RK2. J. Bacteriol. **152:**1078–1090.
36. **Meyer, R. J., and D. R. Helinski.** 1977. Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta **478:**109–113.
37. **Motallebi-Veshareh, M., D. A. Rouch, and C. M. Thomas.** 1990. A family of ATPases involved in the active partitioning of diverse bacterial plasmids. Mol. Microbiol. **4:**1455–1463.
38. **Motallebi-Veshareh, M., D. Balzer, E. Lanka, G. Jagura-Burdzy, and C. M. Thomas.** 1992. Conjugative transfer functions of broad-host-range plasmid RK2 are coregulated with vegetative replication. Mol. Microbiol. **6:**907–920.
39. **Pansegrau, W., E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas.** 1994. Complete nucleotide sequence of Birmingham IncPα plasmids: compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
40. **Prince, A. S., and T. Barlam.** 1985. Isolation of a DNA fragment containing replication functions from IncP2 megaplasmid pMG2. J. Bacteriol. **161:**792–794.
41. **Roberts, R. C., R. Burioni, and D. R. Helinski.** 1990. Genetic characterization of the stabilizing functions of a region of broad-host-range plasmid RK2. J. Bacteriol. **172:**6204–6216.
42. **Roberts, R. C., A. Ström, and D. R. Helinski.** 1994. The *parDE* operon of broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. J. Mol. Biol. **237:**35–51.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Schmidhauser, T. J., M. Filutowicz, and D. R. Helinski.** 1983. Replication of derivatives of the broad-host-range plasmid RK2 in two distantly related bacteria. Plasmid **9:**325–330.
45. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species

of gram-negative bacteria. J. Bacteriol. **164:**446–455.
46. **Schmidhauser, T. J., D. H. Bechhofer, D. H. Figurski, and D. R. Helinski.** 1989. Host-specific effects of the *korA-korB* operon and *oriT* region on the maintenance of miniplasmid derivatives of the broad-host-range plasmid RK2. Plasmid **21:**99–112.
47. **Schreiner, H., and D. H. Figurski.** Unpublished results.
48. **Schreiner, H. C., D. H. Bechhofer, R. F. Pohlman, C. Young, P. A. Borden, and D. H. Figurski.** 1985. Replication control in promiscuous plasmid RK2: *kil* and *kor* functions affect expression of the essential replication gene *trfA*. J. Bacteriol. **163:**228–237.
49. **Sia, E. A., and D. H. Figurski.** Unpublished results.
50. **Sia, E. A., R. C. Roberts, C. Easter, D. R. Helinski, and D. H. Figurski.** 1995. Different relative importances of the *par* operons and the effect of conjugal transfer on the maintenance of intact promiscuous plasmid RK2. J. Bacteriol. **177:**2789–2797.
51. **Simon, R., U. Priefer, and H. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1:**784–791.
52. **Smith, C. A., and C. M. Thomas.** 1987. Comparison of the organization of the genomes of phenotypically diverse plasmids of incompatibility group P: members of the IncPβ sub-group are closely related. Mol. Gen. Genet. **206:**419–427.
53. **Smith, C. A., V. Shingler, and C. M. Thomas.** 1984. The *trfA* and *trfB* promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucleic Acids Res. **12:**3619–3630.
54. **Smith, C. A., and C. M. Thomas.** 1984. Nucleotide sequence of the *trfA* gene of broad host-range plasmid RK2. J. Mol. Biol. **175:**251–262.
55. **Stalker, D. M., C. M. Thomas, and D. R. Helinski.** 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181:**8–12.
56. **Taylor, D. E., and D. E. Bradley.** 1987. Location on RP4 of a tellurite resistance determinant not normally expressed in IncPα plasmids. Antimicrob. Agents Chemother. **31:**823–825.
57. **Theophilus, B. D. M., and C. M. Thomas.** 1987. Nucleotide sequence of the transcriptional repressor gene *korB* which plays a key role in regulation of the copy number of broad host range plasmid RK2. Nucleic Acids Res. **15:**7443–7450.
58. **Thomas, C. M., J. P. Ibbotson, N. Wang, C. A. Smith, R. Tipping, and N. M. Loader.** 1988. Gene regulation on broad host range plasmid RK2: identification of three novel operons whose transcription is repressed by both KorA and KorC. Nucleic Acids Res. **16:**5345–5359.
59. **Thomas, C. M., and D. R. Helinski.** 1989. Vegetative replication and stable inheritance of IncP plasmids, p. 1–25. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press Ltd., London, United Kingdom.
60. **Thomas, C. M., C. A. Smith, J. P. Ibbotson, L. Johnston, and N. Wang.** 1995. Evolution of the *korA-oriV* segment of promiscuous IncP plasmids. Microbiology **141:**1201–1210.
61. **Thomas, C. M., and C. A. Smith.** 1986. The *trfB* region of broad host range plasmid RK2: the nucleotide sequence reveals *incC* and key regulatory gene *trfB*/*korA*/*korD* as overlapping genes. Nucleic Acids Res. **14:**4453–4469.
62. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.
63. **Thomson, V. J., O. S. Jovanovic, R. F. Pohlman, C.H. Chang, and D. H. Figurski.** 1993. Structure, function, and regulation of the *kilB* locus of promiscuous plasmid RK2. J. Bacteriol. **175:**2423–2435.
64. **Trieu-Cuot, C., C. Carlier, M. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
65. **Walter, E. G., C. M. Thomas, J. P. Ibbotson, and D. E. Taylor.** 1991. Transcriptional analysis, translational analysis, and sequence of the *kilA*-tellurite resistance region of plasmid RK2Te$^r$. J. Bacteriol. **173:**1111–1119.
66. **Wilson, J. W., and D. H. Figurski.** Unpublished results.
67. **Wilson, J. W., C.-H. Chang, and D. H. Figurski.** Unpublished data.
68. **Young, C., R. S. Burlage, and D. H. Figurski.** 1987. Control of the *kilA* gene of the broad-host-range plasmid RK2: involvement of *korA*, *korB*, and a new gene, *korE*. J. Bacteriol. **169:**1315–1320.