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Incompatibility Protein IncC and Global Regulator KorB Interact in Active Partition of Promiscuous Plasmid RK2

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Replication of the broad-host-range, IncPα plasmid RK2 requires two plasmid loci: *trfA*, the replication initiator gene, and *oriV*, the origin of replication. While these determinants are sufficient for replication in a wide variety of bacteria, they do not confer the stable maintenance of parental RK2 observed in its hosts. The product of the *incC* gene has been proposed to function in the stable maintenance of RK2 because of its relatedness to the ParA family of ATPases, some of which are known to be involved in the active partition of plasmid and chromosomal DNA. Here we show that IncC has the properties expected of a component of an active partition system. The smaller polypeptide product of *incC* (IncC2) exhibits a strong, replicon-independent incompatibility phenotype with RK2. This incompatibility phenotype requires the global transcriptional repressor, KorB, and the target for *incC*-mediated incompatibility is a KorB-binding site (O_B). We found that KorB and IncC interact in vivo by using the yeast two-hybrid system and in vitro by using partially purified proteins. Elevated expression of the *incC* and *korB* genes individually has no obvious effect on *Escherichia coli* cell growth, but their simultaneous overexpression is toxic, indicating a possible interaction of IncC-KorB complexes with a vital host target. A region of RK2 bearing *incC*, *korB*, and multiple KorB-binding sites is able to stabilize an unstable, heterologous plasmid in an *incC*-dependent manner. Finally, elevated levels of IncC2 cause RK2 to aggregate, indicating a possible role for IncC in plasmid pairing. These findings demonstrate that IncC, KorB, and at least one KorB-binding site are components of an active partition system for the promiscuous plasmid RK2.

The self-transmissible plasmids of incompatibility group P (IncP) are known for their remarkably broad host range. They are capable of promoting conjugative transfer to diverse organisms, including gram-negative and gram-positive bacteria and even some yeast species (14, 31, 35, 36, 67, 87). In addition, IncP plasmids are maintained as stable, autonomously replicating elements in a wide variety of gram-negative hosts (79, 83). The identical IncPα plasmids RK2, RP1, RP4, and R68 (67), as well as the related IncPβ plasmid R751 (86), have been intensively studied to understand the basis for the remarkable replicative promiscuity and segregational stability observed in the various bacterial hosts.

RK2 is a 60,099-bp, self-transmissible IncPα plasmid originally isolated from an antibiotic-resistant *Klebsiella aerogenes* strain cultured from a burn wound (40, 67). Conjugative transfer of RK2 requires at least 19 genes involved in mating pair formation and DNA processing (67). In contrast, replication of RK2 requires a single plasmid-encoded gene, *trfA*, which is necessary (6) and sufficient (77) for replication initiation at the plasmid origin of replication, *oriV*, in all hosts tested (68, 80). Control of initiation is mediated largely through coupled complexes of TrfA and *oriV* (48), and the plasmid is maintained at the moderate copy number of 5 to 10 plasmids per chromosome (24, 94). However, the minimal *oriV-trfA* replicon is not sufficient for the remarkable stability observed of RK2 in its various hosts (77), indicating the existence of additional determinants that act to maintain the plasmid in a growing bacterial population.

Deletion studies of otherwise intact RK2 have shown that both the *kilE* and *par* loci are involved in the stable maintenance of RK2 in different hosts. The *kilE* locus, which contains two operons encoding the *kleABCDEF* genes, is required for the stable inheritance of RK2 in *Pseudomonas aeruginosa* but not in *Escherichia coli* (50, 94). The predicted products of the *kle* genes are not similar to any known or predicted proteins, and the mechanism of stabilization imparted by *kilE* is not known. The *par* locus encodes two plasmid maintenance functions (30, 33, 70). The *parDE* operon specifies a plasmid addiction system that is toxic to plasmidless segregants that emerge after cell division (46, 71). The adjacent and divergently transcribed *parCBA* operon expresses a multimer resolution system (20, 21). Both *par* operons contribute to the stability of RK2, although the relative importance of each operon varies from host to host (79).

Some low-copy plasmids, like P1, F, and R1, contain active partition systems to ensure that a copy of the plasmid segregates to each daughter cell at cell division (37, 44, 64, 93). These systems share common features: an autoregulated operon of two genes and a nearby *cis*-acting sequence that has the properties of a centromere-like element. One of the proteins (e.g., ParA of P1, SopA of F, and ParM of R1) has, or is predicted to have, ATPase activity (18, 19, 44, 62, 90). The second gene of the operon encodes a DNA-binding protein (ParB of P1, SopB of F, and ParR of R1), whose target is the nearby *cis*-acting centromere-like element (17, 27, 44, 61). The genetic properties of these plasmid stability loci, most notably their incompatibility phenotypes, led to a model for active partition that involves plasmid pairing through proteins bound to the *cis* element, proper cellular localization of plasmid pairs

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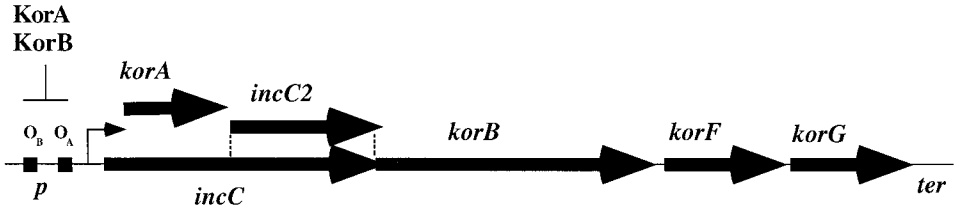


FIG. 1. The *korA* operon of plasmid RK2. The *korA*, *incC*, and *korB* genes (boldface arrows) are described in the text. The *korA* gene is within the *incC* coding sequence but in a different reading frame. *incC2* is the coding region for the small IncC2 polypeptide product that results from an internal translation initiation site in *incC*. *korF* and *korG* code for small basic proteins of unknown function. *p* indicates the promoter; O_A and O_B indicate the operators for the KorA and KorB repressors, respectively; the angled arrow indicates the transcriptional start site. *ter* indicates a putative transcriptional terminator.

at cell division, and active separation of the plasmid pairs into the newly forming daughter cells (3, 4, 65). Recent elegant studies using fluorescence microscopy to visualize F and P1 plasmids in the cell have provided dramatic evidence in support of this model (32, 63). Similar studies have also confirmed the active segregation of bacterial chromosomes (32, 60, 91) and revealed chromosomal determinants closely related to the active partition systems of plasmids. Nevertheless, the composition of the host machinery and the mechanism of active partition for plasmids and chromosomes have remained elusive.

An active partition system has been proposed for plasmid RK2 (62). Meyer and Hinds (59) first identified an incompatibility determinant (IncP1-II) in the region that encodes the global transcriptional repressors KorA and KorB. Sequence analysis subsequently revealed a third gene (designated *incC*) overlapping *korA* in a different reading frame and extending to the beginning of *korB* (84) (Fig. 1). Two polypeptides are expressed from *incC*: the full-length IncC1 protein (38.1 kDa) and a shorter IncC2 protein (27.5 kDa) that is initiated from an internal translational start site (51, 84). The sequences of both IncC polypeptides show significant relatedness with the partition proteins ParA and SopA of P1 and F, respectively (62). This similarity led to the proposal that IncC is a component of an active partition system of RK2. Recent studies from the Thomas laboratory provide strong evidence that *incC* is involved in plasmid stabilization (9, 92).

We have undertaken a systematic analysis of the properties of the *incC* region with respect to incompatibility phenotypes, plasmid stabilization, *cis*-acting elements, and protein-protein interactions to test for an active partition system on RK2. Our results demonstrate that IncC, KorB, and a KorB-binding site are components of an active partition complex.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* strains were BL21(DE3, pLysS) {F$^-$ *hsdS gal dcm ompT* [λD69 ϕ(*lacUV5p*-T7 gene *1*)]} (81); BR2943 {*hsdR17 thi-1 relA1 supE44 endA1 gyrA96 recA1* [λDKC266(P1 *repA*$^+$)]} (from D. Chattoraj); DH5α [*supE44* Δ(*laclZYA-argF*)*U169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 deoR* (ϕ*80dlac lacZ*Δ*M15*)] (34); EKA335 (previously EKA340.2) [*thr-1 leu-6 lacY1 thi-1 tonA21 supE44 rfbD1* Δ*trpE5* Δ(*argF-lac*)*U169 deoC1::*Tn*10*(*Tc*^{s}) *srl::*Tn*10 recA*] (79); EKA13 (*hsdR lacY leuB6* Δ*trpE5 recA1 gyrA*), a spontaneous nalidixic acid-resistant mutant of JA221 (from C. Yanofsky); LS1443 [*pcnB80 zad::*Tn*10 hsdR2 mcrB1 araD139* Δ(*ara-leu*)*7696* Δ*lacX74 galU galK rpsL thi-1*] (from H. Shuman); and M15(pREP4) (Qiagen, Valencia, Calif.). *Saccharomyces cerevisiae* stains were L40 (MAT**a** trp1 leu2 his3 URA3::*lexA-lacZ* LYS2::*lexA* HIS3) (88) and L41 (MATα trp1 leu2 his3 URA3::*lexA-lacZ* LYS2::*lexA* HIS3) (from D. Shore).

The plasmids used in these experiments are described in Table 1 and Fig. 2. The following unpublished plasmids were constructed as indicated: pDB6, by spontaneous Ap^s deletion of pACYC177 (12); pRK21261, by ligation of a *Hin*dIII fragment encoding spectinomycin resistance with *Hin*dIII-cleaved pRK2108 (25); pRK21484, by PCR amplification of the *korB* coding region from pRK2108 using the oligonucleotide primers korBpp1 (5′-GCGGATCCATCGA GGGTAGAATGACTGCGGCTCAAGCCAAGAC-3′) and korBpp2 (5′-CGA GCCAAGCTTGCTCCTTGTAGCGGAACCGTTGTC-3′) followed by end filling of the product using the Klenow fragment of DNA polymerase I, digestion with *Bam*HI and *Hin*dIII, and ligation to *Bam*HI- and *Hin*dIII-digested pQE-8 (Qiagen); pRK21665, by PCR amplification of the *korB* coding region from pRK2108 using the oligonucleotide primers korB-1 (5′-GGCTCAAGCCAAGA CCACCAAG-3′) and korBpp2, followed by ligation of the amplification product to pCRII (InVitrogen, Carlsbad, Calif.); pRK21673, by ligation of the *Eco*RI fragment containing the *korB* coding region from pRK21665 to *Eco*RI-digested pGAD10; pRK21674, by ligation of the *Eco*RI fragment containing the *korB* coding region from pRK21665 to *Eco*RI-digested pBTM116; pRK21841, by PCR amplification of *incC* from pRK2108 using the oligonucleotide primers IncCUP (5′-GGGTGTTATCCATGAAGAAA-3′) and IncCLPS1 (5′-GTCGACAGTC ATTGGGAAATCTCCA-3′) followed by ligation of the amplification product to pCRII; pRK21842, by ligation of the *Eco*RI fragment containing the *incC* coding region from pRK21841 to an *Eco*RI digest of pGAD10; pRK21845, by ligation of the *incC*-containing *Eco*RI fragment from pRK21841 to *Eco*RI-digested pET-17b (Novagen, Madison, Wis.); pRK21984, by PCR amplification of IncC from pRK2526 using the oligonucleotide primers IncC2_upstream (5′-CGCCAAGA AAAAACAGGAAACCAAACG-3′) and IncC2_dnstream (5′-CTTGAGCCGC AGTCATTGGGAAATCTC-3′) followed by ligation of the amplification product to pCR2.1 (InVitrogen); pRK21985, by digestion of pRK21984 with *Hin*dIII and *Xba*I and ligation to *Hin*dIII- and *Xba*I-digested pJAK16, a derivative of pMMB67 (28) (from J. Kornacki); pRK22323, by PCR amplification of O_B3 from pRK2101 (24) using the oligonucleotide primers OB3up (5′-CTGAAATC GGGAAGTGCGAAAAGCATCACCT-3′) and OB3dn (5′-CCCTGCTTCGC AGCCTGGTATTCAGGCTCG-3′), followed by ligation of the amplification product into pCR2.1; pRK22324, by digestion of pRK2362 with *Bss*HII, followed by religation to form an in-frame deletion within *incC*; pRK22327, by digestion of pRK22323 with *Eco*RI and ligation to an *Eco*RI digest of pZeRO (InVitrogen); pRK22329, by digestion of pRK2362 with *Eco*RV and *Hin*dIII, followed by ligation to *Eco*RV- and *Hin*dIII-digested pRK2101; pRK22330, by digestion of pRK22324 with *Eco*RV and *Hin*dIII, followed by ligation to *Eco*RV- and *Hin*dIII-digested pRK2101; and pTR3, by digestion and religation of pZeRO with the compatible end-generating enzymes *Spe*I and *Xba*I.

Media. Media for growth of bacteria were Luria-Bertani (LB) broth and M9-CAA medium (56). M9-CAA medium was supplemented with tryptophan (50 μg/ml) when necessary. The following antibiotics were used at the indicated concentrations: ampicillin, 50 μg/ml; penicillin, 150 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 50 μg/ml; and zeocin, 50 μg/ml. To induce expression of proteins from *tacp* or *trcp* promoters, the medium was supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To detect Lac$^+$ colonies, solid medium contained 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Medium for growth of yeast was yeast extract-peptone-dextrose (YEPD) and synthetic complete (SC) medium (5) lacking histidine, tryptophan, and/or leucine.

DNA procedures. Preparation of DNA from *E. coli* was done by the alkaline lysis protocol (5). Preparation of DNA from *S. cerevisiae* was done according to a glass bead protocol (39). Agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE) have been described previously (74). DNA manipulations with restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were done according to the manufacturers' recommendations. Amplification of DNA by PCR was done with *Taq* DNA polymerase (73). All cloned PCR products were confirmed by nucleotide sequencing. Transformation of bacteria was done by the method of Cohen et al. (13). Transformation of yeast was done by the method of high-efficiency Li acetate transformation (75).

Incompatibility assays. Strains were grown overnight at 37°C in broth with selection for both the *tacp-incC2* plasmid and the test plasmid. Dilutions of the cultures were then plated on medium selecting for the *tacp* plasmid alone (single selection) with and without IPTG, as well as medium selecting for both plasmids (double selection) with and without IPTG. Single-selection medium was supplemented with X-Gal if the test plasmid was Lac$^+$. The presence of the test plasmid was screened for either by taking the ratio of blue to white colonies or by picking individual colonies from single-selection plates and moving them onto medium with the appropriate antibiotic. For each assay, the viable cell count (CFU) of the single selection without IPTG was normalized to an efficiency of plating (EOP)

TABLE 1. Plasmids used in this study

Plasmid	Marker(s)	Relevant genotype	Description	Reference or source
pBR322	Ap^r Tc^r		pMB1 replicon; vector control	74
pBTM116	Ap^r TRP^+	*lexA*(DB)	Cloning vector for *lexA*(DB) fusion in yeast two-hybrid system	88
pDB6	Km^r		P15A replicon; vector control	Bechhofer and Figurski[a]
pGAD10	Ap^r LEU^+	GAL4(AD)	Cloning vector for GAL4(AD) fusion in yeast two-hybrid system	22
pJAK16	Cm^r	*lacI*^{q} *tacp*	IncQ replicon; expression vector	Kornacki[b]
pKJ1	Ap^r		P15A replicon; vector control	69
pRK353	Trp^+		R6K replicon; vector control	49
pRK2013	Km^r	Δ*kilA* Δ*kilE* Δ*kilC* Δ*par* Δ*oriV*	ColE1 replicon; Tra^+ (Fig. 2)	23
pRK2101	Ap^r	*korA*$^+$ *incC*$^+$ *korB*$^+$ *korF*$^+$ *korG*$^+$ *kfrA*$^+$ *upf54.8*$^+$ O_B1^+ O_B2^+ O_B3^+	pMB1 replicon (Fig. 2)	24
pRK2178	Km^r	*incC*$^+$ *korB*$^+$ O_B1^-	P15A replicon; has *korA* promoter but lacks O_B1 (Fig. 2)	8
pRK2300	Km^r	Δ*incC korB*$^+$ O_B1^-	pRK2178 with 465-bp in-frame deletion within *incC* (Fig. 2)	8
pRK2362	Ap^r	*incC*$^+$ *korB*$^+$ O_B1^+	P15A replicon (Fig. 2)	8
pRK2366	Ap^r	*incC*$^+$ *korB*$^-$ O_B1^+	pRK2362 with 14-bp deletion at 3′ end of *korB* (Fig. 2)	8
pRK2526	Ap^r Km^r Tc^s Lac^+	*tetA::lacZYA*	RK2 with *lac* operon insertion in *tetA*	79
pRK21261	Sp^r Trp^+	*korA*$^+$ *incC*$^+$ *korB*$^+$ *korF*$^+$ *korG*$^+$ *kfrA*$^+$ *upf54.8*$^+$ O_B1^+ O_B2^+ O_B3^+	pSM1 replicon (Fig. 2)	This study
pRK21382	Ap^r Km^r Tc^s Lac^+ Sp^r	*tetA::lacZYA* Δ*par1*	pRK2526 with *par* deleted and replaced with Sp^r	79
pRK21408	Trp^+	*lacI*^{q} ϕ[*trcp-korB*]	pRK353 with *korB* expressed from *trcp*	85
pRK21484	Ap^r	ϕ[T7 ϕ*10p-6xhis-korB*]	pQE-8 derivative for expression of *his-korB*	This study
pRK21591	Ap^r Km^r Tc^s Lac^+ Tp^r	*tetA::lacZYA* Δ*trfA*::P1*ori*	pRK2526 with *trfA* deleted and replaced with the P1 *ori* and a trimethoprim resistance marker	Sia and Figurski[c]
pRK21673	Ap^r LEU^+	ϕ[GAL4(AD)-*korB*]	pGAD10 with *korB* fusion for yeast two-hybrid assay	This study
pRK21674	Ap^r TRP^+	ϕ[*lexA*(DB)-*korB*]	pBTM116 with *korB* fusion for yeast two-hybrid assay	This study
pRK21842	Ap^r TRP^+	ϕ[GAL4(AD)-*incC*]	pGAD10 with *incC* fusion for yeast two-hybrid assay	This study
pRK21845	Ap^r	ϕ[T7 ϕ*10p-T7 · TAG-incC*]	pET-17b derivative for expression of *T7-incC*	This study
pRK21985	Cm^r	*lacI*^{q} ϕ[*tacp-incC2*]	pJAK16 with *incC2* coding region expressed from *tacp*	This study
pRK22324	Ap^r	Δ*incC korB*$^+$ O_B1^+	pRK2362 with 465-bp in-frame deletion within *incC* (Fig. 2)	This study
pRK22327	Zeo^r	O_B3^+	pZeRO with O_B3	This study
pRK22329	Ap^r	*korA*$^+$ *incC*$^+$ *korB*$^+$ *korF*$^+$ *korG*$^+$ *kfrA*$^+$ *upf54.8*$^+$ O_B1^+ O_B2^+ O_B3^+	P15A replicon (Fig. 2)	This study
pRK22330	Ap^r	*korA*$^+$ Δ*incC korB*$^+$ *korF*$^+$ *korG*$^+$ *kfrA*$^+$ *upf54.8*$^+$ O_B1^+ O_B2^+ O_B3^+	pRK22329 with 465-bp in-frame deletion within *incC* (Fig. 2)	This study
pRR10	Ap^r	*trfA*$^+$ *oriV*$^+$	Mini-RK2	70
pTR3	Zeo^r		pZeRO with *ccdB* out of frame; vector control	This study

[a] D. H. Bechhofer and D. H. Figurski, unpublished results.
[b] J. A. Kornacki, unpublished results.
[c] E. A. Sia and D. H. Figurski, unpublished results.

of 1.0. The CFU of other sets were then obtained and the relative EOPs were calculated.

Plasmid stability assays. Short-term stability assays (0 to 15 generations) were done as follows. Approximately 10^8 cells were scraped from a fresh selection plate and resuspended in 0.5 ml of LB broth. This sample was diluted 10^4- or 10^5-fold into fresh, nonselective medium and then grown at 37°C. Samples were taken every 2 h to obtain time points every few generations, and then they were plated on both selective and nonselective media to determine the number of generations of growth and percent plasmid retention. For longer-term assays (>15 generations), strains were grown overnight at 37°C in broth with selection for resident plasmids. The cultures were diluted 10^6-fold into nonselective medium, grown to stationary phase, and then diluted as described above into fresh nonselective medium. At each time point, dilutions were plated on nonselective medium, and plasmid retention was measured by picking individual colonies and plating them onto selective medium.

Yeast two-hybrid assay. Derivatives of pBTM116 (vector for LexA DNA-binding domain fusions) were introduced into *S. cerevisiae* haploid strain L41 by transformation and selection for growth on SC medium lacking tryptophan. Derivatives of pGAD10 (vector for GAL4 activation domain fusions) were introduced into *S. cerevisiae* haploid strain L40 with selection for growth on SC medium lacking leucine. To test for interactions between pBTM166 and pGAD10 derivatives, diploid strains were constructed by spotting 5 μl of the L41 and L40 strains together on a YEPD plate. Spots were incubated overnight at 30°C, transferred to sterile velvet, and replica-plated to SC medium lacking tryptophan and leucine to select for the L40/41 a/α strain. Diploid strains containing both the pBTM116 and pGAD10 derivatives were then tested for interaction of fusion products. Broth cultures were grown overnight and β-galactosidase activity was measured as previously described (5).

Purification of His-KorB. The *korB* coding region, beginning with the second codon, was fused in-frame with a 12-codon open reading frame which includes an N-terminal six-His tag downstream of an inducible promoter to generate pRK21484 (described above). The *korB* gene fusion was induced in strain M15 (pREP4). One hundred milliliters of cells were grown in LB broth to an optical density at 600 nm of 0.6 and then were induced by adding IPTG to a final concentration of 1 mM. The culture was incubated at 37°C for 2.5 h, and the cells were collected by centrifugation at 4,000 × *g* for 10 min. The pellet was resuspended in 300 μl of sonication buffer A (50 mM NaH_2PO_4, 300 mM NaCl [pH 8.0]), supplemented with lysozyme (1 mg/ml). The cells were maintained on ice for 5 min; 0.33 ml of 3 M NaCl was added, and the mixture was maintained on ice an additional 5 min. The cells were then sonicated on ice with four 30-s pulses. The sonicate was passed over a Ni-nitrilotriacetic acid-agarose column (Qiagen), and the His-KorB fusion protein was eluted with 40 mM imidazole. The final concentration was approximately 110 μg/ml. His-KorB is competent for binding its DNA target O_B as determined by electrophoretic mobility shift analysis (data not shown). Histidine-tagged glutathione-*S*-transferase (GST-His), for use as a control for binding specificity, was prepared similarly using the GST-His expression plasmid pALEX (gift of S. J. Silverstein).

Preparation of T7-IncC extracts. The *incC* coding region, beginning with the second codon, was fused in-frame to a 34-codon open reading frame whose transcriptional and translational initiation signals were provided by the bacteriophage T7 gene ϕ*10* in the plasmid vector pET-17b (Novagen). This region includes the 12 codons for the leader peptide (T7 · TAG epitope) of T7 gene *10* product. The *incC* fusion protein was designated T7-IncC, and the resulting plasmid was pRK21845 (described above). *T7-incC* was induced in strain BL21 (DE3, pLysS), which contains an IPTG-inducible T7 RNA polymerase gene (81). For induction, cells were grown in LB broth to an optical density at 600 nm of 0.6, and T7 polymerase was induced by adding IPTG to a final concentration of 1 mM. Incubation continued at 37°C for 3 h; cells were then collected by centrifugation at 4,000 × *g* for 10 min; and the pellet was stored at −70°C. The pellet was thawed and resuspended in 300 μl of sonication buffer B (100 mM NaCl, 50 mM NaH_2PO_4, 20 mM Tris-HCl [pH 8.0]) with 1 mM phenylmethylsulfonyl flouride. The suspension was sonicated on ice with two 15-s pulses. PAGE showed that T7-IncC constituted about 50% of the total protein, or approximately 140 μg/ml. The resulting lysate was probed by Western immunoblot

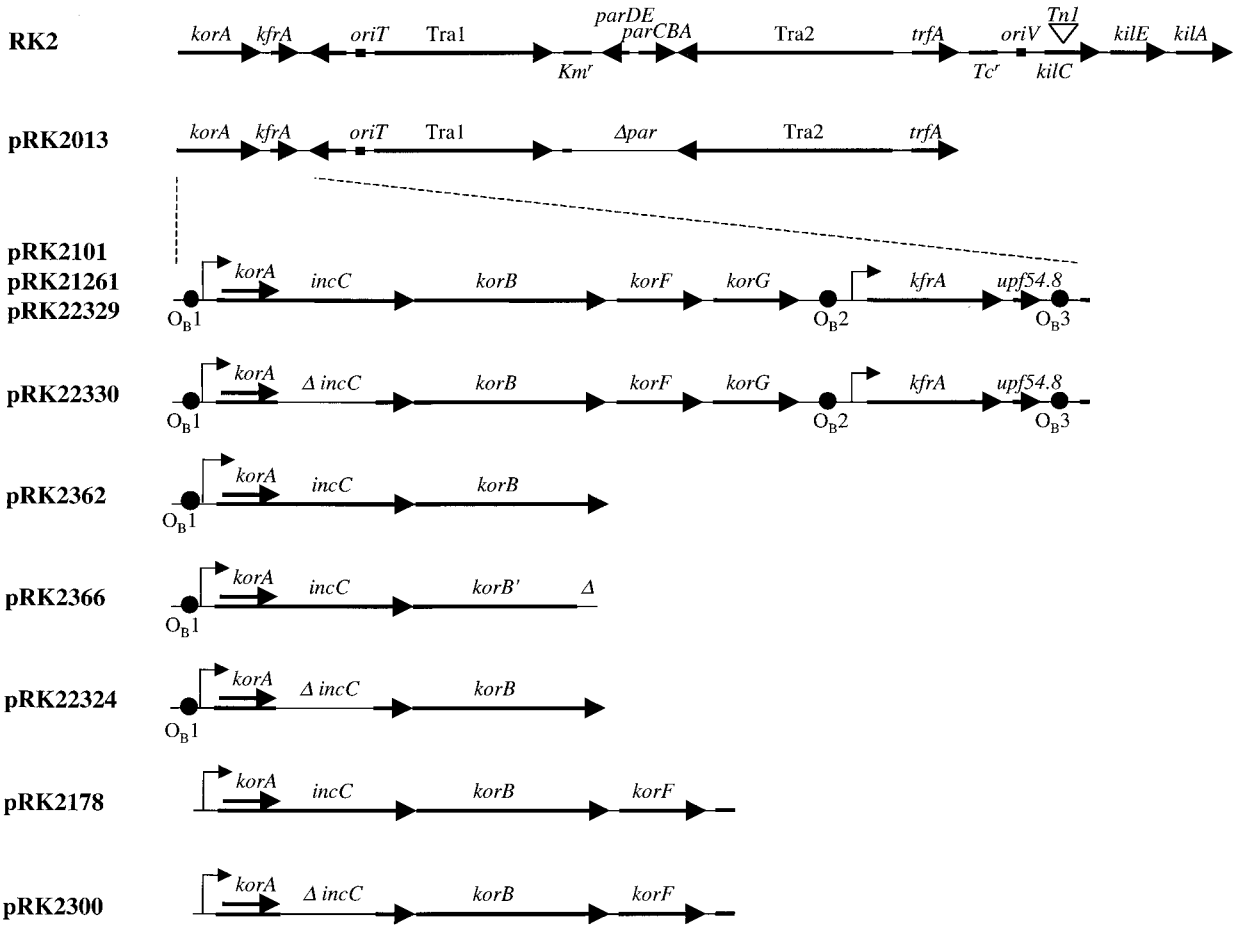


FIG. 2. Plasmids used to study the activities of *incC*. A linear schematic of the RK2 map is shown on the top line with landmark genetic determinants for reference (67). Shown below are the cloned RK2 segments in different plasmid derivatives. Boldface arrows indicate the direction of transcription, angled arrows indicate transcription start sites, and △ indicates a deletion. Tra1 and Tra2 are regions for conjugative transfer; *oriT* is the origin of transfer. Tn*1* is a transposon that encodes resistance to ampicillin; Km^r and Tc^r indicate genes for resistance to kanamycin and tetracycline, respectively. *kfrA* is a gene for a DNA-binding protein of unknown function; *upf54.8* is a putative gene of unknown function. The specific KorB-binding sites (O_B1, O_B2, and O_B3) are indicated by filled circles.

analysis for T7-IncC with the chemiluminescence Amersham Life Sciences (Arlington Heights, Ill.) ECL kit and rainbow molecular weight markers. Anti-T7 · TAG monoclonal antibody directed against the T7 gene *10* leader peptide was obtained from Novagen. The remaining lysate was stored at −70°C for future use. For controls, isogenic vector (pET-17b) extracts were made at the same time.

Immuno-affinity assay. To clear the lysates of proteins that interact nonspecifically with the Sepharose beads, 10 μl each of the T7-IncC and vector control extracts were preadsorbed to 10 μl of protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) in 180 μl of adsorption buffer (20 mM $NaHPO_4$, pH 7.0) at room temperature for 1.5 h. The beads were centrifuged, and the supernatant was used as the source of T7-IncC (or vector extract) for the assay. The anti-T7 · TAG monoclonal antibody was bound to protein A by adding 2 μl of antibody to 10 μl of protein A-Sepharose beads, 180 μl of adsorption buffer, and 10 μl of 10-mg/ml bovine serum albumin. The antibody-bead mixture was incubated on a rocker at 4°C for 1.5 h and washed twice in 200 μl of adsorption buffer. The precleared T7-IncC and vector extracts were each added to the antibody-bead complex and incubated on a rocker at 4°C for 1.5 h and then washed 4 times in 200 μl of adsorption buffer. The precipitated bead complex was resuspended in 180 μl of binding buffer (20 mM HEPES, pH 8.0), and increasing amounts (5 to 50 μl) of purified His-KorB were added. The complex was incubated on a rocker at 4°C for 1.5 h and washed four times in 200 μl of binding buffer. The complex was resuspended in 50 μl of sodium dodecyl sulfate (SDS)-PAGE sample buffer, boiled for 7 min, and then analyzed by Western blotting using the Amersham Life Sciences ECL detection kit and rabbit polyclonal anti-KorB antisera (Cocalico Biologicals, Reamstown, Pa.).

Oligohistidine affinity assay. His-KorB was added to 25 μl of TALON metal affinity resin (Clontech, Palo Alto, Calif.) along with 10 μl of 10-mg/ml bovine serum albumin (as a nonspecific competitor) and adsorption buffer (20 mM $NaHPO_4$, pH 7.0) to give a final volume of 200 μl. The His-KorB-TALON complex was incubated on a rocker at 4°C for 25 min and washed twice in 200 μl of adsorption buffer. Increasing amounts of T7-IncC extract (5 to 50 μl) were added to the complex; the suspension was then incubated on a rocker at 4°C for 25 min and washed four times in 200 μl of adsorption buffer. The resulting complex was resuspended in 50 μl of SDS-PAGE sample buffer, boiled for 7 min, and then analyzed by Western blotting using the Amersham Life Sciences ECL detection kit and the anti-T7 · TAG monoclonal antibody (Novagen).

RESULTS

Elevated expression of IncC2 causes strong incompatibility with RK2. Previous studies have demonstrated that plasmids carrying a region of RK2 encoding *incC* can destabilize RK2 derivatives present in the same cell (59). To determine if *incC* alone is sufficient to confer this incompatibility phenotype, the portion of *incC* that encodes the IncC2 polypeptide (*incC2*) (Fig. 1) was amplified by PCR and cloned downstream of the IPTG-inducible *tacp* promoter. We confirmed that induction of *incC2* in the absence of any other plasmid in the cell has no obvious effect on the growth of the culture or colony formation. We then tested the effect of *incC2* induction on the RK2*lac* plasmid pRK2526, an otherwise wild-type RK2 plasmid with *lacZYA* inserted into the gene for tetracycline resistance. We have previously shown that RK2*lac* is stably maintained in *E. coli* in the absence of selection (79). However, induction of *incC2* in *trans* to RK2*lac* gave evidence of strong incompatibility (Table 2). The EOP of the culture was reduced

TABLE 2. IncC-mediated incompatibility is replicon- independent

Test plasmid	Induced plasmid	Single selection[a]				Double selection		Incompatibility
		−IPTG		+IPTG		EOP[c]		
		EOP	Fraction plasmid [+][b]	EOP[c]	Fraction plasmid [+][b]	−IPTG	+IPTG	
RK2*lac*	Vector	1.0	0.99	1.2	1.0	0.7	0.9	−
	tacp-incC2$^+$	1.0	0.96	0.1	$<10^{-4}$	0.7	2.8×10^{-6}	+
RK2*lac*Δ*par*	Vector	1.0	0.99	1.3	1.0	0.8	0.8	−
	tacp-incC2$^+$	1.0	0.42	1.3	$<10^{-4}$	0.5	3.9×10^{-6}	+
RK2Δ*trfA::P1 ori*[d]	Vector	1.0	1.0	1.0	1.0[e]	1.3	1.0	−
	tacp-incC2$^+$	1.0	1.0	0.4	$<10^{-2}$[e]	1.2	1.6×10^{-5}	+
Mini-RK2	Vector	1.0	1.0	1.0	0.93[e]	0.5	1.1	−
	tacp-incC2$^+$	1.0	0.94	1.0	0.98[e]	1.0	1.4	−

[a] Single selection for induced plasmid, without (−) or with (+) IPTG.
[b] Fraction of colonies that retain the test plasmid.
[c] EOP normalized to single selection without IPTG.
[d] BR2943 was the host strain for this combination; EKA335 was the host for all the other combinations.
[e] Based on screening 100 colonies.

$>10^5$-fold when selection was maintained for both plasmids, indicating that the plasmids could not coexist in the same cell under inducing conditions. This was confirmed by plating the cells on IPTG- and X-Gal-containing medium in the absence of selection for RK2*lac*. The colonies that arose were white (Lac$^-$), indicating that the RK2*lac* plasmid was no longer present. However, even in the absence of selection for RK2*lac*, the EOP was reduced 10-fold. This result might be expected if loss of RK2*lac* triggers the *parDE* plasmid addiction system, which is toxic to plasmidless segregants. The effect of *incC2* induction was therefore tested on the RK2*lac* Δ*par* derivative pRK21382. In this case, the EOP was not reduced and all colonies were Lac$^-$. Essentially the same incompatibility phenotypes were observed for the *tacp* promoter fused to a derivative of the complete *incC* coding region that lacks the translational start site for the *korA* gene and includes the IncC2 translational start site (data not shown). These results show that elevated expression of IncC causes severe destabilization of RK2.

IncC-mediated incompatibility is replicon-independent. We next determined if IncC-mediated incompatibility results from interference with RK2 replication. The RK2Δ*trfA* plasmid pRK21591 has an inactive RK2 replicon, and it replicates using the plasmid P1 replication system, which is insensitive to *incC2* induction (data not shown). Just as with wild-type RK2, induction of *incC2* caused a dramatic loss of RK2Δ*trfA* (Table 2), indicating that IncC-mediated incompatibility is not caused by interference with RK2 replication. As before, the reduction in EOP on single selection is probably due to the *parDE* plasmid addiction system. Conversely, we tested the effect of *incC2* induction on the maintenance of the mini-RK2 plasmid pRR10, which consists entirely of the minimum replication determinants, the *trfA* gene and *oriV*, along with an Ap^r marker. In contrast to the results with wild-type RK2, induction of *incC2* had no effect on the mini-replicon (Table 2). These results show that the target for IncC-mediated incompatibility lies outside the RK2 replication determinants.

IncC-mediated incompatibility requires KorB. Replicon-independent incompatibility is consistent with a role for IncC in active partition. We therefore used the strong incompatibility phenotype to identify other factors that function with IncC. To identify the target for IncC-mediated incompatibility, we examined a variety of plasmids carrying different portions of RK2 (Table 3). Plasmids with large segments of RK2, such as pRK2013 and pRK21261, are sensitive to the IncC-mediated incompatibility. The smallest RK2 segment to confer sensitivity is present on plasmid pRK2362, which carries *korA*, *incC*, and *korB* (Fig. 2). In contrast, plasmid pRK2366, an isogenic *korB* mutant derivative of pRK2362 that has a small deletion at the 3′-end of *korB*, is not affected by induction of *incC2*. This result shows that *korB* is required for sensitivity to IncC-mediated incompatibility and indicates a new function for KorB beyond its role in transcriptional regulation.

The KorB-binding site (O_B) confers sensitivity to IncC-mediated incompatibility. Because KorB is a DNA-binding protein, we tested the possibility that the presence of its binding site (O_B) is required for sensitivity to IncC-mediated incompatibility. KorB binds to a 13-bp palindromic DNA sequence that is present on RK2 in 12 nearly identical, well-distributed copies (O_B1-12), 6 of which are involved in transcriptional regulation (67). All the IncC-sensitive plasmids we tested to this point contained at least one KorB-binding site, including the smallest (pRK2362), which has a single site (O_B1) in the *korA* promoter. We therefore tested plasmid pRK2178, which is comparable to pRK2362, except that it lacks the KorB-binding site in the *korA* promoter (Fig. 2). The results (Table 3) showed that pRK2178 is insensitive to induction of *incC2* in *trans*, indicating that the KorB-binding site may be required for sensitivity to IncC-mediated incompatibility.

To confirm that a KorB-binding site is the target for IncC-mediated incompatibility, we inserted a single site (O_B3) into a plasmid vector (pRK22327) and tested its sensitivity to IncC-mediated incompatibility by placing it in *trans* to the *incC*$^+$ *korB*$^+$ O_B1^+ plasmid pRK2362. Growth of the cells under selection for pRK2362 resulted in rapid loss of the O_B^+ plasmid from the population (Fig. 3). The vector control (pTR3) was not destabilized, indicating that the presence of the KorB-binding site on the plasmid caused it to become incompatible with pRK2362. Isogenic derivatives of pRK2362 lacking *korB* (pRK2366) or *incC* (pRK22324) failed to destabilize the O_B^+ plasmid (Fig. 3). Thus, the destabilization of the O_B^+ plasmid is dependent on *incC* and *korB*, and the KorB-binding site is the target of IncC-mediated incompatibility.

To determine if an O_B site is necessary on both plasmids, we attempted to test the O_B^+ plasmid in the presence of the *incC*$^+$ *korB*$^+$ O_B^- plasmid pRK2178 but were unable to con-

TABLE 3. Sensitivity of RK2 derivatives to IncC-mediated incompatibility

Test plasmid	Relevant properties	Induced plasmid	Relative EOP[a]	Incompatibility[b]
pRK2013	RK2Δ*kilA* Δ*kilE* Δ*kilC* Δ*par* Eight O_B sites	Vector *tacp-incC2*$^+$	1.3 2.4×10^{-5}	− +
pRK21261	*incC*$^+$ *korABFG*$^+$ *kfrA*$^+$ *upf54.8*$^+$ O_B1^+ O_B2^+ O_B3^+	Vector *tacp-incC2*$^+$	1.0 2.2×10^{-5}	− +
pRK2362	*korA*$^+$ *incC*$^+$ *korB*$^+$ O_B1^+	Vector *tacp-incC2*$^+$	0.8 1.0×1.0^{-3}	− +
pRK2366	*korA*$^+$ *incC*$^+$ *korB′* O_B1^+	Vector *tacp-incC2*$^+$	1.0 0.7	− −
pRK2178	*korA*$^+$ *incC*$^+$ *korB*$^+$ *korF*$^+$ O_B1^-	Vector *tacp-incC2*$^+$	0.8 0.9	− −

[a] EKA335 was the host strain. Cells were plated on medium selecting both plasmids and containing or lacking IPTG. The relative EOP was calculated as follows: CFU on medium with IPTG/CFU on medium without IPTG.
[b] −, compatible; +, incompatible.

struct the strain. This incompatibility is independent of *incC* but dependent on *korB*, since it occurred with the isogenic *incC*$^-$ *korB*$^+$ O_B^- plasmid pRK2300, but not the vector control. This mode of destabilization is distinct from IncC-mediated incompatibility. Since KorB is expected to be expressed at high levels from pRK2178 and pRK2300 (see below), this phenotype is similar to the ParB- and SopB-induced silencing of *parS*- and *sopC*-containing plasmids, respectively (55, 72). This phenotype is the subject of another study.

IncC-dependent stabilization of a heterologous plasmid. The IncC-mediated incompatibility phenotypes are consistent with a role for IncC, KorB, and the KorB-binding site in the active partition of RK2. We next tested the prediction that these elements should stabilize an unstable plasmid. The copy number of the ColE1-related replicon pMB1 is reduced to 10% of normal in *pcnB* mutants of *E. coli* (54). As a result, pMB1-derived plasmid vectors, like pBR322, are lost at a significant rate from *pcnB* cells during unselected growth (Fig. 4A). We tested the stability of pRK2101, a pMB1 replicon-containing plasmid carrying the *incC* region on a 6-kb RK2 segment (Fig. 2), and found that it was significantly more stable than pBR322 (Fig. 4A). The stable maintenance of pRK2101 did not result from a significant increase in copy number relative to pBR322. We used a unit copy F plasmid replicon as an internal control in these strains to determine the relative copy numbers of pBR322 and pRK2101. Agarose gel electrophoresis of plasmid DNA showed (i) that both plasmids have copy numbers comparable to that of the F replicon control plasmid and (ii) that the copy number of pRK2101 was at most 50% higher than pBR322 (data not shown). Thus, the lower rate of loss of pRK2101 relative to that of pBR322 suggested that the *incC* region of RK2 can stabilize a heterologous plasmid in a replication-independent manner.

The P15A replicon of vector pKJ1 also exhibits a reduced copy number in a *pcnB* host, even lower than that of a coresident F replicon control plasmid (data not shown). It is more unstable than pBR322 in this host and colonies of pKJ1-containing cells contain a significant fraction of plasmidless cells even on ampicillin selection (Fig. 4B [$t = 0$]). Some plasmidless cells are likely to survive within a colony on ampicillin medium because the plasmid-containing cells produce an ampicillin-degrading β-lactamase. To determine if an *incC*-containing region smaller than 6 kb can stabilize a heterologous P15A plasmid, we examined the stability of the P15A derivative pRK2362, which is *incC*$^+$ *korB*$^+$ O_B1^+, but we were unable to detect stabilization relative to the P15A vector control (data not shown). Plasmid pRK22329 is a P15A derivative that encodes all the determinants present on pRK2101. It is isogenic with pRK2362 but contains the additional downstream genes *korF*, *korG*, *kfrA*, and *upf54.8*, as well as two additional KorB-binding sites (O_B2 and O_B3) (Fig. 2). We found that plasmid pRK22329 was stabilized relative to the P15A vector control (Fig. 4B). An isogenic *incC* derivative (pRK22330), which has an in-frame deletion within *incC* (Fig. 2), was not stabilized (Fig. 4B), indicating that the stabilization was dependent on *incC*. Relative copy numbers were again determined using an

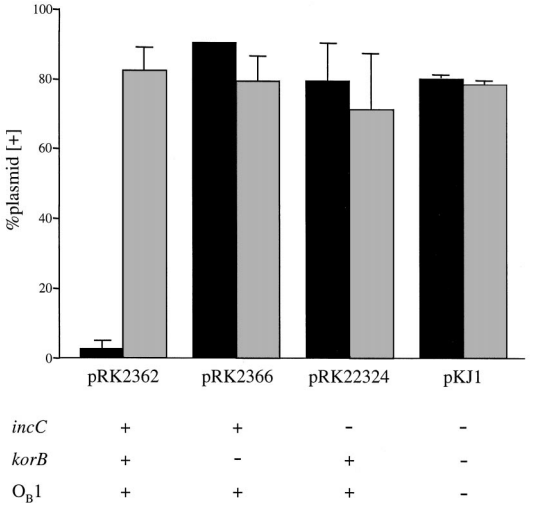


FIG. 3. The KorB-binding site is the target for IncC-mediated incompatibility. The O_B^+ plasmid pRK22327 (black bars) and the vector control plasmid pTR3 (shaded bars) were tested for sensitivity to IncC-mediated incompatibility in *E. coli* LS1443 containing pRK2362, pRK2366, pRK22324, or pKJ1 (vector). The presence (+) or absence (−) of *incC*, *korB*, and O_B1 on these plasmids is indicated at the bottom. After overnight growth under selection, 100% of cells contained both plasmids. Strains were then grown for 20 to 22 generations without selection for pRK22327 or pTR3, as described in Materials and Methods. Shown are the percentages of plasmid-containing cells after unselected growth, as determined by picking colonies and testing for the ampicillin resistance marker on pRK22327 and pTR3. Plotted are the averages of two experiments (error bars, standard deviations).

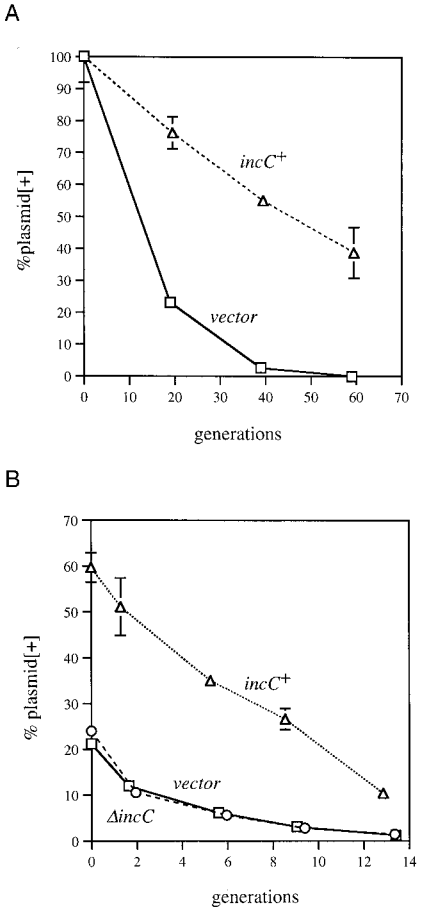


FIG. 4. IncC-dependent stabilization of an unstable plasmid. (A) The pMB1 replicon-based plasmids pRK2101 (*incC*$^+$) (△) and pBR322 (vector) (□) were tested for their maintenance in the *pcnB* strain LS1443. Plasmid loss was assayed by growing cells in the absence of selection for several generations. Cells were plated at regular intervals on medium containing ampicillin to determine plasmid-containing cells and on nonselective medium to determine total cells. (B) The P15A replicon-based plasmids pRK22329 (*incC*$^+$) (△), pRK22330 (Δ*incC*) (○), and pKJ1 (vector) (□) were tested for their maintenance in LS1443, as described for panel A. The P15A replicon is highly unstable in the *pcnB* strain, and even the zero time point shows a high proportion of plasmidless cells. Plotted are averages of three experiments (error bars, standard deviations). The results are highly reproducible, and error bars are visible only for some of the points.

F plasmid replicon as an internal control, as was done above for the pMB1 plasmids. Both the *incC*$^+$ and Δ*incC* plasmids displayed copy numbers comparable to that of the coresident F replicon (data not shown). Thus, *incC* does not appear to stabilize by increasing plasmid copy number. The stabilization of pRK2101 and pRK22329, while significant and highly reproducible, was not complete, and we discuss possible reasons below. Nevertheless, the results are consistent with a role for *incC* in the active partition of RK2.

Interaction of IncC and KorB in vivo and in vitro. Current models for active partition hold that the ATP-hydrolyzing protein and the DNA-binding protein interact to facilitate the formation of plasmid pairs or the positioning of the plasmids in the cell. There is good evidence for the interactions of these proteins in the P1, F, and R1 plasmid systems (10, 18, 38, 44, 47, 95). If IncC and KorB have similar functions in partition, they may be expected to interact.

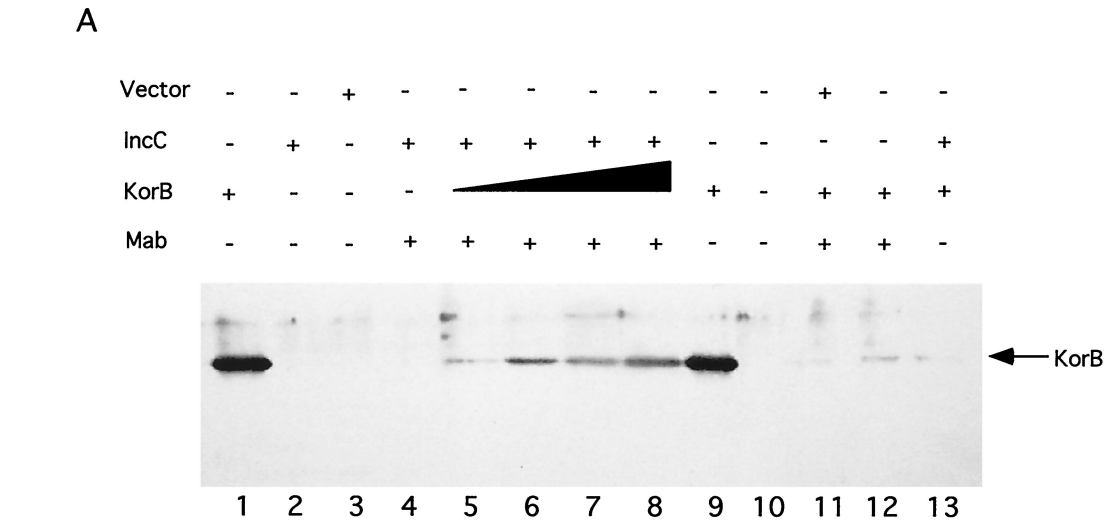
To test for the possible interaction of IncC and KorB, we first used the yeast two-hybrid system (22). The *korB* coding region was fused to the coding region for the LexA DNA-binding [LexA(DB)] domain in plasmid pBTM116, and *incC* was fused to the coding region for the GAL4 transcriptional activation domain [GAL4(AD)] in plasmid pGAD10. As a control, a fusion of GAL4(AD) with *korB* was also made. The fusion proteins were expressed in different combinations with each other and vector controls in a yeast strain containing a *lacZ* reporter gene with LexA-binding sites at the promoter. Quantitative β-galactosidase assays revealed significant *lacZ* expression when both the *lexA*(*DB*)-*korB* fusion and the GAL4(AD)-*korB* fusion were expressed together in the cell, but not when either fusion was expressed alone (Table 4). This result was expected, because KorB is known to form dimers (7). Significant *lacZ* expression also occurred when both the *lexA*(*DB*)-*korB* fusion and the GAL4(AD)-*incC* fusion were expressed together in the cell, but not with either fusion alone (Table 4). These results indicate an interaction between the IncC and KorB moieties of the fusion proteins.

Next we tested for the direct interaction of IncC and KorB proteins in vitro with affinity binding assays. An epitope-tagged version of IncC (T7-IncC) was expressed from a gene fusion, in which the coding region for the T7 · TAG epitope was fused to the second codon at the 5′ end of the *incC* coding region. Likewise, an amino-terminal six-histidine-tagged version of KorB (His-KorB) was expressed from a fusion constructed by cloning *korB* from its second codon into vector pQE-8. In the first experiment, the T7-IncC fusion protein was captured from *E. coli* extracts using protein A-coated Sepharose beads bound to a monoclonal antibody specific for the T7 · TAG epitope. Purified His-KorB was then added to the coated beads. After unbound protein was removed by washing, the proteins on the beads were separated by SDS-PAGE, and Western blot analysis was done using anti-KorB polyclonal antiserum to detect the presence of KorB. His-KorB was found to bind to T7-IncC-coated beads and binding was dependent on the presence of T7-IncC on the beads (Fig. 5A). A purified GST-His fusion did not show detectable binding to the beads (Fig. 5B), indicating that the T7-IncC–His-KorB interaction observed is specific. Cell extracts with wild-type KorB showed results similar to those of His-KorB (data not shown). We did the converse experiment to confirm the interaction. Purified His-KorB was bound to a histidine-affinity resin (TALON), and increasing amounts of a T7-IncC-containing sonicate were added. The complexes were washed to remove unbound protein, and the bound proteins were separated by SDS-PAGE. Western blot analysis was done using the anti-T7 · TAG monoclonal antibody to detect the presence of T7-IncC. T7-IncC was found to bind to His-KorB-coated beads, and binding was dependent on

TABLE 4. Interaction of IncC and KorB in the yeast two-hybrid system[a]

Hybrid		β-Gal (U)
LexA(DB)	GAL4(AD)	
—[b]	—[c]	5 ± 1
—[b]	KorB[d]	7 ± 2
KorB[e]	—[c]	8 ± 2
—[b]	IncC[f]	8 ± 2
KorB[e]	KorB[d]	394 ± 33
KorB[e]	IncC[f]	324 ± 25

[a] As described in Materials and Methods.
[b] pBTM116.
[c] pGAD10.
[d] pRK21673.
[e] pRK21674.
[f] pRK21842.



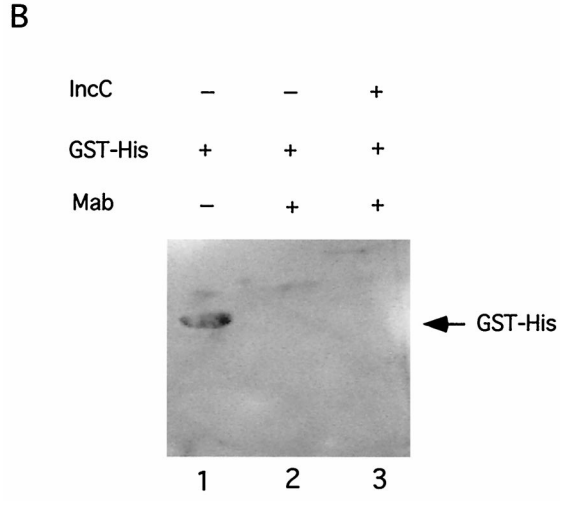


FIG. 5. Binding of KorB to IncC on a solid matrix. (A) T7-IncC was bound to Protein A-Sepharose containing the anti-T7 · TAG monoclonal antibody (Mab), as described in Materials and Methods. Increasing amounts of partially purified His-KorB were added, the beads were washed, and the proteins bound to beads were separated by SDS-PAGE. Western blot analysis was used to assay the presence of His-KorB (indicated by the arrow). Vector, extract made with the T7 · TAG vector as a control; IncC, extract with T7-IncC; KorB, His-KorB; Mab, protein A-Sepharose beads coated with anti-T7 · TAG monoclonal antibody. Control lanes 1, 9, and 11 to 13 contained 50 μl of His-KorB solution (110 μg/ml); experimental lanes 4, 5, 6, 7, and 8 contained 0, 5, 10, 25, and 50 μl, respectively. The His-KorB in lanes 1 and 9 was applied directly to the gel. (B) Control for binding specificity using purified GST-His. Samples were prepared as for panel A, except that approximately 5 μg of GST-His was used in place of His-KorB. The GST-His in lane 1 (1.25 μg) was applied directly to the gel.

the presence of His-KorB (Fig. 6). Taken together, these data show that IncC and KorB proteins physically interact.

Cooverexpression of IncC2 and KorB is toxic to *E. coli* cells. The results above showed that induction of *incC2* is not deleterious to the growth of *E. coli* host cells. Plasmid pRK2300 is a multicopy P15A derivative that contains *korA*, *korB*, and *korF* but lacks *incC* (Table 1; Fig. 2). If the in-frame *incC* deletion is nonpolar, the remaining genes are predicted to be expressed at higher-than-normal levels because the *korA* promoter in this plasmid lacks the operator O_B1 for KorB repression. This plasmid has no effect on the growth of *E. coli*. However, cells containing both pRK2300 and the *tacp-incC2* plasmid pRK21985 have markedly reduced EOP on IPTG-containing medium, even in the absence of selection (Table 5). Thus, the plasmids are toxic to *E. coli* when *incC2* is induced. To determine if *incC* and *korB* are sufficient for the toxicity, we examined the effect of coinduction of *tacp-incC2* (on pRK21985) and *trcp-korB* (on the compatible plasmid pRK21408) (Fig. 7). Coinduction resulted in a marked reduction in *E. coli* colony size, whereas induction of either gene alone had no significant effect. After two days, the colonies are largely nonviable, except for IPTG-insensitive variants (data not shown). Thus, simultaneous overexpression of *incC2* and *korB* is toxic to cell growth.

High levels of IncC2 cause oligocopy RK2 to segregate with low-copy kinetics. We have shown that induction of *incC2* causes the loss of RK2 under nonselective growth conditions. RK2 is present in the cell at 5 to 10 copies per chromosome (24, 94), and we were surprised by how easily RK2 was lost upon induction of *incC2*. We therefore examined the kinetics of RK2 loss from the population after induction. The RK2*lac* Δ*par* plasmid pRK21382 was used to prevent the triggering of toxicity by the *parDE* plasmid addiction system. Upon induction of *incC2*, RK2*lac* Δ*par* was rapidly lost from the population (Fig. 8). The observed plasmid loss curve was similar to the theoretical curves for a nonreplicating plasmid with a copy number of one or two. Massive plasmid degradation was not triggered by induction of *incC2*, because RK2*lac* Δ*par*-containing cells were present after overnight growth in IPTG-containing broth (data not shown). These results indicate that high levels of IncC2 cause the multiple copies of RK2 in the cell to form an aggregate, which then segregates as a unit to generate a plasmidless daughter cell at cell division.

DISCUSSION

We have undertaken a systematic analysis of the *incC* determinant to understand its role in the stable maintenance of the promiscuous plasmid RK2. First we showed that the IncC2 product of the *incC* gene is sufficient to exert replicon-independent incompatibility, a property indicative of plasmid maintenance determinants (3, 65). We then exploited this phenotype to identify other factors that function with IncC. One of these factors was found to be KorB, a protein known previously only as a transcriptional repressor that acts on several

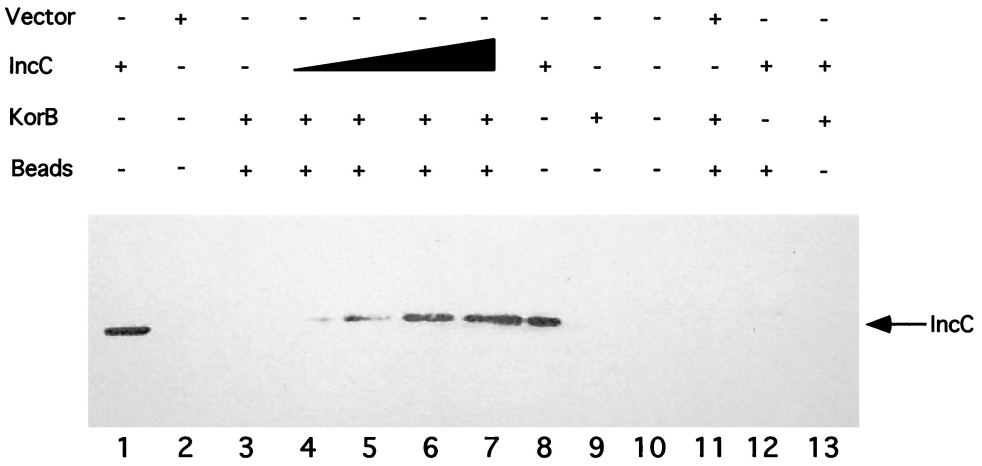


FIG. 6. Binding of IncC to KorB on a solid matrix. His-KorB was bound to TALON beads, as described in Materials and Methods. Increasing amounts of extracts containing T7-IncC were added, the beads were washed, and the proteins were separated by SDS-PAGE. Western blot analysis was used to assay the presence of T7-IncC (indicated by the arrow). Vector, extract made with the T7 · TAG vector as a control; IncC, extract with T7-IncC; KorB, His-KorB; beads, TALON beads. Control lanes 1, 2, and 8 contain 1 μl of the relevant extract; control lanes 11, 12, and 13 contain 50 μl of the relevant extract; experimental lanes 3, 4, 5, 6, and 7 contain 0, 5, 10, 25, and 50 μl of T7-IncC extract (containing approximately 140 μg of T7-IncC per ml), respectively. The T7-IncC in lanes 1 and 8 was applied directly to the gel.

operons of the RK2 *kor* regulon (25, 67). Our studies revealed that KorB is required for IncC-mediated incompatibility and that the KorB and IncC proteins physically interact. A second factor is the *cis*-acting DNA site (O_B) for binding of KorB. A role for O_B was revealed by the finding that a plasmid containing a single copy of O_B is destabilized by IncC-mediated incompatibility. We also showed that the *incC* region of RK2 is able to stabilize an unstable, heterologous plasmid in *cis* in an *incC*-dependent manner. These results lead us to conclude that IncC, KorB, and O_B comprise at least part of an active partition system on plasmid RK2.

In the well-studied active partition systems of plasmids P1 and F, the ATPase (ParA and SopA, respectively), the DNA-binding protein (ParB and SopB, respectively) and the *cis*-acting element (*parS* and *sopC*, respectively) are sufficient to stabilize an unstable plasmid (1, 2, 37, 64, 66). Similar results have been observed for the analogous components of the related partition systems of R1 and NR1 (29, 44, 82). In contrast, a region of RK2 containing *incC*, *korB*, and O_B1 of RK2 was not sufficient to stabilize an unstable plasmid in *E. coli*, and an O_B3-containing plasmid was not stabilized with *incC2* and *korB* in *trans* (data not shown). However, a larger RK2 region that includes *incC*, *korB*, and O_B1 plus additional downstream genes (*korF*, *korG*, *kfrA*, and *upf54.8*) and two other copies of the KorB-binding site (O_B2 and O_B3) showed significant stabilization activity that is dependent on the presence of an intact *incC* gene (Fig. 4). It is possible that one or more of the additional genes or O_B sites is required for stabilization activity. Indeed, Williams et al. (92) have presented intriguing evidence suggesting that O_B1, O_B2, and O_B3 sites are not equivalent and that O_B3 is the preferred site for an *incC*-dependent stabilization activity. However, it is difficult to rule out the effects of structural changes, as different fragments of RK2 in test vectors can affect plasmid maintenance independent of partition functions (76). We are currently seeking to establish a well-defined, manipulable system in which a plasmid containing the required O_B site(s) is stabilized by the controlled expression of the appropriate genes in *trans*.

The observed stabilization of the pMB1 and P15A replicons in a *pcnB* host by the larger *incC-korB*-O_B region (pRK2101 and pRK22329, respectively) was significant relative to the vector and highly reproducible (Fig. 4). Nevertheless, these plasmids were still lost at a significant rate. One explanation is that the replicons occasionally fail to replicate in the *pcnB* host, thus leading to plasmidless segregants regardless of a stabilization mechanism. It is also possible that the copy number is too low in this host for adequate expression of the partition system components or that *E. coli* is not the most suitable host for the RK2 partition system. Another possibility is that other genes may be required to enhance the efficiency of the basic

TABLE 5. *incC2* induction is toxic to *E. coli* in the presence of a *korABF*$^+$ plasmid

Plasmid 1	Relevant property(ies)	Plasmid 2	Relevant property(ies)	Relative EOP[a]		
				Double selection	Single selection for the indicated plasmid	
					incC$^+$[b]	*korB*$^+$[c]
pDB6	Vector	pJAK16	Vector	1.0	1.5	1.3
pDB6	Vector	pRK21985	*tacp-incC2*$^+$	0.8	1.1	1.5
pRK2300	*korA*$^+$ *korB*$^+$ *korF*$^+$	pJAK16	Vector	1.2	1.2	1.6
pRK2300	*korA*$^+$ *korB*$^+$ *korF*$^+$	pRK21985	*tacp-incC2*$^+$	4.7×10^{-4}	6.2×10^{-2}[d]	1.2×10^{-2}[d]

[a] EKA13 was used as the host strain. The relative EOP was calculated as follows: CFU on medium with IPTG/CFU on medium without IPTG.
[b] Chloramphenicol.
[c] Kanamycin.
[d] Large majority of colonies have lost the unselected plasmid.

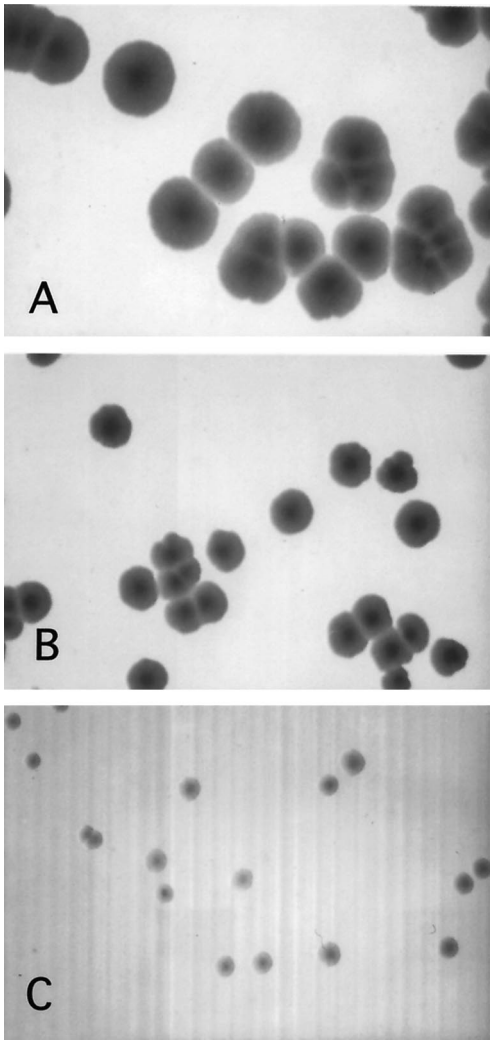


FIG. 7. Simultaneous overexpression of *incC2* and *korB* is toxic to *E. coli*. *E. coli* EKA13 strains contained the following combinations of *incC* and *korB* plasmids and vector controls: pRK21985 (*tacp-incC2*) and pRK21408 (*trcp-korB*), pJAK16 (*incC2* vector control) and pRK353 (*korB* vector control), pRK21985 and pRK353, and pJAK16 and pRK21408. Strains were grown overnight at 37°C with selection for both plasmids and then were plated on medium containing 1 mM IPTG or medium lacking IPTG. Shown are colonies from cells containing pJAK16 and pRK353 on IPTG-containing medium (A) and pRK21985 and pRK21408 on medium lacking IPTG (B) and containing IPTG (C). Magnification is the same for all frames. Results from the other combinations were essentially equivalent to those in panel A, with the exception that the strain with pJAK16 and pRK21408 produced slightly smaller colonies on IPTG.

incC-korB-O_B partition system. In support of this idea, Bignell et al. (9) have recently shown that a larger region of RK2 is even better able to stabilize an unstable plasmid.

The IncC protein was predicted to be involved in partition on the basis of sequence similarity to regions of the ParA and SopA partition proteins of plasmids P1 and F, respectively (62). Both the ParA and SopA proteins have been shown to interact with the cognate DNA-binding proteins ParB and SopB (10, 15, 18, 38, 47, 61, 95). The ParM protein of plasmid R1, which has no sequence relationship with ParA or SopA, but is thought to have a similar function in partition, interacts with the DNA-binding protein ParR (44). Thus, if IncC is involved in active partition, it is predicted to interact with a DNA-binding protein. Our finding that KorB and its binding site O_B are required for IncC-mediated incompatibility suggested that KorB is the interacting protein. Recent studies have shown that IncC can affect the binding of KorB to its target site, suggestive of a physical interaction (43, 52). We show here, both by yeast two-hybrid analysis and by in vitro studies with partially purified proteins, that IncC and KorB directly interact.

The discovery that KorB functions both as an active partition protein with IncC and a global transcriptional repressor is a remarkable finding that distinguishes the RK2 system from any other plasmid partition system. KorB is involved in the control of multiple operons of the *kor* regulon, a feature unique to IncP promiscuous plasmids (25, 67). The regulated operons include genes for replication initiation, conjugative transfer, and stable maintenance in *P. aeruginosa* (67, 94). In addition, KorB is an autorepressor of the *incC-korB* operon along with KorA (Fig. 1). An early clue that KorB might have a function other than that of a transcriptional regulator was that both RK2 and the related, but distinct, IncPβ plasmid R751 have multiple KorB-binding sites distributed around their genomes (67, 86). Only some of these sites are involved in transcriptional regulation. Others are conserved in their location but occur downstream of or within genes and have no obvious function. KorB binds as a dimer to a 13-bp palindromic sequence, and there is evidence that KorB can form tetramers (7) that may be able to couple separated O_B sites. These properties of the IncC-KorB system resemble the Soj-SpoOJ system of *Bacillus subtilis*. SpoOJ is involved in chromosome segregation in vegetative and sporulating cells (11, 41, 78). SpoOJ binds to eight sites on the *B. subtilis* chromosome (53), and recently Soj has been shown to organize the SpoOJ-bound sites into a condensed structure (57). The multiple O_B sites on IncP plasmids may likewise be involved in the production of a specialized, KorB- and IncC-mediated, nucleoprotein structure required for efficient partition.

A prediction of the current plasmid partition model is that plasmid copies pair prior to their segregation into daughter cells (2, 3). Electron microscopic studies have implicated par-

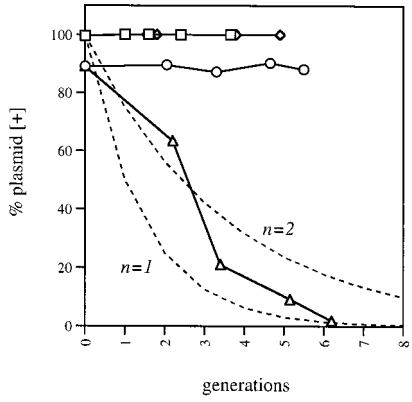


FIG. 8. Elevated IncC2 causes RK2 to segregate as a unit. *E. coli* EKA335 strains contained either pJAK16 (vector) or pRK21985 (*tacp-incC*) in addition to pRK21382 (RK2*lac* Δ*par*). Strains were grown overnight in LB broth with selection for pRK21985 and pRK21382 and then were diluted 1:50 into prewarmed LB broth with selection only for pRK21985 and grown to a cell density of approximately 2×10^8 cells/ml to allow the cells to exit lag phase. At time zero a 10^{-3} dilution of each culture was inoculated into prewarmed medium with or without 1 mM IPTG and with chloramphenicol to select pRK21895 only. At various times, samples were plated on medium with selection for the *tacp-incC2* plasmid and X-Gal to assay RK2*lac* Δ*par* retention. Plasmids RK2*lac* Δ*par* plus pJAK16: □, no IPTG; ◇, with IPTG. Plasmids RK2*lac* Δ*par* plus pRK21985: ○, no IPTG; △, with IPTG. The theoretical curves for loss of a nonreplicating plasmid of copy numbers 1 and 2 ($n = 1$ and $n = 2$, respectively) are shown.

tition proteins of plasmid R1 in the pairing of DNA fragments containing the *cis* element in vitro (45). Remarkable genetic studies on the incompatibility properties of the plasmid P1 *parS* site and smaller, yet functional, derivatives have demonstrated a requirement for equivalent nucleoprotein structures, a result that can best be explained by the pairing model (16, 17, 58). In addition, recent results of fluorescence microscopy on the cellular locations of plasmids P1 and F indicate that the plasmids localize to the division plane of the cell and then segregate to the 1/4 and 3/4 positions prior to cell division (32, 63), a finding consistent with active partition of plasmid pairs. In this study, we found that elevated levels of IncC cause RK2 to be lost at a rate equivalent to that of a plasmid with a copy number of 1 to 2, even though there are at least 10 to 15 copies of RK2 in the cell at cell division. This result indicates that elevated levels of IncC can cause the copies of RK2 to aggregate and therefore segregate as a unit. Aggregation could result from overpairing caused by intermolecular interactions of the multiple KorB sites to form an interlocked plasmid aggregate. We suggest that these results indicate a role for IncC in the pairing of RK2 molecules prior to segregation.

Remarkably little is known about the basic mechanism for directed DNA movement into daughter cells. Because models for partition require an interaction with an as yet unidentified host cell apparatus (3, 26, 42, 89, 93), the broad host range of IncP plasmids makes them particularly interesting. Has the IncC-KorB partition system evolved to exploit universal properties of host cell DNA segregation machinery such that it can function in a wide variety of bacterial hosts, or is it specific only for certain hosts? We have shown here that high levels of IncC and KorB together are toxic to cell growth, and it is reasonable to suggest that they interact with and perturb the machinery for chromosome segregation. It will be interesting to determine if toxicity occurs in other hosts and if it reflects the host range of *incC*-dependent stabilization. The toxicity phenotype may also provide a genetic tool for the identification of components of a host segregation apparatus that interacts with the IncC-KorB partition system. Since the discovery of the *kor* regulon on promiscuous IncP plasmids (25), the genes of the *kilA*, *kilC*, and *kilE* loci have been suggested to encode host-specific functions for stable plasmid maintenance, and studies with *kilE* support this model (94). Given the strong evidence that IncC, KorB, and O_B constitute the basis for a partition system on IncP plasmids, we are investigating the possibility that the gene products of the *kil* loci function through this basic system.

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