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The Centromere Site of the Segregation Cassette of Broad-Host-Range Plasmid RA3 Is Located at the Border of the Maintenance and Conjugative Transfer Modules▽†

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RA3 is a low-copy-number, broad-host-range (BHR) conjugative plasmid of the IncU incompatibility group isolated originally from *Aeromonas* spp. A 4.9-kb fragment of RA3 is sufficient to stabilize an otherwise unstable replicon in *Escherichia coli*. This fragment specifies the *korA-incC-korB-orf11* operon coding for an active partition system related to the central control operon of IncP-1 plasmids and found also in BHR environmental plasmids recently classified as the PromA group. All four genes in the cassette are necessary for segregation. IncC and KorB of RA3 belong to the ParA and ParB families of partitioning proteins, respectively. In contrast with IncP-1 plasmids, neither KorB nor IncC are involved in transcriptional autoregulation. Instead, KorA exerts transcriptional control of the operon by binding to a palindromic sequence that overlaps the putative −35 promoter motif of the cassette. The Orf11 protein is not required for regulation, but its absence decreases the stabilization potential of the segregation module. A region discontiguous from the cassette harbors a set of unrelated repeat motifs distributed over ~300 bp. Dissection of this region identified the centromere sequence that is vital for partitioning. The ~300-bp fragment also encompasses the origin of conjugative transfer, *oriT*, and the promoter that drives transcription of the conjugative transfer operon. A similar set of *cis*-acting motifs are evident in the PromA group of environmental plasmids, highlighting a common evolutionary origin of segregation and conjugative transfer modules in these plasmids and members of the IncU group.

Plasmids from the IncU incompatibility group have been isolated from the fish pathogens *Aeromonas salmonicida* and *Aeromonas hydrophila* in worldwide locations (4, 5, 6, 12, 19, 20, 44), as well as from clinical isolates of *Escherichia coli* and *A. hydrophila* (40, 53). The archetypal IncU plasmid is the low-copy-number, conjugative RA3 plasmid (45,909 bp) isolated from *A. hydrophila* in Japan in 1979 (5), which confers resistance to sulfonamides, streptomycin, and chloramphenicol. RA3 transfers with high frequency from *E. coli* to *Pseudomonas putida* (*Gammaproteobacteria*) and *Ralstonia eutropha* (*Betaproteobacteria*) and with three-magnitude-lower efficiency between *E. coli* and *Agrobacterium tumefaciens* (*Alphaproteobacteria*). RA3 is stably maintained in all tested representatives of these three classes (31). The nucleotide sequence of RA3 (GenBank accession no. DQ401103) (31) revealed the existence of putative functional modules involved in replication, maintenance, and conjugative transfer that are almost identical to those of the tetracycline-resistant IncU plasmid pFBAOT6 (41). The sequence confirms a high degree of conservation in the backbone functions of IncU plasmids suggested previously, on the basis of restriction enzyme analysis (47). Particularly interesting features of the RA3/pFBAOT6 backbone include a replication module with similarity to several unrelated environmental plasmids from a wide diversity of beta- and gammaproteobacteria as well as to plasmids from Gram-positive bacteria, a maintenance region with 50 to 60% identity to the IncP-1 plasmids of Gram-negative species, and a conjugation system homologous to those identified for plasmids pIPO2 and pSB102 (31) belonging to the PromA group of broad-host-range (BHR) plasmids. The putative active partition genes of RA3 are part of the maintenance region and are encoded in the *korA-incC-korB-orf11* operon. Three of the predicted products, the KorA, IncC, and KorB proteins, show high degrees of similarity (53%, 63%, and 53%, respectively) to the equivalent proteins encoded in the central control operon (*cco*) of the RK2 (IncP-1α) plasmid (39). A similar partition cassette adjacent to a conjugative transfer region has been found in pIPO2, pSB102, pTer331, and pMOL98 from the PromA group (54).

Here, we analyze the partition cassette of RA3, identifying the *trans*-acting elements important for its function as well as sequences essential for centromere activity. Furthermore, we demonstrate that KorA is the sole transcriptional autorepres-

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sor of the *korA-incC-korB-orf11* operon and identify the KorA binding site. The product of *orf11* is required for the full stabilization function of the cassette. The RA3 cassette is a new variant of the partition locus identified initially for IncP-1 plasmids and which seems to be widely spread among environmental BHR plasmids isolated from different geographical locations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Escherichia coli* strains used were K-12 strains DH5α [F^- (Φ80d*lacZ*ΔM15) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U19*] (15) and C600K (*thr-1 leu-6 thi-1 lacY1 supE44 ton21 galK*) (35), B strain BL21 [F^- *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (λ DE3)] (Novagen, Inc.), and C strain C2110 (*polA1 his rha*) (kindly supplied by D. R. Helinski). Bacteria generally were grown in L broth (27) at 37°C or on L agar (L broth with 1.5%, wt/vol, agar) supplemented with appropriate antibiotics: benzylpenicillin (sodium salt) (150 μg ml^{-1} in liquid media and 300 μg ml^{-1} in agar plates) for penicillin resistance, with the exception of selection for pOU82 or its derivatives, in which case 150 μg ml^{-1} both in liquid media and L agar were used; kanamycin sulfate (50 μg ml^{-1}) for kanamycin resistance; streptomycin sulfate (20 to 30 μg ml^{-1}) for streptomycin resistance (Sm^r); and chloramphenicol (10 μg ml^{-1}) for chloramphenicol resistance (Cm^r). L agar used for blue/white screening contained IPTG (isopropyl-β-D-thiogalactopyranoside) (0.1 mM) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg ml^{-1}).

Plasmid DNA isolation, analysis, cloning, and manipulation. Plasmid DNA was isolated and manipulated by standard procedures (43). The list of plasmids used and constructed in this study is presented in Table 1. Standard PCRs (37) were performed with pairs of primers numbered 1 to 10 and 21 to 27 (see Table S1 in the supplemental material). Oligonucleotides 28 to 35 and 38 to 43 (see Table S1) were annealed in pairs to form double-stranded DNA fragments with cohesive ends suitable for cloning or were used for *in vitro* protein-DNA binding experiments. PCR primers for open reading frame cloning were designed to include an EcoRI site upstream of the start codon and an SalI site following the stop codon. Fragments for promoter cloning were manipulated to add BamHI sites. PCRs to amplify *korA*, *incC*, and *korB* were performed with an initial denaturation step (95°C for 5 min) and 35 cycles of denaturation (95°C for 30 s), annealing (55°C for 30 s for *korA*, 50°C for 30 s for both *incC* and *korB*), and elongation (72°C for 30, 60, and 90 s, respectively, for $korA_{RA3}$, $incC_{RA3}$ and $korB_{RA3}$). Reactions ended with a final elongation step (72°C for 7 min). All PCR-derived clones were analyzed by sequencing to check their fidelity.

The high-copy-number vector pGBT30 (24), based on the pMB1 replicon with $lacI^q$ and *tacp* followed by a multiple cloning site from pUC18 and λt_{R1}, was used for expression of cloned genes. BamHI-SalI fragments of pGBT30 derivatives with the gene(s) of interest and all upstream control elements were recloned into broad-host-range Cm^r replicon pBBR-MCS1 (30).

Plasmids for protein purification were constructed by inserting the alleles as EcoRI-SalI fragments into a derivative of pET28a Km^r (Novagen, Inc.) (49) modified with the use of a synthetic oligomer so that sequence coding for a His_6 tag and thrombin cleavage site preceded the EcoRI site by six nucleotides (32).

To clone putative KorB binding sites separately, the appropriate pairs of oligonucleotides (28 and 29 or 38 and 39 for an inverted repeat [IR] designated IR-SnaBI and 30 and 31 for a direct repeat [DR2]; see Table S1 in the supplemental material) were first denatured by heating to 100°C and then annealed by cooling slowly to room temperature. These double-stranded fragments, with overhangs corresponding to the overhangs generated by SalI and HindIII or EcoRI and BamHI, were used in a ligation with pGB2 treated with SalI and HindIII or pOU82 cut with EcoRI and BamHI, respectively.

Site-directed mutagenesis *in vitro*. To create nonsense mutations in *korA*, *incC*, *korB*, and *orf11* in pYC16A, as well as modifications in the KorA operator sequence, an *in vitro* site-directed mutagenesis kit (Stratagene) was used with high-fidelity PfuTurbo DNA polymerase. The primers 11 to 18 (see Table S1 in the supplemental material) were designed to introduce stop codons after codon 9 in KorA, codon 17 in IncC, codon 16 in KorB, and codon 18 in Orf11 accompanied by EcoRI, SalI, BamHI, and NcoI recognition sites, respectively. The KorA operator was modified with the use of primers 19 and 20 (see Table S1) designed to introduce an SmaI recognition site. Following 16 temperature cycles (95°C for 30 s; 54°C for 1 min; 68°C for 24 min), the PCR product was treated with DpnI endonuclease to remove template DNA and used for transformations. The plasmid DNA of putative mutants was tested for the presence of restriction sites introduced in the mutagenic primers, and mutagenesis was verified by sequencing.

Sequencing and computer sequence analysis. DNA sequencing was performed using a Pharmacia A.L.F. automatic sequencer (IBB, PAS, Warsaw) and dye terminator kit. Nucleotide and protein sequence analyses were carried out using the DNA Lasergene 99 package (DNASTAR). Sequences were compared to those in GenBank/EMBL databases and studied using the programs BlastN and BlastP (NCBI).

Determination of catechol 2,3-oxygenase activity (XylE). Catechol 2,3-oxygenase activity (the product of *xylE*) was assayed (58) in logarithmically growing bacteria and normalized to the same level as that of the control. One unit of catechol 2,3-oxygenase is defined as the amount needed to convert 1 μmol of catechol in 1 min under standard conditions. Protein concentration was determined using the Bradford method (11).

Purification of His_6-tailed proteins. For protein overproduction and purification, *E. coli* BL21(DE3) was transformed with pET28mod derivative pAKB3.30 or pAKB3.50 encoding N-terminally histidine-tagged (MGSS*HHHHHH*SSGLV PRGSHSEF [the His_6 tag is shown in italics]) KorA or KorB, respectively. Exponentially growing cultures of BL21(DE3) transformants were induced with 0.5 mM IPTG at a cell density of approximately 2×10^8 CFU ml^{-1} and grown for an additional 3 to 4 h, with shaking at 37°C. Cells from 200 ml of culture were harvested by centrifugation and sonicated in 4 ml of sonication buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl for KorB; 50 mM sodium phosphate [pH 8.0], 600 mM NaCl for KorA). The cell lysate–Ni^{2+}-nitrilotriacetic acid (NTA) agarose mixture was stirred for 60 min at 4°C and then transferred to a column. Wash buffer (50 mM sodium phosphate [pH 6.0], 300 mM NaCl, 10 mM imidazole, 10% [vol/vol] glycerol) was passed three times through the column in 5-ml aliquots. For KorA purification, the wash buffer was modified to 50 mM sodium phosphate [pH 6.0], 600 mM NaCl, 10 mM imidazole, and 30% (vol/vol) glycerol. Proteins bound to the column were eluted with a gradient of imidazole (50 mM to 300 mM in 25-mM steps) in 0.5-ml aliquots of elution buffer. The purification procedure was monitored by SDS-PAGE using a Pharmacia Phast system with 12.5% or 20% homogeneous gels.

Analysis of protein-DNA interactions by electrophoretic mobility shift assay (EMSA). (i) Nonradioactive oligonucleotides. Complementary oligonucleotides 32 and 33 for IR1, 34 and 35 for IR2, 40 and 41 for both IRs and accompanying promoter motifs (IR1+IR2), and 42 and 43 for the same region but with IR2 destroyed by multiple nucleotide substitutions (IR1+IR2*) (see Table S1 in the supplemental material) were mixed to obtain 100 pmol μl^{-1} of double-stranded fragment, denatured by heating to 100°C, and incubated at room temperature to anneal. For O_ARK2, self-annealing was performed (see oligonucleotide 36 in Table S1). The protein-DNA binding reactions were performed in binding buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 50 mM NaCl, and 0.2 mg ml^{-1} bovine serum albumin [BSA]) with different amounts of His_6-KorA added in a final volume of 20 μl. Binding mixtures were incubated at 37°C for 15 min, applied to 1.2% or 1.3% agarose gels, and separated in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at room temperature. The gel was stained by ethidium bromide and visualized by UV.

(ii) 5′-end-labeled radioactive DNA fragments. The EcoRI-HindIII fragment of 465 bp from pAKB7.5 containing $parS_{RA3}$ was excised from agarose gels and purified using a GelOut column. The DNA fragment was dephosphorylated and 5′ end labeled with ^{32}P using T4 polynucleotide kinase (Amersham) in a 50-μl reaction mixture containing 2 μg of the dephosphorylated DNA fragment, 5 μl of 10× reaction buffer, 1 unit of T4 polynucleotide kinase, and 50 μCi [γ-^{32}P]ATP (Amersham). The labeled fragment was purified using Qiagen columns and then after appropriate digestion used for the EMSA. The protein-DNA binding reaction was performed at 37°C for 15 min. DNA fragments were separated on 5% native polyacrylamide gel in TBE buffer at 4°C. The dried gels were autoradiographed using a Phosphorimager system (Molecular Dynamics).

Complementation assay. *E. coli* DH5α harboring pOU82 (26), or its derivatives pAKB14.3I, pAKB14.4, pAKB14.5, pAKB14.6, and pAKB14.8, was transformed with pPTOI (34), pAKB4.90, or pAKB7.90. Double transformants were grown overnight at 30°C under double selection and diluted 10^5-fold into fresh medium with kanamycin and 0.1 mM IPTG. The dilution was repeated every 20 generations, and cultures were grown for at least 80 generations in total. The number of generations was calculated after plating the serial dilutions on L agar at the beginning of the experiment and at each time point. For screening of plasmid retention, the cultures were plated on L agar supplemented with X-Gal and IPTG. The percentage of blue colonies indicated the plasmid retention.

Incompatibility and segregation assays. *E. coli* C2110 *polA* harboring pYC16A was transformed with compatible pSC101 replicons (pGB2 derivatives or the pPTOI derivative). Transformation mixtures were plated on appropriate selection plates either for the incoming plasmid only (streptomycin for pGB2 deriv-

TABLE 1. Plasmids used in this study

Designation	Relevant features or description[a]	Copy no.	Reference or source
pALA136	*oriMB1 oriP1*, Cm^r	High/low	1
pBBR-MCS1	IncA/C, Cm^r	Medium	30
pBGS18	*oriMB1*, Km^r	High	48
pET28	*oriMB1*, Km^r, T7*p*, *lacO*, His_6-T7 tag	Medium	Novagen
pET28mod	*oriMB1*, Km^r, T7*p*, *lacO*, His_6 tag (T7 tag deleted, no BamHI site)	Medium	32
pGB2	*oriSC101*, Sp^r/Sm^r	Medium	13
pGBT30	*oriMB1*, Ap^r, $lacI^Q$, *tacp* expression vector	High	24
pGBT37	pGBT30 $tacp\text{-}korA_{RK2}$	High	23
pGBT70	pPTOI $trfAp\text{-}1_{RK2}\text{-}xylE$	Medium	23
pPTOI	*oriSC101*, Km^r, promotorless *xylE*	Medium	34
pOU82	*oriR1*, Ap^r	Medium/low	26
RA3	IncU, Cm^r, Sm^r, Su^r	Low	5
pUC18	*oriMB1*, Ap^r	High	57
pYC16A	4.9-kb (partial Sau3AI) fragment of RA3 (coordinates 4921 to 9854) inserted into BamHI site of pALA136		This study
pYC220	pYC16A derivative deleted of the bulk of *kfrA*		This study
pYC216	pYC220 with deleted fragment HpaI-BamHI (coordinates 9397 to 9854)		This study
pAKB2.30	pGBT30 $tacp\text{-}korA_{RA3}$ (EcoRI-SalI PCR fragment [primers 3 and 4])		This study
pAKB2.40	pGBT30 $tacp\text{-}incC_{RA3}$ (EcoRI-SalI PCR fragment [primers 5 and 6])		This study
pMMB2.50	pGBT30 $tacp\text{-}korB_{RA3}$ (EcoRI-SalI PCR fragment [primers 7 and 8])		This study
pMMB2.60	pGBT30 $tacp\text{-}orf11_{RA3}$ (EcoRI-SalI PCR fragment [primers 9 and 10])		This study
pAKB3.30	pET28mod $T7p\text{-}korA_{RA3}$ (EcoRI-SalI fragment of pAKB2.30)		This study
pAKB3.50	pET28mod $T7p\text{-}korB_{RA3}$ (EcoRI-SalI fragment of pMMB2.50)		This study
pAKB4.10	pPTOI $korAp_{RA3}\text{-}xylE$ (BamHI PCR fragment [primers 1 and 2])		This study
pAKB4.11	pPTOI $korAp_{RA3}\text{-}OAmut\text{-}xylE$ (BamHI PCR fragment [primers 1 and 2])		This study
pAKB4.90	pPTOI *korA incC korB orf11* EcoRV-HpaI fragment of pYC16A (RA3 coordinates 4921–9397)		This study
pMMB5.30	pBBR-MCS1 $tacp\text{-}korA_{RA3}$ (BamHI-SalI fragment of pAKB2.30)		This study
pAKB5.50	pBBR-MCS1 $tacp\text{-}korB_{RA3}$ (BamHI-SalI fragment of pMMB2.50)		This study
pMMB5.60	pBBR-MCS1 $tacp\text{-}orf11_{RA3}$ (BamHI-SalI fragment of pMMB2.60)		This study
pAKB7.5	pBGS18 with 460 bp HpaI-BamHI fragment of pYC16A (RA3 coordinates 9397–9854)		This study
pAKB7.90	pBGS18 *korA incC korB orf11* EcoRV-HpaI fragment of pYC16A (RA3 coordinates 4921–9397)		This study
pAKB8.3I	pGB2 with 302-bp DR1/DR2 EcoRI-HindIII fragment of pAKB10.3 (RA3 coordinates 9397–9700)		This study
pAKB8.4	pGB2 with 307-bp EcoRI-BamHI fragment of pAKB10.4 (RA3 coordinates 9546–9854)		This study
pAKB8.5I	pGB2 with 460-bp EcoRI-HindIII fragment of pAKB7.5 (RA3 coordinates 9397–9854)		This study
pAKB8.6	pGB2 with 230-bp EcoRI-HindIII fragment of pAKB10.6 (RA3 coordinates 9623–9854)		This study
pAKB8.6I	pAKB8.6 derivative with deletion of fragment between NheI and BamHI		This study
pAKB8.7	pGB2 with oligonucleotides 30 and 31 corresponding to IR-DR2		This study
pAKB8.8	pGB2 with oligonucleotides 28 and 29 corresponding to IR-SnaBI		This study
pAKB10.4	pUC18 with 307-bp EcoRI-BamHI PCR fragment (primers 25 and 24) (RA3 coordinates 9546–9854)		This study
pAKB10.6	pUC18 with 230-bp SphI-BamHI PCR fragment (primers 23 and 24) (RA3 coordinates 9623–9854)		This study
pAKB10.3	pUC18 with 302-bp DR1/DR2 (SphI-BamHI PCR fragment [primers 21 and 22]) (RA3 coordinates 9397–9700)		This study
pAKB13.11	pYC16A with mutation in *korAp* at IR1 (PCR mutagenesis with primers 19 and 20)		This study
pAKB13.20	pYC16A with deletion of 1,204-bp fragment (RA3 coordinates of the deleted fragment 8650–9854)		This study
pAKB13.21	pYC16A with deletion of 317-bp HpaI-SnaBI fragment (RA3 coordinates 9397–9714)		This study
pAKB13.22	pYC16A with deletion of 140-bp SnaBI-BamHI fragment (RA3 coordinates 9715–9854)		This study
pAKB13.23	pYC16A with modification of IR-SnaBI motif (inserted oligonucleotide 37)		This study
pAKB13.30	pYC16A $korA_{stop}$ (PCR mutagenesis with primers 11 and 12)		This study
pAKB13.40	pYC16A $incC_{stop}$ (PCR mutagenesis with primers 13 and 14)		This study
pAKB13.41	pYC16A $incC_{stop}$ and $korB_{stop}$ (PCR mutagenesis of pAKB13.50 with primers 13 and 14)		This study
pAKB13.50	pYC16A $korB_{stop}$ (PCR mutagenesis with primers 15 and 16)		This study
pAKB13.60	pYC16A $orf11_{stop}$ (PCR mutagenesis with primers 17 and 18)		This study
pAKB14.3I	pOU82 with 302-bp fragment DR1/DR2 (RA3 coordinates 9397–9700)		This study
pAKB14.4	pOU82 with 307-bp fragment (RA3 coordinates 9546–9854)		This study
pAKB14.5I	pOU82 with 458-bp fragment (RA3 coordinates 9397–9854)		This study
pAKB14.6	pOU82 with 230-bp fragment (RA3 coordinates 9623–9854)		This study
pAKB14.6I	Derivative of pAKB14.6 with deletion of NheI-BamHI fragment (RA3 coordinates 9623–9763)		This study
pAKB14.8	pOU82 with oligonucleotides corresponding to IR-SnaBI (inserted oligonucleotides 38 and 39)		This study
pAKB14.9	pOU82 with 122-bp fragment (RA3 coordinates 9623–9745)		This study

[a] Su^r, resistance to sulfonamide; Sm^r, resistance to streptomycin; Ap^r, resistance to ampicillin; Sp^r, resistance to spectinomycin; Km^r, resistance to kanamycin; Cm^r, resistance to chloramphenicol.

atives and kanamycin for pPTOI derivative) or for both resident and incoming plasmids (supplemented additionally with chloramphenicol) to monitor the differences, if any, in the transformation frequencies. For plasmid segregation analysis, cultures of *E. coli* C2110 *polA* with pYC16A derivatives were grown overnight under selection and then repeatedly diluted 10^5-fold into fresh medium every 20 generations for at least 60 generations. Cultures were plated on L agar from which colonies were transferred onto selective plates for estimation of the number of plasmid-retaining cells.

Transcriptional silencing assay. The derivatives of DH5α strain carrying pGB2 (13) or its derivatives pAKB8.3I, pAKB8.4, pAKB8.5I, pAKB8.6, and pAKB8.8 were transformed with pBBR-MCS1 expression vector and its derivative pAKB5.50 (*tacp-korB*). A total of 100 μl of serially diluted transformation mixtures were plated on selection plates either for incoming or both resident and incoming plasmids. The selective media supplemented with 0.5 mM IPTG were also used to induce KorB production.

RESULTS

Cloning of the RA3 segregation module. Prior to full sequencing of the RA3 plasmid, a library of 4- to 6-kb fragments generated by partial Sau3AI digestion of RA3 was constructed by insertion into the BamHI site of pALA136 to permit screening for plasmid maintenance cassettes, as described previously (16, 17). pALA136 is a dual replicon vector that harbors the moderate-copy-number pMB1 and low-copy-number P1 replication regions (1). The pMB1 replicon allows for ease of plasmid DNA isolation and manipulation, whereas replication switches to the P1 replicon in a *polA* host (*E. coli* C2110 strain). The plasmid is inherently unstable in this background but can be stabilized by the insertion of a plasmid maintenance module. One recombinant derivative of pALA136 (pYC16A) that possessed high segregational stability in the *polA* strain contained a 4.9-kb insert from RA3. Nucleotide sequencing revealed that this region of RA3 includes a cluster of adjacent open reading frames, the *korA-incC-korB-orf11* operon (Fig. 1B), and an open reading frame truncated at its 5′ end and transcribed in the same direction located upstream of *korA-incC-korB-orf11*. The translated product of this open reading frame demonstrates an α-helical structure (31) similar to that of the highly α-helical protein KfrA, encoded within an operon downstream of the *cco* (central control operon) of IncP-1 plasmids (3, 21). KorA (104 amino acids [aa]) is a putative DNA binding protein with a typical α-helix–turn–α-helix (38) DNA recognition motif (see Fig. S1 in the supplemental material). IncC (250 aa) includes a Walker-type ATPase motif (28) typical for members of the ParA family of partition proteins (see Fig. S2 in the supplemental material). KorB (452 aa) is a predicted DNA-binding protein with an α-helix–turn–α-helix motif (38) and other conserved amino acid sequences described for representatives of the ParB family of centromere binding factors (8, 56) (see Fig. S3 in the supplemental material). KorB differs in length by 100 amino acids from its closest homologs, KorB proteins of Inc-P1 plasmids, as it specifies a much longer variable linker between its conserved N- and C-terminal domains, which are likely involved in DNA binding and dimerization, respectively. The extended linker region includes six repetitions of a 19-amino-acid patch (see Fig. S3) that is present just once in the linker of KorB of RK2 (31, 39). The tandem repeats are enriched in glycine and aspartate residues, suggesting that they might form a highly flexible, surface-exposed part of the protein. A fourth open reading frame (*orf11*) is separated from *korB* by 3 bp and encodes a 75-amino-acid polypeptide that shows homology to the predicted products of *orf22* of pIPO2 (50) and *orf14* of pSB102 (45) from the PromA plasmid group (54) (see Fig. S4 in the supplemental material). No significant conserved motifs, e.g., α-helix–turn–α-helix, have been identified in Orf11.

The 1.2-kb region downstream of the *korA-incC-korB-orf11* cluster carries an *mpr* gene on the complementary strand whose predicted product shows 61% similarity to the zinc metallopeptidase of IncN plasmid R46 (GenBank accession no. NC_003292.1). However, no promoter activity upstream of *mpr* has been detected (P. Dolowy and G. Jagura-Burdzy, unpublished data). The 4.9-kb insert in pYC16A ended in a 5′-proximal fragment of *mobC* similar to *orf21* of pIPO2 (50) and *orf15* of pSB102 (45), identified as the first genes in the conjugative transfer regions in IncU and PromA representatives (31, 54). A highly conserved *oriT* sequence in RA3 (J. Szarlak and G. Jagura-Burdzy, unpublished data) is located in the *mobC* promoter region (31).

Mutational analysis of the RA3 stabilization region was undertaken (Fig. 1B and C). Mutations in pYC16A were constructed by site-directed PCR mutagenesis introducing stop codons after the translation start codons of *korA* (pAKB13.30), *incC* (pAKB13.40), *korB* (pAKB13.50), both *incC* and *korB* (pAKB13.41), and *orf11* (pAKB13.60). Transformants of DH5α with pAKB13.40 ($incC_{stop}$) and pAKB13.50 ($korB_{stop}$) formed very small colonies, suggesting that these constructs produced toxic effects on their hosts. Even more pronounced effects were observed with the C2110 *polA* strain, as transformants could not be obtained with either the $incC_{stop}$ or $korB_{stop}$ plasmid. In contrast, the control plasmids pALA136 and pYC16A each produced ~10^5 transformants per microgram of DNA. The presence of the $incC_{stop}$ $korB_{stop}$ double mutation (pAKB13.41) did not cause growth inhibition in DH5α, and the plasmid also was successfully introduced into the *polA* strain. Segregation experiments demonstrated a high degree of pAKB13.41 instability in the *polA* background comparable to that of the empty vector pALA136 (Fig. 1C). The data suggest that *incC* and *korB* are required for segregational stability, and an imbalance in the ratio of partition proteins (in the single mutants) may block plasmid segregation, causing toxicity under conditions that are selective for the plasmid. A plasmid carrying the $orf11_{stop}$ mutation (pAKB13.60) showed a significant decrease in stability compared to pYC16A but was more stable than pALA136 (Fig. 1C). The *orf11* product may play either a regulatory or an accessory role in stabilization. The absence of the KorA putative transcriptional regulator resulted in rapid loss of pAKB13.30. The strong segregational defect in this plasmid might be a result of overexpression of the operon due to a lack of the KorA predicted transcriptional repressor, might indicate a direct involvement of KorA in the partitioning complex, or both. To distinguish between regulatory effects and a role in segregation, the influence of proteins encoded by the operon on regulation of the *korA* promoter (*korAp*) was analyzed.

Regulation of RA3 partition operon expression. The 3′ end of *kfrA* and the 5′ end of the *korA-incC-korB-orf11* gene cluster in RA3 are separated by an intergenic region (80 bp) with 5′-TTGACG-3′ and 5′-TATACT-3′ motifs 16 bp apart that are good matches to the consensus and appear to constitute *korAp* (Fig. 2A). To test for promoter activity in the *kfrA-korA*

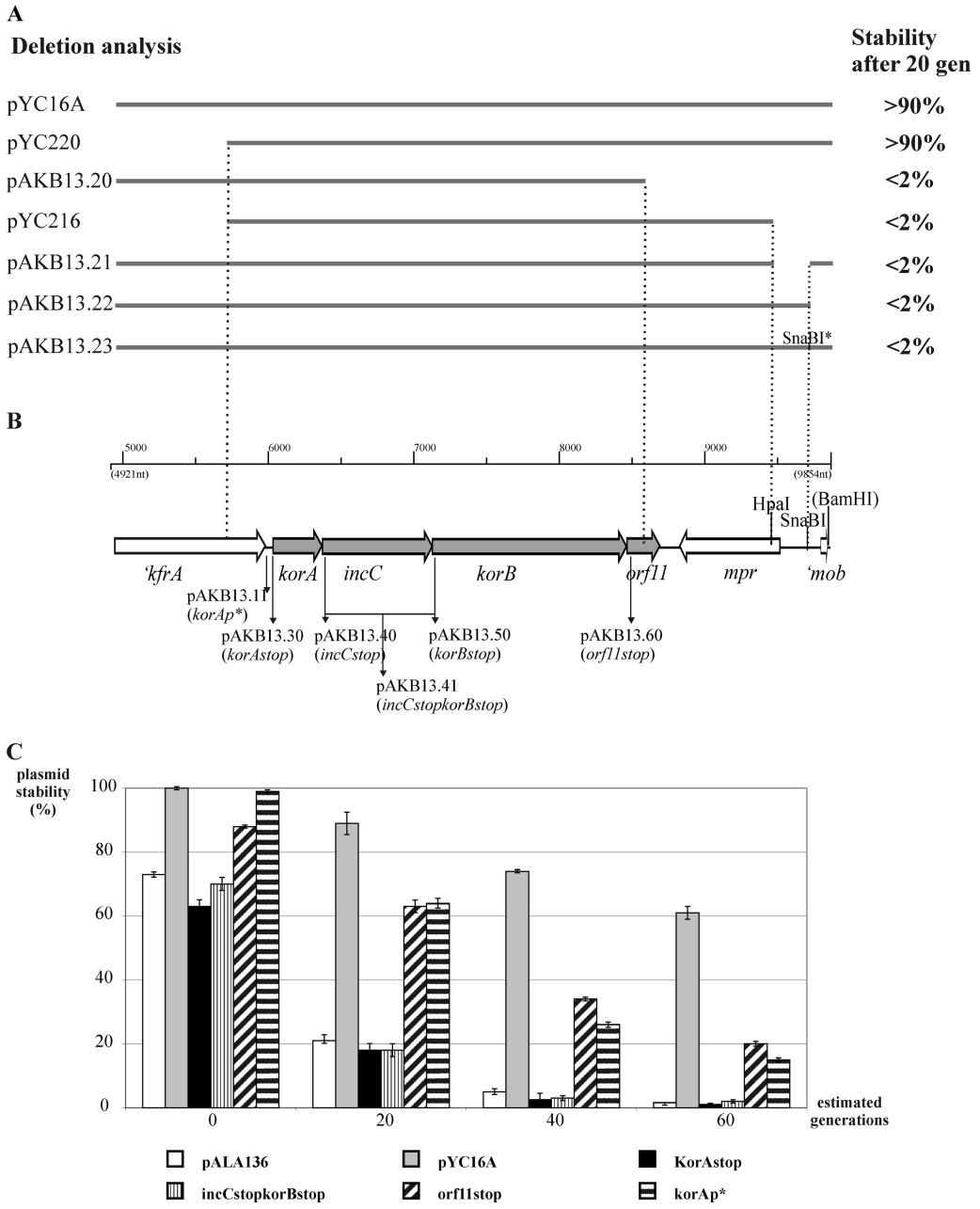

FIG. 1. Organization of the RA3 partition region. Mapping of *parS*. (A) Schematic presentation of deletion derivatives of pYC16A. Plasmid pAKB13.23 has a modified SnaBI (*) restriction site by the insertion of a 20-bp oligonucleotide (see oligonucleotide 37 in Table S1 in the supplemental material). The *E. coli* C2110 *polA* strain was transformed with pYC16A or its deletion derivatives. Transformants were grown overnight in liquid cultures with chloramphenicol, then diluted 10^5 into L broth, and grown without selection. After an estimated 20 generations, the cultures were diluted 10^5 into fresh medium and assessed for plasmid retention. The results are the averages from at least three experiments. (B) Gene organization is shown for the 4.9-kb RA3-derived fragment cloned in pALA136 which conferred enhanced segregational stability (pYC16A). The coordinates correspond to those of the RA3 sequence (GenBank accession number DQ401103). The partitioning operon is represented by gray arrows, whereas open arrows represent other open reading frames in the analyzed region. Truncated forms of *kfrA* and *mob* genes are marked by primes (′). Plasmids below the scheme correspond to pYC16A derivatives with stop codons introduced into particular genes. (C) Segregation of the pYC16A plasmid and its mutated derivatives. *E. coli* C2110 *polA* harboring pALA136, pYC16A, pAKB13.11 with the *korA* operator mutated (*korAp**), pAKB13.30 with the $korA_{stop}$ allele, pAKB13.41 with $incC_{stop}$ $korB_{stop}$, and pAKB13.60 with the $orf11_{stop}$ allele were grown overnight in liquid cultures with chloramphenicol and then diluted 10^5 into L broth without selection. Every 20 generations the cultures were diluted 10^5 into fresh medium and plated in parallel onto L agar to obtain about 100 colonies. The plasmid retention was screened by replica plating. The results are the averages from at least three experiments.

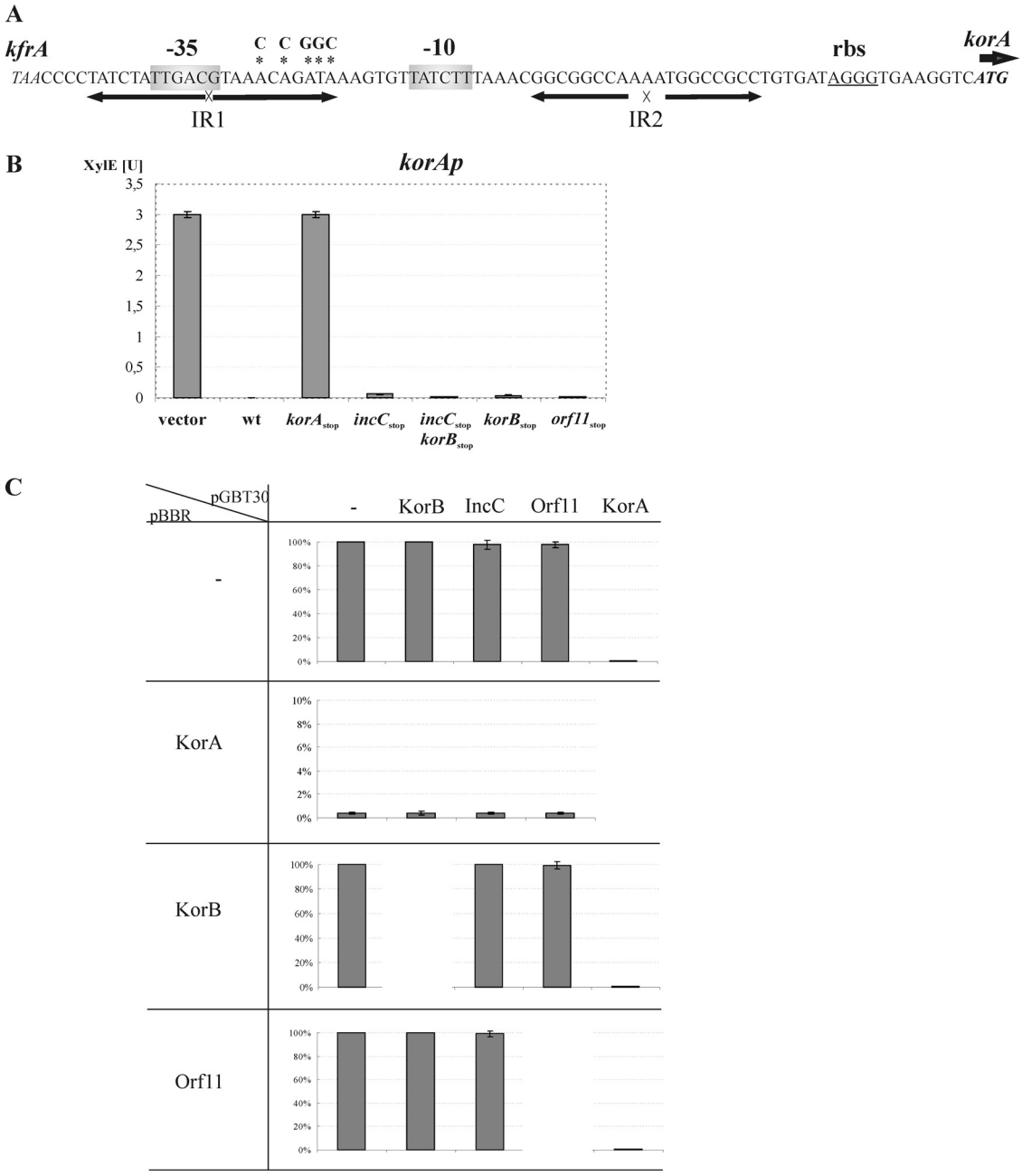

FIG. 2. Regulation of *korAp*. (A) The sequence of the intergenic *kfrA-korA* region containing *korAp*. The *kfrA* stop codon and *korA* initiation codon are in italics. The predicted −10 and −35 motifs and ribosome binding (rbs) sequences are marked. Two inverted repeats found within the promoter sequence are shown as arrows and designated IR1 and IR2. Asterisks indicate nucleotide substitutions in the right arm of IR1. "X" denotes the center of symmetry for a palindromic sequence. (B) Graphical summary of regulation studies of *korAp* transcription. The *korAp* sequence was cloned upstream of the promoterless *xylE* cassette in pPTO1 to obtain pAKB4.10. *E. coli* C600K(pAKB4.10) was transformed with vector pALA136, pYC16A, and its mutated derivatives with stop codons introduced as indicated. XylE activities were assayed in the cleared extracts from logarithmic cultures. The presented results are the averages from at least five experiments. (C) Graphical summary of regulation studies of *korAp-xylE* (pAKB4.10) in the presence of two repressors in *trans*. Genes for putative regulators were cloned into two compatible expression vectors based on pGBT30 and pBBR-MCS1 and introduced into C600K(pAKB4.10) in different combinations. Assays were done in uninduced cultures to minimize the effects of overproduction. The averaged results from at least five experiments are shown as relative values of activities obtained for triple transformants in comparison to the XylE activity of the control strain C600K(pAKB4.10, pGBT30, pBBR1-MCS1).

intergenic region of RA3 and to study the regulation of *korA-incC-korB-orf11* expression, a 312-bp fragment (coordinates 5895 to 6206) encompassing the intergenic region was amplified and cloned into an *xylE* promoter probe vector (pPTOI) (34) to produce plasmid pAKB4.10. Transcriptional activity measured in exponentially growing cultures of C600K(pAKB4.10) indicated a high level of expression (2 to 3 units of XylE). To ascertain whether this promoter activity was

affected by the presence of the stabilization cassette in *trans*, the compatible plasmid pYC16A was introduced into C600K(pAKB4.10). Plasmid content analysis by gel electrophoresis of digested plasmid DNA confirmed the presence of both plasmids. The barely detectable levels of XylE (<0.01 XylE units) in the double transformants suggested that one or more products of the *korA-incC-korB-orf11* gene cluster exerted a strong regulatory effect on the *korAp-xylE* fusion (Fig. 2B). To dissect the role of individual genes in *korAp* repression, the following derivatives of pYC16A were cotransformed into C600K(pAKB4.10): pAKB13.30 ($korA_{stop}$), pAKB13.40 ($incC_{stop}$), pAKB13.50 ($korB_{stop}$), pAKB13.60 ($orf11_{stop}$), and pAKB13.41 ($incC_{stop}$ $korB_{stop}$) (Fig. 2B). Inactivation of *korA* restored XylE activity to the level observed in the control strain. In contrast, inactivation of *incC*, *korB*, and *orf11* individually and *incC-korB* together had no effect on *korAp* activity in *trans* (Fig. 2B), suggesting that neither IncC, KorB, nor Orf11 were involved in transcriptional repression of *korAp*. To examine these observations further, *korA*, *incC*, *korB*, and *orf11* were amplified and cloned under the inducible *tac* promoter into two expression vectors, pGBT30 (26) and pBBR-MCS1 (30). These plasmids were then used in regulatory studies in a three-plasmid compatible system. The presence of *korA* in *trans* to the *korAp-xylE* fusion led to a 200-fold repression of the latter, even without overproduction of the protein (Fig. 2C). The separate or concomitant overproduction of IncC, KorB, and Orf11 in *trans* to the fusion had no effect on *korAp* activity (Fig. 2C). Moreover, no alteration in the repression of *korAp* by KorA was detected in the presence of IncC, KorB, or Orf11 compared to KorA alone. Thus, in contrast with transcriptional regulation of the equivalent genes in IncP-1 plasmids (24, 29, 51), *korAp* of RA3 is regulated only by KorA and no cooperativity occurs between KorA and KorB under the conditions tested.

Identification of the KorA binding site in *korAp*. Inspection of the *korAp* promoter region sequence revealed two palindromic motifs that might possess a regulatory role. One imperfect palindrome (IR1) TATCTATTGACGTAAACA GATA (mismatches in palindrome arms are underlined) spans the probable −35 sequence, and the second inverted repeat (IR2) GGCGGCCAAAATGGCCGCC overlaps the putative transcription start point (Fig. 2A). To assess whether IR1 or IR2 acts as a binding site for KorA, a His-tagged version of the protein was overproduced and purified by nickel affinity chromatography. EMSAs were performed with His_6-KorA and double-stranded oligonucleotides corresponding to either IR1 or IR2. KorA bound strongly to IR1, forming a single complex even at a low-protein concentration applied with an estimated K_{app} of ~0.75 μM (K_{app} was calculated as the protein concentration at which 50% of oligonucleotides was moved to slowly migrating species) (Fig. 3A). In contrast, IR2 was bound only weakly by the protein at the highest concentrations used (~1.5 μM), forming an ill-defined smear. To test whether IR2 altered the affinity of KorA for IR1, two pairs of double-stranded oligonucleotides (62 bp) were analyzed. The oligonucleotides encompassed either both IRs and accompanying promoter motifs (IR1+IR2) or the same region but with IR2 destroyed by multiple nucleotide substitutions (IR1+IR2*). The IR1+IR2 oligonucleotide was bound by KorA with an affinity very similar to that of IR1 alone (K_{app} of KorA for IR1+IR2 was ~0.75 μM). The pattern of KorA binding to the IR1+IR2* sequence was similar to that with IR1+IR2 (Fig. 3A). The data show that KorA binds to IR1 at *korAp* (designated O_A) and that IR2 neither is recognized by the protein nor influences KorA interaction with IR1.

To examine further the contribution of the IR1 palindrome to the regulatory effect of KorA observed *in vivo*, one arm of IR1 was modified without changing the putative −35 motif of *korAp* (Fig. 2A). Site-directed mutagenesis was used to introduce the IR1 mutations into pYC16A to obtain pAKB13.11, and the modified promoter region (mutated *korAp* [*korAp**]) then was amplified and inserted into promoter probe vector pPTOI to produce pAKB4.11 (*korAp*-xylE*). The promoter activity measured in exponentially growing cultures of C600K(pAKB4.11) indicated 0.97 U of XylE activity, that is, ~2-fold less than that for the wild-type promoter. The presence of KorA in *trans* caused a 4-fold repression of *korAp** (0.26 U of XylE) in comparison to the 200-fold repression of wild-type *korAp* (Fig. 3B). RA3 was introduced to the C600K strain with either *korAp-xylE* (pAKB4.10) or *korAp*-xylE* (pAKB4.11). Whereas the expression of *korAp-xylE* in the presence of RA3 in *trans* is repressed 50-fold, the activity of *korAp*-xylE* decreased only 2.5-fold, confirming that KorA is the only RA3-encoded repressor of *korAp*. The role of IR2 in *korAp* regulation remains unclear.

To assess whether the reduced autorepression by KorA and in consequence the derepression of *korAp* influenced plasmid segregation, partition assays of pAKB13.11 bearing *korAp** within the *korA-incC-korB-orf11* cassette were performed. The pAKB13.11 plasmid was unstable, although with a loss rate lower than that for the equivalent plasmid carrying the $korA_{stop}$ mutation (pAKB13.30) (Fig. 1C). Thus, regulation of *korAp* by KorA is fundamental to accurate segregation mediated by the *korA-incC-korB-orf11* cassette.

As the palindromic sequence IR1 shows partial similarity in its central region to the KorA operator of the RK2 plasmid (O_ARK2 [GTTTAGCTAAAC]), $KorA_{RA3}$ and $KorA_{RK2}$ were assessed for recognition of the noncognate sites *in vitro* and *in vivo*. An oligonucleotide corresponding to O_ARK2 was used in the EMSA with purified $KorA_{RA3}$: the binding reaction resulted in an ill-defined smear (Fig. 3A). The specificity of the promoter-repressor interactions was examined further *in vivo* by testing whether the heterologous regulatory proteins supplied in *trans* repressed the expression of noncognate *xylE* promoter fusions. In contrast with the very strong repression of *korAp* of RA3 by $KorA_{RA3}$, $KorA_{RK2}$ exerted no discernible regulatory effect on this promoter (Fig. 3C, right). Conversely, the *trfAp-1* promoter of RK2 that is efficiently repressed by $KorA_{RK2}$ did not respond to $KorA_{RA3}$ (Fig. 3C, left). These experiments reveal that the KorA homologs encoded by IncU plasmid RA3 and IncP-1 plasmid RK2 exhibit species specificity, with respect to operator site recognition and transcriptional repression.

Mapping of the RA3 centromere *parS*. The segregational stability conferred by the presence of the RA3-derived insert fragment in pYC16A (Fig. 1A and C) revealed that the cloned insert contained an intact partition cassette, i.e., both the partition genes and the *cis*-acting centromere-like sequence. As plasmid centromeres are located frequently at the 3′ ends of partition operons, the region between *orf11* and *mobC* was first

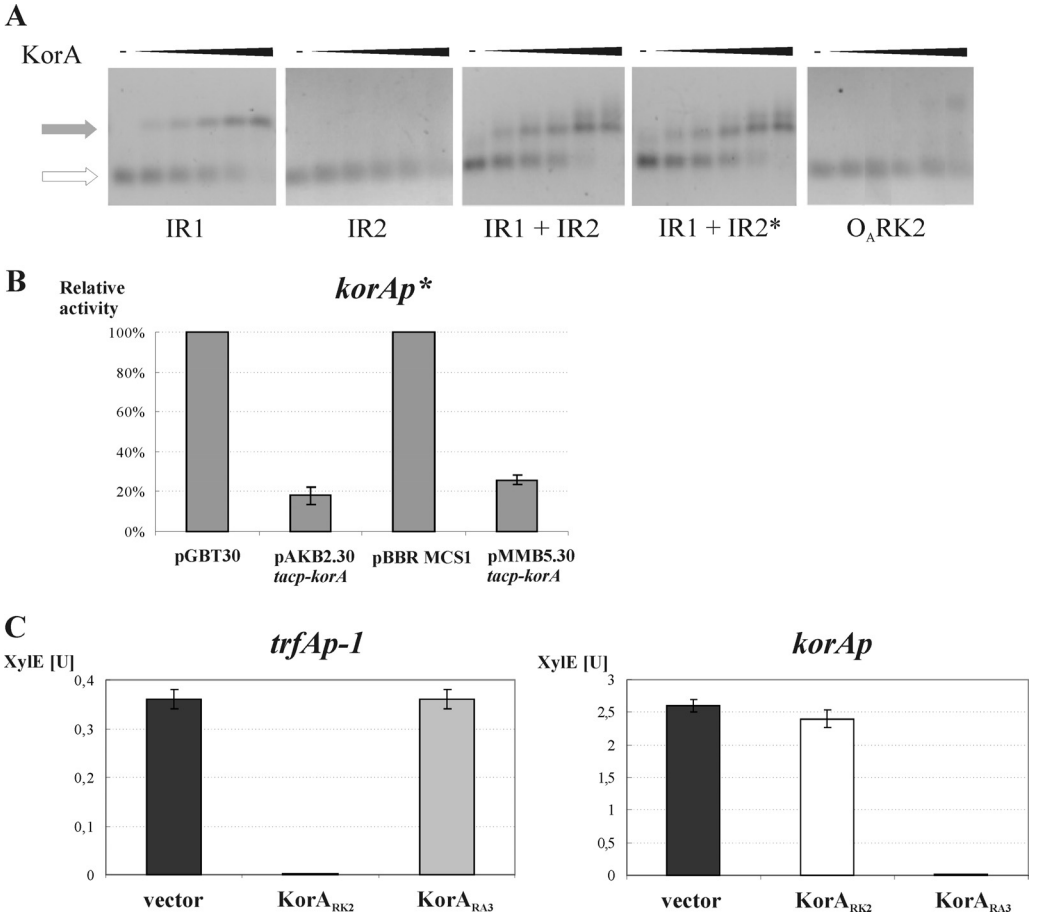

FIG. 3. Specificity of KorA binding. (A) Identification of the KorA binding site. Double-stranded oligonucleotides corresponding to IR1, IR2, IR1+IR2, IR1+IR2*, and O_ARK2 were used in an EMSA with purified His_6-KorA. A total of 5 pmol of the oligonucleotides was added to binding reactions with increasing KorA concentrations (0.25, 0.5, 0.75, 1, and 1.5 μM). Oligonucleotides with no protein added were used in control reactions (−). White arrows indicate input fragments, and gray arrows indicate shifted species. (B) KorA regulation of mutated *korAp* (*korAp**). *korAp** activity was tested in the presence of KorA expressed from a pGBT30 derivative (pAKB2.30) and a pBBR-MCS1 derivative (pMMB5.30). The cultures were grown without IPTG induction, and the results are the averages from at least 5 experiments and presented as relative values compared to the control strains with empty vectors. (C) Cross-reactivity regulation tests for KorA from RA3 and RK2. The activities of RA3 *korAp* and RK2 *trfAp-1* promoters cloned into promoter probe vector pPT01 (plasmids pAKB4.10 and pGBT70 [23], respectively) were analyzed in the presence of plasmids overproducing KorA of RA3 (pAKB2.30) or RK2 (pGBT37 [23]). The double transformants of C600K were grown without and with 0.5 mM IPTG for protein induction, and the assay was conducted in a way similar to that described above. The results are average values from at least three experiments.

studied for centromere activity. To delineate the presence of a centromere in this region, a set of deletions was constructed in pYC16A (Fig. 1A). The removal of most of the already truncated *kfrA* gene had no effect on plasmid segregation (pYC220), whereas deletion of the 3′ end of *orf11* and sequences downstream caused severe instability (pAKB13.20). The removal of a 458-bp HpaI-BamHI fragment from the 3′ end of the insert also led to plasmid loss (pYC216). Moreover, deletion of nonoverlapping 320-bp HpaI-SnaBI (pAKB13.21) or 138-bp SnaBI-BamHI fragments (pAKB13.22) had effects on segregation similar to those of the deletion in pAKB13.20. The insertion of a 20-bp oligonucleotide into the SnaBI restriction site also compromised segregation (pAKB13.23). These data indicate a vital role in the partitioning process for sequences in the vicinity of the SnaBI restriction site between *mpr* and *mobC*.

The presence of a centromere in *trans* causes a strong incompatibility effect on a coresident plasmid carrying the complete partition cassette, leading to missegregation of one of the replicons (7). Plasmids based on a pSC101 vector carrying either the 458-bp HpaI-BamHI fragment that includes the crucial SnaBI site (pAKB8.5I) (Fig. 4A) or the intergenic region bearing *korAp* (pAKB4.10) as a negative control were used in partition-mediated incompatibility tests with the C2110 *polA* (pYC16A) strain. Double transformants were grown without antibiotic selection for ~20 generations and analyzed for pYC16A retention. The presence of the 458-bp HpaI-BamHI fragment in *trans* led to the loss of pYC16A in ~90% of the population. In contrast, the empty vector or the *korAp* region exerted no in *trans* effect on pYC16A maintenance. These results support the location of the putative RA3 centromere (*parS*) in the 458-bp HpaI-BamHI fragment that encompasses the *mpr-mobC* intergenic region.

As the protein products of the RA3 partition operon are

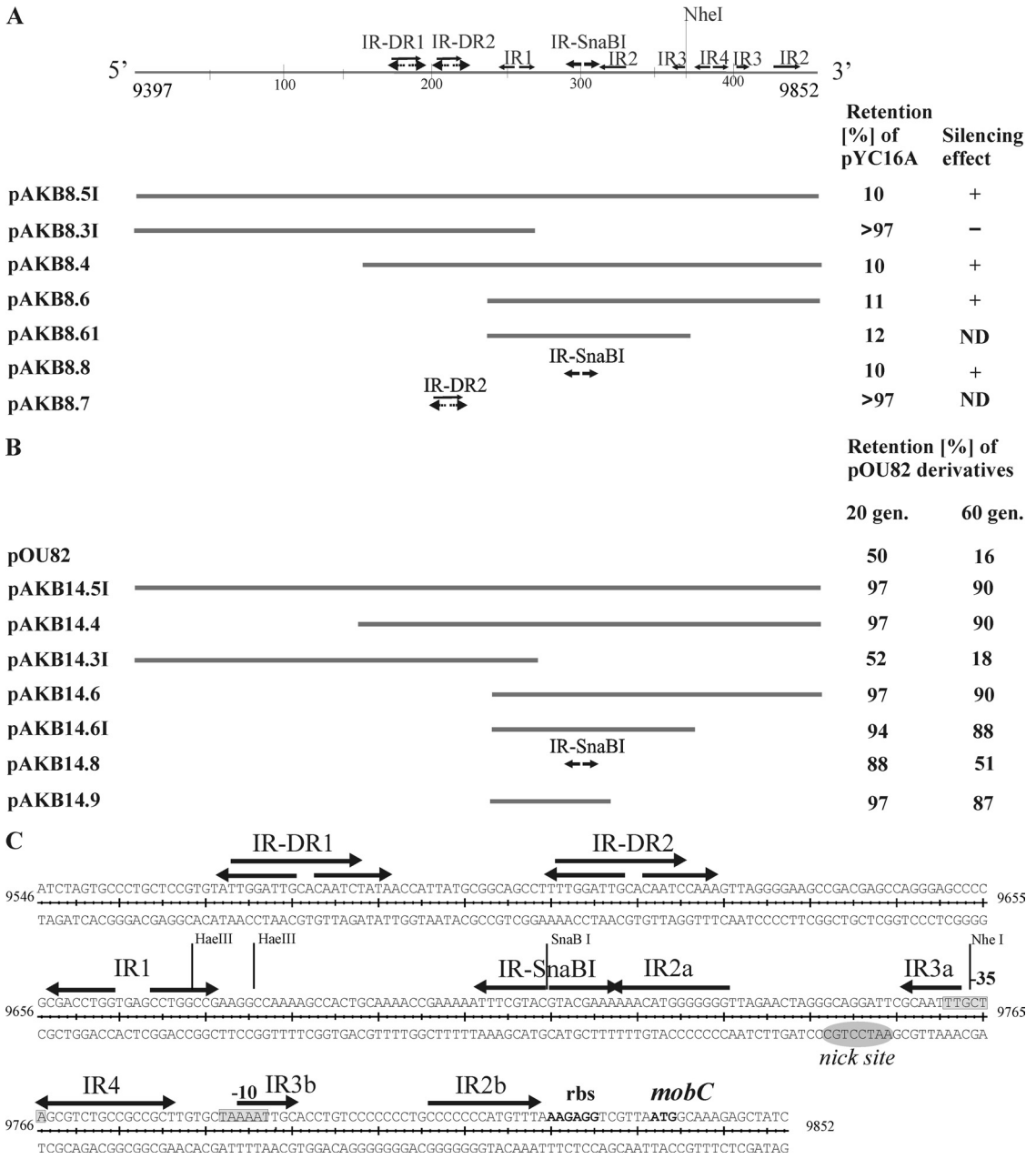

FIG. 4. Dissection of the *parS* region. (A) The coordinates of the fragment are given according to GenBank accession number DQ401103. Identified structural motifs are schematically shown. The fragments of the *parS* region cloned in pGB2 are delineated. IR-DR2 and IR-SnaBI represent double-stranded oligonucleotides inserted in pGB2. The first column on the right shows the retention of pYC16A in C2110 *polA* after ~20 generations of nonselective growth of appropriate double transformants. The results are the averages of at least three experiments with standard deviation values of <4%. The transcriptional silencing effect shown in the second column was tested in transformation assays (8). DH5α with pGB2 or its derivatives were transformed with pBBR-MCS1 or pAKB5.50*tacp-korB*. The transformation mixtures were plated on selective plates for the incoming plasmid (Cm) and for both incoming and resident plasmids (Cm and Sm) with or without 0.5 mM IPTG. The silencing effect appeared as a 10^4-fold drop in the number of transformants obtained on double-selection plates with IPTG in comparison to double-selection plates with no IPTG. The experiments were repeated three times. (B) Complementation assays with subfragments of the *parS* region cloned into pOU82. Partitioning operon *korA-incC-korB-orf11* was cloned into pSC101 derivative to obtain pAKB4.90. *E. coli* DH5α(pAKB4.90) was transformed with pOU82 or its derivatives with *parS* subfragments (according to the scheme in panel A). The retention of pOU82 derivatives in the presence of pAKB4.90 is shown on the right. The results are the averages of at least three experiments, and standard deviation values were <4%. (C) DNA sequence of RA3 centromere-like region. The predicted start codon of *mobC* and ribosome binding sequence (rbs) are in bold; the putative −10 and −35 motifs of the *mobC* promoter are boxed in gray. The predicted nick site sequence (31) is highlighted with a gray ellipse. Direct or inverted repeats are indicated with arrows and labeled. The restriction sites used in the study are shown.

predicted to promote segregation in *trans* of an unstable plasmid with the cognate centromere-like sequence, the 458-bp fragment carrying the putative *parS* site was inserted into pOU82 to form pAKB14.5I (Fig. 4B). The pOU82 plasmid is a single-copy mini-R1 derivative with a *lac* operon, which lacks accessory stabilization functions, and is lost rapidly during growth at 30°C, without selection (26). In parallel, a fragment encoding the *korA-incC-korB-orf11* partition operon deprived of the *parS* sequence was cloned into a medium-copy-number, stable pSC101 derivative (pAKB4.90). *E. coli* DH5α bearing either pOU82 or pAKB14.5I was cotransformed with pAKB4.90. Cultures of double transformants grown without selection for the pOU82 plasmids were diluted and plated on L agar plates with IPTG and X-Gal. The loss of the empty pOU82 vector was estimated by the number of white colonies present: this value increased from ~50% (after ~20 generations of growth without selection) to ~85% (after ~60 generations). The presence of the plasmid carrying the RA3 segregation cassette in *trans* had no effect on the retention of pOU82. However, the presence of the module in *trans* to pAKB14.5I diminished the loss of the latter to <3% after ~20 generations of nonselective growth and to ~10% after ~60 generations. These results indicate that the products of the *korA-incC-korB-orf11* operon promote segregational stability of an otherwise unstable plasmid carrying the cognate *parS* region and demonstrate that all of the *cis*-acting sequences required for segregation reside in the 458-bp fragment. Furthermore, the concentration of the proteins produced in *trans* to *parS* may be important, as the location of the *korA-incC-korB-orf11* cassette on a vector with a copy number higher than that of pSC101 (pAKB7.90) conferred less-efficient segregation of pAKB14.5I (data not shown).

Inspection of the 458-bp HpaI-BamHI fragment that includes *parS* revealed the presence of distinct repeat motifs (Fig. 4C): (i) two 15-bp-long direct repeats (TTGGATTGCA CAATC) embedded in 20-bp inverted repeats (ATTGGATT GCACAATCTATA) and (TTTGGATTGCACAATCCAAA) (mismatches in palindrome arms are underlined) designated IR-DR1 and IR-DR2, respectively; (ii) a 16-bp inverted repeat (TTTCG**TACGTA**CGAAA) designated IR-SnaBI, as it encompasses the SnaBI endonuclease cleavage site (in bold) described above; (iii) a 16-bp imperfect inverted repeat with a 4-bp spacer (CGACCTGG…CCTGGCCG) denoted IR1; (iv) a 26-bp repeat with arms separated by 75 bp (AAACATGGG GGGG…CCCCCCCATGTTT) designated IR2; (v) a 14-bp inverted repeat with 7-bp arms separated by 23 bp (GCAAT TT…AAATTGC) denoted IR3; and (vi) a 16-bp imperfect inverted repeat (AGCGTCTGCCGCCGCT) designated IR4. The region also includes the origin of conjugative transfer *oriT* and the promoter sequence motifs that drive transcription of the *mobC-nic* operon (31) (Fig. 4C). To assess which sequence motifs were important for partition-mediated incompatibility, pGB2 derivatives containing different subfragments (Fig. 4A) were transformed into C2110 *polA* (pYC16A). Double transformants were grown without selection and tested for pYC16A retention on appropriate plates. The results revealed that the presence of IR-SnaBI alone (pAKB8.8) was sufficient to exert incompatibility against pYC16A, whereas subfragments without this motif had no effect on pYC16A retention.

Subfragments surrounding the centromere-like region were also cloned into pOU82 and then cotransformed into DH5α carrying pAKB4.90 that supplies the required partition proteins (Fig. 4B). The segregation values of pOU82 derivatives bearing IR-SnaBI (pAKB14.4, pAKB14.6, pAKB14.6I, and pAKB14.9) in the presence of partition proteins supplied in *trans* were similar to that of pOU82 bearing the intact 458-bp HpaI-BamHI fragment (pAKB14.5I): >90% after ~20 generations and >85% after ~60 generations of growth without selection. A plasmid bearing the left half of the fragment and which lacks IR-SnaBI (pAKB14.3I) was maintained at levels similar to those of the pOU82 empty vector (Fig. 4B). Although a pOU82 derivative with IR-SnaBI alone (pAKB14.8) was not stabilized to the same extent as plasmids with more extensive surrounding sequences, its maintenance after 60 generations of nonselective growth was higher than that for the empty vector. Although IR-SnaBI is required and sufficient for partition-mediated incompatibility, it may not represent the complete *parS* site.

KorB recognizes the region with the IR-SnaBI motif. KorB belongs to the widely spread ParB family of plasmid and chromosome partitioning proteins (10, 31, 56). Since the genetic tests revealed a role for the IR-SnaBI motif and surrounding sequences in proper functioning of the segregation cassette, the ability of KorB to bind to this region was examined. His_6-KorB was used in EMSA experiments with radioactively labeled fragments of the *parS* region containing IR-SnaBI and adjacent sequences (Fig. 5). Whereas a control fragment (Fig. 5A, a) was not bound by KorB, a fragment (Fig. 5A, c) that included IR-SnaBI and motifs downstream was bound with a K_{app} of ~3.0 μM. KorB demonstrated much higher affinity (K_{app} ~1.2 μM) for a fragment (Fig. 5, b) that contains the additional three repeats IR-DR1, IR-DR2, and IR1 upstream of IR-SnaBI (Fig. 5). These preliminary data confirm that KorB is able to specifically interact with the analyzed region and that sequences upstream to IR-SnaBI play an important role in modulating KorB binding affinity to DNA.

KorB binding at IR-SnaBI leads to transcriptional silencing. Several representatives of the ParB family have been shown to silence the expression of nearby genes after binding to their cognate *parS* sequences and spreading on DNA (8, 33, 42). To determine whether KorB binding at the *parS* region also leads to a transcriptional silencing effect, a test system based on the pSC101 derivative pGB2 was used (13). In this system, insertion of *parS* proximal to the promoter for the *repA* replication initiation gene causes a detrimental effect on plasmid replication when the cognate ParB protein is overproduced (8). This effect presumably reflects occlusion of the *repA* promoter by ParB spreading from *parS*. A strain with a pGB2 derivative carrying the 458-bp HpaI-BamHI fragment that includes *parS* (pAKB8.5I) was transformed with a plasmid expressing *korB* from an inducible *tac* promoter in vector pBBR-MCS1. The number of transformants on plates selective for the incoming plasmid was similar to the number on plates selective for both incoming and resident plasmids. However, the colonies formed on double selection plates were minute, suggesting that growth of double transformants was inhibited when selection for both plasmids was maintained. When expression of *korB* was induced, we observed a $>10^4$-fold decrease in the number of transformants on double selection plates. We interpret these results as KorB-mediated transcriptional silencing

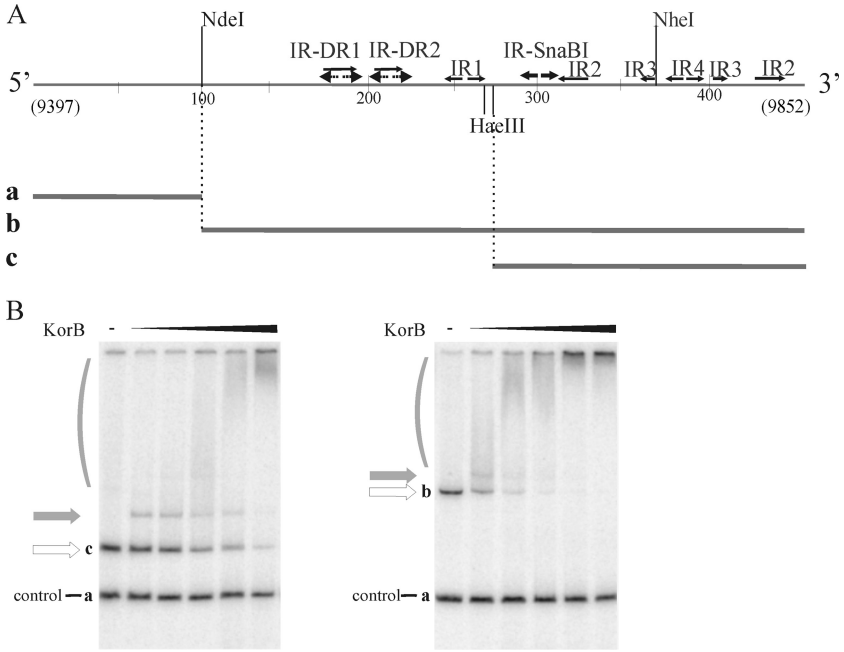

FIG. 5. KorB binding at *parS*. (A) The scheme delineates the RA3 *parS* region. Direct and inverted repeat motifs are indicated with arrows, recognition sites for restriction enzymes used in this study are shown, and fragment coordinates are given in parentheses. Restriction fragments a, b, and c tested in an EMSA are shown (bottom). (B) EMSA analysis of the KorB-*parS* interactions. A 460-bp fragment ^{32}P labeled at the ends was digested either with HaeIII-NdeI (left) or NdeI (right). The restriction fragments were incubated with increasing KorB concentrations (1.2, 2.3, 3.6, 4.8, and 6.0 μM). Fragments with no protein added were used in control reactions (−). White arrows indicate input fragments, whereas gray arrows and brackets indicate shifted species.

of the *repA* promoter on the pGB2 derivative carrying the nearby *parS* sequence (Fig. 4A). No such effect was noted when a strain with pAKB8.5I was transformed with the empty vector pBBR-MCS1 (data not shown). Subfragments of the *parS* region were used to resolve which sequence was necessary for this silencing. Overexpression of *korB* in *trans* to plasmids bearing the IR-SnaBI repeat was sufficient to decrease the number of transformants on double selection plates $>10^4$-fold, whereas there was no effect on the number of transformants when the target plasmid lacked IR-SnaBI (Fig. 4A). The results indicate that the IR-SnaBI motif is necessary and sufficient for KorB-mediated transcriptional silencing.

DISCUSSION

Three products of the partition cassette of BHR plasmid RA3 show homology to proteins KorA, IncC-2, and KorB encoded in the *cco* operons of RK2 (39) and R751 (52), representatives of the IncP-1α and IncP-1β families, respectively. The presence of similar cassettes carrying the regulatory *korA* gene nearby the partition module was also demonstrated in plasmids of the IncP-9 group (14, 46) and in environmental plasmids recently defined as the PromA group, e.g., pIPO2 (50), pSB102 (45), pMOL98 (54), and pTer331 (36). This study shows that the segregation region of RA3 has a number of structural and regulatory properties that clearly distinguishes it from the equivalent regions of RK2 and R751 that have been analyzed most thoroughly. The probable promoter for the RA3 segregation cassette is located in the short (~80-bp) intergenic region between the *kfrA* and *korA* genes and conforms well to the consensus for *E. coli* σ^{70} promoters. The *korA* and *incC* genes are separated by 4 bp, whereas the latter overlaps *korB* by 3 bp. The *orf11* start codon is separated by 2 bp from the *korB* stop codon. Regulatory studies of *korAp* proved it to be a strong promoter that is tightly regulated by KorA. Other products of the operon, KorB, IncC, and Orf11, do not regulate *korAp* expression, either on their own or in combination (Fig. 2C).

There is no cooperativity in repression between KorA and KorB in the RA3 system, which clearly contrasts with the cooperativity for IncP-1 equivalents in which these interactions are highly important in fine-tuning the transcription levels in different hosts (2). KorA is a global regulator of operons involved in vegetative replication and stable maintenance of RK2, regulates seven promoters, and acts either as the primary repressor on operator sites overlapping the −10 motif (class I) or as a secondary, weaker repressor on operators that overlap the −35 sequence (class II) (22). The operator recognized by RA3 KorA is the imperfect palindrome that overlaps the putative −35 sequence of *korAp* (Fig. 2A and Fig. 3A and B). Since this palindromic sequence is unique in the RA3 genome, it implies that KorA does not function as a global regulator in this plasmid, but only as an autorepressor of the partition cassette. Although the location of the operator site in *korAp* of RA3 is similar to that of class II operators that overlap the −35 boxes of promoters in IncP-1 plasmids, KorA of RA3 represses as strongly as its equivalent in IncP-1 plasmids at class I operator locations. Despite the low level of similarity (53%) between KorA proteins of RA3 and RK2, there is a striking resemblance between the operators recognized by the two pro-

teins, confirming a common ancestor. However, *in vitro* and *in vivo* cross-reactivity assays showed an absence of cross-regulation between the KorA proteins and the heterologous binding sites (Fig. 3C). The importance of KorA regulation of *korAp* is reflected by the decreased segregational stability of a plasmid in which the overlapping operator was mutated. The coevolution of regulatory proteins with their binding sites may highlight a significant role for KorA regulatory networks in the differentiation and establishment of plasmid incompatibility groups.

Numerous plasmid-encoded ParA homologues possess an N-terminal DNA binding domain required for autoregulation of partition operon expression (10, 18). The *incC* (*parA*) gene of IncP-1 plasmids encodes two forms of the protein from two alternative translation start points: the longer-form IncC1 (364 and 358 amino acids for RK2 and R751, respectively) and the shortened IncC2 form (258 and 259 amino acids for RK2 and R751, respectively), which is sufficient to act as a partitioning protein (55). IncC1 does not bind specifically to DNA but enhances the binding ability of KorB, functioning as a corepressor in the regulatory network of IncP-1 plasmids (25). Recent studies showed that IncC1 interacts nonspecifically with DNA as homo-oligomers and hetero-oligomers with IncC2, although the purpose of these interactions is still speculative (9). IncC of RA3 (250 amino acids) is equivalent to the IncC2 form of RK2, to which it is 63% similar. Sequence analysis excludes the possibility of two IncC forms being produced in RA3. The production of only the smaller variant of IncC in RA3 shows a new approach to partitioning that differentiates it from the IncP-1 framework. Similar operon structures are found in environmental BHR plasmids of the PromA group (54) that encode only the short IncC variant accompanied by *korA*, *korB*, and homologous open reading frames of unknown function (31).

The RA3 partition cassette includes an open reading frame, *orf11*, which may act as an accessory partitioning factor. In the case of RK2, but not R751, two open reading frames downstream of *korB* encode unrelated short polypeptides, KorF and KorG, with regulatory functions (24). There are short open reading frames of unknown function at similar positions in the putative partition operons of pIPO2 (*orf22* [50]), pSB102 (*orf14* [45]), pTer331(*orf22* [36]), and pMOL98 (*orf58* [54]) (Fig. 6). Although Orf11 of RA3 is smaller than the equivalent proteins of these PromA plasmids, they share similar motifs (see Fig. S4 in the supplemental material) and likely have the same function(s). A stop mutation introduced near the beginning of *orf11* rendered a test plasmid less segregationally stable than the parental plasmid (Fig. 1C). Although the effect of the Orf11 mutation on segregation was not as strong as that observed when $IncC_{stop}$ and $KorB_{stop}$ mutants were examined, the phenotype indicates a role for Orf11 in partition or regulation of segregation operon expression. However, a regulatory role for Orf11 at *korAp* can be excluded since neither knockout nor overexpression of *orf11* affected *korAp* expression (Fig. 2B and C). Further studies of the product of *orf11* will clarify its function in segregation.

Deletion analysis of the insert in pYC16A showed that removal of a 458-bp HpaI-BamHI fragment located discontiguously from the *korA-incC-korB-orf11* operon rendered the plasmid segregationally unstable (Fig. 1A). Additional deletions

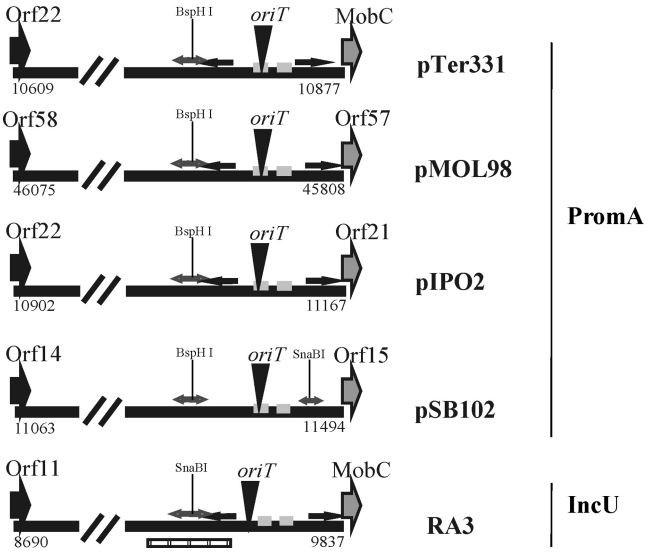

FIG. 6. Organization of the border between maintenance and conjugative transfer modules. Comparison of the *mobCp-oriT* regions of RA3 and plasmids from the PromA group. The scheme illustrates the intergenic regions between the final *orf* of the partition operon and the first gene in the conjugative transfer operon in plasmids pTer331 (EU 315244), pMOL98 (FJ 666348), pIPO2 (NC_003213), pSB102 (NC_003122) from the PromA group and RA3 (DQ 401103) from the IncU group. The predicted promoter motifs are marked with gray boxes, predicted *oriT* motifs are labeled as black triangles, inverted repeat motifs are indicated by black arrows for those with a high GC content and by gray arrows for 14- to 16-bp perfect palindromes, with relevant restriction cleavage sites indicated. Homologous ORFs are marked similarly. The experimentally identified *parS* region of RA3 is highlighted with a striped box.

and an insertion into the SnaBI restriction site in this region proved the importance of sequences traversing this site for segregation. A partition-mediated incompatibility determinant within the RA3 partition region was mapped to the same 458-bp fragment (Fig. 4A). Analysis of the region located between the segregation and conjugative transfer operons (Fig. 4C) revealed a complex set of direct and inverted repeat motifs in close vicinity to the *oriT* site and the promoter for the *mobC-nic* operon. Studies with subsections of this region confirmed that all fragments that included IR-SnaBI or the IR-SnaBI motif alone exerted incompatibility (Fig. 4A). Segregation complementation studies with equivalent fragments and the *korA-incC-korB-orf11* operon provided in *trans* (Fig. 4B) similarly proved that sequences, including IR-SnaBI, functioned as centromeres. Although identified as an incompatibility determinant, the IR-SnaBI motif alone was not as active in the centromere assays as more extensive fragments: IR-SnaBI did not stabilize an unstable test vector (pAKB14.8) to the same extent as the full 458-bp fragment (pAKB14.5I) or a 122-bp subfragment in pAKB14.9 (Fig. 4B). KorB binds specifically to fragments containing parts of the mapped centromere-like region that include IR-SnaBI (Fig. 5). In agreement with the genetic data, the affinity of KorB binding to IR-SnaBI is enhanced by sequences upstream of the motif. Based on these data we conclude that the *parS* site of RA3 comprises the IR-SnaBI motif with adjacent sequences to this inverted repeat (coordinates 9623 to 9745). The IR-SnaBI motif is not unique

to the *parS-mobCp* region in RA3. Two other identical palindromic 16-bp sequences are found in the promoter region of *orf2* and intragenic to *orf25*, respectively. Whereas the function of IR-SnaBI in *orf25* is undefined, two KorB binding sites in the promoter regions *orf2p* and *mobCp* are responsible for KorB repressor functions (P. Dołowy, unpublished data). As shown in this work, the IR-SnaBI motif in the *parS-mobCp* region, although required, is not sufficient for full partition-mediated stabilization of pOU82 (pAKB14.8). Additional sequence determinants (adjacent to IR-SnaBI in the 122-bp fragment of pAKB14.9) which may influence KorB binding at *parS* remain to be investigated.

Since the similarities observed between the PromA group of plasmids and RA3 of the IncU incompatibility group include the stable maintenance and conjugative transfer regions (31), the respective regions separating these modules were compared. The putative segregation cassettes of plasmids from the PromA group contain *ssb*, *korA*, *incC* (short version), *korB*, and conserved short open reading frames of unknown function, *orf14*, *orf22*, *orf22*, and *orf58* in pSB102, pIPO2, pTer331, and pMOL98, respectively (Fig. 6). The cassettes are separated by intergenic regions from the conjugative transfer module. The first open reading frames in the transfer operons are homologous between RA3 (IncU) (31) and PromA plasmids (54). Whereas the distance between *orf11* and *mobC* in RA3 is 1,147 bp, the lengths of the corresponding regions vary in plasmids of the PromA group from 265 bp in pIPO2 to 431 bp in pSB102. Detailed comparison of these sequences highlighted a similar set of distinctive sequence motifs that are present in both RA3 and PromA plasmids (Fig. 6). The putative *oriT* sequences in PromA plasmids overlap −35 motifs in the promoters of the transfer region operons, with the putative nick sites situated between positions −33 and −34 of the promoter. In the case of RA3, *oriT* is located between IR-SnaBI and *mobCp*, with the nick site 13 nt upstream of the −35 motif for *mobCp* (Fig. 4C and 6). A 15-bp arm of a palindromic GC-rich sequence (IR2) is situated next to IR-SnaBI in RA3, with the second arm located downstream of the −10 box of *mobCp*. Identical GC-rich palindromes with separated arms encompassing promoter regions for *mobC* homologs are present in three out of four PromA representatives (Fig. 6). Moreover, a highly conserved 14-bp perfect palindrome with a BspHI cleavage site is located adjacent to the arm of this palindrome in all PromA members at positions similar to IR-SnaBI in RA3. Interestingly, as with IR-SnaBI in RA3, IR-BspHI is present as additional copies on the genomes of PromA group members. Three identical IR-BspHI sites are located, respectively, in the *korB* gene, *oriT* region, and *traN* in both pIPO2 and pTer331. In pMOL98 and pSB102, IR-BspHI is present in two copies, again in the *oriT* regions and *traN* genes. The IR-BspHI sequences in pSB102 differ at two positions from those in other PromA plasmids. Whether these IR-BspHI motifs possess centromeric activity like IR-SnaBI in RA3 needs to be verified experimentally.

The location of important *cis*-acting sites (*parS* and nick in *oriT*) next to each other in the RA3 genome (and presumably in representatives of the PromA group of plasmids) creates the opportunity for a controlled switch between the functions participating in maintenance and establishment of the plasmid in the new host. Further dissection of KorB binding to DNA and of structural elements in the *parS-oriT-mobCp* region of RA3 will allow an improved understanding of how distinct ways of plasmid propagation—segregation and horizontal transfer—influence each other and facilitate the effective spread and maintenance of the IncU class of promiscuous plasmids in many different hosts.

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