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Global Transcriptional Regulation of Backbone Genes in Broad-Host-Range Plasmid RA3 from the IncU Group Involves Segregation Protein KorB (ParB Family)

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The KorB protein of the broad-host-range conjugative plasmid RA3 from the IncU group belongs to the ParB family of plasmid and chromosomal segregation proteins. As a partitioning DNA-binding factor, KorB specifically recognizes a 16-bp palindrome which is an essential motif in the centromere-like sequence *parS*$_{RA3}$, forms a segrosome, and together with its partner IncC (ParA family) participates in active DNA segregation ensuring stable plasmid maintenance. Here we show that by binding to this palindromic sequence, KorB also acts as a repressor for the adjacent *mobC* promoter driving expression of the *mobC-nic* operon, which is involved in DNA processing during conjugation. Three other promoters, one buried in the conjugative transfer module and two divergent promoters located at the border between the replication and stability regions, are regulated by KorB binding to additional KorB operators (O_Bs). KorB acts as a repressor at a distance, binding to O_Bs separated from their cognate promoters by between 46 and 1,317 nucleotides. This repressor activity is facilitated by KorB spreading along DNA, since a polymerization-deficient KorB variant with its dimerization and DNA-binding abilities intact is inactive in transcriptional repression. KorB may act as a global regulator of RA3 plasmid functions in *Escherichia coli*, since its overexpression in *trans* negatively interferes with mini-RA3 replication and stable maintenance of RA3.

Large, low-copy-number plasmids displaying a broad host range (BHR) carry an extended backbone of operons involved in replication, copy number control, maintenance, and in the case of self-transmissible plasmids, also conjugative transfer. Expression of these genetic units is driven by high-activity promoters and therefore imposes a substantial metabolic cost on the bacterial cell. To minimize the metabolic burden to the host, regulatory networks have evolved to diminish the backbone gene expression to a low basal level while still allowing for rapid upregulation of the transcription when needed. The regulation is achieved by autogenous repressors that produce negative-feedback loops, medium-range repressors that control particular modules, and globally acting regulators that bind operators scattered along the plasmid molecule to coordinate expression of all modules. Fine-tuning of gene expression is postulated to be achieved by the action of corepressors and their cooperative binding in the promoter regions, which makes the plasmid highly responsive and facilitates its adaptation (1–4).

BHR plasmids from the incompatibility group IncU are widespread and ubiquitous in various aquatic environments, freshwater, fish farms, and clinical isolates (5–8). The modular-mosaic backbone of the IncU plasmids is extremely well conserved not only in its overall genetic organization but also at the nucleotide sequence level (8–10). The mosaic character of genomes from this group is demonstrated by homology of the functional blocks involved in replication, stable maintenance, and conjugative transfer to the respective functional modules of plasmids from different incompatibility groups. Thus, the stability region of RA3 encodes seven homologs of IncP-1 proteins (11), whereas the RA3 conjugative transfer region clusters with similarly organized modules from the PromA group of plasmids (12–16). The RA3 plasmid (Fig. 1), the group archetype isolated from the aquatic bacterium *Aeromonas hydrophila* (17), is the best-studied IncU representative. Its DNA sequence has been established (GenBank accession no. DQ401103) (10), and individual functional modules have been analyzed (18, 19). The plasmid encodes a number of autoregulators, i.e., RepA and RepB in the replication module (10), KfrA and KorA in the maintenance module (18, 20), and MobC in the conjugative transfer unit (19). KorC, encoded in the stability module, plays the role of a global transcriptional regulator controlling replication, maintenance, and conjugative transfer functions (10, 20).

In this paper, we reveal a regulatory role of the DNA-binding protein KorB (452 amino acids), which recognizes a specific sequence occurring in three RA3 locations (Fig. 1). The *korB* gene is part of the *korA-incC-korB-orf11* active partition operon, which is followed by a *parS* sequence (18). The genetic organization of the RA3 segregation cassette and the structural features of the encoded partition proteins, IncC and KorB, identify it as subtype Ia, an active partition system widely distributed among bacterial plasmids and chromosomes (21–25). KorB of RA3 belongs to the ParB family of plasmid and chromosomal DNA segregation factors that

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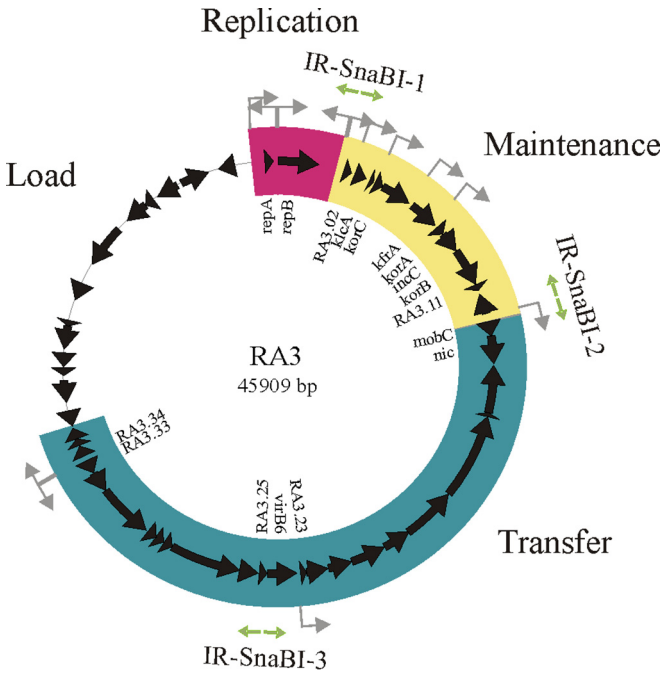

FIG 1 Circular map of plasmid RA3 All identified ORFs are represented by solid arrows indicating the direction of transcription. Functional gene clusters of the plasmid backbone are highlighted with different colors. Experimentally confirmed promoters in the backbone modules are marked with thin gray arrows. The locations of three KorB-binding sites, the IR-SnaBI motifs, are indicated with green arrows.

specifically recognize and bind centromere-like sequences named *parS*, spread along DNA (26–28), and bridge and loop DNA (29, 30), forming a nucleoprotein complex, the segrosome (31). Subsequent interaction with the partner ATPase ParA (or its equivalent IncC in RA3) ensures a proper distribution of the segrosomes during cell division. It has been also postulated that spreading of the plasmid and chromosomal ParB family members after binding to *parS* may lead to transcriptional silencing of nearby promoters (2, 18, 26, 27, 30, 32).

ParB proteins encoded by subtype Ia segregation systems were demonstrated to act as transcriptional regulators, either coautorepressors such as ParB from prophage P1 (33) and SopB of plasmid F (34) or global regulatory proteins exemplified by KorB of RK2 and R751 from the IncP-1 group of plasmids (35, 36). Transcriptomic studies have revealed that chromosomal ParBs of *Pseudomonas aeruginosa* (37) and *Vibrio cholerae* (38) also act as global transcriptional regulators.

Here we demonstrate the activity of the segregation protein KorB in transcriptional repression of four RA3 promoters controlling the replication, stability, and conjugative transfer functions. The mechanism of the KorB action at various distances from the regulated promoters is analyzed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Escherichia coli* strains used were DH5α [F^-(ϕ80d*lacZ*Δ*M15*) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U196*], its Rif^r derivative, BL21 [F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (λDE3)] (Novagen), and BTH101 [F^- *cya-99 araD139 galE15 galK16 rpsL1* (Sm^r) *hsdR2 mcrA1 mcrB1*] (39). Bacteria were grown in L broth (40) at 37°C or on L agar (L broth with 1.5% [wt/vol] agar) supplemented with appropriate antibiotics, i.e., penicillin (Pn) (sodium salt) (150 μg ml^{-1} in liquid media and 300 μg ml^{-1} in agar plates), kanamycin (50 μg ml^{-1}), streptomycin (Sm) (20 μg ml^{-1}), and chloramphenicol 10 μg ml^{-1}. MacConkey agar base (Difco) supplemented with 1% maltose was used for the bacterial adenylate cyclase two-hybrid (BACTH) system. The L agar used for blue/white screening contained 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 μg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Plasmid isolation, analysis, cloning, site-directed mutagenesis, and manipulation. Plasmid DNA was isolated and manipulated by standard procedures (41). Plasmids used and constructed in this study are listed in Table 1. Standard PCRs (42) were performed with RA3 DNA template and pairs of primers listed in Table 2. For amplification of fragment encompassing *orf23*p and its derivative Δ*orf23*p, primer annealing was performed at 54°C for 30 s and elongation at 72°C for 90 s. All PCR-derived clones were sequenced to verify the nucleotide sequence. The pAKB4.81 construct is a derivative of pAKB4.80 with a double-stranded oligonucleotide (self-annealed oligonucleotide 8) containing an EcoRI recognition site inserted in the center of the IR-SnaBI motif. The pAKB4.82 construct with a deletion of one arm of the IR-SnaBI motif was created from pAKB4.81 by removal of EcoRI DNA fragment (one EcoRI site was internal to IR-SnaBI* and the second came from the cloned DNA fragment; the coordinates of this EcoRI site in RA3 are 23480 to 23485). Two expression vectors, the high-copy-number pGBT30 based on the pMB1 replicon and the medium-copy-number pAMB9.37 based on IncA/C replicon, in which open reading frames (ORFs) were cloned under the control of *tac*p and *lacI*^{q}, were used for regulated gene expression. To introduce mutations in the *korB* allele, *in vitro* PCR-based site-directed mutagenesis (Stratagene) was conducted on the pAKB7.7 template with the use of high-fidelity *Pfu* Turbo DNA polymerase. Pairs of complementary primers 6 and 7 (Table 2) were designed to introduce nucleotide substitutions in a particular region of the amplified plasmid DNA, accompanied by introduction of a new restriction site to facilitate screening. Amplified plasmid DNA was treated with DpnI to remove methylated template and then used to transform strain DH5α. Transformants were screened for the presence of the restriction site in the plasmid DNA, and the correctness of mutagenesis was verified by sequencing.

Bacterial transformation. Competent *E. coli* cells were prepared by a standard $CaCl_2$ method (41).

Determination of catechol 2,3-dioxygenase (XylE) activity. The activity of XylE (the product of *xylE*) was assayed spectrophotometrically in cleared supernatants (43) of extracts from actively dividing cells (44). The reaction was initiated by addition of 0.2 mM catechol solution. One unit of catechol 2,3-dioxygenase activity is defined as the amount of enzyme needed to convert 1 μmol of catechol to 2-hydroxymuconic semialdehyde in 1 min. The protein concentration was determined using the Bradford method (45).

Transcriptional silencing assay. Transformants of strain DH5α carrying pGB2 (46) or its derivative pAKB8.5I, pAKB8.81, or pAKB8.10 were transformed with pGBT30 expression vector or its derivative pMMB2.50 *tac*p-*korB* or pJSB5.67 *tac*p-*korB111*. A total of 100 μl of serially diluted transformation mixtures was plated on L-agar plates selective for either the incoming or both the resident and the incoming plasmids. The double-selection plates were also supplemented with 0.5 mM IPTG to induce expression.

Stability assay. Cultures of *E coli* strain DH5α(RA3) with pGBT30 vector as a control or pMMB2.50 *tac*p-*korB* were grown overnight under double selection and then every 20 generations were diluted 10^5-fold into fresh medium supplemented with penicillin and 0.5 mM IPTG. Aliquots of 100 μl of the 10^5-fold diluted cultures were plated on L agar, from which colonies were transferred the next day onto selective plates for estimation of the number of cells retaining plasmid RA3.

Conjugation procedure. Two donor strains, *E. coli* DH5α(pJSB1.24 *tra*)(pAMB9.37 *tac*p) as a control and *E. coli* DH5α(pJSB1.24 *tra*)

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TABLE 1 Plasmids used in this study

Designation	Relevant features	Copy no.	Reference
Plasmids provided by others			
pAKB2.40	pGBT30 *tac*p-*incC*$_{RA3}$	High	18
pAKB3.50	pET28a T7p-*korB*$_{RA3}$	Medium	18
pAKB5.50	pBBR-MCS1 *lacI*^{q}, *tac*p-*korB*$_{RA3}$	Medium	18
pAKB8.5I	pGB2 *parS*$_{RA3}$/*mobC*p$_{RA3}$ (RA3 coordinates 9397–9854)	Medium	18
pAKB8.8	pGB2 IR-SnaBI	Medium	18
pAMB9.37	pBBR-MCS1 *lacI*^{q}, *tac*p, expression vector	Medium	20
pBBR-MCS1	IncA/C, Cm^r, cloning vector	Medium	58
pBGS18	*ori*$_{pMB1}$, Km^r, cloning vector	High	59
pCM132	*ori*$_{pMB1}$, *ori*$_{IncP-1}$, Km^r	High/low	60
pET28a	*ori*$_{pMB1}$, Km^r, T7p, *lacO*, His$_6$ tag, T7 tag	Medium	Novagen
pGB2	*ori*$_{pSC101}$, Sp^r/Sm^r	Medium	44
pGBT30	*ori*$_{pMB1}$, Ap^r, *lacI*^{q}, *tac*p, expression vector	High	42
pJSB1.24	pBGS18 *korC*$_{RA3}$, *tra*$_{RA3}$ (RA3 coordinates 3391–3705 and 9437–33657)	High	52
pJSB4.1	pBBR-MCS1 *lacI*^{q}, *tac*p-*mobC*$_{RA3}$	Medium	19
pJSB4.7	pBBR-MCS1 *lacI*^{q}, *tac*p-*korC*$_{RA3}$	Medium	20
pJSB5.1	pGBT30 *tac*p-*mobC*$_{RA3}$	High	19
pJSB5.7	pGBT30 *tac*p-*korC*$_{RA3}$	High	20
pJSB7.9	pPT01 *mobC*p-*xylE* (RA3 coordinates 9435–9852)	Medium	19
pJSB18	MiniRA3, Tc^r (RA3 coordinates 43327–45909, 1–2300; Tc^r cassette from pKRP12 as HincII fragment)	Low	20
pKGB4	pUT18 with modified MCS^a for ORF cloning as EcoRI-SacI fragments in frame with 5′ end of *cyaT18*	High	35
pKGB5	pKNT25 with modified MCS for ORF cloning as EcoRI-SacI fragments in frame with 5′ end of *cyaT25*	Medium	35
pKNT25	*ori*$_{P15}$, Kn^r, *lac*p-MCS-*cyaAT25*	Medium	37
pKRP12	*ori*$_{pMB1}$, Ap^r, Tc^r cassette	High	61
pKT25	*ori*$_{P15}$, Kn^r, *lac*p-*cyaT25*-MCS	Medium	37
pKT25-zip	pKT25 with leucine zipper of GCN4 in translational fusion with *cyaT25*	Medium	37
pLKB2	pKT25 with modified MCS for ORF cloning as EcoRI-SmaI fragments in frame with 3′ end of *cyaT25*	Medium	62
pLKB4	pUT18C with modified MCS for ORF cloning as EcoRI-SmaI fragments in frame with 3′ end of *cyaT18*	High	62
pMMB2.50	pGBT30 *tac*p-*korB*$_{RA3}$	High	18
pMMB2.60	pGBT30 *tac*p-*orf11*$_{RA3}$	High	18
pMWB6.6	pPT01 *orf02*prev-*xylE*	Medium	20
pPDB11.18	pPT01 *orf02*p-*xylE*	Medium	20
pPT01	*ori*$_{pSC101}$, Kn^r, promoterless *xylE* cassette, promoter-probe vector	Medium	48
pUC18	*ori*$_{pMB1}$, Ap^r, cloning vector	High	63
pUT18	*ori*$_{colE1}$, Ap^r, *lac*p-MCS-*cyaT18*	High	37
pUT18C	*ori*$_{colE1}$, Ap^r, *lac*p-*cyaT18*-MCS	High	37
pUT18C-zip	pUT18C with leucine zipper of GCN4 in translational fusion with *cyaT18*	High	37
Plasmids constructed during this work			
pAKB4.20	pPDB11.18 derivative with IR-SnaBI-1 disrupted by insertion of oligonucleotide 8		
pAKB4.80	pPT01 *orf23*p-*xylE*, 1,403-bp SphI-BamHI fragment from pAKB10.8		
pAKB4.81	pAKB4.80 derivative with IR-SnaBI-3 disrupted by insertion of oligonucleotide 8		
pAKB4.82	pAKB4.81 derivative with deletion of one arm of IR-SnaBI-3 (removal of 100-bp EcoRI fragment)		
pAKB4.90	pPT01 Δ*orf23*p-*xylE*, 1,290-bp SphI-BamHI fragment from pAKB10.9		
pAKB7.7	pBGS18 *korB*$_{RA3}$, EcoRI-SalI fragment from pMMB2.50		
pAKB8.10	pGB2 *orf02*p, HincII-BamHI fragment from pJSB1.70 (RA3 coordinates 2063–2348)		
pAKB8.81	pAKB8.8 derivative with 1.9 kb BamHI fragment (Tc^r cassette from pKRP12) inserted between IR-SnaBI (O$_B$) and *repA* gene of pGB2		
pAKB10.8	pUC18 *orf23p*, 1,403-bp fragment (encompassing *orf25*′, IR-SnaBI-3, *orf24*, and *orf23*p) PCR amplified on RA3 template with primers 1 and 2 and inserted between BamHI and SphI sites (RA3 coordinates 22207–23610)		

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TABLE 1 (Continued)

Designation	Relevant features	Copy no.	Reference
pAKB10.9	pUC18 Δ*orf23*p, 1,290-bp fragment (encompassing *orf25′*, IR-SnaBI-3, and *orf24*) PCR amplified on RA3 template with primers 2 and 3 and inserted between BamHI and SphI sites (RA3 coordinates 22321–23610)		
pAKB15.40	pLKB2 *cyaT25-incC*$_{RA3}$, fragment corresponding to *incC* was PCR amplified on RA3 template with primers 14 and 16 and inserted between EcoRI and SmaI sites		
pAKB15.40N	pKGB5 *incC*$_{RA3}$-*cyaT25*, fragment corresponding to *incC* was PCR amplified on RA3 template with primers 14 and 15 and inserted between EcoRI and SacI sites		
pAKB15.50	pLKB2 *cyaT25-korB*$_{RA3}$, fragment corresponding to *korB* was PCR amplified on RA3 template with primers 11 and 12 and inserted between EcoRI and SmaI sites		
pAKB15.50N	pKGB5 *korB*$_{RA3}$-*cyaT25*, fragment corresponding to *korB* was PCR amplified on RA3 template with primers 11 and 13 and inserted between EcoRI and SacI sites		
pAKB16.40	pLKB4 *cyaT18-incC*$_{RA3}$ with EcoRI-SmaI fragment from pAKB15.40		
pAKB16.40N	pKGB4 *incC*$_{RA3}$-*cyaT18* with EcoRI-SacI fragment from pAKB15.40N		
pAKB16.50	pLKB4 *cyaT18-korB*$_{RA3}$ with EcoRI-SmaI fragment from pAKB15.50		
pAKB16.50N	pKGB4 *korB*$_{RA3}$-*cyaT18* with EcoRI-SacI fragment from pAKB15.50N		
pAKB16.60	pLKB4 *cyaT18-orf11*$_{RA3}$ with EcoRI-SalI fragment from pMMB2.60 with SalI end filled in and cloned between EcoRI and SmaI sites of pLKB4		
pJSB1.67	pAKB7.7 derivative with *korB111* allele (PCR mutagenesis with primers 6 and 7)		
pJSB1.70	pBGS18 *orf02*p, PCR fragment amplified on RA3 template with primers 4 and 5, inserted between EcoRI and SacI sites (RA3 coordinates 2064–2240)		
pJSB5.67	pGBT30 *tac*p-*korB111*, EcoRI-SalI fragment from pJSB1.67		
pJSB6.67	pET28a T7p-*korB111*, EcoRI-SalI fragment from pJSB5.67		
pJSB8.67	pLKB4 *cyaT18-korB111*, EcoRI-SalI fragment from pJSB1.67 with SalI end filled in and cloned between EcoRI and SmaI sites of pLKB4		

[a] MCS, multiple-cloning site.

(pAKB5.50 *tac*p-*korB*), were grown overnight with or without 0.5 mM IPTG. The DH5α Rif^r strain was used as the recipient. Aliquots of 100 μl of overnight cultures of the donor and recipient strains were mixed (1:1) and incubated on L-agar plates for 2 h at 37°C. Cells were scraped and suspended in 2 ml of L broth, and 10-μl aliquots of serial 10-fold dilutions were spotted onto L-agar plates with 100 μg ml^{-1} rifampin and 50 μg ml^{-1} kanamycin to estimate the number of transconjugants. In parallel, 100 μl of the donor strain overnight culture was incubated on an L-agar plate for 2 h at 37°C and treated as described above to estimate the number of donor cells in the conjugation mixture. The

TABLE 2 Oligonucleotides used in this study

No.	Oligonucleotide	Sequence[a]
1	orf23Bam	5′GCGGATCCTATCCTGTGCCTCATCAAAG3′
2	orf23Sph	5′GCGCATGCAACAAGATGACCGCGATTTC3′
3	23DprBam	5′GCGGATCCGTTACATGCTTGCCACGGAG3′
4	Orf02pEco	5′CGGAATTCGGTGGCCCATTTCGTACGTA3′
5	Orf02pSac	5′CGGAGCTCTCGGAGTTCTCCGGTATTGA3′
6	A111Tg	5′GGCGTTACCTGaccGCACAGGAAGCCGGCCTTGATACCG3′
7	A111Td	5′CGGTATCAAGGCCGGCTTCCTGTGCggtCAGGTAACGCC3′
8	oligo EcoRI	5′CATGAATTCATG3′
9	Cy5-FOOT1	5′TGATCATATGCCCGCTGTAG3′
10	Cy5-FOOT2	5′AATTTTAGCACAAGCGGCGG3′
11	korB1	5′CGGAATTCATGAGTGGTAAAGGGGCAGA3′
12	korB3Sma	5′CGCCCGGGTCAGCCCTCGATAACAGCAA3′
13	korBSac	5′CGGAGCTCGGCCCTCGATAACAGCAACAA3′
14	incC1	5′CGGAATTC**ATG**AAAATTGTTACTGTATC3′
15	incC3Sac	5′CGGAGCTCGTTCAGCCACCCCCATTTTTTC3
16	incCSma	5′CGCCCGGGTTTACCACTCATTCAGCCAC3′
17	Cy5-CM132R	5′CGTCAGTAACTTCCACAGTAG3′
18	CM132F8	5′CATCAGGAAAGCAGGGAATTC3′

[a] Restriction enzyme recognition sites are underlined, and start codons are in bold. Substituted nucleotides in primers for PCR mutagenesis are indicated by lowercase letters.

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transfer frequency was calculated as the number of transconjugants per donor cell.

BACTH system. The homo- and hetero-oligomerization of KorB, KorBA111T, IncC, and Orf11 *in vivo* was analyzed using the bacterial adenylate cyclase two-hybrid (BACTH) system in *E. coli* (39). The KorB and IncC proteins were translationally fused via either their N termini (vectors pLKB4 and pLKB2) or C termini (vectors pKGB4 and pKGB5) to CyaT18 or CyaT25 fragments. KorBA111T and Orf11 were fused with the adenylate cyclase fragments via their N termini only. The adenylate cyclase-deficient (*cya*) *E. coli* strain BTH101 was cotransformed with appropriate pairs of BACTH plasmids and plated on MacConkey medium supplemented with 1% (wt/vol) maltose, 0.1 mM IPTG, and selective antibiotics. The plates were incubated for 48 h at 27°C. The appearance of pink colonies indicated the ability to ferment maltose due to the CyaA reconstitution through interactions between the fused polypeptides. The interactions were verified independently by determination of β-galactosidase activity in liquid cultures (47). The double transformants of strain BTH101 were grown overnight at 27°C in L broth with addition of penicillin, kanamycin, and 0.15 mM IPTG, and 0.2 ml of culture was taken for the assay. One unit of β-galactosidase is defined as the amount of enzyme needed to convert 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to *o*-nitrophenol and D-galactose in 1 min under standard conditions.

Protein purification. *E. coli* BL21(λDE3) was transformed with pET28a derivative pAKB3.50 T7p-*korB* or pJSB6.67 T7p-*korB111*. N-terminally His-tagged proteins were overproduced by culturing on L broth with 0.5 mM IPTG as described previously (18), and purification was performed by affinity chromatography on Ni-TED (Macherey-Nagel) columns according to the manufacturer's instructions.

SDS-PAGE analysis of KorC and MobC overproduction. To verify KorC and MobC overproduction in cells used for "roadblock" experiments, *E. coli* strains DH5α(pAKB8.10 *orf02*p) (pAMB9.37 *lacI*^{q}, *tac*p), DH5α(pAKB8.10 *orf02*p)(pJSB4.7 *tac*p-*korC*), and DH5α(pAKB8.5I *parS*/*mobC*p)(pJSB4.1 *tac*p-*mobC*) were grown to an optical density at 600 nm (OD_{600}) of 0.6 on L broth supplemented with streptomycin (20 μg ml^{-1}) and chloramphenicol (10 μg ml^{-1}) and with 0.5 mM IPTG to induce *tac*p. Part of the cultures was used to prepare competent cells for transformation with pMMB2.50 *tac*p-*korB* or pGBT30 vector. In parallel, soluble fractions of 2×10^7 sonicated cells were applied to a 16% denaturing Mini-Protean polyacrylamide gel (Bio-Rad) and separated by SDS-PAGE (41).

SEC-MALS analysis. Half-milliliter aliquots of purified His_6-KorB (0.40 mg ml^{-1}) or His_6-KorBA111T (0.38 mg ml^{-1}) were loaded on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with size exclusion chromatography (SEC) buffer (50 mM sodium phosphate [pH 8.0], 100 mM NaCl) with three in-line detectors: UV (Äkta Purifier; GE Healthcare), multiangle light scattering (MALS) (Dawn Heleos-II; Wyatt Technology), and differential refractometer (Optilab T-rEX; Wyatt Technology). Data processing and molecular mass calculations were performed using ASTRA software (Wyatt Technology). The molecular mass of the His_6-KorB monomer was estimated at 50 kDa by mass spectrometry (Synapt G2 mass spectrometer [Waters], nanoAcquity liquid chromatography system [Waters], Trap Column Acquity ultraperformance liquid chromatography PrST C4 VanGuard [precolumn 300A; 1.7 μm, 2.1 mm, 5 mm]).

Analysis of protein-DNA interactions by electrophoretic mobility shift assay (EMSA). A DNA fragment from RA3 coordinate 9498 to 9804 was amplified on RA3 DNA with Cy5-labeled primers 9 and 10 (Table 2). To obtain a Cy5-labeled nonspecific DNA fragment of similar size (control), PCR was performed on pCM132 (Table 1) DNA as a template with the use of primers 17 and 18 (Table 2). The PCR products were excised from the agarose gel and purified with the Gel-Out kit (A&A Biotechnology). The concentration of the isolated DNA was determined with the NanoDrop 2000 instrument. Portions (20 ng) of the Cy5-labeled DNA fragments were incubated with increasing amounts of KorB or KorBA111T (20, 40, 60, and 70 pmol) and 18 pmol of nonspecific double-stranded oligonucleotides as a competitor DNA in 20 μl of binding buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 50 mM NaCl, 0.2 mg ml^{-1} bovine serum albumin [BSA]) at 37°C for 15 min. The samples were then separated on 5% native polyacrylamide gels in 1× TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) at room temperature. The DNA was visualized using a FluorChemQ MultiImageIII ChemiImager, and the images were captured using Alpha View software (Alpha Innotech). Quantitative analysis of representative results was done with ImageQuant software.

RESULTS

Partitioning protein KorB (RA3) is a transcriptional repressor. KorB of RA3 has been shown earlier to specifically bind the 16-bp palindromic sequence designated IR-SnaBI (TTTCGTACGTAC GAAA), an essential motif in the *parS* region at the border of the stability and conjugative transfer modules (18). Inspection of the RA3 plasmid nucleotide sequence revealed the presence of identical 16-bp palindromes in two other locations (Fig. 1 and 2A). The first one, IR-SnaBI-1 (RA3 coordinates 2075 to 2090) is located in the divergent *orf02*p/*orf02*prev promoter region (20) between the replication module and *orf02*, the first ORF of unknown function in the maintenance region (10). The previously described IR-SnaBI-2 palindrome (RA3 coordinates 9707 to 9722), which is integral to *parS*, is adjacent to the promoter of the *mobC-nic* operon (conjugative module) (19) and close to the nick site in the *oriT* region. The third conserved motif, IR-SnaBI-3 (RA3 coordinates 23579 to 23594), localizes within *orf25*, of unknown function, in the conjugative transfer module (18).

The first two KorB-binding sites are located in previously identified promoter regions, while IR-SnaBI-3 sits in the center of a long multicistronic conjugative transfer operon starting at *orf33*p. Putative transcription initiation signals lie 1.3 kb downstream from this motif. This hypothetical internal promoter precedes *orf23* and is located in the short intergenic region of 112 bp between *orf24* and *orf23* (Fig. 2A), with the −35 motif TTGACT (RA3 coordinates 22264 to 22269) separated by 16 bp from the −10 motif TTTGAT (RA3 coordinates 22243 to 22248). The putative *orf23* promoter region was PCR amplified and cloned into the promoter-probe vector pPT01 (48) as a 1,403-bp DNA fragment spanning the IR-SnaBI-3 motif, *orf24*, and the intergenic region encompassing putative *orf23*p to obtain pAKB4.80. An additional construct bearing a shorter DNA fragment of 1,290 bp lacking the putative *orf23*p (pAKB4.90) was also constructed (Fig. 2A). The promoter activity of those constructs together with the three transcriptional fusions in pPDB11.18 (*orf02*p-*xylE*), pMWB6.6 (*orf02*prev-*xylE*), and pJSB7.9 (*mobC*p-*xylE*) (19) was studied in exponentially growing cultures. The three previously identified promoters showed fairly diverse activity (*orf02*p, 5.5 U of XylE activity; *orf02*prev, 1.6 U; and *mobC*p, 0.5 U), and the putative *orf23* promoter showed an intermediate activity of 1.8 U. No transcription was detected from the pAKB4.90 construct, confirming our prediction that the 112-bp region immediately upstream of *orf23* is the promoter region (Fig. 2B).

Since the distances of the three KorB-binding sites from the −35 promoter motifs are remarkably divergent (46, 85, and 1,317 bp upstream of *mobC*p, *orf02*p, and *orf23*p, respectively, and 150 bp downstream of *orf02*prev), we asked whether KorB could function as a repressor for all these promoters.

The role of KorB as a repressor was tested with the use of a two-plasmid regulatory system (44) comprising expression con-

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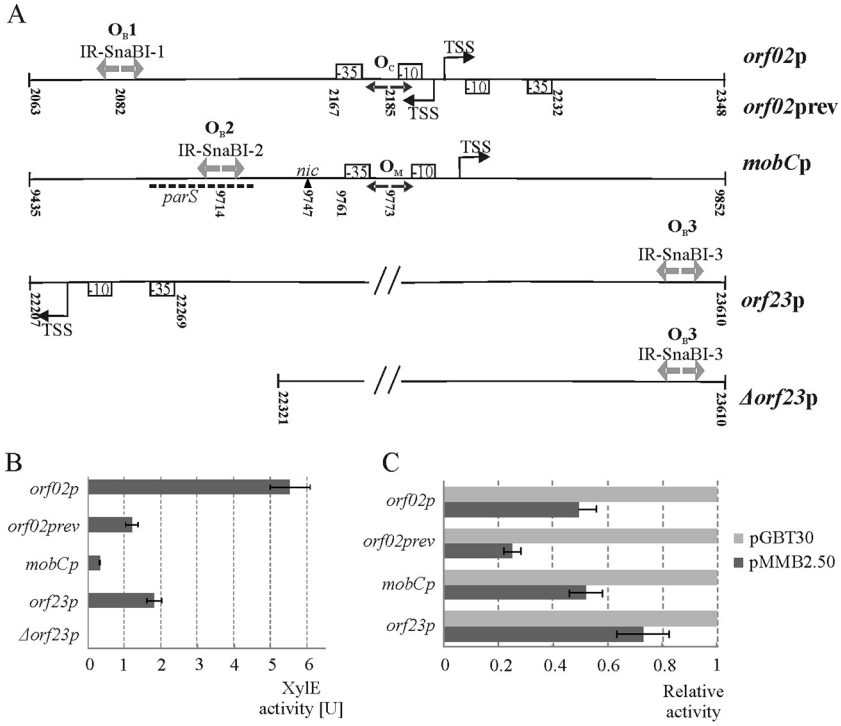

FIG 2 Regulation of RA3 promoters by KorB. (A) Schematic presentation of promoter regions potentially regulated by KorB. The −35 and −10 motifs are boxed. Predicted transcription start sites (TSS) are indicated by thin black arrows. KorB operators (IR-SnaBI motifs) and other defined binding sites, O_M for MobC and O_C for KorC, are also marked. The RA3 *parS* sequence as defined previously (18) is indicated with a dashed line, while the nick site (*nic*) in the *oriT* (10) is indicated by a triangle. The scheme is not drawn to scale, but RA3 nucleotide sequence coordinates are given at both ends of fragments, at the nick site, at the start of −35 motifs, and in the centers of palindromic regulatory sequences. (B) Transcriptional activities of RA3 DNA fragments cloned into promoter-probe vector. Expression of the reporter gene *xylE* was analyzed by measuring the catechol 2,3-oxygenase activity in cell extracts from exponentially growing cultures of DH5α(pPDB11.18) (*orf02*p-*xylE*), DH5α(pMWB6.6) (*orf02*prev-*xylE*), DH5α(pJSB7.9) (*mobC*p-*xylE*), and DH5α(pAKB4.90) (*orf23*p-*xylE*) transformants. Mean values with standard deviations for at least three assays are shown. (C) Activities of promoters in the presence of KorB. DH5α strains with promoter-probe vectors as in panel B, harboring DNA fragments as indicated on the left, were transformed with empty expression vector pGBT30 (control) or with its derivative pMMB2.50 *tac*p-*korB*. XylE activity was assayed in extracts from exponentially growing cultures and is shown as the proportion between control (taken as 100%, gray bars) and *korB*-expressing (black bars) strains. Mean values with standard deviations for at least three assays are shown. A parametric *t* test performed for the data before normalization showed that all the differences were statistically significant, with *P* values in a range from 0.00001 to 0.03.

structs containing the *xylE* reporter gene under the control of the promoter of interest with its adjacent KorB-binding site in a native arrangement and a plasmid encoding the KorB protein or empty vector. We used previously constructed plasmids pPDB11.18 (*orf02*p-*xylE*) and pMWB6.6 (*orf02*prev-*xylE*) (20) for IR-SnaBI-1, pJSB7.9 containing *mobC*p-*xylE* (19) for IR-SnaBI-2, and pAKB4.80 (*orf23*p-*xylE*) for the third KorB-binding motif. *E. coli* DH5α strains carrying any of these plasmids were transformed with the empty expression plasmid pGBT30 (44) or its derivative pMMB2.50 with *korB* under the control of *tac*p. Catechol 2,3-oxygenase activity was assayed in extracts from the double transformants grown without IPTG (leaky *tac*p provides a low level of expression of *korB* gene sufficient for repression and not slowing the growth, the effect observed with KorB overproduction). A pairwise comparison of XylE activity in the transformants with pMMB2.50 and with pGBT30 showed that KorB reduced the activity of all the promoters assayed, but to different extents: almost 5-fold in the case of *orf02*prev, 2-fold for *mobC*p and *orf02*p, and by only 30% for *orf23*p (Fig. 2C). When KorB was overproduced, the repression index increased to 8-fold for *orf02*prev, 3- to 4-fold for *orf02*p and *mobC*p, and 2-fold for *orf23*p.

To verify the role of the IR-SnaBI motif in KorB action, the IR-SnaBI sequences adjacent to *orf02*p and *orf23*p were disrupted by insertion of a 12-bp oligonucleotide to give pAKB4.20 and pAKB4.81, respectively. The comparison of XylE activities in *E. coli* transformants with pPDB11.18 (*orf02*p-*xylE*) versus pAKB4.20 (*orf02*p IR-SnaBI*-*xylE*) and pAKB4.80 (*orf23*p-*xylE*) versus pAKB4.81 (*orf23*p IR-SnaBI*-*xylE*) demonstrated that insertions did not affect transcriptional activities of the modified promoter regions in the absence of KorB. The basic level of KorB in the DH5α(pAKB4.20 *orf02*p IR-SnaBI*-*xylE*)(pMMB2.50 *tac*p-*korB*) and DH5α(pAKB4.81 *orf23*p IR-SnaBI*-*xylE*)(pMMB2.50 *tac*p-*korB*) double transformant cells did not lead to repression (Fig. 3A and B), in contrast to 50% and 30% KorB repression observed for the promoters with unmodified operators in *orf02*p-*xylE* and *orf23*p-*xylE* (Fig. 2C). IPTG induction of *tac*p-*korB* in the double transformants with disrupted KorB operators (O_Bs) decreased XylE activities by 20% (Fig. 3A and B), a much milder repression effect than observed for the unmodified promoters under the same conditions (3-fold repression and 2-fold repression of *orf02*p and *orf23*p, respectively). When instead of disrupting the motif by insertion we destroyed the IR-SnaBI-3 palindrome by removing one of its arms (pAKB4.82), the repressibility of the *orf23* promoter by KorB was completely lost even in the presence of excess KorB (Fig. 3). These results confirmed that the repression by KorB was dependent on its binding to the IR-SnaBI motif

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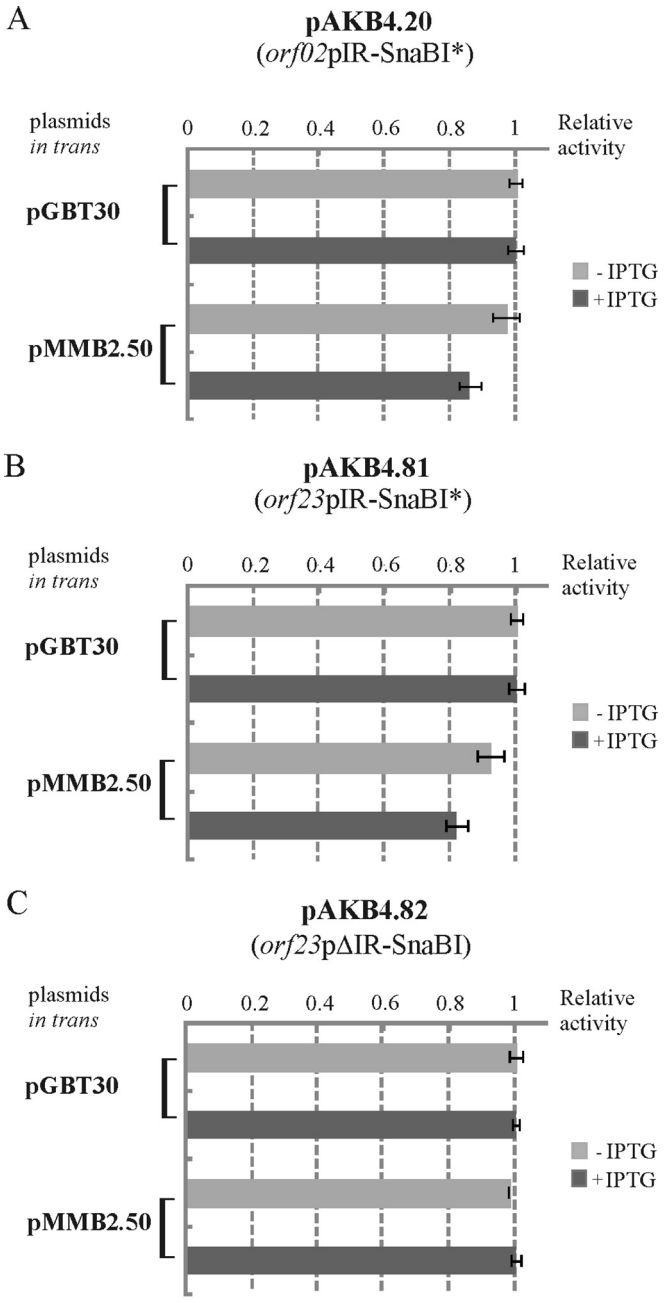

FIG 3 Regulatory effect of $KorB_{RA3}$ on *orf02*p and *orf23*p with modified IR-SnaBI motifs. (A) Activity of *orf02*p IR-SnaBI* (disrupted by a 12-bp insertion) in the presence of KorB in *trans*. Promoter activity was measured in *E. coli* DH5α(pAKB4.20 *orf02*p IR-SnaBI*-*xylE*)(pMMB2.50 *tac*p-*korB*) and *E. coli* DH5α(pAKB4.20 *orf02*p IR-SnaBI*-*xylE*)(pGBT30) as a control, grown in the presence of 0.5 mM IPTG (dark gray bars) or without IPTG added (light gray bars). The results are presented relative to the control; mean values with standard deviations for at least three assays are shown. A parametric *t* test performed for the data obtained from IPTG-induced variants yielded a *P* value of 0.01. (B) Activity of *orf23*p IR-SnaBI* (disrupted by a 12-bp insertion) in the presence of KorB in *trans*. Promoter activity was measured in *E. coli* DH5α(pAKB4.81 *orf23*p IR-SnaBI*-*xylE*)(pMMB2.50 *tac*p-*korB*) and *E. coli* DH5α(pAKB4.81 *orf23*p IR-SnaBI*-*xylE*)(pGBT30) as a control, grown in the presence of 0.5 mM IPTG (dark gray bars) or without IPTG added (light gray bars). The results are shown relative to the control; mean values with standard deviations for at least three assays are shown. A parametric *t* test performed for the data obtained from IPTG-induced variants yielded a *P* value of 0.01. (C) Activity of *orf23*pΔIR-SnaBI with deletion of one arm of IR-SnaBI in the presence of KorB in *trans*. Promoter activity was measured in *E. coli* DH5α(pAKB4.82 *orf23*pΔIR-SnaBI-*xylE*)(pMMB2.50 *tac*p-*korB*) and *E. coli* DH5α(pAKB4.82 *orf23*pΔIR-SnaBI-*xylE*)(pGBT30) as a control, grown in the presence of 0.5 mM IPTG (dark gray bars) or without IPTG added (light gray bars). The results are shown relative to the control; mean values with standard deviations for at least three assays are shown.

in vivo and prompted us to designate the IR-SnaBI sequences KorB operators (O_Bs). The above results together with our previous studies indicating the lack of KorB effect on *korA*p expression (18) clearly show that KorB repression of promoter activity is utterly dependent on the presence of its operator in *cis*.

IncC and Orf11 proteins relieve KorB-dependent repression. Earlier studies on the KorB homolog from the IncP-1 plasmid RK2 have demonstrated an enhancement of KorB DNA binding *in vitro* and of KorB-dependent repression *in vivo* by the cognate IncC-1, the longer version of IncC and the KorB partner protein in partitioning (36, 49). In RA3, the IncC protein (Walker-type ATPase) corresponds to the short IncC-2 variant from RK2, and a third component, Orf11, is required for full activity of the partition system (18). To answer the question whether IncC or Orf11 modulates repression by $KorB_{RA3}$, we tested the activities of two promoters, *mobC*p and *orf02*p, in the presence of KorB and a second protein, IncC or Orf11, produced in *trans*. The *E. coli* DH5α(pPDB11.18 *orf02*p-*xylE*) and DH5α(pJSB7.9 *mobC*p-*xylE*) strains were transformed with pairwise combinations of compatible expression plasmids, i.e., pAKB5.50 *tac*p-*korB* or empty vector pAMB9.37 (pBBRMCS1 derivative) and pMMB2.60 *tac*p-*orf11*, pAKB2.40 *tac*p-*incC*, or empty pGBT30. Promoter activities were measured in cell extracts of strains containing a promoter-probe construct and a pair of expression plasmids without induction of *tac*p. The results (Fig. 4A and B) are shown relative to the XylE activities obtained for DH5α with the respective promoter-probe vector and two empty expression vectors, pGBT30 and pAMB9.37. The IncC and Orf11 proteins did not affect the activity of *orf02*p or *mobC*p in the absence of KorB, but both slightly alleviated the repression of either promoter by $KorB_{RA3}$ (Fig. 4A and B). Notably, such interference with KorB repression was seen not only in the case of *mobC*p, adjacent to the *parS* region, but also for *orf02*p, which is not engaged in partitioning (18). This suggested direct protein-protein interactions, which were further studied using a two-hybrid system.

KorB homo- and heterodimerization analyzed with the BACTH system. *incC*, *korB*, and *orf11* coding sequences were each cloned into four bacterial adenylate cyclase two-hybrid (BACTH) vectors (39, 50) to link the respective proteins via either the N or the C terminus with CyaA fragment T18 or T25. The *E. coli* BTH101 *cyaA* strain was cotransformed with appropriate combinations of two BACTH vectors to test the proteins' interaction, monitored by CyaA reconstitution. Control strains contained at least one empty BACTH vector. The transformants were analyzed on MacConkey agar plates with maltose as a carbon source and in liquid cultures for β-galactosidase activity. Both homodimerization of KorB and IncC and KorB-IncC heterodimerization were observed. The strongest KorB-KorB interaction (Table 3) occurred when KorB was fused via its N terminus with the CyaA fragments and had the C-terminal domain free (1,200 U of β-galactosidase activity); the interaction was 2-fold weaker when one or both KorB fusions were C terminal. Strong IncC-IncC

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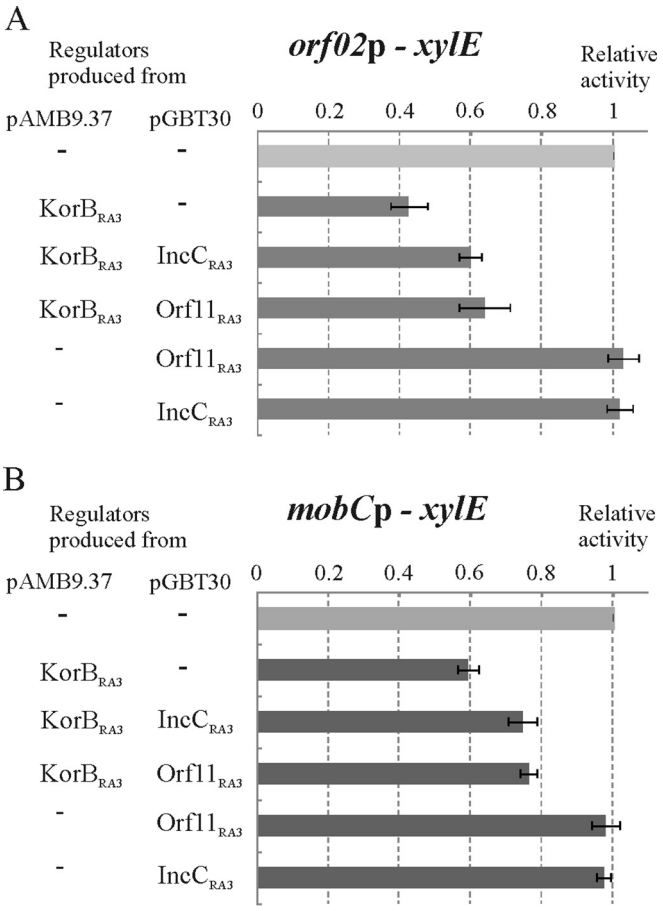

FIG 4 Role of IncC$_{RA3}$ and Orf11$_{RA3}$ proteins in KorB$_{RA3}$-dependent repression. (A) *E. coli* DH5α(pPDB11.18 *orf02*p-*xylE*) was transformed with two compatible expression vectors, pAMB9.37 and pGBT30, or their derivatives overproducing KorB (pAKB5.50), IncC (pAKB2.40), or Orf11 (pMMB2.60). *orf02*p activity measured by the XylE level in the presence of one or two overexpressed proteins produced in *trans* from the expression vectors is shown relative to the activity in DH5α(pPDB11.18)(pAMB9.37)(pGBT30). Mean values with standard deviations for at least three experiments are presented. The data obtained for KorB versus KorB/IncC or KorB/Orf11 were analyzed statistically by a parametric *t* test, yielding *P* values of 0.00009 and 0.0024, respectively. (B) *E. coli* DH5α(pJSB7.9 *mobC*p-*xylE*) was transformed with two compatible expression vectors, pAMB9.37 and pGBT30, or their derivatives overproducing KorB (pAKB5.50), IncC (pAKB2.40), or Orf11 (pMMB2.60). *mobC*p activity measured by XylE level in the presence of one or two overexpressed proteins produced in *trans* from the expression vectors is shown relative to the activity in the control strain DH5α (pJSB7.9)(pAMB9.37)(pGBT30). Mean values with standard deviations for at least three experiments are presented. The data obtained for KorB versus KorB/IncC or KorB/Orf11 were analyzed statistically by a parametric *t* test, yielding *P* values of 0.0009 and 0.0026, respectively.

dimerization (1,500 U of β-galactosidase) was found only for IncC fused via its C terminus with the CyaA fragments. A KorB-IncC interaction was observed only for N-terminally fused KorB, confirming the critical role of a free C-terminal domain for both homo- and heterodimerization of KorB. The interaction was somewhat stronger with C-terminally fused IncC (800 U) than with the N-terminally fused one (550 U); thus, the N terminus of IncC not only is critical for IncC homodimerization but also affects the interaction with KorB. For the Orf11 protein, no interactions with KorB or IncC were detected (data not shown).

KorB action at a distance requires KorB polymerization on DNA. Since the regulation of gene expression by KorB$_{RA3}$ occurs at various distances (46 to 1,317 bp) and positions (upstream/downstream) from the promoter (Fig. 2A), the mechanism of KorB action, similarly to that for other ParB family members (2, 18, 26, 27, 30, 32), could involve spreading on DNA and thereby limiting RNA polymerase (RNAP) access to the promoters (51). Previously we have demonstrated KorB-mediated silencing (18) using either an intact *parS* region (pAKB8.5I) or an oligonucleotide with a KorB binding site (pAKB8.8) cloned close to *repA*p in the test plasmid pGB2 (46). We extended those studies to check whether similar KorB action takes place on another O_B and whether KorB silencing is effective at a long distance. The DNA fragment of 176 bp harboring *orf02*p with O_B1 was cloned into pGB2 (pAKB8.10), whereas a 1.9-kb fragment, originating from pKRP12 (Table 1), was inserted between O_B and *repA*p in the previously tested pAKB8.8 to create pAKB8.81 (O_B+1.9 kb). For the silencing test, DH5α strains carrying empty pGB2, pAKB8.10, or pAKB8.81 were transformed with the expression vector pMMB2.50 *tac*p-*korB* or an empty expression vector (pGBT30) and plated on L agar with penicillin (Pn), selecting for incoming plasmid only, or plated on L agar with Pn and streptomycin (Sm), selecting for both resident and incoming plasmids or for both plasmids under conditions of KorB overproduction (Pn, Sm, and 0.5 mM IPTG). Overproduction of KorB had no effect on the number of transformants of DH5α(pGB2) growing on the different selective media (data not shown), but it drastically (10^4-fold) decreased the number of transformants of DH5α (pAKB8.10) on double-selection plates with IPTG (Fig. 5A). A similar profound drop in the number of transformants on double-selection plates with IPTG was observed when the 1.9-kb insertion (O_B+1.9 kb) separated O_B from the *repA* gene (pAKB8.81), demonstrating the potential for KorB to spread on DNA at long distances (Fig. 5A). No such effect was observed for the control strains transformed with empty pGBT30 (Fig. 5A).

A KorB variant defective in polymerization on DNA but not in DNA binding has no repressor activity. An earlier analysis of

TABLE 3 Analysis of KorB$_{RA3}$ and IncC$_{RA3}$ interactions using the bacterial two-hybrid system[a]

	β-Galactosidase activity (U)[b] with CyaT18 linked to:				
CyaT25 link	pKLB4 empty vector	pAKB16.50 *-KorB	pAKB16.50N KorB-*	pAKB16.40 *-IncC	pAKB16.40N IncC-*
pKLB2 empty vector	111 ± 40	132 ± 40	122 ± 50	150 ± 44	135 ± 33
pAKB15.50 *-KorB	160 ± 53	1,200 ± 60	ND	ND	ND
pAKB15.50N KorB-*	155 ± 47	520 ± 34	600 ± 58	ND	ND
pAKB15.40 *-IncC	158 ± 33	550 ± 37	ND	190 ± 60	500 ± 42
pAKB15.40N IncC-*	130 ± 30	800 ± 60	185 ± 65	ND	1,500 ± 55

[a] The asterisk indicates the CyaA fragment linked to the N terminus (*-) or C terminus (-*) of the analyzed protein.
[b] β-Galactosidase activity was assayed in overnight liquid cultures of double transformants. Results (in units as defined in Materials and Methods) are averages of at least three replicates with standard deviations. ND, not determined.

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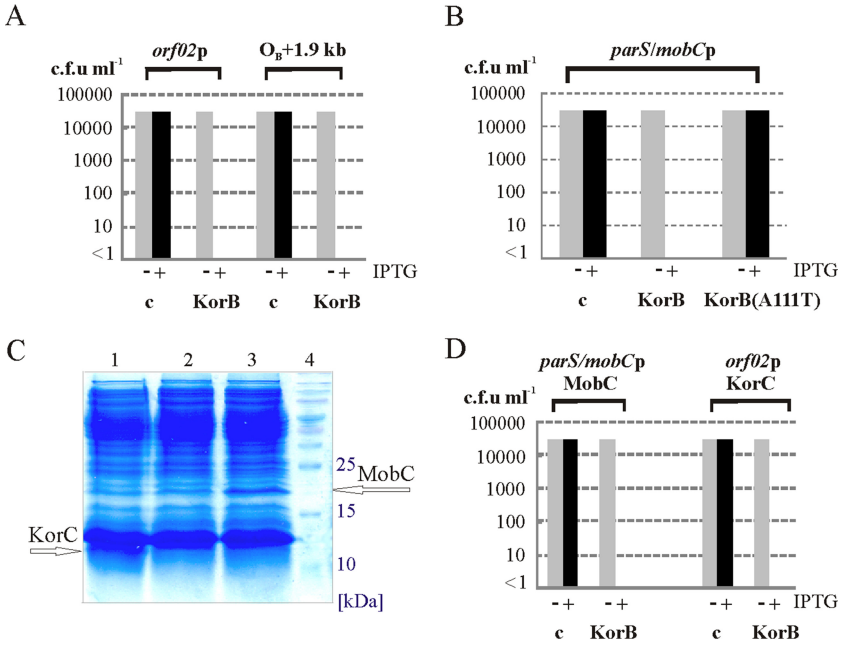

FIG 5 Analysis of $KorB_{RA3}$ spreading. (A) Transcriptional silencing by WT $KorB_{RA3}$. *E. coli* DH5α strains carrying pGB2 derivative pAKB8.5I (*parS*/*mobC*p), pAKB8.10 (*orf02*p), or pAKB8.81 (O_B+1.9 kb) were transformed with empty vector pGBT30 as a control (c) or its derivative pMMB2.50 *tac*p-*korB* (KorB). To estimate the number of transformants, 0.1 ml of undiluted as well as serially diluted transformation mixture was grown on double-selection plates without (gray bars, −) or with (black bars, +) 0.5 mM IPTG. The DNA fragments cloned in pGB2 derivatives present in the analyzed strains are indicated above the graph. Mean numbers of transformants from at least three experiments are shown on a semilogarithmic scale; <1 indicates that no transformants were obtained on double-selection plates with 0.5 mM IPTG after plating 0.8 ml of the undiluted transformation mixture. (B) Transcriptional silencing by the KorBA111T variant. *E. coli* DH5α(pAKB8.5I *parS*/*mobC*p) was transformed with pGBT30 (c) or pMMM2.50 *tac*p-*korB* (KorB) as negative and positive controls or with pJSB5.67 *tac*p-*korB111*. Transformation mixtures were plated on double-selection plates without (gray bars, −) or with (black bars, +) 0.5 mM IPTG. The DNA fragment cloned in the pGB2 derivative present in the analyzed strain is indicated above the graph. Mean numbers of transformants from at least three experiments are shown on a semilogarithmic scale; <1 indicates that no transformants were obtained on double-selection plates with 0.5 mM IPTG after plating 0.8 ml of the transformation mixture. (C) Overproduction of repressor proteins in the competent cells. SDS-PAGE analysis of soluble fractions of extracts from 0.5 mM IPTG-induced cultures of *E. coli* DH5α(pAKB8.10 *orf02*p)(pJSB4.7 *tac*p-*korC*) (lane 1), *E. coli* DH5α(pAKB8.10 *orf02*p)(pAMB9.37 *lacI*^{q}, *tac*p) (lane 2), and *E. coli* DH5α(pAKB8.5I *parS*/*mobC*p)(pJSB4.1 *tac*p-*mobC*) (lane 3). Proteins were stained with Coomassie brilliant blue, and bands corresponding to overproduced proteins, MobC (20 kDa) and KorC (10 kDa migrating slightly slower), are indicated with arrows. Lane 4 contains the protein markers (GPB 260-kDa prestained multicolor protein marker). (D) Transcriptional silencing by KorB in the presence of potential "roadblocks." Competent cells of *E. coli* DH5α(pAKB8.10 *orf02*p)(pJSB4.7 *tac*p-*korC*) and *E. coli* DH5α(pAKB8.5I *parS*/*mobC*p)(pJSB4.1 *tac*p-*mobC*) were prepared from cultures grown in the presence of 0.5 mM IPTG to overproduce KorC and MobC, respectively, as shown in panel C. The cells were then transformed with pGBT30 (c) or pMMB2.50 (*tac*p-*korB*) (KorB), and dilutions of transformation mixtures were plated on triple-selection plates without (gray bars, −) or with (black bars, +) 0.5 mM IPTG. The DNA fragments cloned in pGB2 derivatives and overproduced proteins in the analyzed strains are indicated above the graph. Mean numbers of transformants from at least three experiments are shown on a semilogarithmic scale; <1 indicates that no transformants were obtained on triple-selection plates with 0.5 mM IPTG after plating 0.8 ml of the transformation mixture.

the ParB protein from *P. aeruginosa*, a close homolog of plasmidic KorBs, has defined the protein interface responsible for polymerization on DNA (28). We took advantage of this information to study the impact of polymerization on the repressor action of KorB. Site-directed mutagenesis was used to introduce the amino acid substitution A111T in $KorB_{RA3}$, corresponding to the *P. aeruginosa* ParB polymerization mutation in the conserved box II region (28). The mutant allele was cloned into an expression vector to create pJSB5.67 *tac*p-*korB111*. The KorBA111T protein was tested as described above for the ability to spread on DNA *in vivo* and silence adjacent genes in a pGB2 test vector derivative, pAKB8.5I. KorBA111T had no effect on pAKB8.5I maintenance, indicating a lack of silencing activity (Fig. 5B).

KorBA111T demonstrated dimerization properties *in vitro* and *in vivo* that were similar to those of wild-type (WT) KorB. When examined *in vitro* with the SEC-MALS technique, both variants existed as dimers in solution, since their apparent molecular mass was 100 kDa (Fig. 6A), whereas the molecular mass of the monomer as estimated by mass spectrometry is 50 kDa. The mutant protein KorBA111T also formed dimers with WT KorB in the BACTH system, producing approximately 800 U of β-galactosidase activity (Fig. 6B).

To verify whether the silencing defect of KorBA111T results from its inability to polymerize on DNA or to bind specific *parS* DNA, an EMSA analysis was undertaken with a Cy5-labeled DNA fragment with O_B2 (RA3 coordinates 9498 to 9793) and purified His_6-tagged KorBA111T or WT KorB protein. As a control, a nonspecific DNA fragment was used. DNA-protein complexes were separated by PAGE. No binding to the control fragment was detected in the range of protein concentrations applied (Fig. 6C, upper panel). No significant differences in binding affinity toward the Cy5-labeled fragment with O_B2 were observed between WT KorB and KorBA111T (Fig. 6C, middle and bottom panels). These results clearly indicate that although the mutant KorBA111T is deficient in silencing activity, it retains the DNA-binding ability.

Finally, the repressor activity of KorBA111T was analyzed *in*

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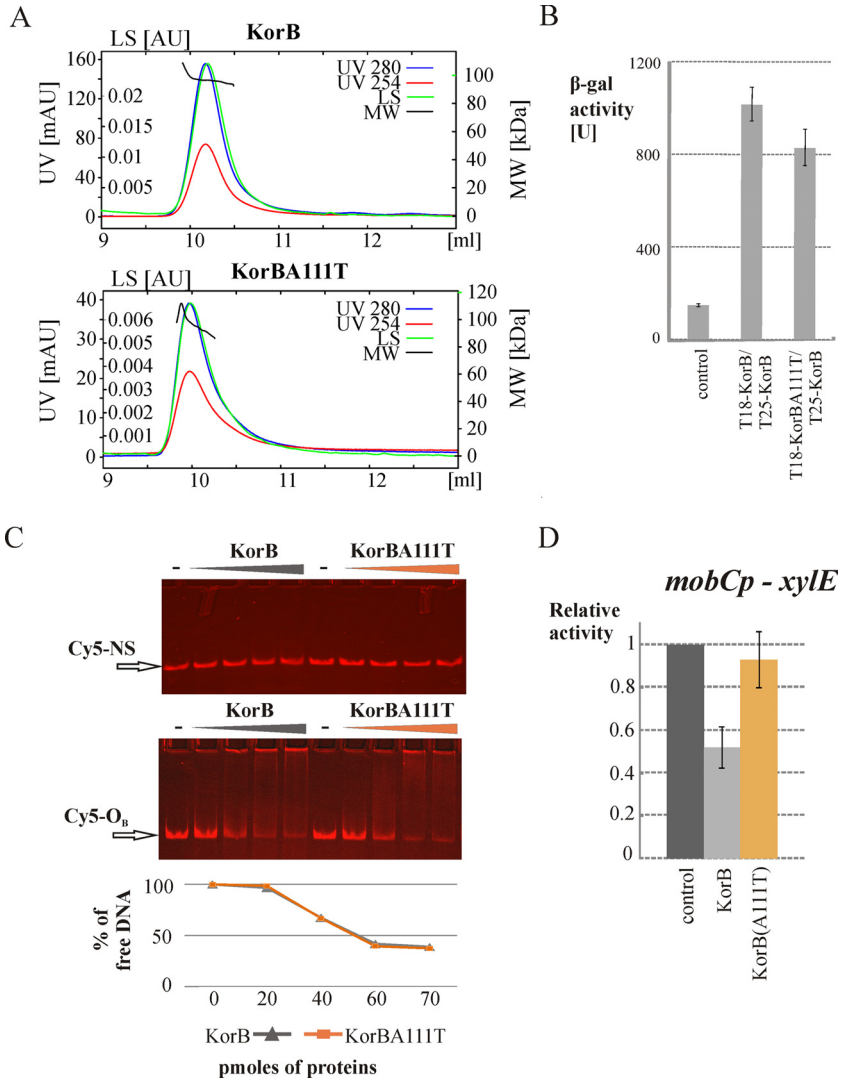

FIG 6 Properties of the spreading-deficient variant KorBA111T. (A) Oligomeric state of WT KorB$_{RA3}$ and mutant KorBA111T analyzed by SEC-MALS. The diagram presents elution profiles at 280 nm of WT KorB (upper panel) and mutant KorBA111T (lower panel). Both proteins elute at an apparent molecular mass of 100 kDa. The molecular mass of His$_6$-KorB monomer is 50 kDa as estimated by mass spectrometry. (B) Analysis of *in vivo* interaction of KorBA111T with WT KorB in the BACTH system. *E. coli* BTH101 *cyaA* was cotransformed with pJSB8.67 *cyaT18-korB111* and pKT25 (empty vector) as a control or pAKB15.50 *cyaT25-korB*. The results obtained for self-interactions of WT KorB (pAKB15.50 and pAKB16.50 *cyaT18-korB*) are included. The transformants were plated on double-selection MacConkey agar plates supplemented with 1% maltose and 0.1 mM IPTG. Strains were assayed for β-galactosidase activity in liquid overnight cultures. Mean values with standard deviations for at least three experiments are presented. The statistical analysis of the data performed by a parametric *t* test indicated a significant difference between KorB/KorB and KorB/KorBA111T (*P* value of 0.00002). (C) DNA-binding activity of WT KorB and KorBA111T. Aliquots of 20 ng of Cy5-labeled DNA probes, nonspecific DNA fragment Cy5-NS (upper panel), and the specific DNA fragment Cy5-O$_B$ (middle panel), were incubated with increasing amounts of KorB or KorBA111T (20, 40, 60, and 70 pmol) in 20 μl of binding buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 50 mM NaCl, 0.2 mg ml^{-1} BSA) at 37°C for 15 min. A negative control (−) comprised all reaction components without the KorB protein or KorBA111T. Samples were separated on 5% native polyacrylamide gels in 1× TBE buffer. Arrows indicate the position of unbound Cy5-labeled DNA fragments. The gel images were scanned and analyzed with ImageQuant. The bottom panel represents the percentage of free specific Cy5-labeled DNA probe plotted against the protein concentration applied. Gray, KorB; orange, KorBA111T. (D) Repressor activity of KorBA111T toward *mobC*p. *E. coli* DH5α(pJSB7.9 *mobC*p-*xylE*) was transformed with expression vector pGBT30 (control strain) or with its derivative pJSB5.67 *tac*p-*korB111*. XylE activity in the presence of KorBA111T (orange bar) was assayed in extracts from exponentially growing cultures and is shown relative to the control (dark gray bar) taken as 100%. Results obtained for WT KorB$_{RA3}$ are included for comparison (light gray bar). Mean values with standard deviations for at least three assays are shown. A parametric *t* test performed for the data before normalization indicated a significant difference between KorB and KorBA111T (*P* value of 0.009).

vivo in the two-plasmid regulatory system. *E. coli* DH5α(pJSB7.9 *mobC*p-*xylE*) was transformed with the expression plasmid pJSB5.67 *tac*p-*korB111* and the empty vector pGBT30 or pMMB2.50 *tac*p-*korB* as controls. The mutant KorBA111T did not repress the *mobC* promoter (Fig. 6D), confirming that the KorB spreading on DNA is critical for its regulatory activity.

Influence of other proteins on KorB spreading. Three KorB-controlled promoters (*orf02*p/*orf02*prev and *mobC*p) are regulated by other plasmid-encoded repressors, raising the question about the possible cooperativity of KorB with those proteins. The divergent promoters *orf02*p and *orf02*prev are both tightly regulated by KorC repressor (repression index, 100-fold at low

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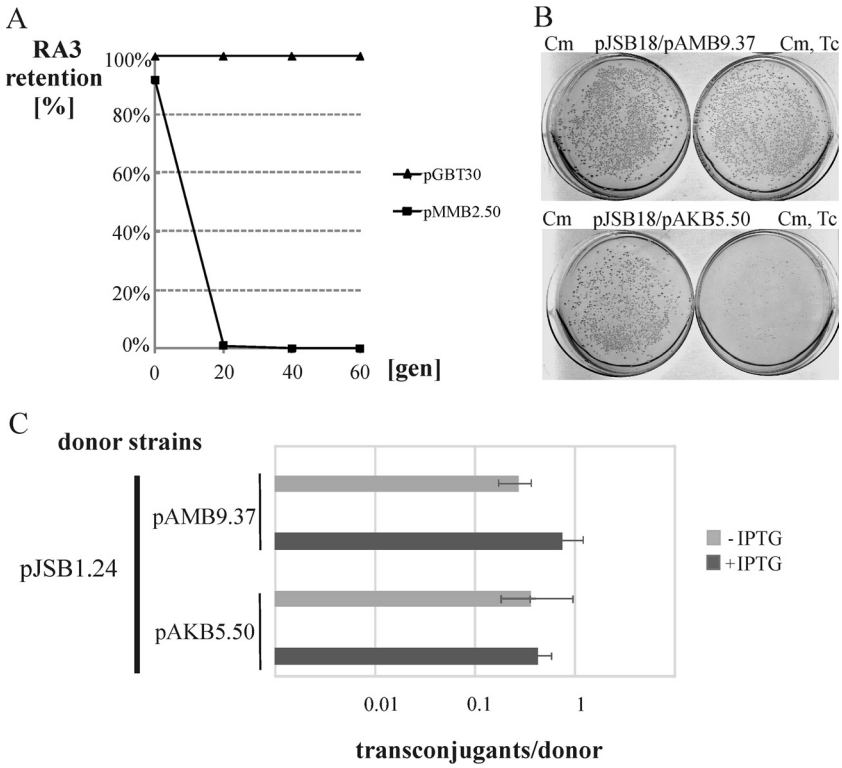

FIG 7 Effect of KorB overproduction on RA3 plasmid maintenance, replication, and conjugation frequency. (A) RA3 stability assay. *E. coli* DH5α(RA3)(pGBT30) and DH5α(RA3)(pMMB2.50 *tac*p-*korB*) were grown overnight in L broth with chloramphenicol and penicillin and then diluted in L broth with penicillin and 0.5 mM IPTG. Approximately every 20 generations (gen), the cultures were diluted into fresh medium and analyzed for RA3 retention. Representative values from one of three experiments are shown. (B) Maintenance of RA3 minireplicon in the presence of excess KorB. *E. coli* DH5α(pJSB18Tc^r) was transformed with pBBR1MCS derivatives (empty expression vector pAMB9.37 *tac*p or pAKB5.50 *tac*p-*korB*), plated on L agar with selection for incoming plasmid only (chloramphenicol) or for both resident and incoming plasmids (tetracycline and chloramphenicol) as indicated above the photographs, and grown at 37°C overnight. (C) Frequency of RA3 plasmid conjugation in the presence of excess KorB. *E. coli* DH5α(pJSB1.24) was transformed with pAKB5.50 *tac*p-*korB* or with empty pAMB9.37 as a control. Double transformants were grown with or without 0.5 mM IPTG and used as donors in conjugation with a DH5α Rif^r strain as the recipient. The frequency of conjugation is indicated on a semilogarithmic scale as the number of transconjugants per donor cell. Mean values with standard deviations for at least three experiments are shown. The significance of the differences between the IPTG-induced control and KorB-expressing variants were evaluated by a parametric *t* test on log-transformed data, giving a *P* value of 0.07 (nonsignificant).

KorC concentration). KorC binds to its operator O_C1 located between the −35 and −10 motifs for *orf02*p (Fig. 2A) and overlapping the predicted transcription start site (TSS) for *orf02*prev (20). The *mobC* promoter has been shown to be shut off by MobC binding to an imperfect palindrome O_M overlapping both the −35 and −10 motifs of *mobC*p (19). Such a strong regulation of those promoters by dedicated repressors precluded direct studies of their cooperativity with KorB, a weak repressor. The putative interference between KorC/MobC and KorB was therefore analyzed using a transcriptional silencing assay.

pGB2 derivatives harboring the *orf02*p (pAKB8.9) or *parS*/*mobC*p (pAKB8.5I) promoter regions were constructed in such a way that the binding of KorC or MobC to their cognate operators would create steric hindrance for polymerizing KorB to reach *repA*p. *E. coli* DH5α(pAKB8.5I) and *E. coli* DH5α(pAKB8.9) were transformed with the expression plasmids pJSB4.1 (*tac*p-*mobC*) and pJSB4.7 (*tac*p-*korC*), respectively. The transformed cells were made competent under conditions that induced the production of MobC and KorC (Fig. 5C) and then transformed with pMMB2.50 (*tac*p-*korB*) or pGBT30 as a control. The transformants were selected directly on triple-selective plates with or without IPTG induction of expression of the regulatory genes. For the both promoter constructs assayed, induction of *korB* expression by IPTG led to silencing of the *repA* gene, resulting in a 10^4-fold decrease in the number of transformants (Fig. 5D), indicating that KorB could reach the distant DNA from its operator O_B despite the presence of a high level of another repressor.

Effects of KorB excess on RA3 biology. KorB-binding sites occur at three locations in the RA3 backbone (Fig. 1), and each site was shown here to be involved in the regulation of gene expression. We decided to check the effects of KorB excess on core biological functions of the RA3 plasmid. First, the effect on RA3 maintenance was tested. *E coli* DH5α(RA3) was transformed with empty pGBT30 vector as a control or pMMB2.50 *tac*p-*korB*. Overnight cultures of such double transformants grown in the presence of chloramphenicol and penicillin were diluted into medium with penicillin only and 0.5 mM IPTG to select for pMMB2.50 or pGBT30 and to induce *tac*p and were passaged in this medium every 20 generations. At each passage, culture samples were checked for RA3 retention. Severe RA3 destabilization (98% cells had lost the RA3 plasmid) was observed already after 20 generations of growth in the presence of KorB excess (Fig. 7A). No loss of

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RA3 following 60 generations on medium lacking chloramphenicol was observed in a control strain harboring the empty expression vector DH5α(RA3)(pGBT30).

The O_B1 operator in the divergent promoter region *orf02*p/*orf02*prev mediates repression by KorB of transcription from *orf02*p toward the stability module but also that from *orf02*prev toward $oriV_{RA3}$ (A. Markowska, unpublished data). To verify the direct impact of KorB on plasmid replication, the RA3 minireplicon pJSB18 (20) without the stability module was used. *E coli* DH5α(pJSB18) was transformed with the BHR expression vector pAMB9.37 (pBBR-MCS1 *lacI*^{q}, *tac*p) or its derivative pAKB5.50 (*tac*p-*korB*). The transformants were selected for the incoming plasmid only or for both the resident and incoming plasmids. Selection for the incoming plasmid yielded similar transformation frequencies of over 10^4 colonies per 1 ml of the transformation mixture for either plasmid. A similar number of well-grown colonies was obtained on double-selection plates for the control transformation with pAMB9.37 (Fig. 7B), while for pAKB5.50, approximately 10-fold-fewer minute colonies per plate appeared, with only a few of them of slightly larger size, indicating strong KorB interference with the replication of the RA3 minireplicon.

The presence of two KorB-binding sites in the conjugative transfer region, one upstream of the *mobC-nic* operon and another in *orf25*, 1 kb upstream of *orf23*p, suggests that KorB could have an impact on the efficiency of conjugative transfer. We therefore checked the effect of KorB excess on the self-transmission of plasmid pJSB1.24, a pBGS18 derivative carrying a functional RA3 conjugative transfer module (52). *E coli* DH5α(pJSB1.24) was transformed with pAMB9.37 (pBBR-MCS1 *lacI*^{q}, *tac*p) as a control or its derivative pAKB5.50 *tac*p-*korB*. Double transformants were grown overnight with or without 0.5 mM IPTG and used as donor strains in the conjugation experiments. No effect of KorB on the efficiency of conjugative transfer of pJSB1.24 was observed, irrespective of the level of KorB overproduction (Fig. 7C), indicating that KorB does not significantly disturb conjugative transfer under the conditions tested.

DISCUSSION

Partition protein $KorB_{RA3}$ acts as transcriptional regulator binding at various distances from promoters. In this paper, we have demonstrated that KorB, an essential component of the active partitioning complex of the conjugative BHR plasmid RA3 (18), has a second important function as a global transcriptional regulator. It was found earlier that KorB specifically binds the palindromic motif IR-SnaBI, the key element in the *parS* sequence. During this study, the three KorB-binding sites in the RA3 genome (Fig. 1) were shown to act as KorB operators (O_Bs) involved in the regulation of four promoters. The O_Bs are distant from each other and are located throughout the plasmid molecule in positions enabling regulation of three major backbone modules, i.e., replication, stability, and conjugative transfer (Fig. 1). Interestingly, KorB can regulate transcription by binding close to or far from the promoter (next to the −35 motif, 85 bp upstream or 150 bp downstream, or as far away as 1,300 bp upstream of the promoter). The KorB operators are not located at typical positions to block RNA polymerase binding; therefore, the question regarding the mechanism of the KorB action arose.

Being a partition protein, KorB can spread along DNA starting from a specific binding site (18), which is not common among transcriptional repressors. The spreading capacity shown for a number of ParB family members belonging to the subtype Ia partition systems (2, 26, 27, 32) is believed to contribute to the formation of a large nucleoprotein complex stabilized by DNA looping and bridging (29, 30, 53). $KorB_{RA3}$ was examined here for spreading on DNA at different distances from the O_B in the test plasmid pGB2 and showed a high potential of gene silencing over a distance of approximately 2 kb (Fig. 5A, pAKB8.81). A homologous partition and regulatory protein, KorB from plasmid RK2 (IncP-1), plays a central role in transcriptional regulation through binding at 12 highly conserved operators positioned at highly diverse distances upstream from promoters (from 10 bp to over 1 kb) in the RK2 genome (2, 49). The $KorB_{RK2}$ action at a distance was proposed to be based on a specific KorB-DNA interaction at the O_B complemented by spreading and looping but also to involve interactions with other global repressors, KorA or TrbA, to block RNA polymerase (2, 54).

Polymerization-deficient $KorB_{RA3}$ is unable to repress transcription. The role of $KorB_{RA3}$ spreading in the repression at various distances was tested using a KorB spreading-deficient mutant with a single amino acid substitution of a conserved alanine residue in box II motif at the N terminus. The KorBA111T variant retained the dimerization and DNA-binding abilities but was unable to repress the *mobC* promoter with O_B2 only 46 bp upstream of the −35 motif. The spreading-dependent mechanism of $KorB_{RA3}$ repressor action can involve short-range and long-range interactions, depending on the promoter-operator distance. In two RA3 promoters, *orf02*p and *mobC*p, the arrangement of the operator sites could be expected to impose some constraints on the KorB spreading, since binding sites for the strong repressors KorC (20) and MobC (19) are located between the O_B operator and promoter motifs. However, we found that oversupply of KorC or MobC did not impede the KorB-exerted silencing effect (Fig. 5C), suggesting an ability of KorB not only to spread along DNA but also to form long-range interactions with DNA. The spreading-bridging model of ParB interactions with DNA (29) explains how a partition protein establishes long-range contact with distant DNA regions and exerts a regulatory effect.

$KorB_{RA3}$ is a global regulator of plasmid replication, stable maintenance, and conjugative transfer. The RA3 KorB-dependent promoters determine expression of crucial diverse plasmid functions such as replication (*orf02*prev), maintenance (*orf02*p), and conjugative transfer (*mobC*p and *orf23*p). These promoters display high activity as measured by reporter gene expression (0.5 to 5.5 U of XylE activity), which is downregulated only 1.5- to 5-fold by KorB (Fig. 2C and D). The strongest (5-fold) repression is exerted by KorB toward *orf02*prev, where the O_B site is in an atypical position, 150 bp downstream of the −35 motif. Three out of the four KorB-dependent promoters are strongly regulated by their primary repressors, KorC (*orf02*p and *orf02*prev) and MobC (*mobC*p); therefore, repression by KorB is apparently required only for the fine-tuning of the promoters, at least in *E. coli*. In the case of *orf23*p, the KorB-exerted repression is also subtle, and we cannot exclude the possibility that this promoter is regulated by another, as-yet-unrecognized protein. However, we could not detect any cooperativity between the primary repressors and $KorB_{RA3}$, in contrast to $KorB_{RK2}$ interacting with two global repressors, KorA and TrbA (3, 54, 55). Cooperativity between repressor proteins may be important for medium-strength repressors in certain circumstances (4) but not valid for strong and weak repressors interfering with transcription at different levels.

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TABLE 4 Conserved KorB operators in IncU and IncU-like plasmids

Plasmid	O_B sequence[a]	Coordinates	Genetic location	Accession no. (reference)
RA3 (IncU)	TTTCGTAC-GTACGAAA	2075–2090	*orf02*p/*orf02*prev[b]	DQ401103 (10)
		9707–9722	*mobC*p[c]	
		23579–23594	*orf23*p[d]	
pMBUI3[e] (IncU)	TTTCGTAC-GTACGAAA	11753–11768	*orf15*p[d]	NC_025092.1 (8)
		25577–25592	*mobC*p[c]	
		33094–33109	*orf38*p[b]	
pMBUI7[e] (IncU)	TTTCGTAC-GTACGAAA	12414–12429	*orf16*p[d]	NC_025095.1 (8)
		26190–26205	*mobC*p[c]	
		33644–33659	*orf39*p[b]	
pP2G1	TTTCGTAC-GTACGAAA	2139–2154	—[b]	NG_040969.1 (64)
		12383–12398	—[c]	
pTOR_01	TTTCGTAC-GTACGAAA	1899–1914	—[b]	NC_019798 (unpublished)
		12383–12398	—[c]	
pAC3	TTTCGTAC-GTACGAAA	2946–2961	—[b]	KM204147 (unpublished)
		9765–9780	—[c]	
pEIB202	TTTCGTgC-GcACGAAA	15053–15068	—[d]	NC_013509 (65)
	TTTCGTgC-GcACGAAA	23426–23441	—[b]	
	TTTCGTAC-GcACGAAA	35901–35916	—[c]	
	TTTCGTgC-GcACGAAA	38871–38886	—[d]	
pEA3	TTTCGTAC-GcACGAAg	2421–2436	—[b]	NC_020920.1 (unpublished)
	TTTCGTAC-tgAatAtA	23504–23519	—[c]	

[a] Lowercase letters in putative KorB operators indicate diversion from the O_B consensus from RA3.
[b] Operator located between replication and stability regions.
[c] Operator located between stability and conjugative transfer regions.
[d] Operator located in conjugative transfer region.
[e] Promoter region identified on the basis of DNA sequence identity with RA3.

Although KorB seems to have a moderate influence on the levels of RA3 gene expression, it revealed a drastic effect on plasmid stability, causing its immediate loss when overproduced in *trans*. This effect could be accounted for by the influence on the *orf02* promoter, predicted to drive transcription of the whole maintenance module (20), or by negative regulation of transcription from *orf02*prev, which is important for RA3 replication initiation (A. Markowska, unpublished data). The latter is likely to play a more important role, since a minireplicon of RA3 lacking the stability module was unable to establish itself in the recipient cells in the presence of KorB (Fig. 7B). A similar negative effect on mini-RA3 replication was exerted by an excess of KorC, another *orf02*prev repressor (20). However, these two regulators differed significantly in the extent of instability of the intact RA3 they induced: after 20 generations in the presence of KorB, excess RA3 was lost in more than 90% of cells (Fig. 7A), whereas it was still retained in 70% of cells after 75 generations in the presence of excess KorC (20). The profound effect of an excess of KorB is most likely related to its function as a partitioning protein and a distorted balance with its partner IncC.

We analyzed direct interactions between the RA3 partition proteins *in vivo* using a BACTH system and detected homodimers of KorB and IncC and KorB-IncC heterodimers. The C terminus of KorB and the N terminus of IncC were important for both homo- and hetero-oligomerization. Our previous study showed an accessory role of Orf11 in plasmid partitioning (18), but neither of the two major partition proteins interacted with Orf11 in the BACTH system. Studies on two IncC forms of RK2 (IncP-1) have demonstrated that the longer IncC-1 but not the shorter IncC-2 potentiates the KorB-operator binding *in vivo* and *in vitro* (49, 56). What is more, the enhancement of the $KorB_{RK2}$ binding to the operators was detected for 11 O_Bs but not for O_B3 (*parS* site), which is vital for active partition (49, 57). In the RA3 plasmid, IncC (equivalent to IncC-2 of RK2) does not help KorB to discriminate between *parS* (*mobC*p) and the other KorB operators. The presence of either IncC or Orf11 diminished the repressive effect of KorB on both promoters tested, *mobC*p and *orf02*p, probably due to the distorted balance between free KorB and that bound in the partition complex.

Since KorB regulates two promoters of the conjugative transfer module by binding to two KorB operators, O_B2 and O_B3, upstream of *mobC*p and *orf23*p, we checked the KorB effect on the self-transmission of pJSB1.24 carrying the conjugative transfer module of RA3 and found that it transfers efficiently between *E. coli* strains regardless of the presence of KorB. An interesting question arises about the possible role of KorB in the regulation of conjugative transfer between hosts other than *E. coli*.

The KorB network is predicted to be common among IncU plasmids. The dual function of KorB in RA3 prompted us to search for homologous KorB-dependent regulatory networks among IncU or IncU-like plasmids (not formally classified into IncU group) encoding highly conserved (88 to 95% similarity) KorB partition proteins. The plasmids pTOR_01 (20,914 bp; NC_019798.1), pP2G1 (26,645 bp; NG_040969.1), pAC3 (15,872 bp; KM204147), pMBUI3 (33,736 bp; NC_025092.1), and pMBUI7 (34,006 bp; NC_025095.1) were analyzed *in silico* and found to contain two or three conserved predicted KorB operators (Table 4). An intriguing finding was also that the location of the conserved palindromic O_Bs followed the RA3 layout, with a few minor differences (Table 4). The conserved genetic localization of the putative KorB operators suggests similar regulatory roles of homologous partition proteins in coordination of plasmid functions. Two additional plasmids, pEA3 (29,595 bp; NC_020920.1) and pEIB202 (43,703 bp; NC_013509), encode KorB homologs

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showing 72% and 58% similarity with $KorB_{RA3}$, respectively, and contain incomplete operators with only one arm fully conserved. In pEIB202, a copy of the operator with one mismatch in the right arm (TTTCGTAC-GcACGAAA) is located the same as O_B2 of *parS* in RA3, suggesting its similar role in active partition. There are also three palindromes with two mismatches (forming a complementary pair), with potential regulatory roles, one between the replication and stability blocks of genes and two in the putative conjugative transfer region. Finally, the pEA3 plasmid contains two putative O_B sites in similar locations as in RA3, one at the border of the predicted replication module and the second downstream of the predicted partition operon; however, these sites diverged more significantly and could be nonfunctional.

Conclusion. A comparison of the KorB-dependent transcriptional regulatory networks of representatives of two BHR plasmid groups, IncU and IncP-1, reveals common features but also substantial differences. KorB proteins from these two groups show the same dual functionality (partition protein and transcriptional regulator) and use spreading/bridging as the mechanism of transcriptional interference, but their regulatory roles are achieved in different ways. The complex multivalent regulatory network of RK2 is based on multiple autorepressors and overlapping regulons with four global regulators with medium-scale repression of 3- to 10-fold (11). $KorB_{RK2}$ controls replication, stable maintenance, and transfer functions by binding 12 O_Bs scattered throughout the plasmid. It acts as a primary or secondary repressor, binding close to or far from the regulated promoters. Most significantly, $KorB_{RK2}$ potentiates its weak 3-fold repression effect by cooperating with other global repressors. The IncU plasmid RA3 encodes several autoregulators and two transcriptional repressors of global function, KorB (this work) and KorC (20), which recognize, respectively, only three and four operators along the plasmid genome but nevertheless control all the backbone functions: replication, stable maintenance, and conjugative transfer. Their regulons overlap only at the divergent promoters at the junction between the replication and stability modules, indicating that regulation at this point is critical for proper expression levels and biological functionality of plasmid RA3. The two global regulators of RA3 seem to act independently from each other. Despite this simple network, RA3 shows stable maintenance in an extremely wide range of hosts by employing the active partition protein KorB as an important regulator of backbone genes.

The present study on KorB of RA3 (IncU group) provides a novel example of a segregation protein acting as a transcriptional regulator by utilizing its intrinsic property of DNA spreading to control gene expression at different distances from the operator site.

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The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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