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Title: Global transcriptional regulator KorC coordinates expression of three backbone modules in the broad-host-range RA3 plasmid of IncU incompatibility group

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Abstract: Broad-host-range conjugative RA3 plasmid of IncU incompatibility group has been isolated from fish pathogen Aeromonas hydrophila. DNA sequencing revealed mosaic modular structure of RA3 with stabilization module showing some degree of similarity to IncP-1 genes whereas conjugative transfer module being highly similar to PromA plasmids. The integrity of mosaic plasmid genome seems to be specified by its regulatory network. In this paper the transcriptional regulator KorC has been analyzed. The KorCRA3 (98 amino acids) is encoded in the stabilization region and it represses five strong promoters by binding to the conserved palindrome sequence, designated OC on the basis of homology to KorC operator sequences in IncP-1 plasmids. Two of KorCRA3 regulated promoters precede the first two cistrons in the stabilization module, and one fires towards replication module. Among two other divergently oriented back-to-back promoters, one is upstream of the long transcriptional unit of 19 orfs, products of which are predicted to be involved in the conjugative transfer process and another controls tricistronic operon encoding proteins of unknown functions. Despite the similarity between binding sites in IncU and IncP-1 plasmids no cross-reactivity between KorC proteins has been detected. The KorC emerges as the global regulator in RA3 coordinating all plasmid backbone functions: replication, stable maintenance and conjugative transfer.

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To
Editor of Plasmid

Dear Dr Rood,

Please find attached the manuscript: "**Global transcriptional regulator KorC coordinates expression of three backbone modules in the broad-host-range RA3 plasmid of IncU incompatibility group**" by Ludwiczak, M., Dolowy, P., Markowska, A., Szarlak, J., Kulinska, A. and Jagura-Burdzy, G. I am hoping you will consider the manuscript appropriate for publication in Plasmid.

Yours sincerely
Grazyna Jagura-Burdzy

***Highlights (for review)**

KorC of RA3 regulates expression of replication, stability and transfer functions
KorC operators from different incompatibility groups of plasmids are highly conserved
Specificity determinants in O_C have been established
KorC mutant analysis led to the identification of HTH motif and dimerization domain

Global transcriptional regulator KorC coordinates expression of three backbone modules in the broad-host-range RA3 plasmid of IncU incompatibility group

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ABSTRACT

Broad-host-range conjugative RA3 plasmid of IncU incompatibility group has been isolated from fish pathogen *Aeromonas hydrophila*. DNA sequencing revealed mosaic modular structure of RA3 with stabilization module showing some degree of similarity to IncP-1 genes whereas conjugative transfer module being highly similar to PromA plasmids. The integrity of mosaic plasmid genome seems to be specified by its regulatory network. In this paper the transcriptional regulator KorC has been analyzed. The $KorC_{RA3}$ (98 amino acids) is encoded in the stabilization region and it represses five strong promoters by binding to the conserved palindrome sequence, designated O_C on the basis of homology to KorC operator sequences in IncP-1 plasmids. Two of $KorC_{RA3}$ regulated promoters precede the first two cistrons in the stabilization module, and one fires towards replication module. Among two other divergently oriented back-to-back promoters, one is upstream of the long transcriptional unit of 19 orfs, products of which are predicted to be involved in the conjugative transfer process and another controls tricistronic operon encoding proteins of unknown functions. Despite the similarity between binding sites in IncU and IncP-1 plasmids no cross-reactivity between KorC proteins has been detected. The KorC emerges as the global regulator in RA3 coordinating all plasmid backbone functions: replication, stable maintenance and conjugative transfer.

1. INTRODUCTION

Conjugative RA3 plasmid (GenBank accession no. DQ401103), representative of IncU incompatibility group, demonstrates very broad-host-range since it self- transfers, replicates and is stably maintained in the representatives of alpha- beta- and gamma-proteobacteria (Kulinska et al., 2008). The factors determining promiscuity of RA3 are under investigation. The sequencing of plasmid DNA revealed its modular-mosaic structure with long blocks of genes putatively engaged in distinct plasmid functions showing similarities to the functional modules of plasmids from different incompatibility groups e.g. stability region of IncP-1 (Pansegrau et al., 1994; Thorsted et al., 1998) or conjugative transfer region of PromA plasmids (van der Auwera et al., 2009).

The stabilization module of RA3 encompasses 10 *orf*s transcribed in the same direction (Fig. 1A). The seven of them encode homologues of IncP-1 products with 30% to 65% similarity at amino acids sequence level, three *orf*s (*orf02, orf04* and *orf11*) have no homologues in the database (Kulinska et al., 2008). The $klcA_{RA3}$ codes for probable antirestriction protein that shares 55% homology with $KlcA_{R751}$, recently shown to act at Type I DNA restriction and modification systems (Serfiotis-Mitsa et al., 2010). The $korC_{RA3}$ codes for the putative transcriptional repressor, 49% and 41% similar to equivalents of RK2 (IncP-1α) and R751 (IncP-1β) plasmids, respectively. The homologues of two putative accessory partition proteins, KfrC and KfrA (69% and 30% similarity to RK2 equivalents, respectively), are encoded upstream of the last part of the stabilization module, the partitioning operon *korAincCkorBorf11* (Kulinska et al., 2011). With the exception of the partition operon, the transcriptional organization of stability regions in representatives of IncU and IncP-1 groups of plasmids differs significantly (Kulinska et al., 2008, Fig. 1A).

The homologues of four putative DNA binding proteins encoded in the stability module of RA3 (KorA, KorB, KorC and KfrA) have defined regulatory roles in the RK2 and R751 biology ranging from autoregulatory to the global repressor functions (Adamczyk et al., 2006; Balzer et al., 1992; Bechhofer et al., 1986; Jagura-Burdzy et al., 1991; 1999b; Jagura-Burdzy and Thomas, 1992; 1994; 1995; Kornacki et al., 1990; Larsen and Figurski, 1994; Macartney et al., 1997; Motallebi-Veshareh et al., 1992; Shingler and Thomas, 1984; Theophilus et al., 1985; Thomas et al., 1988; 1990).

The study initiated on RA3 putative regulatory network demonstrated that $KorA_{RA3}$ has a strong repressor activity as the autoregulator of the *korAincCkorBorf11* partition operon. Since in the RA3 genome the KorA operator occurs only once at *korAp,* it implies that KorA repressor has a very limited role in regulation of gene expression in RA3 plasmid. Another

DNA binding protein, KfrA, plays only the role of a self-repressor of the monocistronic *kfrA* operon (Kulinska and GJB unpublished). The KorB of RA3 acts as the partition protein (B-component of type IA partition system) (Kulinska et al., 2011) and also as the transcriptional repressor for two promoters (Dolowy and GJB unpublished). This work, devoted to KorC of RA3, provides strong evidence that KorC is the most important transcriptional regulator of IncU plasmid backbone gene expression.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Escherichia coli strains used were: DH5α [F^-(*Φ80*d*lacZΔM15*) *recA1 endA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *supE44 relA1 deoR Δ(lacZYA-argF)U196*]; BL21[F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (λ DE3)] (Novagen, 2003); BTH101 **[**F^- *cya-99 araD139 galE15 galK16 rpsL1* (Sm^R) *hsdR2 mcrA1 mcrB1*] (Karimova et al., 1998). Bacteria generally were grown in L broth (Kahn et al., 1979) at 37°C or on L agar (L broth with 1.5% w/v 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, kanamycin 50 μg ml^{-1} for kanamycin resistance, tetracycline 10 μg ml^{-1} for tetracycline resistance and chloramphenicol 10 μg ml^{-1} for chloramphenicol resistance. MacConkey Agar Base (Difco) supplemented with 1 % maltose was used for BACTH system. L agar used for blue/white screening contained IPTG (0.1 mM) and Xgal (40 μg ml^{-1}). Protein synthesis was induced with the use of IPTG (0.5 mM for BL21 and DH5α strains; 0.15 mM and 0.5 mM for BTH101 strain grown in liquid media and agar plates, respectively).

2.2 Plasmid DNA isolation, analysis, cloning and manipulation

Plasmid DNA was isolated and manipulated by standard procedures (Sambrook et al., 1989). The list of plasmids used and constructed in this study is presented in Table 1. Standard PCR reactions (Mullis et al., 1986) were performed with pairs of primers listed in Table 2. All PCR-derived clones were analyzed by DNA sequencing to check their fidelity.

2.3 Site-directed mutagenesis *in vitro*

To create mutations in *korC* an *in vitro* site-directed mutagenesis method (Stratagene, 2006) was used with the high fidelity PfuTurbo DNA polymerase. The primers 19 to 22, 28 and 29 (Table 2) were designed to insert nucleotide substitutions in the particular region accompanied by restriction cleavage site to facilitate screening. PCR reactions to introduce mutations were performed with an initial denaturation step (96°C for 5 minutes) and 18 cycles of denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 68°C for 14 minutes. Reactions ended with a final elongation step (68°C for 25 minutes). The PCR product was treated with DpnI endonuclease to remove template DNA and used for transformations. The plasmid DNA of putative mutant was tested for the presence of restriction site introduced in the mutagenic primers and the effect of mutagenesis was verified by sequencing.

2.4 Bacterial transformation

Competent cells of *E. coli* were prepared by standard $CaCl_2$ method (Sambrook et al., 1989).

2.5 Determination of catechol 2, 3-oxygenase activity (XylE)

XylE activity (the product of *xylE*) was assayed in logarithmically growing strains (Zukowski et al., 1983). One unit of catechol 2, 3-oxygenase is defined as the amount needed to convert 1 μmol of catechol in 1 minute under standard conditions. Protein concentration was determined using the Bradford method (Bradford, 1976).

2.6 Purification of His_6-tailed KorC derivatives

For protein over-production and purification, *E. coli* BL21(DE3) was transformed with one of the constructs: pMWB10.7, pMWB10.24 or pMWB10.25 encoding N-terminally His_6-tagged KorCs. The purification procedure was performed as described previously (Jagura-Burdzy and Thomas, 1995) with the use of sonication buffer (50 mM sodium phosphate pH 8.0; 300 mM NaCl). Protein fractions were analyzed by SDS-PAGE using a Pharmacia PHAST system with 20% homogeneous gels.

2.7 Analysis of protein-DNA interactions by electrophoretic mobility shift assay (EMSA)

The PCR-amplified DNA fragments were excised from agarose gels and purified by Gel-Out kit (A&A Biotechnology). Concentration of the isolated DNA fragments was determined with NanoDrop 2000. The protein-DNA binding reactions were performed in the binding buffer (50 mM Tris-HCl pH 8.0; 10 mM $MgCl_2$; 50 mM NaCl; 0.2 mg ml^{-1} BSA) with increasing amounts of His_6-KorC added in a final volume of 20 μl. Binding reactions were analyzed on 1.2% agarose gels in 0.5xTBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3). The gels were stained with ethidium bromide and DNA was visualized under UV light.

2.8 Cross-linking with glutaraldehyde

His_6-tagged KorC purified on Ni^{2+}-agarose column was cross-linked by the use of glutaraldehyde (Jagura-Burdzy and Thomas, 1995) and separated on 20% (w/v) SDS-PAGE gels. The proteins were transferred onto nitrocellulose membrane and Western blot analysis with anti-His_6-Tag antibodies was performed as described previously (Bartosik et al., 2004).

2.9 Conjugation procedure

E. coli DH5α strain with RA3 plasmid, transformed either with pGBT30 (as a control) or pJSB5.7 (for KorC over-expression), was used as a donor and DH5α Rif^R strain was used as the recipient. 100μl of overnight cultures of donor and recipient strains were mixed (1:1) and incubated on L agar plates for 2 hours at 37°C. Cells were scrapped, re-suspended in L-broth and aliquots of serial 10-fold dilutions were plated onto L agar plates with 100 μg ml^{-1} rifampicin and 10 μg ml^{-1}chloramphenicol to estimate the number of transconjugants. In

parallel 100 μl of donor strain overnight culture was incubated on L agar plate for 2 hours at 37°C, cells were scrapped, diluted and plated on L agar or L agar with antibiotics selective for donor strain. The transfer frequency was calculated as the number of transconjugants per donor cell.

2.10 Bacterial Adenylate Cyclase Two-Hybrid System (BACTH system)

The dimerization of KorC *in vivo* was analyzed using bacterial adenylate cyclase two-hybrid system in *E. coli* (Karimova et al., 1998). KorC protein was translationally fused to C-terminal parts of CyaT18 and CyaT25 fragments encoded on compatible vectors pUT18C and pKT25. *E. coli* BTH101, an adenylate cyclase deficient strain (*cya*), was co-transformed with a mixture of appropriate pairs of BACTH system plasmids and plated on MacConkey medium supplemented with 1 % (w/v) maltose, 0.5 mM IPTG and selective antibiotics. The plates were incubated for 48 h at 27°C. The ability to ferment maltose that manifested in changing color of the medium from pale pink into violet indicated the Cya reconstitution through the interactions between fused polypeptides. Interactions were also verified by determination of β-galactosidase activity in the liquid cultures (Miller, 1972). Double transformants of BTH101 strain were grown overnight in L broth at 27°C with addition of penicillin, kanamycin and 0.15 mM IPTG and 0.2 ml of each culture was taken for the assay. One unit of β-galactosidase is defined as the amount of enzyme needed to convert 1 μmol of ONPG (o-nitrophenyl-β-galactoside) to o-nitrophenol and D-galactose in 1 minute under standard conditions.

2.11 Stability assay

To check the effect of KorC over-expression on RA3 stability *E. coli* DH5α strain with RA3 plasmid was transformed either with pGBT30 (as a control) or pJSB5.7 (for KorC over-expression). Overnight cultures of transformants grown on L broth supplemented with 10 μg ml^{-1}chloramphenicol and 150 μg ml^{-1} penicillin were 10^5 fold diluted into fresh medium supplemented with 150 μg ml^{-1} penicillin and 0.5 mM IPTG. The cultures were diluted repeatedly every 24 hours and simultaneously the diluted cultures were plated on L agar with 300 μg ml^{-1} penicillin. The plates were incubated overnight at 37°C and 100 colonies were re-streaked on L agar supplemented with either 300 μg ml^{-1} penicillin and 0.5 mM IPTG or 300 μg ml^{-1} penicillin, 10 μg ml^{-1}chloramphenicol and 0.5 mM IPTG to estimate a plasmid retention rate.

3. RESULTS

3.1 KorC regulates three promoters in the stability module

The *korC* locates in the stable maintenance module between short *orf04* of unknown function and homologue of *kfrC* from IncP-1 (Fig. 1A). KorC is a relatively small protein (98 amino acids) with two putative α-Helix-turn-α-Helix motifs predicted by I-TASSER server (Fig. 1B; Zhang, 2008). The alignment of amino acids sequences of $KorC_{RA3}$ and its homologues from RK2 (IncP-1α), R751(IncP-1β) and pQKH54 (IncP-1γ) reveals that the least conserved region is around HTH motif previously suggested for $KorC_{RK2}$ (Kornacki et al., 1990) (Fig. 1C). The helices in this motif are separated by long linker in RA3; the linker is conserved in the closest homologue $KorC_{pQKH54}$ and absent in other KorCs.

Promoter search in the 7.7 kb stability region had predicted promoter sequences upstream of *orf02*, *klcA*, *korC*, *kfrA* and *korA* genes (Kulinska et al., 2008). Five DNA fragments with putative promoter sequences have been amplified and cloned into promoter-probe pPT01 vector upstream of the promoter-less *xylE* cassette encoding catechol 2,3-oxygenase (Macartney et al., 1997). All cloned inserts showed high transcriptional activity in the reporter system (1.5 to 6 U of XylE) confirming the presence of strong transcription initiation signals in these regions of RA3 (Fig. 2A). Cloning of the inserts in the opposite direction indicated that the region upstream *orf02* contains two divergently oriented promoter sequences, one firing towards *orf02* (*orf02p*) and another one firing into the repetitive region adjacent to the *repB* gene, designated *orf02prev* and undetected by computer analysis. The close inspection of DNA sequence in this region revealed a few putative promoter motifs. To discriminate between them, the originally cloned fragment was split into two shorter ones by PCR amplification with pairs of primers 08 and 30 or 06 and 31 (Table 2, Fig. 3A) and cloned in the same orientation (for *orf02prev* activity) into the promoter-probe vector to obtain pMWB6.27 and pMWB6.28, respectively. Whereas pMWB6.27 showed no promoter activity firing towards replication module, pMWB6.28 contained *orf02prev* promoter. We arbitrary identified motives recognized by RNAP in this fragment (Fig. 3A) which form divergent face-to- face configuration of promoter sequences for *orf02prev* and *orf02p*.

To check which promoters (if any) are under presumable control of KorC, the *korC* has been cloned under *tacp* into the expression vector pGBT30 compatible with pPT01 (Jagura-Burdzy et al., 1991) to construct pJSB5.7. DH5α strains with resident plasmids carrying analyzed promoter regions in transcriptional fusions with *xylE* were transformed with pGBT30 (vector) and pJSB5.7. The catechol 2,3- oxygenase activity assays in the logarithmic cultures

of double transformants revealed that KorC represses both divergently oriented promoters from the region upstream of *orf02* and the one upstream of *klcA* (Fig. 2A). The level of KorC repression was more than 100-fold even without induction of *tacp* by IPTG. None of other three promoters, *korCp*, *kfrAp* and *korAp* was sensitive to the presence of KorC in the cells (data not shown).

3.2 Identification of KorC binding sites in the stability module

The promoter regions upstream of *orf02* and *klcA* arose seemingly by duplication (Kulinska et al., 2008). The inspection of these promoter sequences showed the inverted repeat TAGGCCATTTTGGCCTA between putative -35 and -10 motifs of *orf02* promoter (overlapping -10 motif by 2 nt) and complementary version of the palindrome TAGGCCAAAATGGCCTA in the same position for *klcAp*. Interestingly, *klcA* promoter is also preceded by additional mutated version of the inverted repeat TAGGCCgATTTGGCCTA (Fig. 3A and B). The comparison of this putative $KorC_{RA3}$ binding site with previously identified operators for $KorC_{RK2}$ and $KorC_{R751}$ (Larsen and Figurski, 1994; Thomas et al., 1988; Thorsted et al., 1998) as well as other sequenced IncP-1 representatives indicated the high degree of similarity (Table 3).

3.3 KorC regulates expression of the conjugative transfer functions

Screening of RA3 genome with such IR sequence revealed an extra copy occurring in the divergent promoter region between *orf33* and *orf34* in the conjugative transfer module (Fig. 3C). Cloning of this divergent promoter region in both orientations upstream of *xylE* into pPT01 (pMWB6.11r for *orf33p-xylE* and pMWB6.11 for *orf34p-xylE*) confirmed that both tested promoters were highly active (1.5 U of XylE) (Fig. 2A). Whereas *orf34p* was repressed by KorC present *in trans* (10-fold repression by KorC at low repressor concentration), *orf33p* repeatedly showed 2-3 fold induction by KorC produced from pJSB5.7. Plasmid DNA isolation from double transformants cultures used for the enzymatic assays revealed unusual profile for pMWB6.11r. The copy number of pPT01 derivative carrying the transcriptional fusion *orf33p-xylE* increased more than 6-fold in the presence of pJSB5.7 in comparison to the cultures with pGBT30 *in trans* (data not shown). Although the rationale behind this transient copy-up phenotype is unclear, we may assume that KorC also represses *orf33p*, possibly not as strongly as other regulated promoters.

The genetic data has been gathered to support this conclusion. The attempts to clone the conjugative transfer module into the high copy number plasmid pBGS18 were unsuccessful leading to rise of minute colonies, unable to grow after passage. However, when the recipient strain DH5α expressed also *korC* from *tacp-korC* transcriptional fusion

(pJSB5.7) the transformants grew normally. The loss of this helper plasmid caused massive rearrangements in the insert, the long operon of 19 orfs transcribed from *orf33p.* Presumably unregulated expression of multicistronic conjugative transfer operon exerts too much metabolic burden on the cells. To obtain stable plasmid with the functional conjugative transfer module it was decided to incorporate the *korC* gene preceded by its own promoter into the vector before cloning the conjugative transfer module. Such construct, pJSB1.24, was stable and proficient in the conjugative transfer with the frequency comparable to the parental RA3 (Bartosik et al., 2012).

We checked whether excess of KorC may disturb the RA3 ability to spread. The donor strain DH5α (RA3) (pJSB5.7) was grown overnight on selective antibiotics and 0.5 mM IPTG. No effect of KorC over-production was observed on conjugation frequency of RA3 plasmid (Fig. 4A) indicating that the complete system is highly balanced and not easily disturbed, at least for approximately 20 generations.

3.4 KorC controls the replication functions of RA3.

The divergent promoters *orf02p-orf02prev* are located in the region between two functional modules: replication and maintenance. The repression of both promoters by KorC implicated the role of KorC not only in the expression of the operon in the stability module but also in the functioning of the replication module. The overnight culture of DH5α (RA3) (pJSB5.7) strain grown in the presence of chloramphenicol and penicillin was diluted repeatedly into the medium with penicillin and 0.5 mM IPTG and every 25 generations checked for RA3 retention. Over-expression of KorC *in trans* to the intact RA3 destabilized the parental plasmid (Fig. 4B). After approximately 75 generations 30% of cells lost RA3 whereas no loss was observed even after 100 generations without KorC excess.

The minireplicon of RA3 (pJSB18) encompassing the replication module and the divergent promoter region *orf02p/orf02prev* was constructed (RA3 coordinates 43327-45909, 1-2300). We used *E. coli* DH5α (pJSB18) as the recipient in transformation with two derivatives of the broad-host-range vector pBBR1MCS: pAMB9.37 (pBBR1MCS-*lacI*^{Q} *tacp*) and pJSB4.7 (pBBR1MCS *lacI*^{Q} *tacp-korC*). Whereas numerous well grown colonies of DH5α (pJSB18) (pAMB9.37) appeared on plates selective for resident and incoming plasmids, only scarce transformants of DH5α (pJSB18) (pJSB4.7) grown as minute colonies appeared on double selection plates (Fig. 4C). The frequencies of transformations of the same recipient strain but with selection for incoming plasmids were very similar for both pAMB9.37 and pJSB4.7 (>10^4 colonies per ml of transformation mixture) indicating that the

presence of KorC was detrimental for ability of RA3 minireplicon to be established. The role of transcription from *orf02prev* in the functioning of replication module e.g. initiation of replication or copy number control is under investigation (Markowska A. and GJB unpublished).

3.5 KorC exists as a dimer in solution

The *korC* was cloned into pET28 derivative under T7p to facilitate purification. Purified His_6-tagged KorC migrated on SDS-PAGE gels as polypeptide of MW 14 kDa. Cross-linking of KorC with increasing concentration of glutaraldehyde demonstrated ability of the protein to form dimers and higher order complexes in solution as shown on Fig. 5A.
The ability of KorC to self-interact was also confirmed *in vivo* in bacterial two hybrid system BACTH (Karimova et al., 1998) by translationally linking *cyaA* domains with *korC.* Dimerization of KorC manifested in the deep purple color of colonies plated and re-streaked on MacConkey agar supplemented with maltose, IPTG, kanamycin and penicillin (data not shown). Self-interaction of KorC was highly effective since it was giving 7000 U of LacZ activity in the liquid cultures, comparable to the activity of LacZ in the BTH101 strain transformed with BACTH plasmids with *cya* domains linked to GCN4 leucine zipper fragments, used as the positive control for strong interactions (Fig. 5B).

3.6 KorC binds to all KorC-regulated promoters with similar affinity *in vitro*

Putative KorC binding sites were numbered sequentially according to their position in RA3 genome: O_C1 maps in the divergent promoter region *orf02p/orf02prev*, O_C2 and O_C3 are localized in *klcAp* and O_C4 occurs in the divergent promoter region *orf33p*/*orf34p*.
DNA fragments of 150 to 300 bp in size containing O_C1, O_C2-O_C3 and O_C4 were PCR-amplified and used in EMSA experiments with the purified His_6-KorC. KorC shifted all three fragments with similar Kapp of about 120 nM (Kapp is defined as the protein concentration at which 50% of probe is shifted), however in the case of *klcAp* fragment with two KorC binding sites (O_C2-O_C3), two distinct retarded species were seen. The first retarded species appeared at low KorC concentration and the second at least at a 5-fold higher concentration of KorC (Fig. 6A). The short fragments with the separated O_C2 and O_C3 were amplified by PCR with the use of pairs of primers 03 and 11 or 04 and 12, respectively (Table 2). Analysis of KorC binding confirmed the same affinity of KorC to fragment with O_C3 as seen for other "perfect" palindromes and lower affinity to the fragment with O_C2 with the mismatch in one of the arms (Fig. 6B). These experiments indicated that there was no co-operativity between KorC molecules bound to two adjacent O_Cs (O_C2-O_C3 fragment).

3.7 Regulation of *klcAp* by KorC

KorC recognizes and binds to two sites in the *klcAp* region, O_C3 overlaps the promoter (between putative -35 and -10 motifs) and the imperfect O_C2 precedes -35 motif by 26 nt (44 nt between the centers of two regulatory palindromes). Whereas O_C3 is bound by KorC *in vitro* with similar affinity as O_C1 and O_C4, O_C2 is recognized and bound at several fold higher concentration of the regulatory protein. To understand the possible role of tandem operators, the *klcA* promoter region was amplified without upstream sequences containing O_C2 and cloned upstream of the promoter-less *xylE* cassette into pPT01 (pMWB6.10). The comparison of transcriptional activities of both versions of *klcA* promoter regions (pMWB6.9 and pMWB6.10) showed that the deletion of upstream sequences had only slight effect on promoter activity. Both versions of *klcAp* were strongly repressed when production of KorC *in trans* was induced by IPTG (>100 fold repression), however, there was a clear difference in the level of repression at low concentration of repressor (Fig. 2B). The short version of *klcA* promoter region deprived of imperfect O_C2 was 7-fold repressed by KorC whereas the longer version with both O_C2 and O_C3 was almost completely shut off under such conditions (more than 100-fold regulation). The presence of low affinity binding site modulates the sensitivity of the *klcA* promoter *in vivo* towards the fluctuations in the repressor concentration.

3.8 KorC mutant analysis

The structural predictions did not univocally determine the location of HTH motif in the KorC moiety (Fig. 1B). It was decided to substitute the glycine residues occurring in two potential DNA binding motifs by negatively charged residues to impair the proper folding (Fig. 1C). Mutant alleles *korC G34G41* and *korC G88G90* were constructed by site-specific mutagenesis of *korC* and inserted into the expression vector pGBT30 under *tacp* (to obtain pMWB7.25 and pMWB7.24, respectively). Subsequently the ability of two forms of KorC was analyzed *in trans* to *orf02prev-xylE* transcriptional fusion (pMWB6.6) in the regulatory two plasmids assay. There was no difference between two KorC derivatives. Both were impaired in the ability to strongly repress *orf02prev* since no regulatory effect was seen in uninduced cultures carrying pMWB7.25 or pMWB7.24 in trans to pMWB6.6 in contrary to WT KorC (Fig. 2C and 2A). The *korC* alleles were also cloned under T7p into pET28mod and His_6-tagged derivatives were purified. Binding to DNA *in vitro* was also impaired in both forms of the protein (data not shown). Since WT KorC exists as a dimer in solution it was necessary to demonstrate whether any modifications affected the ability to dimerize. *In vitro* assay with glutaraldehyde cross-linking of purified proteins KorC G34DG41D and KorC G88EG90E did not distinguish between two KorC derivatives. Both proteins seemed to be able to form dimers

and higher order complexes *in vitro* similarly to WT KorC (Fig. 5A). The mutated *korC* alleles were also cloned into BACTH system to check their abilities for self-interactions *in vivo.* KorC G34DG41D was fully capable of self-interactions and interactions with WT KorC what was demonstrated by deep purple colonies of appropriate BACTH transformants of BTH101 strain. However, KorC G88EG90E interacted neither with itself nor WT KorC (pale pink colonies of double transformants). The β-galactosidase activity assays in the liquid cultures of these transformants confirmed the plate tests (despite the *in vitro* test) (Fig. 5B). It implicated that dimerization domain is located in the C-terminus of KorC and that altered dimerization properties may result in the impairments of DNA binding activity of KorC as observed in the regulatory studies.

The short deletion was introduced into the *korC* allele removing 15 amino acids from C-end (Fig. 1C). The deletion allele was tested in the BACTH system (Fig. 5B) and clearly confirmed the vital role of this part of KorC in forming dimers.

3.9 Lack of cross-reactivity between KorC repressors from IncP-1 and RA3 plasmids

$KorC_{RK2}$ controls three operons *klcA, kleA* and *kleC*, whose products have the auxiliary roles in the stable maintenance (Thomas et al., 1988; Larsen and Figurski, 1994). R751 has lost *kleC* operon hence $KorC_{R751}$ putatively controls only *klcA* and *kleA* (Thorsted et al., 1998). Multiple KorC binding sites are highly conserved in the same plasmid genome, however they slightly differ between RK2 and R751 having transitions G→A at position 5 and C→T at position 13 (Table 3). The comparison of O_C sites from RA3 and IncP-1 plasmids showed transversions in these positions of the palindrome: C occurred at position 5 and G at position 13. It was decided to check if such subtle DNA changes may affect ability of $KorC_{RA3}$ to bind to the operators from IncP-1 plasmids. The *klcAp* fragments from RK2 and R751 were amplified by PCR (pairs of primers 13 and 14, 15 and 16, respectively) and used in EMSA experiments with $KorC_{RA3}$. Since all primary binding sites in RA3 demonstrate similar affinity for KorC for clarity not *klcAp* with two O_Cs but *or0f2p* with single O_C1was used as the control. $KorC_{RA3}$ binding to the heterologous O_Cs from RK2 and R751 was much weaker than to the cognate operator and in the range of protein concentrations where unspecific DNA binding appeared, observed also for $mobC_{RA3}$ promoter region which does not contain O_C (Fig. 6C).

The *klcA* promoter fragments from RK2 and R751 were also cloned into the promoter-probe vector to verify the cross-reactivity *in vivo* (plasmids pMWB6.22 and pMWB6.23, respectively). The presence of pJSB5.7 (*tacp-korC*) *in trans* affected neither $klcAp_{RK2}$-*xylE* nor $klcA_{R751}$-*xylE* expression when non induced (no IPTG added). Slight decrease in XylE activity for $klcAp_{RK2}$-*xylE* was only observed after full induction of *tacp –korC* transcriptional fusion

by the presence of IPTG (Fig. 2D). Both *in vitro* and *in vivo* assays indicated that there is no cross-reactivity between the regulatory protein from IncU and the corresponding binding sites from IncP-1 plasmids (Fig. 2D).

4. DISCUSSION

The broad-host-range conjugative plasmids play an important role in bacterial genomes evolution. The ability to successfully invade and establish themselves in various unrelated hosts is of particular interest. It requires not only "promiscuous" transfer system but also replication system that functions independently of specific host proteins at least to some degree. BHR plasmids use the strong transcriptional signals to provide the sufficient level of gene expression in different hosts. To limit unnecessary metabolic burden on the host they evolved the regulatory networks negatively controlling the transcription events after establishment (Thomas, 2000).

The best studied broad-host-range conjugative IncP-1 plasmids exemplify the most complex multivalent regulatory network, the combination of local autoregulatory circuits and overlapping regulons controlled by five global regulators, KorA, KorB, KorC, TrbA, IncC (Adamczyk and Jagura-Burdzy, 2003). Some of these regulons are limited to certain plasmid function e.g. KorC regulon encompasses two (in R751) or three operons (in RK2) participating in the stable maintenance (Kornacki et al., 1990; Larsen and Figurski 1994; Thomas et al., 1988), whereas others interlink different plasmid functions. TrbA is the repressor of all four conjugative transfer operons in RK2 as well as the *trfA* operon involved in the vegetative replication (Jagura-Burdzy et al., 1992; Zatyka et al., 1994). KorA coordinates replication and stability functions and provides the switch between vertical and horizontal mode of spreading (Jagura-Burdzy and Thomas, 1994; 1995; Thorsted et al., 1996). KorB is accompanied in the repressor functions by IncC (both proteins are also responsible for plasmid partition) and coordinates all plasmid functions: replication, stability and conjugative transfer (Jagura-Burdzy et al., 1999a; 1999b; Kostelidou and Thomas, 2000; Pansegrau et al., 1994; Thorsted et al., 1998). The global regulators KorB and TrbA evolved the ability of repression at the distance, controlling expression of promoters not necessarily adjacent to the binding sites (Jagura-Burdzy et al., 1999b; Jagura-Burdzy et al., 1992; Bingle et al., 2005). The intertwined regulons and self-regulatory circuits provide each promoter with at least two transcriptional regulators. The sensitivity of regulation is potentiated by co-operativity between the regulatory proteins (Bingle et al., 2003, 2008; Jagura-Burdzy et al., 1999a; Kostelidou et al., 1999; Shingler and Thomas, 1984).

The genomic era confirmed that the co-existence of plasmids from different incompatibility groups in the same host facilitated the frequent DNA exchange leading to transfer of short DNA fragments, whole genes, operons and also functional modules. Whereas self-regulated modules (partition operons, toxin-antitoxin units) easily adapt to the new

genetic surroundings, the modules which run away from the regulatory circuit may create the obstacle for plasmid maintenance. RA3 of IncU group is the example of the mosaic modular BHR conjugative plasmid which putatively acquired bits and pieces of the stability functions from IncP-1 plasmids (Fig. 1A) together with the genes encoding homologues of the regulatory proteins: KorA, KorB and KorC. Whereas $KorA_{RA3}$ and $KorB_{RA3}$ have lost their global regulatory character (Kulinska et al., 2008, 2011), $KorC_{RA3}$ emerged as the main transcriptional regulator in RA3 genome. By controlling five promoters it coordinates the expression of all backbone functions. Two of the KorC-dependent promoters drive transcription of operons in the stability module (*orf02p* and *klcAp*), two of them are putatively responsible for expression of conjugative transfer genes (*orf33p* and *orf34p*), fifth (*orf02prev*) fires towards the replication module probably facilitating the replication process. $KorC_{RA3}$ as the single repressor protein not only co-regulates all backbone functions, but also differentiates the level of their expression.

The extent of KorC repression differs from a few fold in *tra* region to more than 100 fold in stability and replication modules (Fig. 2A). It has been demonstrated that KorC binds to the three primary O_Cs (highly conserved sequences with perfect palindromic arms) with similarly high affinity *in vitro* (Fig. 6A and B). The differences in the regulatory effects *in vivo* are most likely the result of localization of the O_C relatively to RNAP recognition sites (Fig. 3). The most potent regulatory effect is achieved by KorC bound between -35 and -10 motifs (*orf02p* and *klcAp*) and also downstream of -10 sequence (*orf02prev*). In the least affected promoters O_C is either far upstream of -35 motif like for *orf33p* or partly overlapping -35 motif like for *orf34p*. So the architecture of the divergent promoter regions and "flexible" localization of repressor binding sites of the same affinity for regulator result in the tightly controlled maintenance systems *versus* transfer operons permanently expressed at low level.

The role of KlcA, homologue of antirestriction $KlcA_{R751}$ protein (Serfiotis-Mitsa et al., 2010) seems to be important in the process of plasmid establishment in the new hosts, however after this initial phase KlcA probably becomes detrimental to the cells so its synthesis must be shut off. The additional lower affinity "secondary" binding site O_C2 evolved in tandem with the primary O_C3 in the *klcAp*. Although there is no co-operativity *in vitro* between KorC molecules bound at O_C2 and O_C3, the duplicated O_Cs increase the sensitivity of *klcAp* response *in vivo* to low concentrations of the repressor.

The homology search for $KorC_{RA3}$ pulled out the identical proteins encoded by other representatives of IncU group supporting previously observed high level of conservation in the backbone functions of these plasmids (Kulinska et al., 2008; Rhodes et al., 2000; 2004;

Sorum et al., 2003). The homology between $KorC_{RA3}$ and tens of homologues from IncP-1 group and a few from IncL/M group varied between 37-49%, with the highest score for KorC encoded by pQKH54 of IncP-1γ. Comparison of KorCs revealed that the most variable region corresponds to the HTH motif previously identified for $KorC_{RK2}$ (Fig. 1C). The long flexible linker between putative regulatory helices is only present in $KorC_{RA3}$ and $KorC_{pQKH54}$. The modifications of glycine residues in this linker (KorCG34DG41D) had no effect on the dimerization ability but destroyed the capacity of $KorC_{RA3}$ to bind and regulate KorC-dependent promoters, confirming that helices 2 and 3 may form atypical HTH motif. Cross-linking of $KorC_{RA3}$ revealed the existence of various oligomeric forms in the solution. Our data clearly shows that the C-terminus of KorC is involved in the self-interactions.

Since evolution of regulatory proteins goes in hand with the evolution of its binding sites, we have searched the database with the short sequences corresponding to RA3 O_Cs. The O_C sites from RA3, RK2 and R751, other annotated IncP-1 plasmids and a few IncL/M representatives demonstrate high degree of conservation (Table 3). The differences between these mainly hypothetical KorC binding sites are limited to the position 5 and 13 opposite to each other in the palindromic arms (several O_C sites with single mismatches have also been found). In IncU plasmids these positions are occupied by C and G respectively, whereas in IncP-1 (and IncL/M) plasmids the transversions occurred, either G appeared at position 5 and C at position 13 or A appeared at position 5 and T at position 13. We tested both of these IncP-1 versions of O_Cs for $KorC_{RA3}$ binding and none have been effective, clearly proving that these two nucleotides in the binding site are main specificity determinants. It was assumed that the 69% level of homology between KorC proteins of R751 and RK2 and binding sites differing only in one pair of nucleotides are sufficient for cross-reactivity (Thorsted et al., 1998), however it would be important to confirm experimentally their exchangeability.

The modular broad-host-range RA3 plasmid of IncU falls into the category of promiscuous plasmids relying on the coordinate expression of all backbone functions despite its mosaicity. Whereas IncP-1 plasmids mastered the complexity of their regulatory networks to be successful, RA3 uses the simplified but highly effective version of once inherited network. The single global regulatory protein KorC binds only to the three regions in the genome. However the combination of localization of the operator sites in the vital promoters and the arrangements of transcriptional signals (divergent promoters regions) facilitates modulation of the expression of particular backbone functions according to the needs of the broad-host range of this conjugative plasmid.

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Figure legends

Figure 1. Stability module of RA3. Predicted structure of $KorC_{RA3}$.

A/ Comparison of stability modules from RA3 of IncU, and two representatives of IncP-1 plasmids: R751 (IncP-1β) and RK2 (IncP-1α). Homologous genes are indicated. Small arrows correspond to the promoter sequences. The sites of insertions are marked with black triangles. **B/** 3D structure of $KorC_{RA3}$ predicted by I-TASSER online server (Zhang, 2008). The helices are numbered for clarity. The putative HTH motives are marked black (helices 2 and 3) and grey (helices 5 and 6). **C/** Alignment of KorC amino acids sequences from plasmids RA3 (IncU), pQKH54 (IncP-1γ), R751 (IncP-1β) and RK2 (IncP-1α). Similar residues in at least 3 proteins are shadowed black, similar residues in two sequences are shadowed grey. Grey boxes above $KorC_{RA3}$ sequence mark α-helices as presented on Fig. 1B. HTH motif identified in $KorC_{RK2}$ sequence (Kornacki et al., 1990) is underlined. Two pairs of glycine residues modified in KorC G34DG41D and KorC G88EG90E are indicated by black and grey circles, respectively.

Figure 2. $KorC_{RA3}$ as the global transcriptional regulator

DH5α strains with various promoter regions cloned into the promoter–probe vector pPT01 were transformed with empty expression vector pGBT30 and its derivatives producing either WT $KorC_{RA3}$ or mutant KorCs. The catechol 2,3,-oxygenase activity assays were performed on the logarithmically growing cultures in the absence and presence of IPTG. The mean values with standard deviation for at least three assays are shown. **A/** Transcriptional activities of promoters preceded by identified O_C sites in RA3 genome: *orf02p* (pPDB11.19), *orf02prev* (pMWB6.6), *klcAp* (pMWB6.9), *orf33p* (pMWB6.11r) and *orf34p* (pMWB6.11) were measured by XylE activities. Enzymatic assays were performed on extracts from the cultures of double transformants grown without IPTG. Dark grey bars correspond to the strains transformed with pGBT30 (KorC-) and light grey bars to the same strains transformed with pJSB5.7 (KorC+). **B/** Activity of XylE expressed from *klcAp* promoter fragments with both O_C2 and O_C3 sites (pMWB6.9) and only O_C3 site (pMWB6.10) assayed in the extracts of double transformants grown without IPTG. Dark grey bars correspond to the strains transformed with pGBT30 (KorC-) and light grey bars to the same strains transformed with pJSB5.7 (KorC+). **C/** $KorC_{RA3}$ mutant derivatives ability to act as the transcriptional regulators. DH5α (pMWB6.6 *orf02prev-xylE*) strain was transformed with appropriate plasmids. KorC G34DG41D and KorC G88EG90E were produced *in trans* to pMWB6.6 from *tacp* of pMWB7.25 and pMWB7.24, respectively. Double transformants were grown without

("-") and with 0.5 mM IPTG ("+"). The XylE activities were expressed relatively to the activity of the control strain DH5α (pMWB6.6)(pGBT30), black bar labeled "Vector". **D/** Cross-reactivity between $KorC_{RA3}$ and $klcAp_{RK2}$ and $klcAp_{R751}$. DH5α (pMWB6.22 $klcAp_{RK2}$-*xylE*) and DH5α (pMWB6.23 $klcAp_{R751}$-*xylE*) strains were transformed with pGBT30 (vector) or pJSB5.7 (*tacp-korC*) and grown in the presence or absence of IPTG. No differences in XylE activities were observed for double transformants carrying pGBT30 (induced and uninduced) and uninduced cultures of double transformants carrying pJSB5.7. The XylE activity from induced cultures of transformants with pJSB5.7 (light grey bars) is shown relatively to uninduced one (dark grey bars).

Figure 3. Localization of KorC operators relatively to the putative promoter sequences. Identified KorC binding sites are boxed with palindromic arms underlined. The predicted promoters' motives are in grey and directions of transcription are indicated by black arrows. **A/** DNA sequence of the divergent *orf02p/ orf02prev* region (RA3 coordinates from 2103 nt to 2236 nt). The grey arrows labeled # 30 and # 31 correspond to the positions of primers used in pairs with # 6 and # 8, respectively to amplify shorter DNA fragments in this region to map position of *orf02prev*. **B/** DNA sequence of the *klcAp* with two KorC operators (RA3 coordinates from 2494 nt to 2627 nt). **C/** DNA sequence of the divergent *orf33p/ orf34p* region (RA3 coordinates from 31052 nt to 31185 nt).

Figure 4. The effect of KorC over-production on RA3 conjugation frequency and stable maintenance

A/ Frequency of conjugation. DH5α (RA3) strain was transformed with pJSB5.7(*tacp-korC*) or with the empty pGBT30 as a control. Double transformants were used as donors in conjugation with DH5α Rif^R strain as the recipient. The frequency of conjugation is indicated on semi-logarithmic scale as the number of transconjugants/ donor cells. The mean values with standard deviation for at least three experiments are shown. **B/** RA3 plasmid stability assay. DH5α (RA3)(pGBT30) and DH5α (RA3)(pJSB5.7) strains were grown overnight on chloramphenicol and penicillin and then diluted to L broth with penicillin and 0.5 mM IPTG. Approximately every 25 generations the cultures were diluted into the fresh medium and analyzed for RA3 retention. Black line demonstrates RA3 retention in the presence of control pGBT30; grey line indicates RA3 retention in the presence of pJSB5.7 (KorC over-production). The mean values with standard deviation for at least three experiments are shown. **C/** DH5α strain with RA3 minireplicon (pJSB18) was transformed with the pBBR1MCS derivatives: empty expression vector pAMB9.37 (*tacp*) and KorC over-

expressing pJSB4.7 (*tacp-korC*). The transformation mixtures were plated on L agar with selection for incoming plasmid (chloramphenicol) and with selection for both resident and incoming plasmids (chloramphenicol and tetracycline). The photographs demonstrate incompatibility between RA3 minireplicon and pJSB4.7.

Figure 5. $KorC_{RA3}$ dimerization ability.

A/ His_6-tagged WT KorC and its mutant derivatives KorC G34DG41D and KorC G88EG90E were incubated at concentration of 0.05 mg ml^{-1} with increasing amounts of glutaraldehyde. The cross-linked species were separated by SDS-PAGE and transferred onto nitrocellulose filters. Western blotting with anti-His antibodies was used to visualize products. Lane 1 - no glutaraldehyde added; lanes 2 - 5: 0.001%, 0.002%, 0.005% and 0.01% glutaraldehyde, respectively). Roman numbers indicate (I) monomers, (II) dimers, (III) trimers, (IV) tetramers and (V) pentamers. M – molecular weight marker (from the bottom: 15 kDa, 25 kDa, 35 kDa, 40 kDa, 55 kDa and 70 kDa). **B/** β-galactosidase activity assay in BTH101 strain of BACTH system. The *korC* alleles were introduced into BACTH vectors indicated under the diagram. Reconstitution of CyaA activity due to the dimerization ability of the analyzed proteins was assayed by β- galactosidase activity (Miller, 1972) in double transformants cultures. As the negative control BTH101 with empty vectors (pUT18C and pKT25) was used, as the positive control BTH101 with plasmids having CyaA fragments linked to CGN4 leucine zippers was used (zip-zip). The results of interactions between WT KorC (allele linked to *T18* in pUT18C in pMWB13.7) and either WT KorC or its mutant derivatives (alleles linked to *T25* in pKT25) are presented. The mean values with standard deviation for at least three assays are shown.

Figure 6. KorC binding ability to DNA fragments *in vitro* (Electrophoretic Mobility Shift Assay).

A/ $KorC_{RA3}$ binding to the PCR-amplified promoter sequences of RA3 containing O_C operators. Panel I – *orf02p/orf02prev* with O_C1 (primers 05 and 06 used for PCR; RA3 coordinates 2063-2348 nt); II – *klcAp* with O_C2 and O_C3 (primers 03 and 04; RA3 coordinates 2336-2704 nt); III – *orf33p/orf34p* with O_C4 (primers 09 and 10; RA3 coordinates 30977-31326 nt). 0.3 pmoles of DNA was added to each binding reaction. Lane 1 - no protein added, lanes 2 - 8: 1 pmole; 2.5 pmoles; 5 pmoles; 7.5 pmoles; 10 pmoles; 12.5 pmoles and 15 pmoles of KorC, respectively. **B/** $KorC_{RA3}$ binding to separated O_C2 and O_C3. Panel I – *orf02p/orf02prev* with O_C1 (primers as above), II – fragment with O_C2 (primers 03 and 11; RA3 coordinates 2336-2569 nt), III – fragment with O_C3 (primers 04 and 12; RA3 coordinates 2550-2704 nt). 0.3 pmoles of DNA was added to each binding reaction. Lane 1 - no protein added, lanes 2-6 - 1 pmole; 2.5 pmoles; 5 pmoles; 7.5 pmoles; 10 pmoles of KorC,

739 respectively. **C/** $KorC_{RA3}$ binding ability to heterologous O_C sites from RK2 and R751 of
740 IncP-1 group. Panel I – *orf02p/orf02prev* with O_C1_{RA3} (primers as above), II – $klcAp_{RK2}$ with
741 O_C1_{RK2} (primers 13 and 14; RK2 coordinates 11775-11502 nt), III – $klcAp_{R751}$ with O_C1_{R751}
742 (primers 15 and 16; R751 coordinates 8410-8626 nt) and IV – $mobCp_{RA3}$ (primers 25 and 26,
743 RA3 coordinates 9435-9852 nt), run as a negative control. 0.3 pmoles of DNA was added to
744 each binding reaction. Lane 1 - no protein added; lanes 2 - 5: 2.5 pmoles; 5 pmoles; 7.5
745 pmoles and 10 pmoles, respectively.
746

747 Table 1. Plasmids used in this study

Plasmids provided by others			
Designation	**Relevant features or description**	**Copy no.**	**Reference or source**
pABB1.0	pBBR1MCS devoid of EcoRI site in Cm casette	Medium	Aneta Bartosik
pAKB4.10	pPT01 *korAp*$_{RA3}$-*xylE*	Medium	Kulinska et al., 2011
pBBR1MCS	IncA/C, Cm^R, cloning vector	Medium	Kovach et al., 1994
pBGS18	*ori*$_{MB1}$, Km^R, cloning vector	High	Spratt et al., 1986
pET28	*ori*$_{MB1}$, Km^R, T*7p*, *lacO*, His$_6$-tag, T7 tag	Medium	Novagen
pET28mod	pET28 without NdeI, BamHI sites and T7 tag	Medium	Lukaszewicz et al., 2002
pGBT30	*ori*$_{MB1}$, Ap^R, *lacI*^{Q}, *tacp* expression vector	High	Jagura-Burdzy et al., 1992
pGEM-T Easy	*ori*$_{MB1}$Pn^R, cloning vector	High	Promega
pJSB1.24	pBGS18 *korC tra*$_{RA3}$ (RA3 coordinates 3391-3705 and 9437-33657)	High	Bartosik et al. 2012
pKT25	*ori*$_{p15,}$ Km^R, *lacp-cyaT25*-MCS,	Medium	Karimova et al., 1998
pKT25-zip	pKT25 with leucine zipper of GCN4 in translational fusion with *cyaT25*	Medium	Karimova et al., 1998
pLKB2	pKT25 with modified MCS	Medium	Mierzejewska et al., 2012
pLKB4	pUT18C with modified MCS	High	Mierzejewska et al., 2012
pPT01	*ori*$_{SC101}$, Km^R, promoterless *xylE*	Medium	Macartney et al., 1997
pUC18	*ori*$_{MB1}$, Ap^R	High	Yanisch-Perron, 1985
pUT18C	*ori*$_{ColE1,}$ Ap^R, *lacp-cyaT18*-MCS	High	Karimova et al., 1998
pUT18C-zip	pUT18C with leucine zipper of GCN4 in translational fusion with *cyaT18*	High	Karimova et al., 1998
RA3	IncU, Cm^R, Sm^R, Su^R	Low	Finbarr Hayes
Plasmids constructed during this work			
Designation	**Description**		
pAKB4.70	pPT01 *kfrAp*$_{RA3}$-*xylE* (SpHI-BamHI fragment amplified by PCR with primers 23 and 24; RA3 coordinates 5895-6206)		
pAMB9.37	pABB1.0 *lacI*^{Q} *tacp* (EcoRI-BamHI fragment from pGBT30)		
pJSB4.7	pBBR1MCS *tacp-korC* (BamHI-SalI fragment from pJSB5.7)		
pJSB5.7	pGBT30 *tacp-korC* (EcoRI-SalI fragment from pJSB1.7)		
pJSB18	miniRA3Tc^R (RA3 coordinates 43327-45909, 1-2300)		

pMWB6.6	pPT01 *orf02prev-xylE* (BamHI-SphI fragment amplified by PCR with primers 7 and 8; RA3 coordinates 2063-2348)
pMWB6.9	pPT01 *klcAp*$_{RA3}$-*xylE* (O_C2-O_C3) (BamHI-SphI fragment amplified by PCR with primers 3 and 4; RA3 coordinates 2336-2704)
pMWB6.11	pPT01 *orf34p-xylE* (BamHI fragment amplified by PCR with primers 9 and 10; RA3 coordinates 30977-31326)
pMWB6.11r	pPT01 *orf33p-xylE* (BamHI fragment amplified by PCR with primers 9 and 10; RA3 coordinates30977-31326; reverse orientation)
pMWB6.22	pPT01-*klcAp*$_{RK2}$-*xylE* (SphI-BglII fragment amplified by PCR with primers 13 and 14; RK2 coordinates 11775-11502)
pMWB6.23	pPT01 -*klcAp*$_{R751}$-*xylE* (SphI-BglII fragment amplified by PCR with primers 15 and 16; R751 coordinates 8410-8626)
pMWB6.27	pPT01*orf02prev*(part 1)-*xylE* (BamHI-SphI fragment amplified by PCR with primers 8 and 30; RA3 coordinates 2063-2223)
pMWB6.28	pPT01*orf02prev*(part 2)-*xylE* (BamHI-BglII fragment amplified by PCR with primers 6 and 31; RA3 coordinates 2150-2348)
pMWB7.24	pJSB5.7 *korC G88G90* generated by PCR site-specific mutagenesis with primers 19 and 20 (substitutions G88E G90E in KorC)
pMWB7.25	pJSB5.7 *korC G34G41* generated by PCR site-specific mutagenesis with primers 21 and 22 (substitutions G34D G41D in KorC)
pMWB7.26	pJSB5.7 *korC A84H85* (NaeI site generated by PCR site- specific mutagenesis with primers 28 and 29)
pMWB10.7	pET28mod *T7p-korC* (EcoRI-SalI fragment from pJSB5.7)
pMWB10.24	pET28mod *T7p-korC G88G90* (MunI-SalI fragment amplified by PCR with primers 27 and 18 from pMWB7.24)
pMWB10.25	pET28mod *T7p- korC G34G41* (EcoRI-SalI fragment from pMWB7.25)
pMWB13.7	pLKB4 *lacp-cyaT18-korC* translational fusion (EcoRI-HincII fragment from pMWB10.7)
pMWB13.25	pLKB4 *lacp-cyaT18- korC G34G41* translational fusion (EcoRI-HincII fragment from pMWB10.25)
pMWB13.26	pLKB4 *lacp-cyaT18-korC*$_{1-83}$ translational fusion (EcoRI-NaeI fragment from pMWB7.26)
pMWB14.7	pLKB2 *lacp-cyaT25-korC* translational fusion (EcoRI-HincII fragment from pJSB5.7)
pMWB14.24	pLKB2 *lacp-cyaT25-korC G88G90* translational fusion (MunI-SalI fragment amplified by PCR with primers 27 and 18 from pMWB7.24)
pMWB14.25	pLKB2 *lacp-cyaT25-korC G34G41* translational fusion (EcoRI-HincII fragment from pMWB7.25)
pMWB14. 26	pLKB2 *lacp-cyaT25-korC*$_{1-83}$ translational fusion (EcoRI-NaeI fragment from pMWB7.26)
pPDB1.18	pGEM-T Easy *korCp* (fragment amplified by PCR with primers 1 and 2; RA3 coordinates 3093-3431)
pPDB1.19	pGEM-T Easy *orf02p* (fragment amplified by PCR with primers 5 and 6; RA3 coordinates 2063-2348)
pPDB11.18	pPT01 *korCp-xylE* (BamHI-SphI fragment from pPDB1.18)
pPDB11.19	pPT01 *orf02p-xylE* (BamHI-SphI fragment from pPDB1.19)

748

749 Table 2. The list of oligonucleotides used in this work

No	Designation	Sequence
01	korCpRA3L	5’ gcgcatgcCTTAAAGGAGGTGCATAGGT 3’
02	korCpRA3R	5’ gcggatccCAATCTTCAGCAAACGGCCT 3’
03	klcApRA3L	5’ gcgcatgcGGGAGCGTGATCGTTACGGT 3’
04	klcApRA3R	5’ gcggatccATTGCAGCCATACGGCGAGG 3’
05	orf02pRA3L	5’ gcgcatgcCCAGGTGGCCCATTTCGTAC 3’
06	orf02pRA3R	5’ cgggatccCGATCACGCTCCCAGGTCAA 3’
07	SnaB2rRA3L	5’ gcgcatgcCGATCACGCTCCCAGGTCAA 3’
08	SnaB2rRA3R	5’ cgggatccCCAGGTGGCCCATTTCGTAC 3’
09	OC4RA3L	5’ cgggatccATCAGAACCACGGCCTTTGCT 3’
10	OC4RA3R	5’ cgggatccgcatgcCTGCCTCACCGCTAATTGAA 3’
11	LeftOcR	5’ gcgtcgacCTATTGTGTCAAGCGGGTAC 3’
12	RightOcL	5’ gcgcatgcGTACCCGCTTGACACAATAG 3’
13	OcRK2F	5’ gcgcatgcACCGAGCTGTAACCGCAGAA 3’
14	OcRK2R	5’ gcagatctATCCAGCCGAATACCAGGGC 3’
15	OcR751F	5’ gcgcatgcACGGGTTGGTCTTGGGTGTT 3’
16	OcR751R	5’ gcagatctATGCTCAGTTGCTGGGTGGT 3’
17	korCRA3L	5’ gcgaattc**ATG**ATTAGACCTGAAACGCT 3’
18	korCRA3R	5’ cggtcgacTTATGTTCGGTCATGGTTTC 3’
19	G8890EF	5’ GGCCCACCTGGCAG*AA*TTCG*A*GGCTATATGGGACGC 3’
20	G8890ER	5’ GCGTCCCATATAGCC*T*CGAA*TT*CTGCCAGGTGGGCC 3’
21	G3441DF	5’ GCAACGAAAAG*AT*CTTAGTAAGCCGCTCAGTG*A*TGTTGATGTTG 3’
22	G3441DR	5’ CAACATCAACA*T*CACTGAGCGGCTTACTAAG*AT*CTTTTCGTTGC 3’
23	prkfrA1	5’ gcggatccgcatgcCTCGCTGATAACCTGGCCCT 3’
24	prkfrA2	5’ gcggatccCTCGCGCACCTGCTCATTG 3’
25	inc230P	5’ gcggatccGATAGCTCTTTGCCATTAAC 3’
26	Sphmob	5’ gcgcatgcTTTTCTCGTTGGAGGGTGAT 3’
27	korCLMun	5’ gccaattg**ATG**ATTAGACCTGAAACGCT 3’
28	84AHCDF	5’ GTCTATCTTG*TGCG*ACCTGG*C*GGCTTCGGGGCTA 3’
29	84AHCDR	5’ TAGCCCCGAAGCC*G*CCAGGT*CGCAC*AAGATAGAC 3’
30	O2pRvinF	5’ cggcatgcGCGGGTGCCCGGTCTTCTTG 3’
31	O2pRvinR	5’ gcagatctCGTAGAGCGCGCTTTTTATTGCC 3’

750 Sequences in capital letters correspond to the RA3 DNA sequence, restriction sites added are
751 underlined, start codons are in bold, nucleotide substitutions in the primers used for site-
752 directed PCR mutagenesis are in italics
753

Table 3. Comparison of the putative KorC binding sites

IncU		
RA3	O_C1[1]	TAGGCCA TTT TGGCCTA[2]
	O_C2	TAGGCC**G** ATT TGGCCTA
	O_C3, O_C4	TAGGCCA AAA TGGCCTA
pFBAOT	O_C1	TAGGCCA TTT TGGCCTA
	O_C2	TAGGCC**G** ATT TGGCCTA
	O_C3, O_C4	TAGGCCA AAA TGGCCTA
pKP048[3]	O_C1	TAGGCCA TTT TGGCCTA
	O_C2	TAGGCC**G** ATT TGGCCTA
	O_C3	TAGGCCA AAA TGGCCTA
IncP-1[4] (subgroup)		
pQKH54 (gamma)	O_C1, O_C2, O_C3	TAGG**A**CA AAA TG**T**CCTA
RK2 (alpha)	O_C1, O_C2, O_C3	TAGG**G**CA TAA TG**C**CCTA
pYS1 (beta)	O_C1, O_C2	TAGG**G**CA AAA TG**C**CCTA
pA1 (beta)	O_C1	TAGG**G**CA AAA TG**C**CCTA
	O_C2, O_C3	TAGG**G**CA AAA TG**T**CCTA
pJAM7	O_C1	TAGG**G**CA AAA TG**C**CCTA
	O_C2	TAGG**G**CA ATT TG**C**CCTA
pB10 (beta)	O_C1	TAGG**G**CA AAA TG**C**CCTA
	O_C2	TAGG**G**CA AAA TG**T**CCTA
pB12 (beta)	O_C1, O_C2	TAGG**G**CA AAA TG**T**CCTA
pB3 (beta)	O_C1, O_C2	TAGG**A**CA AAA TG**T**CCTA
pHP-42	O_C1	TAGG**G**CA AAA TG**C**CCTA
	O_C2, O_C*	TAGG**G**CA ATT TG**C**CCTA
	O_C**	TAGG**G**CA TTT TG**C**CCTA
p9014	O_C1	TAGG**G**CA ATT TG**C**CCTA
R751 (beta)	O_C1, O_C2	TAGG**A**CA AAA TG**T**CCTA
pIJB1 (delta)	O_C1, O_C2, O_C3	TAGG**G**CA AAA TG**C**CCTA
pKJK5 (epsilon)	O_C1, O_C2	TAGG**G**CA ATT TG**C**CCTA
IncL/M		
pCTX-M3	O_C1	TAGG**A**CA AAT TG**T**CCTA
pEL60	O_C1	TAGG**A**CA AAA TG**T**CCTA

[1] For IncU plasmids O_C1 overlaps *orf02p/ orf02prev*, O_C2 and O_C3 are located in *klcAp* and O_C4 is in the transfer region *orf33p/ orf34p*. For IncP-1 O_C1 is located in *klcAp*, O_C2 in *kleAp* and O_C3 in *kleCp* (Oc* and Oc** in pHP-42 precede short orfs of unknown functions upstream of *klcA* operon). In IncL/M plasmids the single O_C is located in the putative *korCp*.

[2] The comparison of palindromic arms revealed three main classes of O_C site labeled in light grey for IncU subgroup, dark grey for RK2 and black for R751 subgroup. The three nucleotides in the centers of the palindromes are AT pairs. Nucleotides in the palindromic arms different from consensus for KorC IncU are indicated in bold and underlined.

[3] pKP048 carries a part of the RA3 replication module and *orf02-klcA-orf04-korC* region (coordinates 763-3809 nt)

[4] Non IncU plasmids are ordered accordingly to the descending similarities between their KorC proteins and KorC of RA3

Figure1

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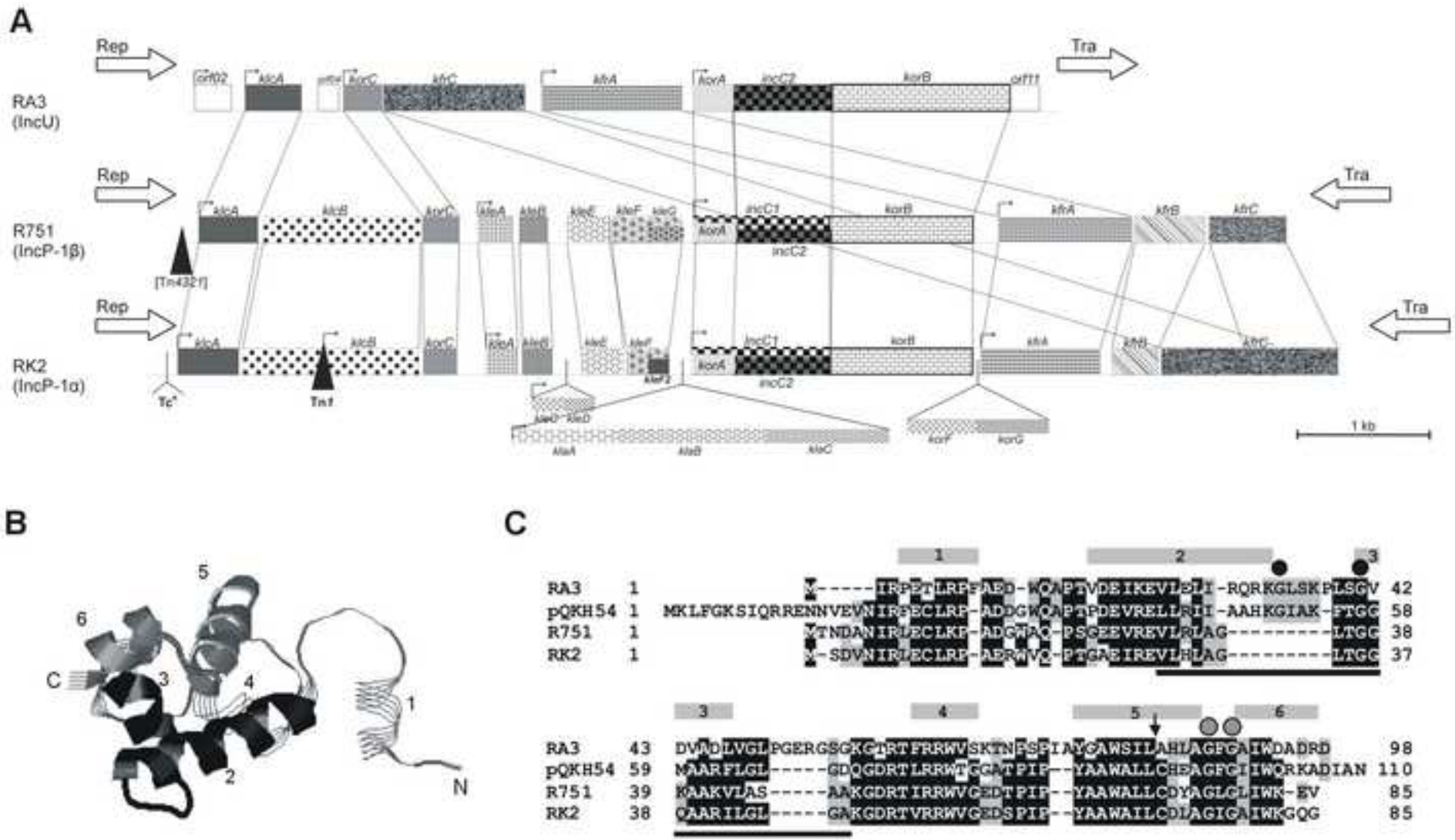

Figure2

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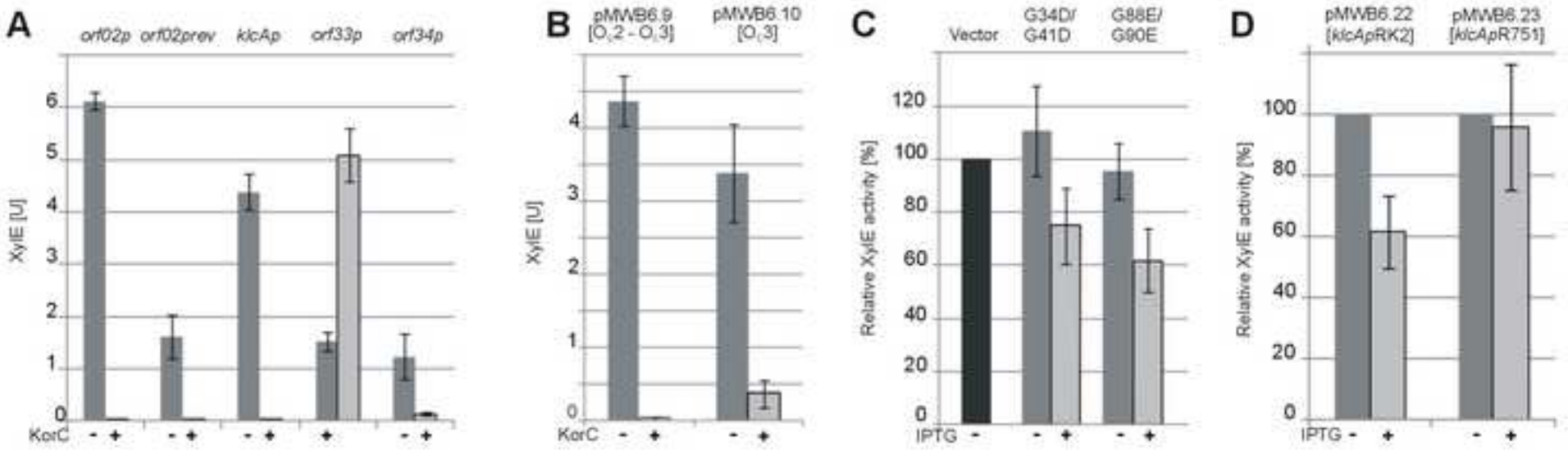

Figure3

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Figure4

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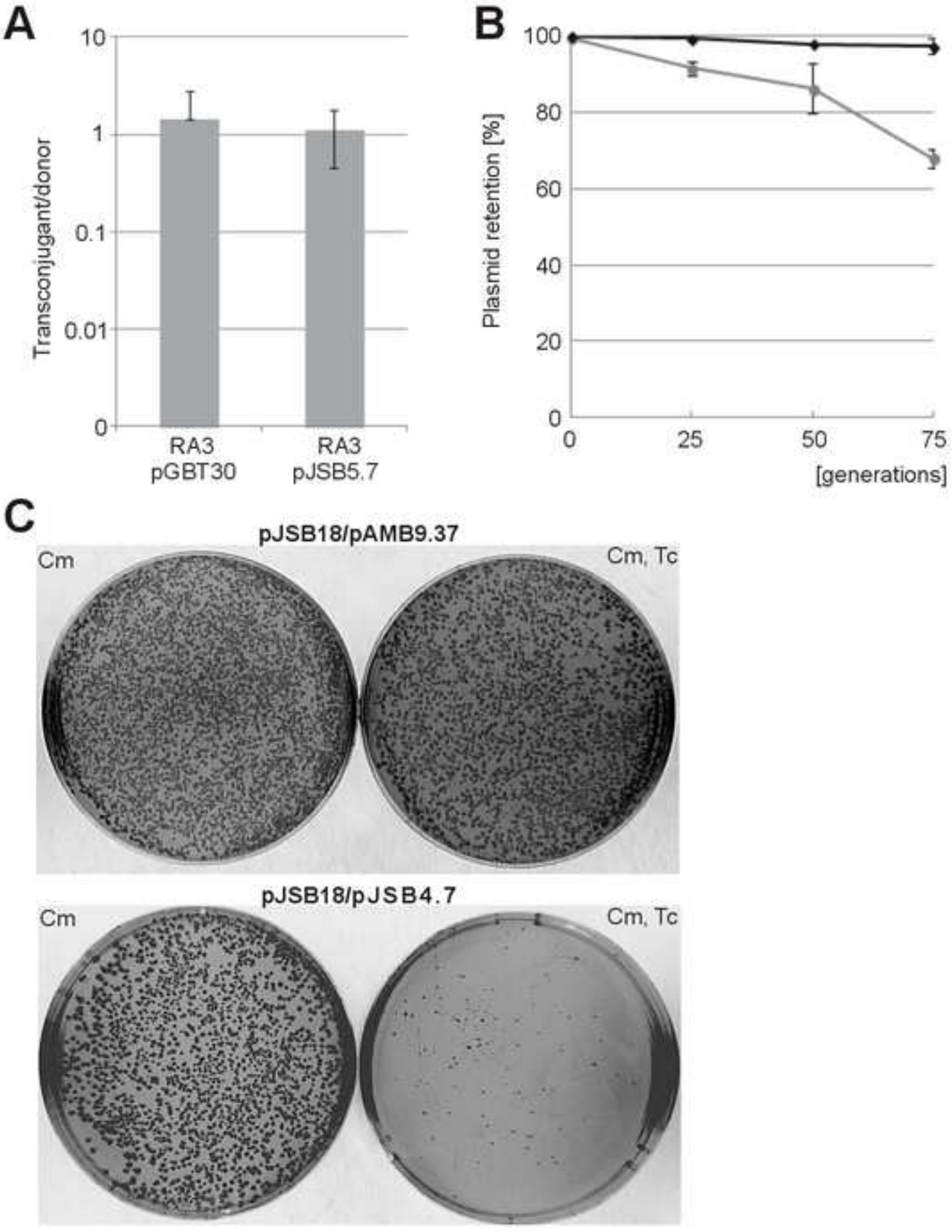

Figure5

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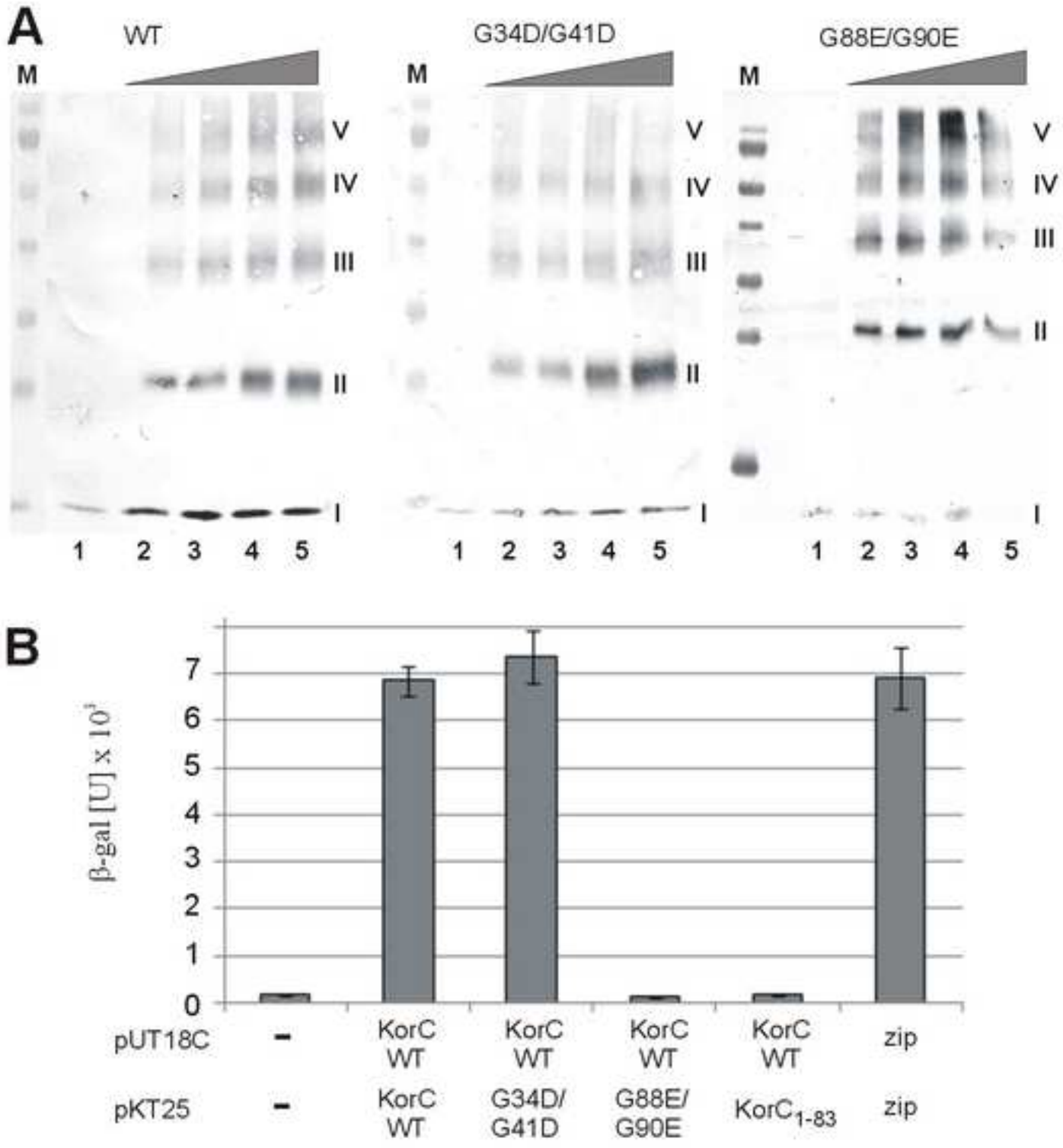

Figure6

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