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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

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

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

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14 Running title: KorC regulon of RA3 plasmid

15

16 Keywords: RA3, broad-host-range IncU plasmid, KorC repressor, global regulation

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18

19 **ABSTRACT**

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

36

37 1. INTRODUCTION

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

47 The stabilization module of RA3 encompasses 10 *orfs* transcribed in the same direction
48 (Fig. 1A). The seven of them encode homologues of IncP-1 products with 30% to 65%
49 similarity at amino acids sequence level, three *orfs* (*orf02*, *orf04* and *orf11*) have no
50 homologues in the database (Kulinska et al., 2008). The *klcA_{RA3}* codes for probable
51 antirestriction protein that shares 55% homology with KlcA_{R751}, recently shown to act at Type
52 I DNA restriction and modification systems (Serfiotis-Mitsa et al., 2010). The *korC_{RA3}* codes
53 for the putative transcriptional repressor, 49% and 41% similar to equivalents of RK2 (IncP-
54 1 α) and R751 (IncP-1 β) plasmids, respectively. The homologues of two putative accessory
55 partition proteins, KfrC and KfrA (69% and 30% similarity to RK2 equivalents, respectively),
56 are encoded upstream of the last part of the stabilization module, the partitioning operon
57 *korAincCkorBorf11* (Kulinska et al., 2011). With the exception of the partition operon, the
58 transcriptional organization of stability regions in representatives of IncU and IncP-1 groups
59 of plasmids differs significantly (Kulinska et al., 2008, Fig. 1A).

60 The homologues of four putative DNA binding proteins encoded in the stability module
61 of RA3 (KorA, KorB, KorC and KfrA) have defined regulatory roles in the RK2 and R751
62 biology ranging from autoregulatory to the global repressor functions (Adamczyk et al., 2006;
63 Balzer et al., 1992; Bechhofer et al., 1986; Jagura-Burdzy et al., 1991; 1999b; Jagura-Burdzy
64 and Thomas, 1992; 1994; 1995; Kornacki et al., 1990; Larsen and Figurski, 1994; Macartney et
65 al., 1997; Motallebi-Veshareh et al., 1992; Shingler and Thomas, 1984; Theophilus et al.,
66 1985; Thomas et al., 1988; 1990).

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

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

77

78 **2. MATERIALS AND METHODS**

79 **2.1 Bacterial strains and growth conditions**

80 *Escherichia coli* strains used were: DH5 α [F⁻(Φ 80dlacZ Δ M15) *recA1 endA1 gyrA96*
81 *thi-1 hsdR17(r_k⁻m_k⁺) supE44 relA1 deoR Δ (lacZYA-argF)U196]; BL21[F⁻ *ompT hsdS_B* (r_B⁻m_B⁻)
82 *gal dcm* (λ DE3)] (Novagen, 2003); BTH101 [F⁻ *cya-99 araD139 galE15 galK16 rpsL1* (Sm^R)
83 *hsdR2 mcrA1 mcrB1*] (Karimova et al., 1998). Bacteria generally were grown in L broth (Kahn
84 et al., 1979) at 37°C or on L agar (L broth with 1.5% w/v agar) supplemented with appropriate
85 antibiotics: benzylpenicillin, sodium salt (150 μ g ml⁻¹ in liquid media and 300 μ g ml⁻¹ in agar
86 plates) for penicillin resistance, kanamycin 50 μ g ml⁻¹ for kanamycin resistance, tetracycline 10
87 μ g ml⁻¹ for tetracycline resistance and chloramphenicol 10 μ g ml⁻¹ for chloramphenicol
88 resistance. MacConkey Agar Base (Difco) supplemented with 1 % maltose was used for
89 BACTH system. L agar used for blue/white screening contained IPTG (0.1 mM) and Xgal (40
90 μ g ml⁻¹). Protein synthesis was induced with the use of IPTG (0.5 mM for BL21 and DH5 α
91 strains; 0.15 mM and 0.5 mM for BTH101 strain grown in liquid media and agar plates,
92 respectively).*

93 **2.2 Plasmid DNA isolation, analysis, cloning and manipulation**

94 Plasmid DNA was isolated and manipulated by standard procedures (Sambrook et al.,
95 1989). The list of plasmids used and constructed in this study is presented in Table 1. Standard
96 PCR reactions (Mullis et al., 1986) were performed with pairs of primers listed in Table 2. All
97 PCR-derived clones were analyzed by DNA sequencing to check their fidelity.

98 **2.3 Site-directed mutagenesis *in vitro***

99 To create mutations in *korC* an *in vitro* site-directed mutagenesis method (Stratagene,
100 2006) was used with the high fidelity PfuTurbo DNA polymerase. The primers 19 to 22, 28
101 and 29 (Table 2) were designed to insert nucleotide substitutions in the particular region
102 accompanied by restriction cleavage site to facilitate screening. PCR reactions to introduce
103 mutations were performed with an initial denaturation step (96°C for 5 minutes) and 18 cycles
104 of denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at
105 68°C for 14 minutes. Reactions ended with a final elongation step (68°C for 25 minutes). The
106 PCR product was treated with DpnI endonuclease to remove template DNA and used for
107 transformations. The plasmid DNA of putative mutant was tested for the presence of restriction
108 site introduced in the mutagenic primers and the effect of mutagenesis was verified by
109 sequencing.

110 **2.4 Bacterial transformation**

111 Competent cells of *E. coli* were prepared by standard CaCl₂ method (Sambrook et al., 1989).

112 **2.5 Determination of catechol 2, 3-oxygenase activity (XylE)**

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

117 **2.6 Purification of His₆-tailed KorC derivatives**

118 For protein over-production and purification, *E. coli* BL21(DE3) was transformed with
119 one of the constructs: pMWB10.7, pMWB10.24 or pMWB10.25 encoding N-terminally His₆-
120 tagged KorCs. The purification procedure was performed as described previously (Jagura-
121 Burdzy and Thomas, 1995) with the use of sonication buffer (50 mM sodium phosphate pH
122 8.0; 300 mM NaCl). Protein fractions were analyzed by SDS-PAGE using a Pharmacia
123 PHAST system with 20% homogeneous gels.

124 **2.7 Analysis of protein-DNA interactions by electrophoretic mobility shift assay (EMSA)**

125 The PCR-amplified DNA fragments were excised from agarose gels and purified by
126 Gel-Out kit (A&A Biotechnology). Concentration of the isolated DNA fragments was
127 determined with NanoDrop 2000. The protein-DNA binding reactions were performed in the
128 binding buffer (50 mM Tris-HCl pH 8.0; 10 mM MgCl₂; 50 mM NaCl; 0.2 mg ml⁻¹ BSA) with
129 increasing amounts of His₆-KorC added in a final volume of 20 μl. Binding reactions were
130 analyzed on 1.2% agarose gels in 0.5xTBE buffer (89 mM Tris-borate and 2 mM EDTA, pH
131 8.3). The gels were stained with ethidium bromide and DNA was visualized under UV light.

132 **2.8 Cross-linking with glutaraldehyde**

133 His₆-tagged KorC purified on Ni²⁺-agarose column was cross-linked by the use of
134 glutaraldehyde (Jagura-Burdzy and Thomas, 1995) and separated on 20% (w/v) SDS-PAGE
135 gels. The proteins were transferred onto nitrocellulose membrane and Western blot analysis
136 with anti-His₆-Tag antibodies was performed as described previously (Bartosik et al., 2004).

137 **2.9 Conjugation procedure**

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

144 parallel 100 μ l of donor strain overnight culture was incubated on L agar plate for 2 hours at
145 37°C, cells were scrapped, diluted and plated on L agar or L agar with antibiotics selective for
146 donor strain. The transfer frequency was calculated as the number of transconjugants per donor
147 cell.

148 **2.10 Bacterial Adenylate Cyclase Two-Hybrid System (BACTH system)**

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

164 **2.11 Stability assay**

165 To check the effect of KorC over-expression on RA3 stability *E. coli* DH5 α strain with
166 RA3 plasmid was transformed either with pGBT30 (as a control) or pJSB5.7 (for KorC over-
167 expression). Overnight cultures of transformants grown on L broth supplemented with 10 μ g
168 ml⁻¹chloramphenicol and 150 μ g ml⁻¹ penicillin were 10⁵ fold diluted into fresh medium
169 supplemented with 150 μ g ml⁻¹ penicillin and 0.5 mM IPTG. The cultures were diluted
170 repeatedly every 24 hours and simultaneously the diluted cultures were plated on L agar with
171 300 μ g ml⁻¹ penicillin. The plates were incubated overnight at 37°C and 100 colonies were re-
172 streaked on L agar supplemented with either 300 μ g ml⁻¹ penicillin and 0.5 mM IPTG or 300
173 μ g ml⁻¹ penicillin, 10 μ g ml⁻¹chloramphenicol and 0.5 mM IPTG to estimate a plasmid
174 retention rate.

175

176 3. RESULTS

177 3.1 KorC regulates three promoters in the stability module

178 The *korC* locates in the stable maintenance module between short *orf04* of unknown
179 function and homologue of *kfrC* from IncP-1 (Fig. 1A). KorC is a relatively small protein (98
180 amino acids) with two putative α -Helix-turn- α -Helix motifs predicted by I-TASSER server
181 (Fig. 1B; Zhang, 2008). The alignment of amino acids sequences of KorC_{RA3} and its
182 homologues from RK2 (IncP-1 α), R751(IncP-1 β) and pQKH54 (IncP-1 γ) reveals that the least
183 conserved region is around HTH motif previously suggested for KorC_{RK2} (Kornacki et al.,
184 1990) (Fig. 1C). The helices in this motif are separated by long linker in RA3; the linker is
185 conserved in the closest homologue KorC_{pQKH54} and absent in other KorCs.

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

204 To check which promoters (if any) are under presumable control of KorC, the *korC* has
205 been cloned under *tacp* into the expression vector pGBT30 compatible with pPT01 (Jagura-
206 Burdzy et al., 1991) to construct pJSB5.7. DH5 α strains with resident plasmids carrying
207 analyzed promoter regions in transcriptional fusions with *xylE* were transformed with pGBT30
208 (vector) and pJSB5.7. The catechol 2,3- oxygenase activity assays in the logarithmic cultures

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

214 **3.2 Identification of KorC binding sites in the stability module**

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

225 **3.3 KorC regulates expression of the conjugative transfer functions**

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

239 The genetic data has been gathered to support this conclusion. The attempts to clone
240 the conjugative transfer module into the high copy number plasmid pBGS18 were
241 unsuccessful leading to rise of minute colonies, unable to grow after passage. However, when
242 the recipient strain DH5 α expressed also *korC* from *tacp-korC* transcriptional fusion

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

251 We checked whether excess of KorC may disturb the RA3 ability to spread. The donor
252 strain DH5 α (RA3) (pJSB5.7) was grown overnight on selective antibiotics and 0.5 mM
253 IPTG. No effect of KorC over-production was observed on conjugation frequency of RA3
254 plasmid (Fig. 4A) indicating that the complete system is highly balanced and not easily
255 disturbed, at least for approximately 20 generations.

256 **3.4 KorC controls the replication functions of RA3.**

257 The divergent promoters *orf02p-orf02prev* are located in the region between two
258 functional modules: replication and maintenance. The repression of both promoters by KorC
259 implicated the role of KorC not only in the expression of the operon in the stability module
260 but also in the functioning of the replication module. The overnight culture of DH5 α (RA3)
261 (pJSB5.7) strain grown in the presence of chloramphenicol and penicillin was diluted
262 repeatedly into the medium with penicillin and 0.5 mM IPTG and every 25 generations
263 checked for RA3 retention. Over-expression of KorC *in trans* to the intact RA3 destabilized
264 the parental plasmid (Fig. 4B). After approximately 75 generations 30% of cells lost RA3
265 whereas no loss was observed even after 100 generations without KorC excess.

266 The minireplicon of RA3 (pJSB18) encompassing the replication module and the
267 divergent promoter region *orf02p/orf02prev* was constructed (RA3 coordinates 43327-45909,
268 1-2300). We used *E. coli* DH5 α (pJSB18) as the recipient in transformation with two
269 derivatives of the broad-host-range vector pBBR1MCS: pAMB9.37 (pBBR1MCS-*lacI^Q tacp*)
270 and pJSB4.7 (pBBR1MCS *lacI^Q tacp-korC*). Whereas numerous well grown colonies of
271 DH5 α (pJSB18) (pAMB9.37) appeared on plates selective for resident and incoming
272 plasmids, only scarce transformants of DH5 α (pJSB18) (pJSB4.7) grown as minute colonies
273 appeared on double selection plates (Fig. 4C). The frequencies of transformations of the same
274 recipient strain but with selection for incoming plasmids were very similar for both
275 pAMB9.37 and pJSB4.7 ($>10^4$ colonies per ml of transformation mixture) indicating that the

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

280 **3.5 KorC exists as a dimer in solution**

281 The *korC* was cloned into pET28 derivative under T7p to facilitate purification.
282 Purified His₆-tagged KorC migrated on SDS-PAGE gels as polypeptide of MW 14 kDa. Cross-
283 linking of KorC with increasing concentration of glutaraldehyde demonstrated ability of the
284 protein to form dimers and higher order complexes in solution as shown on Fig. 5A.
285 The ability of KorC to self-interact was also confirmed *in vivo* in bacterial two hybrid system
286 BACTH (Karimova et al., 1998) by translationally linking *cyaA* domains with *korC*.
287 Dimerization of KorC manifested in the deep purple color of colonies plated and re-streaked on
288 MacConkey agar supplemented with maltose, IPTG, kanamycin and penicillin (data not
289 shown). Self-interaction of KorC was highly effective since it was giving 7000 U of LacZ
290 activity in the liquid cultures, comparable to the activity of LacZ in the BTH101 strain
291 transformed with BACTH plasmids with *cya* domains linked to GCN4 leucine zipper
292 fragments, used as the positive control for strong interactions (Fig. 5B).

293 **3.6 KorC binds to all KorC-regulated promoters with similar affinity *in vitro***

294 Putative KorC binding sites were numbered sequentially according to their position in
295 RA3 genome: O_C1 maps in the divergent promoter region *orf02p/orf02prev*, O_C2 and O_C3 are
296 localized in *klcAp* and O_C4 occurs in the divergent promoter region *orf33p/orf34p*.
297 DNA fragments of 150 to 300 bp in size containing O_C1, O_C2-O_C3 and O_C4 were PCR-
298 amplified and used in EMSA experiments with the purified His₆-KorC. KorC shifted all three
299 fragments with similar K_{app} of about 120 nM (K_{app} is defined as the protein concentration at
300 which 50% of probe is shifted), however in the case of *klcAp* fragment with two KorC binding
301 sites (O_C2-O_C3), two distinct retarded species were seen. The first retarded species appeared at
302 low KorC concentration and the second at least at a 5-fold higher concentration of KorC (Fig.
303 6A). The short fragments with the separated O_C2 and O_C3 were amplified by PCR with the use
304 of pairs of primers 03 and 11 or 04 and 12, respectively (Table 2). Analysis of KorC binding
305 confirmed the same affinity of KorC to fragment with O_C3 as seen for other “perfect”
306 palindromes and lower affinity to the fragment with O_C2 with the mismatch in one of the arms
307 (Fig. 6B). These experiments indicated that there was no co-operativity between KorC
308 molecules bound to two adjacent O_Cs (O_C2-O_C3 fragment).

309

310 **3.7 Regulation of *klcAp* by KorC**

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

327 **3.8 KorC mutant analysis**

328 The structural predictions did not univocally determine the location of HTH motif in the
329 KorC moiety (Fig. 1B). It was decided to substitute the glycine residues occurring in two
330 potential DNA binding motifs by negatively charged residues to impair the proper folding (Fig.
331 1C). Mutant alleles *korC G34G41* and *korC G88G90* were constructed by site-specific
332 mutagenesis of *korC* and inserted into the expression vector pGBT30 under *tacp* (to obtain
333 pMWB7.25 and pMWB7.24, respectively). Subsequently the ability of two forms of KorC was
334 analyzed *in trans* to *orf02prev-xylE* transcriptional fusion (pMWB6.6) in the regulatory two
335 plasmids assay. There was no difference between two KorC derivatives. Both were impaired in
336 the ability to strongly repress *orf02prev* since no regulatory effect was seen in uninduced
337 cultures carrying pMWB7.25 or pMWB7.24 in trans to pMWB6.6 in contrary to WT KorC
338 (Fig. 2C and 2A). The *korC* alleles were also cloned under T7p into pET28mod and His₆-
339 tagged derivatives were purified. Binding to DNA *in vitro* was also impaired in both forms of
340 the protein (data not shown). Since WT KorC exists as a dimer in solution it was necessary to
341 demonstrate whether any modifications affected the ability to dimerize. *In vitro* assay with
342 glutaraldehyde cross-linking of purified proteins KorC G34DG41D and KorC G88EG90E did
343 not distinguish between two KorC derivatives. Both proteins seemed to be able to form dimers

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

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

357 **3.9 Lack of cross-reactivity between KorC repressors from IncP-1 and RA3 plasmids**

358 KorC_{RK2} controls three operons *klcA*, *kleA* and *kleC*, whose products have the auxiliary
359 roles in the stable maintenance (Thomas et al., 1988; Larsen and Figurski, 1994). R751 has lost
360 *kleC* operon hence KorC_{R751} putatively controls only *klcA* and *kleA* (Thorsted et al., 1998).
361 Multiple KorC binding sites are highly conserved in the same plasmid genome, however they
362 slightly differ between RK2 and R751 having transitions G→A at position 5 and C→T at
363 position 13 (Table 3). The comparison of O_C sites from RA3 and IncP-1 plasmids showed
364 transversions in these positions of the palindrome: C occurred at position 5 and G at position
365 13. It was decided to check if such subtle DNA changes may affect ability of KorC_{RA3} to bind
366 to the operators from IncP-1 plasmids. The *klcAp* fragments from RK2 and R751 were
367 amplified by PCR (pairs of primers 13 and 14, 15 and 16, respectively) and used in EMSA
368 experiments with KorC_{RA3}. Since all primary binding sites in RA3 demonstrate similar affinity
369 for KorC for clarity not *klcAp* with two O_Cs but *orof2p* with single O_C1 was used as the control.
370 KorC_{RA3} binding to the heterologous O_Cs from RK2 and R751 was much weaker than to the
371 cognate operator and in the range of protein concentrations where unspecific DNA binding
372 appeared, observed also for *mobC*_{RA3} promoter region which does not contain O_C (Fig. 6C).

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

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

381

382 4. DISCUSSION

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

391 The best studied broad-host-range conjugative IncP-1 plasmids exemplify the most
392 complex multivalent regulatory network, the combination of local autoregulatory circuits and
393 overlapping regulons controlled by five global regulators, KorA, KorB, KorC, TrbA, IncC
394 (Adamczyk and Jagura-Burdzy, 2003). Some of these regulons are limited to certain plasmid
395 function e.g. KorC regulon encompasses two (in R751) or three operons (in RK2)
396 participating in the stable maintenance (Kornacki et al., 1990; Larsen and Figurski 1994;
397 Thomas et al., 1988), whereas others interlink different plasmid functions. TrbA is the
398 repressor of all four conjugative transfer operons in RK2 as well as the *trfA* operon involved
399 in the vegetative replication (Jagura-Burdzy et al., 1992; Zatyka et al., 1994). KorA
400 coordinates replication and stability functions and provides the switch between vertical and
401 horizontal mode of spreading (Jagura-Burdzy and Thomas, 1994; 1995; Thorsted et al., 1996).
402 KorB is accompanied in the repressor functions by IncC (both proteins are also responsible
403 for plasmid partition) and coordinates all plasmid functions: replication, stability and
404 conjugative transfer (Jagura-Burdzy et al., 1999a; 1999b; Kostelidou and Thomas, 2000;
405 Pansegrau et al., 1994; Thorsted et al., 1998). The global regulators KorB and TrbA evolved
406 the ability of repression at the distance, controlling expression of promoters not necessarily
407 adjacent to the binding sites (Jagura-Burdzy et al., 1999b; Jagura-Burdzy et al., 1992; Bingle
408 et al., 2005). The intertwined regulons and self-regulatory circuits provide each promoter with
409 at least two transcriptional regulators. The sensitivity of regulation is potentiated by co-
410 operativity between the regulatory proteins (Bingle et al., 2003, 2008; Jagura-Burdzy et al.,
411 1999a; Kostelidou et al., 1999; Shingler and Thomas, 1984).

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

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

429 The extent of KorC repression differs from a few fold in *tra* region to more than 100
430 fold in stability and replication modules (Fig. 2A). It has been demonstrated that KorC binds
431 to the three primary O_Cs (highly conserved sequences with perfect palindromic arms) with
432 similarly high affinity *in vitro* (Fig. 6A and B). The differences in the regulatory effects *in*
433 *vivo* are most likely the result of localization of the O_C relatively to RNAP recognition sites
434 (Fig. 3). The most potent regulatory effect is achieved by KorC bound between -35 and -10
435 motifs (*orf02p* and *klcAp*) and also downstream of -10 sequence (*orf02prev*). In the least
436 affected promoters O_C is either far upstream of -35 motif like for *orf33p* or partly overlapping
437 -35 motif like for *orf34p*. So the architecture of the divergent promoter regions and “flexible”
438 localization of repressor binding sites of the same affinity for regulator result in the tightly
439 controlled maintenance systems *versus* transfer operons permanently expressed at low level.

440 The role of KlcA, homologue of antirestriction KlcA_{R751} protein (Serfiotis-Mitsa et al.,
441 2010) seems to be important in the process of plasmid establishment in the new hosts,
442 however after this initial phase KlcA probably becomes detrimental to the cells so its
443 synthesis must be shut off. The additional lower affinity “secondary” binding site O_{C2}
444 evolved in tandem with the primary O_{C3} in the *klcAp*. Although there is no co-operativity *in*
445 *vitro* between KorC molecules bound at O_{C2} and O_{C3}, the duplicated O_Cs increase the
446 sensitivity of *klcAp* response *in vivo* to low concentrations of the repressor.

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

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

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

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

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487

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639 **Figure legends**

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

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

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

654 DH5 α strains with various promoter regions cloned into the promoter–probe vector pPT01
655 were transformed with empty expression vector pGBT30 and its derivatives producing either
656 WT KorC_{RA3} or mutant KorCs. The catechol 2,3,-oxygenase activity assays were performed
657 on the logarithmically growing cultures in the absence and presence of IPTG. The mean
658 values with standard deviation for at least three assays are shown. **A/** Transcriptional activities
659 of promoters preceded by identified O_C sites in RA3 genome: *orf02p* (pPDB11.19), *orf02prev*
660 (pMWB6.6), *klcAp* (pMWB6.9), *orf33p* (pMWB6.11r) and *orf34p* (pMWB6.11) were
661 measured by Xyle activities. Enzymatic assays were performed on extracts from the cultures
662 of double transformants grown without IPTG. Dark grey bars correspond to the strains
663 transformed with pGBT30 (KorC-) and light grey bars to the same strains transformed with
664 pJSB5.7 (KorC+). **B/** Activity of Xyle expressed from *klcAp* promoter fragments with both
665 O_C2 and O_C3 sites (pMWB6.9) and only O_C3 site (pMWB6.10) assayed in the extracts of
666 double transformants grown without IPTG. Dark grey bars correspond to the strains
667 transformed with pGBT30 (KorC-) and light grey bars to the same strains transformed with
668 pJSB5.7 (KorC+). **C/** KorC_{RA3} mutant derivatives ability to act as the transcriptional
669 regulators. DH5 α (pMWB6.6 *orf02prev-xyle*) strain was transformed with appropriate
670 plasmids. KorC G34DG41D and KorC G88EG90E were produced *in trans* to pMWB6.6 from
671 *tacp* of pMWB7.25 and pMWB7.24, respectively. Double transformants were grown without

672 (“-“) and with 0.5 mM IPTG (“+”). The Xyle activities were expressed relatively to the
673 activity of the control strain DH5 α (pMWB6.6)(pGBT30), black bar labeled “Vector”.
674 **D/** Cross-reactivity between KorC_{RA3} and *klcAp_{RRK2}* and *klcAp_{R751}*. DH5 α (pMWB6.22
675 *klcAp_{RRK2-xyle}*) and DH5 α (pMWB6.23 *klcAp_{R751-xyle}*) strains were transformed with
676 pGBT30 (vector) or pJSB5.7 (*tacp-korC*) and grown in the presence or absence of IPTG. No
677 differences in Xyle activities were observed for double transformants carrying pGBT30
678 (induced and uninduced) and uninduced cultures of double transformants carrying pJSB5.7.
679 The Xyle activity from induced cultures of transformants with pJSB5.7 (light grey bars) is
680 shown relatively to uninduced one (dark grey bars).

681 **Figure 3. Localization of KorC operators relatively to the putative promoter sequences.**

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

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

692 **A/** Frequency of conjugation. DH5 α (RA3) strain was transformed with pJSB5.7(*tacp-korC*)
693 or with the empty pGBT30 as a control. Double transformants were used as donors in
694 conjugation with DH5 α Rif^R strain as the recipient. The frequency of conjugation is indicated
695 on semi-logarithmic scale as the number of transconjugants/ donor cells. The mean values
696 with standard deviation for at least three experiments are shown. **B/** RA3 plasmid stability
697 assay. DH5 α (RA3)(pGBT30) and DH5 α (RA3)(pJSB5.7) strains were grown overnight on
698 chloramphenicol and penicillin and then diluted to L broth with penicillin and 0.5 mM IPTG.
699 Approximately every 25 generations the cultures were diluted into the fresh medium and
700 analyzed for RA3 retention. Black line demonstrates RA3 retention in the presence of control
701 pGBT30; grey line indicates RA3 retention in the presence of pJSB5.7 (KorC over-
702 production). The mean values with standard deviation for at least three experiments are
703 shown. **C/** DH5 α strain with RA3 minireplicon (pJSB18) was transformed with the
704 pBBR1MCS derivatives: empty expression vector pAMB9.37 (*tacp*) and KorC over-

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

709 **Figure 5. KorC_{RA3} dimerization ability.**

710 **A/** His₆-tagged WT KorC and its mutant derivatives KorC G34DG41D and KorC G88EG90E
711 were incubated at concentration of 0.05 mg ml⁻¹ with increasing amounts of glutaraldehyde.
712 The cross-linked species were separated by SDS-PAGE and transferred onto nitrocellulose
713 filters. Western blotting with anti-His antibodies was used to visualize products. Lane 1 - no
714 glutaraldehyde added; lanes 2 - 5: 0.001%, 0.002%, 0.005% and 0.01% glutaraldehyde,
715 respectively). Roman numbers indicate (I) monomers, (II) dimers, (III) trimers, (IV) tetramers
716 and (V) pentamers. M – molecular weight marker (from the bottom: 15 kDa, 25 kDa, 35 kDa,
717 40 kDa, 55 kDa and 70 kDa). **B/** β-galactosidase activity assay in BTH101 strain of BACTH
718 system. The *korC* alleles were introduced into BACTH vectors indicated under the diagram.
719 Reconstitution of CyaA activity due to the dimerization ability of the analyzed proteins was
720 assayed by β- galactosidase activity (Miller, 1972) in double transformants cultures. As the
721 negative control BTH101 with empty vectors (pUT18C and pKT25) was used, as the positive
722 control BTH101 with plasmids having CyaA fragments linked to CGN4 leucine zippers was
723 used (zip-zip). The results of interactions between WT KorC (allele linked to *T18* in pUT18C
724 in pMWB13.7) and either WT KorC or its mutant derivatives (alleles linked to *T25* in pKT25)
725 are presented. The mean values with standard deviation for at least three assays are shown.

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

728 **A/** KorC_{RA3} binding to the PCR-amplified promoter sequences of RA3 containing O_C
729 operators. Panel I – *orf02p/orf02prev* with O_{C1} (primers 05 and 06 used for PCR; RA3
730 coordinates 2063-2348 nt); II – *klcAp* with O_{C2} and O_{C3} (primers 03 and 04; RA3 coordinates
731 2336-2704 nt); III – *orf33p/orf34p* with O_{C4} (primers 09 and 10; RA3 coordinates 30977-
732 31326 nt). 0.3 pmoles of DNA was added to each binding reaction. Lane 1 - no protein added,
733 lanes 2 - 8: 1 pmole; 2.5 pmoles; 5 pmoles; 7.5 pmoles; 10 pmoles; 12.5 pmoles and 15
734 pmoles of KorC, respectively. **B/** KorC_{RA3} binding to separated O_{C2} and O_{C3}. Panel I –
735 *orf02p/orf02prev* with O_{C1} (primers as above), II – fragment with O_{C2} (primers 03 and 11;
736 RA3 coordinates 2336-2569 nt), III – fragment with O_{C3} (primers 04 and 12; RA3
737 coordinates 2550-2704 nt). 0.3 pmoles of DNA was added to each binding reaction. Lane 1 -
738 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 cassette	Medium	Aneta Bartosik
pAKB4.10	pPT01 <i>korAp_{RA3}-xylE</i>	Medium	Kulinska et al., 2011
pBBR1MCS	IncA/C, Cm ^R , cloning vector	Medium	Kovach et al., 1994
pBGS18	<i>ori_{MB1}</i> , Km ^R , cloning vector	High	Spratt et al., 1986
pET28	<i>ori_{MB1}</i> , Km ^R , T7p, <i>lacO</i> , His ₆ -tag, T7 tag	Medium	Novagen
pET28mod	pET28 without NdeI, BamHI sites and T7 tag	Medium	Lukaszewicz et al., 2002
pGBT30	<i>ori_{MB1}</i> , Ap ^R , <i>lacI^Q</i> , <i>tacp</i> expression vector	High	Jagura-Burdzy et al., 1992
pGEM-T Easy	<i>ori_{MB1}Pn^R</i> , cloning vector	High	Promega
pJSB1.24	pBGS18 <i>korC tra_{RA3}</i> (RA3 coordinates 3391-3705 and 9437-33657)	High	Bartosik et al. 2012
pKT25	<i>ori_{p15}</i> , Km ^R , <i>lacp-cyaT25</i> -MCS,	Medium	Karimova et al., 1998
pKT25-zip	pKT25 with leucine zipper of GCN4 in translational fusion with <i>cyaT25</i>	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	<i>ori_{SC101}</i> , Km ^R , promoterless <i>xylE</i>	Medium	Macartney et al., 1997
pUC18	<i>ori_{MB1}</i> , Ap ^R	High	Yanisch-Perron, 1985
pUT18C	<i>ori_{ColE1}</i> , Ap ^R , <i>lacp-cyaT18</i> -MCS	High	Karimova et al., 1998
pUT18C-zip	pUT18C with leucine zipper of GCN4 in translational fusion with <i>cyaT18</i>	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 <i>kfrAp_{RA3}-xylE</i> (SpHI-BamHI fragment amplified by PCR with primers 23 and 24; RA3 coordinates 5895-6206)		
pAMB9.37	pABB1.0 <i>lacI^Q tacp</i> (EcoRI-BamHI fragment from pGBT30)		
pJSB4.7	pBBR1MCS <i>tacp-korC</i> (BamHI-SalI fragment from pJSB5.7)		
pJSB5.7	pGBT30 <i>tacp-korC</i> (EcoRI-SalI fragment from pJSB1.7)		
pJSB18	miniRA3Tc ^R (RA3 coordinates 43327-45909, 1-2300)		

pMWB6.6	pPT01 <i>orf02prev-xylE</i> (BamHI-SphI fragment amplified by PCR with primers 7 and 8; RA3 coordinates 2063-2348)
pMWB6.9	pPT01 <i>klcAp_{RA3}-xylE</i> (O _{C2} -O _{C3}) (BamHI-SphI fragment amplified by PCR with primers 3 and 4; RA3 coordinates 2336-2704)
pMWB6.11	pPT01 <i>orf34p-xylE</i> (BamHI fragment amplified by PCR with primers 9 and 10; RA3 coordinates 30977-31326)
pMWB6.11r	pPT01 <i>orf33p-xylE</i> (BamHI fragment amplified by PCR with primers 9 and 10; RA3 coordinates 30977-31326; reverse orientation)
pMWB6.22	pPT01- <i>klcAp_{RK2}-xylE</i> (SphI-BglII fragment amplified by PCR with primers 13 and 14; RK2 coordinates 11775-11502)
pMWB6.23	pPT01 - <i>klcAp_{R751}-xylE</i> (SphI-BglII fragment amplified by PCR with primers 15 and 16; R751 coordinates 8410-8626)
pMWB6.27	pPT01 <i>orf02prev</i> (part 1)- <i>xylE</i> (BamHI-SphI fragment amplified by PCR with primers 8 and 30; RA3 coordinates 2063-2223)
pMWB6.28	pPT01 <i>orf02prev</i> (part 2)- <i>xylE</i> (BamHI-BglIII fragment amplified by PCR with primers 6 and 31; RA3 coordinates 2150-2348)
pMWB7.24	pJSB5.7 <i>korC G88G90</i> generated by PCR site-specific mutagenesis with primers 19 and 20 (substitutions G88E G90E in KorC)
pMWB7.25	pJSB5.7 <i>korC G34G41</i> generated by PCR site-specific mutagenesis with primers 21 and 22 (substitutions G34D G41D in KorC)
pMWB7.26	pJSB5.7 <i>korC A84H85</i> (NaeI site generated by PCR site-specific mutagenesis with primers 28 and 29)
pMWB10.7	pET28mod <i>T7p-korC</i> (EcoRI-Sall fragment from pJSB5.7)
pMWB10.24	pET28mod <i>T7p-korC G88G90</i> (MunI-Sall fragment amplified by PCR with primers 27 and 18 from pMWB7.24)
pMWB10.25	pET28mod <i>T7p-korC G34G41</i> (EcoRI-Sall fragment from pMWB7.25)
pMWB13.7	pLKB4 <i>lacp-cyaT18-korC</i> translational fusion (EcoRI-HincII fragment from pMWB10.7)
pMWB13.25	pLKB4 <i>lacp-cyaT18-korC G34G41</i> translational fusion (EcoRI-HincII fragment from pMWB10.25)
pMWB13.26	pLKB4 <i>lacp-cyaT18-korC₁₋₈₃</i> translational fusion (EcoRI-NaeI fragment from pMWB7.26)
pMWB14.7	pLKB2 <i>lacp-cyaT25-korC</i> translational fusion (EcoRI-HincII fragment from pJSB5.7)
pMWB14.24	pLKB2 <i>lacp-cyaT25-korC G88G90</i> translational fusion (MunI-Sall fragment amplified by PCR with primers 27 and 18 from pMWB7.24)
pMWB14.25	pLKB2 <i>lacp-cyaT25-korC G34G41</i> translational fusion (EcoRI-HincII fragment from pMWB7.25)
pMWB14.26	pLKB2 <i>lacp-cyaT25-korC₁₋₈₃</i> translational fusion (EcoRI-NaeI fragment from pMWB7.26)
pPDB1.18	pGEM-T Easy <i>korCp</i> (fragment amplified by PCR with primers 1 and 2; RA3 coordinates 3093-3431)
pPDB1.19	pGEM-T Easy <i>orf02p</i> (fragment amplified by PCR with primers 5 and 6; RA3 coordinates 2063-2348)
pPDB11.18	pPT01 <i>korCp-xylE</i> (BamHI-SphI fragment from pPDB1.18)
pPDB11.19	pPT01 <i>orf02p-xylE</i> (BamHI-SphI fragment from pPDB1.19)

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

No	Designation	Sequence
01	korCpRA3L	5' <u>gcgcatgc</u> CTTAAAGGAGGTGCATAGGT 3'
02	korCpRA3R	5' <u>gcggatcc</u> CAATCTTCAGCAAACGGCCT 3'
03	klcApRA3L	5' <u>gcgcatgc</u> GGGAGCGTGATCGTTACGGT 3'
04	klcApRA3R	5' <u>gcggatcc</u> ATTGCAGCCATACGGCGAGG 3'
05	orf02pRA3L	5' <u>gcgcatgc</u> CCAGGTGGCCCATTTTCGTAC 3'
06	orf02pRA3R	5' <u>cgggatcc</u> CGATCACGCTCCCAGGTCAA 3'
07	SnaB2rRA3L	5' <u>gcgcatgc</u> CGATCACGCTCCCAGGTCAA 3'
08	SnaB2rRA3R	5' <u>cgggatcc</u> CCAGGTGGCCCATTTTCGTAC 3'
09	OC4RA3L	5' <u>cgggatcc</u> ATCAGAACCACGGCCTTTGCT 3'
10	OC4RA3R	5' <u>cgggatccgcgatgc</u> CTGCCTCACCGCTAATTGAA 3'
11	LeftOcR	5' <u>gcgtcgac</u> CTATTGTGTCAAGCGGGTAC 3'
12	RightOcL	5' <u>gcgcatgc</u> GTACCCGCTTGACACAATAG 3'
13	OcRK2F	5' <u>gcgcatgc</u> ACCGAGCTGTAACCGCAGAA 3'
14	OcRK2R	5' <u>gcagatct</u> ATCCAGCCGAATACCAGGGC 3'
15	OcR751F	5' <u>gcgcatgc</u> ACGGGTTGGTCTTGGGTGTT 3'
16	OcR751R	5' <u>gcagatct</u> ATGCTCAGTTGCTGGGTGGT 3'
17	korCRA3L	5' <u>gcgaattc</u> ATG ATTAGACCTGAAACGCT 3'
18	korCRA3R	5' <u>cggtcgac</u> TTATGTTTCGGTTCATGGTTTC 3'
19	G8890EF	5' GGCCACCTGGCAGAATTCGAGGCTATATGGGACGC 3'
20	G8890ER	5' GCGTCCCATATAGCC7CGAA7TCTGCCAGGTGGGCC 3'
21	G3441DF	5' GCAACGAAAAGA7CCTTAGTAAGCCGCTCAGTGATGTTGATGTTG 3'
22	G3441DR	5' CAACATCAACA7CACTGAGCGGCTTACTAAGATCTTTTCGTTGC 3'
23	prkfrA1	5' <u>gcggatccgcgatgc</u> CTCGCTGATAACCTGGCCCT 3'
24	prkfrA2	5' <u>gcggatcc</u> CTCGCGCACCTGCTCATTG 3'
25	inc230P	5' <u>gcggatcc</u> GATAGCTCTTTGCCATTAAC 3'
26	Sphmob	5' <u>gcgcatgc</u> TTTTCTCGTTGGAGGGTGAT 3'
27	korCLMun	5' <u>gccaatg</u> ATG ATTAGACCTGAAACGCT 3'
28	84AHCDF	5' GTCTATCTTG7GCGACCTGGCGGCTTCGGGGCTA 3'
29	84AHCDR	5' TAGCCCCGAAGCCGCCAGGT7GCACAAGATAGAC 3'
30	O2pRvinF	5' <u>cggcatgc</u> GCGGGTGCCCGTCTTCTTG 3'
31	O2pRvinR	5' <u>gcagatct</u> CGTAGAGCGCGCTTTTTATTGCC 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

754 Table 3. Comparison of the putative KorC binding sites

<i>IncU</i>		
RA3	O _{C1} ¹	TAGGCCA TTT TGGCCTA ²
	O _{C2}	TAGGCC <u>G</u> ATT TGGCCTA
	O _{C3} , O _{C4}	TAGGCCA AAA TGGCCTA
pFBAOT	O _{C1}	TAGGCCA TTT TGGCCTA
	O _{C2}	TAGGCC <u>G</u> ATT TGGCCTA
	O _{C3} , O _{C4}	TAGGCCA AAA TGGCCTA
pKP048 ³	O _{C1}	TAGGCCA TTT TGGCCTA
	O _{C2}	TAGGCC <u>G</u> ATT TGGCCTA
	O _{C3}	TAGGCCA AAA TGGCCTA
<i>IncP-I</i> ⁴ (subgroup)		
pQKH54 (gamma)	O _{C1} , O _{C2} , O _{C3}	TAGG <u>A</u> CA AAA TG <u>T</u> CCTA
RK2 (alpha)	O _{C1} , O _{C2} , O _{C3}	TAGG <u>G</u> CA TAA TG <u>C</u> CCTA
pYS1 (beta)	O _{C1} , O _{C2}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
pA1 (beta)	O _{C1}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
	O _{C2} , O _{C3}	TAGG <u>G</u> CA AAA TG <u>T</u> CCTA
pJAM7	O _{C1}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
	O _{C2}	TAGG <u>G</u> CA ATT TG <u>C</u> CCTA
pB10 (beta)	O _{C1}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
	O _{C2}	TAGG <u>G</u> CA AAA TG <u>T</u> CCTA
pB12 (beta)	O _{C1} , O _{C2}	TAGG <u>G</u> CA AAA TG <u>T</u> CCTA
pB3 (beta)	O _{C1} , O _{C2}	TAGG <u>A</u> CA AAA TG <u>T</u> CCTA
pHP-42	O _{C1}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
	O _{C2} , O _{C*}	TAGG <u>G</u> CA ATT TG <u>C</u> CCTA
	O _{C**}	TAGG <u>G</u> CA TTT TG <u>C</u> CCTA
p9014	O _{C1}	TAGG <u>G</u> CA ATT TG <u>C</u> CCTA
R751 (beta)	O _{C1} , O _{C2}	TAGG <u>A</u> CA AAA TG <u>T</u> CCTA
pIJB1 (delta)	O _{C1} , O _{C2} , O _{C3}	TAGG <u>G</u> CA AAA TG <u>C</u> CCTA
pKJK5 (epsilon)	O _{C1} , O _{C2}	TAGG <u>G</u> CA ATT TG <u>C</u> CCTA
<i>IncL/M</i>		
pCTX-M3	O _{C1}	TAGG <u>A</u> CA AAT TG <u>T</u> CCTA
pEL60	O _{C1}	TAGG <u>A</u> CA AAA TG <u>T</u> CCTA

755

756 ¹ For *IncU* plasmids O_{C1} overlaps *orf02p/ orf02prev*, O_{C2} and O_{C3} are located in *klcAp* and O_{C4} is in
757 the transfer region *orf33p/ orf34p*. For *IncP-1* O_{C1} is located in *klcAp*, O_{C2} in *kleAp* and O_{C3} in *kleCp*
758 (O_{C*} and O_{C**} in pHP-42 precede short orfs of unknown functions upstream of *klcA* operon). In
759 *IncL/M* plasmids the single O_C is located in the putative *korCp*.

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

764 ³ pKP048 carries a part of the RA3 replication module and *orf02-klcA-orf04-korC* region (coordinates
765 763-3809 nt)

766 ⁴ Non *IncU* plasmids are ordered accordingly to the descending similarities between their KorC
767 proteins and KorC of RA3

Figure 1
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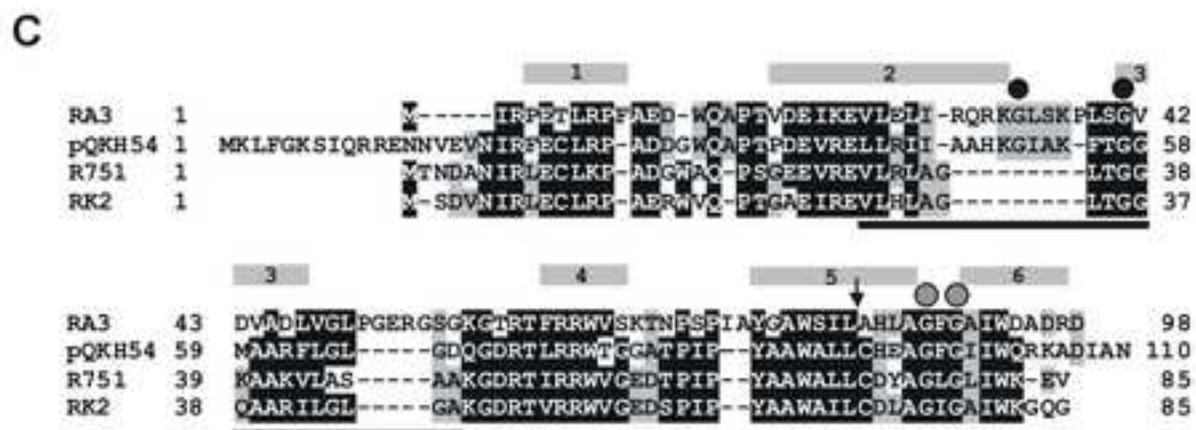
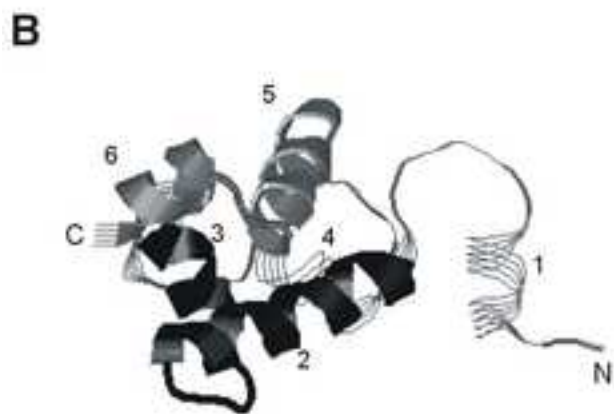
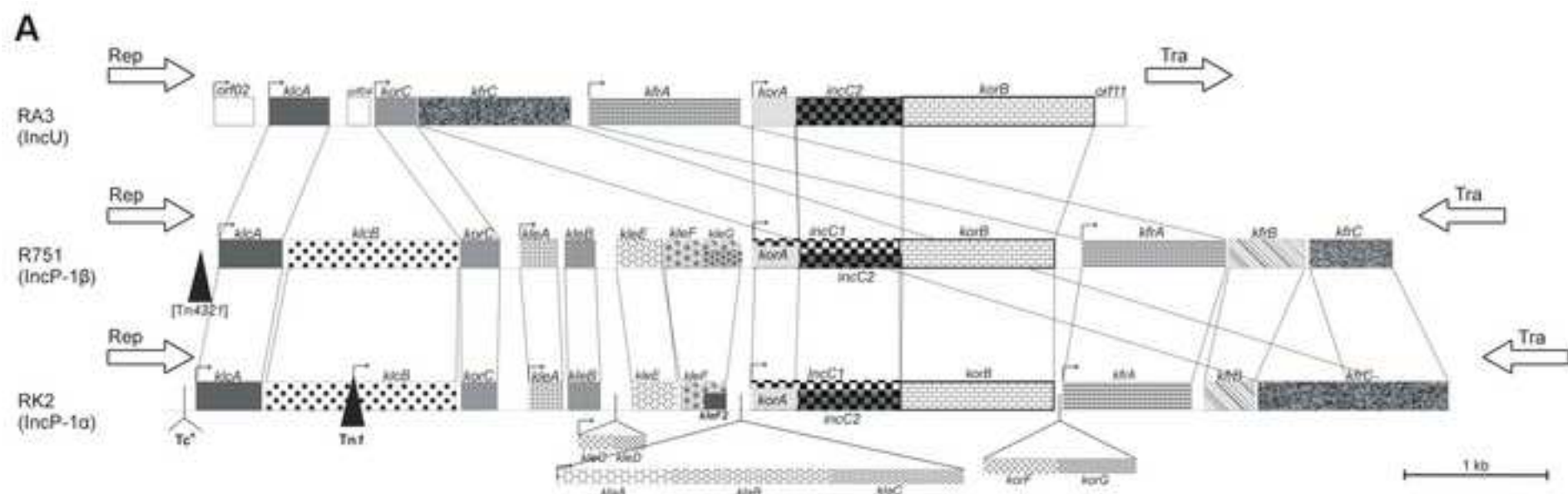


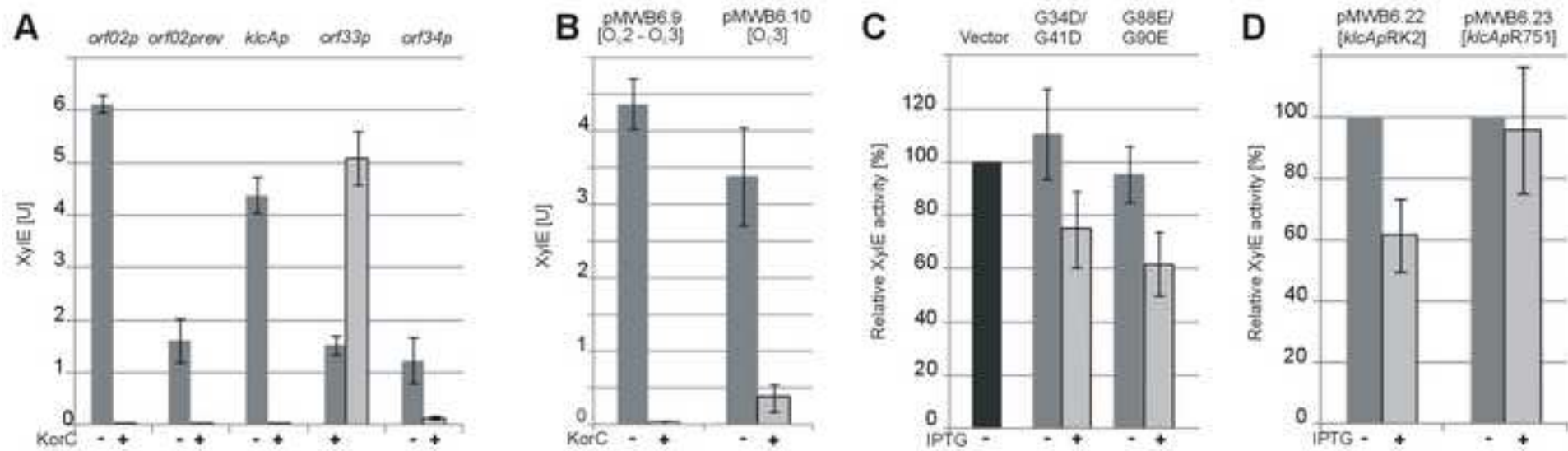
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Figure3

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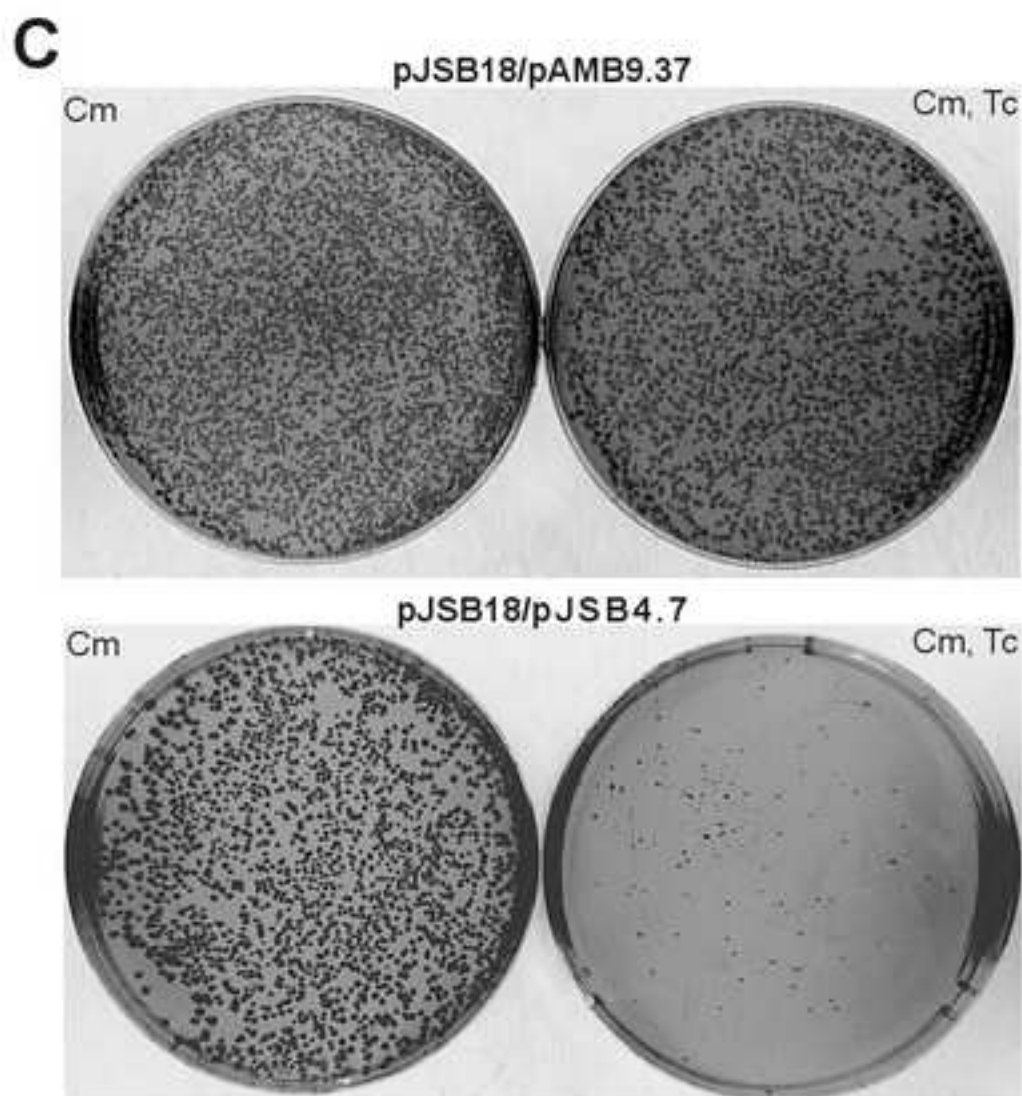
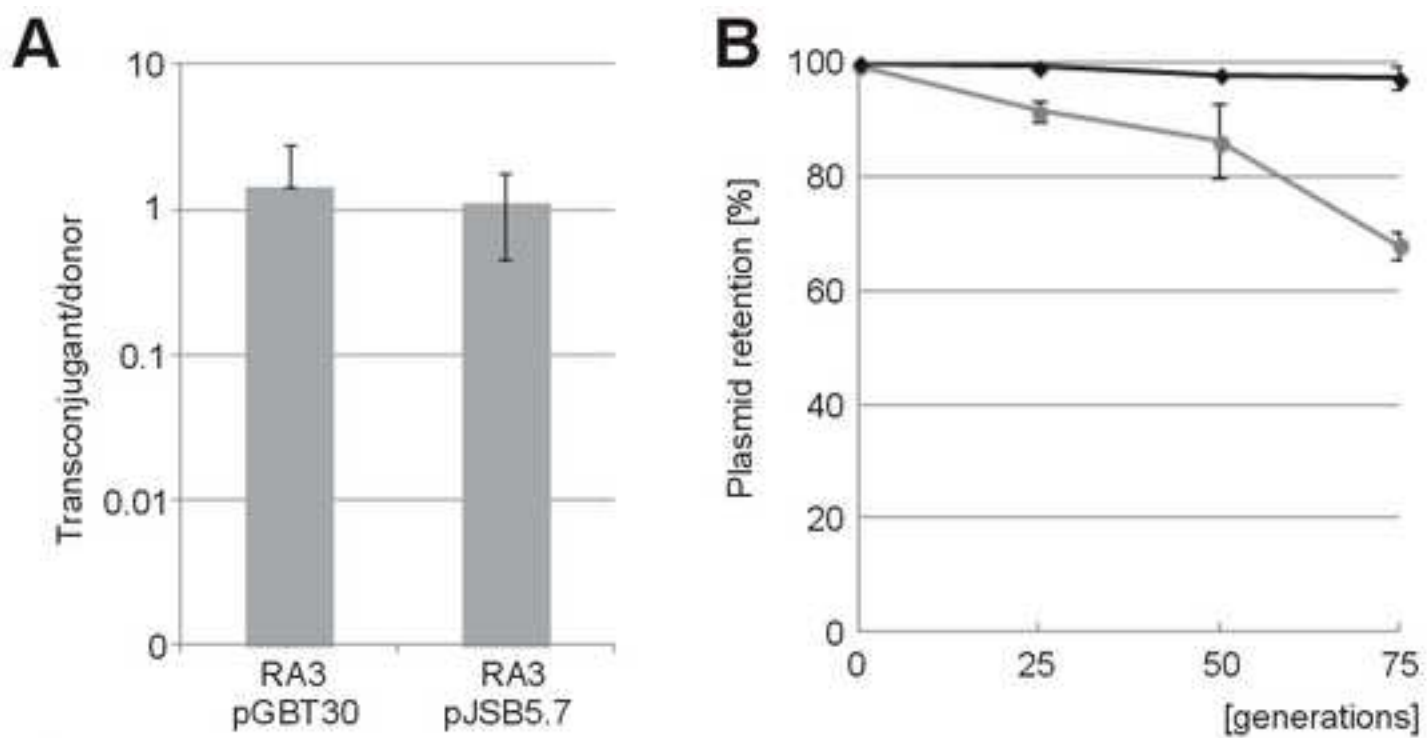


Figure5
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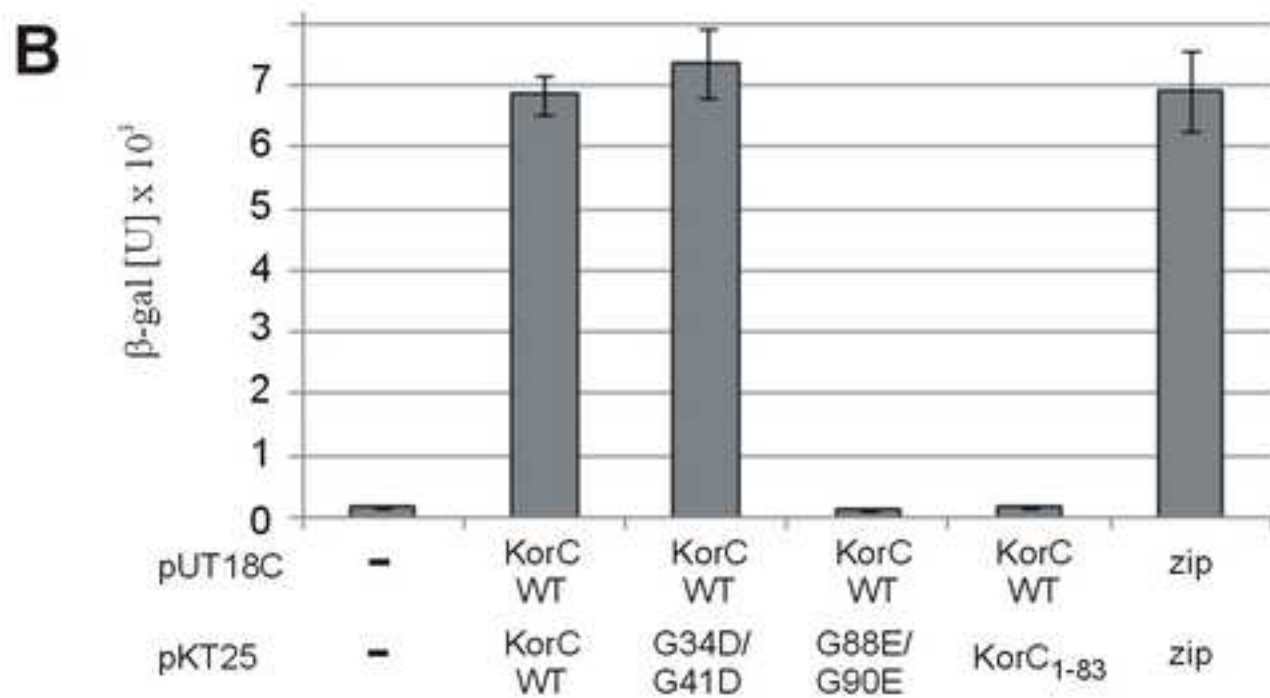
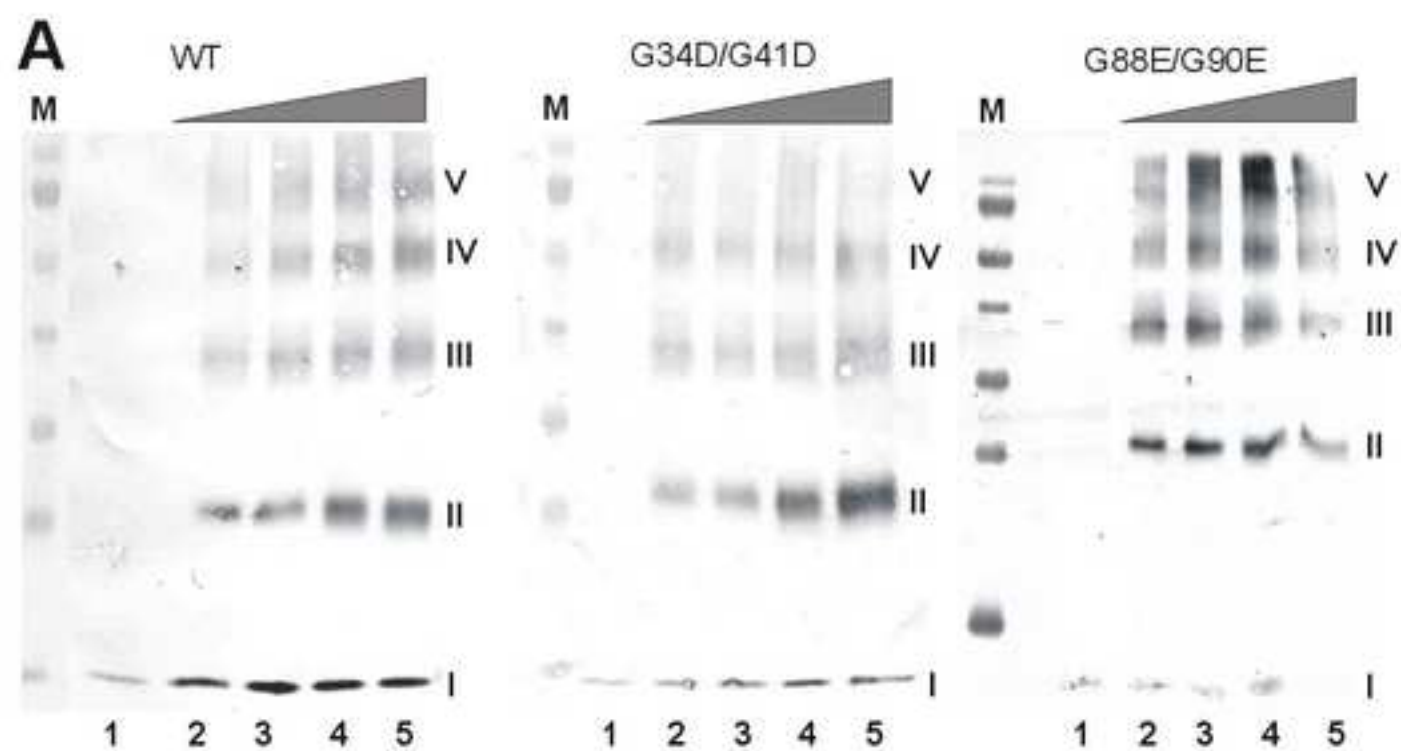


Figure6
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