Divergence and conservation of the partitioning and global regulation functions in the central control region of the IncP plasmids RK2 and R751

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The central control region (Ctl) of IncP plasmids is associated with two phenotypes: the coordinate expression of replication and transfer genes; and the ability to increase the segregational stability of a low-copy-number test plasmid. This region of the IncP β plasmid R751 shows significant sequence divergence from the IncP α plasmid RK2 sequence, and two genes, *korF* and *korG*, present in the IncP α region are missing in the IncP β Ctl. In other respects the organization of the Ctl is basically the same. Although the two key global regulatory genes *korA* and *korB* are highly conserved, studies on their ability to repress transcription from a variety of IncP α and IncP β plasmid promoters suggest differences in operator recognition by KorA and synergy with other repressors. The products of *kfrA*, *upf54.8* and *upf54.4* genes are conserved; KfrA shows least conservation and, while retaining the ability to act as a transcriptional repressor, appears to have completely different DNA-binding specificity. The genes required for the plasmid segregational stabilization (partitioning) phenotype – *incC*, *korB* and the KorB operator O₃ – are conserved and contribute to a more efficient plasmid stabilization than the IncP α equivalents. This may indicate that the Ctl plays an especially important role in partitioning of IncP β plasmids, since they lack the second stability region (*par/mrs*) found in IncP α plasmids.

Keywords: plasmid R751, IncP β plasmids, plasmid partitioning, transcriptional repressor KorA, transcriptional repressor KorB

INTRODUCTION

IncP plasmids have been studied because of their ability to transfer between and stably maintain themselves in almost all Gram-negative bacterial species (Thomas & Smith, 1987; Thomas & Helinski, 1989; Guiney & Lanka, 1989; Pansegrau *et al.*, 1994). Their conjugation system, which is related to many other conjugation systems including the *vir* system of Ti plasmids which transfers T DNA from *Agrobacterium* species to plant cells (Lessl *et al.*, 1992b; Pansegrau *et al.*, 1993), can

promote transfer from Gram-negative bacteria to Gram-positive bacteria and to yeast (Trieu-Cuot *et al.*, 1987; Heinemann & Sprague, 1989; Sikorski *et al.*, 1990).

A unique feature of IncP plasmids is the coordinate regulation of their replication and transfer functions by three global regulators: KorA, KorB and TrbA (reviewed by Pansegrau *et al.*, 1994; Zatyka *et al.*, 1994). KorA and KorB are encoded within the central control operon (Bechhofer & Figurski, 1983; Theophilus & Thomas, 1987), while TrbA is encoded at the start of the *trb* operon (Jagura-Burdzy *et al.*, 1992), which contains most of the genes required for mating-pair formation (Lessl *et al.*, 1992a, 1993). The plasmid region encompassing the central control operon also shows a stability (Par) phenotype: when linked to an unstable low-copy-number plasmid it will decrease its rate of loss (Motallebi-Veshareh *et al.*, 1990). This phenotype was

Abbreviations: Ctl, central control region.

Plasmid gene nomenclature: all genes are designated by the names of their homologues in RK2.

The GenBank accession number for the sequence reported in this paper is L13688.

deliberately studied after sequence alignments showed that IncC (the first gene in the central control operon) and KorB belonged to an ubiquitous family of proteins encoded by plasmids and chromosomes and associated with partitioning or cell division (Motallebi-Veshareh *et al.*, 1990). The family of Par proteins has grown steadily since it was recognized 6 years ago and there is increasing interest in what these proteins do (Williams & Thomas, 1992; Wake & Errington, 1995; Lobocka & Yarmolinsky, 1996). In IncP plasmids they, or at least KorB, have clearly been recruited to coordinate gene expression as well. Studying the evolution of these and associated genes within the IncP family will help to elucidate their structure and function.

Southern blot analysis of *Hae*II restriction fragments of IncP plasmids using the *oriT* region of RK2 as a probe first revealed subdivision of the plasmids into α and β subgroups (Yakobson & Guiney, 1983). Similar analyses using *oriV* and part of the *trfA* gene from RK2 (Chikami *et al.*, 1985) and the *pri* region from RP4 (Lanka *et al.*, 1985) confirmed this. Although the IncP β plasmid R751 (Jobanputra & Datta, 1974) and IncP α plasmid RK2 have very different restriction maps, the basic organization of their replication and transfer functions is the same (Villaroel *et al.*, 1983; Pansegrau & Lanka, 1987; Smith & Thomas, 1987).

The conservation of the backbone functions of RK2 and R751 extends to a degree of functional cross-reactivity between the two plasmid groups: a combination of the Tra1/*oriT* region of RK2 and the Tra2 region of R751 produced a functional conjugal transfer system, as did the reverse combination (Meyer & Shapiro, 1980); the IncP β plasmids R751, R906 and R772 have been shown to suppress the host-lethal effects of the RK2 *kilA* and *kilB* loci (Figurski *et al.*, 1982); RK2 *korA* and *korB* can suppress the R751 equivalent of the *kilB* locus; R751 *trfA* can allow replication to initiate at RK2 *oriV*; and the presence of RK2 *trfA* *in trans* allows replication initiation at R751 *oriV* (Smith & Thomas, 1985).

In this paper we describe the organization and sequence of the R751 central control region (Ctl), the cross-reactivity of control functions with those of RK2 and the conservation of the partitioning functions in R751. The studies reveal similarities and significant differences in both regulation and partitioning. These differences will help future studies unravel some of the mechanisms of action of these proteins.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strains used were K12 strains C600K (*thr-1 leu-6 thi-1 lacY1 supE44 ton-21 galK*), MV10 (C600*trpR* Δ *trpE5*) and C strain C2110 (*polA1 his rha*) obtained from Professor D. R. Helinski, Department of Biology, UC San Diego, California, USA). The plasmids used or constructed during the study are shown in Table 1. Bacteria were generally 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 antibiotics as appropriate: benzylpenicillin, sodium salt (100 mg ml⁻¹ in liquid media and

300 mg ml⁻¹ in agar plates) or ampicillin (100 mg ml⁻¹) for penicillin resistance, kanamycin sulphate (50 mg ml⁻¹) for kanamycin resistance, tetracycline hydrochloride (20 mg ml⁻¹) for tetracycline resistance and streptomycin sulphate (50 mg ml⁻¹) for streptomycin resistance.

Isolation, analysis and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline SDS method of Birnboim & Doly (1979) with slight modifications (Smith & Thomas, 1983), and manipulated and analysed using standard techniques (Sambrook *et al.*, 1989). Digestion of plasmid DNA with restriction enzymes was carried out under conditions recommended by the suppliers. Bacteria were made competent and transformed as described by Cohen *et al.* (1972). DNA sequencing was carried out by Alta Bioscience using the chain-termination method (Sanger *et al.*, 1977) and an ABI 373 automated DNA sequencer. Sequences were aligned and analysed using programs of the Wisconsin package (Devereux *et al.*, 1984).

Cloning using the polymerase chain reaction. Standard PCR reactions (Mullis *et al.*, 1986) were performed as previously described by Jagura-Burdzy *et al.* (1992). Fragments were amplified on a pCT691 (hybrid of pRK229 and pACYC184, joined at the *SalI* sites: Thomas *et al.*, 1988) template for RK2 and a pCT4712 template for R751. All PCR-derived clones were checked by DNA sequencing. The primers used were:

RK2 *kfrAp*, D4662DM 5'-CCCAAGCTTGGGGGAACGC-GGCCTCGACTG-3', D4663DM 5'-CGGAATTCGGCC-GCTGGCGTCGTTG-3';

RK2 *korAp*, D8139CT 5'-GGGAATTCTTGTGGGCTG-CGAGTGTGCG-3', D8140CT 5'-GGGATCCTCCTGGAAC-TGGCTTTCGG-3';

R751 *korAp*, D8213DM 5'-GAGGATCCTGCAGTGTTC-TACGACCAGGACG-3', D8214DM 5'-CAGGATCCGAA-TTCAGCGAAGTGACAACTCG-3';

R751 *kfrAp*, D7151DM 5'-CTGGCTCAACTACGAGGATG-3', D7151DM 5'-TCGCCTCGCTGATGGTGGTG-3';

R751 *korAp-incC*, D8213DM 5'-GAGGATCCTGCAGTGTTC-TACGACCAGGACG-3', D8091CT 5'-GGGTGCGACT-CATTGCGGATCTCCATCTTC-3'.

Construction of R751 *incC* and *korB* mutants. To construct *incC* and *korB* mutants, DNA of pCT4734 was digested with *BsmI* or *BspEI* respectively. The *BsmI*-digested DNA was treated with T4 DNA polymerase and religated to produce a 2 bp deletion within *incC* (pCT4734C). The *BspEI*-digested DNA was treated with DNA polymerase Klenow fragment in the presence of dNTPs and then religated in the presence of unphosphorylated *PstI* linkers (5'-GGCTGCAGCC-3') to give a 14 bp insertion in *korB* (pCT4734B). The *HindIII* fragments from pCT4734B and pCT4734C were ligated to stability vector pOG04.

For regulation analyses a *korB* mutation was created in the context of the complete R751 Ctl. The *korAp-incC* region was amplified by PCR and the 1.3 kb product purified, digested with *BamHI* and *SalI* and introduced into pUC18 to give pDM2.16. *EcoRI/BsmI* digestion of pDM2.16 gave a fragment which was used to replace the equivalent *EcoRI/BsmI* fragment from pCT4734B, generating a complete R751 Ctl carrying the *korB* mutation (pCT4712B).

Construction of a modified *xylE* promoter-probe plasmid. Promoter-probe vector pPT01, a derivative of pGBT43 (Jagura-Burdzy *et al.*, 1992) in which the *EcoRI* site is deleted, was digested with *BamHI*, treated with DNA polymerase

Table 1. Plasmids used in this study

Plasmid	Size (kb)	Replicons	Selectable marker*	Other properties	Reference/source†
R300B	8.6	IncQ	Sm ^r Su ^r		Barth & Grinter (1974)
pALA1029	8.5	pMB1, P7	Ap ^r	P7 <i>par</i>	Ludtke <i>et al.</i> (1989)
pCT415	17.0	IncQ	Sm ^r	RK2 <i>klaC-kfrA</i>	Thomas & Hussain (1984)
pCT470	15.4	pMB1	Ap ^r	pBR322 plus R751 <i>SalI</i> fragment from kb 0 to 11	Smith & Thomas (1985)
pCT654	5.0	pMB1	Ap ^r	RK2 <i>korAp</i>	Theophilus <i>et al.</i> (1985)
pCT691	28	p15a	Tc ^r Cm ^r	pACYC184 and pRK229 (RK2 Δ) cointegrate	Thomas <i>et al.</i> (1988)
pCT720	15.5	IncQ	Sm ^r Tc ^r	RK2 <i>klaAp</i>	Thomas <i>et al.</i> (1988)
pGBT37	6.8	pMB1	Ap ^r	<i>lacI^q tacp</i> -RK2 <i>korA</i>	Jagura-Burdzy <i>et al.</i> (1992)
pGBT43	9.7	pSC101	Km ^r	<i>xylE</i> promoter probe	Jagura-Burdzy <i>et al.</i> (1992)
pMMB25	14.9	IncQ	Ap ^r Sm ^r	<i>lacI^q tacp xylE</i>	Bagdasarian <i>et al.</i> (1984)
pMMV811	7.4	pMB1	Ap ^r	<i>lacI^q tacp</i> -RK2 <i>korB</i>	Williams <i>et al.</i> (1993)
pOG4	12.7	pMB1, P7	Ap ^r	RK2 Ctl	Williams <i>et al.</i> (1993)
pWS131	6.4	pMB1	Ap ^r	<i>lacI^q tacp</i> -RK2 <i>kfrA</i>	Thomas <i>et al.</i> (1990)
pUC18	2.7	pMB1	Ap ^r	<i>lacZ</i> cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pCT470R	15.4	pMB1	Ap ^r	pCT470 with inversion of R751-derived <i>PstI</i> fragment	
pCT4704	10.0	pMB1	Ap ^r	R751 <i>korC-upf54.8</i> , pCT470R deletion derivative	
pCT4712	8.5	pMB1	Ap ^r	R751 <i>klaB'-upf54.8</i> , pCT4704 deletion derivative	
pCT4720	4.5	pMB1	Ap ^r	R751 <i>kfrA'-upf54.8</i> , pCT4704 deletion derivative	
pCT4734	7.2	pMB1	Ap ^r	R751 <i>korA'-upf54.8</i> , pCT4704 deletion derivative	
pCT4754	6.3	pMB1	Ap ^r	R751 <i>incC'-upf54.8</i> , pCT4704 deletion derivative	
pDM1.1	11.6	IncQ	Sm ^r	pMMB25 minus Ap ^r and <i>xylE</i> cassettes	
pDM1.2	14.6	IncQ	Sm ^r Tc ^r	pDM1.1 plus Tc ^r originating from pCT720	
pDM1.21	16.0	IncQ	Tc ^r	<i>lacI^q tacp</i> -RK2 <i>korB</i> , derivative of pDM1.2	
pDM2.1E	4.1	pMB1	Ap ^r	pUC18 plus RK2 <i>korB</i> from pMMV811	
pDM2.15	3.1	pMB1	Ap ^r	pUC18 plus R751 <i>kfrAp</i>	
pDM2.15E	3.1	pMB1	Ap ^r	As pDM2.15 with <i>EcoRI</i> linkers in <i>HindIII</i> site	
pDM2.16	4.0	pMB1	Ap ^r	pUC18 plus R751 <i>korAp-incC</i>	
pDM2.6E	3.2	pMB1	Ap ^r	pUC18 plus RK2 <i>kfrAp</i> , <i>EcoRI</i> linkers in <i>HindIII</i> site	
pDM3	9.7	pSC101	Km ^r	<i>xylE</i> promoter probe, derivative of pPT01	
pDM3.1	10.4	pSC101	Km ^r	RK2 <i>korAp-xylE</i> promoter probe	
pDM3.5	10.1	pSC101	Km ^r	R751 <i>korAp-xylE</i> promoter probe	
pDM3.6	10.1	pSC101	Km ^r	R751 <i>kfrAp-xylE</i> promoter probe	
pDM300	10.2	pSC101	Km ^r	RK2 <i>kfrAp-xylE</i> promoter probe	
pOG04	6.0	pMB1, P7	Ap ^r	Stability vector, pALA1029 deletion derivative	
pOG0412	11.5	pMB1, P7	Ap ^r	R751 Ctl	
pOG0434	10.5	pMB1, P7	Ap ^r	R751 <i>incC2-upf54.4</i>	
pOG0434B	10.5	pMB1, P7	Ap ^r	<i>korB</i> mutant of pOG0434	
pOG0434C	10.5	pMB1, P7	Ap ^r	<i>incC</i> mutant of pOG0434	
pPT01	9.7	pSC101	Km ^r	<i>xylE</i> promoter probe, derivative of pGBT43	

* Antibiotic-resistance markers are denoted as Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Cm, chloramphenicol; Sm, streptomycin; and Su, sulphonamide.

† Where no reference is shown, plasmids were derived in this study.

Klenow fragment in the presence of dNTPs and religated in the presence of unphosphorylated *EcoRI* linkers (5'-CCGAATTCGG-3'). This generated a unique *EcoRI* site upstream of the *xylE* cassette (pDM3).

Construction of an IncQ expression vector and RK2 *korB* expression plasmid. The *xylE* cassette was removed from pMMB25 by *EcoRI* digestion and religation. The Ap^r marker was subsequently removed from this deletion derivative by

PstI digestion and religation to give pDM1.1. A *PstI* fragment carrying the RK2 Tc^r determinant (originating from pCT720) was introduced into pDM1.1 to give IncQ expression vector pDM1.2. Plasmid pDM2.1E is a pUC18 derivative in which RK2 *korB* has been introduced as an *EcoRI/HindIII* fragment from pMMV811, and unphosphorylated *EcoRI* linkers (see above) subsequently introduced into the *HindIII* site. The pDM2.1E *EcoRI* fragment carrying *korB* was introduced into pDM1.2 to give expression plasmid pDM1.21.

Construction of RK2 *korA* and *kfrA* promoter-probe plasmids.

The pCT654 *EcoRI* fragment which carries RK2 *korAp* was cloned into pDM3 to give pDM3.1. The RK2 *kfrAp* region was amplified using primers D4662DM and D4663DM, and introduced as an *EcoRI*/*HindIII* fragment into pUC18, giving pDM2.6. Insertion of unphosphorylated *EcoRI* linkers (see above) into the *HindIII* site gave pDM2.6E. The pDM2.6E *EcoRI* fragment carrying *kfrAp* was introduced into pDM3 to give pDM300.

Construction of R751 *korA* and *kfrA* promoter-probe plasmids.

The PCR-amplified *korAp* (primers D8213DM and D8214DM) was digested with *Bam*HI and introduced into the *xylE* promoter-probe vector pDM3 to give pDM3.5. The *kfrAp* region was amplified using primers D7150DM and D7151DM and introduced into the pUC18 *Hinc*II site, giving pDM2.15. Insertion of unphosphorylated *EcoRI* linkers (see above) into the *HindIII* site gave pDM2.15E. The pDM2.15E *EcoRI* fragment carrying the *kfrAp* was introduced into pDM3 to give pDM3.6.

Determination of Xyle activity. Catechol 2,3-dioxygenase (the product of *xylE*) activity was assayed in exponentially growing cells (OD₆₀₀) as described by Zukowski *et al.* (1983). One unit of enzyme activity is defined as the amount required to convert 1 µmol substrate to product in 1 min under standard conditions. Where appropriate *tacp* was induced by addition of 1 mM IPTG to the exponential-phase culture. Protein concentration was assayed by the biuret method (Gornall *et al.*, 1949). Plasmids in the assay strains were checked by isolation and gel electrophoresis after restriction.

Estimation of the ability of R751 segments to stabilize a low-copy-number plasmid. A shuttle vector, pOG04, was derived by deletion of the P7 *par* system from pALA1029. The vector replicates at high copy number in a *polA*⁺ host from pMB1 origin and at low copy number in a *polA* host from P7 origin. In *polA* strain C2110, pOG04 is lost during exponential growth at the rate expected of a randomly segregating plasmid present at 4–6 copies per cell at division. Derivatives bearing wild-type and mutant Ctl regions from R751 were constructed and selected in a high-copy-number strain and then transformed into C2110 to test their stability. The transformants were subcultured in selective medium to OD₆₀₀ 0.8–1.2, diluted 10⁻⁶ into nonselective medium (*t*₀) and grown for about 25 generations (*t*_{end}). Viable counts were calculated at both *t*₀ and *t*_{end} and the fraction of ampicillin-resistant colonies at both time-points calculated by replica-plating on L agar containing 100 mg ampicillin ml⁻¹. The mean apparent percentage loss of plasmid (*L*_a) was calculated using the formulae $L_a = (1 - R^{1/g}) \times 100$, where $R = (\% \text{ Ap}^r \text{ at } t_{\text{end}}) / (\% \text{ Ap}^r \text{ at } t_0)$ and g (generations) = $\log_e[x_{\text{end}}/x_0] / 0.693$, where x_0 = viable count at *t*₀ and x_{end} = viable count at *t*_{end}.

Visualization of polypeptide products by *in vitro* transcription and translation of plasmid DNA. Plasmid-encoded polypeptides were labelled with [³⁵S]methionine (DuPont NEN) using *in vitro* transcription/translation (Promega kit for circular molecules) on CsCl-purified plasmid DNAs (2 mg). Polypeptides were separated by SDS-PAGE and detected by phosphorimaging.

Analysis of protein-DNA interactions by gel retardation.

DNA fragments were amplified by PCR using primers described above and labelled with terminal transferase and [α -³²P]ddATP. Radioactive fragments were incubated with previously purified RK2 KorA and KorB under the conditions previously described by Jagura-Burdzy & Thomas (1995) and Williams *et al.* (1993) respectively. Samples were analysed as described in Jagura-Burdzy & Thomas (1995).

RESULTS AND DISCUSSION**Creation of a deletion series through the R751 central control region**

Analysis of the region of R751 encoding the central control operon started with cloning, into pBR322, the *Sal*I fragment that runs from kb coordinate 0 to 11 on the standard map of R751 (Fig. 1), giving pCT470 (Smith & Thomas, 1985). In attempting to delete the central *Pst*I fragment from the clone this fragment was inverted (generating pCT470R), thus placing the expression of *korC* under the control of the constitutive pBR322 *tet* promoter, which may be an advantage when this region is joined to a high-copy-number plasmid. Bal31 deletions were generated from the unique *Sst*II site of pCT470R and a *Hind*III linker was inserted. One of these deletion derivatives had lost all the DNA to within approximately 100 bp of the KorB operator in the *upf54.4/upf54.8* intergenic region. This derivative was designated pCT4704; it still has *korC*, *kleABEF*, *korA*, *incC*, *korB*, *kfrA* and *upf54.8*. To remove DNA from the *korC* end, pCT4704 was digested to completion with *Bam*HI and then partially digested with *Sau*3AI before ligation. This generated a family of deletions starting at

Fig. 1. Map of R751 and derived plasmids with the Ctl. (a) The genetic organization of R751 showing the location of the Ctl, the *cis*-acting and *trans*-acting factors required for vegetative replication (*oriV* and *trfA* respectively), the two regions carrying genes involved in conjugative transfer (Tra1 and Tra2) and including the *cis*-acting site required for transfer (*oriT*), and the two transposable elements. The *Sal*I (S) fragment introduced into pBR322 to produce pCT470 is indicated; other *Sal*I sites are not shown. (b) The deletion series for the R751 Ctl. pCT470R (identical to pCT470 except for an inverted *Pst*I fragment) was deleted to produce pCT4704, from which the Ctl deletions illustrated were generated. Restriction enzyme sites are indicated: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; and Ss, *Sst*II.

Fig. 2. Genetic organization of the RK2 and R751 Ctl. The DNA sequence conservation of R751 with RK2 is indicated. Numbers indicate plasmid kb coordinates and O_B indicates the location of KorB operator sequences. DNA is illustrated as a solid black line, mRNA as ~, putative rho-independent terminators as $\square$ and polypeptide ORFs as stippled boxes.

Fig. 3. Dot-matrix comparison of the Ctl of R751 with that of RK2.

the *Bam*HI site and moving progressively into the R751 DNA. pCT4712, pCT4734, pCT4754 and pCT4720 had deletion end-points in the *kleB*, *korA*, *incC* and *kfrA* genes respectively. Key plasmids in this series, illustrated in Fig. 1, were used to investigate the R751 functions in this region.

Nucleotide sequence of the central control region of plasmid R751

The nucleotide sequence of the region from *korA* to *upf54.4* was determined by a combination of shotgun cloning and sequencing of *Sau*3AI fragments from this region, sequencing of deletion derivatives, and sequencing with custom-designed oligonucleotides on a pCT470 template. The sequence is deposited in GenBank under accession number L13688. The main ORFs and other features of the sequence are presented in Fig. 2 and along the axes in Fig. 3. The G + C content of the R751 DNA is 66.5 mol% on average, varying from 48 mol% to 80 mol% in a window of 100 bp. When the G + C content was plotted it was very clear that there were local A + T-rich regions where promoters were located (data not shown).

Dot-matrix comparison showed blocks of conservation with IncP α sequences. However, there were a number of significant differences, particularly areas where sequences present in RK2 are absent from R751 (Fig. 3). The most obvious differences are the absence from R751 of *korF*, *korG* and the N-terminal region of *upf54.4*.

The overall percentage identity of the R751 genes with each equivalent RK2 gene is shown in Fig. 2. It varies from 79% for *korA* to 54% for *kfrA*. The *kfrA* gene stands out as peculiar in terms of high G + C content (72 mol%) and high divergence between RK2 and R751 genes. The dot-matrix comparison shows that there are many internally repeated sequences within the *kfrA* gene (Fig. 3). These G + C-rich repeats could form the basis of illegitimate recombination to create deletions or duplications by unequal crossing over, or result in slipped mispairing to cause local expansion or contraction of the DNA.

The *korA* promoter region shows identical organization to that of RK2, with a KorB operator upstream of the -35 region and a KorA operator overlapping the -10 region. However, at the *kfrA* promoter in R751 the KorB operator is directly adjacent to the KorA operator

Fig. 4. (a) Alignment of the RK2 and R751 *kfrAp* regions. KorA (O_A), KorB (O_B), KfrA (O_K) operators, promoter -10 and -35 sequences, transcriptional start points ($\rightarrow$) based on primer extension mapping in RK2, putative rho-independent terminators ($\leftarrow$), and translational signals, Shine-Dalgarno (SD) and start codons are shown. (b) Comparison of the O_A and O_B sequences in the RK2 and R751 *korAp*, *kfrAp*, *trfAp* and at O_B 3 in the intergenic region between *upf54.8* and *upf54.4*. N/A indicates that a KorA operator is not present in this region.

The core operators for KorA and KorB, O_A and O_B, are generally conserved between the two plasmids (Fig. 4b). However, there is considerable divergence in the flanking sequences of O_A. At R751 *kfrAp* the O_B does not have the T → C transition which is present in RK2, and the

Interestingly, *upf54.4* and *upf54.8*, for which there are no identified functions, are more highly conserved than either *incC* or *kfrA*. The conservation in *upf54.8* varies from perfect alignment to gaps with no similarity, and the predicted product shows sequence similarity to TraA of the IncP α plasmids (Pansegrau *et al.*, 1994), which is not present in the IncP β genome (Thorsted *et al.*, 1996). The *upf54.4* ORF lacks the first 200 aa present

Table 2. Regulation of *korAp*, *kfrAp* and *trfAp* by repressors of RK2 and R751

pMB1 plasmid <i>in trans</i>	Genetic loci, pMB1 replicon	IncQ plasmid <i>in trans</i>	Mean relative catechol 2,3-dioxygenase activity*				
			RK2 <i>korAp</i> pDM3.1	R751 <i>korAp</i> pDM3.5	RK2 <i>kfrAp</i> pDM300	R751 <i>kfrAp</i> pDM3.6	RK2 <i>trfAp</i> pGBT58
pGBT37†	RK2 <i>korA</i>	None	0.003 ± 0.001	0.12 ± 0.06	0.13 ± 0.01	0.38 ± 0.06	0.004 ± 0.001
pMMV811†	RK2 <i>korB</i>	None	0.24 ± 0.10	0.51 ± 0.10	0.12 ± 0.04	0.17 ± 0.04	0.09 ± 0.02
pWS131†	RK2 <i>kfrA</i>	None	ND	ND	< 0.002	1.1 ± 0.35	ND
pCT415‡	RK2 <i>korA incC korB</i> <i>korF korG kfrA</i>	None	0.008 ± 0.004	< 0.002	0.13 ± 0.03	0.86 ± 0.17	0.012 ± 0.004
pCT4712§	R751 <i>korA incC korB kfrA</i>	None	0.006 ± 0.002	0.003 ± 0.002	0.36 ± 0.04	0.009 ± 0.005	0.007 ± 0.004
pCT4712B§	R751 <i>korA incC kfrA</i>	None	0.16 ± 0.03	0.06 ± 0.005	0.69 ± 0.08	0.012 ± 0.001	0.09 ± 0.02
pCT4712B	R751 <i>korA incC kfrA</i>	pDM1.2 vector	ND	0.04 ± 0.01	ND	ND	0.88 ± 0.24
pCT4712B	R751 <i>korA incC kfrA</i>	pDM1.21 RK2 <i>korB</i>	ND	0.011 ± 0.005	ND	ND	1.23 ± 0.25
pCT4734§	R751 <i>korB incC2 kfrA</i>	None	1.23 ± 0.22	2.07 ± 0.11	0.97 ± 0.16	0.003 ± 0.002	ND
pCT4754§	R751 <i>korB kfrA</i>	None	1.18 ± 0.30	1.31 ± 0.08	1.09 ± 0.10	0.009 ± 0.006	ND

ND, Not done.

* Mean relative activities for at least four experimentally determined values ± 95 % confidence limits.

† Catechol 2,3-dioxygenase activity of an induced culture relative to an induced control culture containing expression vector pGBT30 and the appropriate promoter-probe plasmid.

‡ Catechol 2,3-dioxygenase activity of culture relative to a control culture containing R300B and the appropriate promoter-probe plasmid.

§ Catechol 2,3-dioxygenase activity of culture relative to a control culture containing pCT4720 and the appropriate promoter-probe plasmid.

|| Catechol 2,3-dioxygenase activity of culture relative to a control culture containing pCT4720, and the appropriate IncQ and promoter-probe plasmids.

in the RK2 equivalent but otherwise shows considerable identity. A particular feature of these proteins is the high glutamine content. Given the degree of divergence that has taken place in this region, the conservation of these ORFs between the two subfamilies argues for a significant role in plasmid survival.

Functional conservation and divergence of the global regulatory genes *korA* and *korB*

Both KorA and KorB are key regulators of the IncP plasmids and, as expected, the DNA sequence analysis shows that they are highly conserved. Nevertheless, there are enough sequence differences to make it possible that the specificity of binding to DNA may not be identical for the members of each pair. To assess the extent of functional (regulatory) conservation we studied the effect of the regulatory genes from RK2 and R751 on the RK2 *trfAp*, *korAp* and *kfrAp* and the R751 *korAp* and *kfrAp*. To make this possible, *xylE* promoter-probe plasmids were constructed using PCR to amplify and clone *kfrAp* of RK2 and the R751 *korAp* and *kfrAp*. The RK2 *korAp* was obtained from plasmid pCT654. We used RK2 *korA* (pGBT37), *korB* (pMMV811) and the whole Ctl (pCT415) to demonstrate that repression was as expected for the RK2 promoters, and that the R751 promoters were sensitive to KorA and KorB as predicted by the presence of O_A and O_B sequences within the promoter regions. Table 2 shows that RK2 KorA and

KorB repressed R751 *korAp* (pDM3.5) and *kfrAp* (pDM3.6), but less strongly than the equivalent RK2 promoters. Flanking bases play an important role in operator specificity and binding strength for RK2 KorA (Jagura-Burdzy & Thomas, 1995). Examination of the O_A sequences revealed that the bases flanking the core of the operator in R751 differed from those found in RK2 (Fig. 4b). These differences may represent a significant divergence between the two plasmids.

RK2 KfrA had no effect on R751 *kfrAp* (see below). Nevertheless, weak repression was observed for the whole RK2 Ctl (pCT415) on R751 *kfrAp* (pDM3.6). This must have been due to KorA and/or KorB expressed from the autogenously regulated *korAp*, indicating the importance of KfrA repression at *kfrAp* when *korA*, *korB* and *kfrA* are in the normal regulatory context.

When we studied the effect of the R751 regulatory genes it was clear that the complete Ctl present on pCT4712 caused severe repression of all promoters except RK2 *kfrAp* (pDM300). This can be explained if R751 KfrA has little effect on RK2 *kfrAp*. Inactivation of *korB* (pCT4712B) gave a significant reduction in the observed repression. The level of repression of the RK2 *korAp* (pDM3.1) was clearly reduced more than that for R751 *korAp* (pDM3.5), which again suggested differences in specificity. Placing RK2 *korB* *in trans* to the R751 *korB* mutant (pCT4712B) resulted in increased repression,

but not restoration of the level observed for the intact R751 operon. Assays with two deletion derivatives (pCT4734 and pCT4754) which had lost *korAp* and *korA* and placed *korB* transcription under the control of the pBR322 *tet* promoter showed that there was little residual repression that could be attributed to KorB.

The results show that while there is clearly cross-reaction between the repressors of the two plasmids and their targets, there seem to be clear differences in the effect of the repressors on the heterologous promoter. This suggests that there has been divergence of the operator recognition between the two plasmids.

Gel-retardation assays of RK2 KorA and KorB affinities on RK2 and R751 operators

The transcriptional regulation studies described above suggested that RK2 KorA and KorB had a stronger effect on RK2 promoter/operators than on R751 promoter/operators. To test whether this was due to different affinities of the proteins for the RK2 and R751 operators we labelled DNA fragments containing either *korAp* or *kfrAp* and used gel-retardation analyses to study the binding of purified RK2 KorA and KorB to these fragments. The labelled *kfrAp* fragments for both RK2 and R751 were digested with *Mbo*II and *Nci*II respectively to produce smaller DNA fragments for retardation.

Specific binding of RK2 KorA and KorB to the DNA containing their operators in the *kfrAp* fragments is demonstrated in Fig. 5(a). No difference was observed for KorA at either *kfrAp* operator. KorB may have a slightly higher affinity for its operator in R751 *kfrAp*. More significantly, KorB forms a second retarded complex with R751 *kfrAp*, but not with RK2 *kfrAp*. These differences may reflect either or both the facts that in R751 the O_B inverted repeat is 15 bp compared with 13 bp in RK2, and that the R751 operator does not have the T → C transition present in RK2, which we think reduces affinity for KorB.

When the *korAp* fragments were studied, RK2 KorA was demonstrated to bind the RK2 *korAp* with high affinity and the R751 *korAp* with much lower affinity (Fig. 5b). RK2 KorB was shown to have high binding affinity for both the RK2 and R751 *korAp* regions and in both cases formed the more slowly migrating complexes observed for RK2 (Fig. 5b). Thus, there seem to be significant differences in KorA–DNA interactions which are due to differences in the target sites.

The specificities of R751 and RK2 *kfrA* genes are completely different

The *kfrA* genes of R751 and RK2 show the greatest divergence of all the ORFs present in this region of both plasmids. In addition, the operator identified in RK2 as the binding site for KfrA (Jagura-Burdzy & Thomas, 1992) is not conserved in R751 (Fig. 4a). To test the prediction that there is no cross-reactivity between KfrA

Fig. 5. Gel retardation using RK2 KorA and KorB proteins with fragments carrying the RK2 and R751 *kfrAp* and *korAp* regions. (a) Retardation of *kfrAp* fragments. 0 indicates that neither KorA nor KorB is present. The values for KorA correspond to: 1, 1 µg; 2, 7 µg; 3, 9 µg; and for KorB to: 1, 18 ng; 2, 54 ng; 3, 540 ng. (b) Retardation of *korAp* fragments. 0 indicates that neither KorA nor KorB is present. The values for KorA correspond to: 1, 1 µg; 2, 3.3 µg; 3, 10 µg; and for KorB to: 1, 18 ng; 2, 54 ng; 3, 180 ng; 4, 540 ng.

proteins and the heterologous *kfrA*s, we determined the effects of plasmids carrying each gene against appropriate reporter plasmids (Table 2). The results showed that the *tacp-kfrA*_{RK2} plasmid pWS131 represses the *kfrAp*_{RK2}-*xylE* plasmid (pDM300) strongly but has no effect on the *kfrAp*_{R751}-*xylE* plasmid (pDM3.6). Conversely the *kfrA*_{R751} plasmid pCT4754 represses pDM3.6 strongly but has no effect on pDM300.

We predict that the inverted repeat overlapping the *kfrAp* of R751 is the operator for the KfrA of R751, since this is the location of the KfrA operator in RK2 *kfrAp*. This inverted repeat is very interesting because each half consists of direct repeats. Given the conservation of the other replication and regulation functions between RK2 and R751, the divergence of the *kfrA* genes suggests that their function must be auxiliary, so that a mutation which inactivates *kfrA* or its operator may not have a disastrous effect on plasmid survival.

R751 exhibits a more efficient partitioning phenotype than IncPα plasmids

One of the key phenotypes of the Ctl in IncPα plasmids is the stability it confers on an unstable, low-copy-number heterologous replicon. To determine whether this phenotype is conserved in R751 we cloned the *Hind*III fragment containing this region from pCT4712

Fig. 6. Plasmid stability analysis. The mean apparent percentage loss (L_a) for each plasmid is presented, including 95% confidence limits, as calculated from at least three independent experiments.

into stability test vector pOG04 to give pOG0412. Fig. 6 shows that the R751 Ctl (pOG0412) gave a stronger stabilization than the equivalent RK2 region (pOG4). Estimates of plasmid copy number by quantitative hybridization showed that the stabilization was not due to an increase in copy number (data not shown). This suggests that the R751 Ctl encodes a more efficient stability system than the RK2 Ctl. A deletion derivative (pCT4734) lacking the beginning of *korA* and *incC1* was created in which the *korA* ORF was fused in phase with the *tet* ORF of pBR322. The remaining R751 Ctl was transferred to pOG04 as a *Hind*III fragment (pOG0434). Stability analysis revealed that this region was still able to stabilize pOG04 and suggested that O_B1 , *korA* and *incC1* are not needed for the stability system.

To check that the stabilization observed was similar to that found in the IncP α plasmid RK2, we inactivated *incC* (pOG0434C) and *korB* (pOG0434B) in the context of pCT4734 as described in Methods. Stability analyses (Fig. 6) confirmed that both *incC2* and *korB* were needed for the stability phenotype.

Conclusions

The results confirm the previous observation that the basic organization of the IncP α and IncP β plasmids is the same. They show that different regions have diverged to varying extents, which appears to reflect both functional constraints and inherent instability of particular gene sequences. The lack of *korF/korG* in R751 could be interpreted as loss of functions present in a common ancestor. However, since no known *parAparB* homologues of *incC* and *korB* have been shown to be accompanied by *korF/korG* homologues it is equally plausible that they were more recently acquired, perhaps in a similar way to that proposed as a possibility for the *klaAtelAtelB* genes in the RK2 *kilA* operon (Walter *et al.*, 1991; Goncharoff *et al.*, 1991; Thomas *et al.*, 1995). Either way it shows that *korF* and *korG* are dispensable. Although *KorA* and *KorB* and their operators are conserved, there appear to be significant differences in

the specificity of repressor-operator interactions and possibly repressor-repressor synergy. Based on previous analysis of *KorA*-operator interaction (Jagura-Burdzy & Thomas, 1995) we proposed that this reflects changes in the sequences flanking the core operator. Such changes should be able to occur without disruption of essential interactions.

The R751 Ctl displays a stronger Par^+ phenotype than the equivalent RK2 region. This may correlate with the absence of a second partitioning function equivalent to the *parABCDE* region found in RK2.

The divergence between the plasmids may provide a natural means of exploring regions important for partitioning, particularly those which specify the interactions between subcomponents.

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