The *kfrA* gene is the first in a tricistronic operon required for survival of IncP-1 plasmid R751

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The *kfrA* gene of the IncP-1 broad-host-range plasmids is the best-studied member of a growing gene family that shows strong linkage to the minimal replicon of many low-copy-number plasmids. KfrA is a DNA binding protein with a long, alpha-helical, coiled-coil tail. Studying IncP-1 β plasmid R751, evidence is presented that *kfrA* and its downstream genes *upf54.8* and *upf54.4* were organized in a tricistronic operon (renamed here *kfrA kfrB kfrC*), expressed from autoregulated *kfrAp*, that was also repressed by KorA and KorB. KfrA, KfrB and KfrC interacted and may have formed a multi-protein complex. Inactivation of either *kfrA* or *kfrB* in R751 resulted in long-term accumulation of plasmid-negative bacteria, whereas wild-type R751 itself persisted without selection. Immunofluorescence studies showed that KfrA_{R751} formed plasmid-associated foci, and deletion of the C terminus of KfrA caused plasmid R751 Δ C₂*kfrA* foci to disperse and mislocalize. Thus, the KfrABC complex may be an important component in the organization and control of the plasmid clusters that seem to form the segregating unit in bacterial cells. The studied operon is therefore part of the set of functions needed for R751 to function as an efficient vehicle for maintenance and spread of genes in Gram-negative bacteria.

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INTRODUCTION

Sequencing of bacterial genomes has revealed not only that some chromosomes are very similar to megaplasmids in their replication and stable inheritance mechanisms, but also that most standard *oriC*-based chromosomes carry a range of stable inheritance functions, already studied in some detail on bacterial plasmids (Gerdes *et al.*, 2000a). This includes active partitioning systems of the *parAB* family (Gerdes *et al.*, 2000b; Bignell & Thomas, 2001), multimer resolution systems of the *xerC/D* family (Summers & Sherratt, 1984; Blakely *et al.*, 1993) and a variety of post-segregational killing systems (Gerdes *et al.*, 2000a; Zielenkiewicz & Ceglowski, 2001). Thus, studies on bacterial plasmids, particularly large, low-copy-number plasmids, can be of direct relevance for understanding key events of the bacterial cell cycle. Genomic approaches also reveal genes that are conserved between many systems and cluster with known replication and stable inheritance functions,

but do not yet have a role assigned. One such gene family has as its archetype the *kfrA* gene of IncP-1 α plasmid RK2 that we studied many years ago, but for which we did not establish a phenotype, although we speculated that it may be involved in active partitioning (Jagura-Burdzy & Thomas, 1992; Williams & Thomas, 1992). KfrA_{RK2} is 308 aa in length, is a site-specific DNA binding protein, and is predicted to be almost 100 % α -helical, which is consistent with its circular dichroism (CD) spectrum (Jagura-Burdzy & Thomas, 1992). It is predicted to consist of an N-terminal, globular, DNA binding domain and an extended coiled-coil tail. Database searching revealed similarity to proteins of the myosin/kinesin family and, recently, to prokaryotic structural maintenance of chromosomes (SMC) and MukB-like proteins (Hirano, 1998; Soppe, 2001). However, there is no easily identified nucleotide-binding site in KfrA, so it seems unlikely to be able to provide the force-generating element of a molecular motor.

Recent accumulation of plasmid sequences has revealed other members of the *kfrA* family, located in or close to a cluster of stable inheritance functions. In both pFBAOT6 (Rhodes *et al.*, 2004) and pIP02 (Tauch *et al.*, 2002), this gene is close to stable inheritance functions very similar

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; PAS, Polish Academy of Sciences; WT, wild-type; YTH system, yeast two-hybrid system.

The DNA sequence and predicted amino acid sequence of the R751 region containing *kfrA*–*upf54.8* and the 5' end of *upf54.4* are available as supplementary data with the online version of this paper.

to the active partitioning/central control region of RK2 (Pansegrau *et al.*, 1994; Thorsted *et al.*, 1998). In other plasmids, the *kfrA* orthologue is clustered with replication functions (pMOL98, GenBank accession no. AJ 345055; pSa, Close & Kado, 1992), with partitioning functions (pXAC33, accession no. NC 003921; pKDSC50, accession no. NC 002638; Haneda *et al.*, 2001), or a mixture of replication and stable inheritance determinants (pRA2, accession no. NC 005909; Kwong *et al.*, 1998, 2000; pO157, accession no. NC 002128; Burland *et al.*, 1998). This prompted us to re-examine the IncP-1 plasmids to see if we could establish a role for this gene in stable inheritance. The IncP-1 plasmids are, so far, the only system in which the operons encoding *parA*(*incC*) and *parB*(*korB*) homologues and the associated *parS* sequences (Motallebi-Veshareh *et al.*, 1990; Williams *et al.*, 1998) have been shown to require additional plasmid functions for full effectiveness (Thorsted *et al.*, 1998). Thus, in both RK2 and R751, the central control region (consisting of central control operon (*cco*) *korA**incC**korB* and orphan *kfrA*, *upf54.8* and *upf54.4* genes; Fig. 1) provides a significant partitioning phenotype, but this is increased markedly by the addition of the *kbc* and *kde* regions, and it has been shown that the effect of these regions depends on an intact central control region (Thorsted *et al.*, 1998). It is possible that the *kfrA* operon provides an independent stable inheritance mechanism or that it enhances that activity of another mechanism. This forms the basis for trying to establish what role, if any, the genes flanking the central control operon play in IncP-1 plasmid stability.

The stability of RK2 (IncP-1 α) relies on active partitioning (Motallebi-Veshareh *et al.*, 1990; Williams *et al.*, 1998), assisted by *kbc* and *kde* functions whose role is not understood (Macartney *et al.*, 1997). It also encodes a multimer resolution system (*parABC*) (Roberts & Helinski, 1992; Easter *et al.*, 1998) and a post-segregational killing system (*parDE*) (Easter *et al.*, 1998; Roberts *et al.*, 1993; Johnson *et al.*, 1996). R751 (IncP-1 β) lacks the whole *parABCDE* region. Thus, the stable inheritance of R751 is based only on the presence of the central control region, assisted by a more basic set of *kbc* and *kde* operons (Thorsted *et al.*, 1998). Its *kde*

region is shorter than in RK2, because it does not contain the *kdeCD* genes. On the other hand, the *kbc* operon of R751 has a functional *kbcA* gene which is inactivated in RK2 by Tn1 insertion (Pansegrau *et al.*, 1994). Therefore, it seems likely that the central control region of R751 provides the basis for the definition of the minimal requirements for this region; the central control operon codes only for *korA*, *incC* and *korB*, and not for the two genes *korF* and *korG* that are present in IncP-1 α . The *upf54.4* ORF is also 50 % shorter, encoding a protein lacking the N-terminal 231 aa, as compared with RK2. Despite these differences, R751 is even more stably maintained than RK2, at least in *Escherichia coli* and *Pseudomonas* species, so we have chosen R751 as a simpler model than RK2 to analyse the role of the orphan genes *kfrA*, *upf54.8* and *upf54.4*.

Comparison between DNA and amino acid sequences of R751 and RK2 in this region (Fig. 1) showed low conservation in the *kfrA* ORFs (50 % DNA identity, 38 % amino acid identity) but high conservation in the *upf54.8* and *upf54.4* of both representatives (70 % DNA identity, 68 and 64 % amino acid identity, respectively). Despite this unusually low conservation of the primary sequences of KfrA_{RK2} and KfrA_{R751}, both proteins are highly α -helical and have an almost identical predicted secondary structure, with an N-terminal globular part and a long, coiled-coil α -helical tail (Jagura-Burdzy & Thomas, 1992; data not shown for KfrA_{R751}). The only function identified so far for the whole region (*kfrA*, *upf54.8*, *upf54.4*) is for KfrA_{RK2} and concerns the autoregulation of transcription from *kfrAp* (Jagura-Burdzy & Thomas, 1992). The previous study of KfrA_{RK2} by Williams *et al.* (1998) showed that an internal in-frame deletion of *kfrA*, in a pOG04 derivative (hybrid replicon P7-pMB1 with stabilization functions of the central control region of RK2), did not affect the stability of the plasmid when it had a low copy-number in a *polA1* strain. Although RNA polymerase binding sites have been identified in the *upf54.8* and *upf54.4* region of RP4 (=RK2) by electron microscopy (Pansegrau *et al.*, 1994), DNA sequence analysis of both RP4 and R751 in this region has failed to identify strong candidate promoters, raising the possibility

Fig. 1. Genetic organization of the studied region of R751. Comparison of the central control regions of RK2 (IncP-1 α) and R751 (IncP-1 β). ORFs are boxed, transcripts are indicated by arrows and broken lines, terminators are shown by the hairpin-loop symbol, and promoters for *korA* and *kfrA* are indicated as well as the binding sites for KorA (O_A), KorB (O_B) and KfrA (O_K). The numbers in the central box show the level of DNA sequence identity between the corresponding genes from RK2 and R751 and predicted amino acid sequences of their products. A question mark in parentheses signifies a lack of data about operon organization before we started our analysis.

that all these genes constitute an operon expressed from the *kfrA* promoter. On the other hand, the sequence of a putative Rho-independent terminator, previously identified in RP4 between *kfrA* and *upf54.8* (Pansegrau *et al.*, 1994), is conserved in R751 (Supplementary data). In order to define better what role, if any, *kfrA*, *upf54.8* and *upf54.4* play in the stable inheritance of R751, we have addressed a number of questions in this paper. Do the three ORFs belong to the same operon and how is the expression of the operon regulated in R751? Can the genes in this region be inactivated and what effect will the mutations have on R751 biology? What is the function of the unusual α -helical KfrA and, possibly, the other two proteins? Do KfrA, Upf54.8 and Upf54.4 interact with each other to fulfil their biological function?

METHODS

Bacterial strains and growth conditions. *E. coli* strains used were K12 strains DH5 α F[−] (Φ80*dlacZ*Δ*M15*) *recA1* *endA1* *gyrA96* *thi-1* *hsdR17*(r_k[−] m_k⁺) *supE44* *relA1* *deoR* Δ(*lacZYA-argF*)U196, C600K *thr-1* *leu-6* *thi-1* *lacY1* *supE44* *ton21* *galK* (McKenney *et al.*, 1981), NEM259 *met*, *trpR*, *supE*, *supF*, *hsdR*[−] (W. Brammar, University of Leicester, UK), *E. coli* B strain BL21 F[−] *ompT* *hsdS_B* (r_B[−] m_B[−]) *gal dcm* (λ DE3) (Novagen), and *E. coli* C strains C2110 *polA1* *his rha* (D. R. Helinski, University of California, San Diego, CA, USA) and C2110Rif^R (this work). Bacteria were generally grown in L broth (Kahn *et al.*, 1979) at 37 °C. Some experiments were performed at 25 or 42 °C. L agar (L broth with 1.5%, w/v, agar) was supplemented with antibiotics as appropriate: benzylpenicillin sodium salt (150 μg ml^{−1} in liquid media and 300 μg ml^{−1} in agar plates) for penicillin resistance; kanamycin sulphate (50 μg ml^{−1}) for kanamycin resistance; tetracycline hydrochloride (10–25 μg ml^{−1}) for tetracycline resistance; and streptomycin sulphate (30 μg ml^{−1}) for streptomycin resistance. Rifampicin was used at 25 μg ml^{−1}. The L agar used for blue/white screening contained 0.1 mM IPTG and X-Gal at 40 μg ml^{−1}.

Yeast strains and growth conditions. *Saccharomyces cerevisiae* strain L40 MAT a, *trp1*, *leu2*, *his3*, *ade2*, *LYS2::lexA-HIS3*, *URA3::lexA-lacZ* was used for transformation (Clontech) (Fields & Song, 1989). Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose). Plasmid-containing yeast strains were grown in YNB medium (0.67%, yeast nitrogen base and 3%, glucose) supplemented with a mixture of appropriate nutrients lacking tryptophan (for selection of pBTM116 derivatives), leucine (for selection of pGAD424 derivatives) or both as required (for selection of double transformants). Agar was added to a concentration of 2% (w/v) for the plates. To grow transformants for the β-galactosidase filter-lift assay, YNB agar was supplemented with 0.5% instead of 3% (w/v) glucose. For the β-galactosidase assay using liquid cultures, the yeast cells were grown to exponential phase in YNB medium with 0.5% (w/v) glucose and then transferred to YNB medium with 3% (w/v) ethanol for several hours. For testing the expression of the His reporter gene in strain L40, YNB plates were supplemented with a mixture of amino acids (Clontech) lacking tryptophan, leucine and histidine. Plates were incubated at 30 °C for 3–4 days.

Plasmids. The plasmids used in this study are listed in Table 1.

For expression of cloned ORFs, we used the high-copy-number Pn^R vector pGBT30 (Jagura-Burdzy *et al.*, 1991), based on the pMB1 replicon with *lacI^Q* and *tacp* separated from λ*t_{R1}* by the multiple cloning site of pUC18 (Fig. 2C). A *Pst*I–*Sal*I fragment of pGBT30

derivatives, with the ORF of interest and all upstream control elements, was recloned into a broad-host-range Smr^R IncQ-based replicon, pGBT400 (Jagura-Burdzy *et al.*, 1999b), or Tc^R broad-host-range pBBR1MCS-3 (Kovach *et al.*, 1995). Sequencing of the initial PCR product corresponding to *kfrA*_{R751} revealed an extra codon ACC (an extra Thr at amino acid position 246) (supplementary data) relative to the published sequence, but none of the studies performed here suggested that this was a mutant that had lost any activity. We sequenced two other independently amplified *kfrA* ORF clones and an identical change was found. It may be that the difference was due to an error in the original sequencing. The internal *kfrA* deletion (Δ*kfrA*) was created by removal of an internal *Not*I fragment encoding 70 aa between A136 and A207. C- and N-terminal *kfrA* deletions were obtained using the unique *Not*I restriction site in pMAB424.11, a pGAD424 derivative with Δ*kfrA*. The *Not*I linearized plasmid was treated with a DNA PolI Klenow fragment and then ligated either with an oligonucleotide carrying a *Sal*I site and stop codons in all three frames (next section), to create Δ*CkfrA*, or with an oligonucleotide carrying an *Eco*RI recognition site, followed by an ATG codon in-frame with the rest of KfrA, to create Δ*NkfrA*. *Eco*RI digestion and religation removed the N-terminal part of *kfrA*. All constructions were checked by sequencing of the new junction point.

A plasmid for KfrA purification was constructed by inserting the *kfrA* allele into a derivative of pET28a Km^R (Studier & Moffatt, 1981; Novagen), modified with the help of the synthetic oligomer, so that a His₆-tag and thrombin cleavage site preceded, almost directly, the *Eco*RI site (Lukaszewicz *et al.*, 2002). Purified His-tagged KfrA was N-terminally extended by the sequence MGSSH₆SSGLVPRGSHSEF.

E. coli–*S. cerevisiae* shuttle plasmids used in the yeast two-hybrid (YTH) system, based on vectors pBTM116 (Invitrogen) and pGAD424 (Fields & Song, 1989; Ma & Ptashne, 1987; Clontech Matchmaker), fused the polypeptides encoded by *Eco*RI–*Sal*I inserts to the C terminus of the LexA binding domain (BD) and GAL4 activation domain (AD), respectively. The system provided Ap^R selection in *E. coli* and LEU⁺ (pGAD424) or TRP⁺ (pBTM116) selection in the yeast cells.

Plasmid DNA isolation, analysis, cloning and manipulation.

Plasmid DNA was isolated by standard procedures (Birnboim & Doly, 1979). Digestion of plasmid DNA with restriction enzymes was carried out under conditions recommended by suppliers, and digested DNA was run on 0.8–2% (w/v) agarose gels. Standard PCR reactions (Mullis *et al.*, 1986) were performed as described by Jagura-Burdzy & Thomas (1994), with the pairs of primers listed in Table 2.

The PCR reaction to amplify the ORF for *kfrA* was performed with a hot start denaturation step (98 °C for 5 min) and 35 cycles of denaturation (94 °C for 30 s), annealing (52 °C for 30 s) and elongation (72 °C for 90 s). The reaction ended with a final elongation step (72 °C for 7 min). The PCR reaction mixture was supplemented with *N,N*-dimethylformamide to a final concentration of 2% (v/v).

PCR products were usually cloned into the pGEM-T vector (Promega) and later recloned as *Eco*RI–*Sal*I fragments into appropriate vectors. All PCR-derived clones were analysed by DNA sequencing to check their fidelity.

Sequencing and computer sequence analysis. DNA sequencing was performed using an Applied Biosystems 377 automated DNA sequencer (Alta Bioscience, School of Biochemistry, University of Birmingham, UK) or a Pharmacia ALF automatic sequencer (IBB, PAS) using dye terminator kits supplied by the manufacturer. DNA and amino acid sequence analysis was carried out using the GCG Wisconsin Package version 8.0. Sequences were compared to GenBank/EMBL databases and studied using the programs BLASTN and BLASTP (NCBI), BLAST (Swiss server) and Coil tool (Expasy).

Table 1. Plasmids used in this study

Designation	Relevant features	References
pAKE600	<i>ori</i> _{MBI} , <i>oriT</i> _{RK2} , Ap ^R , <i>sacB</i>	El-Sayed <i>et al.</i> (2001)
pBBR1MCS-3	Broad-host-range cloning vector, Tet ^R	Kovach <i>et al.</i> (1995)
pBTM116	Shuttle vector <i>E. coli</i> / <i>S. cerevisiae</i> , <i>ori</i> _{MBI} /2 µ, Ap ^R / <i>trp1</i> , LexA _{BD}	Invitrogen
pET28a	<i>ori</i> _{MBI} , Km ^R , T7p, <i>lacO</i> , His tag, T7 tag	Novagen
pET28mod	<i>ori</i> _{MBI} , Km ^R , T7p, <i>lacO</i> , His tag	G. Jagura-Burdzy
pGAD424	Shuttle vector <i>E. coli</i> / <i>S. cerevisiae</i> , <i>ori</i> _{ColE1} /2 µ, Ap ^R / <i>leu2</i> , GAL4 _{AD}	Fields & Song (1989)
pGBT30	<i>ori</i> _{MBI} , Ap ^R , <i>lacI</i> ^Q , <i>tacp</i> expression vector	Jagura-Burdzy <i>et al.</i> (1991)
pGBT314	pGAD424 derivative, GAL4 _{AD} - <i>incC</i> _{R751}	G. Jagura-Burdzy
pGBT400	<i>ori</i> _{IncQ} , Sm ^R , <i>lacI</i> ^Q , <i>tacp</i> expression vector	Lukaszewicz <i>et al.</i> (2002)
pGBT401	<i>ori</i> _{IncQ} , Sm ^R , <i>lacI</i> ^Q , <i>tacp</i> - <i>korA</i> _{RK2}	Jagura-Burdzy <i>et al.</i> (1999b)
pGEM-T	<i>ori</i> _{MBI} , Ap ^R	Promega
pPT01	<i>ori</i> _{SC101} , Km ^R , promoterless <i>xylE</i>	Macartney <i>et al.</i> (1997)
pR751Tet ^R	IncP-1β, Tet ^R cassette from RK2	Thorsted <i>et al.</i> (1998)
pUC18	<i>ori</i> _{MBI} , Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pWS131	<i>ori</i> _{MBI} , Ap ^R , <i>lacI</i> ^Q , <i>tacp</i> - <i>kfrA</i> _{RK2}	Thomas <i>et al.</i> (1990)
pKK38	<i>ori</i> _{MBI} , Ap ^R , <i>lacI</i> ^Q , <i>tacp</i> - <i>korA</i> _{R751}	Kostelidou & Thomas (2002)
Plasmids constructed during this work		
pUC18 derivative		
pUC7/8	<i>kfrA</i> promoter amplified with primers #7 and #8, inserted in <i>Sma</i> I	
pGBT30 derivatives (expression vector)		
pMAB30.1	<i>kfrA</i> _{R751} amplified with primers #9 and #10, inserted as <i>Eco</i> RI- <i>Sa</i> II fragment	
pMAB30.11	Δ <i>zkfrA</i> allele (<i>Not</i> I internal 210 bp fragment removed), inserted as above	
pMAB30.12	Δ <i>CkfrA</i> allele encoding N-terminal 136 aa of KfrA (N-KfrA), inserted as above	
pMAB30.13	Δ <i>NkfrA</i> allele encoding C-terminal 136 aa of KfrA (C-KfrA), inserted as above	
pMAB30.2	<i>korB</i> _{R751} amplified with primers #11 and #12, inserted as above	
pMAB30.3	<i>incC1</i> _{R751} amplified with primers #13 and #14, inserted as above	
pMAB30.4	<i>upf54.8</i> _{R751} amplified with primers #3 and #4, inserted as above	
pMAB30.5	<i>upf54.4</i> _{R751} amplified with primers #5 and #6, inserted as above	
pPDB30.6	<i>korAkorB</i> _{R751} fragment amplified with primers #20 and #11, inserted as above	
pPDB30.14	Δ <i>C2kfrA</i> allele encoding C-terminal KfrAΔ187–323, inserted as above	
pGBT400 derivatives (expression vector)		
pMAB40.1	<i>Pst</i> I- <i>Sa</i> II fragment of pMAB30.1 carrying <i>lacI</i> ^Q , <i>tacp</i> - <i>kfrA</i> _{R751}	
pMAB40.2	<i>Pst</i> I- <i>Sa</i> II fragment of pMAB30.2 carrying <i>lacI</i> ^Q , <i>tacp</i> - <i>korB</i> _{R751}	
pBBR1MCS-3 derivative (expression vector)		
pPDB3.6	<i>Pst</i> I fragment of pPDB30.6 carrying <i>lacI</i> ^Q , <i>tacp</i> - <i>korAkorB</i> _{R751}	
pET28mod derivative (protein overexpression)		
pMAB28.1	<i>Eco</i> RI- <i>Sa</i> II fragment of pMAB30.1	

Table 1. cont.

Designation	Relevant features	References
pPT01 derivatives (promoter-probe vector)		
pMAB01	<i>Bam</i> HI fragment (<i>kfrAp</i>) from pUC7/8 cloned upstream of the <i>xylE</i> cassette	
pMAB03.4	PCR product (primers #3 and #4) subcloned as a <i>Bam</i> HI fragment	
pMAB05.6	PCR product (primers #5 and #6) subcloned as a <i>Bam</i> HI fragment	
pMAB08.5	PCR product (primers #5 and #8) subcloned as a <i>Bam</i> HI fragment	
pMAB08.51	PCR product (primers #5 and #8) amplified from R751 Δ C ₂ <i>kfrA</i> , subcloned as a <i>Bam</i> HI fragment	
pGAD424 derivatives (YTH)		
pMAB424.1	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB28.1 carrying <i>kfr</i> A _{R751}	
pMAB424.2	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.2 carrying <i>korB</i> _{R751}	
pMAB424.3	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.3 carrying <i>incC</i> I _{R751}	
pMAB424.4	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.4 carrying <i>upf</i> 54.8 _{R751}	
pMAB424.5	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.5 carrying <i>upf</i> 54.4 _{R751}	
pMAB424.11	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.11 carrying $\Delta\alpha$ <i>kfrA</i> _{R751}	
pBTM116 derivatives (YTH)		
pMAB116.1	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB28.1 carrying <i>kfr</i> A _{R751}	
pMAB116.4	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.4 carrying <i>upf</i> 54.8 _{R751}	
pMAB116.5	<i>Eco</i> RI– <i>Sal</i> I fragment of pMAB30.5 carrying <i>upf</i> 54.4 _{R751}	
pAKE600 derivatives (suicide plasmid)		
pMAB6 Δ 15	PCR fragments amplified with primers #5/#6 (region downstream of <i>kfrA</i>) and #7/#8 fragment (<i>kfrA</i> promoter)	
pMAB6.12	<i>Eco</i> RI– <i>Pst</i> I fragment from pMAB30.12	
pMAB6.11	<i>Eco</i> RI– <i>Sal</i> I fragment from pMAB30.11	
pMAB6.41	PCR fragment amplified with primers #3 and #6, modified by insertion of oligonucleotide #15 into <i>Hinc</i> II site at 5' end of <i>upf</i> 54.8 ORF	
R751Tet^R derivatives		
R751 Δ C ₂ <i>kfrA</i>	Mutant allele Δ C ₂ <i>kfrA</i>	
R751 $\Delta\alpha$ <i>kfrA</i>	Mutant allele $\Delta\alpha$ <i>kfrA</i>	
R751 Δ <i>upf</i> 54.8	Mutant allele Δ <i>upf</i> 54.8	

***E. coli* transformation and PCR-mediated screening of transformants.** Competent cells of *E. coli* were prepared by the standard CaCl₂ method (Sambrook *et al.*, 1989). To screen for the recombinant plasmids, each transformant colony picked from the selective plate was resuspended in 200 μ l water and boiled for 2 min. After centrifugation, 5–10 μ l supernatant (depending on the plasmid copy

number) was added to a 25 μ l PCR reaction mixture. Alternatively, bacterial colonies were directly transferred into 25 μ l PCR reaction mixture supplemented with 0.5 μ l *N,N*-dimethylformamide.

Bacterial conjugation. Overnight cultures of the donor and recipient strain were grown in selective medium. One millilitre of each

culture was drawn into a syringe mounted on a Millipore filter. The bacterial suspensions were filtered through a sterile nitrocellulose membrane, which was then placed face-up onto an L agar plate and incubated at 37 °C overnight. The filter was transferred to a tube containing 1 ml L broth and vortexed to resuspend the bacteria. Serial dilutions of the suspension were plated out on L agar with appropriate selection and allowed to grow for 48 h at 37 °C.

Yeast transformation. Yeast transformation was performed using the PEG/LiAc standard method (Chen *et al.*, 1992). Either single or double transformations were conducted and transformants selected and stored on minimal YNB agar.

Determination of LacZ activity in yeasts. β -Galactosidase activity was monitored by the filter-lift assay with X-Gal as a substrate and by the quantitative liquid culture assay (Clontech) using ONPG as substrate. The protein concentration was estimated using the Bradford assay (Bradford, 1976). The specific activity of β -galactosidase activity was expressed in standard units where one unit represents the amount of enzyme that hydrolyses 1 nmol ONPG (mg protein)⁻¹ min⁻¹ at 30 °C.

Determination of catechol 2,3-oxygenase activity (XylE) in bacteria. Catechol 2,3-oxygenase activity (the product of *xylE*) was assayed in exponentially growing bacteria (Zukowski *et al.*, 1983) and normalized to the same plasmid level as the control. One unit of catechol 2,3-oxygenase is defined as the amount needed to convert 1 μ M catechol in 1 min under standard conditions. Protein concentration was determined using the Bradford method (Bradford, 1976).

Purification of His₆-tailed KfrA. Exponentially growing *E. coli* BL21(λ DE3)(pMAB28.1) was induced with 0.5 mM IPTG at a cell density of $\sim 2 \times 10^8$ c.f.u. ml⁻¹ and grown for an additional 2–3 h with shaking at 37 °C (Arnold, 1991). The cells from 300 ml culture were harvested by centrifugation and sonicated. The cell lysate/Ni²⁺-nitrilotriacetic acid/agarose mixture was stirred for 60 min at 42 °C and then transferred to a column. The purification procedure of His₆-tailed KfrA was monitored by SDS-PAGE using a Pharmacia Phast gel system.

Preparation of anti-KfrA antiserum. Purified His₆-KfrA protein (1 mg ml⁻¹) was injected into a rabbit. The blood collected from the rabbit was allowed to clot at room temperature before removing the antiserum. Anti-KfrA antibodies were affinity-purified on His₆-KfrA bound to Affi-gel 15 (Bio-Rad), using a method described by Reznikov *et al.* (1996) with modifications introduced by Bignell *et al.* (1999). The antibodies were stored at -20 °C in 50% (v/v) glycerol.

Estimation of KfrA concentration in the cells carrying R751Tet^R by Western blotting. Growth of *E. coli* NEM259(R751Tet^R) was monitored by OD₆₀₀. The culture was diluted and plated on L agar to establish c.f.u. ml⁻¹. Bacteria were harvested from an appropriate volume depending on the phase of growth to collect similar numbers of cells. The cells were washed in water and resuspended in 200 μ l sonication buffer (50 mM phosphate buffer, pH 8.0, 300 mM NaCl). Bacteria were kept on ice and disrupted by sonication in short bursts for 2 min. Crude extracts were cleared by centrifugation at 4 °C in a microfuge for 15 min at 12 000 g. Protein concentration was measured using the Bradford method (Bradford, 1976). Proteins from the cleared extracts were separated on 12.5% (w/v) SDS-PAGE gels (Laemmli, 1970) and electroblotted (semi-dry transfer unit) for 1–2 h onto nitrocellulose membranes (Protron; Schleicher & Schuell). Probing of the blots was carried out using the amplified alkaline phosphatase goat anti-rabbit immunoblot assay (Promega) with anti-KfrA antiserum at a dilution of 1:100 000 as the primary antibody. The cell extracts were compared to a range of concentrations of purified His₆-KfrA on the same gel. Band intensity on Western blots was determined using Image Quant (Molecular Dynamics).

Immunofluorescence microscopy. The fixation and permeabilization of cells and subsequent staining for immunofluorescence were carried out as described by Bignell *et al.* (1999). Ten microlitres of affinity-purified anti-KfrA and anti-KorB antibodies were used as the primary antibodies (1:5000 dilution for anti-KfrA and 1:2000 dilution for anti-KorB antibodies in 2%, w/v, BSA/PBS) followed by 10 μ l anti-rabbit IgG FITC-conjugate solution (6.9 μ g ml⁻¹ in 2%, w/v, BSA/PBS) (Sigma). The coverslip was placed onto a microscope slide in a solution of 4',6-diamidino-2-phenylindole (DAPI)/Vectorshield mounting medium (Vector Laboratories) (ratio of 1:4 DAPI 1 μ g ml⁻¹ to Vectorshield). Cells were studied with an Olympus IX70 inverted reflected light fluorescence microscope fitted with a Sensys charge-coupled device (CCD) camera (Photometrics). Images were captured and manipulated on a Macintosh G3 with the Smartcapture I program (Digital Scientific).

Introduction of mutant alleles into R751Tet^R backbone by reverse genetics. *E. coli* strain NEM259(R751Tet^R) was transformed with suicide vector pAKE600 derivatives (El-Sayed *et al.*, 2001). Double transformants were conjugated with the recipient strain C2110Rif^R in which the suicide vector based on pMB1 *ori* was unable to replicate. Putative conjugants with R751Tet^R-suicide vector cointegrated plasmids were selected on L agar with rifampicin, penicillin and tetracycline. After double restreaking, Rif^RPen^RTet^R colonies were used to inoculate L broth containing 10% (w/v) sucrose and tetracycline to facilitate the removal of the suicide vector insertion from R751Tet^R. Survivors were plated on L agar with 10% (w/v) sucrose and tetracycline and checked for the Pen^S phenotype. Suc^RTet^RPen^S colonies were analysed by colony PCR to determine whether allele exchange had occurred and, where necessary, the PCR products were gel-purified and digested with *SalI* (Δ CkfrA, Δ upf54.8). To confirm the knockout of the *kfrA* gene in the R751Tet^R backbone protein, extracts were analysed by Western blotting with anti-KfrA antibodies.

Determination of plasmid stability. Plasmid stability tests were performed as described by Williams *et al.* (1998) and Macartney *et al.* (1997). *E. coli* strain NEM259 was transformed with plasmid R751Tet^R (or its derivatives) and transformants were selected on L agar plates containing tetracycline. The transformants were grown in selective medium and then diluted 10²-fold into non-selective L broth. Samples of the culture were collected every 12 h (at which point the cultures were diluted 10⁵-fold and transferred into fresh medium), diluted and plated out onto L agar and then transferred onto L agar with tetracycline. The c.f.u. were counted to determine the proportion of bacteria retaining the plasmid.

RT-PCR reactions. One microgram of total RNA (isolated by Qiagen RNeasy kit), suspended in diethyl pyrocarbonate-treated water, was mixed with 2.5 pM gene-specific downstream primer #1 annealing to the 3' end of *upf54.4*, and the reverse transcriptase reaction was performed with 200 U SuperScript RT. RNA was removed by treatment with RNase and cDNA was purified using GlassMAX spin cartridges as recommended by the manufacturer (GibcoBRL). Purified cDNA was used as a template for PCR with primer pairs as in Fig. 2(B, C).

RESULTS

kfrA, *upf54.8* and *upf54.4* constitute a transcriptional unit

From the original studies on IncP-1 α plasmid RK2 it appears that the *kfrA* gene forms an autogenously regulated, monocistronic operon, leaving completely open the benefit that it may provide for RK2 (Thomas *et al.*, 1990). However, there

Table 2. Primers for PCR reactions

Restriction enzyme recognition sites are underlined and start/stop codons are in bold type.

Primer code	Primer name	Sequence
#1	<i>upf54.4</i> _{R751}	5'-C <u>GTC-GAC-GGA-TCC</u> -TTT-TTA-TGC-GGG-CCT-GGC-TG-3'
#2	<i>upf54.4</i> _{R751}	5'-CC- <u>GAA-TTC-ATG</u> -AAA-CGA-CTC-ACC-ATC-CCC-3'
#3	<i>upf54.8</i> _{R751}	5'-CC- <u>GTC-GAC-GGA-TCC</u> -GGG-ATG-GTG-AGT-CGT-TTC-AA-3'
#4	<i>upf54.8</i> _{R751}	5'-GG- <u>GAA-TTC-ATG</u> -AAA-CAAC-GCC-TCT-TGG-3'
#5	<i>upf54.8</i> _{R751}	5'-CG- <u>GGA-TCC</u> -GTT-CAT-GAC-CAA-GAG-GCG-T-3'
#6	<i>upf54.8</i> _{R751}	5'-CC- <u>GAA-TTC</u> -TGG-AAA-CCG-TCA-TGG-CGC-AA-3'
#7	<i>kfrA</i> _{R751}	5'-CC- <u>GGA-TCC</u> -TTC-ATC-GCC-TCG-CTG-ATG-G-T-3'
#8	<i>kfrA</i> _{R751}	5'-CC- <u>GGA-TCC</u> -GCC-TGG-CTC-AAA-TAC-GAG-GA-3'
#9	<i>kfrA</i> _{R751}	5'-CG- <u>GTC-GAC</u> -TCA-GGT-CTT-TTT-GCG-TCC-GC-3'
#10	<i>kfrA</i> _{R751}	5'-CG- <u>GAA-TTC-ATG</u> -GCG-ATC-TCG-AAG-GAG-CA-3'
#11	<i>korB</i> _{R751}	5'-CG- <u>GTC-GAC-GGA-TCC</u> -TCA-GCC-CTC-CAG-CAG-CGC-3'
#12	<i>korB</i> _{R751}	5'-CG- <u>GAA-TTC-ATG</u> -AGC-GCC-AAG-ACC-AAC-GC-3'
#13	<i>incC</i> _{R751}	5'-CG- <u>GAA-TTC-ATG</u> -AAA-ACA-CTG-GTC-ACG-G-3'
#14	<i>incC</i> _{R751}	5'-CG- <u>GTC-GAC-GGA-TCC</u> -TCA-TTG-CGC-GAT-CTC-C-3'
#15	oligo <i>SalI</i>	5'- TGA-CCT-AGT -CGA-CTA-GGT-CA-3'
#16	oligo <i>EcoRI</i>	5'-CG-CAT- <u>GAA-TTC-ATG</u> -CG-3'
#17	oligo end <i>korB</i> _{R751}	5'-TGG-AGG-GCT-GAC-ACG-CCC-AG-3'
#18	oligo <i>XbaI</i> -rbs	5'- <u>CTA-GAA</u> -ATG-AAG-GAG-AG-3'
#19	oligo <i>EcoRI</i> -rbs	5'- <u>AAT-TCT</u> -CTC-CTT-CAT-TT-3'
#20	oligo <i>KorA</i> _{R751}	5'-CG- <u>GAA-TTC-ATG</u> -AAG-AAA-CGG-CTA-ACG-GA-3'

are no experimental data confirming termination of transcription after *kfrA* or independent transcription of downstream genes *upf54T.8* and *upf54.4*. To test the possibility that all three genes form a single operon (and may have a common function), reporter gene fusions were first constructed with *xylE* and subsegments of this region (Fig. 2A and supplementary data for primer positions). Strong transcriptional activity was detected for the region containing the predicted *kfrAp* (pMAB01; nucleotides 2201–2597, supplementary data), whereas activity was only very weak for a fragment spanning the end of *kfrA* and the start of *upf54T.8* (pMAB03.4; nucleotides 3666–4062) and hardly detectable for another fragment running from *upf54.8* to *upf54.4* (pMAB05.6; nucleotides 3432–3691). When the cloned segment started upstream of *kfrA* and ended within *upf54.8* (pMAB08.5; nucleotides 2201–3691), much higher transcriptional activity was observed compared to the *upf54.8* fragment alone (pMAB03.4), indicating that transcription originating within, or upstream of, *kfrA* was responsible for most of the *upf54.8* transcription. To further locate this activity, a 408 bp internal deletion in *kfrA* was created, running from nucleotides 3025 to 3432 (pMAB08.51). This resulted in a further fourfold increase in transcription into *upf54.8*, confirming that the promoter responsible for *upf54.8* transcription must be upstream of nucleotide 3025, close to the beginning of *kfrA* at nucleotide 2467 (Supplementary data). Although this deletion did not abolish KfrA autorepressor activity (see below), the increase in XylE activity in pMAB08.51 versus pMAB08.5 suggests that the promoter activity measured was under the control of KfrA. No obvious promoters, other than

kfrAp, were found in the remaining R751 DNA present in pMAB08.51, and also no similarities to the putative KfrA binding site identified in *kfrAp*. Therefore, the simplest explanation is that transcription from *kfrAp* ran through into *upf54.8*. This may have added to the weak promoter activity detected in the intergenic region between *kfrA* and *upf54.8*.

Independent evidence for co-transcription of *kfrA*, *upf54.8* and *upf54.4* came from isolation of total RNA from strain NEM259(R751Tet^R) and PCR on cDNA obtained with reverse transcriptase and primer #1 (supplementary data) complementary to the 3' end of *upf54.4*. PCR products of expected sizes were obtained with primer pairs spanning both the *upf54.4*–*upf54.8* and *upf54.8*–*kfrA* junctions, as well as for the whole of *kfrA*, indicating that the cDNA, and hence mRNA, covered the whole region (Fig. 2A, B). As a negative control, we performed RT-PCR on the *korB*–*kfrA* intergenic region, which contained a predicted strong transcriptional terminator preceding *kfrAp* (primers 7/17) and on the *kfrA* promoter region (primers 7/8), and obtained no products. All these primer pairs amplified the appropriate fragments from the R751 DNA template in parallel PCR reactions. We therefore conclude that while *upf54.8* and *upf54.4* may have been weakly expressed independently of *kfrAp*, the majority of their transcription was part of a tricistronic unit starting with *kfrA*.

Regulation of transcription from *kfrAp*

The DNA sequence of *kfrAp*_{R751} contains highly conserved sequences, known to bind the global regulators of IncP-1

Fig. 2. Transcriptional organization of *kfrAp*_{R751}. (A) *kfrA* region of R751. Fragments below the diagram were amplified using the indicated primers and R751Tet^R as the template, with the exception of clone #5#8 in pMAB08.51, for which the template used in this PCR reaction was R751Tet^R Δ C₂*kfrA*. The fragments cloned upstream of the *xyIE* cassette in the promoter-probe vector pPTO1 gave the plasmids listed on the left (for plasmid description see Table 1). The promoter activities detected, expressed as units of catechol oxygenase activity, are summarized in the first column on the right. The second column on the right shows which pairs of primers gave products of the expected size after RT-PCR with cDNA synthesized from RNA from *E. coli* NEM259(R751Tet^R), with the use of primer #1 annealing to the 3' end of *uptf54.4*. ND, Not determined. (B) RT-PCR products of the *kfrA-uptf54.8-uptf54.4* region obtained from cDNA from cells of strain NEM259(R751Tet^R). As positive controls, the same pairs of primers were used with R751 DNA as the template (dsDNA panel). M, 1 kb DNA ladder; –, no template added. (C) Compatible plasmids used in the regulatory studies. C600K[–] was first transformed with pMAB01(*kfrAp-xyIE*) and then either one, or both, of the pGBT30 and pGBT400 derivatives were introduced to C600K[–] (pMAB01). Both expression vectors had exactly the same transcriptional and translational signals to overproduce the regulatory proteins. They differed by copy number, so the experiments with two regulators *in trans* were done with reciprocal combinations of ORFs, on either medium- or high-copy-number vectors.

plasmids KorB (O_B) (Bignell *et al.*, 1999; Williams *et al.*, 1993; Jagura-Burdzy *et al.*, 1999a; Kostelidou & Thomas, 2000) and KorA (O_A) (Theophilus *et al.*, 1985; Jagura-Burdzy & Thomas, 1995) located in tandem upstream of the predicted -35 region (Supplementary data). Here, O_B and O_A are separated by only 3 nt (16 nt between their centres of symmetry). While similar close spacing is also observed at *klcAp*_{R751}, previous studies on simultaneous binding and cooperativity of KorA and KorB have been confined to promoters where the centre-to-centre distance between O_B and O_A is 32–34 nt. To determine the sensitivity of *kfrAp*_{R751} to KorA and KorB, reporter gene assays were performed with the regulatory genes, inducible by IPTG, provided *in trans* on compatible plasmids (Fig. 2C). Both KorA and KorB alone showed only two- to fourfold repression but gave about 100-fold repression (Table 3) when present together, giving a cooperativity index of 10, which indicates a 10-fold higher repression than expected from the product of the repression indices of both proteins individually. Thus, close spacing of O_A and O_B did not prevent their cooperative interaction and also did not cause them to interfere with each other. It may be of significance that the centres of symmetry of these operator sequences are actually located 16 bp apart and therefore should be on opposite faces of the DNA helix (Bingle *et al.*, 2005).

We previously showed for RK2 that *kfrAp* is autoregulated and that the target for KfrA_{RK2} is an operator, O_K , overlapping the -10 region (Jagura-Burdzy & Thomas, 1992;

Thomas *et al.*, 1990). The long inverted sequence is also present in *kfrAp* of R751 and this was tentatively named putative KfrA_{R751} operator O_K (Thorsted *et al.*, 1998). The *kfrA*_{R751} ORF was inserted into pGBT30 and pGBT400 derivatives under *tacp* control, and the effect of KfrA_{R751} overproduction on the activity of *kfrAp*_{R751} (*kfrAp*–*xylE* transcriptional fusion in pMAB01) was analysed in double transformants. Table 3 shows that when KfrA_{R751} was expressed *in trans* from *tacp*, significant repression was observed even without induction by IPTG (0.28 U Xyle in the presence of pMAB30.1 *in trans* to pMAB01 in comparison to 3.55 U in the presence of vector pGBT30 under the same conditions). In contrast, no effect of KfrA_{RK2} on *kfrAp*_{R751} was seen [*E. coli* C600K(pMAB01)(pWS131)], confirming that the O_K sequences changed autoregulator specificity. The regulatory effects of Upf54.8 (pMAB30.4) and Upf54.4 (pMAB30.5) were tested in the same way, but with negative results (Table 3). The presence of Upf54.8 or Upf54.4 did not increase the repression of *kfrAp* exerted by KfrA from pMAB40.1 (data not shown).

To determine how KfrA_{R751}, KorA_{R751} and KorB_{R751} might interact at *kfrAp*_{R751}, we provided *korA* or *korB* at the same time as *kfrA*. Surprisingly, under uninduced conditions, when either KorA or KorB was present with KfrA, the activity of *kfrAp* was two- to fourfold higher than when KfrA was present alone (Table 3). The effect seemed not to depend on the level of *korA/korB* gene dosage versus *kfrA* gene dosage (*tacp*–*kfrA* was expressed either from

Table 3. Effects of different regulators on expression of *kfrAp*_{R751}

Plasmids <i>in trans</i> to pMAB01*	Relevant regulatory gene(s)	Xyle activity (U) of <i>kfrAp</i> – <i>xylE</i> transcriptional fusion†			
		IPTG concentration (mM)			Relative repression at 0.5 mM IPTG‡
		0	0.05	0.5	
pGBT30	None	3.55	3.55	3.8	1
pMAB30.1	<i>kfrA</i> _{R751}	0.28	0.12	0.08	45
pWS131	<i>kfrA</i> _{RK2}	3.6	ND	3.7	1
pKK38	<i>korA</i> _{R751}	ND	ND	1.9	2
pMAB30.2	<i>korB</i> _{R751}	ND	1.6	0.85	4.2
pKK38 + pMAB40.2	<i>korA</i> _{R751} + <i>korB</i> _{R751}	0.6	0.1	0.04	95
pKK38 + pMAB40.1	<i>korA</i> _{R751} + <i>kfrA</i> _{R751}	0.5	0.17	0.26	14
pMAB30.1 + pMAB40.2	<i>kfrA</i> _{R751} + <i>korB</i> _{R751}	0.6	0.18	0.23	15.6
pMAB30.4	<i>upf54.8</i> _{R751}	4.1	ND	3.6	1.1
pMAB30.5	<i>upf54.4</i> _{R751}	4.0	ND	3.5	1.1
pPDB3.6	<i>korA</i> _{R751} <i>korB</i> _{R751}	0.15	ND	0.02	190
pPDB3.6 + pMAB30.1	<i>korA</i> _{R751} <i>korB</i> _{R751} + <i>kfrA</i> _{R751}	0.07	ND	0.01	360

*Regulatory genes were cloned under *tacp* either on high-copy-number pGBT30 (pMB1 origin) or medium-copy-number derivatives of RSF1010 (IncQ replicon), or pBBR1MCS. All vectors contained the *lacI*^Q gene to facilitate tighter transcriptional control. Plasmids were transformed into *E. coli* C600K[−] strain. When two regulators were present *in trans* no difference was observed in the level of repression related to the gene dosage.

†Transcriptional fusion *kfrAp*–*xylE* was present on pMAB01 (derivative of pPT01 vector). The enzymic assays were performed on extracts from exponentially growing cells (OD₆₀₀ 0.4–0.6) after IPTG induction for 2.5–3 h at the indicated concentrations of inducer.

‡Relative repression indicates the Xyle activity of strain C600K[−](pMAB01)(pGBT30) grown on 0.5 mM IPTG divided by the Xyle activity of the appropriate transformant.

medium-copy-number plasmid pMAB40.1 or high-copy-number plasmid pMAB30.1). These results suggest some sort of interference between these repressors. Western blotting extracts from the cultures did not indicate any significant fluctuations in the level of KfrA production in the presence or absence of other regulators. The presence of three repressors, KorA, KorB (pPDB3.6) and KfrA (pMAB30.1), together completely shut off expression from *kfrAp*.

Repression by deletion derivatives of KfrA

From the previous section it can be seen that repression of transcription is a key property of KfrA. The predicted secondary structure of KfrA_{R751}, 343 aa, revealed a distinct N-terminal region that might form a globular head linked to a long, coiled-coil, α -helical structure (data not shown). No typical DNA binding motifs were detected. This is very similar not only to RK2 KfrA but also to the protein TlpA of pLT2, a protein involved in regulating temperature-responsive gene expression (Koski *et al.*, 1992; Hurme *et al.*, 1994, 1996, 1997). To determine which parts of KfrA are needed for DNA binding, mutant alleles of *kfrA* were created with extensive deletions: $\Delta\alpha kfrA$, encoding KfrA, lacking 70 aa between A136 and A207 from the long α -helical tail (KfrA Δ 137–206); $\Delta CkfrA$, encoding the N-terminal 136 aa (KfrA1–136); ΔC_2kfrA , encoding the N-terminal 186 aa linked in-frame to the C-terminal 20 aa (KfrA Δ 187–323); and $\Delta NkfrA$, encoding the C-terminal 137 aa (KfrA207–343).

To test the possibility that KfrA might control a temperature-dependent switch, the deletion alleles of *kfrA*

were expressed from *tacp* *in trans* to the *kfrAp*–*xylE* reporter plasmid (pMAB01) at 25, 37 and 42 °C. At all temperatures, WT KfrA showed very strong repression and this activity appeared to be enhanced even at 42 °C. The C-terminal fragment KfrA207–343 showed no repression at any temperature. At 25 °C, both KfrA1–136 and KfrA Δ 137–206 were able to repress the *krfA* promoter, although KfrA1–136 demonstrated a significant drop in repression compared to wild-type (WT) KfrA or KfrA Δ 137–206. At the higher temperatures, KfrA1–136 lost most of its repressor activity. Interestingly, KfrA Δ 137–206 showed quite clear temperature sensitivity at low levels of induction, with significant levels of repression at 37 °C but not at 42 °C. However, at higher levels of induction, KfrA Δ 137–206 still caused repression at high temperature and, indeed, was an even more potent transcriptional repressor than WT KfrA, at least at 37 °C (Table 4). This indicates that the DNA binding domain is in the protein segment amino acids 1–136, but that the rest of the protein contributes to stabilization of the active form of the protein, so that inactivation can occur at higher temperature in the mutants. The *kfrA* allele producing KfrA Δ 187–323 with 137 aa removed was also tested (pPDB30.14) and found to have repressor activity comparable to WT KfrA when overproduced (data not shown).

KfrA, Upf54.8 and Upf54.4 interact with each other

The YTH system was used to test for interactions between KfrA_{R751} and other proteins encoded by the same operon as KfrA, and with proteins encoded by the adjacent central control operon and involved in the active partitioning of

Table 4. Influence of temperature on autorepression ability of KfrA and its truncation products

For key to symbols, see footnotes to Table 3.

Plasmids <i>in trans</i> to pMAB01*	Relevant KfrA product	IPTG concn (mM)	XylE activity (U) of <i>kfrAp</i> – <i>xylE</i> transcriptional fusion†: temperature of growth		
			25 °C	37 °C	42 °C
pMAB30.1	None	0	3.55	3.55	3.60
		0.5	3.70	3.8	3.70
		Relative repression‡	1	1	1
		0	0.20	0.28	0.04
		0.5	0.04	0.08	0.03
pMAB30.11	KfrA Δ 137–206	Relative repression	93	45	123
		0	0.02	0.02	3.70
		0.5	0.02	0.02	0.02
		Relative repression	185	190	185
		0	4.30	3.70	3.70
pMAB30.12	KfrA1–136	0.5	0.46	1.30	2.40
		Relative repression	8	3	1.5
		0	4.00	3.60	3.9
		0.5	3.9	3.70	3.8
		Relative repression	1	1	1

Fig. 3. Stability of R751 mutant derivatives. (A) Schematic representation of mutated *kfrA* and *upf54.8* alleles introduced by reverse genetics into an R751Tet^R background. The expressed parts of the encoded proteins are marked in black. (B) Western blot of extracts from *E. coli* NEM259(R751Tet^RΔC₂*kfrA*) and NEM259(R751Tet^R) with anti-KfrA antibodies. Similar numbers of cells (2×10^8 , collected at OD₆₀₀ 0.6) were loaded. Column M shows biotinylated molecular mass markers. (C) Stability data for R751Tet^R and its three derivatives. Overnight cultures of *E. coli* NEM259(R751Tet^R or its mutant derivatives), grown on tetracycline, were diluted 10⁵-fold and left to grow without selection for 12 h (~20 generations), and then diluted into fresh medium. At the time of dilution, the appropriate samples were plated on L agar plates to obtain single colonies, which were subsequently checked for the ability to grow on plates with tetracycline. Plasmid retention was calculated as the percentage of Tet^R clones in relation to the total number of colonies.

IncP-1 plasmids. To study relevant protein–protein interactions, *kfrA*, *incC1*, *korB*, *upf54.8* and *upf54.4* were cloned into the shuttle vectors pBTM116 and pGAD424 to create translational fusions with the C termini of LexA and the GAL4 activation domain (GAL4_{AD}). *S. cerevisiae* L40 was used to detect protein–protein interactions on the basis of activation of the *lacZ* and *HIS* genes under control of GAL4-dependent promoters, with LexA binding sites upstream.

The YTH data demonstrated that KfrA was able to dimerize *in vivo*. In liquid cultures of double transformants of strain L40(pMAB116.1)(pMAB424.1), a high level (90 U) of β -galactosidase activity was detected, in comparison with <0.2 U in transformants with control plasmids, suggesting very strong protein–protein interactions. Experiments with glutaraldehyde cross-linking of purified His₆-KfrA confirmed the ability of KfrA_{R751} to form dimers and other higher-order complexes (data not shown).

No interactions were detected between KfrA and either IncC1 or KorB, which are core components of the active partitioning system encoded by the adjacent operon. These therefore provide negative controls (<0.2 U β -galactosidase). However, the YTH system did show that there were weak interactions between KfrA and Upf54.8 (2–5 U β -galactosidase) but not between KfrA and Upf54.4 (data not shown). Both Upf proteins self-interacted (Upf54.4 dimerization gave rise to ~30 U β -galactosidase, whereas Upf54.8 was much weaker with 3–5 U) and interacted with each other in the YTH system (30–90 U β -galactosidase, depending on translational fusion). It is therefore possible that although KfrA did not interact with Upf54.4 directly, all three proteins may have formed a complex, with Upf54.8 being the linker between the other two components.

Mutations in KfrA and Upf54.8 lead to plasmid instability

To establish the role of intact KfrA in R751 biology, mutant *kfrA* alleles were introduced into the R751Tet^R backbone as described in Methods. In essence, for each mutation, 400 bp arms flanking the mutation were generated by PCR, introduced together into a suicide plasmid, which was then forced to integrate by homologous recombination into R751 (Fig. 3A). Finally excisants were selected and screened by PCR and Western blotting with anti-KfrA antibodies for those that had retained the mutant allele. This was attempted with a total deletion of *kfrA* as well as two previously characterized deletion alleles, $\Delta\alpha kfrA$ and $\Delta CkfrA$. The procedure worked smoothly for the $\Delta\alpha kfrA$ allele and nine out of 10 clones after the last step had exchanged WT *kfrA* for the mutation. However, for the $\Delta CkfrA$ allele, only one in 50 analysed clones demonstrated the loss of WT *kfrA* and, while further analysis indicated the presence of a deletion in *kfrA*, this did not exactly match the allele which was supposed to recombine, producing a larger polypeptide on SDS-PAGE than was expected (M_r 23 000 rather than 17 000 estimated from the product of plasmid pMAB30.12) (Fig. 3B). PCR amplification followed by DNA sequencing

Fig. 4. Intracellular level of KfrA_{R751}. (A) Western blot with anti-KfrA_{R751} antibodies. Similar numbers of cells from logarithmic cultures (10⁸) were loaded on the gel in all tracks, with the exception of track NEM259(R751Tet^R), marked S, which corresponds to 2 × 10⁹ cells from the stationary culture (track L represents 10⁸ cells of the same strain but from a logarithmic culture). Cells with plasmids overproducing KfrA_{R751} (pMAB30.1) and KfrA_{RK2} (pWS131) were induced for 3 h with 0.5 mM IPTG. Purified His₆-KfrA (1.28 µg) and biotinylated molecular mass standards (track M) were also run on this 12% SDS-polyacrylamide gel. (B) A culture of strain NEM259(R751Tet^R) was diluted 100-fold and grown on L broth at 37 °C. Growth was monitored by OD₆₀₀ and c.f.u., and samples were collected, sonicated and analysed by SDS-PAGE with dilutions of purified His₆-KfrA as standards. This gel is representative of many experiments. The samples from logarithmic cultures correspond to 10⁸ cells and that from the stationary culture to 5 × 10⁸ cells. The OD₆₀₀ at which the cells were collected is indicated above the tracks. The stationary culture had OD₆₀₀ 2.0.

showed that the derivative had a deletion from 187Q to 323L, linking the C-terminal 20 aa to the first 186 aa. This derivative was designated R751ΔC₂kfrA. The regulatory

ability of KfrAΔ187–323 was tested by cloning the PCR product, under *tacp*, into pPDB30.14, and was shown to give a similar autorepression effect as WT KfrA (data not shown). The difficulty in obtaining R751ΔCkfrA suggested that the allele we tried to introduce, encoding KfrA1–136 with significantly impaired regulatory properties, was somehow disadvantageous for R751. This conclusion is also strengthened by the fact that we were unable to construct an R751ΔkfrA null plasmid; none of the 100 derivatives screened had lost *kfrA* completely.

The two R751Tet^R derivatives that were obtained (R751Tet^RΔ₂kfrA and R751Tet^RΔC₂kfrA) were introduced into *E. coli* NEM259 and checked for their stability phenotype. The strains were grown for approximately 60 generations without tetracycline selection. Every 20 generations, the cultures were diluted by a factor of 10⁵ into fresh medium. The results of this test are presented in Fig. 3(C), demonstrating the loss of R751 Tet^RΔC₂kfrA, but not R751Tet^RΔ₂kfrA. The presence of R751Tet^RΔC₂kfrA in the host did not inhibit the growth of bacteria more than R751Tet^R (data not shown). Therefore, it seems unlikely that, in the absence of antibiotic selection, the overgrowth of plasmid-free segregants, relative to the cells with R751Tet^RΔC₂kfrA, was the only reason for the observed loss of plasmid stability (especially since neither R751Tet^R itself nor R751Tet^RΔ₂kfrA demonstrated significant segregation).

The instability of R751 carrying a mutation in *kfrA* could have been due to a need for KfrA in a complex directly involved in stable inheritance/plasmid survival or an indirect effect due to disrupted regulatory circuits. To distinguish between these possibilities, the production of the truncated KfrA from R751ΔC₂kfrA was analysed by Western blotting. Standardized samples of bacteria carrying R751 and R751ΔC₂kfrA showed lower production of KfrAΔ187–323 than KfrA (as expected from regulatory studies) (Fig. 3B), indicating that the level of expression of the *kfrA*–*upf54.8*–*upf54.4* operon was not dramatically disturbed in the R751ΔC₂kfrA mutant, and that plasmid instability was therefore most likely due to a direct role of KfrA.

A mutant allele for *upf54.8* was also constructed by introducing a stop codon after the tenth codon into the coding sequence of *upf54.8* (pMAB6.41). Using the suicide technique, the wild-type *upf54.8* in R751Tet^R was successfully exchanged for the mutated allele at the expected frequency with no problems. The stability test on R751Tet^RΔ*upf54.8* showed that the mutated plasmid became unstable to a similar extent as with the impaired *kfrA* allele (ΔC₂kfrA) (Fig. 3C). The *upf54.8*–*upf54.4* region in RK2 has previously been suggested to be a part of the *tra* region (Krishnapillai, 1988) and occasionally referred to as *traN*–*traO*. The constructed mutant R751Tet^RΔ*upf54.8* was highly proficient in conjugative transfer between *E. coli* strains, as well as between *E. coli* and *Pseudomonas aeruginosa*. Attempts to construct an *upf54.4* deletion allele have not been successful.

Intracellular concentration of KfrA

Polyclonal rabbit antibodies raised against KfrA were applied to estimate KfrA concentrations in the cells with plasmid R751. The specificity of anti-KfrA antibodies was tested against *E. coli* extract. A single protein band was detected only when the strain contained R751. There was only very insignificant cross-reactivity between anti-KfrA_{R751} antibodies and KfrA_{RK2}. No signal was observed with an extract from *E. coli* (RK2) (data not shown) and only a very weak signal with highly overproduced KfrA_{RK2} from pWS131 (*tacp-kfrA_{RK2}*), as demonstrated in Fig. 4(A). Overproduction of KfrA_{RK2} was confirmed by Coomassie staining (data not shown). Thus, the 40 % homology at the primary amino acid sequence level was not sufficient for anti-body cross-reactivity. The purified KfrA_{R751} demonstrated a range of multimers, even without the presence of cross-linking agent. Western blotting was applied to estimate the level of KfrA_{R751} in bacteria from logarithmic and stationary-phase cultures, to determine whether levels were high enough for detection by immunofluorescence, and to compare these levels with those of other regulatory proteins that have already been studied. Previous studies on RK2 proteins (KorA and KorB) showed dependence of the protein level on the stage of culture growth (G. Jagura-Burdzy & C. M. Thomas, unpublished observations). Extracts from *E. coli* NEM259(R751Tet^R) demonstrated that actively growing cells contained up to 7000 monomers of KfrA per cell (the number of cells estimated on the basis of c.f.u.), whereas stationary-phase bacteria contained about 500 monomers of KfrA per cell (Fig. 4B). This drop in number of molecules per cell cannot be explained completely by the smaller size of stationary-phase bacteria, which had decreased approximately twofold, as estimated from the micrographs. This indicates that either production of KfrA was switched off as bacteria approached the end of the exponential phase, or KfrA was degraded. However, no products of KfrA proteolysis were observed, suggesting that there was a transcriptional block in KfrA production in the transition-phase cells. Thus, the fall in KfrA level may have been due to dilution of the protein after production was reduced in bacteria that continued to divide.

Fig. 5. Localization of KfrA in cells with R751 by immunofluorescence. (A) Representative cells of *E. coli* NEM259(R751Tet^R) stained by DAPI and incubated with the primary anti-KfrA or anti-KorB rabbit antibodies and secondary FITC-conjugated, anti-rabbit antibodies. The top panel shows the cells of strain NEM259(R751Tet^R) incubated with the primary anti-KfrA_{R751} rabbit antibodies. The bottom panel shows the cells from the same culture incubated with the primary anti-KorB_{RK2} rabbit antibodies. (B) Cells of strain NEM259(R751ΔC₂kfrA) were DAPI-stained and incubated with the primary anti-KfrA or anti-KorB rabbit antibodies and then FITC-conjugated, anti-rabbit antibodies. Cells were collected from logarithmic culture grown for only 3 h without selection, so no plasmid loss should have been observed. Representative small and large cells are shown, stained either with DAPI alone or with DAPI and FITC combined. (C) Magnification of part of panel (B).

KfrA forms foci in the presence of R751

To determine possible localization of KfrA_{R751} in particular parts of the bacterial cell, and to obtain more information on the defect caused by KfrA Δ 187–323 (R751 Δ C₂kfrA), we used immunofluorescence with anti-KfrA antibodies to visualize KfrA_{R751} protein. In earlier studies, use of anti-KorB antibodies showed that IncP-1 plasmids form one to four foci, dependent on the stage of cell growth, despite the fact that the plasmid copy number per cell is seven to 15 (Bignell *et al.*, 1999; Pogliano *et al.*, 2001). In our experiments, we used anti-KorB and anti-KfrA antibodies. No signals were detected in bacteria without plasmid R751, while very strong and specifically localized foci were observed in bacteria with R751 and the KfrA single binding site (Fig. 5A). Ninety percent of cells contained one to four KfrA foci per cell. A similar distribution of foci was observed with anti-KorB antibodies, suggesting co-localization of KfrA with R751 plasmid clusters.

The visualization experiments were repeated with exponentially growing cultures of *E. coli* NEM259(R751Tet^R Δ C₂kfrA). The culture was grown without selection for five to six generations, so the R751 derivative was still expected to be present in most of the bacteria. KorB and KfrA foci were visible in almost all cells, but two different conclusions could be drawn. First, most of the cells were of normal size or only slightly elongated and, in those cells, the KfrA and KorB foci were much smaller, more numerous and localized close to the pole (Fig. 5B, C). Second, there was a significant percentage of long filamentous cells (forming up to 3 % of the population) which had regularly distributed nucleoids and large DNA-free zones between nucleoids filled with KorB/KfrA/plasmid molecules.

DISCUSSION

In this paper we provide evidence that three R751 genes, *kfrA*, *upf54.8* and *upf54.4*, form a unit that is transcribed from *kfrAp* and regulated by KorA, KorB and KfrA. The *kfrA*_{RK2} gene was originally deduced to be monocistronic (Thomas *et al.*, 1990), mainly on the basis of the presence of a predicted mRNA secondary structure downstream of *kfrA* that may constitute part of a transcriptional terminator, but this picture needs to be reviewed in the light of the studies on R751. While the low level of transcription into *upf54.8*_{R751} is consistent with there being a transcriptional terminator between *kfrA* and *upf54.8*, the latter gene clearly does not have a strong promoter of its own and transcription from *kfrA* is able to pass through into the downstream gene. Circumstantial evidence for such operon organization has already been provided by *in vitro* transcription/translation studies on this region from RK2 as a DNA template: much higher expression of *upf54.8* and *upf54.4* is observed if *kfrA* is mutated (D. R. Williams & C. M. Thomas, unpublished observations). Further work will be necessary in both IncP-1 α and β systems to establish whether there is any transcriptional termination after *kfrA* and before *upf54.8* which would result in higher mRNA levels for *kfrA* relative to the

two downstream genes. Our reporter gene assays suggest that any promoter immediately upstream of *upf54.8* is very weak, but it is conceivable that even a weak promoter may be significant under some circumstances. Nevertheless, because of the organization, coregulation and physical interaction between the proteins and the phenotypes reported in this paper, we conclude that the *upf* genes are part of the coregulated plasmid backbone functions of the IncP-1 plasmids, and are probably part of the machinery that ensures the efficient survival of the plasmid as a mobile genetic element. In recognition of this, we suggest that the *upf54.8* and *upf54.4* genes should be renamed *kfrB* and *kfrC*, respectively.

A key part of this newly defined operon is the autoregulation by KfrA. The putative target for KfrA is probably an extended inverted repeat overlapping the –10 region of *kfrAp* (Supplementary data). One could therefore expect KfrA_{R751} to be at least dimeric. This is consistent with the self-association demonstrated using the yeast two-hybrid system and confirmed by chemical cross-linking studies (data not shown). The predicted structure of KfrA_{R751} implicates the whole of the extended α -helical, coiled-coil structure in dimerization. The removal of an internal stretch of 70 aa (KfrA Δ 136–207) or even 137 aa (KfrA Δ 187–323) from the α -helical tail did not impair the autoregulatory properties of KfrA_{R751}, at least at 37 °C (when overproduced), in the dissected *kfrAp*–*xylE* reporter gene assays with *tacp*–*wtkfrA* or mutants *in trans*. Nevertheless, deletion of 137 internal aa did have a slight effect in pMAB08.51, which was consistent with it causing fourfold derepression of transcription from *kfrAp*. The N-terminal part of KfrA (KfrA1–136) is sufficient for DNA binding and a basal level of repression, but its temperature sensitivity indicates decreased stability relative to full-length KfrA or derivatives containing more of the α -helical, coiled-coil domain.

The introduction of the Δ *akfrA* allele (KfrA136–207) into R751 had no effect on plasmid stability. A similar observation has been made previously (Williams *et al.*, 1998) for a short in-frame deletion of *kfrA*_{RK2} in the P7–pMB1 hybrid derivative, which was the basis of the conclusion that KfrA_{RK2} did not play any role in the stability of the test plasmid. Attempts to introduce a null *kfrA* allele into R751 were unsuccessful. However, the reverse genetic method used to introduce the *kfrA* Δ 137–343 allele led to formation of a new deletion allele, *kfrA* Δ 186–323, in R751 (R751 Δ C₂kfrA). The failure to introduce the *kfrA* Δ 137–343 mutation into the R751 background may be due to either loss of repressor activity (it causes a 30-fold decrease in repression when in excess) or some other function that is essential for plasmid or cellular survival. However, almost all the extra DNA that was present in mutant R751 Δ C₂kfrA was missing from the Δ *akfrA* allele which was successfully introduced into R751. It therefore seems more likely that the failure to recombine *kfrA* Δ 137–343 into R751 was due to the effect of this deletion on gene expression. As already mentioned, the *kfrA* Δ 187–323 allele, which arose spontaneously in R751 Δ C₂kfrA,

seems to increase transcription from *kfrA* into *kfrBC* when tested in the promoter-probe vector (pMAB05.81). In the natural context of R751, this mutation does not seem to have a drastic effect on production of truncated KfrA, suggesting that the presence of KorA and KorB, which also repress *kfrAp*, suppresses this slight defect. Nevertheless, the inability to completely inactivate *kfrA* in R751 implies that, despite *kfrAp* being subject to repression by KorA and KorB, KfrA is the lead regulator and is necessary to maintain correct expression levels.

Our estimate of intracellular KfrA concentration is about 7000 monomers per cell, which would give a concentration well in excess of 1 μ M. Fluorescence microscopy indicated that the majority of these KfrA molecules were present in the same type of plasmid DNA-protein foci as previously identified for KorB of the IncP-1 α plasmids (Bignell *et al.*, 1999). Since there are no additional copies of the putative KfrA binding sequence elsewhere in the genome, other than at *kfrAp*, this implies either protein-protein interaction or presence of multiple, less specific, binding sites. So long as the protein is distributed evenly around the genome, there should still be a small fraction of the promoter unoccupied at any time, thus allowing enough expression to replenish the levels of KfrA. The very weak promoter for *kfrB* and *kfrC* may also ensure synthesis of these proteins under steady-state conditions.

The nature of the KfrA-DNA complex formed should be of interest and will be the subject of future work. One of the functions of the KfrABC complex appears to be to aid in the formation of clusters containing multiple copies of the plasmid since, in its absence, the observed foci of KorB bound to O_B sites in the IncP-1 genome and KfrA foci split into multiple, smaller signals. This could reflect the fact that individual molecules are no longer sticking together. The structure of KfrA is reminiscent of SMC-like proteins (Soppa, 2001; Rhodes *et al.*, 2004) and, as such, it may be important in linking protein-plasmid DNA complexes together, perhaps helping them to condense into more compact units and this, in turn, may be essential for their correct localization within the bacterial cell.

Thus KfrA may act as a specific plasmid nucleoid organizer that is important for stable inheritance and plasmid survival. This would be consistent with the instability phenotype observed in the R751 Δ C₂*kfrA* and R751 Δ *kfrB* mutants constructed, and the importance of *kfrC* for the plasmid may be reflected by our inability to create a mutation in this gene. The kinetics of R751 Δ C₂*kfrA* and R751 Δ *kfrB* plasmid loss may not be typical for defects in the stability determinants. The strains with R751Tet^R Δ C₂*kfrA* and R751Tet^R Δ *kfrB* do not have an impaired growth rate when compared to the strain with stable R751Tet^R and the R751Tet^R Δ *akfrA* derivative. The Δ C₂*kfrA* mutation does not seem to disturb significantly the level of expression of the *kfrA* operon, although we cannot exclude an effect on the general level of expression of other genes, as it leads to the defect in plasmid clustering and, putatively, DNA condensation.

Introduction of the *kfrA* allele on a medium-copy plasmid *in trans* to *E. coli* NEM(R751Tet^R) led to 90 % loss of R751 after 10 generations (data not shown), so the attempt to complement the Δ C₂*kfrA* mutation by supplying extra WT KfrA was declined. Previous studies suggest that the IncP-1 active partitioning system requires additional functions to work efficiently (Thorsted *et al.*, 1998). It is conceivable that KfrABC could be part of this apparatus, but we were unable to demonstrate any direct interactions between KfrA and either KorB or IncC. The negative results may reflect a real lack of protein-protein interactions, improper folding of the hybrid proteins, or interactions taking place only under certain conditions, such as DNA binding or in the presence of other proteins within a multi-protein complex. On the other hand, we were able to demonstrate interactions between KfrA and KfrB and between KfrB and KfrC. This, therefore, defines additional likely components of the plasmid foci. A challenge for the future will be to isolate those complexes still bound to DNA and to determine which other host- or plasmid-encoded proteins are present. A catalogue of such proteins is probably essential before one attempts to predict a role for such complexes, because a prediction based on an incomplete picture is likely to be unreliable.

All the systems in which KfrA-like proteins are encoded also carry a type 1 ParAB active partitioning system, according to the classification of Gerdes *et al.* (2000b). The localization of the putative *kfrAs* is close to either *rep/oriV* regions or partitioning/stabilization cassettes. If the IncP-1 system is a good model, then we might expect to be able to identify homologues/analogues of KfrB and KfrC. Examination of ORFs next to *kfrA* homologues in different plasmids indicates that some of these may be part of the same transcriptional units as *kfrAs*. Further biochemical characterization of the products of *kfrA*, *kfrB* and *kfrC* of IncP-1 plasmids is under way and may shed some light on the functions of these proteins in plasmid stability.

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REFERENCES

- Arnold, F. H. (1991). Metal-affinity separations: a new dimension in protein processing. *Biotechnology* **9**, 151–156.
- Bignell, C. R. & Thomas, C. M. (2001). The bacterial ParA-ParB partitioning proteins. *J Biotechnol* **91**, 1–34.
- Bignell, C. R., Haines, A. S., Khare, D. & Thomas, C. M. (1999). Effect of growth rate and *incC* mutation on symmetric plasmid distribution by the IncP-1 partitioning apparatus. *Mol Microbiol* **34**, 205–216.
- Bingle, L. E., Macartney, D. P., Fantozzi, A., Manzoor, S. E. & Thomas, C. M. (2005). Flexibility in repression and cooperativity by KorB of broad host range IncP-1 plasmid RK2. *J Mol Biol* **349**, 302–316.

- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513–1523.
- Blakely, G., May, G., McCulloch, R., Arciszewska, L. K., Burke, M., Lovett, S. T. & Sherratt, D. J. (1993). Two related recombinases are required for site-specific recombination at *dif* and *cer* in *E. coli* K12. *Cell* 75, 351–361.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.
- Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J. & Blattner, F. R. (1998). The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Res* 26, 4196–4204.
- Chen, D. C., Yang, B. C. & Kuo, T. T. (1992). One-step transformation of yeast in stationary phase. *Curr Genet* 21, 83–84.
- Close, S. M. & Kado, C. I. (1992). A gene near the plasmid pSa origin of replication encodes a nuclease. *Mol Microbiol* 6, 521–527.
- Easter, C. L., Schwab, H. & Helinski, D. R. (1998). Role of the *parCBA* operon of the broad-host-range plasmid RK2 in stable plasmid maintenance. *J Bacteriol* 180, 6023–6030.
- El-Sayed, A. K., Hotherhall, J. & Thomas, C. M. (2001). Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. *Microbiology* 147, 2127–2139.
- Fields, S. & Song, O. (1989). A novel genetic system to detect protein–protein interactions. *Nature* 340, 245–246.
- Gerdes, K., Ayora, S., Canosa, I. & 10 other authors (2000a). Plasmid maintenance systems. In *Horizontal Gene Pool. Bacterial Plasmids and Gene Spread*, pp. 49–85. Edited by C. M. Thomas. Amsterdam: Harwood Academic Publishers.
- Gerdes, K., Moller-Jensen, J. & Bugge Jensen, R. (2000b). Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* 37, 455–466.
- Haneda, T., Okada, N., Nakazawa, N., Kawakami, T. & Danbara, H. (2001). Complete DNA sequence and comparative analysis of the 50-kilobase virulence plasmid of *Salmonella enterica* serovar *Choleraesuis*. *Infect Immun* 69, 2612–2620.
- Hirano, T. (1998). SMC protein complexes and higher-order chromosome dynamics. *Curr Opin Cell Biol* 10, 317–322.
- Hurme, R., Namork, E., Nurmiäho-Lassila, E. L. & Rhen, M. (1994). Intermediate filament-like network formed *in vitro* by a bacterial coiled coil protein. *J Biol Chem* 269, 10675–10682.
- Hurme, R., Berndt, K. D., Namork, E. & Rhen, M. (1996). DNA binding exerted by a bacterial gene regulator with an extensive coiled-coil domain. *J Biol Chem* 271, 12626–12631.
- Hurme, R., Berndt, K. D., Normark, S. J. & Rhen, M. (1997). A proteinaceous gene regulatory thermometer in *Salmonella*. *Cell* 90, 55–64.
- Jagura-Burdzy, G. & Thomas, C. M. (1992). *kfrA* gene of broad host range plasmid RK2 encodes a novel DNA-binding protein. *J Mol Biol* 225, 651–660.
- Jagura-Burdzy, G. & Thomas, C. M. (1994). KorA protein of promiscuous plasmid RK2 controls a transcriptional switch between divergent operons for plasmid replication and conjugative transfer. *Proc Natl Acad Sci U S A* 91, 10571–10575.
- Jagura-Burdzy, G. & Thomas, C. M. (1995). Purification of KorA protein from broad host range plasmid RK2: definition of a hierarchy of KorA operators. *J Mol Biol* 253, 39–50.
- Jagura-Burdzy, G., Ibbotson, J. P. & Thomas, C. M. (1991). The *korF* region of broad-host-range plasmid RK2 encodes two polypeptides with transcriptional repressor activity. *J Bacteriol* 173, 826–833.
- Jagura-Burdzy, G., Macartney, D. P., Zatyka, M., Cunliffe, L., Cooke, G. D., Huggins, C., Khanim, F. & Thomas, C. M. (1999a). Repression at a distance by the global regulator KorB of promiscuous IncP plasmids. *Mol Microbiol* 32, 519–532.
- Jagura-Burdzy, G., Kostelidou, K., Pole, J., Khare, D., Jones, A., Williams, D. R. & Thomas, C. M. (1999b). IncC of broad-host-range plasmid RK2 modulates KorB transcriptional repressor activity *in vivo* and operator binding *in vitro*. *J Bacteriol* 181, 2807–2815.
- Johnson, E. P., Strom, A. R. & Helinski, D. R. (1996). Plasmid RK2 toxin protein ParE: purification and interaction with the ParD antitoxin protein. *J Bacteriol* 178, 1420–1429.
- Kahn, M. R., Kolter, R., Thomas, C. M., Figurski, D., Meyer, R., Remault, E. & Helinski, D. R. (1979). Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. *Methods Enzymol* 68, 268–280.
- Koski, P., Saarilahti, H., Sukupolvi, S., Taira, S., Riikonen, P., Osterlund, K., Hurme, R. & Rhen, M. (1992). A new alpha-helical coiled coil protein encoded by the *Salmonella typhimurium* virulence plasmid. *J Biol Chem* 267, 12258–12265.
- Kostelidou, K. & Thomas, C. M. (2000). The hierarchy of KorB binding at its 12 binding sites on the broad-host-range plasmid RK2 and modulation of this binding by IncC1 protein. *J Mol Biol* 295, 411–422.
- Kostelidou, K. & Thomas, C. M. (2002). DNA recognition by the KorA proteins of IncP-1 plasmids RK2 and R751. *Biochim Biophys Acta* 1576, 110–118.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175–176.
- Krishnapillai, V. (1988). Molecular genetic analysis of bacterial plasmid promiscuity. *FEMS Microbiol Rev* 4, 223–237.
- Kwong, S. M., Yeo, C. C., Chuah, D. & Poh, C. L. (1998). Sequence analysis of plasmid pRA2 from *Pseudomonas alcaligenes* NCIB 9867 (P25X) reveals a novel replication region. *FEMS Microbiol Lett* 158, 159–165.
- Kwong, S. M., Yeo, C. C., Suwanto, A. & Poh, C. L. (2000). Characterization of the endogenous plasmid from *Pseudomonas alcaligenes* NCIB 9867: DNA sequence and mechanism of transfer. *J Bacteriol* 182, 81–90.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lukaszewicz, M., Kostelidou, K., Bartosik, A. A., Cooke, G. D., Thomas, C. M. & Jagura-Burdzy, G. (2002). Functional dissection of the ParB homologue (KorB) from IncP-1 plasmid RK2. *Nucleic Acids Res* 30, 1046–1055.
- Ma, J. & Ptashne, M. (1987). A new class of yeast transcriptional activators. *Cell* 51, 113–119.
- Macartney, D. P., Williams, D. R., Stafford, T. & Thomas, C. M. (1997). Divergence and conservation of the partitioning and global regulation functions in the central control region of the IncP plasmids RK2 and R751. *Microbiology* 143, 2167–2177.
- McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C. & Rosenberg, M. (1981). A system to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase. *Gen Amplific Anal* 2, 383–415.
- Motallebi-Veshareh, M., Rouch, D. A. & Thomas, C. M. (1990). A family of ATPases involved in active partitioning of diverse bacterial plasmids. *Mol Microbiol* 4, 1455–1463.
- Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol* 51, 263–273.

- Pansegrau, W., Lanka, E., Barth, P. T. & 7 other authors (1994). Complete nucleotide sequence of Birmingham IncP alpha plasmids. Compilation and comparative analysis. *J Mol Biol* **239**, 623–663.
- Pogliano, J., Ho, T. Q., Zhong, Z. & Helinski, D. R. (2001). Multicopy plasmids are clustered and localized in *Escherichia coli*. *Proc Natl Acad Sci U S A* **98**, 4486–4491.
- Reznek, O., Alper, S. & Losick, R. (1996). Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Genes Cells* **1**, 529–542.
- Rhodes, G., Parkhill, J., Bird, C., Ambrose, K., Jones, M. C., Huys, G., Swing, J. & Pickup, R. W. (2004). Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. *App Envir Microb* **70**, 7497–7510.
- Roberts, R. C. & Helinski, D. R. (1992). Definition of a minimal plasmid stabilization system from the broad-host-range plasmid RK2. *J Bacteriol* **174**, 8119–8132.
- Roberts, R. C., Spangler, C. & Helinski, D. R. (1993). Characteristics and significance of DNA binding activity of plasmid stabilization protein ParD from the broad host-range plasmid RK2. *J Biol Chem* **268**, 27109–27117.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Soppa, J. (2001). Prokaryotic structural maintenance of chromosomes (SMC) proteins: distribution, phylogeny, and comparison with MukBs and additional prokaryotic and eukaryotic coiled-coil proteins. *Gene* **278**, 253–264.
- Studier, F. W. & Moffatt, B. A. (1981). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Summers, D. K. & Sherratt, D. J. (1984). Multimerization of high copy number plasmids causes instability: CoIE1 encodes a determinant essential for plasmid monomerization and stability. *Cell* **36**, 1097–1103.
- Tauch, A., Schneiker, S., Selbitschka, W. & 13 other authors (2002). The complete nucleotide sequence and environmental distribution of the cryptic, conjugative, broad-host-range plasmid pIPO2 isolated from bacteria of the wheat rhizosphere. *Microbiology* **148**, 1637–1653.
- Theophilus, B. D. M., Cross, M. A., Smith, C. A. & Thomas, C. M. (1985). Regulation of the *trfA* and *trfB* promoters of broad host range plasmid RK2: identification of sequences essential for regulation by *trfB/korA/korD*. *Nucleic Acids Res* **13**, 8129–8142.
- Thomas, C. M., Theophilus, B. D., Johnston, L., Jagura-Burdzy, G., Schilf, W., Lurz, R. & Lanka, E. (1990). Identification of a seventh operon on plasmid RK2 regulated by the *korA* gene product. *Gene* **89**, 29–35.
- Thorsted, P. B., Macartney, D. P., Akhtar, P. & 9 other authors (1998). Complete sequence of the IncPbeta plasmid R751: implications for evolution and organisation of the IncP backbone. *J Mol Biol* **282**, 969–990.
- Williams, D. R. & Thomas, C. M. (1992). Active partitioning of bacterial plasmids. *J Gen Microbiol* **138**, 1–16.
- Williams, D. R., Motallebi-Veshareh, M. & Thomas, C. M. (1993). Multifunctional repressor KorB can block transcription by preventing isomerization of RNA polymerase-promoter complexes. *Nucleic Acids Res* **21**, 1141–1148.
- Williams, D. R., Macartney, D. P. & Thomas, C. M. (1998). The partitioning activity of the RK2 central control region requires only *incC*, *korB* and KorB-binding site O(B)₃ but other KorB-binding sites form destabilizing complexes in the absence of O(B)₃. *Microbiology* **144**, 3369–3378.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zielenkiewicz, U. & Ceglowski, P. (2001). Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. *Acta Biochim Pol* **48**, 1003–1023.
- Zukowski, M. M., Gaffney, D. F., Speck, D., Kauffman, M., Findeli, A., Wisecup, A. & Lecoq, J. P. (1983). Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. *Proc Natl Acad Sci U S A* **80**, 1101–1105.