Functional dissection of the ParB homologue (KorB) from IncP-1 plasmid RK2

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Received September 11, 2001; Revised and Accepted November 21, 2001

ABSTRACT

Active partitioning of low-copy number plasmids requires two proteins belonging to the ParA and ParB families and a cis-acting site which ParB acts upon. Active separation of clusters of plasmid molecules to the defined locations in the cell before cell division ensures stable inheritance of the plasmids. The central control operon of IncP-1 plasmids codes for regulatory proteins involved in the global transcriptional control of operons for vegetative replication, stable maintenance and conjugative transfer. Two of these proteins, IncC and KorB, also play a role in active partitioning, as the ParA and ParB homologues, respectively. Here we describe mapping the regions in KorB responsible for four of its different functions: dimerisation, DNA binding, repression of transcription and interaction with IncC. For DNA binding, amino acids E151 to T218 are essential, while repression depends not only on DNA binding but, additionally, on the adjacent region amino acids T218 to R255. The C-terminus of KorB is the main dimerisation domain but a secondary oligomerisation region is located centrally in the region from amino acid I174 to T218. Using three different methods (potentiation of transcriptional repression, potentiation of DNA binding and activation in the yeast two-hybrid system) we identify this region as also responsible for interactions with IncC. This IncC–KorB contact differs in location from the ParA–ParB/SopA–SopB interactions in P1/F but is similar to these systems in lying close to a masked oligomerisation determinant.

INTRODUCTION

A fundamental part of the bacterial cell cycle is the partitioning of chromosomes and plasmids to either side of the cell division plane. For low-copy number plasmids it is well established that this is a better-than-random process driven by an active partitioning apparatus (1). More recent data with Escherichia coli

(2–4), Bacillus subtilis (5–8), Caulobacter crescentus (9), Streptomyces coelicolor (10) and Pseudomonas putida (11) have indicated that chromosomal segregation also appears to depend on active processes. Remarkably, one of the gene systems that drives active partitioning of many plasmids is also found on most bacterial chromosomes, except for those of the Enterobacteriaceae (12–14). The key features of the plasmid and chromosomal systems are two trans-acting genes, generically termed parA and parB, and a cis-acting site termed the centromere-like sequence by analogy to eukaryotic chromosomes (1). The ParB protein is a DNA-binding protein, which binds to the centromere-like sequence (15,16). The ParA protein is an ATPase whose activity is essential for partitioning (17–20). These Par functions, possibly together with as yet unidentified chromosomal functions, appear to be responsible for localisation of plasmid or chromosomal oriC region to the regions where DNA replication takes place and for the movement between these zones (2,21,22). The interaction between ParA and ParB appears to be a vital part of this process (21,23–26). Recent studies have roughly mapped the zones of interactions between the F and P1 ParA/ParB proteins (27,28) but it is uncertain whether the conclusions from these studies can be extrapolated to other systems.

The IncP-1 plasmid Par system (19,29–31) has many interesting features not least because it also serves as a central control region which coordinates the expression of basic plasmid genes involved in plasmid replication, transfer and stable inheritance (30,32,33). Also, in the phylogenetic trees, the IncP-1 ParA and ParB homologues cluster with the chromosomally encoded members of the two families and away from other plasmid-encoded homologues (12). For simplicity, an alignment of KorB from IncP-1α and IncP-1β plasmids only with chromosomal ParB from P.putida and Pseudomonas aeruginosa are shown in Figure 1 to illustrate the sequence conservation and the structural features of these proteins. The Pseudomonas proteins are chosen because this genus appears most likely to be the natural host of the IncP-1 plasmids (34).

The IncP-1 ParB homologue KorB binds to 12 sites (Oq1–Oq12) in the RK2 plasmid genome (35–37). Class I sites (Oq1, Oq10 and Oq12) lie immediately upstream of the –35 region of the promoters they are associated with (korAp, trfAp, klaAp), while Class II sites (Oq2, Oq9, Oq10 and Oq11) lie up to 189 bp upstream/downstream of a transcription start point (kfrAp,
In vitro, at all O₅s except O₅₉ (37,39). With respect to partitioning, we know that IncC₂ (254 amino acids) and KorB are needed for partitioning but that at least during growth in rich medium additional genes encoded in the kle, kla and kle regions are required for full stability (21,31). Nevertheless, IncC is essential to ensure that the foci that KorB forms on plasmid DNA are distributed regularly in the bacterial cell as expected for active partitioning (21). Whereas other plasmid-encoded partitioning proteins are known to have autoregulatory functions (1), such complex regulatory involvement of partitioning proteins has only been reported so far for other IncP-1 plasmids (31,41) and the chromosomal homologues of IncC and KorB such as Soj and SpoOJ of B.subtilis (25,26,42). There is no evidence that IncC has the ability to bind DNA. The co-regulatory effect it exerts on the activity of many plasmid promoters is absolutely dependent on the KorB protein, implying a direct interaction between these two proteins or modification of DNA structure by KorB to facilitate IncC binding (39). Direct interaction between KorB and IncC has recently been reported (43) and is likely to be the basis for IncC-related displacement of IncP-1 as well as heterologous plasmids encoding a KorB-binding site so long as KorB is also produced (39,44).

To provide further understanding of the ParB family and the subfamily that includes the chromosomal homologues, we have used deletion analysis to define the subregions of KorB. We describe mapping of the DNA-binding segment, definition of two KorB–KorB interaction zones and the region required for interaction with IncC. The localisation of the latter is different from the ParA/SopA recognition patch on ParB of P1 or SopB of F (27,28).

**MATERIALS AND METHODS**

**Bacterial strains and growth**

Escherichia coli K12 strains used were C600K (thr-1 leu-6 thi-1 lacY1 supE44 ton21 galK) and BL21 F-ompT hsdS15(rB–mB–) gal dcm (phage DE3) (Novagen, Inc.). Bacteria were generally grown in L broth (45) at 37°C or on L agar (L broth with 1.5% w/v agar) supplemented with antibiotics as appropriate: benzyl penicillin, 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 and streptomycin sulphate (30 µg ml⁻¹) for streptomycin resistance.

**Yeast strains and growth conditions**

Saccharomyces cerevisiae strain Y187 MATα ura3-52, his3-200, ade2-101, trpl-901, leu2-3112, met+, gal4Δ, gal80Δ, URA3::GAL1 UAS-GAL1 TATA- lac Z was used for β-galactosidase tests, and Y190 MATα ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3112, gal4Δ, gal80Δ, URA3::GAL1 UAS-GAL1 TATA-lac Z, cly2, LYS2::GAL1 UAS-HIS3 TATA-His3 was used for complementation of HIS auxotrophy in the presence of 3-amino-1,2,4-triazole (3-AT). 3-AT was used to repress the basal activity of the His3 reporter gene, which otherwise results in non-specific background growth in the absence of exogenous histidine.

Yeast cells were grown in YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose). Plasmid-containing yeast strains were grown in YNB medium (0.67% yeast nitrogen base, 3% glucose) supplemented with the mixture of appropriate nutrients lacking tryptophan, leucine or both, as required. Agar was added to a concentration of 2% for plates. To grow transformants for β-galactosidase filter-lift assay, YNB agar was supplemented with 0.5% glucose instead of 3% glucose. For β-galactosidase assay the liquid cultures, the yeast cells were grown to logarithmic phase in YNB medium with 0.5% glucose and then transferred to YNB-ethanol (3%) medium for

**Figure 1.** Alignment of KorB from two subgroups of IncP-1 plasmids and ParB proteins of P. putida and P. aeruginosa. The regions of homology between four proteins are shown in pink. The similarities between KorB₉₂ (IncP1-P. putida) and KorB₉₂ (IncP1-B. subtilis) are shown in yellow. Putative helix–turn–helix motif and linker regions are underlined. The change in the codon for R₂₅₄ introduces a HI site is marked with an arrow. The symbols above the amino acids indicate the extent of deletions: black diamonds, deletions from N-terminus; open diamonds, deletions from C-terminus; black and white arrows, internal deletions from upstream and downstream of BamHI site, respectively. The green rectangle above the sequence shows region mapped in this work as ‘IncC interaction domain’ whereas blue rectangle corresponds to the secondary dimerisation domain.

trbBp, trbAp, kleAp; note O₅₁₀ is Class I relative to trfAp and Class II relative to trbAp). KorB, bound at all these sites, can repress transcription from the adjacent promoter. The role of Class III (intergenic and intragenic) KorB-binding sites (O₅₋₃–O₅₈) is still unclear although the role of O₅₅ in the active partitioning has been suggested (38).

The ParA homologue IncC can potentiate transcriptional repression by KorB at Class I and Class II sites (39). incC codes for two forms of IncC, IncC₁ and IncC₂, differing at the N-terminus by 105 amino acids (40). IncC₁(364 amino acids) stabilises KorB–DNA complexes formed in vitro at all O₅s except O₅₉ (37,39). With respect to partitioning, we know that IncC₂ (254 amino acids) and KorB are needed for partitioning but that at least during growth in rich medium additional genes encoded in the kle, kla and kle regions are required for full stability (21,31). Nevertheless, IncC is essential to ensure that the foci that KorB forms on plasmid DNA are distributed regularly in the bacterial cell as expected for active partitioning
several hours. For testing the expression of the His reporter gene in Y190, YNB plates also contained 75 mM 3-AT. Plates were incubated at 30°C for 3–4 days.

**Plasmids**

The plasmids used are described below except for standard vectors (46).

**Plasmids for regulatory studies were as follows.** The transcriptional fusions to monitor the activity of RK2 promoters were constructed by insertion of PCR-amplified promoter fragments into the BamHI site of pGBT43 (10.4 kb KmR plasmid based on the pSC101 replicon) upstream of promoterless xylE cassette (30,47). pGBT30 (48) is an expression Pl vector based on the pMB1 replicon with lacF and tacp separated from λΔg by the multiple cloning site from pUC18. To make use of the associated tacp SD sequence, the ATG codon of the inserted open reading frame (ORF) must directly follow the EcoRI site. ORFs were amplified by PCR with flanking EcoRI and SalI sites. pGBT36 was created by inserting the PCR product of the incC1 ORF as an EcoRI–SalI fragment into pGBT30. The upstream primer eliminated the ATG codon for KorA without changing the amino acid sequence of IncC (39). pGBT301 has the corB under the control of tacp (33).

Expression vector pGBT400 is an 11.6 kb IncQ SmR derivative (30,47). pGBT30 (48) is an expression Pn R vector based on the pMB1 replicon with lacF and tacp separated from λΔg by the multiple cloning site from pUC18. To make use of the associated tacp SD sequence, the ATG codon of the inserted open reading frame (ORF) must directly follow the EcoRI site. ORFs were amplified by PCR with flanking EcoRI and SalI sites. pGBT36 was created by inserting the PCR product of the incC1 ORF as an EcoRI–SalI fragment into pGBT30. The upstream primer eliminated the ATG codon for KorA without changing the amino acid sequence of IncC (39). pGBT301 has the corB under the control of tacp (33). pGBT400 was created by amplification of the lacF–tacp fragment (lacfF tacp) with lacF and tacp separated from λΔg by the multiple cloning site from pUC18.

**Plasmid DNA isolation, analysis, cloning and manipulation of DNA**

Plasmid DNA was isolated by standard procedures (46). Large-scale plasmid purification was carried out by means of standard alkaline SDS extraction followed by CsCl ethidium bromide density gradient centrifugation. Digestion of plasmid DNA with restriction enzymes was carried out under conditions recommended by suppliers and run on agarose gels of concentration 0.8–2.0% (w/v). DNA sequencing was performed by internal sequencing facility (IBB, Warsaw) and AluBioscience (Birmingham) using the Dye-terminator method in conjunction with an ABI 377 automated DNA sequencer. Standard PCR reactions (50) were performed as described previously (51). Fragments were amplified on a PCR690 (52) template for RK2. All PCR-derived clones were analysed by DNA sequencing to check their fidelity. DNA fragments (PCR amplified) for gel retardation studies were 3′-end-labelled with terminal transferase (Boehringer Manheim) and [α-32P]ddATP (Amersham).

**Yeast transformation**

Yeast transformation was performed using the standard PEG/LiAc method recommended by Clontech. Either single or double transformations were conducted and transformants selected and stored on minimal YNB agar.

**Determination of β-galactosidase activity in yeasts**

β-Galactosidase activity was monitored by the filter-lift assay with X-gal and quantitative liquid culture assay using o-nitrophenyl-β-d-galactopyranoside (ONPG) as a substrate (Clontech manual).

**Determination of catechol 2,3-oxygenase activity**

Catechol 2,3-oxygenase (the product of yxIE) activity was assayed in logarithmically growing bacteria (OD600nm = 0.6) as described previously (53). Protein concentration was assayed by the Biuret method (54). Plasmid DNA was isolated from assayed cultures and XyIE activities were normalised to the same plasmid level as the control.

**Purification of His6-tailed polypeptides**

Exponentially growing BL21 strains with pET28 derivatives (49,55) were induced with 0.5 mM IPTG at a cell density of ∼2 × 10^8 c.f.u./ml, grown for an additional 2 h with shaking at 37°C. The bacteria were harvested by centrifugation and sonicated. Over-produced His-tagged proteins were purified as recommended for soluble native proteins by Qiagen on Ni-agarose columns with an imidazole gradient in phosphate buffer.
buffer at pH 6.0. Purification was monitored by SDS–PAGE using a Pharmacia PHAST gel system.

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

A synthetic OG palindrome (37) 5′-gaattcTTTAGCGGCTAA-Aaagctt-3′ was cloned between EcoRI–HindIII into pUC18 (pKK113). Using PCR and primers, 5′-CGAAAGGGGGAT-GTGCTGC-3′ and 5′-GCTTCCGGCTCGTATGTTG-3′, annealing at positions 313 and 531 nt, respectively, on the pUC18 map, a fragment of 187 bp was amplified and labelled with terminal transferase. This labelled PCR product was either used directly for EMSA in the presence of 2 µg of salmon sperm DNA or cleaved with HaeIII to produce 78 and 109 bp fragments, the latter with OG, and used for EMSA with C-terminally truncated KorB polypeptides without non-specific DNA. Radioactive fragments were incubated with purified HisKorB (wild-type or truncated derivatives) and wild-type HisIncC and then separated by PAGE on 5% gels under conditions described previously (52).

Cross-linking with glutaraldehyde

His-tagged polypeptides purified on Ni-agarose columns were cross-linked with the use of glutaraldehyde (52) and separated on SDS–PAGE gels by Phast system (Pharmacia).

RESULTS

Construction of korB deletion mutants

Deletion mutants were constructed to define the functional domains of KorB protein (Fig. 2). Using PCR and suitable restriction sites in the korB sequence, N-terminal deletions ranging from 15 to 173 amino acids were obtained. BAL31 digestion (33) was applied to get the C-terminal deletions removing up to 101 amino acids. A BamHI site was conveniently incorporated into the variable region between the two conserved domains of KorBRK2 and KorBR751 without changing the amino acid sequence (see Fig. 1). By linking the EcoRI–BamHI or BamHI–SalI fragments with PCR products, short internal deletions from 10 to 37 amino acids in length were created going from this site towards the N-terminus (upstream) or the C-terminus (downstream) of KorB. Finally, we combined the N-terminal deletion of 173 amino acids with two C-terminal deletions to produce short internal KorB fragments of 84 and 107 amino acids. The korB alleles were then incorporated into pGBT30 for overproduction and regulatory studies in vivo, into the pET28 derivative for purification of His-tagged products and into the shuttle vectors pGAD424 and pGBT9 for the yeast two-hybrid system to create fusions with the GAL4 AD (ADGAL4) and DBD (DBDGAL4), respectively. We tried to determine whether korB deletion derivatives produced stable products. The extracts from IPTG-induced cultures of transformants of BL21(DE3) with pET28-korB alleles were separated by SDS–PAGE and the proteins of the right size were visualised. Representative extracts are shown in Figure 3. Using this criterion all the derivatives used, with the exception of the N-terminal 15 amino acid deletion, produced an apparently stable product of the expected size. Truncated product of ΔN15 is also visible on the gel but in smaller quantities.
Repressor activity of truncated KorB products and its enhancement by IncC

To establish whether truncated forms of KorB were still able to repress transcription through binding to Class I operators, we placed pGBT30 derivatives with different korB alleles in trans to a pSC101 replicon with a korAp–xylE transcriptional fusion (pDM3.1), along with a third plasmid based on the IncQ replicon initially carrying no IncP-1 gene (pDM1.1) but later to carry incC (pPOLE1) (see below). The effectiveness as a repressor is given by the repression index (RI),—the ratio between the repression activity of the korAp in the IPTG-induced cultures without korB derivatives (the empty expression vectors pGBT30 and pDM1.1) and with korB derivatives (the empty pDM1.1). All deletion derivatives tested affected the ability to repress, either by reducing or enhancing this activity (Table 1). The key points of the results are as follows. First, it was possible to make successive deletions from the N-terminus as far as amino acid 150 without abolishing repression. The shortest deletions, NA15 and NA30, reduced repressor activity but deletions of 60, 110 and 150 amino acids gave enhanced repressor activity. Deletion of 173 amino acids abolished repression. Secondly, from the C-terminus at least 101 amino acids could be removed without loss of repressor activity. Thirdly, with internal deletions, removal of amino acids 255–284 did not abolish repression but removal of amino acids 219–255 did abolish activity. These results are summarised in Figure 2.

To determine whether IncC can enhance repression by the korB deletion derivatives we included POLE1 in the three-plasmid system because this plasmid carries incC. The results in Table 1 express the repression in the presence of IncC as RlP—the ratio of XylE activity when only IncC is over-produced compared with XylE activity when the korB derivative and incC are expressed together under IPTG induction. Expression of IncC without korB present had no effect on korAp activity. The results showed that despite the differences in repression by the N-terminal deletion derivatives, their effect in all cases was potentiated 2–3-fold by the presence of IncC (RlP/RlA in Table 1). Similarly, repression by the longest C-terminal deletion derivative was still potentiated 2–3-fold by the presence of IncC. Finally, the same effect was observed for the internal deletion of amino acids 255–284. Those deletions that did not show repression (NA15 and NA37up) were also tested, but the presence of incC did not elicit any repressor activity. This indicated that we can delete to 123 and 173 amino acids from korAp activity. This result suggests that the biological activity of wild-type IncC in the three-plasmid system.

Table 1. Potentiation of in vivo repressor activity of KorB by IncC protein monitored with korAp–xylE transcriptional fusion reporter plasmid pDM3.1 (30)

<table>
<thead>
<tr>
<th>korB derivative in transa</th>
<th>Repression index (RI)</th>
<th>Potentiationb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RlP without IncC (pDM1.1)</td>
<td>RlP with IncC (pPOLE1)c</td>
</tr>
<tr>
<td>None</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Wild-type KorB</td>
<td>3.7</td>
<td>12.4</td>
</tr>
<tr>
<td>NA15</td>
<td>1.8</td>
<td>4.2</td>
</tr>
<tr>
<td>NA30</td>
<td>2.7</td>
<td>6.0</td>
</tr>
<tr>
<td>NA60</td>
<td>10.1</td>
<td>17.2</td>
</tr>
<tr>
<td>NA68</td>
<td>3.3</td>
<td>8.3</td>
</tr>
<tr>
<td>NA90</td>
<td>3.6</td>
<td>8.3</td>
</tr>
<tr>
<td>NA110</td>
<td>7.3</td>
<td>25.9</td>
</tr>
<tr>
<td>NA150</td>
<td>11.1</td>
<td>23.1</td>
</tr>
<tr>
<td>NA173</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>BA11up</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>BA37up</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>BA10down</td>
<td>2.2</td>
<td>5.5</td>
</tr>
<tr>
<td>BA20down</td>
<td>3.1</td>
<td>5.8</td>
</tr>
<tr>
<td>BA30down</td>
<td>3.2</td>
<td>10.2</td>
</tr>
<tr>
<td>CA78</td>
<td>2.1</td>
<td>4.7</td>
</tr>
<tr>
<td>CA101</td>
<td>3.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>

&a korB deletions were cloned into pGBT30 (44) where they were expressed from tacp. Strain C600K with pDM3.1 plasmid, based on the pSC101 replicon, and carrying korAp–xylE transcriptional fusion (30) was set up with relevant korB derivatives and either with pDM1.1 (an IncQ based expression vector) or pPOLE1 (pDM1.1 derivative with incC under control of tacp; 39). Strain with pDM3.1, pGBT30 and pDM1.1 was used as a control. Cells were grown in the presence of 1 mM IPTG for 3 h to induce the expression of regulatory proteins.

b Repression index (RlP) was calculated as the ratio between the XylE activities in extracts of C600K (pDM3.1/pDM1.1/pGBT30) and C600K (pDM3.1/pPOLE1/pGBT30) (Res). This index gives the value of KorB (and korB deletion derivatives) repressor activity in the three-plasmid system.

c Repressor index (RlP) was calculated as the ratio between the XylE activities in extracts of C600K (pDM3.1/pPOLE1/pGBT30) and C600K (pDM3.1/pPOLE1/pGBT30-korB alleles). This index gives the value of KorB (and korB deletion derivatives) repressor activity in the three-plasmid system.

d Potentiation is defined as the ratio between RlP and RlA, and indicates the factor by which IncC protein enhances the in vivo repressor activity of either wild-type or mutant KorB proteins.

The wild-type KorB and some of the products of deletion derivatives were purified as N-terminally His-tagged proteins.
from BL21/pET28 extracts. The pUC18 fragment of 187 bp with a centrally located oligonucleotide corresponding to the consensus OB was radioactively labelled and used as the target in the mobility shift assays (Fig. 4).

$K_{\text{app}}$ was established experimentally for a selection of truncated KorB products alone and in the presence of IncC. The data are summarised in Table 2.

### Table 2. IncC enhancement of KorB DNA-binding activity in vitro

<table>
<thead>
<tr>
<th>Purified His-KorB and its deletion derivatives used</th>
<th>$K_{\text{app}}$ of KorB (nM) in the presence of IncC</th>
<th>$K_{\text{app}}$ of KorB (nM) in the presence of IncC</th>
<th>$K_{\text{app}}/K_{\text{app}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type KorB</td>
<td>1125</td>
<td>385</td>
<td>2.9</td>
</tr>
<tr>
<td>N∆30</td>
<td>313</td>
<td>60</td>
<td>5.2</td>
</tr>
<tr>
<td>N∆60</td>
<td>167</td>
<td>34</td>
<td>4.9</td>
</tr>
<tr>
<td>N∆90</td>
<td>650</td>
<td>160</td>
<td>4.1</td>
</tr>
<tr>
<td>N∆150</td>
<td>135</td>
<td>27</td>
<td>5.0</td>
</tr>
<tr>
<td>N∆173</td>
<td>No binding</td>
<td>No binding</td>
<td>ND</td>
</tr>
<tr>
<td>B∆11up</td>
<td>61</td>
<td>13</td>
<td>4.7</td>
</tr>
<tr>
<td>B∆37up</td>
<td>520</td>
<td>346</td>
<td>1.5</td>
</tr>
<tr>
<td>B∆10down</td>
<td>64</td>
<td>20</td>
<td>3.2</td>
</tr>
<tr>
<td>B∆20down</td>
<td>29</td>
<td>16</td>
<td>1.8</td>
</tr>
</tbody>
</table>

$K_{\text{app}}$ was estimated experimentally in EMSA as the concentration of purified His-tagged KorB derivatives needed to shift 50% of the radioactive fragment containing OB. $K_{\text{app}}$ for N∆173 could not be experimentally established, no binding to DNA was observed at 10 nM.

$K_{\text{app}}$ was estimated experimentally as the concentration of products of korB alleles needed to shift 50% of the labelled fragment in the presence of 581 nM IncC.

The presence of IncC in the reaction mixture increased the affinity of KorB for DNA 2–6-fold in the cases where KorB could bind to DNA (Table 2). The internal deletion BΔ37up retained the ability to interact with IncC. This limited the region of KorB necessary for interaction with IncC to amino acids E151 to T218 (Fig. 1).

Table 2 shows that any $K_{\text{app}}$ calculated on this basis is not comparable with the other proteins and is not therefore included in Table 2.

Application of the yeast two-hybrid system to define the domain in KorB responsible for interaction with IncC

ORFs for KorB and IncC were cloned into vectors for the yeast two-hybrid system to create fusion proteins with GAL4 DBD (DBDGAL4) and AD (ADGAL4). Both IncC and KorB proteins were linked via their N-termini to GAL4 domains. IncC may code for two forms of IncC so we fused both products to GAL4 separately through N-terminal parts (IncC1 and IncC2). The interactions between the proteins were demonstrated by the colony-lift test for $\beta$-galactosidase activity followed by $\beta$-galactosidase assay on the liquid cultures of Y187 derivatives and confirmed by histidine prototrophy of Y190 double transformants in the presence of 3-AT. The interactions detected using this approach were quite weak but significant. Representative interactions are shown in Figure 5A and the summary of the results is presented in Figure 5B. From the IncC–KorB interaction pattern we concluded that when KorB was linked to the ADGAL4 it lost its ability to fold properly. This

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Figure 4. EMSA with representative N-terminal and internal deletion derivatives on DNA fragments containing a consensus OB site. The first two tracks on each gel demonstrate the conditions of DNA fragment incubation with no protein or IncC on its own. The numbers above the black lines refer to the concentration (nM) of His-tagged KorB truncation products used in the presence and absence of IncC. For NA150KorB, where indicated, IncC was added at 258 nM. For B∆11upKorB and B∆20downKorB, where indicated, IncC was added at 581 nM. Carrier DNA was added to the incubation mixtures at final concentration of 0.1 mg/ml.
and 107 amino acids (from I174 to D280). These two short polypeptides fused to DBD\textsubscript{GAL4} were still able to interact with AD\textsubscript{GAL4}–IncC. To map the interaction domain even further, we used korB internal deletion derivatives in the two-hybrid system. We chose two deletion derivatives, BA11up and BA37up, having removed amino acids from between K244 and D256, and T218 and D256, respectively. Both deletion derivatives produced hybrid proteins DBD\textsubscript{GAL4}–KorB capable of interactions with AD\textsubscript{GAL4}–IncC. The fragments of KorB from M1 to D173 and from D256 to G358 showed no interactions with IncC. These results allowed us to limit the patch on KorB capable of interaction with IncC to the 45 amino acid segment from I174 to T218 in the KorB sequence (Fig. 1).

**Dimerisation domains of KorB analysed by the yeast two-hybrid system**

KorB molecules fused to both GAL4 domains gave only a weak positive signal in contrast to their established ability to dimerise (33,35,36). However, these interactions were significant enough to confirm the presence of a dimerisation domain in the C-terminus of KorB. Deletion of 101 amino acids from the C-terminus (DBD\textsubscript{GAL4}–KorB\textsubscript{CA101}) removed the ability to interact with wild-type KorB fused to AD\textsubscript{GAL4}, whereas the C-terminus 103 amino acids (DBD\textsubscript{GAL4}–KorB\textsubscript{NA255}) retained this ability (Fig. 6). The ability of other KorB fragments to interact with themselves and wild-type KorB was also tested. Both internal fragments of 107 amino acids (from I174 to D280) and 84 amino acids (from I174 to P257) showed strong self-interactions and cross-interactions between them. We could detect hardly any interactions of internal fragments with wild-type KorB (Fig. 5A) or with segments truncated either at the N-terminus (NA\textsubscript{173}) or C-terminus (CA\textsubscript{101}).

**Glutaraldehyde cross-linking of the products of mutant korB alleles**

The oligomerisation ability of purified His-tagged proteins corresponding to some of the deletion derivatives described in this paper was tested by glutaraldehyde cross-linking. Previously, we have shown (33) that CA\textsubscript{17} results in a monomeric form of truncated KorB. C-terminal deletions of up to 101 amino acids showed the same effect whereas N-terminal deletions up to 255 amino acids gave products that could be cross-linked (Fig. 7). The C-terminal 103 amino acids of KorB form very stable dimers and higher order complexes. The complexes are so stable that they can be detected even without glutaraldehyde under SDS–PAGE denaturing conditions. We also His-tagged and purified the internal parts of KorB (products of the deletions NA\textsubscript{173}–CA\textsubscript{78} and NA\textsubscript{173}–CA\textsubscript{101}). Both fragments showed the ability to be cross-linked by glutaraldehyde confirming the presence of the potential secondary dimerisation domain in KorB, although this effect appears significantly less strong than that observed with the C-terminal 103 amino acids (only NA\textsubscript{173}–CA\textsubscript{78} is shown in Fig. 7).

**DISCUSSION**

In this paper we have attempted to define key functional regions of KorB using a combination of genetic and in vitro methods. As described in the Introduction, KorB, the global regulatory protein and partitioning protein of IncP-1 plasmids (30,41), is known to bind DNA and function as a repressor in a
way that depends on its oligomeric state (32). Its role in repression is modulated by IncC (33) and its role in partitioning is dependent on IncC (21,38), with which it is known to interact directly (33,39,43). The results we have presented above have helped us to localise regions either sufficient for or involved in each of these properties.

With respect to DNA-binding ability we have localised, by deletion analysis, the key region as lying between amino acids E151 and T218. Deletion of amino acids 1–173 destroyed DNA binding consistent with the position of the previously identified putative HTH motif (from K171 to T190) (56), which is present in all ParB family members (14). C-terminal deletions leaving amino acids 1–257 still bound DNA and the internal deletions covering T218 to D285 did the same. The estimated affinity for DNA (\(K_{\text{app}}\)) of the various deletion derivatives varies over a wide range, suggesting that confirmation of the resulting proteins may differ and thus influence the stability of the protein–DNA complexes. Confirmation may be especially critical for KorB–DNA interactions as KorB is predicted to have a net negative charge so the juxtaposition of the acid residues relative to the DNA phosphate backbone should determine the degree of electrostatic repulsion.

For N-terminal deletion derivatives that still bind DNA there is a rough correlation between affinity for DNA and the ability to repress transcription at Class I O\(_B\). This contrasts with the ability to repress through a Class II O\(_B\), which is lost in all but the shortest N-terminal deletions (our unpublished results). We still do not understand exactly how KorB represses transcription at a distance so it would be premature to speculate what role the N-terminal domain plays. However, these results, along with previous ones (33), do point to much of the whole native protein structure of KorB being more essential for its other tasks—long range repression and possibly active partitioning—than for perhaps the simpler task of transcriptional repression through binding to proximal operators. The most striking of these results is that deletion of the region T218 to R255 gave a protein still able to bind DNA but no longer able to repress transcription at any of the promoters tested. This indicates that DNA binding alone is not sufficient for transcriptional repression even at Class I O\(_B\), implying that direct interaction between KorB and RNAP may be necessary. This would be consistent with the observation that KorB does not prevent RNAP from binding but does stop isomerisation from closed to open complex (36).

With respect to oligomerisation, we have shown previously that the C-terminus is important for repression at a distance and for a multimeric state of KorB in vitro (33). Deletion of 255 amino acids from the N-terminus generated a 103 amino acid segment that could strongly dimerise as judged by cross-linking with glutaraldehyde in vitro (Fig. 7) and interactions in the yeast two-hybrid system in vivo (Fig. 6). Sequence alignments show that this C-terminal segment is highly conserved and is separated from the DNA-binding segment by a much more variable region (Fig. 1). The idea that all or part of this region forms a dimerisation domain is attractive.

Interestingly, the studies with the yeast two-hybrid system suggested that there is another segment that has potential to promote oligomerisation when both the N- and C-terminal regions are removed. These results have been confirmed also by glutaraldehyde cross-linking in vitro (Fig. 7). Deletion of 101 amino acid residues from the C-terminus renders KorB unable to form dimers in cross-linking reactions, but deletion of a further 173 amino acids from the N-terminus allows formation of dimers (oligomers) although with much lower efficiency than observed for the C-terminus under the same conditions. This may indicate that the secondary oligomerisation domain can function after a conformational change in KorB structure due, for example, to interaction with DNA, IncC or another macromolecule in the cell. A similar inhibitory
function of one dimerisation domain over another was also observed for ParB of P1 (28).

Finally, we defined the patch in KorB sufficient for interactions with IncC. Combined in vitro and in vivo assays allowed us to localise this region to the central 45 amino acids in which the HTH motif has also been localised. So far, the domains of interactions with the ParA component of the partitioning apparatus have been defined in the N-terminal parts of ParB of P1 (the first 29 amino acids) (28) and SopB of F (N-terminal 180 amino acids) (27). So whereas the localisation of the primary dimerisation domain in ParB members of the family is highly conserved (27,33,56,57) there is no universal localisation of the domain of interaction with ParA. This may seem surprising in light of the apparent co-evolution of these two proteins as part of a ubiquitous stable inheritance mechanism. However, it seems likely that there has been selection for diversity in the specificity of the partitioning complexes. Given the differences in the centromere-like sequences, we might expect a corresponding diversity in the protein–DNA architecture of the partitioning complexes. In addition, the particular location in KorB of this interaction domain makes sense of the fact that IncC potentiates KorB DNA binding apparently by modulating the interaction of KorB with flanking sequences on either side of the core operator (37). IncC may distort KorB and make it more capable of productive contacts with these flanking regions. This coarse mapping of functional regions in KorB will now allow more detailed dissection of the different functions performed by KorB and will provide a fine analysis of the relationship between structure and function.

ACKNOWLEDGEMENTS

M.L. was supported in part by a Scholarship from the Darwin Trust of Edinburgh. K.K. was supported by project grant 0408040 from The Wellcome Trust. The collaboration between G.J.B. and C.M.T. during this work was funded by a Research Initiative Grant from The Wellcome Trust (056022) which also provided partial support for A.A.B. G.D.C. was supported by a BBSRC Mres Studentship. This work was carried out in the context of EU Concerted Action MECBAD (Mobile Elements Contribution to Bacterial Adaptability and Diversity).

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