The partitioning activity of the RK2 central control region requires only incC, korB and KorB-binding site O₈3 but other KorB-binding sites form destabilizing complexes in the absence of O₈3

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INTRODUCTION

The process that leads to the accurate distribution of genomes prior to cell division in bacteria is of interest because of the central role it plays in the cell cycle (Hiraga, 1992; Rothfield, 1994; Wake & Errington, 1995). The machinery involved may provide novel targets for antimicrobial agents. Studies with Bacillus subtilis have identified two genes, spoOJ and soj, which have been implicated in chromosome segregation into the pre-spore during sporulation (Sharpe & Errington, 1996). This appears to be achieved by anchoring the oriC region of the chromosome near the cell pole so that the rest of the chromosome can be spooled into the pre-spore compartment (Wu et al., 1995; Lewis & Errington, 1997). Fluorescent tagging has shown both the oriC region and Par proteins in polar locations during sporulation and vegetative growth (Webb et al., 1997; Lin et al., 1997). Defects in chromosome partitioning occur even during vegetative growth when these genes are disrupted (Ireton et al., 1994). Therefore, it is proposed that Soj and SpoOJ form part of a general mitotic apparatus for directing chromosome movement.
[Glaser et al., 1997]. Homologues of either one or both SpoOJ and Soj are encoded by all bacterial chromosomes that have thus been studied to date. In Caulobacter crescentus the homologous proteins move in the bacteria in a way that is also strongly suggestive of a mitotic cycle (Mohl & Gober, 1997). In Escherichia coli the Soj homologue MinD is encoded away from oriC but is required for correct placement of septa apparently through a recognition of key sites in the internal cellular architecture (reviewed by Lutkenhaus & Addinall, 1997). Nevertheless, E. coli chromosomal oriC regions have been observed to migrate in a fashion indicative of a cycle with similarity to that of B. subtilis and C. crescentus (Gordon et al., 1997).

These chromosomally encoded proteins belong to families, designated ParA and ParB, originally discovered on bacterial plasmids and associated with genetic loci responsible for the better than random segregation of low-copy-number plasmids, a phenomenon known as active partitioning (Austin, 1988; Nordström & Austin, 1989; Williams & Thomas, 1992). Recent evidence suggests that the best studied low-copy-number plasmids F and P1 actively segregate to quarter/three-quarter positions of the E. coli cell rather than the poles as observed for the chromosome (Gordon et al., 1997; Niki & Hiraga, 1997). Nevertheless, it seems likely that study of the plasmid-encoded ParA/ParB proteins and their associated cis-acting sequence, referred to as centromere-like regions, will help to uncover the nature of this bacterial mitotic apparatus (reviewed by Williams & Thomas, 1992). Sequence comparison showed that many plasmids carry related genes. The ParB proteins bind specifically to the cis-acting sequences (Mori et al., 1989; Watanabe et al., 1989), although the nature of this element seems variable in size and organization (Lane et al., 1987; Martin et al., 1987, 1991). Conserved motifs suggested that the A proteins may be ATPases (Motalebi-Veshareh et al., 1990), a proposal which has since been confirmed for P1 ParA and F SopA (Davis et al., 1991; Watanabe et al., 1992). The ParA ATPase activity is necessary for partitioning (Davis et al., 1996). However, the way that these proteins direct the better than random segregation of plasmid DNA molecules remains unclear and further analysis of individual systems is needed to build up a picture of the properties of this family of proteins.

The central control region (ctl) of broad-host-range IncP plasmid RK2 encodes five polypeptides of which two, IncC and KorB, show significant sequence similarity to SopA/ParA and SopB/ParB of F and P1, respectively (Motalebi-Veshareh et al., 1990). Inactivation of incC leads to decreased stability of mini IncP plasmids, indicating that the region may encode partitioning functions similar to those of F and P1 (Motalebi-Veshareh et al., 1990). However, because the region is more complex than those of F and P1 we thought its study might provide additional insights. This was particularly prompted by the presence of the korA gene encoding a DNA-binding protein with an extended coiled-coil domain reminiscent of components of force-generating motor proteins (Jagura-Burdzy & Thomas, 1992).

The ctl region by our definition here runs from korAp at the start of the korA-korB operon to the bi-directional terminator between upf54.4 and traM (Fig. 1). It contains three operons, the korAB operon encoding korA, incC, korB, korF and korG, the korA monocistronic operon and the upf54.8-upf54.4 bicistronic operon (reviewed by Pansegrau et al., 1994), although during the course of this work our observations sug-
gested that expression of upf54.8 and upf54.4 depends on transcription from kfrAp (D. R. Williams, unpublished), implying that kfrAp, upf54.8 and upf54.4 form a single operon. KorA (101 aa) is a global regulator that binds to seven operators on the RK2 genome (Bechhofer & Figurski, 1983; Jagura-Burczy & Thomas, 1993). Two of these KorA operators are in the ctl region, at kfrAp and kfrAp, both of which are repressed by KorA (Theophilus et al., 1985; Thomas et al., 1990). incC encodes the putative ATPase related to SopA and ParA (Motallebi-Veshareh et al., 1990; Thomas & Smith, 1986), but contains two translational starts so that it produces two polypeptides, IncC1 (364 aa) and IncC2 (259 aa), both of which should have ATPase activity. KorB (358 aa), which belongs to the SopB/ParB family (Motallebi-Veshareh et al., 1990), is a second global regulator, binding to 12 operators in the RK2 genome (Theophilus & Thomas, 1987; Kornacki et al., 1987; Balzer et al., 1992; Williams et al., 1993). Three of these, O_R1, O_R2 and O_R3, are in the ctl region near kfrAp, near kfrAp and between upf54.8 and upf54.4, respectively. KorF (173 aa) and KorG (175 aa) are putative histone-like proteins (Jagura-Burczy et al., 1991). KrFA (314 aa) has been described above. Little is known about the additional two proteins encoded by upf54.8 and upf54.4, although they are quite highly conserved between RK2 and R751 (Macartney et al., 1997).

The purpose of the work described in this paper was to check that this region can stabilize heterologous replicons, and to determine which genes are necessary for this activity. However, the phenotypes of some of the mutants generated suggest that the multiple binding sites that exist for KorB provide a degree of complexity which may help to identify the components essential for active partitioning and their mechanism of action.

**METHODS**

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strains used were K-12 strains C600K (thr-1 leu-6 thi-1 lacY1 supE44 ton21 galK), MV10 (C600 trpR trpE5) and TG1 (supE hsdS5 thi Δ(lac-proAB) F'etrAD36 proABlacY lacZAM15); Sambrook et al., 1989), and E. coli C strain C2110 (polA1 his rha) (all strains except TG1 were from Professor D. R. Helinski, Department of Biology, UC San Diego, CA, USA). Bacteria were generally grown in L broth (Kahn et al., 1979), at 37 °C, or on L agar (L broth with 1.5% agar supplemented with appropriate benzyl penicillin, sodium salt (100 mg mL^-1 in liquid media and 300 mg mL^-1 in agar plates), or ampicillin (100 mg mL^-1) to select for penicillin resistance. Previously described plasmids used during the course of this study were pAL1029 (Ludtke et al., 1989), pOG04 derived from pAL1029 by replacing the EcoRI to HindIII par region by the short EcoRI to HindIII segment from pBR322 (Macartney et al., 1997) and pOG4 derived from pAL1029 by replacing the P7 par genes with the RK2 ctl genes (Macartney et al., 1997). The structures of pOG4 and pOG4 are shown in Fig. 2. All plasmids constructed during the course of this work are related to pOG4 or were involved in constructing derivatives of pOG4. These are described below.

**Isolation, analysis and manipulation of plasmid DNA.** Plasmid DNA was isolated on small and large scales by standard procedures (Sambrook et al., 1989). Digestion of plasmid DNA with restriction enzymes was carried out under conditions recommended by suppliers and run on a 0.8% or 1% (w/v) agarose gel. Bacterial transformations were carried out using standard CaCl2 treatment. DNA sequencing was carried out by Alta Bioscience using the Dye-terminator method (derived from Sanger et al., 1977) in conjunction with an ABI 373 automated DNA sequencer. Sequences were aligned and analysed using programs of the Wisconsin package (Devereux et al., 1984). DNA PCR amplified fragments were labelled with terminal transferase and [a-32P]ddATP.

**Construction of derivatives of pOG4.** Mutations were introduced by a variety of strategies: exploitation of spontaneous deletions that had been defined by DNA sequence analysis; cutting at unique sites; oligonucleotide-directed mutation with the Sculptor kit from Amersham. Since the whole region being manipulated is about 5 kb we found that it was best to introduce mutations into subcloned fragments which could then be spliced back into the full context after the mutation had been obtained and checked by sequencing. Attempts to
carry out oligonucleotide-directed mutation on the full region tended to generate deletions at the single-stranded stage and subsequently required large amounts of sequencing to check for secondary mutations. In other cases unique sites were obtained in the region of interest only when a subclone was used lacking segments that had additional copies of the desired site. Construction of each mutant was quite complicated, so the intermediate plasmids are not listed in full below.

Removal of O2, korA and the start of incC so that IncC1 is no longer produced was achieved by replacing the EcoRI (at RK2 co-ordinate 60099 kb) - BspEI (in korA) fragment with Tsps091 fragments bearing the tet promoter from pBR322 and a transcriptional terminator (giving pOG4.100). incC was inactivated (so that neither IncC1 nor IncC2 was produced) by a spontaneous 70 bp internal deletion which was described previously (Thomas, 1986; Thomas & Smith, 1986). It was spliced into the ctf region using the Sphi and SacII (SstII) sites that lie upstream and downstream of the deletion, respectively (giving pOG4.1). korB was inactivated by a 2 bp deletion generated by digestion with SacII, removal of the 5’ overhang with T4 DNA polymerase and ligation (giving pOG4.1). korB was inactivated by disruption of the AupI site to create a stop codon at triplet 66 within the orf (giving pDM204.3). korG was inactivated by oligonucleotide-directed mutagenesis of the first bases of triplets 20 and 21 in the orf to create an XbaI site (giving pDM205.3). A double mutant with both the korB and the korG mutations was made by cloning the appropriate Sphi fragment from pDM204.3 into the context of pDM205.3 (giving pDM209.3). kfa was inactivated by a spontaneous deletion of 3 bp that occurred between repeated sequences that included Sphi sites (giving pOG4.53). up6, up6 was inactivated by a 4 bp deletion caused by digestion with ApaI, removal of the 5’ overhang with T4 DNA polymerase and ligation (giving pOG4.7). O2 was inactivated by use of oligonucleotide-directed mutagenesis, which changed the sequence from 5’ CCGTAAAATA 3’ to 5’ CC(G/CG)ATTTAAA 3’ (giving pOG4.020). O3 was inactivated by oligonucleotide-directed mutagenesis, which deleted the central region of the sequence to leave a DraI site (5’ TTTTTAAA 3’; pOG4.003). The logic for nomenclature of derivatives with one of the three KOR-binding sites inactivated or deleted was to add .010, .020 or .03 to the name of the parent plasmid depending on which O was affected.

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

**Estimation of the ability of RK2 segments to stabilize a low-copy-number plasmid.** Loss rates were determined as previously described (Macartney et al., 1997). The tests employed a shuttle vector, pOG04, which was derived by deletion of the P7 par system from pAL1029 (Ludtke et al., 1989). The vector carries both pMB1 and P7 origins and therefore replicates at high copy number in a PolA+ strain (pMB1 origin) and at low copy number in a PolA- strain (pOL-independent P7 origin). In PolA+ strain C2110 pOG04 is lost during exponential growth at the rate expected of a randomly segregating plasmid present at four to six copies per cell at division. Derivatives bearing wild-type and mutant ctf regions from RK2 were constructed and selected in a PolA- strain and then transformed into C2110 to test their stability. The transformants were subcultured in selective medium to an OD600 ~ 1.0, diluted 10–4 into non-selective medium (t0) and grown for 8 h until they had undergone about 25 generations (tG). Viable counts and the percentage of ampicillin-resistant colonies were calculated for both t0 and tG. The mean apparent percentage loss of plasmid was calculated using the formulae L (loss rate) = (1 − R1/G) × 100, where g (generations) = 1.44 × log10(x0/xG) (xG is the viable count at the start of the experiment and x0 is the viable count at the end of the experiment), and R = % AmpR bacteria at tG/% AmpR bacteria at t0 (tG is the time at the end of the experiment while t0 is the time at the beginning of the experiment).

**Estimation of relative plasmid copy number.** Relative plasmid copy number was estimated as follows. To extract total DNA, 0.7 ml bacterial culture and 0.7 ml aqueous phenol that had been equilibrated with 50 mM Tris/HCl, pH 8.0, 10 mM NaEDTA (TE) were mixed thoroughly for 30 s in a 1:5 ml microfuge tube. After centrifugation for 5 min in a microfuge (12500 r.p.m.) the aqueous (top) layer was removed and mixed thoroughly with phenol/chloroform (24:1 with isoamyl alcohol) for 30 s. After centrifugation again the aqueous layer was removed, avoiding any carry-over of the phenol/chloroform layer, and then precipitated by addition of 2 vol 2-propanol, mixing and leaving at room temperature for 2 min. After spinning in a microfuge for 5 min the DNA pellet was washed with ice-cold 70% (v/v) ethanol, dried and resuspended in 50 μl TE. A standard amount of DNA was digested with EcoRI and run on a 1% (w/v) agarose gel, before Southern blotting and probing by standard methods (Sambrook et al., 1989). A DNA fragment with part of the trp operon was used as the probe for chromosomal DNA while a fragment containing the bla gene was used as the probe for the plasmid DNA. After standard washing the filters were placed in a phosphorimager cassette which was scanned after overnight exposure. The image was analysed using Imagequant software and the pixels in the plasmid and chromosomal bands were compared to give a plasmid to chromosome ratio which was used as a measure of the relative copy number.

**RESULTS**

**The RK2 ctf region stabilizes a low-copy-number P7 replicon**

We have previously demonstrated the need for incC of the RK2 ctf region for stable inheritance of IncP replicons (Mottalebi-Veshareh et al., 1990). To allow easier dissection of this system we exploited a shuttle vector previously used by Austin and co-workers (Ludtke et al., 1989) that consists of pBR322 joined to the replicon from phage/plasmid P7. The pBR322 section allows easy plasmid construction in a PolA+ host while the P7 replicon allows replication at low copy number in a PolA- host. However, in such a PolA- host the rate of loss is so high if the plasmid lacks an active partitioning system that selection for the plasmid favours mutants with higher copy number, which therefore rapidly dominate. Therefore, tests on the stability of derivatives of these plasmids need to be performed as soon as possible after their introduction into the PolA- strain. Our starting point was pALA1029, which possesses both the P7 replicon and the P7 active partitioning system. In pOG04 the P7 par is deleted and in pOG4 it is replaced by the RK2 ctf region (Fig. 2). After introduction into PolA- strain C2110, stability was estimated as described in Methods. The results are
Table 1. Relative plasmid copy numbers of the plasmids studied in this work

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Relative copy number†</th>
<th>95% confidence limits (n)‡</th>
</tr>
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<tbody>
<tr>
<td>pALA1029 (P7 par)</td>
<td>4.96</td>
<td>2.58 (3)</td>
</tr>
<tr>
<td>pOG04 (unstable control)</td>
<td>2.09</td>
<td>0.19 (6)</td>
</tr>
<tr>
<td>pOG4 (RK2 ctl)</td>
<td>1.95</td>
<td>1.54 (3)</td>
</tr>
<tr>
<td>pOG14 (RK2 ctl incC)</td>
<td>2.66</td>
<td>0.29 (3)</td>
</tr>
<tr>
<td>pOG41 (RK2 ctl korB)</td>
<td>2.08</td>
<td>0.37 (3)</td>
</tr>
<tr>
<td>pDM204.3 (RK2 ctl korF)</td>
<td>2.21</td>
<td>0.69 (3)</td>
</tr>
<tr>
<td>pDM205.3 (RK2 ctl korG)</td>
<td>1.72</td>
<td>0.20 (4)</td>
</tr>
<tr>
<td>pDM209.3 (RK2 ctl korF korG)</td>
<td>2.32</td>
<td>0.43 (4)</td>
</tr>
<tr>
<td>pOG453 (RK2 ctl kfrA)</td>
<td>2.92</td>
<td>0.37 (3)</td>
</tr>
<tr>
<td>pOG47 (RK2 ctl upf54.8)</td>
<td>1.99</td>
<td>0.51 (7)</td>
</tr>
<tr>
<td>pOG4.100 (RK2 ctl korA O6,1)</td>
<td>1.85</td>
<td>0.68 (6)</td>
</tr>
<tr>
<td>pOG4.020 (RK2 ctl O2)</td>
<td>1.88</td>
<td>0.64 (4)</td>
</tr>
<tr>
<td>pOG4.003 (RK2 ctl O3)</td>
<td>1.88</td>
<td>0.37 (7)</td>
</tr>
<tr>
<td>pOG4.103 (RK2 ctl O3,1 O5,3)</td>
<td>1.70</td>
<td>0.55 (6)</td>
</tr>
<tr>
<td>pOG4.023 (RK2 ctl O5,2 O5,3)</td>
<td>2.36</td>
<td>0.48 (4)</td>
</tr>
<tr>
<td>pOG41.003 (RK2 ctl korB O6,3)</td>
<td>1.28</td>
<td>0.18 (4)</td>
</tr>
</tbody>
</table>

* Plasmid genotypes: pALA1029 has the P7 partitioning system; pOG4 and below have the RK2 ctl with mutations as indicated by the genes listed.
† Determined as described in Methods.
‡ n, Number of samples for which a copy number determination was performed.

shown in Fig. 3. The RK2 ctl region clearly decreases loss rate but not as much as the P7 par. However, to correct for plasmid copy number variation we carried out a Southern blot of EcoRI-digested total DNA separated by agarose gel electrophoresis and then hybridized with chromosomal and plasmid probes. Copy number was estimated by comparison with a segment of the trp operon. The trp operon is encoded at 27.5 min on the genome, close to the replication terminus, so that the total number of trp gene copies should approximate to the number of completed chromosomes in the bacterial cell. For exponentially growing bacteria in L broth at 37 °C the mean generation time is 25–30 min so that when the bacteria divide there should still only be two copies of the terminus. Thus very roughly our relative copy number of two should correspond to a copy number of approximately four per cell. We found that the combination of the P7 rep/P7 par has a twofold higher copy number than P7 rep/RK2 ctl (Table 1). Although we have not measured absolute copy numbers precisely, data previously obtained for the P1/P7 replicon with an active partitioning system suggest that the copy number should be in the region of two to three prior to cell division. Our results suggest a rather higher value. A twofold decrease in copy number as observed when the P7 par system is removed or replaced by the RK2 system would be expected to dramatically increase the rate of plasmid loss. Thus much of the increased
stability of pALA1029 compared to pOG4 may be due to its higher copy number, which could arise from an interaction between the two related systems. Therefore, we decided that this shuttle vector demonstrated the stabilizing effect of the RK2 cdt region and provided an adequate system for studying the effect of mutations on this phenotype.

Mutagenesis of each protein-coding gene from korA to upf54.8

In the RK2 cdt region there are a number of cistrons, in addition to korA, incC and korB, which have not yet been assigned a function. Therefore, we hypothesized that the IncP cdt region may provide more partition functions than the F and P1/7 region and thus give greater insights into the partition process. To test this hypothesis with respect to encoded polypeptides we exploited spontaneous mutations or generated site-directed mutations in each of the ORFs as described in Methods. This was done using subcloned sections of the cdt region to limit the number of restriction sites present and to minimize the region that went through a single-stranded stage during in vitro site-directed mutagenesis. The mutagenized region was then sequenced to ensure that only the desired changes had been introduced. The mutated region was spliced back into the complete region. In vitro transcription/translation was performed on each mutant cloned cdt region to check that only the gene product targeted had been affected by the mutation (data not shown). Interestingly, the results showed clearly that the repressors synthesized by the region reach sufficient in vitro concentrations to inhibit further transcription of the operons present. Thus when repressor genes were inactivated, the level of the polypeptide products synthesized in vitro was considerably elevated compared to wild-type template. Checking the mutants in this way also proved valuable because we detected secondary mutations in a number of clones. For example, in at least one upf54.8 mutant we discovered that IncC1 was no longer produced either. Such clones were rejected in favour of clones that exhibited the expected polypeptide profile. However, when the desired mutation had already been confirmed in the target region the easiest strategy was then to splice the desired mutation back into a known wild-type region.

incC and korB are the only protein-encoding genes needed for partitioning

Each plasmid into which we had introduced the specific mutations described above was then established in C2110 (PolA-) along with appropriate controls and the loss rate for each plasmid was determined. We found that only the mutations in incC and korB resulted in loss of the Par+ phenotype (Fig. 3). These mutations returned the plasmid loss rate to the region of 4–6% per generation, similar to the level of instability observed for pOG04. Mutations in korF, korG, kfrA, upf54.8 and upf54.4 had no significant effect on loss rate (data not shown). Since it was possible that korF and korG may be able to substitute for each other we created a korFkorG double mutant but this did not show any increased rate of loss either (data not shown). The relative copy number of each plasmid was tested at the start of the experiment as described in Methods with the results shown in Table 1. While variation in copy number was observed between different plasmids, there was no correlation between these small variations and the rate of plasmid loss. Therefore, it appears that only two plasmid-encoded proteins are needed for the stability phenotype.

IncC1 is dispensable when IncC2 is still produced

The start of the korA/korB operon is complicated because korA and incC overlap. However, incC has two translational starts, producing IncC1 and IncC2. One start precedes the korA start while the other overlaps the stop codon for korA. To determine the importance of this part of the operon we created a deletion/fusion which removes korAp, most of korA, the start of incC and places the remains of the korA orf in-frame with the tet orf from pBR322. The tet promoter provides transcription for the operon. We found that this deletion did not result in loss of the Par+ phenotype (data not shown). Therefore KorA is not required, nor is IncC1. We can conclude that only IncC2 and KorB are needed for active partitioning.

cis-acting sites needed for partitioning

By analogy with the F and P1 systems we expected that the cis-acting sequences – the centromere-like region – should contain KorB-binding sites. In the cdt region there are three such sites which we have designated O_b1 (at korAp), O_b2 (at kfrAp) and O_b3 (between upf54.8 and upf54.4) (Fig. 1). We determined the effect of removing each of these in turn. O_b2 and O_b3 were inactivated by site-directed mutation while O_b1 was removed by deletion along with korAp as described in the previous section. Again we determined the sequence of mutated regions, checked that the polypeptide profile was complete and then tested the effect on stability. We found that inactivation of O_b1 and O_b2 had no effect on the Par+ phenotype but that inactivation of O_b3 resulted in complete loss of the stabilizing effect conferred by the cdt region (Fig. 3). In fact, mutagenesis of O_b3 gave a much higher rate of appearance of plasmid-free segregants than any of the other plasmids with a single mutation. This effect was not due to an effect on copy number, which remained similar to the other plasmids. Therefore we propose that the O_b3 region is the candidate centromere-like region.

The relative orientation of incC/korB and O_b3 does not affect the stability phenotype

During the dissection of the RK2 cdt region we isolated a series of mutations arising from the insertion of the transposon Tn1723 (KmR). This transposon has EcoRI sites 15 bp from each end so that it is possible to remove
the majority of the transposon but leave behind an EcoRI site at the original point of insertion. One such mutation was found in the kfrAp region. In attempting to construct a pOG4 mutant derivative of this region we generated an inversion of the korA–korC region which is flanked by EcoRI sites. Testing the stability phenotype of this region we found that it was no less stable in maintaining the plasmid than the parent from which it was derived (data not shown), indicating that the orientation of incC and korB relative to O₃ is not important for the stability phenotype.

**Suppression of the extreme instability of the O₃ mutant**

Inspection of the data in Fig. 3 shows that in contrast to the effect of inactivating incC or korB, inactivation of O₃ results in a loss rate that is considerably greater than that for pOG04, the control plasmid that lacks a partitioning apparatus and should therefore be distributed at random. However, the apparent level of plasmid DNA in the bacteria is not reduced (Table 1). This suggests that the mutation reduces the number of physically separate units which are available to segregate prior to cell division. Since a favoured model for partitioning involves KorB-mediated pairing, it seemed possible that although only one region with a KorB-binding site should be able to promote active segregation, other binding sites may promote pairing. Applying this to the RK2 ctl region and the data presented so far we hypothesized that pairing occurs at O₃, O₂, or O₁ but that separation depends on O₃ alone. In this case the absence of O₃ would result in the plasmid becoming unstable because pairs of plasmids would be held together by KorB at O₃, O₂, and thus reduce the number of segregating units. We would then expect that the high instability of the O₃ mutant should be suppressed by deleting other O₃ sites or inactivating the protein which mediates pairing. We therefore created hybrids in which the O₃ mutant was combined with O₁⁻, O₂⁻, and korB⁻. Starting from pOG4.003 we inactivated either O₁ (pOG4.103) or korB (pOG41.003) and observed instability return to the level for the unstable vector control (Fig. 3). However, inactivation of O₂ had little effect (pOG4.023). This correlates with our recent studies which showed that O₂ has 10-fold lower affinity for KorB than O₁ or O₃, probably due to the mismatch in one half operator site compared to the consensus sequence (C. M. Thomas, unpublished). Copy number determinations indicated that the increased stability was not the result of an increase in copy number (Table 1).

**DISCUSSION**

In this paper we describe the mutational analysis of all the cistrons and KorB-binding sites in the ctl region of plasmid RK2 with respect to the ability of this region to stabilize an unstable, low-copy-number, heterologous replicon. We had expected to find that, in contrast to other plasmid systems so far characterized, the RK2 system would show a dependence on more plasmid-encoded proteins than just the ParA and ParB homologues IncC and KorB. However, the results show clearly that to achieve the degree of stability that we observe with the region cloned, we only need IncC and KorB. However, for a number of reasons we can not at this stage rule out any role for the additional proteins in this region in plasmid stability. First, it may be that the region we have been studying lacks additional functions which are needed to allow the other genes tested here to play their full role. This would be consistent with the fact that the stabilization that we see is not as efficient as that achieved by the P7 par system used as a positive control in these experiments. This idea would also be consistent with recent observations that overexpression of korC destabilizes RK2, especially in Pseudomonas aeruginosa (Wilson et al., 1997). The korC regulated operons form part of a block of functions between ctl and oriV which until recently had no demonstrated function but which are conserved in many respects between IncPα plasmid RK2 and IncPβ plasmid R751 (Thomas et al., 1988, 1995). A role in stable inheritance would therefore make considerable sense and recent data with R751 showed clearly that inclusion of this additional region, along with the ctl region, results in almost no loss of a low-copy-number test plasmid (Thorsted et al., 1998). Second, the observation that overexpression of korC has a greater destabilizing effect in P. aeruginosa than in E. coli also raises the possibility that a phenotype may be observed for some of our mutants if they were studied in species other than E. coli. This would imply that the system carries with it functions which are also provided by the host but where the host version can be used in some species but not others. However, to test this possibility would necessitate using a different low-copy-number plasmid than one based on the P7 replicon because this has a rather restricted host range.

The second important observation we report is that IncC2 is sufficient for partitioning, as we found previously for IncPβ plasmid R751 (Macartney et al., 1997). This raises the question as to the role of IncC1 but is consistent with the fact that many systems only encode a homologue of IncC2 but not IncC1. The chromosomal homologues are generally like IncC2 (for examples, Mohl & Gober, 1997; Sharpe & Errington, 1996) while among plasmids one finds one or the other but normally not both (reviewed by Williams & Thomas, 1992). The F and P1 systems are the best examples encoding a protein with the extra N-terminal region like IncC1. Our observations suggest that the N-terminal region present in IncC1 modulates KorB binding to DNA (C. M. Thomas, unpublished) and therefore this may be a feature particularly associated with the global regulation encoded in the central control region rather than with its role in active partitioning. The fact that IncC2 does function in partitioning increases its attraction as a model for the structure and function of the chromosomal homologues as compared to the F and P1 systems.

The finding that the KorB-binding site O₃ is sufficient
and necessary to give a Par$^+$ phenotype is exciting for a number of reasons. First, it confirms the expectation that a KorB-binding site is an important part of the centromere-like sequence of RK2. Second, it indicates that it is a specific site, which is important since it is difficult to conceive of a situation in which more than one site can be used unless there is co-ordination between the two sites so that they partition in the same direction. We clearly need to show that the other sites present on the complete RK2 genome (O$_B^1$–O$_B^{12}$) are not able to substitute for O$_B^3$ but the specificity demonstrated here fits with what one would expect: namely, that only one site can perform this role in active partitioning. Third, a role for O$_B^3$ in active partitioning is the first demonstration of a role for one of the O$_B$s which does not appear to regulate transcription (see Pansegrau et al., 1994). At present we do not know why O$_B^3$ should behave differently from the other O$_B$s. O$_B^3$ is different from O$_B^1$ and O$_B^2$ because its two halves define a 15 bp inverted repeat which is thus longer than either of the others. However, other O$_B$s are of a similar length so this can not make O$_B^3$ unique. Another possibility is suggested by the finding that IncC potentiates KorB binding at all O$_B$s other than O$_B^3$ (C. M. Thomas & K. Kostelidou, unpublished). The significance of this observation awaits further investigation.

However, perhaps the most interesting observation is that inactivation of O$_B^3$ creates a region which destabilizes the unstable vector and that this destabilization is suppressed by deletion of O$_B^1$ or by inactivation of KorB. The model that we have put forward to explain this is shown in Fig. 4. The key point is that the deletion of O$_B^3$ decreases the number of segregating units because KorB bound at O$_B^1$ promotes plasmid pairing but can not promote separation. A second explanation, that deletion of O$_B^3$ results in derepression of a gene with an inhibitory product (Upf54.4), seems unlikely because it is hard to see how deletion of O$_B^1$ or inactivation of korB could suppress this effect. A third explanation might be that the conformation of the plasmid DNA is critical for the segregation process or for replication and that the complexes formed by O$_B^1$ and KorB interfere with this. Similar explanations have been proposed for the par region of pSC101, which seems to work through DNA-gyrase-mediated effects on plasmid DNA conformation which affect plasmid copy number (Ingmer & Cohen, 1993; Conley & Cohen, 1995). However, this does not provide an explanation for how KorB and O$_B^3$ actually increase the stability of the test plasmid. We also estimated changes in copy number very carefully and the data suggest that changes in copy number are not the explanation. We therefore consider the mechanism proposed in Fig. 4 as the most simple one. However, when we consider the loss rates in Fig. 3, they do not fit the predictions from the proposed changes in the copy number of segregating units. The copy number prior to cell division that is consistent with
the calculated 4% loss for pOG04 is between four and five. A reduction in copy number by twofold due to pairing would be expected to give a loss rate of between 25 and 12.5% per generation, which is much higher than the 8% loss per generation observed. This could be explained if the partitioning system is not working with complete efficiency, as is clearly the case since the complete system in pOG4 does not achieve the reduction in loss rate observed for pALA1029, which relies on the P7 system; i.e. the complexes formed may break down sufficiently frequently to prevent the effective copy number being reduced by a factor of two. However, we think that the model fits sufficiently well to justify further tests to identify pairing between plasmids mediated by the partitioning apparatus in vitro and in vivo. The same conclusions have also been reached with other plasmids, most notably plasmid R1, where recent evidence suggests strongly that pairing can be mediated by sequences which function as a centromere (Jensen et al., 1998).

If the model is correct then it illustrates the advantage of the IncP system with multiple O's which allow the separation of pairing and segregation steps in the prokaryotic equivalent of the mitotic cycle. We do not wish to imply that pairing at O sites other than the one involved in provision of centromere-like activity is an essential part of partitioning – it clearly is not since the normal degree of stabilization is observed when only O3 is present. Nor do we wish to imply that it is therefore an artefact. Rather, we suggest that it could be an event that is not essential for partitioning but may play a role in the control of expression of genes for replication, transfer and stable inheritance so that recently replicated molecules are reduced in activity until they have undergone the partitioning event. In this way the global regulation and active partitioning activities of KorB could be intermeshed.

The fact that inactivation of incC does not result in increased instability suggests that, in the absence of IncC, the process does not get stuck at the proposed intermediate (‘paired’) stage. This could be because in the context studied here, IncC is needed to form the stable complexes which cause the instability observed. This would be the case either if the putative ATPase activity of IncC is required to promote formation of the complexes that destabilize the plasmid, or if IncC is simply a structural component of the complex, which breaks down more rapidly in its absence and therefore does not result in an effective copy number decrease. Further studies involving point mutations to inactivate the ATP binding and ATPase active sites are under way to test these possibilities.

ACKNOWLEDGEMENTS

D.R.W. was supported by project grants from the MRC (G9231237CB), D.P.M. was supported by an MRC Studentship for training in research methods. Automated DNA sequencing was performed in Alta Bioscience using an ABI373 machine purchased with a grant from The Wellcome Trust (038654/Z/93). The phosphorimager was purchased with grants from The Wellcome Trust (037160/Z/92) and the MRC (G9216078MB).

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Received 7 July 1998; revised 24 August 1998; accepted 3 September 1998.