

# Molecular analysis of the pRA2 partitioning region: ParB autoregulates *parAB* transcription and forms a nucleoprotein complex with the plasmid partition site, *parS*

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## Summary

The partitioning locus (*par*) of plasmid pRA2 belongs to a recently discovered subgroup of plasmid partitioning systems that are evolutionarily distinct from the P1, F and R1/NR1 prototypes. The pRA2 *par* region was effective in stabilizing both pRA2 and F mini-replicons. Analysis of the nucleotide sequence revealed three potential coding regions that were designated *parA*, *parB* and *parC*. Through mutagenesis, *parA* and *parB* were found to be essential for partitioning function, whereas *parC* did not appear to be required. Using transcriptional reporter systems, it was demonstrated *in vivo* that ParB repressed *par* promoter activity by 60-fold and that ParA had little effect on transcriptional activity. Primer extension analysis revealed that the *par* transcriptional start point was located 47 nucleotides upstream of the *parA* translational start codon. Based on this information, putative –10 and –35 transcriptional signals were identified, and their subsequent deletion resulted in a dramatic reduction in promoter activity. The *par* promoter region was also demonstrated to exert incompatibility towards a plasmid with an active pRA2 *par* system. Nested deletions in this region allowed the incompatibility determinant, designated *parS*, to be localized. Recombinant ParA and ParB proteins were overexpressed and purified by affinity chromatography. Through *in vitro* binding experiments, purified ParB was shown to interact specifically with the *par* promoter region. DNase I footprinting revealed that ParB not only binds to the conserved sequence 5'-TCA AA(T/C) (G/C)CT CAA (A/T)A, which is present in three copies in the *par* promoter region, but also binds to the pRA2

partitioning site, *parS*. It appears that ParB has a dual role in pRA2 partitioning, being responsible for both the regulation of *par* transcription and the formation of a partition nucleoprotein complex at *parS*.

## Introduction

Bacterial plasmids depend on stabilization systems in order to maintain their presence within a growing bacterial population. Enhanced plasmid stabilization can be achieved through the mechanisms of plasmid multimer resolution, active partitioning and post-segregational killing (Nordström and Austin, 1989; Helinski *et al.*, 1996; Gerdes *et al.*, 2000a). Low-copy-number plasmids usually possess a partitioning system that is capable of actively segregating plasmid units during the process of cell division (Hiraga, 1992; Williams and Thomas, 1992)

Typical plasmid partitioning cassettes are composed of two *trans*-acting proteins and a *cis*-acting DNA sequence, termed the plasmid partition site. In all cases examined so far, the two partitioning genes are organized as an operon, being tandemly transcribed from a common transcriptional promoter. The first gene to be transcribed gives rise to a protein belonging to a superfamily of evolutionarily related proteins involved in plasmid and chromosome segregation. Members of the ParA superfamily are characterized by the presence of a conserved ATP-binding motif (Motallebi-Veshareh *et al.*, 1990; Bork *et al.*, 1992; Koonin, 1993), the activity of which is essential for plasmid partitioning (Davey and Funnell, 1994; 1997; Davis *et al.*, 1996; Jensen and Gerdes, 1997). The proteins of the second gene bind directly to the plasmid partitioning sites (Davis and Austin, 1988; Funnell, 1988; Mori *et al.*, 1989), which are normally composed of direct or inverted iterated sequences. Partition sites can be found downstream of the *par* coding regions, as in the plasmids F and P1 (Davis and Austin, 1988; Funnell, 1988; Mori *et al.*, 1989), or can be associated with the partition promoter region, which is the case for plasmids R1/R100 (Dam and Gerdes, 1994) and pTAR (Gallie and Kado, 1987). In the case of plasmid RK2/RP4 and linear plasmid N15, multiple partitioning sites are dispersed throughout the plasmid genome and

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can be found at a considerable distance from the partitioning operon (Williams *et al.*, 1998; Ravin and Lane, 1999). Different plasmids containing the same partitioning site exert incompatibility towards one another. During the process of plasmid partitioning, plasmids containing identical partitioning sites cannot be differentiated and, therefore, the stabilizing effect normally exerted by the partitioning system is abolished. This ultimately gives rise to cells containing one or the other plasmid, but not both (Austin and Nordström, 1990).

Partitioning systems can be divided broadly into two types depending on whether the ATP-binding domain of the upstream gene is a Walker-type ATPase motif (type I) as in plasmids P1 and F or belongs to the actin/hsp70 superfamily of ATPases (type II) as in plasmid R1/R100 (Gerdes *et al.*, 2000b). The type I partition systems can be further divided into two subgroups: type Ia and type Ib. In comparison with type Ia systems, the type Ib ATPases lack the N-terminal DNA-binding domain that appears to be required for autoregulation (Davis *et al.*, 1992; Hayes *et al.*, 1994; Radnedge *et al.*, 1998). The downstream genes of type Ia and type Ib systems are evolutionarily unrelated (Gerdes *et al.*, 2000b; Hayes, 2000). Those from type Ia have a bifunctional role in the formation of the partitioning nucleoprotein complex at the plasmid partition site and also as an enhancer of *par* repression (Friedman and Austin, 1988; Mori *et al.*, 1989). The role of the downstream gene in type Ib systems has not been characterized extensively; however, ParB from plasmid pTAR has been shown to be involved in transcriptional repression by binding to the *par* promoter region, and this repression was found to be modestly augmented by ParA (Kalnin *et al.*, 2000).

Plasmid pRA2 is the endogenous plasmid from the metabolically versatile environmental isolate *Pseudomonas alcaligenes* NCIB 9867 (Hopper and Chapman, 1971). Analysis of the complete pRA2 nucleotide sequence revealed the presence of a *parA* homologue and two closely linked open reading frames (ORFs), *parB* and *parC* (Kwong *et al.*, 2000). In this study, we characterize the components of the pRA2 partitioning system and demonstrate that ParB has two roles – as a transcriptional repressor of the *par* operon as well as in the formation of a nucleoprotein complex at the plasmid partitioning site, *parS*.

## Results

### *The pRA2 partitioning region increases the stability of homologous and heterologous replicons*

The partitioning (*par*) region of plasmid pRA2 was located between the *Pac25I* restriction–modification (R–M) system and the transposable element, Tn5563 (Fig. 1A).

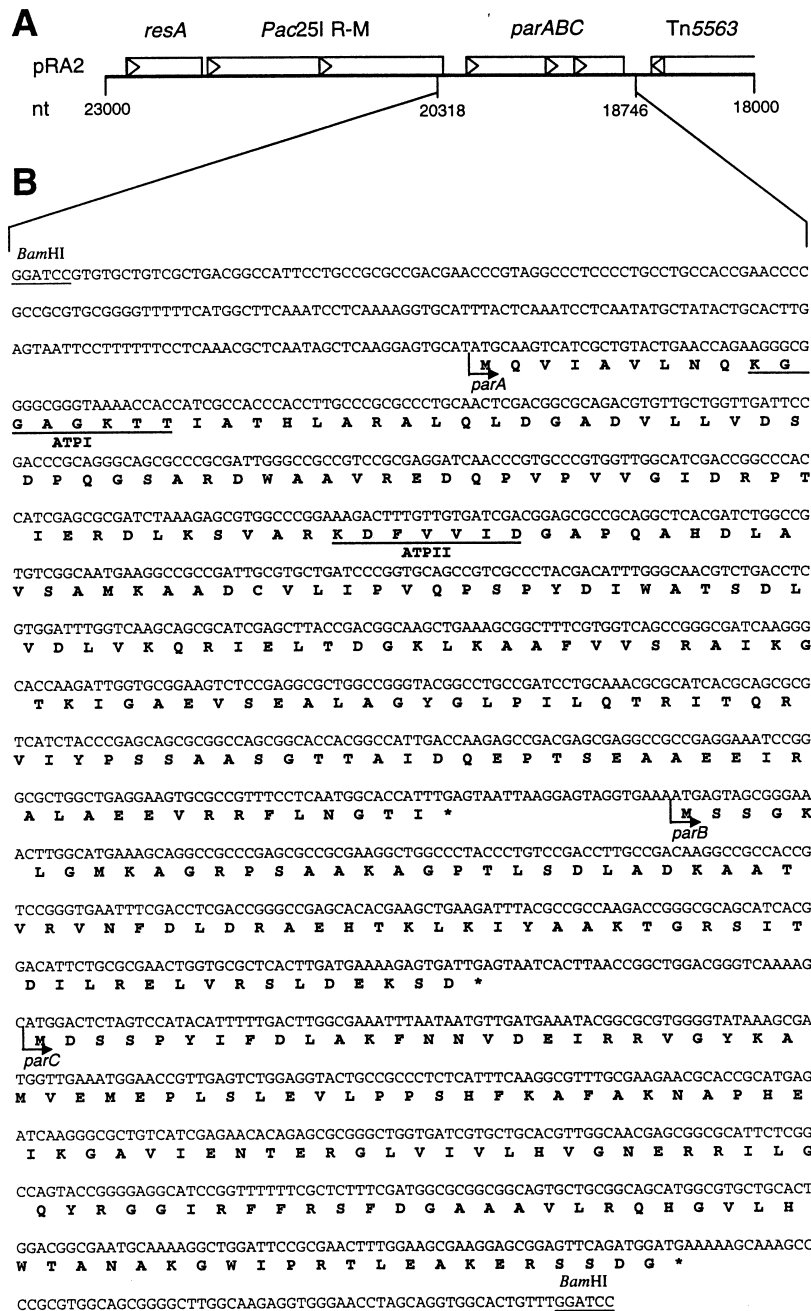
Owing to the lack of restriction sites in this region, pRA2 *par* was isolated as a 1.6 kb *Bam*HI fragment (Fig. 1B) by polymerase chain reaction (PCR). The pRA2 shuttle vector, pVK182 (Kwong *et al.*, 1998), contains the pRA2 mini-replicon cloned into pUC18, and it was found to replicate at two or three copies per chromosome in *Pseudomonas* (unpublished results). pVK182 was found to be segregationally unstable, with plasmid retention in *Pseudomonas putida* KT2440 determined to be 22% after 100 generations of non-selective growth (Fig. 2A). The pRA2 *par* region was subsequently cloned into pVK182, yielding plasmid pVK200. The proportion of cells retaining pVK200 was 87% after 100 generations of non-selective growth in *Pseudomonas putida* KT2440 (Fig. 2A). Plasmid isolations of pVK200 suggested that there was no significant increase in pVK200 copy number.

As pVK182 and pVK200 could also replicate in *Escherichia coli* DH5 $\alpha$  using the pUC replicon, and thus at high copy number, the segregational stability of the plasmids was also examined in this host. Under non-selective growth conditions, plasmid pVK182 was present in only 12% of cells after 100 generations, yet pVK200 was present in 98% of cells after the same non-selective growth conditions (Fig. 2B). Partitioning cassettes may be more advantageous to the survival of low-copy-number plasmids, as they appear to occur naturally on low-copy-number plasmids and not on high-copy plasmids. However, it is evident from our experiments that low plasmid copy number is not essential for the stabilization effect exerted by pRA2 *par*, as it effectively stabilized both low-copy- and high-copy-number replicons.

In order to establish that the isolated pRA2 *par* cassette was complete and that no other factors encoded by the pRA2 replicon were involved, the pRA2 *par* region was cloned into an unrelated replicon, FK14 (plasmid F mini-replicon), yielding FK143. The stability of the FK14 and FK143 plasmids was also examined in *E. coli* DH5 $\alpha$ . The retention of FK14 was 12%, whereas the retention of FK143 was 89% after 100 generations of non-selective growth (Fig. 2C). Again, no apparent increase in plasmid copy number was observed. These results suggested that the cloned 1.6 kb pRA2 *par* region was complete and functional in both *Pseudomonas* and *E. coli* hosts.

### *Mutational analysis of the par coding regions*

Single base deletions or insertions were sought for mutagenesis of the *par* coding regions. In addition to these frameshifts, primers were designed to introduce a premature stop codon (TGA) into each of the ORFs (the same stop codon used by all three *par* ORFs). The *parA* mutation involved a single nucleotide insertion, converting Trp-47 (TGG) of ParA to TGA. The *parB* and *parC* mutations both involved single nucleotide deletions,



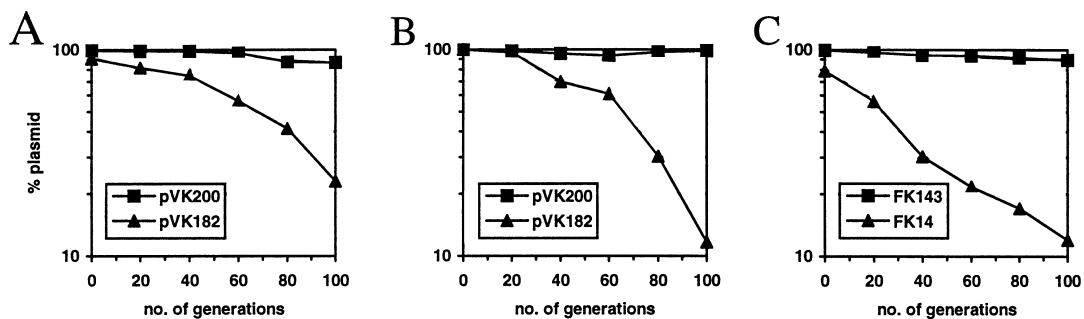
**Fig. 1.** A. Genetic map of plasmid *pRA2* from co-ordinates 23 000 to 18 000, displaying the location of the *par* region, *parABC*, downstream of the *Pac25I* R–M system and adjacent to the mobile element *Tn5563*. B. Nucleotide sequence of the cloned 1.6 kb *pRA2 par* region and the predicted amino acid sequences of the coding regions, *ParA*, *ParB* and *ParC*. The conserved ATP-binding motifs (ATPI and ATPII) that are characteristic of the *ParA* superfamily are underlined.

converting Met-8 (ATG) of *ParB* and Leu-31 (TTG) of *ParC* to TGA. Mutagenesis was performed in the *pALTER* vector (Promega), and the entire *par* fragment was sequenced before cloning into the *pVK182* test plasmid. When the mutant plasmids were analysed for stability in *Pseudomonas* and *E. coli* hosts, it was found that plasmids with the *parA*<sup>-</sup> genotype (*pVK200A*) had lost the ability to stabilize *pVK182* but, in contrast, *parC*<sup>-</sup> mutations (*pVK200C*) resulted in no loss of plasmid stability (Table 1). We were unable to obtain clones containing the *parB* mutation. Restriction analysis of

plasmids isolated from *parB*<sup>-</sup> transformants showed that intramolecular deletions were occurring constantly. Only when the *parB* mutation was introduced into a plasmid already containing the *parA* mutation was the *parB*<sup>-</sup> genotype non-deleterious. Plasmids containing both the *parA* and the *parB* mutations (*pVK200AB*) were segregationally unstable (Table 1).

#### Identification of the *par* transcriptional start site

Primer extension analysis was performed to locate the



**Fig. 2.** Stabilization of homologous and heterologous replicons by pRA2 *par*. Cultures were grown in LB for 100 generations in the absence of plasmid selection and then analysed for plasmid retention. Plasmids pVK182 and FK14 are unstable mini-replicons derived from plasmids pRA2 and F respectively. When the pRA2 *par* region was cloned into these plasmids, they became pVK200 and FK143 respectively. pRA2 *par* was capable of stabilizing pVK182 in *P. putida* KT2440 (A) and *E. coli* DH5 $\alpha$  (B) as well as plasmid FK14 in *E. coli* DH5 $\alpha$  (C). The data presented are the average of at least three independent experiments.

primary transcriptional start point of the *par* transcript. Total RNA was isolated from *P. alcaligenes* NCIB 9867 and *E. coli* harbouring the plasmid pPAR64, which contains the *par* promoter region and the N-terminal 32 amino acid residues of ParA fused to the *lacZ* reporter gene. A single primer extension product was obtained corresponding to a transcriptional start point 47 nucleotides upstream of the *parA* start codon at the guanidine residue on the coding strand (Fig. 3). No primer extension product could be detected using the RNA isolated from *P. alcaligenes* NCIB 9867. In its natural context, pRA2 *par* is contained on a low-copy-number plasmid, and the *par* transcript may also be tightly regulated. Both these factors would have reduced the sensitivity in detecting the primer extension product from *P. alcaligenes* RNA.

#### Localization of the *par* promoter

A search for transcriptional promoter sequences upstream of the *par* transcript revealed the presence of  $-10$  and  $-35$  sequences that matched the consensus sequence of the *E. coli*  $\sigma^{70}$  transcriptional promoter. Analysis of the transcriptional activity from the putative *par* promoter region was performed by fusing the region to the promoterless *lacZ* gene contained in plasmid pQF52 (McLean *et al.*, 1997), resulting in plasmid pPAR64 (Fig. 4). pPAR64 exhibited levels of  $\beta$ -galactosidase of  $\approx 1190$  Miller units, whereas pQF52 without the *par* promoter consistently produced  $< 5$  Miller units, indicating that this DNA fragment did contain a transcriptional promoter, which was designated  $P_{par}$ . The promoter region was subjected to partial deletion using exonuclease III, followed by S1 nuclease treatment and religation. A set of nested deletions from the 5' region was obtained (Fig. 4) and analysed for levels of  $\beta$ -galactosidase. The most noticeable difference in promoter activity was observed for deletion clones SX41 and SX81, in which transcriptional activity dropped sharply from a

relative activity of 76% to 0% in the latter clone (Fig. 4). Clone SX81 had an additional 28 bp deleted from the 5' end, and this deletion removed the predicted  $-10$  and  $-35$  promoter sequences that were present in clone SX41.

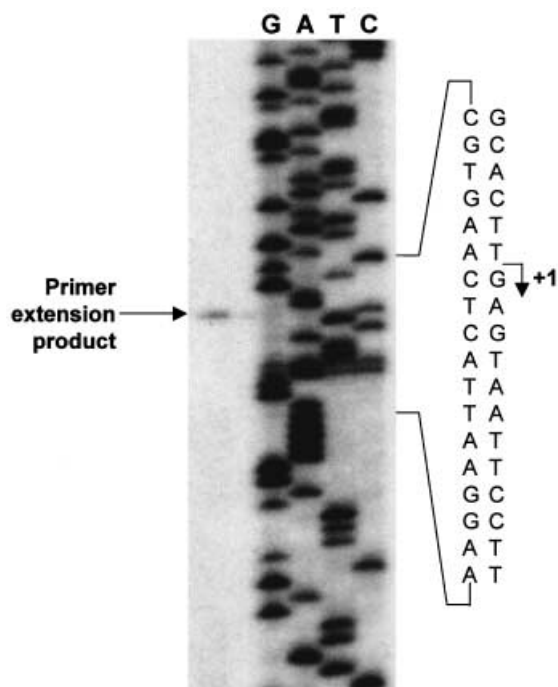
#### Identification of the putative centromeric site, *parS*

The set of nested deletions that was generated in the *par* promoter region was also useful in the identification of the *cis*-acting site required for pRA2 partitioning. During the process of partitioning, the Par proteins usually recognize and form a nucleoprotein complex at a specific plasmid site that is required *in cis*. If the same *cis* site is present on two co-resident plasmids, they will then show incompatibility, as both plasmids will be substrates for the same partitioning proteins (Austin and Nordström, 1990). Each of the nested deletion clones in plasmid pQF52 ( $Ap^R$ ) (Fig. 4) were co-transformed with plasmid FK143 ( $Km^R$ ) into *E. coli* DH5 $\alpha$ . Transformants that were both  $Ap^R$  and  $Km^R$  were grown in the presence of ampicillin for 25 generations and then tested for plasmid FK143 retention by the ability to grow on  $Km$ . As expected, FK143 (F replicon) was completely compatible with the pQF52 vector (ColE1 replicon) and expressed the normally high

**Table 1.** Mutational analysis of the pRA2 *par* open reading frames.

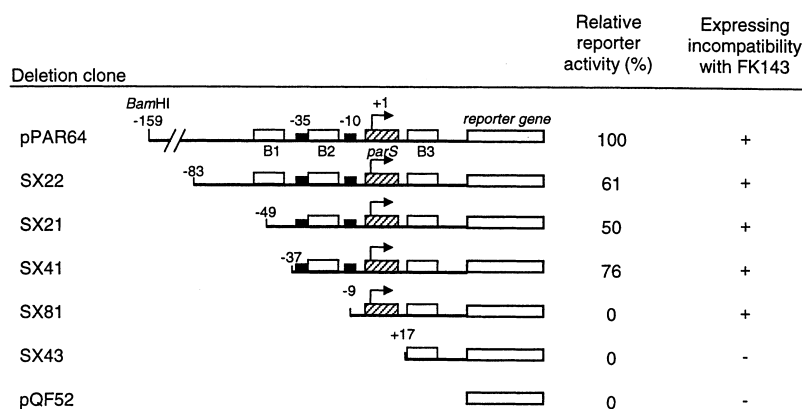
Plasmid	<i>par</i> genotype	% plasmid after 100 generations
pVK200	A <sup>+</sup> B <sup>+</sup> C <sup>+</sup>	87
pVK200A	A <sup>-</sup> B <sup>+</sup> C <sup>+</sup>	24
pVK200C	A <sup>+</sup> B <sup>+</sup> C <sup>-</sup>	91
pVK200AB	A <sup>-</sup> B <sup>-</sup> C <sup>+</sup>	21
pVK182	A <sup>-</sup> B <sup>-</sup> C <sup>-</sup>	22

The potential *par* coding regions, *parA*, *parB* and *parC*, were disrupted by introducing ochre stop codons through site-directed mutagenesis. *par* fragments containing the mutations were cloned into pVK182, and the segregational stability of the resulting plasmids was determined in *P. putida* KT2440.



**Fig. 3.** Primer extension analysis of the *par* transcript. Total RNA was extracted from *E. coli* bearing the plasmid pPAR64. The labelled primer PEX2 (5'-GTG GTT TTA CCC GCC CCG C) was used in the reverse transcription reaction and for the DNA sequencing ladder. Only one reverse transcription product was obtained, and it corresponded in size to the guanine (G) residue marked +1 on the coding strand.

levels of plasmid stability seen when it was the sole plasmid present. In contrast, FK143 was unstable in the presence of pPAR64, which contained the *par* promoter region, thus implying that the *par* promoter region also contained an incompatibility determinant. Incompatibility analysis of the deleted derivatives revealed that clone SX81, which was deleted up to position -9 (Fig. 4), still showed incompatibility with FK143, whereas clone SX43, deleted to position +17 (Fig. 4), was completely



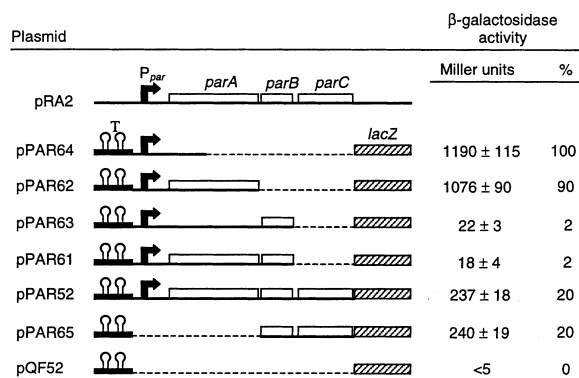
**Fig. 4.** Schematic diagram and selected properties of deletion clones obtained in the *par* promoter region. The pPAR64 parental plasmid was constructed by cloning the *par* promoter region from positions -159 to +143 into plasmid pQF52. pPAR64 therefore contains the first 32 codons of *parA* transcriptionally fused to the promoterless *lacZ*. Deletions from the 5' end were generated by exonuclease III digestion. The deletion end-points of clones are shown with respect to the +1 position of the *par* transcript (depicted by an arrow). The putative -10 and -35 transcriptional signals are shown as a dark box, and ParB binding sites B1, B2, B3 and *parS* are shown as open boxes and a hatched box respectively. The deletion clones were analysed for  $\beta$ -galactosidase activity and for the expression of incompatibility towards plasmid FK143.

compatible with FK143. Clones SX81 and SX43 differed by only 25 bp, and it appeared that this 25 bp region contained important sequences necessary for the formation of a plasmid partitioning complex. The region containing the putative *pRA2* partitioning site was designated *parS*.

#### *ParB* represses transcriptional activity of $P_{par}$

To determine the effects of the *par* gene products on the upstream transcriptional promoter, clones were constructed with combinations of the *par* genes located between  $P_{par}$  and the promoterless *lacZ* (Fig. 5). When only *parA* was present (pPAR62), the level of transcription from  $P_{par}$  was not significantly different from that from the construct that contained the *par* promoter alone (pPAR64). However, when *parB* was present (pPAR63), transcriptional levels were reduced from 1190 Miller units to an average of 22 Miller units of  $\beta$ -galactosidase, implying that ParB was involved in *par* transcriptional repression. When both *parA* and *parB* were present (pPAR61), similar levels of transcription to those seen with *parB* alone were observed, indicating that ParA did not significantly effect *par* transcriptional regulation. Interestingly, when the region downstream of *parB* was also included (pPAR52), the clone produced levels of  $\beta$ -galactosidase of about 240 Miller units, and this activity remained even in the absence of the *par* promoter (pPAR65). These observations suggested that there was another region, downstream of *parB* and within *parC*, that possessed transcriptional promoter activity.

To illustrate further the repressor activity of ParB, an additional transcriptional reporter system was constructed, in which  $P_{par}$  was fused to the *xyIE* reporter gene (pRK230). The *xyIE* fusion system could be assayed in *E. coli* strain BL21(DE3)pLysS: pET-30a/*parB*, in which ParB expression can be controlled by IPTG



**Fig. 5.** Schematic diagram of the pRA2 *par-lacZ* transcriptional fusion constructs in pQF52. The wild-type organization of the *par* genes, as they occur on plasmid pRA2, are shown at the top. DNA fragments containing genetic combinations of the *par* region were cloned upstream of the promoterless *lacZ* (hatched box) and downstream of the two transcriptional terminators (T) present in pQF52. The *par* promoter is represented by a black arrow, whereas the *par* genes that are complete and functional are depicted as white boxes. The levels of β-galactosidase activity presented are the average results of at least four independent experiments. The relative levels of β-galactosidase activity (%) are also shown.

levels, and circumvents the inability to assay β-galactosidase in this strain which is *lac*<sup>+</sup>. The *xyIE* reporter construct uses the vector pRK415, which is an IncP replicon and is therefore compatible with the pET-30a expression vector (ColE1) and pLysS (pACYC). It was found that growth in the presence of increasing amounts of IPTG (0–50 mM), which induces ParB expression, repressed *xyIE* transcription (catechol-2,3-dioxygenase activity) and therefore repressed P<sub>par</sub> activity. The level of catechol-2,3-dioxygenase activity was dependent upon the amount of IPTG added to cultures (Table 2). We also observed that, in cultures in which ParB was not induced, the *par* promoter was repressed fivefold in comparison with strains without pET-30a/ParB, showing that the *par* promoter is sensitive to the basal levels of ParB produced in the absence of IPTG induction.

**Table 2.** The effect of ParB levels on *par* promoter activity.

Plasmids	Concentration of IPTG in growth media (μM)	Catechol-2,3-dioxygenase	
		Specific activity (nmol min <sup>-1</sup> mg <sup>-1</sup> protein)	Relative activity (%)
pRK230	0	94 000 ± 6700	100
pRK230 + pET-30a/ParB	0	16 900 ± 1200	18.0
	10	5890 ± 170	6.3
	20	3070 ± 120	3.3
	30	1970 ± 80	2.1
	40	1790 ± 70	1.9
	50	1820 ± 60	1.9

Plasmid pRK230 contains the *par* promoter transcriptionally fused to the *xyIE* (catechol-2,3-dioxygenase) reporter gene. Plasmid pET-30a/ParB contains *parB* under the control of an IPTG-induced T7 promoter. *E. coli* BL21(DE3)pLysS (Cm<sup>R</sup>) was transformed with pRK230 (Tc<sup>R</sup>) and pET-30a/ParB (Km<sup>R</sup>) and grown in the presence of increasing concentrations of IPTG. Cultures were harvested, and cell extracts were analysed for catechol-2,3-dioxygenase activity. Experiments were performed in triplicate and the standard deviation is shown.

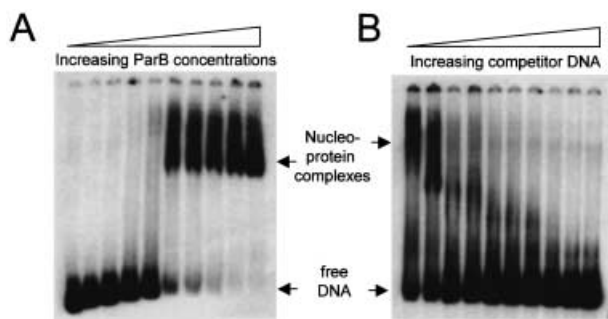
### Expression and purification of polypeptides

The potential *par* coding regions, *parA*, *parB* and *parC*, were cloned under the control of the IPTG-induced T7 promoter. The expression clones were expected to produce recombinant gene products that were translationally fused to an N-terminal region containing six consecutive histidine residues for subsequent purification. For optimal expression of the soluble forms of ParA and ParB fusion proteins, the cultures were best grown at 32°C in LB until mid-log phase, at which time they were induced with IPTG at a final concentration of 1 mM. Cells were harvested after 3 h of induction. The ParC fusion protein could not be detected by SDS-PAGE despite various growth and induction conditions.

The ParA and ParB fusion proteins were purified by affinity chromatography on Ni-NTA agarose columns using an imidazole gradient in phosphate buffer. SDS-PAGE analysis of the collected fractions indicated that the majority of the tagged proteins did not elute until a concentration of 0.5 M imidazole was reached for both ParA and ParB. The salt and imidazole were subsequently separated from the purified proteins by gel filtration.

### ParB binds specifically to the *par* promoter region

As the overexpression of ParB dramatically reduced transcription from P<sub>par</sub>, ParB was therefore responsible for the autoregulation of *parAB*. Electrophoretic mobility shift assays (EMSAs) were performed by incubating purified ParB fusion protein with a γ-<sup>32</sup>P-labelled, 271 bp DNA fragment that contained P<sub>par</sub>. Binding reactions were performed in the presence of 2 μg of polydeoxyinosinic–deoxycytidylic acid [poly-(dI–dC)] non-specific competitor DNA. The majority of the labelled DNA was retarded in 4% polyacrylamide gels when 500 ng of ParB was included in the binding reaction (Fig. 6A). By



**Fig. 6.** Electrophoretic mobility shift assays demonstrating specific binding of ParB fusion protein with the *par* promoter *in vitro*. A. Increasing levels of ParB (from left to right: 0, 30, 60, 125, 250, 500, 1000, 2000, 3000 and 6000 ng) were incubated with a labelled 271 bp DNA fragment containing the *par* promoter in a total volume of 20  $\mu$ l. B. Increasing ratios of unlabelled to labelled *par* promoter DNA (from left to right: no competitor, 1:1, 2:1, 4:1, 8:1, 12:1, 16:1, 24:1, 32:1, 50:1) were incubated with a fixed amount of ParB (500 ng) in a total volume of 20  $\mu$ l. Unbound DNA fragments were separated from nucleoprotein complexes in 4.0% polyacrylamide gels.

adding increasing amounts of unlabelled specific competitor DNA, i.e. the same 271 bp  $P_{par}$ -containing fragment that had not been labelled with  $\gamma$ - $^{32}$ P, less labelled DNA was retarded (Fig. 6B), thus demonstrating that the ParB binding was specific. In contrast, incubation of labelled  $P_{par}$  with purified ParA, with or without ATP (1 mM), did not have any influence on the mobility of the labelled DNA (data not shown).

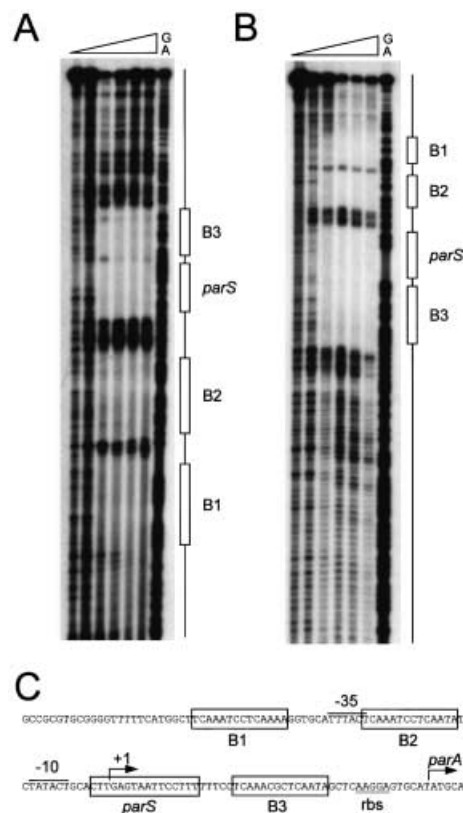
#### *ParB* interacts with *parS* and three repeats found in the *par* promoter region

The specific DNA sequences in the *par* promoter region with which ParB interacts were identified by DNase I footprinting. After labelled  $P_{par}$  DNA had been incubated with ParB, several regions of the non-coding strand were observed to be protected from DNase I nuclease activity (Fig. 7A). The same regions were protected from DNase I cleavage on the coding strand (Fig. 7B). Three of the protected regions corresponded to three directly repeated sequences found in the promoter region (Fig. 7C). The three direct repeats, designated B1, B2 and B3, had the consensus sequence 5'-TCA AA(T/C) (G/C)CT CAA (A/T)A and started at positions -54, -29 and +19 with respect to the *par* transcriptional start point. The DNase I-protected regions extended for several nucleotides beyond the conserved sequences, and this may have been caused by steric hindrance as a result of ParB binding. In addition to the three directly repeated sequences, a fourth sequence, located within *parS*, was also found to be protected from DNase I cleavage by ParB. The protected sequence within *parS*, 5'-CTT GAG TAA TTC CTTT (Fig. 7C), did not show any sequence similarity to the other three ParB binding sites. When the

concentration of ParB in the binding reaction was limiting, each of the four binding sites was partially protected (Fig. 7A and B), indicating that ParB does not have a preference or higher affinity for one of the binding sites. The ParB binding sites are in positions that would be very likely to inhibit accessibility of RNA polymerases to  $P_{par}$ . DNase I protection experiments were also performed with ParB (20  $\mu$ g) and/or ParA (equimolar) and/or ATP (1 mM). Under these conditions, no detectable differences were observed in the DNase I-protected regions (data not shown).

## Discussion

The *par* locus isolated from the *P. alcaligenes* NCIB 9867 *pRA2* plasmid was capable of stabilizing a mini-*pRA2* replicon in *P. putida* KT2440 as well as a mini-F replicon in *E. coli* DH5 $\alpha$ . The well-studied partitioning loci from



**Fig. 7.** DNase I footprinting analysis of the ParB-*par* promoter interaction. The interaction of ParB was examined on the non-coding strand (A) and coding strand (B) of the same DNA fragment.  $\alpha$ - $^{32}$ P-labelled *par* promoter DNA was incubated with increasing concentrations of purified ParB protein (from left to right: 0, 1, 2, 5, 10 and 20  $\mu$ g), and binding reactions were then subjected to partial DNase I digestion. The nicked fragments were separated on a denaturing sequencing gel together with the G+A sequencing ladder of the same labelled DNA fragment. Four individual ParB binding sites were identified in the *par* promoter region and designated B1, B2, B3 and *parS*.

plasmids P1, F and R1/NR1 are found on plasmids replicating in *E. coli*. Partitioning loci have also been characterized from plasmids that were isolated from other bacteria, such as pTAR from *Agrobacterium tumefaciens* (Gallie and Kado, 1987), plasmid TP228 from *Salmonella newport* (Hayes, 2000) and the broad-host-range plasmid RK2/RP4 that was initially isolated from *Pseudomonas aeruginosa* (Motallebi-Veshareh *et al.*, 1990). The partitioning system from pTAR is capable of stabilizing plasmids in *Agrobacterium* and several species of Rhizobiaceae (Gallie *et al.*, 1985; Gallie and Kado, 1987), but does not appear to be functional in *E. coli* (Kalnin *et al.*, 2000). The partitioning loci from TP228 and RK2/RP4 have been shown to promote plasmid stability in *E. coli* (Motallebi-Veshareh *et al.*, 1990; Williams *et al.*, 1998; Hayes, 2000), but this effect has not been verified in other species. As the pRA2 *par* locus has been demonstrated to be functional in both *P. putida* and *E. coli*, it may prove useful in helping to elucidate matters concerning plasmid partition host range. Some factors that may limit partition host range could include the requirement for host-specific cofactors, such as integration host factor (IHF), or the presence of specific host cell structures that interact directly with the partitioning complex, or it could simply be a matter of host promoter recognition that may lead to the prevention of *par* gene expression.

The pRA2 *par* locus contains two genes, *parA* and *parB*, which are both essential for full plasmid partitioning function. Using transcriptional reporter genes, ParB was shown to repress transcription of *par*. Plasmids containing the *par* locus with a mutation in *parA* were not stabilized, indicating that ParA is essential. However, we were unable to clone the *parB* mutation into the test plasmid pVK182 when the *parA* wild-type allele was present. As ParB is required for *par* repression, disruption of *parB* would have led to overexpression of ParA, and the high level of expression seemed to inhibit cell growth. The *parA*<sup>+</sup>*parB*<sup>-</sup> genotype could be obtained when the 5' region of the *par* transcript was fused to the *lacZ* gene in pQF52, resulting in plasmid pPAR62. However, it was observed that *E. coli* cells transformed with pPAR62 grew much more slowly on plates and in broth compared with cells containing plasmids with a *parA*<sup>+</sup>*parB*<sup>+</sup> genotype.

Through the course of our experiments, we obtained evidence that seems to suggest that *parC* is not a coding region. First, we established that *parC* is not required for plasmid stability and that recombinant ParC could not be overexpressed. But the most telling observation was found by cloning combinations of the *par* genes into the reporter plasmid, pQF52. From these experiments, we identified a promoter, designated P<sub>par</sub>, upstream from the *parA* coding region. However, these experiments also suggest the presence of a weaker promoter downstream of *parB* and within the predicted *parC* coding region. No

ORF that could be influenced by this weaker promoter was detected in the region immediately downstream. It should be noted, however, that Tn5563 is located in a position adjacent to *parC* (Fig. 1), and its past transposition into pRA2 may have led to genetic rearrangements at the site of insertion, leaving no discernible trace of any ORFs that could have been transcribed from the promoter within *parC*. In addition, we located a putative *rho*-independent terminator, characterized by a stem-loop secondary structure preceding a poly T sequence, 33 nucleotides downstream of *parB* and overlapping the predicted *parC* start codon. Therefore, the location of transcriptional signals in the *par* region suggests that the pRA2 partitioning system is composed of only two genes, *parA* and *parB*, and as they are transcribed from the same promoter, P<sub>par</sub>, they would appear to constitute an operon.

pRA2 ParA is predicted to contain a Walker-type ATP-binding motif (Koonin, 1993) and lack a helix-turn-helix DNA-binding domain at its N-terminus that is associated with ParA/SopA repressor activity in plasmids P1 and F (Davis *et al.*, 1992; Hayes *et al.*, 1994; Radnedge *et al.*, 1998). The pRA2 ParB is evolutionarily unrelated to the ParB/SopB proteins of P1 and F, and the plasmid partitioning site, *parS*, is located upstream of the two coding regions. Based on these characteristics, pRA2 *par* has been classified as a type Ib partitioning system (Gerdes *et al.*, 2000b). The pRA2 *parA* and *parB* gene products are most closely related to the ParF (31% identity) and ParG (25% identity) proteins of plasmid TP228 (Hayes, 2000) and to the Par proteins of plasmid pTAR (Gallie and Kado, 1987; Kalnin *et al.*, 2000), with 28% and 15% identity between ParA and ParB homologues respectively.

Autoregulation of type Ia *par* operons is achieved by ParA/SopA interaction with the promoter region, and the ParB/SopB proteins act as repressor cofactors (Friedman and Austin, 1988; Mori *et al.*, 1989). In contrast, autoregulation of the type Ib systems, of which plasmid pTAR and pRA2 are representatives, appears to be achieved solely through ParB binding to the promoter, and ParA has little if any effect on the levels of transcription (Kalnin *et al.*, 2000). This type of autoregulation is similar to the type II partitioning system of R1/NR1. In R1/NR1, the product of the downstream gene, *parR*, is responsible for autoregulation, whereas the upstream gene, *parM*, is not involved (Jensen *et al.*, 1994).

Another feature that distinguishes the type Ia and type Ib partitioning systems is the location of the plasmid partitioning site. The pRA2 *cis*-acting partition element, *parS*, was located using incompatibility studies. It was determined that the 25 bp region, found between positions -9 and +17 relative to the transcriptional start site, was important for the expression of *par*-mediated incompatibility, suggesting that this region contained the

sequences that are necessary for a functional pRA2 partitioning site. In type Ia systems, the partition sites *parS/sopC* are located downstream of the coding regions (Mori *et al.*, 1986; Davis and Austin, 1988; Funnell, 1988), whereas in plasmids R1/NR1 (type II) and pTAR (type Ib), the partition site is associated with the promoter region (Gallie and Kado, 1987; Dam and Gerdes, 1994). The partition sites for plasmids R1/NR1 and pTAR are highly repeated sequences containing 10 11 bp and 11 7 bp repeats respectively. Deletion analysis of the R1/NR1 partition site revealed that all 10 repeats are required to exert full incompatibility (Breüner *et al.*, 1996). The *sopC* site of F is composed of 12 43 bp iterons, and all 12 appear to be essential for maximal centromere efficiency (Mori *et al.*, 1986). In contrast, the pRA2 partitioning site is flanked by three 14 bp iterated sequences, and deletion of two of these repeats (B1 and B2) did not abolish incompatibility, implying that these two repeats are not required for the formation of a functional partitioning complex. Nevertheless, it cannot be ruled out that B1 and B2 may be required for full centromere-like activity.

Several regions of the *par* promoter region were protected from DNase I digestion in the presence of purified ParB protein. Three of the protected regions, which were designated B1, B2 and B3, correspond to the consensus sequence 5'-TCA AA(T/C) (C/G)C TCA A(A/T)A that was found to be directly repeated three times within the *par* promoter region (Fig. 7C). Another ParB binding site with a completely different recognition sequence was identified and located within the 25 bp incompatibility determinant, thus corroborating the evidence concerning the location of the pRA2 partitioning site, *parS*. Although deletion of the ParB binding sites B1 and B2 still allowed expression of partition-mediated incompatibility, the close proximity of B1, B2 and B3 to the putative plasmid partition site cannot rule out their possible involvement. The binding sites B1, B2 and B3 are likely to act as operator sites and, from their positions in the promoter region (Fig. 7C), it is probable that saturation of these sites would effectively inhibit the interaction of cellular transcriptional components with the -10 and -35 promoter sequences and thus lead to transcriptional repression.

ParB binding to *parS* occurs even in the absence of ParA, and it is therefore likely to be one of the initial events in the formation of a partition complex in which ParA is also assumed to participate. Perhaps the ParB-*parS* complex is recognized by ParA in a similar fashion to P1 partitioning (Youngren and Austin, 1997; Bouet and Funnell, 1999; Erdmann *et al.*, 1999). No differences were observed in the ParB-protected DNase I footprint when ParA or both ParA and ATP were provided in addition to ParB. As in P1, ParA may not contact the *parS* region directly; however, we concede that the ParA protein used

in this study is recombinant and may not accurately reflect the activity of ParA in its natural context. In plasmid P1, IHF promotes binding of ParB to the plasmid partitioning site (Davis and Austin, 1988; Funnell, 1988). It may also be possible that there is a requirement for certain host factors that promote formation of a *parS*-ParB-ParA complex *in vivo*.

In addition to the *par* locus, plasmid pRA2 has several other loci that may contribute to overall plasmid stability. The *Pac25I* R-M system is located immediately upstream from the *par* promoter (Fig. 1). Type II R-M systems have been shown to reduce plasmid loss (Kulakauskas *et al.*, 1995; Kusano *et al.*, 1995; Naito *et al.*, 1995) through post-segregational killing of plasmid-free segregants. In some of our preliminary experiments, the *Pac25I* R-M system was found to promote plasmid stability and can produce extremely stable plasmids when cloned in combination with the pRA2 *par* system (unpublished results). pRA2 also possesses two genes encoding resolvases, namely *resA*, found immediately upstream of the R-M system, and *tnpR*, found within Tn5563. It has not been determined whether these two resolvases contribute to pRA2 stability; however, resolvases from the Tn3 family have been shown to contribute to plasmid stability by converting plasmid multimers into monomers (Swinfield *et al.*, 1991; Easter *et al.*, 1998). The presence of multiple stability systems could explain why the *par* region, by itself, does not stabilize the pRA2 mini-replicon to a level comparable with the complete pRA2 plasmid, and why interruption of the *Pac25I* endonuclease gene that was carried out previously did not significantly reduce pRA2 stability (Kwong *et al.*, 2000). Other plasmids are also known to encode multiple systems that work together to promote plasmid stability (Nordström and Austin, 1989; Williams and Thomas, 1992; Helinski *et al.*, 1996; Gerdes *et al.*, 2000a) and, clearly, such a situation is beneficial.

## Experimental procedures

### Bacterial strains and growth conditions

*Escherichia coli* DH5 $\alpha$  [F<sup>-</sup>  $\phi$ 80 *dlacZ* $\Delta$ M15  $\Delta$ (*lacZYA-argF*)U169 *deoR* *recA1* *endA1* *hsdR17*(*r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>*) *phoA* *supE44*  $\lambda$ <sup>-</sup> *thi1* *gyrA96* *relA1*; Woodcock *et al.*, 1989] was used as the plasmid host for the majority of cloning procedures in this study. Plasmid stability assays were conducted in *E. coli* DH5 $\alpha$  and *P. putida* KT2440, which is a restriction-deficient derivative of *P. putida* mt-2 (Bagdasarjan *et al.*, 1981). For site-directed mutagenesis, the *E. coli* hosts JM109 (*endA1* *recA1* *gyrA96* *thi* *hsdR17* (*r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>*) *relA1* *supE44*  $\Delta$ (*lac-proAB*) [F<sup>'</sup> *traD36* *proAB* *lacI<sup>f</sup>*  $\Delta$ M15]; Yanisch-Perron *et al.*, 1985) and ES1301 *mutS* (*lacZ53* *thyA36* *rha-5* *metB1* *deoC* IN(*rrnD-rrnE*) *mutS201::Tn5*; Promega) were used. Proteins were overexpressed in *E. coli* BL21(DE3)pLysS [F<sup>-</sup> *ompT* *hsdSB* (*r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>*) *dcm* *gal*  $\lambda$ (DE3) pLysS Cm<sup>R</sup>; Novagen]. The  $\beta$ -galactosidase and

catechol-2,3-dioxygenase assays were conducted in DH5 $\alpha$  and BL21(DE3)pLysS hosts respectively. *E. coli* and *P. putida* strains were incubated at 37°C and 32°C, respectively, unless stated otherwise. Cultures were grown in Luria–Bertani broth (LB) [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride] with aeration or on LB agar plates, which were prepared by adding Oxoid purified agar (1.5% w/v) to LB before sterilization. When necessary, antibiotics were supplemented at the following concentrations: ampicillin (Ap), 100  $\mu\text{g ml}^{-1}$ ; kanamycin (Km), 25  $\mu\text{g ml}^{-1}$ ; tetracycline (Tc), 15  $\mu\text{g ml}^{-1}$ ; chloramphenicol (Cm), 34  $\mu\text{g ml}^{-1}$ .

### DNA manipulation

Plasmid DNA was prepared, manipulated and transformed by standard methods (Sambrook *et al.*, 1989). Restriction enzymes and T4 ligase were purchased from New England Biolabs. DyNAzyme EXT DNA polymerase (Finnzymes) or Vent DNA polymerase (New England Biolabs) was used in PCR amplifications. The introduction of mutagenic primers was achieved using the Altered Sites II *in vitro* mutagenesis system (Promega) and the mutagenic primers FSMA (5'-CCC GCG ATT GAG GCC GCC GTC), FSMB (5'-GAA ACT TGG CAT GAA AGC AGG) and FSMC (5'-AAT GGA ACC GTT GAG TCT GGA). DNA sequencing was conducted using the ABI Taq Dye Deoxy cycle sequencing kit and an ABI 373 DNA sequencer (PE Applied Biosystems).

### Plasmids

Plasmid pRA2 was isolated from *P. alcaligenes* NCIB 9867 and purified through a caesium chloride–ethidium bromide density gradient (Sambrook *et al.*, 1989). Plasmid pVK182 is a relatively unstable pRA2/pUC-based shuttle vector capable of replication in *Pseudomonas* and *E. coli* (Kwong *et al.*, 1998). The pRA2 *par* region was isolated from pRA2 by PCR, using the primers parR (5'-GGA TCC GTG TGC TGT CGC TGA; *Bam*HI) and parF (5'-GGA TCC AAA CAG TGC CAC CTG; *Bam*HI). The 1.6 kb *par* region was then cloned into the *Bam*HI site of plasmid pVK182, resulting in plasmid pVK200. DNA sequencing established that no nucleotide misincorporations were present in the cloned *par* region.

Plasmid FK14 was constructed to assess the ability of pRA2 *par* to increase the segregational stability of a low-copy-number plasmid in *E. coli*. PCR amplification of JM109 genomic DNA using primers F1 (5'-GGA AGC TTA CAC ACG ATG CCT G; *Hind*III) and F2 (5'-GGA TCC GGT ACC TCA GTC ACT TAT TAT C; *Bam*HI) led to the isolation of the plasmid F replicon as a 1.5 kb fragment. The blunt-ended mini-F replicon was then ligated to a 2.2 kb Km<sup>R</sup> gene cassette from mini-Tn5 (de Lorenzo *et al.*, 1993) that had been isolated with *Hind*III and treated with Klenow fragment. The resulting plasmid, FK14 (3.7 kb), confers kanamycin resistance and replicates at low copy number in *E. coli* hosts. The pRA2 *par* region, which was isolated as a 1.6 kb fragment, was cloned into the *Bam*HI site of FK14, resulting in plasmid FK143.

For overexpression of *par* polypeptides, the predicted *par* coding regions were PCR amplified using primers parA-F

(5'-CAT GCC ATG GAT ATG CAA GTC ATC GCT; *Nco*I) and parA-R (5'-CCC AAG CTT CAA ATG GTG CCA TTG A; *Hind*III) for *parA*; parB-F (5'-CAT GCC ATG GCA ATG AGT AGC GGG A; *Nco*I) and parB-R (5'-CCC AAG CTT CAA TCA CTC TTT TC; *Hind*III) for *parB*; and parC-F (5'-CAT GCC ATG GAC TCT AGT CC; *Nco*I) and parC-R (5'-CCC AAG CTT CAT CCA TCT GAA CT; *Hind*III) for *parC*. PCR fragments containing each of the genes were cloned into the *Nco*I and *Hind*III sites of pET-30a (Novagen). The DNA sequence of each clone was confirmed before protein expression was carried out.

The *lacZ* transcriptional fusions were constructed in the vector pQF52 (McLean *et al.*, 1997). PCR amplification of *par* fragments that contained different combinations of the *par* genes was achieved using primers parR and parC-R, parR and parB-R, parR and parA-R, parR and ParAB1 (5'-GGA TCC GCT TTC AGC TTG CCG T; *Bam*HI), parB-F and parC-R. The PCR products were then cloned into pQF52, resulting in plasmids pPAR52, pPAR61, pPAR62, pPAR64 and pPAR65 respectively. pPAR63 was generated in the same fashion as pPAR61, except that the template DNA used contained the *parA* ochre mutation and not the wild-type allele. For the generation of nested deletion clones, SX22, SX21, SX41, SX81 and SX43, pPAR64 was digested with *Sph*I and *Xho*I before partial exonuclease III digestion followed by treatment with S1 nuclease and religation. The extent of deletion was determined by DNA sequencing.

Plasmid pRKC230 has the pRA2 *par* promoter driving expression of the catechol-2,3-dioxygenase *xylE* reporter gene from *P. putida* NCIB 9869 (Ng *et al.*, 1994). The promoterless *xylE* was PCR amplified from *P. putida* genomic DNA using the primers pphB-F (5'-GCT CTA GAA ACA TTA AGA GGT G; *Xba*I) and pphB-R (5'-GGG GTA CCG AAG TGC TTG ATC TC; *Kpn*I). The pRA2 *par* promoter was amplified from pPAR64 using primers pQF52-F (5'-CCC AAG CTT CTG CTC TGA TGC CG; *Hind*III) and DEF2 (5'-ACC CAA GCT TCT GCG CCG TCG AGT TG; *Hind*III). By isolating P<sub>*par*</sub> from pPAR64, the double transcriptional terminators of pQF52 found upstream of P<sub>*par*</sub> were retained. The two blunt-ended PCR products were purified, ligated and subjected to amplification using only primers pQF52-F and pphB-R. The resulting 1.5 kb PCR product was cloned into pGEM-TEasy (Promega), and colonies expressing high levels of catechol-2,3-dioxygenase (C230) were identified by spraying with 0.1 M catechol, after which they turned a deep yellow colour. The P<sub>*par*</sub>-*xylE* reporter fragment was cleaved from pGEM-TEasy by *Eco*RI digestion and cloned into pRK415 (Keen *et al.*, 1988; IncP, Tc<sup>R</sup>), giving rise to pRKC230. *E. coli* transformed with pRKC230 also exhibited high C230 activity, as determined by the catechol spray test.

### Plasmid stability and incompatibility analysis

Test plasmids were transformed into bacterial hosts and spread onto LB plates containing the appropriate antibiotics. A fresh transformant was then used to inoculate a flask of LB containing antibiotic(s), and this culture was grown to late log phase. A small portion of the culture was then diluted 10<sup>3</sup>-fold into prewarmed media lacking antibiotics and grown to late log phase, at which time a sample of the culture was spread onto non-selective media. Single colonies were tested for

plasmid retention by patching onto selective media. The dilution and plating steps were repeated for 100 generations.

For incompatibility analysis, the stability of plasmid FK143 in the presence of *par* promoter nested deletion clones (SX22, SX21, SX41, SX81 and SX43) was determined according to the method of Martin *et al.* (1987). In this assay, FK143 grown in the presence of a compatible plasmid (pQF52) was retained 100% of the time, whereas in the presence of an incompatible plasmid (pPAR64), the retention rate was 40–60% after 25 generations of non-selective growth.

#### Protein expression and purification

Experiments to identify *par* polypeptides were performed by cloning the predicted coding regions of *parA*, *parB* and *parC* under the control of the T7 promoter of pET-30a (Novagen). In this system, recombinant proteins are expressed with an N-terminal fusion containing a 6×His tag for affinity purification. Expression clones containing the individual *par* genes were transformed into *E. coli* BL21(DE3)pLysS, and single colonies were used to inoculate 50 ml of LB media. The culture was grown to an optical density (600 nm) of 0.5, and protein expression was then induced by adding IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation, washed in native buffer (20 mM Tris, pH 7.8, 500 mM NaCl) and resuspended in 5 ml of the same buffer. The cells were sonicated while being chilled on ice, and the insoluble material was pelleted by centrifugation. The soluble proteins were analysed by PAGE (Sambrook *et al.*, 1989) under denaturing conditions.

All steps in the purification procedure were performed at 4°C. Soluble protein extract (5 ml) was mixed with 2 ml of Ni-NTA resin (Qiagen) and gently shaken for 10 min. The resin was washed with 4 × 4 ml of native buffer followed by 8 × 4 ml of wash buffer (20 mM Tris, pH 6.0, 500 mM NaCl). Non-specific proteins were removed with an imidazole gradient (0–350 mM) in wash buffer. The proteins were eluted with 5 ml of elution buffer (20 mM Tris, pH 6.0, 500 mM NaCl, 0.5 M imidazole), and 1 ml fractions were collected. Fractions containing the purified proteins were subjected to gel filtration chromatography, and the purified proteins were collected in storage buffer (20 mM Tris, pH 7.5, 20% glycerol) and kept at –20°C.

#### Reporter gene assays

Levels of β-galactosidase activity were assayed according to the method of Miller (1972) using SDS and chloroform to permeabilize the cells. Catechol-2,3-dioxygenase activity in cell-free extracts was measured according to the method of Zukowski *et al.* (1983). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

#### Primer extension analysis

The bacterial strain DH5α containing plasmid pPAR64 was grown to late log phase. Total RNA was extracted using Trizol reagent (Gibco BRL) according to the manufacturer's

instructions. Primer extension was performed essentially as described by Ausubel *et al.* (1987) using the primer PEX2 (5'-GTG GTT TTA CCC GCC CCG C), which was end-labelled with γ-<sup>32</sup>P and 40 μg of total RNA. AMV reverse transcriptase and the Sequenase version 2.0 DNA sequencing kit were purchased from AP Biotech.

#### DNA-binding experiments

A 271 bp DNA fragment encompassing the *par* promoter was PCR amplified using primers DEF1 (5'-CGC GGA TCC CGT AGG CCC TCC C; *Bam*HI) and DEF2 (ACC CAA GCT TCT GCG CCG TCG AGT TG; *Hind*III). The PCR product was gel purified and end labelled with γ-<sup>32</sup>P using T4 polynucleotide kinase (Gibco BRL).

Electrophoretic mobility shift assays were performed essentially as described previously (Chodosh *et al.*, 1986). Briefly, end-labelled DNA was incubated with 2 μg of poly-(dI–dC) (Sigma) and increasing amounts of partially purified ParB fusion protein in binding buffer [10 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM dithiothreitol (DTT), 10% glycerol] and incubated on ice for 40 min. Binding reactions were then loaded onto a 4% polyacrylamide (acrylamide–bisacrylamide, 29:1), 0.25× TBE gel that had been prerun at 200 V for 2 h. Nucleoprotein complexes were separated from unbound DNA by applying 200 V for 4 h. The gel was dried and exposed to Hyperfilm (AP Biotech) for 1–2 h before image development.

DNase I footprinting was performed essentially as described by Brenowitz *et al.* (1986). The same *par* promoter DNA fragment that was used in EMSAs was also used for DNase I footprinting; however, the fragment was labelled at only one end. The DNA fragment was digested to completion with the appropriate restriction enzyme (*Bam*HI or *Hind*III), and then the cleaved terminus was labelled in a Klenow end-filling reaction using [α-<sup>32</sup>P]-dATP (AP Biotech). DNA–protein binding reactions were incubated under the same binding conditions used for EMSAs. After incubation, binding reactions were brought to a total volume of 200 μl with assay buffer [10 mM Tris, pH 8.0, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM DTT, 100 mM KCl, 50 μg ml<sup>-1</sup> BSA, 2 μg ml<sup>-1</sup> poly-(dI–dC)] and then digested with the appropriate amount of DNase I (Sigma). DNA fragments were separated on denaturing 8% polyacrylamide sequencing gels. The Maxam–Gilbert G+A sequencing ladder was prepared by the rapid method (Sambrook *et al.*, 1989).

#### Accession number

The complete pRA2 nucleotide sequence has been deposited in GenBank under the accession no. U88088. The pRA2 *par* region that is presented in Fig. 1 corresponds to nucleotides 20 318–18 746 on the complementary strand of the deposited sequence.

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