Regulatory Cross-talk in the Double par Locus of Plasmid pB171*

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The double par locus of Escherichia coli virulence factor pB171 consists of two adjacent and oppositely oriented par loci of different types, called par1 and par2. par1 encodes an actin ATPase (ParM), and par2 encodes an oscillating, MinD-like ATPase (ParA). The par loci share a central cis-acting region of ≈200 bp, called parC1, located between the two par loci. An additional cis-acting region, parC2, is located downstream of the parAB operon of par2. Here we show that ParR of par1 and ParB of par2 bind cooperatively to unrelated sets of direct repeats in parC1 to form the cognate partition and promoter repression complexes. Surprisingly, ParB repressed transcription of the noncognate par operon, indicating cross-talk and possibly epistasis between the two systems. The par promoters, P1 and P2, affected each other negatively. The DNA binding activities of ParR and ParB correlated well with the observed transcriptional regulation of the par operons in vivo and in vitro. Integration host factor (IHF) was identified as a novel factor involved in par2-mediated plasmid partitioning.

Bacterial plasmids have been used extensively as model systems in the study of DNA segregation. This is because plasmids encode centromere-like loci, also called partitioning (par) loci, that ensure stable propagation of their replicons (1, 2). Plasmid-borne par loci invariably consist of two proteins encoded by a bicistronic operon and one or more cis-acting centromere regions where the proteins act. The first gene in the operon (called parA, parF, or parM) encodes an ATPase. The second gene (called parB, parG, or parR) encodes an adaptor protein that binds to its cognate centromere and thereby forms the “partition complex” that, in turn, is recognized by the ATPase. Based on the ATPase, all par loci are divided into two types: Type I loci, which encode Walker box ATPases related to the MinD family, and Type II loci, which encode actin-like ATPases (3–5). Based on gene sizes and arrangement, Type I loci are subdivided in Type Ia and Ib. Type Ib ATPases are generally smaller than those of Type Ia, which include ParA of plasmid P1 and SopA of plasmid F. The Type Ib ATPases lack the DNA-binding helix-turn-helix (HTH) domain found in the N-terminal part of the longer Type Ia ATPases (5, 6). Thus, contrary to the Type Ia ATPases, the Type Ib ATPases do not themselves specifically bind DNA. The molecular mechanism specified by Type II loci is well understood (1, 7, 8). By contrast, the molecular mechanism behind the common and more efficient Type I loci has been more difficult to understand (2, 9).

The Escherichia coli virulence plasmid, pB171, has two par loci designated par1 (Type II) and par2 (Type I) with a peculiar genetic arrangement. The oppositely oriented par1 and par2 loci share a common cis-acting region, parC1, of ≈200 bp only (see Fig. 1, A and B) (10). parC1 contains 17 6-bp direct repeats (called B1 to B17) organized in two clusters. As described previously (10), parC1 expresses both par1- and par2-specific incompatibility, indicating that the full-length parC1 fragment contains centromere-like sites for both par loci. The parC2 region downstream of parB also expresses par2-specific incompatibility and contains 18 6-bp direct repeats (B18–B35) that are related to the B repeats in parC1. In contrast, parC2 does not express par1-specific incompatibility (10). Based on sequence similarity, the B repeats could be divided into two subclasses (I and II) (see Fig. 1, B and C) (10). Individual repeats belonging to the two subclasses of B repeats are interspersed among each other in parC1 and parC2. In analogy with other Type I par loci (2, 9), the genetic organization of parC1 and parC2 suggests that ParB binds to the B repeats. parC1 also harbors the P1 and P2 promoters that transcribe par1 and par2 (Fig. 1, A and B) (10).

ParA of par2 is a Walker box ATPase, which forms filaments that oscillate in spiral-shaped structures over the nucleoid (10, 11). In the presence of par2, plasmids localize to midcell and/or cell quadrants, a pattern that is dependent on ParA spiral formation and oscillation (11). Recently, we proposed a working model for how an oscillating and filament-forming protein can localize plasmids primarily at midcell and quarter-cell positions (12).

The unusual genetic arrangement of the double par locus of pB171 stimulated an analysis of parC1 and parC2. We show here that both ParB and ParR bind to parC1 and that ParB binds to parC2. ParB recognizes the 6-bp direct repeats located within parC1 and parC2. Interestingly ParR recognizes two 10-bp direct repeats located in parC1 just upstream of parM. The ability of ParB and ParR to bind to parC1 is consistent with their transcriptional regulation of the two par operons, i.e. ParR regulates the par1 operon and parB the par2 operon. Surprisingly, however, ParB represses the par1 operon as efficiently as ParR, suggesting that par2 is epistatic to par1. This is, to our

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knowledge, the first example of cross-talk regulation between two different par loci.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Plasmids are listed in Table 1. The following E. coli K-12 strains were used: MC1000, F− Δ ara-leu Δ lac rpsL15 (13); Top10, F− mcrA Δ(mrr-hsdRMS-mcrBC) d800lacZΔM15 lacX74 recA1 deoR araD139 Δ ara-leu7697 galU galK rpsL15 (Stp3) endA1 nupG6; CH1,182, hubA16::aphA; CH1,192, hubA16::aphA himA82::TetR (not Δgall-lys); MG1655, bkg Δ(lav-arg) Δ(gall-lys) galP211 both CH1,182 and CH1,192 are MG1655 derivatives.

Construction of Plasmids Containing parC1-lacZ Fusions—Thirteen mini-R1 derivatives containing parC1 fragments with varying numbers of repeats cloned in front of lacZ in the transcriptional fusion vector pRB200 were constructed (see Table 1). In the pGE3100 series the P1 promoter reads into transcriptional fusion vector pRBJ200 were constructed (see Table 1). Varying numbers of repeats cloned in front of mcrBC (pGE107), eno, parR (pGE108), and the DNA sequences of the oligonucleotides are given in Tables 2–4. A Shine-Dalgarno sequence (from parMR of R1, included in the upstream primer) was cloned in front of the parR and parB open reading frames. Upstream primers harbored a SacI site and downstream primers a HindIII site. These sites were subsequently used to clone the PCR fragments into the corresponding sites located downstream of the arabinose-inducible PBAD promoter of vector pBAD33. The pBAD33 derivatives were transformed into strains of MC1000 already harboring the pOU254 derivatives. The growth rate of almost all strains (except control strains containing the pBAD33 vector) decreased slightly upon arabinose addition, especially strains expressing ParB.

Construction of Plasmids Used for Overexpression of His6−ParR and His6−ParB—ParR and ParB were amplified from plasmid pBAD33 by using PCR with the following DNA primers: parR, B171-20 + B171-21; parB, B171-22 + B171-23 (sequences are given in Table 1). Upstream primers contained His6 codons inserted between the start codon and the second codon of the ParR and ParB reading frames, respectively. The PCR products were digested with restriction enzymes BamHI and XhoI and cloned into the BamHI and SalI sites of plasmid pMG25 thereby creating plasmids pGE121 (P1,4/O3::his6-parR) and pGE122 (P1,4/O3::his6-parB). DNA sequences of all PCR-amplified constructs were verified by DNA sequencing by using the CEQ2000 sequencing system provided by Beckman.

**Protein Purification**—E. coli strain Top10 harboring either pGE223 (P1,4/O3::his6-parB) or pGE221 (P1,4/O3::his6-parB) was grown in LB medium at 37 °C to an A600 of 0.8 before expression of recombinant protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Induction was continued for 4 h. All of the following steps of the purification procedure were performed on ice or in a 4 °C cold room. Harvested cells were resuspended in 4 ml of lysis buffer/200 ml of culture. Egg
white lysozyme was added to resuspended cells at a final concentration of 1 mg/ml and incubated for 30 min. Cells were then sonicated three times for 20 s. The lysate was cleared by centrifugation at 10,000 rpm for 30 min. The cleared lysate from 1 liter of culture was mixed with 1.0 ml of nickel-nitriilotriacetic acid-agarose matrix (Qiagen) and incubated with rotation for 1 h for binding of the histidines in the His<sub>6</sub> tag to the nickel ions. The mixture was then allowed to settle in a column. The column was washed twice with 4 ml of wash buffer. Flow-through was collected for further analysis. Recombinant proteins were eluted four times with 0.5 ml of elution buffer. Each elution fraction was stored separately. 5 µl of all samples were analyzed by SDS-PAGE (4% stacking gel, 12.5% separation gel) followed by Coomassie Brilliant Blue staining of the gel. Lysis buffer consisted of 50 mM Na<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 30 mM imidazole, pH 8.0; wash buffer consisted of 50 mM Na<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 40 mM imidazole, pH 8.0; elution buffer consisted of 50 mM Na<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0.

LacZ Assay—Cells of strain MC1000 harboring one mini-R1 plasmid (pOU254 derivative) and one expression plasmid (pBAD33 derivative) were grown at 35 °C in AB medium supplemented with 0.5% glycerol, 0.1% casamino acids, 0.1 µg/ml thiamin, and antibiotics (50 µg/ml chloramphenicol and 30 µg/ml ampicillin). The generation time in this medium was about 50 min. Overnight cultures were diluted 50-fold into fresh medium with antibiotics and grown to an A<sub>450</sub> of ~0.05. Then, 0.2% arabinoose was added, and the cultures were grown for 3 h to an A<sub>450</sub> of ~0.4. Samples were collected for β-galactosidase activity measurements carried out according to Miller (14). The stability of the R1 plasmids containing the DNA fragment of interest.

Electrophoretic Mobility Shift Assay—DNA oligonucleotides were <sup>32</sup>P-end-labeled in a kinase reaction. 20 pmol of oligonucleotide was mixed with 1.5 µl of 10× polynucleotide kinase (PNK) buffer (1 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 70 mM DTT, pH 8.0), 2 µl of 10 µCi/µl [γ-<sup>32</sup>P]ATP (3000 Ci/mmol), 1 unit of PNK buffer of PNK, and H<sub>2</sub>O to a total volume of 15 µl. The reaction mixture was then incubated for 30 min at 37 °C followed by 10 min at 70 °C. End-labeled oligonucleotides were then used in a PCR reaction with an additional oligonucleotide designed to obtain the DNA fragment of interest.

Oligonucleotides used in construction of truncated parC1 fragments for the ParB electrophoretic mobility shift assay (EMSAs)<sup>2</sup> are: fragment a, B171-39 + B171-40, 256 bp; fragment b, B171-39 + B171-83, 216 bp; fragment c, B171-39 + B171-41, 189 bp; fragment d, B171-39 + B171-42, 161 bp; fragment e, B171-39 + B171-44, 126 bp; fragment f, B171-39 + B171-45, 99 bp; fragment g, B171-39 + B171-46, 69 bp; fragment h, B171-40 + B171-49, 223 bp; fragment i, B171-40 + B171-50, 193 bp; fragment j, B171-40 + B171-51, 165 bp; fragment k, B171-40 + B171-52, 144 bp; fragment l, B171-40 + B171-53, 130 bp; fragment m, B171-40 + B171-54, 102 bp (oligonucleotide sequences are listed in Table 3).

Primers used in construction of DNA fragments containing ihf1 and ihf2 are: ihf1, B171-SP7 + B171-SP10, 194 bp; ihf2, B171-10 + B171-11, 222 bp. The sequences of these oligonucleotides are listed in Table 4.

Primers used in construction of DNA fragments containing P1-parS are: P1-parS, B171-68 + B171-69. Primers used in construction of DNA fragments containing pUC19 DNA are: 171SR14 + 171SR16, 199 bp (oligonucleotide sequences are listed in Table 4).

Oligonucleotides used in construction of truncated parC1 fragments for the ParR EMSA are: fragment a, B171-PE2 + B171-99, 355 bp; fragment b, B171-PE2 + B171-100, 345 bp; fragment c, B171-PE2 + B171-101, 335 bp; fragment d, B171-PE2 + B171-102, 325 bp; fragment e, B171-PE2 + B171-103, 315 bp; fragment f, B171-PE2 + B171-104, 305 bp; fragment g, B171-PE2 + B171-105, 295 bp; fragment h, B171-PE2 + B171-106, 285 bp; fragment i, B171-PE2 + B171-107, 275 bp; fragment j, B171-108 + B171-109, 265 bp; fragment k, B171-108 + B171-110, 256 bp; fragment l, B171-108 + B171-111, 246 bp; fragment m, B171-108 + B171-112, 236 bp; fragment n, B171-108 + B171-113, 226 bp; fragment o, B171-108 + B171-114, 216 bp; fragment p, B171-108 + B171-115, 206 bp (see Table 2).

The standard reaction mixture (20 µl) for the ParB/ParR experiments contained 10 mM Tris-base (pH 7.5), 50 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM DTT, and 0.1 µg/µl sonicated salmon sperm DNA. In EMSA experiments performed with integration host factor (IHF), the standard reaction mixture (20 µl) contained 25 mM Tris-base (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM DTT, 1 mg/ml bovine serum albumin, and 0.1

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**TABLE 2**

| Primers for construction of truncated ParR binding site in EMSA |
|---------------------------------------------------------------|
| **Direction of truncation** | **Primer name** | **Primer sequence** |
|---------------------------|----------------|--------------------|
| par2, cw                   | B171-99       | 5′-GATACTGTTGATGCATCTGAGCCTT-3′ |
|                           | B171-100      | 5′-AAGCCCTGCTCTATTAGGAGCA-3′ |
|                           | B171-101      | 5′-GCTTCTGTGCTGGAGGATCGAGGCTG-3′ |
|                           | B171-102      | 5′-GTATTTAAGGGGCTATAGATGAC-3′ |
|                           | B171-103      | 5′-GTATCTGTTTCTGGTTGCAAG-3′ |
|                           | B171-104      | 5′-GCTCGATGTCACGCTCGTGG-3′ |
|                           | B171-105      | 5′-GGCTGATGTCAGCACTGCT-3′ |
|                           | B171-106      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-107      | 5′-GATTGCTCGTTTACAGCAGC-3′ |
|                           | B171-108      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-109      | 5′-GCTCGATGTCACGCTCGTGG-3′ |
|                           | B171-110      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-111      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-112      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-113      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-114      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-115      | 5′-GCTCTAGTTTATGATTTAT-3′ |
|                           | B171-116      | 5′-GCTCTAGTTTATGATTTAT-3′ |

<sup>2</sup> The abbreviations used are: EMSA, electrophoretic mobility shift assay; HMW, high molecular weight; DTT, dithiothreitol; IHF, integration host factor; TBE, Tris borate-EDTA.
μg/μl sonicated salmon sperm DNA. The standard reaction mixture was mixed on ice followed by the addition of 32P-end-labeled DNA fragments. Protein concentrations are given in the figure legends. We used a concentration of 5 nM 32P-labeled DNA fragments. Protein concentrations are given in the table.

TABLE 3

| Direction of truncation<sup>a</sup> | Primer name | Primer sequence |
|-----------------------------------|-------------|-----------------|
| par2, cw                           | B171-40     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par2, ccw                          | B171-39     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-49     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-50     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-51     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-52     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-53     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-54     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-55     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-56     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-57     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-58     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-59     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-60     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-61     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-62     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-63     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-64     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-65     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-66     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-67     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-68     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-69     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| par1, ccw                          | B171-70     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| ParM-up                            | B171-71     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| ParM-down                         | B171-72     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| 171SR1                            | B171-73     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| 171SR16                           | B171-74     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |

<sup>a</sup> cw, clockwise; ccw, counterclockwise.

TABLE 4

| Additional oligonucleotides used for PCR |
|-----------------------------------------|
| Primer name | Primer sequence |
|-------------|-----------------|
| B171-10     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-11     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-19     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-20     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-21     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-22     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-23     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-24     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-25     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-26     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-27     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-28     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-29     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-30     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-31     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-32     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-33     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-34     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-35     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-36     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-37     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-38     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |
| B171-39     | 5'-CCCCCGGATCCGACGTCTAGCTTACGCTTAGGAGAATCTACG-3' |

DNA Binding and Promoter Regulation by ParR and ParB

Generation of Biotinylated Double-stranded parC1 Fragments for Surface Plasmon Resonance (SPR)—Biotinylated parC1-containing DNA fragments were produced by PCR using a 5'-end-biotinylated B171-39 clockwise primer and B171-40 counterclockwise primer (Table 3) with pGE2 as template DNA. B171-39 was biotinylated. The PCR product was purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) as recommended by the manufacturer.

SPR Measurements—SPR was carried out using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) with streptavidin-coated SA sensor chips (Biacore AB). Biacore measures the binding of molecules to ligands immobilized on sensor chips as real-time changes in the optical SPR phenomenon that is caused by changes in the refractive index near the sensor chip. The refractive index changes are linearly dependent on mass binding to the sensor chip, and hence the amount of analyte that binds the immobilized ligand can be calculated. Because the injections of analyte supply constant concentrations, the binding kinetics is monitored. When the flow is changed to buffer without analyte, the dissociation kinetics is monitored, as bound analyte leaves the immobilized ligand. For the binding studies, 150
DNA Binding and Promoter Regulation by ParR and ParB

FIGURE 1. The double par locus of plasmid pB171. A, the double par locus encodes two divergently transcribed par loci, par1 and par2, of different types (see the Introduction). The par loci share a common cis-acting central region, par1, which encodes the two par promoters, P1 and P2. A second cis-acting region, par2, is located downstream of par1. Two IHF binding sites, ihf1 and ihf2, are located within parA and at the end of parB, respectively. ihf1 is located on the (−)-strand, and ihf2 is located on the (+)-strand. B, enlargements of the parC1 and parC2 regions that contain 17 and 18 6-bp direct repeats (iterons) of related sequences, respectively. The 35 iterons were divided into two subclasses, class I (red numbers) and class II (blue numbers). R1 and R2 denote two direct repeats of identical sequence just upstream of ihf1, ihf2, and at the end of parC2, respectively. Two IHF binding sites, ihf1 and ihf2, are shown in brackets. C, consensus sequences of the R and B iterons. Alternative bases of the B repeats are shown in brackets. D, sequences of the two IHF binding sites. Bold letters show the minimum IHF binding site (15), and arrows indicate the direction of the sites. The tandem stop codons of parB are shown in red, and the three left-hand B iterons of parC2 that overlap with ihf2 are indicated by the three shorter arrows (right panel).

mm KCl, 4 mM MgCl2, 1 mM DTT, 0.005% Tween 20, 20 mM HEPES, pH 7.5, was used as running buffer. 200 resonance units of the biotinylated parC1 PCR product was captured on flow cell 2 by injecting the cell with running buffer supplied with 0.5 mM NaCl, whereas flow cell 1 was left blank for reference subtraction. Analysis of ParB binding to immobilized parC1 was conducted as follows. A continuous flow of running buffer at 10 μl/min over the two flow cells created a stable base line. ParB diluted to the specified concentration in running buffer was then injected over the two flow cells. To release the ParB complex from the immobilized DNA, the flow was increased to 40 μl/min, and two 15-s pulses of 6 M guanidinium hydrochloride were injected over both flow cells leaving the immobilized DNA ready for another binding cycle. The SPR response is dependent on mass bound to the sensor chip. Thus the relative numbers of ParB binding per immobilized parC1 were calculated according to

\[ n = \frac{R_{\text{analyte}} \times M_r \text{ligand}}{R_{\text{ligand}} \times M_r \text{analyte}} \]  

(Eq. 1)

where \( R_{\text{analyte}} \) is the SPR response contributed by the analyte (i.e. ParB) binding to the immobilized ligand (parC1 or ParB captured by parC1), \( R_{\text{ligand}} \) is the response contributed by the immobilized ligand, and \( M_r \text{ligand} \) and \( M_r \text{analyte} \) are the molecular weights of the ligand and analyte, respectively.

Calculation of the Number of ParB Molecules per Iteron in parC1—This calculation is as follows: \( R(\text{ParB}) = 450; R(\text{parC1}) = 200; M_r(\text{His}_6-\text{ParB}) = 10,882.5 \text{ g/mol}; M_r(\text{parC1}) = 165,472.4 \text{ g/mol}. \)

\[ n = \frac{R(\text{parC1}) \times M_r(\text{parC1})}{R(\text{ParB}) \times M_r(\text{His}_6-\text{ParB})} \]

\[ = \frac{450 \times 165,472.4}{200 \times 10,882.5} = 34.2 \approx 34 \]

(Eq. 2)

Thus, at 100 nM ParB, 34 molecules of ParB binds to parC1, corresponding to two ParB molecules per direct repeat in parC1; this suggests that one repeat binds one ParB dimer. \( R \) denotes response units in the SPR experiments.

In Vitro Transcription Reactions—PCR fragments for P1 and P2 transcription were obtained using the primers B171-SP3/B171-39 and B171-40/B171-SP9 resulting in fragments of 1174 bp and 1114 bp, respectively (Tables 3 and 4). The standard reaction mixture contained 4 μl of 5× transcription buffer (Promega), 2 μl of 100 mM DTT, 0.8 μl of RNA-guard, 4 μl of NTPmix (2.5 mM ATP, GTP, UTP), 2.4 μl of 100 μM CTP, 1 μl of template DNA (stock concentration, 20 ng/μl), 1 μl of [α-32P]CTP (10 μCi/μl), and 1 unit of DNA polymerase in a total volume of 25 μl. Where indicated protein was added to a concentration of 1 μM for ParB and 10 μM for ParR. Samples were incubated for 60 min at 37 °C followed by
the addition of 1 μl of DNase (1 unit) and were then incubated an additional 15 min at 37 °C. Reactions were stopped by the addition of formamide loading buffer and boiled for 10 min at 99 °C followed by denaturing PAGE on a 5% polyacrylamide gel. The gel was dried on paper and exposed to α32P-sensitive screen overnight for imaging on a PhosphorImager.

### A: P1-lacZ fusions: 

| DNA Fragment Ends | P1 | P2 |
|-------------------|----|----|
| a | lacZ (R1) | B1 - 13 | P2 |
| b | lacZ (R1) | B14 - 17 |
| c | lacZ (R1) | P2 |
| d | lacZ (R1) | P2 |
| e | lacZ (R1) | P2 |
| f | lacZ (R1) | P2 |

### B: P2-lacZ fusions: 

| DNA Fragment Ends | P1 | P2 |
|-------------------|----|----|
| a | lacZ (R1) | B1 - 13 | P2 |
| b | lacZ (R1) | B14 - 17 |
| c | lacZ (R1) | P2 |
| d | lacZ (R1) | P2 |
| e | lacZ (R1) | P2 |
| f | lacZ (R1) | P2 |

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**FIGURE 2. Regulation of the P1 and P2 promoters in vivo.** PCR-generated DNA fragments were inserted into pOU254, a low-copy-number R1 transcriptional fusion vector. P1 and P2 indicate the promoters of the par1 and par2 operons, respectively. Transcriptional start points are indicated with broken arrows. The numbers at the DNA fragment ends are sequence coordinates, with +1 corresponding to the transcriptional start point of P2. Numbers within the DNA bars indicate B iterons 1–17 and R1 and R2 iterons upstream of parM. Numbers in the first column are LacZ Miller units expressed by the unrepressed lacZ fusion plasmids. Numbers in the two following columns represent repression -fold when parR and parB were present in trans in p15 replicons (pGE107 and pGE207, respectively). Cells of MC1000 carrying the appropriate plasmids were grown at 35 °C in glycerol minimal medium containing antibiotics and 0.2% arabinose to induce the pBAD promoter upstream of the parR and parB genes.
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Oligonucleotides Used for PCR—These primer sequences are listed in Tables 2–4.

RESULTS

The Divergent P1 and P2 Promoters Are Negatively Coupled—As described above, parC1 contains the B1–B17 repeats located in two clusters that are potential operators for ParB binding (Fig. 1B). Further inspection of the parC1 region revealed two 10-bp direct repeats, R1 and R2, separated by 31 bp (corresponding to three helical turns of the DNA helix). R1 and R2 are located between the P1 promoter and parM, and the distance between R2 and B1 is only 9 bp (Fig. 1B). To investigate whether the R and B repeats are involved in regulation of the divergent P1 and P2 promoters in parC1, we constructed two series of lacZ transcriptional fusions containing either the entire parC1 region or truncations thereof (Fig. 2). In the absence of ParR and ParB, P1 and P2 had comparable, strong activities (Fig. 2, Aa and Ba). In the P1 series, deletion of P2 resulted in increased P1 activity (Fig. 2A, c and d). Likewise, deletion of P1 from the P2 series also increased P2 activity (Fig. 2B, c–e). Deletion of the −10 and −35 boxes of P1 or P2 resulted in loss of promoter activity in both cases, consistent with previous primer extension analysis of P1 and P2 (10). ParR did not affect P2 activity (Fig. 2Bb). P2 Is Regulated by ParB, whereas P1 Is Regulated by both ParR and ParB—ParR or ParB were donated in trans, and changes in P1 and P2 activities were determined. ParR repressed P1 activity 3.5-fold (Fig. 2Aa). Repression of P1 by ParR was not seriously affected by the progressive deletion of B repeats (Fig. 2A, b–e), and the construct containing only R1, R2, and B1–B4 was fully reproducible (Fig. 2Ae). A promoter-less lacZ fusion containing R1 and R2 only did not respond to ParR (Fig. 2Ag). ParB in trans repressed P1 to a similar extent (Fig. 2A, a–e), and the activity from the promoter construct containing only R1, R2, and B1–B4 was still repressed. These results are consistent with the proposal that ParR represses P1 via binding to R1 and R2 and that ParB represses P1 via binding to the B repeats, notably B1–B4. This conjecture was supported by in vitro experiments described later.

In the intact parC1 fragment, ParB, repressed P2 activity 3.5-fold (Fig. 2Ba). The P2 activity of constructs carrying progressive deletions into the B1–B13 repeats were still repressible by ParB (Fig. 2B, b–e), and the construct that had B14–B17 only was also repressed (Fig. 2Bf). Somewhat unexpectedly, the constructs in which R1 and R2 were deleted were more responsive to repression by ParB.

ParR Also Binds Cooperatively to parC1—Similarly, we assessed the ability of ParR to bind to parC1 and parC2. As seen from Fig. 4B, ParR shifted parC1 DNA at a much lower concentration than that required to shift parC2 or control DNA (Fig. 4, Bc and Ba, respectively). At the highest ParR concentration, two shifted bands were seen (Rc1 and Rc2 in Fig. 4Bb), indicating the formation of distinct higher order com-

![FIGURE 3. Regulation of P1 and P2 in vitro.](image-url)
plexes. The HMW complexes seen with ParB binding were not observed with ParR, perhaps reflecting a biological difference in the mode of action of the proteins. The Hill coefficient of ParR binding to $\text{parC}_1$ was 2.7, which indicates cooperativity.

Comparison of ParB and ParR DNA Binding Affinities—To compare the binding affinities of ParB and ParR for $\text{parC}_1$, the percentage of shifted $\text{parC}_1$ DNA was plotted as a function of the protein concentration to determine the $K_d$ values of ParR and ParB. The inset graph is an enlargement of the ParB binding curves.

Two-stage Binding of ParB to $\text{parC}_1$—To characterize further the in vitro interactions between ParB and $\text{parC}_1$, a series of SPR experiments were accomplished (see “Experimental Procedures”). A DNA fragment encoding $\text{parC}_1$ was immobilized on a streptavidin sensor chip and assayed for binding of 100 nM ParB. Biotinylated double-stranded $\text{parC}_1$ fragments were immobilized on a streptavidin sensor chip and examined for concentration-dependent ParB binding. The amount of free protein was approximately equal to that of total protein. This allows for the estimation of the concentration of DNA-binding protein i.e. ParB and ParR, required for 50% complex formation, which is a measure of the apparent dissociation constant, $K_d$. The $K_d$ values for binding of ParB and ParR to $\text{parC}_1$ were determined to 0.5 and 20 μM, respectively, showing that ParB binds with an ~40-fold higher affinity than ParR (Fig. 4C).

Two-stage Binding of ParB to $\text{parC}_1$—To characterize further the in vitro interactions between ParB and $\text{parC}_1$, a series of SPR experiments were accomplished (see “Experimental Procedures”). A DNA fragment encoding $\text{parC}_1$ was immobilized on a streptavidin sensor chip, and subsequently ParB in various concentrations was injected over the chip. ParB bound avidly to $\text{parC}_1$ (Fig. 5). At 100 nM ParB, a response corresponding to 34 molecules of ParB per $\text{parC}_1$ or one ParB dimer per direct repeat in $\text{parC}_1$ was detected (Fig. 5B).
Next, we analyzed the effect on ParB binding when parC1 was truncated in the direction of P1 (Fig. 6, fragments h–m). Again, as the number of 6-bp iterons was reduced, the slowly migrating band in the top of the gel gradually disappeared, and a less shifted band became evident. The intensity of a shifted band entering the gel increased as more and more iterons were removed (Fig. 6, fragments j–l). Importantly, ParB bound to the fragment containing B1–B4 repeats that overlaps with the P1 promoter (Fig. 6, fragment l). ParB was not able to bind to the 68-bp fragment containing R1 and R2, supporting the notion that the binding sites for ParB are the 6-bp iterons (Fig. 6, fragment m). Again, these results are consistent with the in vivo P1 and P2 promoter analysis (Fig. 2).

Identification of ParR Binding Sites—Deletion of the region harboring R1 and R2 completely abolished ParR binding to the parC1 DNA (Fig. 7A). Thus R1 and R2 may be ParR recognition sites. Therefore, a more detailed truncation analysis was performed on the region encoding R1 and R2 using successive deletions of 10 bp. ParR-binding was not affected by deletion of the region 5’ of the R1 site (Fig. 7B, fragments a–c). For fragments a and b, one shifted band was observed, whereas two were observed for fragment c (denoted as Rc1 and Rc2 in Fig. 7B). When the 5’ 5 bp of R1 were deleted, only one specific band with a migration corresponding to Rc1 was observed (Fig. 7B, fragment d). Thus, partial deletion of R1 clearly affected ParR binding. Deletion of the region between R1 and R2 did not abolish ParR binding (Fig. 7B, fragments e–g). By contrast, deletion of the 5’ 5 bp of R2 completely abolished ParR binding (Fig. 7B, fragments h and i).

When truncated in the opposite direction, ParR binding was not affected by deletion of the region 3’ of R2 (Fig. 7C, fragment j–l). For fragments j–l, two distinct shifted bands, Rc1 and Rc2, were observed. When the 5’ 5 bp of R2 were deleted, only one specific band with a migration corresponding to Rc1 was observed (Fig. 7C, fragment m). Thus, partial deletion of R2 affected ParR binding. Again, deletion of the region between R1 and R2 did not affect ParR binding (Fig. 7C, fragments n–p). However, deletion of 5 bp of R1 completely abolished ParR binding (Fig. 7C, fragments q and r). The results described above show that ParR specifically recognizes the R1 and R2 sequences and that one binding site is sufficient for efficient ParR binding. These results are also consistent with the in vivo analysis of P1 regulation (Fig. 2).

IHF Binds to Two Sites within par2—Inspection of the DNA sequence revealed two potential IHF binding sites within par2. Both sites exhibit 100% identity with the minimum consensus...
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IHF recognition sequence (15). The two sites were designated ihf1 and ihf2, respectively. ihf1 is located within parA on the (−)-strand, 504 bp downstream of the parA start codon. ihf2 is located on the opposite strand and overlaps with 2 bp of the second stop codon of parB (Fig. 1, A and D). Both ihf1 and ihf2 exhibit similarity to other strong IHF binding sites (data not shown), hence indicating that ihf1 and ihf2 might act as bona fide IHF binding sites (Fig. 1D). 32P-end-labeled DNA fragments of ~200 bp, containing either ihf1 or ihf2, were synthesized and used in gel-shift experiments with purified IHF. As a positive control, parS, the cis-acting site of the P1 par locus, which contains a known IHF binding site, was included in the analysis. Because parC1 contains no sequence homology to known IHF binding sites, it was used as a negative control. As expected, IHF bound to parS from P1 (Fig. 8D) but not to parC1 of pB171 (Fig. 8C). Interestingly, IHF bound to both the ihf1- and ihf2-containing DNA fragments (Fig. 8, A and B). Consistently, par2-containing plasmids were moderately but reproducibly less stable in an ihf deletion strain as compared with an isogenic wild-type strain (data not shown).

FIGURE 7. Determination of ParR binding site. Mobility shift analysis was performed to determine the ParR binding site in parC1. The left panels show an overview of the respective DNA fragments used in the gel shifts, and the right panels show the gel analysis. A, mobility shift experiment of ParR binding to parC1 (primers B171-40 + B171-39) and parC1ΔR1/R2 (primers B171-41 + B171-39). In parC1ΔR1/R2, the region encoding the two 10-bp direct repeats, R1 and R2, had been deleted in the DNA fragment used. ParR was added in the following concentrations: 0, 12.5, 25, and 50 μM. B and C, gel shifts showing ParR binding to DNA fragments truncated in the region harboring R1 and R2. At each truncation, 10 bp was deleted. DNA fragments were incubated in the absence (−) or presence (+) of ParR (25 μM). Letters a–r refer to the respective DNA fragments used in the gel-shift experiments. B, truncation in the direction of par2. C, truncation in the direction of par1.

DISCUSSION

We show here that ParR regulates the parMR operon via binding to the two 10-bp R repeats upstream of parM and that ParB regulates the parAB operon via binding to the B repeats. This type of transcriptional regulation is typical for Type Ib and Type II loci (2, 9). Surprisingly, however, ParB regulates the parMR operon as efficiently as ParR. The B repeats in parC1 and parC2 bind ParB highly cooperatively in vitro (Fig. 4). The SPR analysis is consistent with one dimer of ParB binding to each of the 17 direct repeats in parC1 (Fig. 5A), in agreement with ParB behaving as a dimer in solution (16). At ParB concentrations higher than 100 nm, the SPR analysis yielded a biphasic response, indicating a two-stage binding process (Fig. 5B). The reason for the two-stage binding can only be speculated upon. One possibility is that ParB binds to the two types of B repeats (Fig. 1C) with different affinities. Another explanation that we favor assumes that all repeats are rapidly saturated and that ParB then extends from the repeats covered with ParB via ParB-ParB interactions. The formation of such higher order nucleoprotein complexes is consistent with the HMW bands seen in the gel-shift analysis (Fig. 4A). The gel-shift and SPR analyses are also consistent with the observation that ParB regulates P2 in vivo (Fig. 2) and in vitro (Fig. 3). The deletion analysis in Fig. 2B shows that a DNA fragment containing four B repeats is sufficient for ParB binding. We conclude that ParB recognizes the B repeats in parC1 and parC2 and thereby generates a nucleoprotein complex that functions in P2 promoter repression and likely plasmid partitioning. It was more surprising to learn that ParB also represses P1. However, this observation is consistent with the fact that the −10 and −35 sequences of P1 are embedded in the B repeats (Fig. 2). Thus, ParB binding to the B1−B4 repeats may prevent RNA polymerase to access P1 by steric hindrance.

ParR bound to the R1 and R2 repeats in parC1 in vitro (Figs. 6 and 7). ParR binding was also cooperative although considerably less avid than ParB binding to the B repeats. ParR did not
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FIGURE 8. Binding of IHF to ihf1 and ihf2 in par2. 32P-End-labeled DNA fragments were incubated in the absence (−) or with increasing concentrations of IHF. A, binding of IHF to a 200-bp DNA fragment containing ihf1. B, binding of IHF to a 200-bp DNA fragment containing ihf2. C, negative control, binding of IHF to parC1. D, positive control, binding of IHF to parS of P1. IHF concentrations: lane 1, 0 μM; lane 2, 31 μM; lane 3, 62 μM; lane 4, 93 μM; lane 5, 124 μM.

bind to the B repeats (Fig. 4Bc), and ParB did not bind to the R repeats (Fig. 6). ParR regulated P1 in vivo, consistent with the R operator sites located between P1 and the start of parM. ParR of plasmid R1 binds to 10 direct repeats in the parC locus (7) and thereby facilitates DNA pairing and repression of transcription (17, 18). It is likely that ParR of pB171 have similar functions.

Many divergent promoters have been described. In most cases, transcriptional interference between divergent promoters is positive (19, 20), and only few examples of negative interference are known (21). The deletion analysis presented in Fig. 2 shows that the P1 and P2 promoters are negatively coupled, i.e. one promoter affects negatively the activity of the other. Our analysis thus adds a new example to the short list of divergent promoters exhibiting negative interference. The mechanism of transcriptional interference is not known. Unexpectedly, deletion of the R repeats resulted in a highly increased, ParB-mediated repression-fold of P2 (Fig. 2B). The reason for this effect can only be speculated upon, but it is possible that RNA polymerase binding to the full-length fragment competes with ParB binding to B1–B4 and thereby reduces P2 repression.

Interestingly, IHF binds to two sites in par2 (Figs. 1, A and D, and 8). In plasmid P1, IHF binds to parS and induces a strong bend in the DNA (22, 23). The bend is likely to facilitate interactions between P1 ParB molecules bound to sequences in parS flanking the IHF site (23). Plasmid stability measurements showed that IHF had a moderate positive effect on par2 efficiency (data not shown). We speculate that binding of IHF to ihf1 and ihf2 might induce a bend in the DNA such as to spatially join parC1 and parC2 in a higher order partition complex.

Both par loci of pB171 are active, i.e. par1 stabilizes mini-R1 by ~15-fold, whereas par2 stabilizes mini-R1 by ~150-fold, showing that par2 is significantly more efficient than par1 (10). The higher efficiency of Type I par loci is correlated with a much broader phylogenetic distribution (5). Furthermore, ParB repressed par1 transcription as efficiently as ParR (Figs. 2 and 3). This result is consistent with the hypothesis that par2 is epistatic to par1.

It was pointed out by Eugene Koonin (24, 25) that almost all biological functions have evolved independently at least twice (the classical example is aminoacyl-tRNA synthetases with identical substrate specificities but unrelated tertiary folds) and that the corresponding analogous genes can replace each other in a process called nonorthologous gene displacement. The Walker box and actin partition ATPases belong to ancient, nonorthologous gene families and therefore must have evolved independently. This fact raises the possibility that Type I and Type II loci undergo nonorthologous gene displacement. The higher prevalence of Type I loci is correlated with a higher efficiency of plasmid stabilization. The double par locus of pB171 may thus represent an evolutionary snapshot revealing one par locus in the process of replacing another.

Even though most plasmids have one par locus only, plasmid pB171 is not the only plasmid that has two. Thus, plasmid R27 also has two par loci, par1 (Type I, encoding a Walker box ATPase) and par2 (Type II, encoding an actin ATPase) located several kilobases apart (26). As in the case of pB171, the Type I locus of R27 positioned plasmids at mid- and quarter-cell positions and was more efficient than the Type II locus. These observations are also consistent with the nonorthologous gene displacement hypothesis. To our knowledge, plasmids carrying two par loci of the same type have yet to be identified.

In conclusion, we show here that ParB binds to the B repeats in parC1 and parC2 and thereby is able to form the partition complex at which ParA acts. Likewise, ParR binds to the R repeats in parC1, thereby probably forming a paired plasmid complex at which ParM can act. Surprisingly, however, ParB repressed transcription of par1, indicating that par2 is epistatic to par1 and might possibly replace par1 by nonorthologous gene displacement.

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