Centromere Pairing by a Plasmid-encoded Type I ParB Protein*

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The par locus of Escherichia coli plasmid pB171 encodes two trans-acting proteins, ParA and ParB, and two cis-acting sites, parC1 and parC2, to which ParB binds cooperatively. ParA is related to MinD and oscillates in helical structures and thereby positions ParB/parC-carrying plasmids regularly over the nucleoid. ParB ribbon-helix-helix dimers bind cooperatively to direct repeats in parC1 and parC2. Using four different assays we obtain solid evidence that ParB can pair parC1- and parC2-encoding DNA fragments in vitro. Convincingly, electron microscopy revealed that ParB mediates binary pairing of parC fragments. In addition to binary complexes, ParB mediated the formation of higher order complexes consisting of several DNA fragments joined by ParB at centromere site parC. N-terminal truncated versions of ParB still possessing specific DNA binding activity were incompetent in pairing, hence identifying the N terminus of ParB as a requirement for ParB-mediated centromere pairing. These observations suggest that centromere pairing is an important intermediate step in plasmid partitioning mediated by the common type I loci.

Bacterial plasmids are useful model systems to study DNA segregation because they encode partition (par) loci that ensure active segregation of plasmid copies to each daughter cell before cell division (1–4). In general, par loci are organized as bicistronic operons that encode two trans-acting proteins and one or more cis-acting centromere-like sites (5). The first gene invariably codes for an ATPase, whereas the second gene codes for a protein that binds to specific sequences in the centromere(s) and forms the partition complex. The partition complex is recognized by the ATPase that actively moves and positions the plasmids via interactions with the partition complex (1, 3).

Prokaryotic par loci were divided into two unrelated classes (5): Type I par loci encode ATPases that have a variant of the Walker A box (e.g. ParA of P1 and pB171, SopA of F, ParF of TP228) (6), whereas Type II par loci encode ATPases that belong to the actin superfamily (ParM of R1) (7). Based on gene similarity, Type I ATPases were further divided into two subgroups (5); thus, type Ib ATPases (ParA of pB171, ParF) are generally smaller than the Type Ia ATPases (P1 ParA, SopA). Type Ia ATPases have an N-terminal DNA binding helix-turn-helix domain that is absent in the smaller type Ib ATPases. Type Ib ATPases bind to the par promoter region and thereby auto-regulate par operon transcription. In contrast, type Ib operon transcription is autoregulated by the cognate ParB proteins (8–10).

The molecular mechanism by which the type II par locus of plasmid R1 segregates plasmid DNA is well understood. Thus, ParM is an actin-like ATPase that interacts with the centromere-binding protein ParR. ParR is a ribbon-helix-helix (RHH)2 protein that binds cooperatively to 10 direct repeats in the parC centromere and forms the partition complex (11).3 When bound to parC, ParR mediates plasmid pairing in vitro (12). The subcellular symmetrical localizations of plasmids carrying par of R1 are consistent with paired plasmids as intermediates in the DNA segregation process (13, 14). ParM forms long, actin-like filaments that recognize the ParR/parC complex and push the plasmids to opposite cell poles (14–17). Thus, the type II par locus of plasmid R1 specifies a simple prokaryotic mitotic apparatus.

The molecular mechanism behind DNA segregation mediated by the common type I loci is less well understood. To better understand this process we analyze the type Ib par2 locus of plasmid pB171. This par locus encodes Walker Box ATPase ParA, DNA-binding protein ParB, and two centromere sites, parC1 and parC2, which flank the parAB operon. ParB binds cooperatively as dimers to 17 and 18 six-base pair direct repeats (iterons) in parC1 and parC2, respectively (8). Because the parAB promoter is located within parC1, ParB auto-regulates parAB expression (8).

ParA ATPase is related to MinD and oscillates over the nucleoid in spiral-shaped filamentous structures (18, 19) and has been suggested to form ATP-dependent filaments (20). Oscillation, but not filament formation, required the presence of both ParB and a ParB binding site (parC1 or parC2). Interestingly, the subcellular plasmid localization pattern mediated by par2 of pB171 is quite distinct from that mediated by par of R1; in cells with one or two plasmids, par2 positions plasmids at

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2 The abbreviations used are: RHH, ribbon-helix-helix; HMW, high molecular weight; BTH, bacterial two-hybrid; TB, transfer buffer; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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mid-cell or at quarter-cell positions, respectively. In cells with three or more plasmids, the plasmids are distributed regularly along the length of the nucleoid (20). Similarly, the ParA homolog of F (SopA) oscillates, forms filaments in vivo and in vitro, and distributes plasmids regularly within the cell (21, 22). It is reasonable to assume that ParA and SopA filament formation provides the physical driving force for the observed directional plasmid movement and positioning (19, 20, 22). The molecular mechanism that results in the observed regular plasmid distribution is not yet understood, but the evidence accumulating indicates that the unrelated type I and II par loci function by different molecular mechanisms.

The most popular models that have been proposed to explain plasmid partitioning involves partitioning via the par loci. So far, plasmid pairing has only been demonstrated directly (and only in vitro) for the type II par locus of plasmid R1 (12). We show here that ParB of plasmid pB171 is a dimeric protein that recognizes parC-encoding DNA fragments through its C-terminal RHH motif. Importantly, we obtain direct evidence that ParB mediates pairing of parC DNA fragments in vitro and assign this property of ParB to its N-terminal end.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Bacterial strains and plasmids are listed in Table 1.

**Construction of His<sup>6</sup>-ParBΔ19N and His<sup>6</sup>-ParBΔ39N Overexpression Plasmids** pSR112 and pSR113—Genes parBΔ19N and parBΔ39N were amplified by PCR from plasmid pGE223 using the following DNA primers: parBΔ19/N, B171-92 and B171-21; parBΔ39/N, B171-93 and B171-21. Upstream primers had six histidine codons between the start codon and the second codon of the ParB reading frames. PCR products were digested with BamHI and XhoI and cloned into the BamHI and PstI sites located downstream of the isopropyl 1-thio-β-D-galactopyranoside-inducible P<sub>AI/04/03</sub> promoter of plasmid pMG25, thus generating pSR112 (P<sub>AI/04/03;his<sup>6</sup>-parBΔ19/N) and pSR113 (P<sub>AI/04/03;his<sup>6</sup>-parBΔ39/N).

**TABLE 1**

| Strains/plasmids | Relevant genotype / description | Resistance | Primers used in PCR | Reference/source |
|------------------|--------------------------------|------------|---------------------|-----------------|
| Top10            | F<sup>−</sup> mcrA Δ(mrr-hsdRMS-mcrBC) Δ800lacZAM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU1 galK1 rpsL1 (Str<sup>α</sup>) endA1 supG | Amp        | Laboratory collection |                 |
| BTH101           | F<sup>−</sup>, cya-99 araD139 galE15 galK16 rpsL1 mcrA1 mcrB1 | Kan        | Laboratory collection |                 |

**Construction of Plasmids**—Bacterial Two-hybrid (BTH) Assay—To analyze protein-protein interactions between ParB, ParBΔ19N, and ParBΔ39N, we constructed plasmids expressing in-frame fusions to fragments of T18 and T25 of Bordetella pertussis adenylate cyclase. Genes parBΔ19N and parBΔ39N were amplified by PCR from plasmid pB171 using the following DNA primers: parBΔ19/N, B171-92 + B171-21; parBΔ39/N, B171-93 + B171-21. PCR products were cloned between Sall and Kpnl of pUT18C and between BamHI and Kpnl of pK25, thus resulting in the hybrid-expressing plasmids pK25-ParBΔ19N, pUT18C-ParBΔ19N, pK25-ParBΔ39N, and pUT18C-ParBΔ39N.

**BTH Assay**—Plasmids expressing fusion proteins to T18 and T25 fragments of B. pertussis adenylate cyclase were co-transformed into Escherichia coli strain BTH101. Cells were plated on LB (Luria-Bertani) medium agar plates containing 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5 mM isopropyl-1-thio-D-galactopyranoside, 100 μg/ml ampicillin, and 25 μg/ml kanamycin. Bacteria expressing interacting fusion proteins formed blue colonies, whereas bacteria expressing non-interacting fusion proteins resulted in white colonies (23). As a control we included pUT18C-Zip and pK25-Zip.

**Protein Purification**—E. coli strain Top10 harboring pGE223, pSR112, or pSR113 was grown in 2 liters of 2 X Tryp-Boose extract (Bacto) at 37°C to an A<sub>450</sub> of 0.5 at which recombinant protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mm. After induction, cells were incubated additional 4 h at 37 °C, then harvested and frozen in liquid nitrogen. Frozen cells were resuspended in 50 mM Na<sub>2</sub>PO<sub>4</sub> 300 mM NaCl, 30 mM imidazole, pH 8.0, and treated with DNase1 and egg white lysozyme (final concentration of 1 mg/ml of each) for 1 h followed by sonication. The lysate was cleared by centrifugation at 25,000 × g for 40 min at 4 °C. The cleared lysate was loaded onto a 5-ml HiTrap HP Ni<sup>2+</sup> column and washed comprehensively in lysis buffer. Protein was eluted in a linear gradient of
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50–500 mM imidazole in buffer (50 mM NaPO₄, 300 mM NaCl, pH 8.0).

Western Blotting—Increasing concentrations of ParB, ParBΔ19N, and ParBΔ39N were loaded on a Tricine-polyacrylamide gel (4% stacking and 12.5% separation) and run for ~1 h at 140 V. After completion of the PAGE, an Immobilon P membrane was saturated in 100% methanol followed by equilibration for 5 min in transfer buffer (TB; 39 mM glycine, 48 mM Tris base, 0.0375% SDS, 20% methanol). The gel was likewise equilibrated for 5 min in TB. Subsequent to the equilibration, a blotting sandwich was made composed of three pieces of Whatman No. 3MM paper soaked in TB, the equilibrated Immobilon P membrane, the equilibrated protein gel, and again 3 pieces of Whatman No. 3MM paper soaked in TB. All air bubbles between the different layers in the blotting sandwich were carefully removed. Proteins were then blotted and fixed onto the Immobilon P membrane in a semidy electrol blotter in a blotting sandwich with a current of 0.8 mA/cm² for 50 min. After electro-blot the membrane was blocked in TTBS with 5% low fat dry milk (TTBS: 0.05% Tween 20, 20 mM Tris-HCl, 137 mM NaCl, pH 7.6) for 4 h at room temperature and then washed 2 × 5 min in TTBS. Membranes were incubated for 2 h in TTBS containing 2% low fat dry milk and affinity-purified rabbit anti-ParB antibodies. Membranes were then washed 2 × 5 and 1 × 15 min in TTBS, then re-incubated in a 2500× dilution of the horseradish peroxidase-conjugated pig-α-rabbit IgG secondary antibody in TTBS containing 2% low fat dry milk for 1 h followed by washing for 1 × 15 and 4 × 5 min in TTBS. Membranes were then developed using chemiluminescence, the ECL + detection system (Amersham Biosciences) according to the manufacturer’s recommendations. Chemically treated membranes were wrapped in plastic foil, and films were developed in a darkroom.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay experiments were carried out essentially as described (8). Cy3- and Cy5-endlabeled PCR fragments were used throughout the experiments and were obtained using either a 5′-Cy3 or 5′-Cy5 end-labeled oligonucleotide in a PCR reaction with an additional oligonucleotide designed to obtain the construct of interest. We used a concentration of 5 nM DNA fragments throughout. The reactions were then incubated for 15 min at ambient temperature. ParB, ParBΔ19N, and ParBΔ39N were added in the following concentrations: 0.66, 1.33, and 2.66 µM. After incubation, glycerol was added to a concentration of 5%, and reactions were analyzed by electrophoresis on a 0.5× Tris borate-EDTA (TBE, pH 7.5) 5% polyacrylamide gel in 1×TBE running buffer (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA, pH 8.3) at 150 V for 1.5 h. The gel was scanned on a Typhoon Trio instrument (Amersham Biosciences). Primers used in PCR: Cy3-parC1 DNA: B171-130 and B171-131-Cy3; Cy3-parC2 DNA: B171-133 and B171-134-Cy3; Cy5-pUC DNA: 171SR14-Cy5 and 171SR16.

Fluorescence Polarization—Fluorescence polarization measurements were carried out with a PerkinElmer LS55 fluorescence spectrometer. Fluorescein end-labeled 155-bp DNA fragments were obtained using a 5′-end-labeled oligonucleotide in a PCR reaction. Two different DNA fragments were tested: (i) a parC1 encoding fragment (primers B171-140-fluorescein and B171-39) and (ii) as a negative control a nonspecific pUC DNA fragment (primers 171SR14 and 171SR17-fluorescein). Binding studies were carried out by titrating ParB, ParBΔ19N, or ParBΔ39N into 390 µl of reaction buffer 20 mM Tris base, pH 7.5, 100 mM KCl, 2 mM MgCl₂, and 2.5 nM labeled DNA. Samples were excited at 492 nm, and emission was measured at 520 nm using an excitation and emission slit width of 10.0 nm and an integration time of 1.0 s.

Ligation Frequency Assay—DNA fragments used were amplified by PCR from plasmid pB171. For each assay we constructed two DNA fragments of different length labeled with Cy3 and Cy5. PCR fragments were then digested for 3 h using StuI or HincII to create blunt-ended fragments ready for ligation. The standard reaction mixture contained 15 µl of 20 mM Tris-base, pH 7.5, 100 mM KCl, 2 mM MgCl₂, and 0.1 µg/µl sonicated salmon sperm DNA. Cy3- and Cy5-labeled DNA was added to at concentration of 5 nM each. ParB, ParBΔ19N, and ParBΔ39N were added in concentrations as indicated in the figure legends. Reactions were incubated for 15 min at ambient temperature. Then 2 µl of 10× ligation buffer and 5 units of T4 DNA ligase were added. Water was added to obtain a total volume of 20 µl, and reactions were incubated an additional 2 h at ambient temperature. DNA was purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and analyzed by PAGE on a 5% gel which was scanned on a Typhoon Trio instrument (Amersham Biosciences). Primers used in PCR: Cy3-parC1: B171-130 and B171-131-Cy3 (Stul); Cy5-parC1: B171-116 and B171-132-Cy5 (HincII); Cy3-parC2: B171-133 and B171-134-Cy3 (Stul); Cy5-parC2: B171-135-Cy5 and B171-136 (Stul).

Electron Microscopy; Rotary Shadowing—A 1324-bp PCR product encoding the parC1 region was constructed using the primer pair B171-SP4 and B171-SP10. Standard reactions for rotary shadowing were performed in a total volume of 15 µl of 20 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 90 ng of parC1 DNA was added and, when indicated in the figures ParB, ParBΔ19N, or ParBΔ39N was added to a concentration of 20–1000 nM. The samples were incubated 5 or 15 min at room temperature and diluted 20-fold in reaction buffer. 3-µl samples were applied for 30 s on glow-discharged carbon-coated grids and rinsed with 0.002% uranyl acetate, blotted dry with filter paper, and further dried with a hairdryer. Rotary shadowing was performed in an Edwards E306A coating system using a platinum source, which was tilted in an angle of 6° to the sample at a sample-to-source distance of 8 cm. Electron microscopy was carried out at 80 kV using a Philips EM208 transmission electron microscope. Images were obtained at a magnification of 20,000–50,000-fold. Negatives were scanned using a MRC–KZA scanner.

Oligonucleotides Used for PCR—Oligonucleotides used for PCR are listed in Table 2.

RESULTS

The C-terminal RHH Motif of ParB Mediates ParB Dimerization and DNA Binding—Inspection of the ParB sequence revealed a RHH motif in the C terminus that exhibits similarity with the DNA recognition motif of the dimeric transcriptional repressor CopG (Fig. 1A). The C terminus of ParB also exhibits similarity with ParG, a ParB homolog encoded by...
TABLE 2
DNA oligonucleotides used for PCR

| Oligonucleotide name | Sequence |
|---------------------|----------|
| B171-21             | 5'-CCCCTTCACTTACATGTTATTTTAAATGATTG-3' |
| B171-39             | 5'-CCCAAACCCCTCTAATTTGCTGTCGACGTTG-3' |
| B171-56             | 5'-CCGCCCTCGGCTGACGTCGACGTTG-3' |
| B171-92             | 5'-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-93             | 5'-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-122            | 5'-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-123            | 5'-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-130            | 5'-AGCCCTCACTTACATTGCCACCAATATTCA-3' |
| B171-131-Cy3        | 5'-Cy3-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-132-Cy5        | 5'-Cy5-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-133            | 5'-CCCCAGGCCTACCTTTGTTCCCACCAACTACA-3' |
| B171-134-Cy3        | 5'-Cy3-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-135-Cy5        | 5'-Cy5-CCCCCGTGATCTGCTGACGTTG-3' |
| B171-136            | 5'-CCCCAGGCCTACCTTTGTTCCCACCAACTACA-3' |
| B171-140-fluorescein| 5'-Fluorescein-ACCATGATTACGCCAAGCTTGCATGC-3' |
| B171-SP4            | 5'-GTGCCGCCAATATCAACTACCAGCG-3' |
| B171-SP10           | 5'-GTGCCGCCAATATCAACTACCAGCG-3' |
| 171SR14             | 5'-GTCGGCGACGTTCTCTGTC-3' |
| 171SR14-Cy5         | 5'-GTCGGCGACGTTCTCTGTC-3' |
| 171SR16             | 5'-GTCGGCGACGTTCTCTGTC-3' |
| 171SR17-fluorescein | 5'-Fluorescein-ACCATGATTACGCCAAGCTTGCATGC-3' |

**FIGURE 1.** C-terminal RHH motif dimerizes ParB. A, alignment of ParB with ParG of TP228 (10) and CopG of pLS1 (24). Green boxes indicate α-helices, whereas purple boxes indicate β-sheets. B, non-denaturing Western blot of full-length and truncated ParB showing that all three species dimerize. Protein concentrations were 1, 1.5, and 2.0 μM. C, non-denaturing Western blot of full-length and truncated ParB showing that all three species dimerize. Protein concentrations were 1, 1.5, and 2.0 μM.

**TABLE 3**
BTH analysis of ParB derivatives

| Protein | ParB | ParBΔ19 | ParBΔ39 |
|---------|------|---------|---------|
| Dimer   |      |         |         |
|         | 1    | 3       | 2       |
| Lane    | 7    | 9       |         |
| Protein | ParB | ParBΔ19 | ParBΔ39 |
| Monomer |     |         |         |
|         | 1    | 3       | 2       |
| Lane    | 7    | 9       |         |

Salmonella newport plasmid TP228 that also contains a RHH motif in its C terminus (10). Perhaps unexpectedly, the similarity between ParB and ParG is less than that between ParB and CopG (Fig. 1A).

We constructed two N-terminal truncated versions of ParB (ParBΔ19N and ParBΔ39N) in which the 19 and 39 N-terminal amino acid residues were deleted (Fig. 1B). Non-denaturing Western blotting analysis showed that purified ParB, ParBΔ19N, and ParBΔ39N formed dimers (Fig. 1C). ParB self-interaction in vivo was investigated by BTH analysis. ParB interacted with itself (Table 3). Moreover, ParBΔ19N interacted with itself and both ParB and ParBΔ39N. Similarly, ParBΔ39N interacted with itself and both ParB and ParBΔ19N (Table 3). Thus, as with other members of this class of DNA-binding proteins, the RHH domain mediates dimerization of ParB (10, 24–26). As a control we included pUT18C-Zip and pKT25-Zip are positive controls.

Dimers of full-length ParB bind cooperatively to the 17 and 18 six-base pair repeats in parC1 and parC2 (8). To directly assess the ability of the truncated ParB derivatives to bind parC1 and parC2, we performed electrophoretic mobility shift assays with Cy3-labeled DNA fragments encoding parC1 and parC2 (Fig. 2). As a negative control, a Cy5-labeled nonspecific DNA fragment was included in the binding reactions. Full-length ParB shifted parC1 and parC2 very efficiently even at...
very low concentrations (Fig. 2, A and B). Interestingly, full-length ParB and the parC-encoding fragments formed high molecular weight nucleoprotein complexes almost incapable of migrating into the gel (Fig. 2, A and B), also seen in a previous analysis (8). By inference, the HMW bands consists of parC DNA and ParB. ParBΔ19N and ParBΔ39N specifically bound to parC1 (Fig. 2A) and parC2 (Fig. 2B) at concentrations similar to that of full-length ParB. However, the shortened versions of ParB did not result in the formation of HMW complexes, indicating that the N-terminal 19 amino acids of ParB are required for this phenomenon.

To verify the electrophoretic mobility shift assay results, we measured fluorescence polarization of fluorescein-labeled parC1 DNA or control DNA in the presence of increasing concentrations of ParB, ParBΔ19N, and ParBΔ39N (Fig. 2C). As seen, ParB, ParBΔ19N, and ParBΔ39N specifically increased the fluorescence polarization signal from parC1 DNA. Thus, this result confirms that the C-terminal end of ParB containing the RHH motif is sufficient for specific DNA binding. Importantly, the change in fluorescence polarization signal was significantly higher for full-length than for the truncated ParB versions, perhaps reflecting the formation of HMW complexes similar to those seen in the gel-shift analysis.

ParB Stimulates Ligation of parC1-encoding DNA Fragments—We speculated that the HMW complexes described above might reflect a ParB-mediated ligation reaction of parC-DNA. To pursue this further, we employed a DNA ligation frequency assay. If ParB pairs parC-DNA, then the proximity of the DNA ends in the presence of ParB will increase the frequency by which T4 DNA ligase ligates parC-encoding fragments into dimers (due to the end-labeling method used, only dimers can form in this assay) (12, 27). In the assay we used two fragments of different length that were labeled at their ends with Cy5 (long fragment) or Cy3 (short fragment), respectively (Fig. 3). Blunt-ended DNA fragments were incubated with T4 DNA ligase in the presence or absence of ParB. In the presence of T4 DNA ligase, three different ligation products were expected: (i) Cy5-parC1-Cy5-parC1 (green), (ii) Cy5-parC1-Cy3-parC1 (yellow) and (iii) Cy3-parC1-Cy3-parC1 (red). Due to the different sizes of the two DNA fragments, the presence of a yellow band with a mobility between those of (i) and (iii), a mix of Cy5 and Cy3 signal, confirms that the three bands in fact are ligation products. The addition of T4 DNA ligase alone mediated the formation of the expected three binary ligation products (lane 2 in Figs. 3, A, C, and D). As seen, the addition of increasing amounts of ParB resulted in an increased DNA ligation frequency (Fig. 3A). Quantification of the reaction revealed that the ligation frequency depended highly on the ParB concentration, especially at low ParB concentrations (Fig. 3B). As expected, ParB without T4 DNA ligase did not stimulate ligation (Fig. 3C). In a further control ParB did not stimulate the ligation of a linear pUC DNA fragment (Fig. 3D), hence demonstrating the specificity of the assay.

The N-terminal End of ParB Is Required for Stimulation of parC DNA Ligation—Next we tested ParBΔ19N and ParBΔ39N in the DNA ligation assay. Again, full-length ParB significantly stimulated the ligation frequency assay reaction (compare Fig. 4A, lanes 5 and 6 with lane 2). By contrast, ParBΔ19N increased the ligation frequency considerably less than full-length ParB (Fig. 4A, lanes 9 and 10), and ParBΔ39N did not stimulate ligation at all (Fig. 4A, lanes 13 and 14).

ParB also increased the ligation frequency between two different parC2 DNA molecules (compare Fig. 4B, lanes 5 and 6 with lane 2). Again, ParBΔ19N stimulated the ligation frequency less than full-length ParB (Fig. 4B, lanes 9 and 10), and ParBΔ39N had no effect (Fig. 4B, lanes 13 and 14), hence supporting the involvement of ParB N terminus in parC2 DNA pairing. To test if ParB also could stimulate the ligation of two different parC regions (parC1 and parC2), we used a mixture of parC1- and parC2-encoding DNA molecules. As seen in Fig. 4C, the effects of full-length and truncated versions of ParB were very similar to those described for homologous parC regions. Fig. 4D shows a quantification of the ligation reactions in Fig. 4, A–C, to further underpin the involvement of the N terminus of ParB in centromere pairing.

ParB Mediates Centromere Pairing in Vitro—The above results are consistent with the proposal that ParB can pair DNA molecules encoding parC1 or parC2 centromeres. To obtain more direct support for this suggestion, we performed rotary shadowing electron microscopy imaging of the ParB-parC1 complex (Fig. 5). For the imaging, we used a 1324-bp DNA
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FIGURE 3. ParB stimulates ligation of parC DNA fragments by T4 ligase. Shown are ligation products in the presence (concentration bar) or absence (−) of ParB. Two blunt-ended parC DNA fragments of different length were used; the longest was Cy5-labeled (green), and the shortest was Cy3-labeled (red). Five different bands were observed and are indicated by arrows. A, effect of ParB on T4 DNA ligase-mediated ligation. ParB was added in the concentrations 0, 0.033, 0.066, 0.133, 0.266, 0.400, 0.533, 0.66, 1.33, 2.00, 2.66, and 4.00 μM. The slower moving green and red bands represent ligation products between two Cy5-labeled and two Cy3-labeled parC fragments, respectively, whereas the yellow band represents ligation products between Cy5- and Cy3-labeled parC fragments. B, amount of ligated product in Fig. 3A plotted versus the ParB concentration. Band intensity was analyzed using ImageQuant 5.0 software. The amount of ligated product in the absence of ParB was normalized to 100. Values are the averages of three independent experiments. C, control showing the effect of ParB in the absence of T4 Ligase. ParB was added at 0, 2.00, 2.66, and 4.00 μM. D, control showing that ParB does not stimulate ligation of control DNA (pUC fragment). ParB was added at 0, 2.00, 2.66, and 4.00 μM.

fragment with the parC1 region close to the center. The addition of ParB resulted in the formation of a nucleoprotein complex at the expected location on the DNA (Fig. 5B). No such complexes were observed in the absence of ParB (Fig. 5A) or with control DNA lacking parC1 (data not shown).

In addition to single DNA molecules with ParB bound at parC1, we also observed DNA molecules connected via ParB bound to parC1 in large nucleoprotein complexes (Fig. 5, C–L). At 200 nM ParB and 7 nM parC1 DNA, 64% of DNA molecules were monomeric, and 36% were assembled in paired and higher order complexes (Fig. 6). Of the 36%, a third were joined as di-, tri-, or quaterners, whereas two-thirds were joined in higher order complexes. At lower ParB concentrations (e.g. 50 nM) almost all complexes were binary, whereas at higher ParB concentrations (e.g. 1000 nM), almost all parC fragments were present in higher order complexes, consistent with the HMW complexes seen in the gel-shift analysis (Fig. 2). The ParB-mediated complexes joined at parC consisted of both uneven (Fig. 5G, three joined molecules) and even (Fig. 5f, four joined molecules) numbers of DNA molecules.

N-terminal End of ParB Is Required for Centromere Pairing—Finally, we performed rotary shadowing electron microscopy imaging of mixtures containing parC1 DNA and ParBΔ19N or ParBΔ39N. In both cases we observed nucleoprotein complexes at the expected location on the DNA (Fig. 7, A–D), similar to the parC1 complexes seen with full-length ParB. This observation supports the conclusion that the C-terminal RHH domain of ParB binds to parC DNA. However, the N-terminal truncated versions of ParB did not mediate parC DNA fragment pairing, even at high protein concentrations. Thus, ParB dimerization and centromere binding requires its C-terminal end containing the RHH-motif, whereas ParB-mediated centromere pairing requires its N-terminal end.

DISCUSSION

Previously, we showed that ParB of plasmid pB171 binds cooperatively as a dimer to the 6-bp direct repeats in the parC1 and parC2 centromere regions (8). Here we present direct evidence that ParB pairs parC1 DNA fragments in vitro (Fig. 5) and that the pairing reaction requires the N-terminal 19 amino acids of ParB (Figs. 4–6). DNA fragment-pairing was correlated with the formation of HMW complexes (Fig. 2), the ability to increase anisotropy (Fig. 2), and the ability to stimulate the ligation of parC-encoding DNA fragments (Figs. 3 and 4). Thus, we conclude that ParB mediates centromere pairing in vitro and that its N-terminal end is required for pairing. How ParB-mediated centromere pairing occurs is not known. Fig. 8A presents a working model invoking that ParB binds DNA as a dimer, that the C-terminal RHH-motif is responsible for dimerization and DNA binding, and that the N terminus is free to facilitate centromere pairing (Fig. 8, B and C). We do not exclude that ParB adopts a configuration that favors centromere pairing when bound to the repeats in parC. However, the repetitive nature of the ParB dimers bound to DNA may also itself favor the pairing reaction.

We also attempted to obtain evidence for ParB-mediated plasmid-pairing in vivo using a ParB overproduction assay. However, even slight overproduction of ParB inhibited replication of the parC1-encoding test plasmid, and the results were not conclusive (data not shown). Thus, direct evidence for partition-mediated plasmid pairing in vivo is still lacking. However, a number of additional observations supports that ParB proteins encoded by type I par loci mediate plasmid pairing. First, ParB of plasmid P1 is a dimer that contains three separate DNA binding sites, one formed by the dimer interface and two helix-turn-helix motifs, one in each monomer (2). These individual DNA binding sites rotate freely about a flexible linker, enabling them to bridge adjacent DNA duplexes. Thus, the
structure of P1 ParB-parS is consistent with the proposal that ParB pairs two separate DNA molecules. Experimentally, it has been very difficult to obtain direct evidence for the existence of paired plasmid complexes in vivo. However, an elegant study from Michael Yarmolinsky's laboratory (28) yielded evidence supporting that P1 par mediates plasmid pairing. These
authors used plasmid dimers containing two parS sites. Induction of ParB in the presence of novobiocin resulted in the accumulation of positive supercoils in the plasmid. Several control experiments, including plasmids with two LacI binding sites, suggested that the change in plasmid supercoiling was best explained by ParB-mediated intramolecular pairing between two parS sites. Recently, it was shown that SopB (the ParB homolog of F) stimulates the formation of multimers of plasmids carrying sopC (29). The multimers were formed by homologous recombination, and this observation is, therefore, consistent with the suggestion that SopB pairs sopC-carrying plasmids.

We also show that ParB of pB171 binds to DNA via its C-terminal RHH domain (Figs. 1 and 2). The RHH domain mediates ParB dimerization (Fig. 1C), as in the cases of other RHH proteins (10, 24–26). Dimerization via the RHH domain is consistent with the observation that ParB dimers bind stoichiometrically to the direct repeats in parC1 and parC2 (8).

S. newport plasmid TP228 encodes the Type Ib partition locus parFGH. ParF is an ATPase that in vitro forms filaments in the presence of ATP (30). ParG (the ParB homolog) is a dimer with two intertwined C termini with a RHH architecture and two unstructured N termini (10). The unstructured N termini of ParG stimulate ParF ATPase activity, filamentation, and filament bundling in vitro (30). Recently, it was shown that the N-terminal arginine 19 of ParG was crucial for in vitro stimulation of ParF ATPase activity (31). Arg-19, however, was not essential for stimulation of ParF filamentation or bundling. Thus, the N-terminal end of ParG possesses two distinguishable functions in vitro. We speculate that ParB of pB171 may play a similar role in ParA filament dynamics. Here we have described an additional function for the N-terminal end of ParB; that is, its requirement for ParB-mediated parC centromere pairing.

It is not yet known how type I par loci secure plasmid segregation before cell division. However, the observations described here and elsewhere suggest that centromere pairing is an indispensable intermediate in the process. Recently, we described a model for how the oscillating ParA ATPase encoded by par2 of pB171 mediates regular distribution of plasmids over the nucleoid (20). In that model ParA filaments push apart par2-carrying plasmids, and the oscillation of the ParA filaments averages the force exerted on each plasmid, thereby conferring the observed regular distribution. It is, thus, possible that plasmids paired by ParB/parC are intermediates upon which the dynamic ParA filaments act, thereby securing that the plasmids are moved in opposite directions. Two other models have been forwarded to explain how a filament-forming and oscillating ATPase can segregate plasmid DNA (21, 22). None of these models, however, invoke plasmid pairing as an intermediate step.
In a recent cytological study we showed that two different replicons (R1 and F) both carrying par2 of pB171 did not pair at a detectable level (32). The lack of pairing and the concomitant random order of the two different plasmids distributed along the length of the cell yielded a novel explanation for partition-mediated incompatibility (two plasmids are incompatible when they cannot coexist stably in a cell line).

The lack of pairing of two different plasmids carrying the same par locus may seem to contradict the results shown here. One explanation may be that pairing actually does occur but that it is too short-lived to be detected with conventional microscopic techniques. Another explanation might be that pairing occurs solely between homologous replicon molecules immediately after replication. Thus, when a plasmid carrying parC has replicated, ParB binds immediately to the two new parC sites and pairs the sister replicons. These sister replicons are then separated by the oscillating and filament forming ParA ATPase. Further experiments are required to discriminate between these possibilities.
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