Molecular Anatomy of ParA-ParA and ParA-ParB Interactions during Plasmid Partitioning*

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Background: Small homodimeric δ₂ (ParA) and ω₂ (ParB) and parS mediate pSM19035 partitioning.

Results: The δ₂ ATPase is a modular protein.

Conclusion: δ₂ and δ₂-ω₂ interacting domains are juxtaposed.

Significance: ATP, nonspecific DNA, and ω₂ bound to parS induce multiple transitions on δ₂.

Firmicutes multidrug resistance inc18 plasmids encode parS sites and two small homodimeric ParA-like (δ₂) and ParB-like (ω₂) proteins to ensure faithful segregation. Protein ω₂ binds to parS DNA, forming a short left-handed helix wrapped around the full parS, and interacts with δ₂. Protein δ₂ interacts with ω₂ and, in the ATP-bound form, binds to nonspecific DNA (nsDNA), forming small clusters. Here, we have mapped the ω₂-δ₂ and δ₂-δ₂ interacting domains in the δ₂ that are adjacent to but distinct from each other. The δ₂ nsDNA binding domain is essential for stimulation of ω₂-parS-mediated ATP hydrolysis. From the data presented here, we propose that δ₂ interacts with ATP, nsDNA, and with ω₂ bound to parS at near equimolar concentrations, facilitating a δ₂ structural transition. This δ₂ “activated” state overcomes its impediment in ATP hydrolysis, with the subsequent release of both of the proteins from nsDNA (plasmid unpairing).

Faithful segregation of newly replicated genomes to daughter cells requires specialized partitioning machinery. In eukaryotic cells, the well known microtubule-based mitotic spindle apparatus, which guides the separating chromosomes to the different ends of the dividing cell, drives the chromosome segregation process (1, 2). In contrast, the mechanism of chromosome separation and plasmid partition in bacterial cells is not well understood. Three main types of systems have been described that ensure faithful partition of low copy number plasmids (reviewed in Refs. 3–6). Of these, the ParAB system is the most widespread in plasmids and is the only type present also in bacterial chromosomes (3–6). Unraveling the molecular basis of this plasmid partitioning system is therefore important to help us to understand chromosomal segregation.

The ParAB partition system requires three or more components: two trans-acting dimeric proteins (ParA and ParB), one or more cis-acting parS site(s), and, in most cases, the host’s chromosomal DNA (3–6). The Walker box ParA ATPases can be subdivided into two distinct families based on their size or structure. The large (Lg)3 Walker box ATPases (e.g. Escherichia coli P1-ParA (Lg) or F-SopA (ParA-Lg) plasmids) bind and hydrolyze ATP, and then ParA-ADP specifically binds to its cognate site to regulate expression of the ParAB locus. When hydrolysis is delayed, ParA-ATP* binds nonspecific DNA (nsDNA), forming a carpet on the DNA in vitro (7–11). This gradient-like distribution of the ParA protein on the nucleoid is an essential step for faithful plasmid segregation in vivo (12). The small (Sm) Walker-box ParA ATPases, which lack the specific DNA binding domain, can be divided into two subgroups. The first group includes Streptococcus pyogenes pSM19035-δ₂ and Bacillus subtilis chromosome-encoded Soj (ParA-Sm), which in the ATP-bound form bind to nsDNA (13, 14). DNA binding by the small ParA ATPases (pSM19035-δ₂ and Soj) is a critical step for faithful segregation of plasmids and chromosomes (13, 15). In contrast, the small ParA ATPases of the second group (e.g. Salmonella Newport TP228-ParF plasmid) form bundles in the absence of DNA (16, 17).

The structurally unrelated ParB centromere-binding proteins can also be classified into two groups. The first group includes large dimeric helix-turn-helix ParB proteins (e.g. P1-ParB, F-SopA, and chromosome-encoded Spo0J) that, upon binding to parS, spread over many kb, bridging, looping, and condensing the nsDNA (18–22). The second group includes the small dimeric ribbon-helix-helix ParB-like proteins (e.g. TP228-ParG and pSM19035-ω₂). Protein ω₂, upon site-specific binding to parS DNA, which comprise 7–10 contiguous iterons or heptads (Fig. 1A), assembles as a left-handed helix wrapped around the full parS site without significant spreading onto nsDNA and without distortion of the parS DNA (14, 23–25).

The interaction of the ParA and ParB components, which leads to proper separation of plasmid copies, has been extensively studied in plasmids of the γ-Proteobacteria class. These studies provide the foundation for filament- and non-filament-based modes of plasmid segregation. In the filament-based modes, ParA (Sm), when bound to ATP, assembles into fila-

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3 The abbreviations used are: Lg, large; DSS, disuccinimidyl suberate; IF1 and IF2, interface 1 and 2, respectively; nsDNA, non-specific DNA; Sm, small; ATPγS, adenosine 5'-O-(thiotriphosphate); PDB, Protein Data Bank.
ments or bundles, and the partition complexes are mobilized by linear contractile filaments reminiscent of the spindle mechanism in eukaryotes (thread pushing or pulling model) (Fig. 2A) (16, 17). Alternatively, ParA (Sm), in the ATP-bound form, assembles by forming nucleoprotein filaments, and the partition complexes are mobilized by helical or linear filaments as a cargo (filament-pulling model) (Fig. 2B) (26, 27). In the non-filament-based models (diffusion-ratchet and DNA relay models), ParA dimers or small oligomers bind to the nucleoid (Fig. 2C) (9–12, 28, 29). In the diffusion-ratchet model, a propagating ParA (Lg) ATPase gradient is the driving force for movement of the partition complexes (9–11), whereas in the DNA relay model, the forces that drive segregation are generated by a ParA (Sm) gradient and the elastic forces within the DNA molecule (29). The segregation machinery of plasmids and bacteria of the Firmicutes phylum, which are evolutionarily separated by more than 1,500 million years from Proteobacteria (a genetic distance larger than the one between humans and plants), is less understood. It is likely that the characterization of the mechanism of active partitioning of low copy number plasmids of the Firmicutes phylum might contribute to the general understanding of ParAB-mediated plasmid and chromosome segregation in bacteria.

Plasmids of the ubiquitous inc18 vancomycin-, methicillin-, or erythromycin-resistant family encode two small homodimeric ParA-like (δ2) and ParB-like (ω2) proteins and 2–6 parS sites in order to ensure faithful segregation (30). Plasmid pSM19035 is the best characterized member of this family. The pSM19035 parS sites overlap the promoter regions of the copS, δ, and ω genes (Fig. 1A) (25, 31–33). In vitro, ω2 and δ2ΔN19, which lacks the unstructured N-terminal region, transiently bind with high affinity and cooperatively (KD(app) = 5 ± 1 nM) to parS DNA, forming short-lived partition complexes (14, 32).

The structure of δ(−14) (having 14 extra N-terminal residues) bound to ATPγS and Mg2+ includes all 284 residues of the WT δ protein (15). ATP-bound δ2S possesses a U-shaped structure, with one arm and a part of the joining region representing a monomer (Fig. 1B). The Walker domains face the cleft, and the bottom of the U-shaped dimer is negatively charged, whereas the C-terminal upper tips are positively charged (Fig. 1B) (15). Previous mutagenesis data showed that the nsDNA binding domain lies at the tip of the arm (Fig. 1B) (14). Protein δ2 shows a low but significant degree of sequence identity with the P1-ParA2 (Lg) (~24%) and the Soj (ParA-Sm) (~22%) ATPases, but the sequence identity drops to <20% when compared with the TP228-ParF (ParA-Sm) ATPase. However, the quaternary structures of these four ParA ATPases, three from Gram-negative and one from Gram-positive bacteria, are conserved (Fig. 1C).

Protein δ2 binds to nsDNA, forming short dynamic complexes. These complexes have a blob shape containing up to 5 ± 1 δ2/blob rather than forming filaments or nucleoprotein filaments (15, 24). Likewise, free δ2 in solution (i.e. in the absence of nsDNA) forms discrete blob-shaped structures containing 2–3 δ2 molecules rather than long bundles (24).

In vitro experiments have shown that at substoichiometric ω2/δ2 molar ratios, the interaction of apo-δ2 with the unstructured N-terminal end of ω2 bound to parS DNA increases the binding affinity of ω2 for parS DNA by ~8-fold, leading to a long-lived complex (14). Protein δ2 bound to nsDNA, upon interacting with a ω2-parS complex, loses its association with nsDNA and relocates with ω2-parS to form a ternary complex (parS/ω2/δ2) (24). In vivo, however, the interaction of a ternary (parS/ω2/δ2) complex with a dynamic δ2/nsDNA complex leads to plasmid-nucleoid pairing (parS/ω2/δ2/nsDNA) (34). At equimolar ω2/δ2 molar ratios, however, ω2 stimulates δ2-mediated ATP hydrolysis, causing subsequent dislodging of δ2 from paired complexes, via a poorly understood mechanism (15).

At present, the δ2 regions involved in oligomerization and in the interaction with ω2 are poorly defined, and they have been mapped in this work. We show here that the δ2/ω2 and δ2/δ2 interacting domains are adjacent to but distinct from each other and suggest some functional transitions that are crucial for the activation of the δ2 ATPase activity.

**Experimental Procedures**

**Strains and Plasmids**—The B. subtilis strain and the plasmids used for segregation studies are indicated in Table 1. The plasmids used for overexpression of proteins were propagated in E. coli BL21(DE3) (pLysS) and are listed in Table 1.

The δ gene encodes two co-linear polypeptides, a 298-residue (δ(−14)) and a WT 284-residue product. The plasmid pCB746-borne WT δ gene was used for site-directed mutagenesis and discrete deletions from the N- and C-terminal regions. The exposed and positively charged Lys residues at the C-terminal end of δ2 were substituted by Ala (δ2K242A, δ2K259A/K260A) or Ser (δ2K248S), and the negatively charged residue 211 was replaced by Ala (δ2D211A), as described previously (34). Many of the initially designed C-terminal deletion mutants were insoluble. To overcome this inconvenience, δ2 variants containing or lacking residues within random coil regions around the initially programmed C-terminal deletion were constructed. Finally, the codons at positions 255, 227, 197, and 164 of the WT δ gene were fused to His6 codons, leading to a series of constructs truncated from the 3′-end.

**Plasmid Copy Number and Plasmid Stability Test**—The number of plasmid copies per cell was estimated by quantitative PCR. Normalization was done with two distinct chromosomal genes as described earlier (35). The number of plasmid-containing cells was determined by replica-plating onto chloramphenicol-supplemented LB plates. The frequency of plasmid loss was calculated as described previously (35).

**Chemicals, Enzymes, Proteins, and DNA**—All chemicals were pro-analysis grade and purchased from Roche Diagnostics (Mannheim, Germany). DNA restriction and modification enzymes and nucleotides were from New England Biolabs (Frankfurt, Germany) and Sigma-Aldrich (Madrid, Spain). Ultrapure acrylamide was purchased from Serva (Heidelberg, Germany). The ω2 or δ2 variants were purified as described for...
WT \( \omega_2 \) and \( \delta_2 \) proteins (15, 31, 33). pBC30-borne \( \text{parS} \) DNA (source of \( \text{parS} \) DNA) was purified as described (31). \( \text{parS} \) DNA is expressed as moles of DNA molecules, and this was estimated using a molar extinction coefficient of 6,500 M\(^{-1}\) cm\(^{-1}\) at 260 nm. The protein concentrations were determined by absorption at 280 nm using molar extinction coefficients of 2,980 M\(^{-1}\) cm\(^{-1}\) for \( \omega_2 \) and 38,850 M\(^{-1}\) cm\(^{-1}\) for \( \delta_2 \) and its variants. Concentrations of all the proteins are expressed as moles of protein homodimers. All structural images were generated using the PyMOL Molecular Graphics System, version 1.5.0.4 (Schrödinger, LLC).

**Protein Cross-linking**—The cross-linking agent DSS was used to study protein-protein interactions as described previously (36). Cross-linking was performed by incubating \( \text{parS} \) DNA with \( \delta_2 \) or its mutant variants in buffer B (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10 mM MgCl\(_2\)) supplemented or not with 1 \( \mu \)M ATP for 15 min at 37 °C. The assays were performed in the presence (+) or absence (−) of \( \omega_2 \) prior to the addition of the indicated concentrations of DSS at various \( \omega_2/\delta_2 \) molar ratios. Alternatively, \( \text{parS} \) DNA was preincubated with one of the proteins (5 min, 37 °C), and the preformed complex was incubated with the second protein (5 min). Then DSS was added, and reactions were left for 10 min at 37 °C and then stopped by the addition of 10 \( \mu \)l of stop buffer C (50 mM Tris-HCl, pH 7.5, 400 mM glycine, 3% β-mercaptoethanol, 2% SDS, 10% glycerol) and subjected to SDS-PAGE. Cross-linked protein bands were excised from the gel and identified by tryptic digestion coupled to MALDI-TOF-TOF analysis as described (14).

**Protein-DNA Complexes**—For EMSA, gel-purified 423-bp \( \alpha-\text{32P} \)-labeled HindIII-KpnI \( \text{parS} \) DNA was incubated with various amounts of WT \( \omega_2 \), WT \( \delta_2 \) (or its variants), or both proteins together in buffer B containing or lacking 1 \( \mu \)M ATP or 1 \( \mu \)M ADP for 15 min at 37 °C in a 20-μl final volume. The reaction was stopped by the addition of loading buffer (1 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol), and the samples were subjected to 4% or 6% PAGE. Gel electrophoresis was conducted using 1 × TAE as running buffer, at 200 V, 4 °C, and the gels were dried prior to autoradiography.

To obtain \( K_{D_{\text{app}}} \) values, the concentration of free DNA and protein-DNA complexes was densitometrically determined under non-saturating conditions from differently exposed autoradiographs of EMSA gels. Protein concentrations that transfer 50% of the labeled DNA into complexes are approximately equal to the \( K_{D_{\text{app}}} \) under conditions where the DNA concentration is much lower than the \( K_{D_{\text{app}}} \).

**ATPase Activity Assays**—The ATPase activity was assayed by thin layer chromatographic separation of the reaction products as described (15). The indicated proteins in the presence or absence of \( \text{parS} \) DNA were incubated for 180 min at 37 °C in buffer B containing 5 mM ATP in a 20-μl reaction mixture.

**Results**

The C-terminal Region of \( \delta_2 \) Contributes to Plasmid Segregation—To investigate the contribution of the C-terminal region of \( \delta_2 \) to faithful partitioning, a plasmid stability test was used. A set of plasmid-borne WT and mutant \( \omega_2 \) and \( \delta_2 \) genes, transcribed from their native promoters (P), termed \( P_\omega \) (\( \text{parS} \)) and \( P_\delta \) (\( \text{parS} \)) (Fig. 1A), were tested. Under the growth conditions used in this study, each cell contained, on average, ∼8 ± 1 plasmid copies, and the different mutations (see Table 1) did not affect plasmid copy number (data not shown).

As already observed, the \( \omega \) and \( \delta \) gene products, the presence of at least one \( \text{parS} \) site, \( \omega_2\delta_2 \) interaction, ATP binding and hydrolysis, and the nsDNA binding domain of \( \delta_2 \) were necessary and sufficient for stabilization of an otherwise unstable plasmid in \( B. \) subtilis cells (Table 2) (15, 37).

In the filament pulling model, the polymerization onto nsDNA as shown for pB171-ParA2 (Sm) is essential (26). The structure and mutagenesis analysis of the symmetric \( \delta_2 \) ATPase revealed that nsDNA binding domain occurs at the tip of the U (Fig. 1B) (14). If \( \delta_2 \) polymerizes on nsDNA, it is likely that one of the external arms (e.g. the negatively charged C-terminal end) of \( \delta_2 \) might be in close proximity with the opposite arm (e.g. the positively charged N-terminal end) of another \( \delta_2 \) (Figs. 1B and 2B). To test this hypothesis, \( \delta_2 \) variants lacking the N- (\( \delta\Delta N20 \)) or C-terminal (\( \delta\Delta C255 \)) regions were constructed. Protein \( \delta\Delta C255 \) also lacks the putative IF2 domain predicted to be involved in bundle formation (Fig. 2A) (17).

When the \( \delta_2 \) gene was replaced by the \( \delta\Delta N20 \) variant, whose product lacks the first 20 residues, or by \( \delta\Delta C255 \), whose product lacks the last 29 residues (15), plasmid segregation was only marginally affected by ∼1.5- and 3-fold, respectively (Table 2). However, when the \( \delta_2 \) gene was replaced by the \( \delta\Delta C197 \) or \( \delta\Delta C164 \) genes, which not only lacked the negatively charged C-terminal domain but also lacked the nsDNA binding domain, faithful plasmid segregation was abolished (Table 2). These results suggest that the DNA binding domain is essential, but the N- and C-terminal ends are dispensable for accurate plasmid partitioning. These data were not consistent with the filament-based models (Fig. 2, A and B).

The \( \delta \) Dimer of Dimer Interacting Domain Does Not Overlap with the nsDNA Binding Domain—To further evaluate the contribution of the C-terminal region to the different activities of \( \delta \) (i.e. ATPase, nsDNA binding, and \( \omega_2\delta_2 \) interaction), the exposed and positively charged Lys residues at the C-terminal end of \( \delta_2 \) (\( \delta K242A, \delta K248S \), and \( \delta K259A/K260A \)) were substituted by Ala or Ser, the genes were cloned, and their products were overexpressed and purified (Fig. 3). To ascertain the importance of these basic residues for DNA binding, the exposed and negatively charged Asp-211 residue was replaced by Ala. The mutated gene was cloned, and its product (\( \delta D211A \)) was purified.

In the presence of ATP, dimers and higher order oligomers were observed with \( \delta D211A, \delta K242A \), or \( \delta K248S \) upon the addition of the DSS cross-linking agent, but \( \delta K259A/K260A \) only formed dimers (Fig. 3A) (14).

Proteins \( \delta K242A, \delta K248S \), and \( \delta K259A/K260A \) bound and hydrolyzed ATP at efficiencies comparable with the WT \( \delta_2 \) or \( \delta D211A \) (Fig. 3B). The addition of \( \omega_2 \) bound to \( \text{parS} \) DNA significantly stimulated the \( \delta_2 \), \( \delta D211A \), \( \delta K242A \), or \( \delta K248S \)-mediated ATP hydrolysis ∼4-fold (Fig. 3A). However, the stimulation was less evident (∼1.7-fold) in the case of the \( \delta K259A/K260A \) variant.

Wild-type \( \delta_2 \) binds nsDNA with a \( K_{D_{\text{app}}} \) of 140 ± 31 nM, and \( \delta_6D60A \), which binds but does not hydrolyze ATP, binds nsDNA with a \( K_{D_{\text{app}}} \) of 75 ± 19 nM (Table 3) (14, 15). ATP\textsubscript{\text{S}}
**FIGURE 1.** Relevant genome organization, critical residues for δ₂ interaction with nsDNA, and structural comparison of ParA ATPases. A, genome organization of the duplicated part of plasmid pSM19035. The rep (involving copS, RNA III, repS, and the leading (oriS) and lagging (ssAI) replication origins) and segB loci (segB1 (δ, ω, and ε) and segB2 (δ, ω, and parS sites, P_{copS}, P_ω, and P_ε)) are highlighted. The promoters (P), the mRNAs, and the genes of the relevant regions are shown and denoted as boxes, wavy lines, and rectangles, respectively. The upstream region of the promoters of the copS (P_{copS}), δ (P_δ), and ω (P_ω) genes, which constitute the cis-acting centromere-like parS sites, are enlarged. The variable number of contiguous 7-bp iterons (heptads) and their relative orientation are symbolized by arrows (→ or ←). The promoters repressed by ω₂ (double red spheres) are indicated. B, mapping of the nsDNA binding domain in δ₂. Electrostatic potential surface representation of δ₂ in the ATP·P·Mg²⁺-bound form (PDB code 2OZE) displayed using PyMOL. The surface charge of δ₂ is negative near the bottom of the U and positive at the tips of the arms of the U. The relevant α-helices involved in nsDNA binding (in green) map to the tips of the arms of its U-shaped structure. Relevant residues for nsDNA binding, residue Asp-60 (involved in ATP hydrolysis), and ATP·P·Mg²⁺ for one of the monomers are indicated, but they are repeated twice in the dimer structure. A lateral view highlighting the U-shaped form of δ₂ is shown. C, superimposition of full-length monomer structures of δ (in green) and *T. thermophilus* Soj (ParA-Sm) (in blue) (PDB code 2BEK), with P₁-ParA (in yellow) (PDB code 3EZ6) and with TP228-ParF (ParA-Sm) (in orange) (PDB code 3KOH). The superimpositions were done using PyMOL.
or ADP failed to promote δ2 binding to nsDNA (Table 3). Removal of a negatively charged residue (δ2D211A) from the nsDNA binding domain increased binding to nsDNA with K_D(app) of 25 ± 1.5 nM (Table 3). Removal of positively charged residues (δ2K242A, δ2K248S, or δ2K259A/K260A) reduced binding to nsDNA as measured by EMSA (K_D(app) of >1.6 μM) (Fig. 3, C and D). However, electron microscopic analysis showed that the δ2K242A mutant bound to the nsDNA with an efficiency comparable with WT δ2, which suggests that the mutation does not affect the on rate of protein-nsDNA complex formation (34). It is likely that the δ2K242A-nsDNA complex has a higher off rate than the WT δ2-nsDNA complex (see below).

Table 2

| Par genes | ParB-like | ParA-like | Stability |
|-----------|-----------|-----------|-----------|
| None      | None      | <1"      |
| δ         | δ         | <1"      |
| ω         | ω         | <1"      |
| δN19      | δ         | 100<1°   |
| ω         | δK36A     | <1"      |
| ω         | δD60A     | <1"      |
| ω         | ΔΔN20     | 67.8     |
| ω         | ΔΔC255    | 29.4     |
| ω         | ΔΔC197    | <1       |
| ω         | ΔΔC164    | <1       |

* This information was reported previously (15), but the experiments were reproduced here for comparison.

Deletion of the nsDNA Binding Domain of δ Does Not Affect Dimer-Dimer Interaction.—The δ genes coding for the previously mentioned deletion variants from the C-terminal end (Table 1) were cloned, and their products were overexpressed and purified. Proteins δ2ΔC255 (which lacks the negatively charged C-terminal end and part of the predicted IF2 (17)), δ2ΔC227 (which also lacks the ATP-interacting residues), δ2ΔC197 (which also lacks the nsDNA binding domain), and δ2ΔC164 (which also lacks part of the hypothetical IF1 region) were analyzed under in vitro conditions (Fig. 4A).

Effect of the different variants of the pSM19035 partitioning system in the faithful segregation of an unstable vector

Effect of the different variants of the pSM19035 partitioning system in

TABLE 2

| B. subtilis strains | Genotype/relevant properties | Source |
|---------------------|-------------------------------|--------|
| pCB30               | pUC18-borne parS2 (Pp)        | (35)   |
| pT712               | expression vector             | (33)   |
| pT712o              | pT712-borne ω                 | (33)   |
| pT712o ΔN19         | pT712-borne ΔN19              | (33)   |
| pCB746              | pT712-borne δ                 | (15)   |
| pCB855              | pT712-borne AD60A             | (15)   |
| pCB853              | pT712-borne ΔK242A            | (14)   |
| pCB854              | pT712-borne ΔK242S            | This work |
| pCB857              | pT712-borne ΔK259K260A        | This work |
| pCB869              | pT712-borne M119C D189C       | This work |
| pCB913              | pT712-borne ΔM255             | This work |
| pCB914              | pT712-borne ΔM227             | This work |
| pCB915              | pT712-borne ΔM197             | This work |
| pCB916              | pT712-borne ΔM164             | This work |

* Mid-copy number (7–9 copies/cell) pH14 derivatives bearing the δ and ω genes (or their variants) under the control of their own promoters (Pp (parS2) and Pp (parS2); see Fig 1).

The ATPase activity of the δ2 variants (δ2ΔC227, δ2ΔC197, and δ2ΔC164) was not stimulated by the addition of parS DNA or parS bound to ω2 (Fig. 4C). Upon addition of ω2 bound to parS DNA, the ATPase activity of δ2ΔC255 was slightly stimulated (Fig. 4C). This is consistent with crystallographic data that show that three δ residues, Lys-238, Ser-240, and Tyr-265, which map to the C terminus, are also involved in ATP binding. Ser-240 forms hydrogen bonds with the adenosine, and Lys-238 and Tyr-265 form hydrogen bonds with the amino group of the two ATP γS molecules in the δ2-ATP γS structure (see below) (15). Thus, all three residues have activities that are crucial for full δ2-mediated ATP hydrolysis, and δ2ΔC255, which only lacks Tyr-265, shows an intermediate phenotype (Fig. 4C).

The δ2ω2 Interacting Domain Does Not Map to the C-terminal End of δ2.—Protein ω2 binds to parS, forming transient ω2-parS complexes (K_D(app) of 5 ± 1 nM, half-life <1 min) (24, 32). The interaction of ω2 bound to parS with δ2 or apo-δ2 led to longer-lived ω2-parS complexes (K_D(app) 0.7 ± 0.1 nM, half-life ~34 min) (14). It is likely that a poorly defined region of δ2ω, upon interacting with the unstructured N-terminal domain of ω2, facilitates structural transitions that enhance the stability of ω2-parS complexes (14). To test whether ω2 interacts with δ2 through its C-terminal domain and whether such protein-protein interaction facilitates the binding of δ2 or ω2 to parS DNA, two different approaches were undertaken. First, in the presence of ATP, preformed ω2-parS DNA complexes were incubated with limiting δ2, δ2K242A, δ2K248S, or δ2K259A/K260A concentrations. The preformed ω2-parS complexes enhanced ternary complex formation with WT δ2 (ω2-parS δ2) to K_D(app) 5 A. Volante and J. C. Alonso, unpublished results.
40 ± 4.2 nm as compared with $K_{D(app)}^{\omega_2} = 140 ± 31$ nm with $\delta_2$-nsDNA alone (Table 3). When WT $\delta_2$ was replaced by $\delta_2$K242A, $\delta_2$K248S, or $\delta_2$K259A/K260A, the preformed $\omega_2$parS complexes enhanced the formation of ternary complex from $K_{D(app)}^{\omega_2}$ of >1600 nm to $K_{D(app)}^{\delta_2}$ of 40–85 nm (Table 3). Second, in the presence of limiting $\omega_2$ concentrations (0.75 nm), C-terminal deletion mutants, which lacked the DNA binding domain, cannot form ternary complexes but may help $\omega_2$ to bind to parS DNA. In the presence of limiting $\omega_2$, $\delta_2$, $\delta_2$C255, $\delta_2$C227, $\delta_2$C197, or $\delta_2$C164 enhanced $\omega_2$parS complex formation from $K_{D(app)}^{\omega_2}$ of 5 ± 1 nm to $K_{D(app)}^{\delta_2}$ of $\approx$0.75 nm (Fig. 4, D and E). The interaction of $\omega_2$ with $\delta_2$C164 facilitated the formation of $\omega_2$parS complexes, but these protein-DNA complexes were found to be less stable in comparison with the WT complex (Fig. 4, E and F). These results suggest that the deletion mutants still interact with $\omega_2$ and facilitate $\omega_2$parS complex formation. To compare these results, the data presented in Fig. 4, D and E, were quantified. As revealed in Fig. 4F, the concentration of $\delta_2$ (or its mutant) sufficient to facilitate $\omega_2$parS DNA complex formation was significantly enhanced, with a $K_{D(app)}^{\omega_2}$ of 30–50 nm, which is 3–5-fold below the $K_{D(app)}^{\delta_2}$ of 140 ± 31 nm. It is likely, therefore, that the $\delta_2$ variants interact with $\omega_2$ with an efficiency similar to that of WT $\delta_2$ (Fig. 4F). These data altogether support the conclusion that (i) interaction of the preformed $\omega_2$parS DNA complex with $\delta_2$K242A, $\delta_2$K248S, or $\delta_2$K259A/K260A facilitates ternary complex formation; (ii) the C-terminal region of $\delta_2$ (residues 164–284) is dispensable for interaction with the $\omega_2$parS DNA complexes; (iii) removal of the 120 C-terminal residues from $\delta_2$ still enhances $\omega_2$parS complex formation, but the complexes formed are less stable (Fig. 4E); and (iv) the $\delta_2$ concentration required for interaction with $\omega_2$ bound to parS DNA (Fig. 4, D and E) or to facilitate $\omega_2$parS complex formation (Fig. 4F) is 3.5–4-fold lower than for interaction with nsDNA.

The Central Domain of $\delta_2$ Interacts with $\omega_2$. To identify the region(s) of $\delta_2$ involved in the interaction with $\omega_2$, both proteins were cross-linked in vitro by DSS in the presence of parS DNA, and the different protein bands were gel-purified (Figs. 5A (a–g) and 6A (a–f), subjected to limited proteolysis, and analyzed by MALDI-TOF-TOF as described previously (14).
With this technique, under high sequence coverage, the regions not involved in protein-protein interaction should be proteolyzed by trypsin, and the generated polypeptides should be detected with the expected molecular mass. On the other hand, if trypsin does not reach the covalently bound protein-protein regions, the expected polypeptide(s) should be absent. Alternatively, the interacting polypeptides, due to cross-linking among them, will have a higher molecular mass and therefore

FIGURE 3. The $\delta_2$ nsDNA binding domain and the $\omega_2$, $\delta_2$, or $\delta_2$, interacting domain do not overlap. A, $\delta_2$ variants form dimers in solution. Equal amounts of $\delta_2$, $\delta_2$D211A, $\delta_2$DK242A, $\delta_2$DK248S, or $\delta_2$K259A/K260A (3 $\mu$g) were preincubated for 5 min at 37 °C in buffer B. Then increasing concentrations of DSS (0.01, 0.05, 0.1, 0.25, and 0.5 nM) were added, and the reaction was further incubated for 10 min. The products of the cross-linking reactions were separated by 15% SDS-PAGE. Running positions of the monomer (1), dimer (2), trimer (3), and tetramer (4) are indicated. B, ATPase activity of $\delta_2$ protein and its variants ($\delta_2$D211A, $\delta_2$K242A, $\delta_2$K248S, and $\delta_2$K259A/K260A) alone (1 $\mu$M; white bars), in the presence of $\omega_2$ (1.5 $\mu$M; gray bars), or in the presence of both $\omega_2$ and parS DNA (1.5 $\mu$M and 25 nM, respectively; black bars). Values are the average of three or more independent experiments, and error bars indicate S.E. C and D, binding to nsDNA of the different $\delta_2$ variants. The 423-bp $\alpha^32$P-labeled parS DNA (0.1 nM) was incubated with increasing amounts of $\delta_2$ (0.009–0.3 $\mu$M), $\delta_2$K242A and $\delta_2$K248S (0.15 nM to 2.4 $\mu$M), and $\delta_2$K259A/K260A (0.3–4.8 $\mu$M) for 15 min at 37 °C, in buffer B containing 1 mM ATP, and complexes were detected by EMSA and autoradiography.
observed (Fig. 5 and followed by MALDI-TOF-TOF analysis (Fig. 6). Indeed, in the crystal structure, a tide coverage corresponding to the 139–215 and 242–265 were not detected (Fig. 5). The cross-linked product, we cannot rule out the possibility that the cleavage site at position 116. Because we have not identified the ωδ complexes might be stabilized by residues 47–52 of ω, because this region was also not detected when both proteins were incubated (Fig. 5B). When δ peptides missing in the gel-purified 40 kDa band e were compared with those of bands d (δ) and f (ω) (coverage >60%), the two discrete segments, encompassing residues 88–119 and 216–223, were also not detected, suggesting that these regions may correspond to the ω-interacting region (Fig. 5, B and C). Within the 88–119 missing interval, there is a potential trypsin cleavage site at position 116. Because we have not identified the cross-linked product, we cannot rule out the possibility that the 88–119 or the 88–116 region could be targeted by ω. Consistent with this, the region consisting of residues 88–116 or 88–119, comprising the α1–α6 interval, is surface-exposed in the δ2 structure (Fig. 5C).

Protein δ2ΔC227 was incubated with ωparS DNA, and then DSS was added. A novel band of ~34 kDa (denoted as band e), which was composed of one ω and one δC227 monomer, was observed at substoichiometric ω:δC227 ratios (Fig. 5A, lanes 7 and 8). In band e, four discrete regions of δ and two of ω were not observed by MALDI-TOF-TOF analysis (Fig. 5B). When the peptides absent in this gel-purified 34 kDa band were compared with those absent in bands d and f, again the region detected previously with the WT protein (88–119 or 88–116 interval) was missing. The other potentially interacting region, the 216–223 interval, was present when δ2 was replaced by δ2ΔC227. Therefore residues 216–223, which were previously attributed to DNA binding, were not further evaluated. It is likely that the main ωδ interacting domain maps to the central region of δ2 or δ2ΔC227 (residues 88–119; the α4, α5, β3, β4, and 89%), in the presence of nsDNA (Fig. 5 and lanes 3 and 4). When the order of addition was altered or the relative molar ratio of ω/δ was modified (3:1 or 0.3:1), bands e (as in Fig. 5A) and e′ were observed (Fig. 6A, lanes 3 and 6). Limited trypsin proteolysis and MALDI-TOF-TOF analysis of this gel-purified band e (~40 kDa; Figs. 5A and 6A) showed that this novel polypeptide was composed of ω (7.9 kDa) and δ (32.6 kDa) cross-linked in a 1:1 stoichiometry with sequence coverage of 65.8% for δ and 81.3% for ω. The gel-purified band e′ (~48 kDa) (Fig. 6A, lanes 3 and 6) was composed of two ω monomers and one δ.

When bands d and f (~32 and ~64 kDa), which corresponded to δ monomers and dimers, respectively, were analyzed, six (band d) and seven (band f) discrete regions of δ were not observed. In these bands, the sequence coverage was 57.0 and 54.5%, respectively (Fig. 5B). Of these, only one discrete region, encompassing residues 1–7, was not observed in the ~64-kDa protein (band f). The δ polypeptides encompassing residues 31–36 (Walker A), 67–71 (Walker A’), 116–119, 139–215, 240–265, and 275–284 were not detected in bands d and f.

In the presence of preformed ωparS DNA complexes, at about stoichiometric ωδ/δ ratios, δ and ω formed a new discrete band e (Fig. 5A, lanes 3 and 4). When the order of addition was altered or the relative molar ratio of ω/δ was modified (3:1 or 0.3:1), bands e (as in Fig. 5A) and e′ were observed (Fig. 6A, lanes 3 and 6). Limited trypsin proteolysis and MALDI-TOF-TOF analysis of this gel-purified band e (~40 kDa; Figs. 5A and 6A) showed that this novel polypeptide was composed of ω (7.9 kDa) and δ (32.6 kDa) cross-linked in a 1:1 stoichiometry with sequence coverage of 65.8% for δ and 81.3% for ω. The gel-purified band e′ (~48 kDa) (Fig. 6A, lanes 3 and 6) was composed of two ω monomers and one δ.

When band e was analyzed, six discrete regions of δ and two discrete regions of ω were not observed (Fig. 5B). Polypeptides 1–10 and 47–52 (coverage >77%) of ω were missing. We propose that the unstructured N-terminal region of ωb bound to parS, upon interaction with δ2 (Fig. 5B), undergoes a structural transition that might involve α-helix formation, as suggested by protein structure predictions (34). These ωδ complexes might be stabilized by residues 47–52 of ω, because this region was also not detected when both proteins were incubated (Fig. 5B). When δ peptides missing in the gel-purified 40 kDa band e were compared with those of bands δ (δ) and f (ω) (coverage >60%), the two discrete segments, encompassing residues 88–119 and 216–223, were also not detected, suggesting that these regions may correspond to the ω-interacting region (Fig. 5, B and C). Within the 88–119 missing interval, there is a potential trypsin cleavage site at position 116. Because we have not identified the cross-linked product, we cannot rule out the possibility that the 88–119 or the 88–116 region could be targeted by ω. Consistent with this, the region consisting of residues 88–116 or 88–119, comprising the α1–α6 interval, is surface-exposed in the δ2 structure (Fig. 5C).

Protein δ2ΔC227 was incubated with ωparS DNA, and then DSS was added. A novel band of ~34 kDa (denoted as band e), which was composed of one ω and one δC227 monomer, was observed at substoichiometric ω:δC227 ratios (Fig. 5A, lanes 7 and 8). In band e, four discrete regions of δ and two of ω were not observed by MALDI-TOF-TOF analysis (Fig. 5B). When the peptides absent in this gel-purified 34 kDa band were compared with those absent in bands d and f, again the region detected previously with the WT protein (88–119 or 88–116 interval) was missing. The other potentially interacting region, the 216–223 interval, was present when δ2 was replaced by δ2ΔC227. Therefore residues 216–223, which were previously attributed to DNA binding, were not further evaluated. It is likely that the main ωδ interacting domain maps to the central region of δ2 or δ2ΔC227 (residues 88–119; the α4, α5, β3, β4, and

TABLE 3

| Protein | Nucleotide cofactor | Addition of ωδ | Kωδapp, kM |
|---------|-------------------|---------------|------------|
| δ2b     | None              | No            | >3,000     |
| δ2b     | ATP               | No            | >3,000     |
| δ2b     | ADP               | No            | >3,000     |
| δ2b     | ATP               | Yes           | 40 ± 4.2   |
| δ2Δ600b | ATP               | No            | 72 ± 19    |
| δ2Δ600b | ATP               | Yes           | 20 ± 5.3   |
| δ2D211A | ATP               | No            | 25 ± 1.5   |
| δ2D211A | ATP               | Yes           | 12 ± 0.9   |
| δ2K242A | ATP               | No            | ~1,000     |
| δ2K242A | ATP               | Yes           | 50 ± 2.4   |
| δ2K248S | ATP               | No            | ~1,700     |
| δ2K248S | ATP               | Yes           | 45 ± 6.8   |
| δ2K292A/K260A | ATP | Yes | >3,000     |

a A linear (α32P-labeled) 423-bp-long HindIII-KpnI parS2 DNA (0.1 nM) segment containing seven hexadeps ((→→→→→→→)→) (see Fig. 1) was incubated with increasing concentrations of the indicated protein for 15 min at 37 °C in buffer B containing or not containing 1 mM ATP, ATPβS, or ADP, and the Kωδapp was determined. The Kωδapp values (kM) are the average of at least three independent experiments and are within a 10% S.E.

b δ2 binding to DNA was reported elsewhere (14, 15) and determined here again for a direct comparison. When indicated, parS2 DNA was preincubated with ωδ (24 nM), and then increasing concentrations of the indicated δ2 were added, and δ2-mediated complex formation or ternary complex formation was analyzed. Samples were separated by 6% PAGE, and the formation of parSωδ-δ2 or δ2parSωδ parS complexes was quantified as described under "Experimental Procedures."
FIGURE 4. The δ DNA binding domain is not essential for interaction with ω2, and for dimer-dimer interaction. A, constructed C-terminal deletions of δ. The Walker A, A′, and B boxes are highlighted in red, and the nDNA binding domain is highlighted in green, and the hypothetical IF1 and IF2 regions are denoted by dotted lines. The ω2·C255 and δ·C197·ΔC164 interacting domains mapped in this report are shown in purple. The position of the last residue present in each variant is indicated. B, proteins δ, δ·ΔC255, δ·ΔC227, δ·ΔC197, or δ·ΔC164 (3 μg) formed dimers and higher order oligomers in solution when incubated with the cross-linking reagent DSS (0.5 mM), as shown by 15% SDS-PAGE. C, ATPase activity of δ, and its variants (δ·ΔC255, δ·ΔC227, or δ·ΔC197 and δ·ΔC164) alone (1 μM white bar), in the presence of ω2 (1.5 μM gray bar), or in the presence of both ω2 and parS DNA (1.5 μM and 25 nM, respectively; black bar). D and E, interaction of δ variants with ω2 and parS DNA. In D, the 423-bp α·32P-labeled HindIII-KpnI parS DNA (0.1 nM) was incubated with different amounts of ω2 (0.75 and 6 nM), a fixed amount of δ·ΔC255, or δ·ΔC227 (140 nM) for 15 min at 37 °C, in buffer containing 1 mM ATP. In E, α·32P-labeled parS DNA was incubated with a fixed amount of δ·ΔC197 or δ·ΔC164 (140 nM) for 15 min at 37 °C in buffer B containing 1 mM ATP. Protein-DNA complexes were detected by EMSA. The free DNA (FD) and the formed complexes are indicated. F, the δ DNA binding domain is not essential for interaction of ω2 with parS DNA. The percentage of ω2·parS DNA complex formed in the presence of limiting ω2 concentrations (0.75 nM) and increasing concentration of δ·ΔC164 is shown. Values are the average of three or more independent experiments.
and α6 interval) and is oriented opposite the face of the DNA binding domain (Figs. 5C and 7C). In contrast, using yeast or bacterial two-hybrid systems, it was suggested that the ParA-ParB interacting domain of the F-SopA (ParA-Lg) or the (pSM19035-Δc27) ParA ATPase maps to the C-terminal end (i.e., residues 198–284) (38, 39). Because the previous results suggested that Δc27 still interacts with ω or with itself is highlighted in the crystal structure in purple (α4-α5-β3-β4-α6). In this structure, ATP-S-Mg²⁺ is shown in orange and yellow, and residue Asp-60 (Walker B') is shown in green (PDB 2OZE, displayed using PyMOL). The experiments were performed more than three independent times.

The Central Region of Δ₂ Is Involved in Dimer-Dimer Interface—To define the region(s) of Δ₂ involved in dimer formation, Δ₂ was preincubated with preformed ω₂-parS DNA complexes, the ternary complexes were treated with DSS, and the newly covalently bound protein was analyzed as described above (Fig. 5A, termed band g). A comparison of bands g (~98 kDa, Δ₂-Δ) with bands d (~32 kDa, Δ) and f (~64 kDa, Δ₂) revealed that one discrete region, encompassing residues 68–119, was not detected in band g (Fig. 5B). This region constitutes the α3–α6 interval in the crystal structure and is an exposed surface distant from the DNA binding domain (Figs. 5B and 7C). However, the 68–119 interval partially overlaps the ω6 interacting domain (residues 88–119; interval α4–α6) (Figs. 5B and 7C). The Δ₂-Δ interacting region is close to the ATP binding and hydrolysis domains (Walker A' (residues 51–61) and Walker B (residues 142–147)) (Fig. 7, A and C).

Discussion

pSM19035 partitioning, which depends on the dynamic interaction between the Δ₂ ATPase, ω₂-parS, and the host chromosome, is a multistep process with discrete functional and/or structural transitions necessary for faithful segregation. The small Walker box ATPase Δ forms dimers that are stabilized by

FIGURE 5. A central domain of Δ₂ is required for ω₂-Δ₂ interaction. A, DSS cross-linking assay showing the interaction between ω₂ (1 μg) and Δ₂ (or Δ₂Δc27), at 0.5:1 and 1:1 ω₂/Δ₂ ratios, in the presence of parS DNA. Cross-linked products were separated by 10–15% SDS-PAGE. Bands a–g were gel-purified and subjected to limited trypsin proteolysis, followed by MALDI-TOF-TOF identification of the polypeptides. Δ, the identified peptide sequences are shown in light (ω) or dark gray (Δ) boxes, and the missing regions are shown in dotted lines. C, the relevant region of Δ involved in the interaction with ω or with itself is highlighted in the crystal structure in purple (α4-α5-β3-β4-α6). In this structure, ATP-S-Mg²⁺ is shown in orange and yellow, and residue Asp-60 (Walker B') is shown in green (PDB 2OZE, displayed using PyMOL). The experiments were performed more than three independent times.
a hydrophobic patch that occupies about 2197 A²/subunit of otherwise solvent-accessible surface and augmented by two reciprocal intersubunit salt bridges formed between Arg-119 and Asp-189 of each monomer (see Ref. 15). Protein δ₂₂, upon binding to nsDNA, forms discrete clusters on the nsDNA (4–6 δ₂₂/blob) (24), perhaps by interacting through the 68–119 region with other δ₂ molecules (see Fig. 7, A and C). In contrast, using a bacterial two-hybrid assay, the δ monomer-monomer interface was mapped to residues 104–105 (39), and using yeast two-hybrid and immunoprecipitation assays, the chromosome-encoded Pseudomonas aeruginosa ParA (Sm) monomer-monomer interface was mapped to an interval equivalent to residues 89–105 of δ₂ (40). To reconcile the apparent discrepancy between the crystal structure of δ₂₂-ATPγS-Mg²⁺ (15), our data (Fig. 5B), and those published by other groups (39, 40), we propose that the bacterial or yeast two-hybrid assays cannot discriminate between the monomer and the dimer interface and that the authors were mapping the dimer-dimer interface (see Fig. 7A, center).

The interaction of the N-terminal region of ω₂ (residues 1–9) with the central region of δ₂ (residues 88–119) leads to a discrete ternary (ω₂-parS-δ₂) complex (Figs. 5C and 7A (left) and C). However, if parS DNA was omitted or ω₂ was replaced by ω₂ΔN19, such protein-protein interaction was not observed (14, 33). We propose that the unstructured N-terminal region of ω₂ bound to parS, upon interaction with δ₂₂, undergoes a structural transition with residues 1–9 folding into an α-helix, as predicted by different protein folding prediction programs (34). Such interaction, at stoichiometric ω₂ and δ₂₂ concentrations, may facilitate δ₂₂-mediated ATP hydrolysis and δ₂₂ disassembly from the paired (parS-ω₂-δ₂₂-nsDNA) complexes. In the presence of an excess of ω₂, the ATPase activity of δ₂₂ is inhibited (15), and the plasmid can be engaged in a new pairing complex with a δ₂ in the nucleoid.

While the functional characterization of the ω₂-δ₂ interacting domains was being carried out during this study, the interacting domain of chromosome-encoded P. aeruginosa ParA (Sm) with ParB (Lg) was published (40). These authors mapped the ParB-ParA interacting region to within an interval equivalent to residues 89–105 of δ₂, by yeast two-hybrid and immunoprecipitation assays (40). This is in good agreement with the ωδ interacting region reported here (Fig. 5B). However, it was unexpected because equivalent regions in both small ParA ATPases share very little sequence identity (<15%). Furthermore, plasmid- and chromosome-encoded large ParB proteins, which are of the helix-turn-helix family, bind specifically and nonspecifically to DNA and spread over many kb, leading to bridging, looping, and condensing of the nsDNA (18–21). Con-

FIGURE 6. A central domain of δ is required for δ-δ and δ₂-δ₂₂ interaction. A, the δ₂-δ₂₂ interaction requires the presence of ω₂ and parS DNA. Preformed ω₂-parS DNA (ω₂ (3 µg), lanes 1–3) or δ₂₂-parS DNA complexes (lanes 4–6) were incubated with δ₂ or ω₂ as indicated (at a 3:1 ω₂/δ₂ ratio in lane 3 or at a 0.5:1 ω₂/δ₂ ratio in lane 6), and the specific interactions were analyzed using DSS. Cross-linked products were resolved by 10–15% SDS-PAGE. The stained protein bands e (~40 kDa) and e’ (~48 kDa) were gel-purified and subjected to limited trypsin proteolysis and MALDI-TOF-TOF analysis of the polypeptides. Tetrameric to hexameric complexes are marked (lanes 4–6). B, δ-δ interaction in the absence of nsDNA. The DSS cross-linked product (lane 2) was separated by 10–15% SDS-PAGE. Band h was gel-purified and subjected to limited trypsin proteolysis, followed by MALDI-TOF-TOF identification of the polypeptides. C, the identified peptide sequences are shown in boxes, and the missing regions are shown in dotted lines. The N- and C-terminal regions are denoted.
versely, the plasmid-encoded small ParB proteins (e.g. \( \omega_2 \)), which are ribbon-helix-helix proteins, specifically interact with \( \text{parS} \) without significant spreading (24). It is likely that the unrelated \( \text{P. aeruginosa} \) ParB (Lg) and \( \omega_2 \) might expose an equivalent domain recognized by an equivalent region in the ParA (Sm) ATPase.

The results presented here are inconsistent with the ParAB filament-based (thread-pushing or -pulling (Fig. 2A)) or filament-pulling (Fig. 2B) models. In the thread-pushing or -pulling model, the central (IF1; see Fig. 4A) and the C-terminal regions (IF2) of the TP228-ParF (ParA-Sm) (Fig. 7B) should be involved in bundle formation, as depicted in Fig. 2A (16, 17). However, \( \delta_2\Delta C164 \), which lacks IF2 and part of IF1, forms dimers and small oligomers (Fig. 4B). We have shown by atomic force microscopy that upon ATP binding, \( \delta_2 \) free in solution forms discrete blob-shaped structures containing 2–3 \( \delta_2 \) molecules (24) rather than long bundles in the absence of nsDNA. It is likely that DNA-independent bundle formation (Fig. 2A) and \( \delta_2 \) blobs on nsDNA (Fig. 2C) are two mutually exclusive states, and \( \delta_2 \) bundles, if formed in vitro, might be for storage purposes, as proposed for RecA (41), rather than for plasmid segregation. Indeed, many of these large or small ParA proteins might form bundles (e.g. F-SopA), but such assembly is inhibited in the presence of nsDNA (42).

In the filament-pulling model, pB171-ParA\(_2\) (Sm) binds and polymerizes on nsDNA, with the filamentous pull-apart by depolymerization (Fig. 2B) (26, 27). If pB171-ParA\(_2\) (Sm) interacts with and polymerizes on nsDNA, as depicted in Fig. 2B, the N- and C-terminal domains should play a crucial role in plasmid segregation. However, deletion of the N-terminal (\( \delta_2\Delta N20 \)) or C-terminal (\( \delta_2\Delta C255 \)) domain did not abrogate accurate plasmid segregation (Table 2). Alternatively, once the tip of U-shaped ParA\(_2\) (Sm) binds nsDNA, ParA\(_2\) (Sm) may polymerize through unknown regions. To accommodate our results, we have to assume that ParA\(_2\) polymerization onto nsDNA leads to double filaments, because the dimer of dimer interface maps at the bottom of the U-shaped protein (Fig. 7A, center) and is free for interacting with a preformed filament. Protein \( \delta_2 \) fails to form nucleoprotein filaments (15, 24). However, pseudofilaments on nsDNA were observed in the presence of \( \omega_2 \) and a very large excess of \( \omega_2 \) (>300-fold over its \( K_{\text{D(app)}} \)) (15). Dissection of these \( \langle \omega_2\delta_2 \rangle \text{nsDNA filaments} \) revealed that they were composed of discrete \( \delta_2 \) blobs stabilized by the nonspecific interaction of \( \omega_2 \) with nsDNA (34).

Our data support non-filament-based models and are compatible with the diffusion-ratchet or DNA relay models. Because these models diverged at later stages (11, 29) and we are analyzing the early ones, they are considered as a single model (Fig. 2C). From previous data and data presented here, we conclude that (i) the unstructured N-terminal region of \( \omega_2 \) (denoted in red in Fig. 2C), which is dispensable for binding to \( \text{parS} \) DNA, is involved in the interaction with \( \delta_2 \) (25, 33); (ii) \( \delta_2 \) upon ATP binding, binds to nsDNA and forms discrete blob-shaped structures containing 5–6 \( \delta_2 \) rather than filaments on the nsDNA \( \text{in vitro} \) (14, 24), and it is homogeneously distributed on the nucleoid \( \text{in vivo} \) (34); (iii) the interaction of \( \delta_2 \) bound to nsDNA with \( \omega_2 \) bound to \( \text{parS} \) DNA promotes a structural transition in both proteins (see Figs. 5C and 7A); (iv) this protein-protein complex captures and moves the plasmids from any cytosolic position toward

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**FIGURE 7. Summary of the nsDNA, \( \omega_2 \), \( \delta_2 \), and \( \delta_2\delta_2 \) binding motifs of \( \delta_2 \) identified in this work.** A, ribbon diagram of \( \delta_2 \) with key motifs highlighted in green, cyan, purple, and brown. From left to right, \( \delta_2 \) was rotated 90° to highlight the different motifs. Boxes of broken lines indicate the interacting regions. B and C, sequence alignment of relevant regions of the small ParA ATPases (\( \delta_2 \), Thermus aquaticus Soj and plasmid TP228 ParF). B, nsDNA binding motif (\( \alpha_10 \) to part of \( \alpha_12 \); green). C, sequence alignment of the \( \delta_2 \) region involved in \( \delta_2\delta_2 \) (\( \alpha_3 \) to \( \alpha_4 \); cyan) and \( \omega_2 \) (\( \alpha_4 \) to \( \alpha_6 \); purple) interaction derived from our cross-linking experiments (see Figs. 5A and 6A). Sequence alignments were generated using ClustalW and displayed using Jalview version 2.7.
the nucleoid (Fig. 2C) (14, 24, 34); and (v) at equimolar ratios, ω2 bound to parS enhances δ2-mediated ATP hydrolysis and directs the Brownian ratchet of the diffusing partition complexes (Fig. 2C).

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