Molecular anatomy of the *Streptococcus pyogenes* pSM19035 partition and segrosome complexes

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**ABSTRACT**

Vancomycin or erythromycin resistance and the stability determinants, $\delta_2$ and $\omega\varepsilon\zeta$, of Enterococci and Streptococci plasmids are genetically linked. To unravel the mechanisms that promoted the stable persistence of resistance determinants, the early stages of *Streptococcus pyogenes* pSM19035 partitioning were biochemically dissected. First, the homodimeric centromere-binding protein, $\omega_2$, bound parS DNA to form a short-lived partition complex 1 (PC1). The interaction of PC1 with homodimeric $\delta_2$ even in the apo form (Apo-$\delta_2$), significantly stimulated the formation of a long-lived $\omega_2$·parS complex (PC2) without spreading into neighbouring DNA sequences. In the ATP·Mg$^{2+}$ bound form, $\delta_2$ bound DNA, without sequence specificity, to form a transient dynamic complex (DC). Second, parS bound $\omega_2$ interacted with and promoted $\delta_2$ redistribution to co-localize with the PC2, leading to transient segrosome complex (SC, parS·$\omega_2$·$\delta_2$) formation. Third, $\delta_2$, in the SC, interacted with a second SC and promoted formation of a bridging complex (BC). Finally, increasing $\omega_2$ concentrations stimulated the ATPase activity of $\delta_2$ and the BC was disassembled. We propose that PC, DC, SC and BC formation were dynamic processes and that the molar $\omega_2$/$\delta_2$ ratio and parS DNA control their temporal and spatial assembly during partition of pSM19035 before cell division.

**INTRODUCTION**

Accurate distribution of a newly replicated genome to daughter cells at cell division is a precise process, however this process is prone to occasional error. Low-copy number plasmids of the Inc18 family such as pSM19035, make use of at least two active stabilization systems, partition and toxin–antitoxin (TA), rather than relying on random segregation of plasmid monomers (1–3). pSM19035 encodes three loci (Rep, Par and TA) whose expression is regulated by the homodimeric centromere binding protein (CBP) $\omega_2$ [(4), Figure 1A]. The toxin of the TA locus, which consists of two trans-acting proteins (the $\varepsilon_2$ antitoxin and $\zeta$ toxin), inhibits the growth of cells that lose the plasmid (1,2). The Rep locus comprises a small antisense RNA and homodimeric CopS (CopS2), both involved in regulation of plasmid copy number, and the RepS protein which activates replication [(4), Figure 1A]. The par locus consists of two sets of three cis-acting parS centromeres and two homodimeric trans-acting proteins, $\delta_2$ (ParA-like) and $\omega_2$ (ParB-like), which allow the plasmid to be actively segregated to daughter cells [(1,2), Figure 1A and B]. Given the genetic linkage between the stability determinants $\delta$–$\omega$ and $\omega$–$\varepsilon$–$\zeta$ and erythromycin and/or vancomycin resistance in Enterococci and Streptococci (5), the characterization of both loci is relevant to understanding the persistence of resistance determinants in Firmicutes. To understand how plasmids are segregated, we have studied the early stages of the pSM19035 partition mechanism.

The partition machinery of low-copy number plasmids and bacterial chromosomes is of two main types: type I (ParAB) and type II (ParMR) (6–9). The majority of plasmids and bacterial chromosomes carry a partition locus of the ParAB type. ParAB systems are further subdivided into those whose ParA has an N-terminal extension needed for autoregulated expression (type Ia) and those whose proteins, in addition to lacking the ParA extension, are relatively small (type Ib) (6–9). Several GFP-fusion derivatives of ParA have been localized in the cell (6–8). In the absence of their cognate ParB, each

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Figure 1. Genome organization and proposed structure of the $\omega_2\cdot\text{parS}$ complex. (A) pSM19035 map. Duplicated sequences are indicated by the thick line, and unique non-repeated (NR) sequences by the thin line. The arrowheads on the thick lines denote the arbitrarily chosen polarity of the inverted repeated sequences. One arm of the repeated region is denoted in grey and is not described. The outer thin arrows indicate the replication and segregation loci. The replication origin (light blue box) and direction of replication (denoted by inner arrows) are indicated. The upstream region of the promoters of $\text{copS}$, $\delta$ and $\omega$ genes (red boxes), which constitute the six cis-acting centromere-like parS sites, are enlarged. The variable number of contiguous 7-bp heptad (iterons) repeats are symbolized by direct or inverse filled triangle. The promoters repressed by $\omega_2$ (red balls) are indicated. (B) The parS sites consist of a variable number of contiguous iterons with the sequence 5'-WATCACW-3', where W is A or T. The boxes denote the -35 and -10 boxes of the promoters of the copS, $\delta$ and $\omega$ genes, and the bent arrows denote the +1 of the transcripts. (C) The structural model of $\omega_2$ bound to parS1 DNA. The overall structure of the PC ($\omega_2\cdot\text{parS}$ DNA), with $\omega_2$ forming a left-handed matrix around straight DNA is shown. The iterons are denoted as arrows.
of them decorates the nucleoid. In the presence of their ParB counterpart, ParAs are re-located, moving along and even between nucleoids (10–16). This oscillation of the ParA proteins is similar to that observed with MinD which oscillates between the cell poles in association with the membrane (17). Deconvolution of oscillation images suggests that: (i) ParB proteins dynamically regulate ParA oscillation; (ii) the ParA proteins form spiral structures on DNA; and (iii) ParA mutations which block ATP binding prevent nucleoid association (11–14, 18).

Segmentation of pSM19035 requires a type Ib ParAB system, composed of the NTPase δ2, the CBP ω2 and two sets of three parS centromere sites [(3,12,19), Figure 1A and B], comprising 9, 7 and 10 contiguous heptads of sequence 5’-WATACW-3’ (where W is an A or a T) in direct or inverse orientation [(3,12,19), Figure 1B]. These parS sites overlap the promoter region of the δ2, ω2 and cosP genes, respectively [(3,20), Figure 1B].

The δ2 monomer is a 71-residue polypeptide with an unstructured N-terminal domain (residues 1–19) and a ribbon–helix–helix-fold (residues 20–71) (21–23). The crystallographic structures of δ2DN19 in complex with two repeats in direct or inverted orientation and AFM analysis of δ2*parS complexes have allowed us to propose the architecture of the partition complex (PC) [(24,26), Figure 1C]. In this complex, the ω2 DNA-binding site faces inward, and successive ω2 molecules are displaced relative to their neighbours by 7-bp so as to assemble as a left-handed helix that wraps around parS DNA, without bending or twisting it [(24,26), Figure 1C]. The PC formed by δ2 do not spread significantly beyond the parS site, unlike those formed by large CBPs such as P1-ParB and F-SopB or the medium-sized CBP Spo0J of Bacillus subtilis that spread in a sequence-independent manner up to several kilobases upon binding to their cognate site(s) (27–30).

The δ2 ATPase, whose monomer is a 284-residue long polypeptide, is essential for better-than-random plasmid segregation (1,12). In the presence of ATP·Mg2+, δ2 binds DNA in a sequence-independent manner (12). Note that unless stated otherwise the δ2 ATPase or its mutant variants are in the ATP-bound form and denoted as δ2, δ2D60A, δ2K242A, δ2K248S or δ2K259A/K260A, respectively.

The ω2*δ2 interactions are key events of the partition mechanism, but in vitro analyses have shown the outcome to depend on the ratio of the two proteins. At low ω2/δ2 ratios, ω2 bound to parS enhances the ATPase activity of δ2 and promotes plasmid pairing (26). At equimolar ω2/δ2 ratios, ω2 stimulates ATP hydrolysis by δ2 and promotes disassembly of the paired complexes (12). At high ω2/δ2 ratios, ω2 promotes δ2 polymerization onto DNA (12). In the ATP bound form, the small ATPases (δ2, Soj, etc. 260 ± 50 residues long) and the Vibrio cholerae large ATPase ParA2, bind and polymerize on DNA in a sequence independent manner (12,31,32). In contrast, when bound to ATP, the large ATPases (370 ± 50 residues long) and few small ATPases, as ParF of pTP228 or ParA of pB171, form bundles of polymers in the absence of DNA or any other surface (33–38).

Cytological studies have shown that ParA binding to DNA and interaction with ParB, mediates pairing and plasmid movement in opposite directions (12,14–16,18,39,40). Indeed, the interaction of CBP bound to its cognate site with nucleoid-bound NTPase causes the re-localization of the latter in vivo (12,15,16,18,26). Atomic force microscopy (AFM) revealed that δ2 bound to DNA non-specifically, was detached from DNA upon interaction with the PC and relocalized to form the segrosome complex (SC, Figure 2E) (26). Interaction of two SCs via δ2 then forms a bridging complex (BC, Figure 2E) (12,26). The following step of unpairing, δ2 polymerization on and depolymerization from DNA require ATP hydrolysis (12,26). Protein δ2D60A, which binds but does not hydrolyse ATP, led to accumulation of BCs (Figure 2D and E), but Apo-δ2K36A, which neither binds nor hydrolyses ATP, did not bind DNA (12,26).

To elucidate the early stages of pSM19035 partitioning we performed detailed biochemical analyses of these protein–DNA complexes. We report here that the paired partition complexes presumably needed to initiate plasmid segregation are not formed by random collisions of freely diffusing molecules but are constructed through a series of defined stages. Such deliberate assembly could facilitate regulation of partition in accordance with conditions prevailing in the cell.

MATERIALS AND METHODS

Chemicals, enzymes, proteins, DNA and reagents

All chemicals were p.a. grade and purchased from Roche Diagnostics (Mannheim, Germany). DNA restriction, DNA modification enzymes and nucleotides were from Boehringer (Mannheim, Germany). Ultrapure acrylamide was from Serva (Heidelberg, Germany). The broad protein molecular weight marker was obtained from Gibco-BRL (Barcelona, Spain). Proteins ω2, ω2Δ19, ω2T29A, δ2, δ2K36A and δ2D60A and pBC30-borne parS DNA, which is the source of parS DNA, were purified as described (3,12,26). Similar results were obtained with the three parS sites (12,26, data not shown). Here, only experiments with parS2 DNA containing seven contiguous iterons or heptads, herein parS DNA, are described. Plasmid pCB746-bearing δ gene was used for site-directed mutagenesis: AAA codons 242, 248 or 259 and 260 of wild-type (wt) δ gene coding for Lys, were exchanged for GCA, which encodes for Ala, or TCA encoding Ser. The His-tagged protein variants δ2D211A, δ2K242A, δ2K248S or δ2K259A/K260A were purified as described for wt protein (12). The concentration of DNA was expressed as moles of DNA molecules and was determined using a molar extinction coefficient of 6500 M−1 cm−1 at 260 nm. The protein concentrations were determined by absorption
Figure 2. Complexes formed by $\omega_2$ and $\delta_2$ binding to parS DNA. (A) The 423-bp [\(\gamma^{32}\)P]-HindIII-KpnI parS DNA (0.1 nM) was incubated with increasing concentrations of $\omega_2$ (3 and 6 nM), $\omega_2\Delta N19$ (4 and 8 nM), $\omega_2\Delta T29A$ (5 and 10 nM), $\delta_2$ (140, 280 and 560 nM) or in the presence of $\delta_2$ (140 nM, indicated by plus) and increasing amounts of $\omega_2$, $\omega_2\Delta N19$ or $\omega_2\Delta T29A$. (B) parS DNA (0.1 nM) was incubated with increasing amounts of $\omega_2$ (1.5, 3 and 6 nM), $\delta_2$ (140, 280 and 560 nM), $\delta_2\Delta 60A$ (35, 70 and 140 nM) or a constant amount of $\omega_2$ (1.5 nM, indicated by plus) and increasing concentrations of $\delta_2$ or $\delta_2\Delta 60A$. (C) The 183-bp [\(\gamma^{32}\)P]-BamHI-HindIII non-parS DNA (0.1 nM) was incubated with increasing concentrations of $\delta_2$ (120, 240 and 480 nM, lanes 5–7) or the 423-bp [\(\gamma^{32}\)P]-parS DNA (0.1 nM) with increasing concentrations of $\omega_2$ (3–12 nM, lanes 2–4). non-parS DNA was pre-incubated with $\delta_2$ (120 nM) and then parS DNA and increasing concentrations of $\omega_2$ (3–12 nM) were added. (D) non-parS DNA was incubated with increasing concentrations of $\delta_2\Delta 60A$ (37, 75 and 150 nM, lanes 7–9) or parS DNA with increasing concentrations of $\omega_2$ (3–12 nM, lanes 2–4). non-parS DNA was pre-incubated with $\delta_2\Delta 60A$ (75 nM) for 5 min, and then parS DNA and increasing concentrations of $\omega_2$ (3–12 nM) were added and the reaction incubated for 15 min at 37°C in buffer A containing 1 mM ATP. The absence of a component is indicated by minus, and the presence of a fixed amount by a plus or variable concentration by a triangle, respectively. (E) Protein $\omega_2$ bound to parS DNA led to the formation of a partition complex (PC); $\delta_2$ bound to PC leading to segrosome complex (SC) formation; and the interaction of two SCs leading to bridging complex (BC) formation. (F) Protein $\delta_2$ bound to DNA leading to dynamic complex (DC) formation; $\omega_2$ binding to DC led to a transient complex (TC); and the interaction of two TCs led to pseudo-bridging complex (‘BC’) formation. FD, protein-free DNA.
at 280 nm using molar extinction coefficients of 2980 M\(^{-1}\) cm\(^{-1}\) for \(\delta_2\), \(\delta_2\Delta N19\) and \(\delta_2\)T29A, and 38 850 M\(^{-1}\) cm\(^{-1}\) for \(\delta_2\), \(\delta_2\)K36A, \(\delta_2\)D60A, \(\delta_2\)D211A, \(\delta_2\)K242A, \(\delta_2\)K248S or \(\delta_2\)K259A/K260A. Concentrations are expressed as mol of protein dimers.

Limiting proteinase K (ProK, 0.5–2 μg/ml) was used to partially proteolyse free \(\delta_2\) or DNA-bound \(\delta_2\), and the resulting products were separated using 15% SDS–polyacrylamide gel electrophoresis (PAGE). Tryptic digestion of gel-purified protein bands and their spotting onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems, Foster City, USA) were performed as described (41). The MALDI-TOF-TOF measurements of spotted peptide solutions were carried out on a Proteome-Analyzer 4700 (Applied Biosystems, Foster City, USA) as described previously (41).

Protein–DNA complexes

For electrophoretic mobility shift assays (EMSA), gel-purified 423-bp \([\alpha^32P]\)-HindIII-KpnI parS DNA or 183-bp \([\alpha^32P]\)-BamHI-HindIII non-parS DNA (0.1 nM) was incubated with various amounts of wt \(\delta_2\) (or its variants), wt \(\delta_2\) (or its variants), or both proteins in buffer A (50 mM Tris–HCl pH 7.5, 10 mM MgCl\(_2\), 50 mM NaCl) containing or lacking 1 mM ATP or ADP for 15 min at 37°C in 20 μl final volume as previously described (3,12). The reaction was stopped by addition of loading buffer (1 mM EDTA, 0.1% [v/v] bromophenol blue and 0.1% [v/v] xylene cyanol) and was then separated using 4 or 6% PAGE. PAGE conducted in running buffer 1 x TAE at 45 V at 4°C, and the gels were dried prior to autoradiography as described (3).

DNase I footprinting was performed as previously described (3,19). Briefly \([\alpha^32P]\)-HindIII-KpnI parS DNA (1 nM) was incubated with wt \(\delta_2\) (or its variants), \(\delta_2\) (or its variants) or both proteins under the same conditions as the EMSA experiments (3,19). After 15 min incubation at 37°C, the footprint was started by DNaseI addition. After 2 min, the reactions were stopped by addition of loading buffer, separated in 6% denaturing (d) PAGE and autoradiographed. As size control markers, ladders obtained from differently exposed autoradiographs of EMSA densitometrically determined under non-saturating conditions from EMSA and DNase I footprint experiments, the concentration of free DNA and protein–DNA complexes was transferred 50% of the free labelled DNA into complexes or from differently exposed autoradiographs of EMSA and DNase I footprinting gels. Protein concentrations that transfer 50% of the free labelled DNA into complexes or protect 50% from DNase I digestion are approximately equal to the \(K_{Dapp}\) under conditions where the DNA concentration is much lower than the \(K_{Dapp}\).

To determine the dissociation half-life of protein–DNA complexes, protein were incubated with \([\alpha^32P]\)-HindIII-KpnI parS DNA in buffer A containing 1 mM ATP, when indicated, for 15 min at 37°C in a 100 μl final volume as previously described (3,12). A 50-fold excess of unlabelled DNA was then added to the pre-formed protein–DNA complexes, and samples were collected at varying times and the solution was filtered through a nitrocellulose membrane filter (Millipore, type HAWP 0.45 μm) as previously described [42, Supplementary Figure S1]. While free DNA passed through the filter the radiolabelled DNA bound to the protein was retained on the filter (42). Filters were dried and the amount of radioactivity bound to the filter was determined by scintillation counting. The DNA retained on filter was corrected for the retention of radiolabelled DNA in the absence of protein. The specific activity of the input labelled DNA was measured as 10% trichloroacetic acid precipitable material.

RESULTS

Protein \(\omega_2\) forms a discrete complex on parS DNA while \(\delta_2\) non-specifically binds DNA

To elucidate features of the early stages of plasmid segregation, the binding of \(\omega_2\) to centromeric parS DNA was studied. In the presence or absence of ATP, \(\omega_2\) bound with high affinity and specificity to parS DNA (\(K_{Dapp} \sim 5 \pm 1\) nM) to form a partition complex (PC) (Figure 2A, lanes 2 and 3; and Figure 2B–D, lanes 2–4). The PC formed was confirmed by AFM (Figure 2E and Supplementary Figure S2A). Protein \(\omega_2\Delta N19\), which lacks the first 19-residues, bound parS DNA with similar affinity (\(K_{Dapp} \sim 7 \pm 1\) nM) (Figure 2A, lanes 4 and 5), but the \(\omega_2\)T29A variant (Figure 2A, lanes 6 and 7), which contains an essential mutation in the DNA binding motif, bound parS DNA with low affinity (\(K_{Dapp} \sim 1.5\) μM) (24,25).

It has previously been shown that in their apo form, Apo-\(\delta_2\) or Apo-\(\omega_2\)D60A failed to bind or to polymerize onto DNA in the nM range [12], \(K_{Dapp} > 1.5\) μM. Similar results were observed when the proteins were in the ADP bound form (ADP-\(\delta_2\) or ADP-\(\omega_2\)D60A) (12). Protein \(\delta_2\) bound cooperatively to parS or non-parS DNA with similar affinity (Figure 2A–C, \(K_{Dapp} \sim 1.5\) μM) (24,25).

To explain the differences of the \(K_{Dapp}\) of both proteins, we hypothesize that either \(\delta_2\)D60A binds DNA faster than \(\delta_2\) or the latter protein upon ATP hydrolysis increases the off rate leading to a dynamic association and dissociation complex (DC, \(\delta_2\)•DNA). To address these possibilities, \(\delta_2\) or \(\omega_2\)D60A, at \(K_{Dapp}\), were pre-incubated with parS DNA, and the half-life of the preformed complex was measured in the presence of a 50-fold excess of cold parS DNA as competitor by filter binding assays. When the cold DNA was omitted, there was no apparent time-dependent decrease in the protein•parS DNA complexes (data not
shown). As shown in Supplementary Figure S1, the time-dependent decrease of the retained parS DNA was used to calculate the half-life of protein–DNA complexes. The half-life of δ2·DNA was ~10 min, which was ~3-fold longer for the δ2D60A·DNA complex (~28 min). It is likely that: (i) δ2 binding to DNA specifically requires ATP; (ii) δ2 and δ2D60A bind DNA with similar affinities and (iii) ATP hydrolysis makes the short δ2-DNA filament dynamic, leading to DC formation (Figure 2A). The DC formed was confirmed by AFM (Figure 2F and Supplementary Figure S2B).

Proteins δ2 and δ2 bind parS DNA forming a ternary complex

Stable physical interactions in solution have not been detected between δ2 and Ω0 (12). To determine whether parS DNA, δ2 and Ω0 (or its variants, Ω2ΔN19 or Ω2T29A) formed ternary complexes, EMSA studies were performed. In the presence of parS DNA, sub-saturating Ω2 (2- to 4-fold lower than $K_{D_{\text{app}}}$) and saturating δ2 (2- to 4-fold higher than $K_{D_{\text{app}}}$) concentrations formed a low-mobility complex, termed the segrosome complex (SC) (Figure 2A, lane 9; and Figure 2B, lane 13). SC formation was confirmed by AFM analysis (Figure 2E). When the Ω2T29A variant, which does not bind DNA at the range of concentrations tested here, replaced wt Ω0, the slow moving complex also accumulated (Figure 2A, lane 13). However, when the Ω2ΔN19 variant, which binds parS DNA but fails to interact with δ2, was used, only diffuse low-mobility DC was observed (Figure 2A, lanes 10 and 11). It is likely that DNA-bound δ2 loads both Ω2 onto parS DNA and Ω2T29A onto DNA but fails to interact with Ω2ΔN19.

At Ω2 concentrations below $K_{D_{\text{app}}}$ (e.g. 1.5 nM), formation of PCs (Ω2·parS DNA) were not observed (Figure 2B, lane 2), but in the presence of limiting δ2 or δ2D60A concentrations, PCs were readily formed (Figure 2B, lanes 11 and 14). Sub-saturating or saturating δ2 concentrations increased ternary complex formation (Ω2·parS·δ2) and led to the accumulation of SC (Figure 2B, lanes 12 and 13). High-order complexes, formed by two or more SCs, leading to BC, were also confirmed by AFM analysis (Figure 2E and Supplementary Figure S2C). Unlike wt protein, δ2D60A accumulated bands that migrated slower (BC; Figure 2B, lanes 14–16). Although Ω2 shows significantly greater binding affinity when compared to δ2 or δ2D60A, it appears that the latter two were able to markedly enhance the affinity of Ω2 for parS DNA. It is likely that both Ω2 and δ2 interact and cooperate to circumvent the energetic and spatial constraints required for Ω2 binding to parS DNA.

Protein Ω2 binding to parS DNA promotes dislodging of DNA-bound δ2

Previous studies revealed that δ2 is an ATP-dependent DNA binding protein whose activities are controlled by Ω2 (12). To re-evaluate the hypothesis that δ2 interacts with Ω2 and facilitates the interaction with parS DNA, EMSA studies were performed with parS and non-parS DNAs. Protein δ2 or δ2D60A was pre-bound to non-parS DNA (Figure 2C and D, lanes 5–7), and then preformed Ω2·parS DNA was added to the reaction mixture. Protein δ2 or δ2D60A pre-bound to non-parS DNA interacted poorly with parS DNA (Figure 2C and D, lane 8). At limiting Ω2 concentrations, δ2 was dislodged from non-parS DNA (Figure 2C, lanes 8 and 9). At sub-saturating Ω2 concentrations, the PC and SC accumulated (Figure 2C, lanes 10 and 11), suggesting that Ω2 bound to parS DNA promotes the re-localization of δ2 towards parS DNA to form a SC, as shown by the accumulation of free non-parS, and the slow moving SCs (Figure 2C). However, when wt δ2 was replaced by δ2D60A, the accumulation of free non-parS DNA was decreased (Figure 2D), suggesting that dislodging might require ATP hydrolysis. Under this condition, the accumulation of BCs was observed. It is possible that proteins bound to both DNA molecules led to BC formation, where two or more SCs paired (Figure 2D, lanes 10 and 11; and Figure 2E). The formation of BCs was confirmed by AFM analysis (26).

Protein Ω2 binding to parS DNA promotes δ2 re-localization

To further evaluate whether both proteins interact and Ω2 promotes re-localization of δ2, enzymatic footprinting experiments were performed. Binding of δ2 or Ω2ΔN19 to DNA specifically protected parS sequences from DNase I cleavage, with only limited spreading (<15 nt) on non-specific sequences (Figure 3A, lanes 6, 8 and 10). At limiting protein concentrations (seven δ2/parS DNA molecule), a globular-shaped δ2 bound DNA in a sequence-independent manner (Supplementary Figure S2B). In contrast, at saturating protein concentrations (>75 protein molecules/parS DNA molecule), δ2 or δ2D60A polymerized onto parS DNA and protected extended regions from DNase I digestion in a concentration-dependent manner (Figure 3A, lane 4; and Figure 3B, lanes 6 and 9).

When sub-saturating Ω2 concentrations were added to pre-formed DCs (δ2·parS DNA complexes) the Ω2 cognate site became protected from DNase I, even in the presence of saturating δ2 concentrations (Figure 3A, lanes 11 and 12; and Figure 3B, lanes 10–12). However, δ2 bound to parS DNA was poorly re-localized by Ω2ΔN19 (Figure 3A, lanes 13 and 14), suggesting that specific contacts between δ2 and Ω2 are determined by the N-terminal 18 amino acid residues of Ω2 (24,25). When sub-saturating Ω2 concentrations were added to pre-formed δ2D60A·parS DNA complexes, the Ω2 cognate site was also protected from DNase I. Protein δ2 bound to parS DNA partially redistributed δ2D60A next to it (Figure 3B, lanes 13–15). It is likely that δ2 bound to parS DNA redistributes δ2 to adjacent regions, to form a SC (Figure 2E, 26).

The DNA binding domain of δ2 maps in its C-terminus

Recently it has been shown that the ParA-like proteins (e.g. pSM19035·δ2, F-SopA, P1-ParA or chromosomal-encoded Soj) in the ATP bound form bind DNA through its C-terminus (12, this work, 31,32,36,43). To
define functional δ₂ regions and to examine whether binding to DNA protects structural domains of δ₂, limited proteolysis together with mass spectrometry experiments were performed. Limited ProK proteolysis, of δ₂ unbound or DNA-bound, revealed that the C-terminal fragment (band d) became less sensitive to ProK digestion upon DNA binding (Figure 4A, lanes 3, 5 and 7). The N-terminal folded core (band a) of δ₂ became more sensitive to proteolysis in the DNA bound form (Figure 4A, compared bands a, b and c). Limiting trypsinolysis of the gel-purified a–d polypeptide bands in conjunction with mass spectrometry analysis allowed us to identify these bands (Figure 4B). The polypeptide stabilized in the presence of DNA corresponded to the C-terminal end (Figure 4B). A structural comparison of these regions from different ATPases revealed that there are charged residues, but they are poorly conserved (Figure 4C). An analysis of the residues implicated in ATP-Soj₂, P1-ATP-ParA₂ or F-ATP-SopA₂ sequence-independent DNA binding (32,36,43) and the surface-exposed charged residues of δ₂ suggested a potential role for residues D211, K242, K248 and K259/K260 in DNA binding.

These residues were replaced by Ala or Ser, and the resulting products were purified and biochemically analysed. In the ATP-bound form, the δ₂ variant D211A had unimpaired sequence-independent DNA binding relative to wt δ₂ (data not shown). As revealed in Figure 4D, the δ₂K242A mutant bound DNA with ~30-fold lower affinity (KDapp > 3 μM) relative to wt δ₂. Similar results were observed with the δ₂K248S or δ₂K259A/K260A variants (data not shown). The DNA binding defect presented by δ₂K242A, δ₂K248S or δ₂K259A/K260A was specific because all of them formed dimers in solution and were able to bind and hydrolyse ATP (data not shown), suggesting that these mutants were properly folded.

**Interaction of δ₂ with o₂ markedly increases PC formation**

Previously, it was assumed that o₂ was present in two molecular states, parS-bound and free in the cytosol, and that all molecules in the system were competent for parS binding (19,24). Protein o₂ specifically bound parS DNA with a KDapp ~5 ± 1 nM, but no binding to parS
Apo-δ₂ or Apo-δ₂D60A increased formation of ω₂*parS DNA complexes at least 6- to 8-fold (Figure 5A, lanes 13, 14, 16 and 17). In this experiment, we cannot rule out that δ₂ or δ₂D60A formed transient complexes with DNA in the presence of ω₂ and that such interaction increases the accumulation of PCs. To test this hypothesis, δ₂ was replaced by δ₂K242A, which is deficient in DNA binding (Figure 4D). In the presence of limiting ω₂ concentrations (~6-fold lower than the $K_{Dapp}$), addition of Apo-δ₂K242A (or δ₂K242A at ~100-fold lower than the $K_{Dapp}$) facilitated ω₂ binding to parS DNA (Figure 5B, lanes 11–13, $K_{Dapp}$ 0.7 ± 0.1 nM). Similar results were observed when δ₂K36A, which cannot bind or hydrolyse ATP nor bind DNA, was used (data not shown). It is likely that a transient and synergistic interaction between δ₂ and ω₂ increases the ω₂ $K_{Dapp}$ at least ~7-fold, and such an effect occurs even in the absence of Apo-δ₂ binding to DNA. It is worth mentioning that: (i) ω₂ binds its cognate site with a stoichiometry of 1 (19, 24), (ii) the parS used contains seven heptads, and in the above experiments the parS concentration was 0.1 nM, suggesting that the $K_{Dapp}$ could be even smaller and (iii) the ω₂*δ₂ interaction, which might also involve determinants in the C-terminal region of δ₂, was not affected by the K242A mutation in δ₂.

To address whether δ₂ or its variant increased the on or off rate of the reaction, the dissociation rate of the ω₂*parS was measured both in the presence or absence of δ₂K242A. Previously, it was shown by surface plasmon resonance that the ω₂*parS complex is short-lived (~50 s) (19). parS DNA was incubated with half-saturating ω₂ concentrations (6 nM) or with ω₂ and Apo-δ₂K242A (100 nM). As expected, the half-life of the ω₂*parS complex was short-lived, but increased ~10-fold to ~34 min in the presence of Apo-δ₂K242A (Supplementary Figure S3). Since the addition of Apo-δ₂K242A decreased the dissociation rate of the PC, it was assumed that Apo-δ₂ or Apo-δ₂K242A transiently interacted with the unstructured N-terminal domain of ω₂, facilitating domain folding and/or a more extended ω₂ structural change, leading to an ω₂ variant (ω₂*) with an structured N-terminal end. We suspect that upon a transient δ₂*ω₂ interaction, there are two PC states: a transient (PC1, ω₂*parS DNA, Figure 2E) and a stable (PC2, ω₂*parS DNA, Figure 7) one.

To test whether limiting δ₂ or δ₂K242A concentrations also facilitated PC2 formation, EMSA experiments were performed. Protein δ₂ stimulated PC and SC formation (Supplementary Figure S4, lanes 13 and 14), whereas δ₂K242A could only stimulate PC2 formation (Supplementary Figure S4, lanes 16 and 17), suggesting that stable SC formation required δ₂ to interact with DNA.

The ω₂ and δ₂ interaction facilitates DC and TC formation on DNA

Previously, it was shown that: (i) at low ω₂:δ₂ ratios (0.3:1), ω₂ bound to parS DNA stimulates the ATPase activity of δ₂ and (ii) at high ω₂:δ₂ ratios (4:1), δ₂ polymerizes onto DNA (12). To re-evaluate the hypothesis that ω₂, at parS, promotes changes in δ₂ and facilitates
SC formation, EMSA studies were performed with non-parS DNA. Limiting ω2 (≤250-fold lower than KDapp, for non-specific DNA) did not bind DNA lacking its cognate site (Figure 6A, lanes 2–4), and δ2, at sub-saturating concentrations, promoted DC formation (Figure 6A, lane 5). Addition of limiting ω2 concentrations to pre-formed DC (Figure 2F) facilitated the formation of a slow-moving transient complex (TC) (Figures 2F and 6A, lanes 6–8). The TC, which resembles the SC, is a very transient complex formed in the absence of parS DNA. In the presence of both proteins and DNA a discrete band that moved slower than the TC was formed, this new complex appeared to be a pseudo BC and was termed ‘BC’ (Figure 2F). The accumulation of TC and ‘BC’ was less evident when limiting ω2 concentrations were incubated with non-parS DNA (TC) and ‘BC’ was less evident when limiting ω2 concentrations were incubated with non-parS DNA (Figure 2A, condition 1 and 3). Second, the interaction between PC1 and Apo-δ2 leads to the formation of a stable PC2, but PC2’s interactions with DNA-bound δ2 leads to δ2 re-localization of the DC towards PC2 and SC formation (Figure 7A, conditions 1 and 2). Third, the interaction of δ2 in the SC, with a second SC leads to the formation of a dynamic BC (plasmid pairing complex) (Figure 7A, condition 2). Finally, ω2-bound to parS stimulates the ATPase activity of δ2, BC disassembly, and δ2 polymerization (Figure 7B). ATP hydrolysis at the end of the filament led to ADP-δ2 release from DNA. PC2 interaction with the new end of the filament moves the plasmid, like a cargo, towards the cells poles (Figure 7B). In previous

**DISCUSSION**

To gain insights into the molecular mechanisms that ensure the accurate distribution of a newly replicated genome to daughter cells at cell division by the type Ib ParAB system, the process was analysed in four different stages as summarized in Figure 7. First, ω2 binding to parS DNA and δ2 binding to non-specific DNA lead to transient PC1 and DC formation, respectively (Figure 7A, conditions 1 and 2). Second, the interaction between PC1 and Apo-δ2 lead to the formation of a stable PC2, but PC2’s interactions with DNA-bound δ2 leads to δ2 re-localization of the DC towards PC2 and SC formation (Figure 7A, conditions 1 and 2). Third, the interaction of δ2 in the SC, with a second SC leads to the formation of a dynamic BC (plasmid pairing complex) (Figure 7A, condition 2). Finally, ω2-bound to parS stimulates the ATPase activity of δ2, BC disassembly, and δ2 polymerization (Figure 7B). ATP hydrolysis at the end of the filament led to ADP-δ2 release from DNA. PC2 interaction with the new end of the filament moves the plasmid, like a cargo, towards the cells poles (Figure 7B). In previous
BamHI-HindIII concentrations of o

increasing concentrations of d

then incubated with increasing respectively. (Protein d

reports, the late stages (dynamic plasmid pairing, and polymerization and de-polymerization) of pSM19035 partitioning were addressed (12,26). In this report we have dissected the early stages, the transient and the stable partitioning were addressed (12,26). In this report we have

protein dimerization or ATP hydrolysis

Like B. subtilis Soj or V. cholerae ParA2, δ2 in concert with ω2 bound to parS polymerizes on DNA forming nucleoprotein filaments (12,31,44). Interaction of δ2 with DNA led to diffuse migrating bands that could be attributed to polymerization and subsequent depolymerization of DC by ATP hydrolysis. However, the interaction with limiting ω2 facilitates TC formation on non-parS DNA (Figure 7A, condition 3). This is consistent with the observation that δ2D60A, which binds but does not hydrolyse ATP, forms a stable non-parS·δ2·ω2·δ2·non-parS DNA complex. However, ParF of pTP228 and the large ParA ATPases follow a different path, because these ATPases form bundles in the absence of any surface (15,35–37).

Protein δ2 binds DNA

Limited proteolysis experiments revealed that δ2 has several regions that become protected upon DNA binding, suggesting that DNA binding have local consequences and induce conformational changes in the protein (Figure 7, ATP-δ2*). The residues required for non-specific DNA interaction in δ2, ATP-Soj, or ATP-SopA are not conserved but map generally to the C-terminal region [(32,43), Figure 4]. Single point mutations in ω11, as in residues K242 (δ2-K242A), abrogate DNA binding, without affecting protein dimerization or ATP hydrolysis (Figure 4D, data not shown). An equivalent mutation in

Soj (e.g. ATP-Soj2R218A), only marginally (2- to 2.5-fold) reduces the DNA binding affinity relative to wt ATP-Soj2, but the ATP-Soj2R218E variant shows no binding to DNA (32). These finding suggested that: (i) ATP induced transient δ2 conformational change, which might be stabilized upon DNA binding and (ii) the basic residues in the C-terminal region contact the DNA phosphate backbone. Type Ia ParA ATPases, such as P1-ParA2 or F-SopA2, when bound to ATP, mediate segregation by interacting with parS-bound ParB (6–9). ATP-ParA2 or ATP-SopA2 also contains a basic region in the C-terminus that contacts DNA in a sequence-independent manner (36,43). This basic region of P1-ParA is equivalent to the DNA binding motif of δ2. Indeed, the P1-ParA2K375A/R378A double mutation, in the ADP bound form, essentially abrogated DNA binding (36). Similarly, the δ2K259A/ K260A variant also abrogates DNA binding (data not shown).

Centromere recognition in pSM19035 includes six copies of parS DNA containing several copies of unspaced iterons, and a small size CBP, ω2 (19,23). parS DNA forms a transient complex with ω2 (PC1), with high affinity and cooperativity. PC1 leads to a contiguous left-handed helical nucleoprotein complex that does not distort the contour length of right-handed parS DNA (24,26). DNA titration experiments with increasing numbers of iterons (heptads) and stoichiometric studies of the PC1 revealed that each iteron recruits one ω2 molecule. Each ω2 being displaced relative to its neighbour by 7-bp and left-handed rotated by 252° (19,24). The overall structure of the PC1, in linear or supercoiled DNA, revealed the formation of a discrete structure with ω2 wrapping around straight B-form parS DNA, without significant spreading, compaction, shortening or distortion of the DNA (24,26). At the PC1, the ω2 DNA-binding domain is facing inward (Figure 1C). The interaction of δ2 or Apo-δ2 with PC1 stimulate the assembly of the longer-lived PC2 (see below). Unlike ω2-mediated PC1 or PC2 formation, the large (e.g. P1-ParB or F-SopB) and middle size (e.g. chromosomal-encoded Spo0J) CBPs, which recognize their cognate target via a helix-turn-helix domain, spread onto and around parS up to several kilobases of DNA in a centromere-dependent manner upon binding to parS DNA (27–30).
We propose that δ₂ positively controls the dynamic activities of ω₂ on parS DNA. Protein ω₂ binds parS DNA to form PC1. A transient interaction between Apo-δ₂ and PC1 markedly stabilizes the latter (>12-fold) leading to the accumulation of the PC2 intermediate (ω₂*·parS DNA) (Figure 7A, condition 1), δ₂·PC1 interaction leads to SC and BC formation (Figure 7A, condition 2). This is consistent with the observations that: (i) the PC is a highly dynamic structure (with a PC1 half-life <1 min) (19) and (ii) the Apo-δ₂K242A or δ₂K242A...
variant, which abrogates DNA binding, or Apo-δ2K36A, which abrogates ATP binding and hydrolysis and DNA binding, markedly enhanced PC2 formation (Figures 5 and 7A, condition 1, data not shown). It is likely that δ2 binds to parS DNA and forms the transient PC1. Therefore, the interaction of Apo-δ2 or δ2 with the unstructured N-terminal domain of δ2 induces conformational changes in the latter to facilitate PC2, SC or BC formation, respectively. Unlike δ2·parS, pB171-ParB binds to the centromere and forms discrete PCs and large, high-order complexes consisting of several DNA fragments joined by ParB at the centromere site (BC complex or plasmid pairing) in the absence of pB171-ParA (45).

Protein δ2 facilitates SC and BC formation on parS DNA

We propose that δ2 also controls the dynamic activities of δ2. Different δ2:δ2 ratios and the presence of parS DNA play a critical role in the regulation of the different stages of plasmid segregation. At stoichiometric concentrations of both proteins, δ2 binding to parS DNA promotes dislodging of δ2 from non-parS DNA and re-localization towards PC2 leading to SC and BC formation (Figures 2C and 7A, condition 2). It is likely that this dynamic redistribution resembles ParA oscillation from non-parS DNA (the nucleoid) to ParB-bound parS DNA. The δ2·δ2 interaction induces conformational changes in both proteins. The interaction of two SCs leads to the formation of a BC, with subsequent change in the δ2:δ2 ratios (12,26). Indeed, the unstructured N-terminal domain of δ2 is required to control δ2-mediated ATP hydrolysis and formation of the transient SC and BC (Figure 7A, condition 2). The BC, which resembles specific plasmid pairing (26), was dislodged upon PC2 stimulated ATPase activity of δ2, leading to SC formation (Figure 7B).

Protein δ2 facilitates TC and ‘BC’ formation on non-parS DNA

At limiting protein concentrations, δ2 binds cooperatively to DNA forming discrete bead-like transient DCs of variable length (26). Limiting δ2 (>250-fold lower than K_Diss for parS DNA), upon interaction with δ2 bound to non-parS DNA facilitating TC and ‘BC’ formation (Figure 7A, condition 3). It is likely that δ2, at the transient DC, should load δ2 onto non-parS DNA. Indeed, δ2-bound non-parS DNA (DC) facilitates δ2 loading onto non-parS DNA, TC and ‘BC’ formation. However, in the presence of limiting δ2 concentrations, only TC formation was detected. Formation of ‘BCs’, which have similar apparent mobility to that of genuine BCS at parS regions (Figures 2B and 6A), is dynamic, with δ2 stimulating δ2 release from non-parS DNA. The interaction of both proteins leads to BC on parS and ‘BC’ formation on non-parS DNA, suggesting a genuine interaction rather than a random collision of free particles. This is consistent with the observation that δ2 facilitates plasmid pairing (‘BC’) in the presence of δ2T29A that only binds DNA in a sequence-independent manner (26).

Molecular model explaining the role of SC and BC formation

A synergistic interaction between δ2·parS (PC1) and δ2·DNA (DC), promotes δ2 relocation leading to PC2, SC and BC formation, ensuring plasmid pairing. Upon disassembly of the BC, δ2 polymerization and depolymerization move the plasmids towards the poles leading to accurate segregation (12,26, this work). We propose a sequential, multistep mechanism to position and move the plasmids to cell quarters. In the first step, δ2 binds cooperatively and with high affinity to parS DNA to form a transient left-handed nucleoprotein complex, PC1 (Figure 7A, condition 1), and δ2 binds non-parS DNA forming a transient DC (Figure 7A, conditions 2 and 3) (12,24,44). In step 2, the interaction of δ2 with PC1 leads to PC2, SC and BC formation (Figure 7A, condition 2); however, when ATP is omitted, the interaction of Apo-δ2 with PC1 significantly stimulates the accumulation of the long-lived PC2 intermediate (Figure 7A, condition 1). In step 3, δ2 bound to parS interacts with δ2 bound to non-parS to promote dynamic instability of the DC (δ2·non-parS DNA) leading to δ2 redistribution and co-localization of the PC2 and SC formation (Figure 7A, conditions 1 and 2). In step 4, at low δ2:δ2 ratios, the interaction of δ2 in the PC2 with δ2 in the SC, facilitates BC formation (Figure 7A, condition 2). In step 5, δ2, which has dual effects on δ2 binding to DNA, significantly stabilizes the TC to form ‘BC’ between two non-parS DNA molecules (Figure 7A, condition 3). In step 6, at low δ2:δ2 ratios, δ2 enhances the bulk ATPase activity of δ2, facilitates the release of ADP-δ2 from DNA, and stimulates disassembly of the BC or ‘BC’ (12). Finally, upon disassembly of the pairing complex, the local δ2 concentration increases in one of the partners leading to a left-handed δ2·DNA filament onto chromosomal or plasmid DNA (12,44). At high δ2:δ2 ratios, δ2-bound to parS DNA inhibits the δ2 ATPase and chases protein δ2 off the DNA (re-localization and/or depolymerization) (12). Protein δ2 polymerization on and depolymerization from pSM19035 or chromosomal DNA moves the plasmid, as a PC2 cargo, towards the cell poles by an unknown mechanism (Figure 7B). We propose that PC1, DC, PC2, SC and BC formation and δ2 polymerization–depolymerization, modulated by PC2, are dynamics processes. The molar δ2:δ2 ratio and parS DNA controls the temporal and spatial partition of pSM19035 before cell division.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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