Streptococcus pyogenes pSM19035 requires dynamic assembly of ATP-bound ParA and ParB on parS DNA during plasmid segregation

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ABSTRACT

The accurate partitioning of Firmicute plasmid pSM19035 at cell division depends on ATP binding and hydrolysis by homodimeric ATPase δ₂ (ParA) and binding of ω₂ (ParB) to its cognate parS DNA. The 1.83 Å resolution crystal structure of δ₂ in a complex with non-hydrolyzable ATPγS reveals a unique ParA dimer assembly that permits nucleotide exchange without requiring dissociation into monomers. In vitro, δ₂ had minimal ATPase activity in the absence of ω₂ and parS DNA. However, stoichiometric amounts of ω₂ and parS DNA stimulated the δ₂ ATPase activity and mediated plasmid pairing, whereas at high (4:1) ω₂:δ₂ ratios, stimulation of the ATPase activity was reduced and δ₂ polymerized onto DNA. Stimulation of the δ₂ ATPase activity and its polymerization on DNA required ability of ω₂ to bind parS DNA and its N-terminus. In vivo experiments showed that δ₂ alone associated with the nucleoid, and in the presence of ω₂ and parS DNA, δ₂ oscillated between the nucleoid and the cell poles and formed spiral-like structures. Our studies indicate that the molar ω₂:δ₂ ratio regulates the polymerization properties of (δ₂•ATP•Mg²⁺)₂ on and depolymerization from parS DNA, thereby controlling the temporal and spatial segregation of pSM19035 before cell division.

INTRODUCTION

Accurate distribution of newly replicated chromosomes before cell division is imperative for the stable transmission of genetic information. In eukaryotic cells, after chromosomal DNA condensation and alignment at mid-cell, microtubule fibers anchored via the kinetochore to the centromere pull the sister chromatids apart (1,2). In bacterial cells the mechanism that moves the newly replicated chromosomes and plasmids to opposite sides of the division plane requires a genuine partition system (ParA and ParB, ParM and ParR, or TubZ and TubR) (2–6).

For active and faithful segregation, most bacterial chromosomes and low-copy-number plasmids have evolved genuine partitioning (par) loci. The par loci contain one or more cis-acting DNA segment(s) (parS) and encode two trans-acting proteins: an ATPase motor protein and a centromere binding protein (3–5,7,8). Three evolutionary different plasmid partition systems have been identified: the tubulin-like (TubZ or type III), the actin-like (ParM or type II), and the Walker-box (ParA or type I) ATPases (4–6,9,10). The ParA system, which is the most common and conserved one, can be subdivided into two subfamilies (ParA-Ia and ParA-Ib) (4). The mechanism of action of ParA systems is less clear than that of the other mentioned systems, although a similar mechanism to the one observed with the actin-like systems has been suggested (3–5,7,8). Among the Proteobacteria phylum a large number of plasmid- and chromosome-encoded partition systems have been studied, e.g. plasmids P1, F and RK2 encode ParA-Ia ATPases (P1-ParA, F-SopA and RK2-IncC), while plasmids pB171 and pTB228 and the Caulobacter crescentus (Ccr) chromosome encode ParA-Ib ATPases (pB171-ParA, pTB228-ParF and CcrParA). However, among the Firmicutes phylum the ParA ATPases studied thus far (e.g. Streptococcus pyogenes pSM19035 and the Bacillus subtilis chromosome) encode ATPase of the ParA-Ib (δ₂ and BsuSoj) subfamily [(3–5), this work]. The ParA-Ia

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ATPases feature an N-terminal helix-turn-helix (HTH) motif that specifically interacts with DNA to repress the expression of the par loci, while the ParA-Ib ATPases bind non-specifically to DNA [e.g. δ2 and Thermus thermophilus Soj (TthSoj)] (3–5).

The ParB proteins are divided into three discrete subfamilies (ParB-I, -II and -III) (3–5). The ParB-I proteins (e.g. P1-ParB, F-SopB or RK2-KorB), and ParB-II proteins (e.g. BsuSpo01 or TthSpo01) recognize parS DNA via an HTH fold and ‘spread’ around the parS site. The ParB-III proteins (e.g. pB171-ParB, pTB228-ParG, pSM19035-ω2) work in concert with ParA-1b ATPases and recognize parS (parC) DNA via a ribbon-helix-helix (RHH) motif. The cis-acting parS DNA consists of one (e.g. P1-parS, F-sopC), two (e.g. pB171-parC) or several copies of the centromeric parS site (e.g. pSM19035-parS, Bsu-parS) (3–5,8).

Plasmid partitioning has mainly been studied in species of the γ Proteobacteria phylum (3–5,8). The polymerization of (δ•ATP•Mg2+)2 on DNA (see below), which is in stark contrast to ParA ATPases of γ proteobacterial plasmids that form protocilia in the absence of ParB and DNA, suggests that the dynamic movement of plasmid and bacterial chromosomes during faithful segregation in Firmicutes may not necessarily follow similar mechanisms as found for plasmids of γ Proteobacteria [5, this work]. Indeed, the evolutionary distance between B. subtilis or Streptococcus pyogenes (Firmicutes) and E. coli (γ Proteobacteria) exceeds that between plants and animals, and this raises the question whether bacteria of these two phyla share the same mechanism of plasmid partitioning. Here, we address this question by studying the segregation of plasmid pSM19035 originally isolated from the Firmicute and human pathogen S. pyogenes.

Plasmid pSM19035 replicates via a theta mechanism and is maintained stably at 1–3 copies per cell in B. subtilis, as well as in a wide range of species of the Firmicutes phylum (11–13). The par locus of pSM19035 encodes two trans-acting proteins, δ (ParA-Ib type) and ω (ParB-III type) and harbors six cis-acting parS sites ([14–16], this work). Protein δ, which occurs as a homodimer (δ2), shares sequence identity with bacterial and archaeal Walker-box ATPases, namely ThsSoj and Pyrococcus furiosus MinD (PfuMinD) ([11], Figure S1 in the Supplementary Data available with this article online). Protein ω, which occurs as a homodimer (ω2), acts as a multifunctional repressor of genes involved in copy number control, plasmid addiction and accurate segregation ([15], this work). Repressor ω2 negatively controls promoter utilization by binding cooperatively and with high affinity to the promoter regions upstream of copS, δ and ω genes (PcopS, Pδ and Pω). These regions, which function as the cis-acting parS sites (parS1 or Pω, parS2 or Pδ and parS3 or PcopS. Figure 1A and B), contain 10, 7 and 9 unspaced heptads with sequence S'-WATCACW-3' in (↔ or ←→) orientations (15). The affinities of ω2 for the cognate sites PcopS, Pω and Pδ are similar with a kD of ~6 nM ([17], Figure 1B). The minimal cooperative yet high affinity binding sites for ω2 are two contiguous heptads in direct (↔→→) or inverted (←→→→) orientations (minimal centromere) (17).

The N-terminal region of protein ω2 is unstructured (18,19). Crystal structures have been determined for protein ω2 in which the monomers lack the first 20 N-terminal amino acid residues (ω2ΔN20) (20) and for ω2ΔN19 in complex with two diheptads in (↔→→) and (←→→→) orientations (21). Chemical and enzymatic footprint data of ω2 binding to the centromere reveal a continuous protein super-structure consistent with the crystal structures (21). Extrapolating from these structures, ω2ΔN19 molecules assemble as a left-handed protein helix that wraps parS sites consisting of multiple DNA heptad repeats (Figure 1C). The abilities of ω2ΔN19 and wild-type (wt) ω2 to bind to parS DNA in vitro and to repress transcription in vivo are comparable, but substitution of Thr29 in (that binds specifically to the central G–C base pair of the heptads) for Alanine (ω2T29A) abolishes DNA binding (18,19,21).

Here, we provide the first crystal structure of a plasmid-encoded ParA-Ib type protein in the ATPγS-bound state (δ•ATP•γS•Mg2+)2 and show that plasmid pairing, polymerization of (δ•ATP•Mg2+)2 on and depolymerization from parS DNA, which is dependent on wt ω2 bound to parS DNA, is fine tuned by the stoichiometry of ω2 and δ2. This is consistent with the dynamic assembly of a partition apparatus through the interaction of (δ•ATP•Mg2+)2 with ω•parS complexes and supports a model proposed here for DNA segregation in Firmicutes that is mediated by δ2 (ParA) plus ω2 (ParB) and differs from that in γ proteobacteria plasmids.

MATERIALS AND METHODS

Plasmid stability test

The bacterial strains and plasmids used are listed in Supplementary Table S1. The numbers of plasmid-containing cells were determined by replica plating onto chloramphenicol-containing plates. The theoretical frequency of plasmid loss (Lθ = 2−n) is the probability of plasmid-free cells arising per division, with n being the number of copies of the plasmid per cell at cell division (12). The frequency of plasmid loss (L) was calculated as L = 1 − (P)½, where P is the number of cells bearing plasmids after growth for g generations.

Protein expression and purification

Proteins ω, ωΔN19 or ωT29A were expressed in E. coli BL21(DE3) pLysS cells and purified as described (17,18). Proteins δ, δK36A, δD60A or δΔ14 (having 14 extra N-terminal residues when compared to δ) were expressed in E. coli ER2566 cells and purified by sequential heparin POROS 20HE (buffer A, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA) containing 0.05 to 1 M NaCl concentrations, anion-exchange PL-SAX (buffer A containing increasing NaCl concentrations) and gel-filtration chromatography (buffer B, 20 mM Tris–HCl, pH 8.0, 200 mM NaCl). The protein concentrations were determined by absorption at 280 nm using molar extinction coefficients of 2980 M−1 cm−1 for ωω, ωΔN19 and ωT29A, and 38 850 M−1 cm−1 for δ2, δK36A and δD60A, and concentrations are specified for protein dimers.
Co-crystallization, data collection and structure determination

Crystals in space group P6_522 grew at 18°C in sitting drop vapour diffusion setups from 1 ml protein solution (14 mg δ_2/ml, buffer B with 2 mM ATP_5S and 5 mM MgCl_2) mixed with 1 μl of buffer C (1 M Hepes and 3% (v/v) ethanol pH 7.0). The mother liquor was supplemented with glycerol to a final concentration of 25% (v/v) prior to flash freezing the crystals in liquid N_2.

X-ray diffraction data were collected at 100 K at Protein Structure Factory beamline BL1 of Freie Universität Berlin at BESSY and processed with DENZO/Scalepack (22). The structure was determined by molecular replacement using a monomer of the TthSoj protein structure (pdb code 2BEJ) as a search model. The model of δ was built and water molecules were located with ARP/wARP (23). Restrained refinement cycles in REFMAC5 (24) converged at an R factor (R_free) of 19.2% (21.8%) (Table 1). Atomic coordinates and structure factors have been deposited with the Protein Data Bank under accession code 2OZE.

ATPase activity assay

ATPase activity was assayed by thin-layer chromatographic separation of the reaction products. Reaction mixtures (20 μl) contained buffer D [50 mM Tris–HCl, pH 7.5, 10 mM MgCl_2, 50 mM NaCl], 1 μM δ_2, δ-K36A or δ-D60A, 10 μM ATP, 0–2.5 nM parS DNA, 0–4.2 μM ω_2 or 1.4 μM ω_2, ω_2ΔN19 or ω_2T29A and were incubated for up to 180 min at 37°C.

Polymerization of protein δ_2

Dynamic light scattering (DLS) was measured in a 1.5 mm path length quartz cuvette at 90° angle in arbitrary units using a Laser Spectroscatter 201 (RIINA GmbH, Berlin) with 350 nm emission wavelength and plotted using the FL Solutions computer programme and Savitsky-Golay smooth data processing. Aliquots of HindIII-linearized 3.1 kb pUC57-borne parS DNA (25 nM) were incubated for 2 min on ice with protein δ_2 or variants δ-K36A or δ-D60A (1 μM) and different concentrations of ω_2 (0, 0.24, 0.48, 0.96, 1.8 or 3.6 μM) or ω_2Δ19N (2 μM) in
buffer E (50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTE, 5% glycerol). After pre-incubation of the proteins/DNA complex for 1 min, ATP, ADP or ATP/S was added to 1 mM final concentration and light scattering was measured as above at 30 s intervals for 5 min and subsequently every 2 min at room temperature. The measured intensity or count rate was the amount of scattered light expressed as photons detected per second and converted to particle size using the Stokes–Einstein relation. The intensity is given in arbitrary units (AU).

Filament formation of protein δ₂ or variants δ₂K36A or δ₂D60A (1 μM) in the presence or absence of fixed (1 μM) or variable concentrations of ω₂, parS DNA or ATP•Mg²⁺ was measured in a 1 cm path length quartz cuvette as the change in turbidity using a Hitachi F2500 scanning fluorometer equipped with a thermo-cuvette holder at constant temperature (37°C). Relative light scattering intensities were recorded. Linear 3.1 kb pUC57-borne parS DNA (25 nM) was pre-incubated with protein δ₂ (1.2 μM) and different ω₂ concentrations (0, 0.24, 0.48, 0.96, 1.8 or 3.6 μM) in buffer E for 1 min at 37°C. Then, ATP was added to 1 mM final concentration, and the samples were used for light scattering. In presence or absence of linear pUC57-borne parS DNA (2 nM), wt δ₂ or variants δ₂K36A or δ₂D60A (1 μM) and ω₂ (1 μM) were pre-incubated in buffer E for 1 min at 37°C. The ATP was added to a 1 mM final concentration and the samples were used for light scattering.

### Fluorescence and electron microscopy

Aliquots of *B. subtilis* cultures grown overnight in LB medium at 30°C were diluted in fresh medium to OD₅₆₀ ~ 0.05. IPTG (10 μM final concentration) was added to OD₅₆₀ ~ 0.2 cultures to induce the synthesis of (δ-GFP)₂ or (δK36A-GFP)₂, and incubation was continued until OD₅₆₀ ~ 0.6. Samples of the cells present were fixed and visualized as described (25). Images were acquired using an Olympus BX61 fluorescence microscope with an Olympus DP70 color CCD camera. Z-stacks of 20–25 images, separated by 0.1 μm, were collected and image deconvolution was performed using Huygens Professional software (Scientific Volume Imaging). DNA was stained using 0.2 μg DAPI/ml before microscopy.

**EcoRI**-linearized 3.1 kb pCB30 or pUC57 DNA (2 nM) harboring parS DNA was incubated with the desired protein(s) (see figure legends) for 15 min at 37°C in buffers D or E, respectively, in the presence or absence of 1 mM ATP, as previously described (26). The DNA–protein complexes were visualized by electron microscopy (EM) after negative staining with 1% uranyl acetate (27) or after fixation with 0.2% (v/v) glutaraldehyde for 10 min at room temperature. The procedures for adsorption of the complexes to mica, rotational shadowing with platinum and EM image evaluation have been described previously (28).

### RESULTS

**Crystal structure of (δ•ATPγS•Mg²⁺)₂**

The crystal structure of δ₂ bound to the non-hydrolyzable ATP analogue ATPγS and Mg²⁺ was determined using the structure of the *TthSoj* monomer in molecular replacement. The crystal asymmetric unit contains one δ•ATPγS•Mg²⁺ complex that forms a dimer (δ•ATPγS•Mg²⁺)₂ with the two subunits related by a crystallographic C₂ axis (Figure 2A and B). The dimer is stabilized by a hydrophobic surface patch that buries 2197 Å² of otherwise solvent accessible surface area per subunit, augmented by two reciprocal inter-subunit salt bridges formed between R119 and D189 of each monomer. The structure, refined at 1.83 Å resolution, includes all 284 residues of the wt protein δ. The recombinant version of this protein that was also used in genetic assays ([δ₁₄]₂, Table S1 in the Supplementary Data) carries additional 14 residues at the N-terminus that are disordered in the crystal structure (Table 1).

Gel filtration and chemical cross-linking (Figure S2) confirmed that δ₂ and the Walker A mutant δ₂K36A (see below) form dimers in solution even in the absence of a nucleotide or the presence of ADP.

The δ monomer contains an eight stranded β-sheet surrounded by 12 α-helices (Figure 2A). The N-terminal α-helix (α₁) is not conserved in other Walker-box ATPases and shields the outward facing edge of the β-sheet (Figure 2B). The (δ•ATPγS•Mg²⁺)₂ complex is U-shaped and each arm of the U represents one subunit with an ATP-binding site occupied by ATPγS facing the cleft of the U (Figure 2A and B).
The surface charge of \((\delta\bullet\text{ATP}\gamma\text{S}\bullet\text{Mg}^{2+})_2\) is negative near the bottom of the U and positive at the tips of the arms of the U (Figure 2D). The tip regions therefore most likely bind DNA and/or the negatively charged ‘bottom’ region of an adjacent \(\delta\) when assembled into a \(\delta\) polymer (see below).

The closest structural relatives of the \(\delta\) monomer structure are \(Tth\text{Soj}\) and \(Pfu\text{MinD}\) although the underlying primary sequences exhibit only 25 and 14% identity, respectively (Figure S1 in the Supplementary Data). Superimposition of \(\delta\) with 232 C\(\alpha\) atoms of \(Tth\text{Soj}\) and 219 C\(\alpha\) atoms of \(Pfu\text{MinD}\) shows root mean square deviations of 2.2 A\(\delta\) and 3.2 A\(\delta\), respectively, indicating a high degree of structural similarity between these proteins. However, the structure of the dimer \((\delta\bullet\text{ATP}\gamma\text{S}\bullet\text{Mg}^{2+})_2\) is significantly different from the hydrolysis-deficient \((Tth\text{Soj}D44A\bullet\text{ATP}\gamma\text{S}\bullet\text{Mg}^{2+})_2\) variant.

\(Tth\text{Soj}\) and \(Pfu\text{MinD}\) dimerize only in the presence of ATP. In the dimers, each ATP molecule interacts with both monomers and becomes completely buried within the dimer interface (7,29). Based on these structures, it appears that ADP could only be released after dissociation of \((Tth\text{Soj})_2\) or \((Pfu\text{MinD})_2\) into monomers, whereas the wide and open cleft in the U-shaped \((\delta\bullet\text{ATP}\gamma\text{S}\bullet\text{Mg}^{2+})_2\) allows free exchange of ATP and ADP without dissociation of the subunits.
The nucleotide-binding site

The ATPase activity in δ2 are positively charged (Figure 2E). The adenine N1 and amino group N6 of the two ATPyS form hydrogen bonds to S240Oγ and Y265Oγ/K238O, respectively (Figure 2F). The C2’-endo puckered ribose is not engaged in hydrogen bonds but K36-S37-K38 within the Walker A motif at the N-terminus of helix α2 hydrogen bond with their peptide NH groups to α- and β-phosphates of ATPyS, whereas K36Nη forms salt bridges with the β- and γ-phosphates. Mg2+ is octahedrally coordinated by β- and γ-phosphate oxygen atoms, by S37Oγ and by three water molecules (Figure 2E and F).

ATP hydrolysis requires a catalytic water molecule (Wcat) positioned in-line with the Pγ-Oβ bond (Wcat-Pγ-Oβ). In Walker-type ATPases, this Wcat is activated for nucleophilic attack on the γ-phosphate by an amino acid side chain in the Walker B motif that acts as catalytic base. However, in δ1ATPγS·Mg2+δ2, the position expected for Wcat is occupied by P150 within the Walker B motif (Figure 2F) but D60 of the Walker A motif hydrogen bonds to and likely activates Wcat, which may in turn attack the γ-phosphate group of ATP (Figure 2F). This atypical positioning of amino acids likely participating in catalysis explains the relatively low ATPase activity of δ2 (see below).

The two above mentioned residues K36 and D60 (Figure S1) were considered to be engaged in the ATPase activity and replaced by Alanine to form δ2K36A and δ2D60A (see below).

The ATPase activity of δ2 is fine-tuned by δ2 levels in the presence of parS DNA and ATP

Since δ2, δ2, and cis-acting parS DNA interact and regulate pSM19035 segregation (see below), we investigated how the presence or absence of δ2 and/or parS DNA affect the enzymatic activity of δ2. Only experiments with parS2 DNA are described here because similar results were obtained with parS1 or parS3 DNA (Figure 1). The ATPase activity of δ2 was low and δ2 had no ATPase activity (Figure 3A). The ATPase activity of δ2 was not stimulated by the addition of parS DNA (δ2+parS, Figure 3A). Addition of stoichiometric amounts of δ2 stimulated the ATPase activity of δ2 by about 50% (δ2+δ2+parS, Figure 3A). However, supplementation of parS DNA (δ2+δ2+parS) resulted in a 3-4-fold stimulation of the ATPase activity of δ2, which was reduced to ~2-fold when non-parS DNA was added (δ2+δ2+non-parS, Figure 3A). However, when δ2 was added at nanomolar concentrations, the ATPase activity of δ2 was only stimulated by parS DNA (Figure 3C, see below).

Stimulation of the ATPase activity of δ2 was marginal when δ2 was replaced by δ2T29A or δ2ΔN19 (δ2+δ2T29A+parS; δ2+δ2T29A+parS, Figure 3B). The ATPase activity of variants δ2K36A or δ2D60A amounted to ~30% of wt δ2 activity and no stimulation of their ATPase activity was observed in the presence of δ2 and parS DNA (δ2K36A+δ2+parS or δ2D60A+δ2+parS) (Figure 3B).

The stimulatory effect of increasing δ2 concentrations on the ATPase activity of δ2 in the presence of parS or non-parS DNA was also assayed (Figure 3C). In the presence of parS DNA and δ2, δ2 molar ratios from 0.9:1 to 1.4:1 the δ2-catalyzed ATP hydrolysis was stimulated with the peak around 1.4μM δ2, and the stimulation declined when the δ2:δ2 ratio was further increased from 1.4:1 to 4.2:1 (Figure 3C). The observed characteristics of ATPase activity stimulation and alleviation of δ2 is genuinely associated with δ2 and parS DNA. In contrast, when parS DNA was replaced by non-parS DNA, increasing δ2 concentrations stimulated the ATPase activity of δ2 almost linearly (Figure 3C).

Pairing of parS regions by proteins δ2 and δ2

The complexes formed by δ2 and parS DNA, in the absence or presence of δ2 or δ2AN19 or δ2T29A, were visualized by EM at low protein concentrations (Figure 4B–D). The substrate was the linear 3.1-kb pCB30 DNA containing parS DNA located at 320 bp from one end (Figure 4A). In presence of ATP·Mg2+, δ2 (100 nM) assembled to form discrete clusters on ~85% of the DNA molecules (n = 200) at random locations (Figure 4B), whereas ~40% of the DNA molecules (n = 250) that were incubated only with δ2 showed clusters of δ2 bound to parS on the plasmid (Figure 4C). The parS DNA region on linear DNA was not significantly disturbed by δ2 binding, consistent with the prediction based on crystal structures that protein δ2 would wrap around parS sites without significantly bending the DNA double helix [21, Figure 1C].

At 100 nM δ2 and 1 nM parS DNA but in the absence of δ1ATP·Mg2+δ2 only ~1% of the δ2+DNA complexes (n = 300) contained two DNA molecules that were paired at the position where δ2 was bound at the parS region. The frequency of these complexes was not increased by raising the δ2 concentration. However, when 1 nM parS containing DNA was incubated with 100 nM δ2, 60 nM δ2 or 1 nM ATP·Mg2+, ~20% of the parS DNA molecules were paired with DNA molecules juxtaposed at their δ2·parS regions (n = 200, Figure 4D), indicating that δ1ATP·Mg2+δ2 is required for plasmid pairing (Figure S4A). Plasmid pairing was absent at a 2:1 δ2:δ2 molar ratio (Figure S4B). Replacing δ2 with δ2AN19 or δ2T29A or δ2 with δ2K36A abolishing DNA pairing (Figure S4C–S4E).

δ2 polymerization on parS DNA is dependent on δ2 and ATP

DLS data in Figure 5A show that δ1ATP·Mg2+δ2 (1 μM) polymerizes onto linear 3.1-kb parS DNA in the presence of a 2:1 molar ratio of δ2:δ2. No δ2 polymers were formed when either parS DNA was omitted (see below), protein δ2 was substituted by δ2AN19, or ATP was substituted by ADP (Figure 5A).

ATP binding but no hydrolysis is required for δ2 polymerization on parS DNA in the presence of δ2 because ATPyS satisfied the cofactor requirement (Figure 5A). In presence of ATP or ATPyS, the size increment of the polymers showed a sigmoidal pattern consistent with cooperative polymerization and levelled off after ~60 min at room temperature. It remained at this level in the presence of ATPyS (Figure 5A, blue line) contrasting the presence of ATP, where polymerization decreased to initial
Figure 3. Stimulation of the ATPase activity of $\delta_2$ by $\omega_2$ and DNA. (A) ATPase activity in reaction mixtures containing 1 $\mu$M $\delta_2$ alone or with 2.5 nM parS DNA ($\delta_2 + \text{parS}$) or 1.4 $\mu$M $\omega_2$ alone or after incubation of 1 $\mu$M $\delta_2$ and 1.4 $\mu$M $\omega_2$ ($\delta_2 + \omega_2$) or additionally with parS DNA ($\delta_2 + \omega_2 + \text{parS}$) (average of five experiments, $n = 5$) or with non-cognate DNA ($\delta_2 + \omega_2 + \text{non-parS}$) ($n = 4$). (B) ATPase activity of $\delta_2$ after incubation of 1 $\mu$M $\delta_2$ and 1.4 $\mu$M $\omega_2$ in 90' light scattering, we investigated the component requirements for $\delta_2$ polymerization (Figure 5C). In presence of ATP, no polymerization was observed when $\omega_2$ ([$\delta_6\text{ATP}\text{Mg}^{2+}$]$_2$ + parS) DNA or parS DNA ($\delta_6\text{ATP}\text{Mg}^{2+}$]$_2$ + $\omega_2$) were omitted or in presence of ATPS when protein $\omega_2$ was omitted ($\delta_6 + \text{parS} + \text{ATP-S}$) (Figure 5C), suggesting that $\delta_2$ polymerization on DNA requires the interaction with $\omega_2$. To further evaluate the effect of the nucleotide cofactor, the $\delta_6$D60A and $\delta_6$K36A variants were also analyzed. As shown in Table S2, protein $\delta_6$ or $\delta_6$D60A bound with ~12-fold higher affinity to parS DNA in the presence of ATP than ADP, while binding of $\delta_6$K36A to parS DNA was weak, regardless of the presence of ATP or ADP. Wild type $\delta_2$ and variant $\delta_6$D60A feature similar polymerization kinetics ($\delta_2 + \omega_2 + \text{parS}$ vs $\delta_6$D60A + $\omega_2 + \text{parS}$) (Figure 5C), albeit the equilibrium was reached earlier in case of $\delta_6$D60A and the formed filaments were shorter. In contrast, variant $\delta_6$K36A showed
 replicating and segregationally unstable vector pHPl3 (Table S1). This vector was reported to replicate far from mid-cell and its replication imposes a metabolic burden that compromises its maintenance in host cells (30). The frequency of plasmid-loss was measured in B. subtilis cultured in LB medium at 30°C. Plasmids bearing a parS site and genes that directed the synthesis of o2 and δ2 (pCB706) or o2 and (δ-GFP)2 (δ2 with C-terminally fused GFP, pCB702) were retained in progeny cells at ~10-fold higher frequencies (Table S3) than predicted if the plasmid were randomly distributed (see Materials and methods section). However, random distribution was observed if plasmids lacked either the o2 or δ gene or carried genes that encoded variants δK36A, oΔN19 or oT29A (Table S3). This indicates that the integrity of the ATP binding site of δ2. DNA binding and the N-terminus of o2 and parS DNA are essential for correct pSM19035 partitioning.

Dynamic movement of protein (δ-GFP)2 depends on parS DNA and o2

To gain insight into the molecular mechanism by which the fully functional (δ-GFP)2 (see above) contributes to plasmid segregation, we imaged its cellular localization in the presence or absence of o2. The GFP signal overlapped with that of DAPI-stained DNA in 90% of the cells of a B. subtilis strain containing a pCB578-borne δ-gfp gene transcribed from its own Pδ (parS1) promoter (Figure 6). When o2 was also present (pCB702) it repressed the δ-GFP synthesis by ~70-fold compared to the absence of o2 (15). The low (δ-GFP)2 signal was no longer statically associated with the nucleoid but was dynamically located near the cell poles and/or associated with the nucleoid. Image deconvolution showed that in the presence of parS DNA, o2 and (δ-GFP)2 a spiral-like structure was formed within the cytosol of B. subtilis cells (Figure 6B). It is of interest that unlike (δ-GFP)2 that only formed a spiral-like structure in presence of parS DNA and o2 under auto-regulated conditions, spiral-shaped filaments formed by other ParA (pB171-ParA or F-SopA) neither required ParB (pB171-ParB or F-SopB) nor parS (pB171-parC or F-sopC) DNA (31,32).

To vary the intracellular concentration of (δ-GFP)2 independently of the o2 concentration, we placed the δ-gfp gene under the transcriptional control of the LacI repressor and integrated a single copy of this construct into the amy locus of the B. subtilis genome (Figure 7A). In the absence of a plasmid-borne parS site and of gene o2, (δ-GFP)2 was seen to co-localize at low IPTG concentration (10 μM) with the nucleoid in ~90% of the cells (n = 300), suggesting that (δ-GFP)2 binds non-specifically to DNA (Figure 7B and 7B’) and the fluorescence signal of (δ-GFP)2 was 2- to 3-fold higher than the one from cells bearing the pCB578-borne parS1 site and gene δ-gfp (Table S1). Under this condition, the presence of a parS region and o2 led to (δ-GFP)2 re-localization (‘oscillation’) near one cell pole in ~60% of the cells (n = 300) that had one nucleoid, or co-localized with one nucleoid in ~70% of the cells (n = 200) that had two nucleoids (Figure 7C and 7C’). Similar results were reported for the chromosomally encoded BsuSoj in the presence of BsuSapo0J and parS sites (25,33).

**Figure 4.** Electron micrographs of protein-DNA complexes. In the illustrated cartoons parS DNA, located 320 bp from one end (orange) of 3.1-kb linear pCB30 DNA, was incubated with o2 (red spheres) and/or δ2 (blue spheres), in the presence of 1 mM ATP and the complexes formed were visualized by EM. Linear DNA (1 nM) alone (A), plus δ2 (100 nM) (white arrows) (B), plus o2 (60 nM), showing o2-parS complexes (black arrows) (C), (D) Linear parS DNA (1 nM) incubated with δ2 (100 nM) and o2 (60 nM) in the presence of 1 mM ATP with paired molecules indicated by empty arrows. The bars indicate 500 nm.

A near-linear increase in light scattering or slowly assembled on parS DNA in the presence of o2 and ATP (δ2K36A + o2+ parS; Figure 5C).

**Plasmid segregation requires (δ•ATP•Mg2+)2, o2 and parS DNA**

To test the importance of proteins o2 and δ2 and a cis-acting parS site for plasmid segregation, we combined the respective genes and variants in the rolling-circle localization and integrated a single copy of this construct into the amy locus of the B. subtilis genome (Figure 7A). In the absence of a plasmid-borne parS site and of gene o2, (δ-GFP)2 was seen to co-localize at low IPTG concentration (10 μM) with the nucleoid in ~90% of the cells (n = 300), suggesting that (δ-GFP)2 binds non-specifically to DNA (Figure 7B and 7B’) and the fluorescence signal of (δ-GFP)2 was 2- to 3-fold higher than the one from cells bearing the pCB578-borne parS1 site and gene δ-gfp (Table S1). Under this condition, the presence of a parS region and o2 led to (δ-GFP)2 re-localization (‘oscillation’) near one cell pole in ~60% of the cells (n = 300) that had one nucleoid, or co-localized with one nucleoid in ~70% of the cells (n = 200) that had two nucleoids (Figure 7C and 7C’). Similar results were reported for the chromosomally encoded BsuSoj in the presence of BsuSapo0J and parS sites (25,33).
At time zero, we added 1 mM of ATP to incubated on ice in buffer E and DLS was measured at room temperature.

At levels comparable to (δ-GFP)$\text{2}$, the (δK36A-GFP)$\text{2}$ signal was distributed throughout the cells regardless of the presence or absence of a parS sequence and/or gene φ (Figure 7E, 7E′, 7F and 7F′). This indicates that the binding of δ$_2$ to DNA and oscillation in the cell requires ATPase activity. Indeed, variant (δK36A-GFP)$\text{2}$ bound to parS or non-parS DNA with a 6- to 7-fold lower affinity than wt δ$_2$ (Table S2).

**DISCUSSION**

ATPase δ$_2$, structure and properties

We showed that the ATPase δ$_2$ from the Firmicute plasmid pSM19035 and EcoMinD and ThhSoj from Gram-negative bacteria have similar monomer structures, but form dimers under different conditions. EcoMinD and ThhSoj are monomers in solution with or without ADP•Mg$^{2+}$ and binding of ATP•Mg$^{2+}$ induces formation of structurally similar dimers (7,29). In contrast, δ forms a dimer regardless of the presence of ADP•Mg$^{2+}$ or ATP•Mg$^{2+}$ or absence of a nucleotide cofactor (Figure S2) that is structurally different from the above two dimers (Figure 2B).

The ATPase activity of δ$_2$ is regulated by binding of the ω$_2$•parS DNA complex to δ$_2$. When the concentrations of ω$_2$ were increased up to 1.4:1 ω$_2$:δ$_2$ ratios the ATPase activity was stimulated but above this ω$_2$:δ$_2$ ratio, the stimulation diminished when the concentration of ω$_2$ is further increased up to a ratio of 4.2:1.

**Dynamic assembly of (δ•ATP•Mg$^{2+}$)$_2$ on ω$_2$•parS**

Our results show that pSM19035-borne parS sequence(s), ATP binding and hydrolysis by δ$_2$, and the N-terminus and the DNA binding ability of ω$_2$ are essential components of the genuine pSM19035 partition system, because the mutation or deletion of either one of these components abrogates plasmid segregation. Protein (δ-GFP)$\text{2}$ localized within the nucleoid of B. subtilis cells, whereas catalytically inactive (δK36A-GFP)$\text{2}$ was distributed throughout the cytoplasm, arguing for ATP•Mg$^{2+}$-dependent nucleoid localization (Figure 7E and F). Under native regulation, 1.2 μM δ$_2$ and the indicated ω$_2$ concentration were pre-incubated at 37°C in buffer E. Polymerization was initiated by the addition of 1 mM ATP (denoted by an arrow). Light scattering was measured at 37°C Values are averages of three independent experiments. (C) Polymerization of protein δ$_2$ requires ω$_2$•parS DNA and ATP•Mg$^{2+}$, as shown by an increase of light scattering. Reaction mixtures containing 3.1-kb parS DNA (20 nM), 1 μM δ$_2$, ω$_2$D60A or δ$_2$K36A and 2 μM ω$_2$ were pre-incubated at 37°C in buffer E. Polymerization was initiated by the addition of 1 mM ATP (denoted by an arrow). Light scattering was measured at 37°C. Values are averages of four independent experiments.

Figure 5. Polymerization of δ$_2$ in the presence of parS DNA, ω$_2$ or ω$_2$ΔN19 and ATP, ADP or ATP$\gamma$S. (A) Dependence of light scattering (AU) on time (min). Reaction mixtures containing linear 3.1-kb parS DNA (20 nM), 1 μM δ$_2$ and 2 μM ω$_2$ or ω$_2$ΔN19 proteins were pre-incubated on ice in buffer E and DLS was measured at room temperature. At time zero, we added 1 mM of ATP to δ$_2$ and ω$_2$ (red line) and to δ$_2$ and ω$_2$ΔN19 (green line), 1 mM ATP$\gamma$S to δ$_2$ and ω$_2$ (blue line) or 1 mM ADP to δ$_2$ and ω$_2$ (black line). (B) Light scattering increases with increasing ω$_2$:δ$_2$ ratio. Reaction mixtures containing 3.1-kb parS DNA (20 nM),
Bars indicate 2 μm. (A) The top illustration shows the structure of pCB578-borne P5 (parS1) and δ-GFP gene in B. subtilis cells. The lower panels show the localization of (δ-GFP)_2 fusion protein in the absence of protein δ2. Images captured in different optical planes were subjected to 2D deconvolution. Bars indicate 2 μm. (B) The top illustration shows the structure of pCB702-borne P5 (parS1) and δ-GFP gene, Pm (parS2) and ω2 gene in B. subtilis cells, and the repression by protein ω2 is indicated by ellipsoids. The lower panels show the spiral-like organization of (δ-GFP)_2 fusion protein in the presence of parS DNA, (δ-GFP)_2 and ω2. Images captured in different optical planes were subjected to 2D deconvolution. Bars indicate 2 μm.

Figure 6. Subcellular localization of (δ-GFP)_2 in the presence or absence of δ2 and parS DNA. (A) The top illustration shows the structure of pCB578-borne P5 (parS1) and δ-GFP gene in B. subtilis cells. The lower panels show the localization of (δ-GFP)_2 fusion protein in the absence of protein δ2. Bars indicate 2 μm. (B) The top illustration shows the structure of pCB702-borne P5 (parS1) and δ-GFP gene, Pm (parS2) and ω2 gene in B. subtilis cells, and the repression by protein ω2 is indicated by ellipsoids. The lower panels show the spiral-like organization of (δ-GFP)_2 fusion protein in the presence of parS DNA, (δ-GFP)_2 and ω2. Images captured in different optical planes were subjected to 2D deconvolution. Bars indicate 2 μm.

In vivo, spiral-shaped filaments formed by γ proteobacterial plasmids of the ParA-Ia or ParA-Ib families required only ParA (pB171-ParA or F-SopA) (31,32). This apparent paradox between the segregation mechanisms of plasmids of Proteobacteria and Firmicutes can be reconciled if one type of filaments were inactive (representing a storage form) and the other type were the active form. Alternatively, considering that the phylogenetic divide between γ Proteobacteria and Firmicutes is large (more than 1.5 billion years), we might assume that the ParA partition systems of these evolutionarily distant bacteria evolved different mechanisms to secure plasmid segregation during cell division.

The catalytic activity of δ2 is influenced by the concentration of δ2

Pairing of parS sites from two pSM19035 plasmids requires both, (δ•ATP•Mg^{2+})_2 and ω2 in the nM range (Figure 4D) to organize a structure that facilitates plasmid segregation. Indeed, the presence of ω2 alone gave rise to only few (~1%) presumably short-living and/or unstable pairs and failed to promote centromere pairing of pSM19035. Plasmid pairing at parS regions was observed at a ω2:δ2 ratio of 0.6:1 (Figure 4D), but no pairing was observed at a ω2:δ2 ratio of 1.2:1 (Figure S4B). The given ω2 concentration also affects the ATPase activity of δ2. Molar ω2:δ2 ratios of 0.09:1 to 1.4:1 stimulate ATP hydrolysis, whereas higher ratios (2.8:1 to 4.2:1) have the opposite effect and diminish the δ2 ATPase activity (Figure 3C). Moreover, we have shown here that the ω2 concentration needs to exceed a threshold to stimulate/initiate δ2 nucleoprotein filament formation (Figure 5B). In contrast, in vitro pairing of plasmids of the γ Proteobacteria requires only the centromere binding protein (R1-ParR or pB171-ParB) in the μM range and its cognate parC site, but is independent of its ParA partner (28,34). This was also observed in vivo by supercoil trapping for Pl-ParB•parS (35).

Formation of nucleoprotein filaments and proteofilaments have different requirements

In vitro, ParA filament formation has a different requirement for plasmids of γ Proteobacteria than those of the Firmicutes phylum. Protein (δ•ATP•Mg^{2+})_2 of Firmicutes polymerized onto DNA at high ω2:δ2 ratios, and formed nucleoprotein filaments that in turn depolymerized when ATP was hydrolyzed. When new ATP was added, polymerization was reinitiated (Figure 5A). Filament formation by δ2 onto DNA was not observed
Figure 7. Subcellular localization of (δ-GFP)$_2$ in *B. subtilis* cells in the presence or absence of parS and protein ω2. (A) Illustrations showing the structure of the (δ-GFP)$_2$ expression cassettes integrated into the *B. subtilis* chromosome, and plasmids that had or lacked a parS2 sequence and encoded ω or the ωAN19 variant. (B–F) Images of cells with fluorescence from (δ-GFP)$_2$, or (δK36A-GFP)$_2$, and (B to F) images of the same cells stained with DAPI to show DNA. Cells contained (δ-GFP)$_2$, (B), (δ-GFP)$_2$, ω2 and parS DNA (C), (δ-GFP)$_2$, ωAN19 and parS DNA (D), (δK36A-GFP)$_2$, (E) and (δK36A-GFP)$_2$, ω2, and parS DNA (F) were taken from exponentially growing cultures in the presence of 10 μM IPTG. Scale bar (in C) is 2 μm.
when $\omega_2$ or ATP was omitted or when $\omega_2$ was replaced by $\omega_2$AN19. Like protein $\delta_2$, chromosomally encoded $TthSoj$—from the *Deinococcus-Thermus* phylum—also forms nucleoprotein filaments onto DNA, but does not require its ParB partner Spo0J (29). Recently it was shown that one of the arginine residues (R189 in *BsuSoj*) that is conserved among chromosome-encoded ParA-Ib type ATPases lies on the surface of the Soj dimer and is essential for binding to DNA (36). In $\delta_2$, the equivalent position features S212 (Figure S1).

Dynamic assembly and disassembly of *EcoMinD* resembles that of $\delta_2$ or the *TthSoj* systems. *EcoMinD* assembled only on the surface of phospholipid vesicles and formed filaments in the presence of ATP$\cdot$Mg$^{2+}$ (37). Addition of *EcoMinE* promoted *EcoMinD* disassembly through stimulation of its ATPase activity (37,38).

*In vitro*, proteofilaments formed by proteobacterial plasmids of the ParA-Ia or ParA-Ib families required only ParA (pB171-ParA or F-SopA) but neither required ParB (pB171-ParB or F-sopB) nor parS (parC or sopC) DNA (31,32). Even in the absence of DNA and its ParB partner, purified ParA-Ia or ParA-Ib ATPases of proteobacterial plasmids are stimulated by the addition of ATP and form surface-independent proteofilaments and bundles that continuously increase in length (39–41). Replacement of ATP by ATP$\cdot$S had different outcomes since it failed to induce polymerization in case of F-SopA but greatly stimulated polymerization in case of pTP228-ParF (40,41).

**Modelling $\omega_2$parS(6ATP)$_2$ segregation**

Many of the models proposed for ParA-type partitioning systems of plasmids isolated from γ Proteobacteria share some features in common with the ParM-type system (9,42,43) because ParB promotes pairing of ParB$\cdot$parS complexes, and ParA forms multi-stranded proteofilaments formed in the absence of ParB and parS DNA (32,34,40,41,44,45). There is no evidence for filament disassembly in the presence of their ParB homologs, which markedly stimulate (≈30-fold) ATP hydrolysis, and a large excess (>10-fold) of ParB-like protein does not exert a negative effect on the activity of the ParA ATPase (46). These features neither apply for plasmids isolated from Firmicutes nor for chromosome-encoded ParA systems ([5], this work).

On the basis of all our findings with the pSM19035 Par system, we propose a model that is distinct to those developed for Proteobacterial plasmids. In *B. subtilis* cells, the best studied member of the Firmicutes, plasmids move dynamically within the cytosol, and the replisome is recruited to the subcellular position of the plasmid rather than to a central stationary position of the chromosomally associated replisome (30). Replication and segregation of pSM19035 are regulated by the intracellular concentration of $\omega_2$ (15). Upon plasmid replication, $\omega_2$ binds to and wraps around the parS sites on pSM19035 DNA to mediate both, transcriptional regulation and segregation ([15,21], Figure 1C). In an artificial system, which contains only one parS site, $\omega_2$ and parS form a parS$\cdot$$\omega_2$ complex with only limited extension (spreading) on non-specific sequences (15,17). This complex stimulates the recruitment of (6ATP$\cdot$Mg$^{2+}$)$_2$, and the resulting parS$\cdot$$\omega_2$ (6ATP$\cdot$Mg$^{2+}$)$_2$ nucleoprotein super-structure leads to intermolecular pairing of parS regions and accurate positioning of plasmid copies. In the natural context, with six consecutive parS sites, multiple parS$\cdot$$\omega_2$ complexes can be formed and organized into higher-order complexes.

We postulate that once the plasmids are paired, the local intracellular concentration of $\omega_2$ increases, the ATPase activity of $\delta_2$ decreases, plasmid pairing is lost and polymerization of (6ATP$\cdot$Mg$^{2+}$)$_2$ onto plasmid DNA is stimulated. Protein (6ATP$\cdot$Mg$^{2+}$)$_2$ nucleation onto plasmid DNA at the $\omega_2$parS DNA complex, thereby generates one end of the nucleoprotein filament [parS$\cdot$$\omega_2$ (6ATP$\cdot$Mg$^{2+}$)$_2$]. From this initial assembly site, polymerization of (6ATP$\cdot$Mg$^{2+}$)$_2$, leads to the formation of a nascent $\delta_2$ filament that depends on the presence of ATP, but is independent of ATP hydrolysis, to reach the end where another parS$\cdot$$\omega_2$ (6ATP$\cdot$Mg$^{2+}$)$_2$ complex is located. The decrease of the $\omega_2$: $\delta_2$ molar ratio at the site where the $\delta_2$ filament encounters another parS$\cdot$$\omega_2$ complex will stimulate the ATPase activity of $\delta_2$ (Figure 3A) and after ATP hydrolysis (6ADP$\cdot$Mg$^{2+}$)$_2$ will dissociate from the nucleoprotein complex, beginning proximal to the $\omega_2$parS region. A parS$\cdot$$\omega_2$ diffusion towards the adjacent (6ATP$\cdot$Mg$^{2+}$)$_2$ molecule in the nucleoprotein filament can then re-initiate the cycle of ATP hydrolysis and propagation along the DNA lattice towards opposite ends, with subsequent retraction of the $\delta_2$ nucleoprotein filament. The disassembly of filaments could contract the spiral-like structure (Figure 6) by moving the same cargo (individual $\omega_2$parS region) in a step-wise motion, and generate a force that moves the plasmid DNA outwards along the cell axis. The proposed model depends on changes in the intracellular concentration of $\omega_2$, the dislodging of $\delta_2$ from the nucleoid and the assembly and disassembly of $\delta_2$ rather than on anchoring of the $\delta_2$ nucleoprotein filament at the cell quarters or at midcell (47). Although many questions remain, the data reported here favor pSM19035 pairing before $\delta_2$ nucleoprotein filament dynamics, thereby securing plasmid movement from the replication site to opposite cell poles. A similar mechanism may be employed by some chromosomally encoded partitioning systems (5).

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank D. Rudner (Harvard University) for providing plasmid pDR111, C. Böttcher (Freie Universität Berlin) for help with EM. This work was partially supported by grants BFU2006-01062 from Ministerio de Educación y Ciencia-Dirección General de Investigación (MEC-DGI) to J.C.A. and by EU-grant QLK2-CT-2002-01079 to J.C.A. and W.S. Crystallographic atomic coordinates and structure factors have been deposited with the Protein.
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