ParAB Partition Dynamics in Firmicutes: Nucleoid Bound ParA Captures and Tethers ParB-Plasmid Complexes

Virginia S. Lioy1a, Andrea Volante1, Nora E. Soberón1ab, Rudi Lurz2, Silvia Ayora1, Juan C. Alonso1*

1 Department of Microbial Biotechnology, Centro Nacional de Biotecnología, CNB-CSIC, Darwin Str. 3, 28049 Madrid, Spain, 2 Max Planck Institute for Molecular Genetics, Ihnestrasse 73, D-1000 Berlin, Germany

a Current address: Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, 91190, France
b Current address: Centro Nacional de Investigaciones Oncológicas, Madrid, Spain
* jcalonso@cnb.csic.es

Abstract

In Firmicutes, small homodimeric ParA-like (δ2) and ParB-like (ω2) proteins, in concert with cis-acting plasmid-borne parS and the host chromosome, secure stable plasmid inheritance in a growing bacterial population. This study shows that (ω:YFP)2 binding to parS facilitates plasmid clustering in the cytosol. (δ:GFP)2 requires ATP binding but not hydrolysis to localize onto the cell’s nucleoid as a fluorescent cloud. The interaction of (δ:CFP)2 or δ2 bound to the nucleoid with (ω:YFP)2 foci facilitates plasmid capture, from a very broad distribution, towards the nucleoid and plasmid pairing. parS-bound ω2 promotes redistribution of (δ:GFP)2, leading to the dynamic release of (δ:GFP)2 from the nucleoid, in a process favored by ATP hydrolysis and protein-protein interaction. (δD60A:GFP)2 accumulates foci or patched structures on the nucleoid. We propose that (δ:GFP)2 binding to different nucleoid regions and to ω2-parS might generate (δ:GFP)2 gradients that could direct plasmid movement. The iterative pairing and unpairing cycles may tether plasmids equidistantly on the nucleoid to ensure faithful plasmid segregation by a mechanism compatible with the diffusion-ratchet mechanism as proposed from in vitro reconstituted systems.

Introduction

In eukaryotes, much insight has been gained into how chromosomes are segregated. In contrast, much less is known in prokaryotes. The ParAB partition system, which is the only type present in bacteria, is the most widespread system among low-copy number plasmids. This system relies on four components: ParA and ParB proteins, cis-acting plasmid-borne parS DNA and the host genome [1,2]. In general the ParA and ParB proteins are subdivided in two...
subfamilies based on their size [3]. The large ParA ATPases (e.g., P1-ParA or F-SopA) contain two DNA binding domains: an N-terminal sequence-specific and a C-terminal non-specific (ns) DNA binding domain [4–6]. The small ParA ATPases, which lack the N-terminal sequence-specific DNA binding motif, bind nsDNA by forming either filaments (e.g., pB171-ParA) [7], discrete blobs (e.g., pSM19035-ω2) [8], or they might form bundles (e.g., TP228-ParF) in the absence of any support [9].

The ParB centromere binding proteins (CBPs) are subdivided also into two structurally unrelated groups. The first group includes large (or medium size) dimeric helix-turn-helix proteins (e.g., P1-ParB, F-SopB and chromosomal-encoded ParB) that bind to parS and to nsDNA to form large nucleoprotein complexes [10–14]. These CBPs, upon binding to parS, co-operatively spread over nsDNA many kilobases (kb) and promote bridging, looping and condensation of nsDNA [10–12,15]. The second group includes small dimeric ribbon-helix-helix ParB proteins (e.g., pSM19035-ω2, TP228-ParG, pB171-ParB). pSM19035-ω2 specifically binds to parS to form ordered helical structures without significant spreading into nsDNA [8,16,17].

The interaction of the ParA and ParB components, which leads to proper separation of plasmid copies, has been extensively studied in plasmids and bacteria of the Proteobacteria phylum. These studies provide the foundation for filament- and non-filament-based modes of plasmid and bacterial chromosome segregation. In the filament-based modes, small ParA, when bound to ATP (ParA-ATP), assembles into bundles, and the partition complexes are mobilized by linear contractile filaments in a manner reminiscent of the spindle mechanism in eukaryotes (thread pushing or pulling model) [9,18]. Alternatively, ParA assembles by forming nucleoprotein filaments, and the partition complexes are mobilized by contractile helical or linear filaments as a cargo (filament-pulling model) [7,19]. In the non-filament-based mode (diffusion-ratchet and DNA relay models), small or large ParA-ATP binds to the nucleoid as dimers or small oligomers [8,20–25]. In the diffusion-ratchet model, a propagating large ParA ATPase gradient is the driving force for movement of the partition complexes [22–24], whereas in the DNA relay model, the forces that drive segregation are generated by the small ParA gradient and the elastic forces within the DNA molecule [25]. Very little is known about the mechanisms that lead to accurate segregation of small ParA- and ParB-like proteins in plasmids of the Firmicutes phylum. It was previously shown that the almost absolute segregational stability of plasmids belonging to the inc18 family requires at least two active stabilization systems, the partition (ParAB or SegB2) and toxin-antitoxin (SegB1) systems (Fig 1A). Plasmids of the inc18 family (pSM19035 being its representative) require homodimeric small ParA-like δ (δ3) and small ParB-like ω (ω2) products as well as parS to ensure faithful segregation (Fig 1B) [26]. In pSM19035, the parS sites, which comprise 7 to 10 contiguous heptads, overlap with the promoter (P) regions of the δ (Pδ or parS1), ω (Pω or parS2) and cop (Pcop or parS3) genes (Fig 1B) [8,16,17,27]. Faithful segregation of a plasmid bearing the ω gene, transcribed from its own promoter Pω, is not significantly impaired in comparison with its natural context if the expression of the (gfp) gene (integrated into the bacterial chromosome and transcribed from an IPTG-inducible promoter, Phsp) mimics its native concentration [28]. It is likely therefore that a single parS site may be sufficient for stable plasmid segregation, but in its natural context parS1, parS2 and parS3 are present (Fig 1A).

The structure of CBP ω2 or its variant ω2ΔN19, which lacks the first 19 amino acids, bound to minimal sub-sites allowed us to understand how ω2 binds to parS DNA (Fig 1C) [16,29]. In vitro, ω2 or ω2ΔN19 transiently binds with high affinity and co-operativity to parS DNA (apparent dissociation constant [KDapp] 5 ± 1 nM) [30–32]. The interaction of the unstructured N-terminal end of ω2 with δ3 (even in the apo form) increased the binding affinity of ω2 for parS DNA ~8-fold (KDapp 0.7 ± 0.1 nM) and the half-life of the ω2-parS DNA complex >20-fold [17].
Fig 1. Genome organization, δ₂ structure and proposed ω₂-parS complex. (A) Plasmid pSM19035 map indicating the duplicated (thick arrows) and the unique non-repeated sequences (thin lines). The replication origin (yellow box) and direction of replication (denoted by arrows) are indicated. The upstream region of the promoters of the copS, δ and ω genes (red boxes), which constitute the six parS sites, are enlarged. The variable number of 7-bp repeats (iterons) is symbolized by filled arrowheads (▸ or ◅). The promoters repressed by ω₂ (red balls) are indicated. The SegB1 (ω₂, ε₂ and ζ) and the SegB2 (δ₂, ω₂) loci are indicated. For simplicity all these features are colored and highlighted in the duplicated region located at the right of the plasmid, but the same applies for the other long inverted repeat. (B) The parS sites consist of a variable number of contiguous iterons present in three different promoter regions.
The boxes denote the -35 and -10 consensus sequences and the bent arrows denote the +1 position of the transcripts. (C) Model of seven $\omega_2$ bound to parS2 DNA based on the crystal structures determined for $[\omega_2\Delta N19]_2$ (-----) and $[\omega_2\Delta N19]_2$ (------) complexes (PDB 1IRQ, 2BNW, 2BNZ and 2CAX) [16]. DNA is shown in grey with the -35 and -10 sequences in yellow, and $\omega_2$ in surface representation (one monomer is purple, the other violet). (D) Electrostatic potential surface representation of $\delta_2$ in the ATP$\gamma$S-Mg$^{2+}$ bound form (PDB 2OZE) displayed using PyMOL. The surface charge of $\delta_2$ is negative (red) near the bottom of the U, and positive (blue) at the tips of the arms of the U. The relevant region involved in nsDNA binding maps at the tips of the arms of its U-shaped structure. The localization of two residues involved in nsDNA binding used in this work are indicated by dotted lines (each one located in one monomer).

Protein $\delta_2$ has a U-shaped structure, with each of the arms and the joining region representing one monomer (Fig 1D). The C-terminal nsDNA binding domain lies at the tip of the arms of the U structure (highlighted by the D211 and K242 residues in Fig 1D) [27]. In vitro, wild type [wt] $\delta_2$ bound to ATP-Mg$^{2+}$ (denoted as ATP) binds to nsDNA, forming discrete complexes. These complexes, which show spherical or blob shapes rather than a nucleoprotein filament, contain up to $5 \pm 1 \delta_2$/blob as shown by atomic force microscopy (AFM) [8]. In the absence of nsDNA, however, wt $\delta_2$-ATP free in solution forms discrete blob shaped structures containing $2-3 \delta_2$/blob, rather than long bundles [8]. The interaction of $\delta_2$-ATP bound to nsDNA with wt $\omega_2$ bound to parS facilitates plasmid-nucleoid pairing in vitro [8,27]. Biochemical analysis also showed that stoichiometric $\omega_2$ concentrations stimulate the ATPase activity of $\delta_2$, resulting in dissociation of $\delta_2$ from nsDNA and plasmid-nsDNA unpairing [17,27].

We report here that in vivo ($\omega_2$:YFP)$_2$ binding to a plasmid-borne parS site causes discrete clustering of plasmid copies and that ($\delta$:GFP)$_2$ bound to the Bacillus subtilis genome forms dynamic clouds over the nucleoid. The interaction of ($\delta$:GFP)$_2$ bound to the nucleoid with wt $\omega_2$ or ($\omega$:YFP)$_2$ bound to parS captures and tethers plasmids at the nucleoid, as reported from in vitro analyses [8,27]. Then, the $\omega_2$:parS complex stimulates the $\delta_2$ or ($\delta$:GFP)$_2$, ATPase activity, and ATP hydrolysis facilitates the disassembly of $\delta_2$. The iterative assembly/disassembly cycles may transduce the chemical energy produced by the motor protein into unidirectional plasmid movement.

Materials and Methods

Strains and plasmids

The B. subtilis strains used are listed in S1 Table. In BG1311, the 3´-end of the lacI gene was fused to gfp gene, to render the lacI-gfp gene that was placed under the control of the xylose-inducible promoter, and integrated, by a double crossover event, as a unique sequence at the amy locus of BG214 cells. In BG1469 and BG1447, the promoter-less $\omega$:yfp and $\omega$:N19:yfp genes were placed under the transcriptional control of the IPTG-inducible Phsp promoter, and integrated, by a double crossover event, as unique copy at the amy locus of BG214 cells. The plasmids used for localization studies, based in the pH14 vector, were grown in B. subtilis and are listed in S1 Table. The $\delta$ gene encodes two co-linear polypeptides, a 298-residues ($\delta_{14}$) and a 284-residues product. The structure of $\delta_{14}$ (having 14 extra N-terminal residues) bound to ATP$\gamma$S and Mg$^{2+}$ includes all 284 residues of the wt $\delta$ protein [27]. The plasmid-based wt $\delta$ gene and its variants were under the control of its own promoter ($P_\delta$), which overlaps with the parS1 site, and the $\omega$ gene and its variants were under the controls of its own promoter ($P_\omega$), which overlaps with parS2 (S1 Table, Fig 1B). The plasmids used for overexpression, based in the pT712 vector, were grown in E. coli ER2566 (Biolabs), and are listed in S1 Table.

Plasmid copy number, plasmid stability test, $\beta$-galactosidase assays and in vivo $\omega_2$ and $\delta_2$ concentrations

The number of plasmid copies per cell was estimated by hybridization and by quantitative PCR and normalization with two distinct chromosomal genes as previously described [21,33]. To
determine the frequency of plasmid loss, cells bearing plasmids were grown for more than 100 generations in S7 minimal medium (MMS7). After 8 h incubation at 30°C (~12 generations), a fraction of the culture was diluted into pre-warmed fresh antibiotic-free MMS7 medium, and grown again for 8 h at 30°C. This dilution and growth was repeated until the 100 generations were reached. The number of cells containing plasmid (conferring chloramphenicol resistance) was determined at different time intervals by plating appropriate dilutions in LB plates and then replica plating onto chloramphenicol-containing plates. The relative loss rate is expressed as a percentage and calculated as $L = \left( L_N - L_X \right) / \left( L_N - L_P \right) \times 100$, where $L_N$ is the loss rate per cell generation of negative control (empty vector), $L_X$ is the empirical loss rate of vector-bearing $\delta_2$ and $\omega_2$ variants, and $L_P$ is loss rate per cell generation of positive control (vector-bearing $\delta$ and $\omega$ wt genes).

The promoter-less lacZ read from the $P_\delta$ promoter ($P_\delta$ lacZ fusions), integrated into the amyE locus (BG508 strain), was used for in vivo transcription experiments. $\beta$-galactosidase assays were performed as described [30] except that the centrifuged $B$. subtilis cells were resuspended and lysed by the addition of 0.1% sodium dodecyl sulfate (SDS) (final concentration 0.0025%) and chloroform (final concentration 2%). The activity is expressed in Miller units after small modifications as described [30].

To quantify protein levels, $B$. subtilis cells bearing plasmid-borne $\delta$, $\omega$, $\delta$ and $\omega$ gene(s) (or their mutant variants), under their native (or IPTG-induced) control (see S1 Table), were grown in LB to an OD560 = 0.4 at 37°C with agitation in the presence of chloramphenicol (5 $\mu$g/ml). The cells were harvested, resuspended in buffer A (50 mM Tris HCl [pH 7.5], 300 mM NaCl, 5% glycerol) and lysed by sonication. Extracts containing equal concentrations of protein from each condition alongside purified $\omega$ and $\delta$ protein standards (5 to 500 ng) were separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Mouse polyclonal anti-$\delta_2$ and rabbit polyclonal anti-$\omega_2$ antibodies were obtained using standard techniques [27]. Immunoblots were transferred and probed with the antibodies as described previously [34]. Protein $\omega$ and $\delta$ bands, on developed immunoblots, were quantified with a scanning densitometer (Quantity One software, BioRad). Purified $\omega$ and $\delta$ protein standards yielded a linear relationship between antibody signal and the protein concentration. The amount of $\omega$ and $\delta$ protein in each sample was interpolated from the standard curve obtained with purified protein, and the in vivo concentration of $\omega$ and $\delta$ was estimated considering a cell volume of 1.2 femtoliters and based on 5 x 10$^7$ colony-forming unit (CFU)/ml at an OD560 of 0.4. Since >95% of BG214 cells were singlets and doublets, a correlation of CFU per cell averaged to 1.6.

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 New England Biolabs and Sigma. Ultrapure acrylamide was from Serva (Heidelberg, Germany). The broad protein molecular weight marker was obtained from GIBCO-BRL (Barcelona, Spain). Proteins $\omega_2$, $\delta_2$, $\delta_2$D60A, $\delta_2$D211A, $\delta_2$D60A $\delta_2$D211A, and $\delta_2$K242A and pBC30-borne parS2 DNA, which is the source of parS DNA, were purified as described [27,30,32].

The concentration of DNA was expressed as moles of DNA molecules and was determined 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 $\delta_2$, $\delta_2$D60A, $\delta_2$D211A, $\delta_2$D60A $\delta_2$D211A and $\delta_2$K242A. Concentrations are expressed as moles of protein dimers. It must be noted that unless stated otherwise, $\delta_2$ or its mutant variants in the ATP bound form are denoted as $\delta_2^*$, in the presence of ADP-Mg$^{2+}$ as $\delta_2$-ADP, and in the absence of a nucleotide co-factor as apo-$\delta_2$, respectively.
Protein-DNA complexes

For electrophoretic mobility shift assays (EMSA), gel-purified 423-bp $[^{32}P]-\text{HindIII-KpnI}$ parS2 DNA (0.1 nM) was incubated with various amounts of wt $\omega_2$, wt $\delta_2$ (or its variants), or both proteins in buffer B (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM MgCl$_2$) containing or lacking 1 mM ATP or ADP for 15 min at 37°C in 20 µl final volume. The reactions were stopped by addition of loading buffer (1 mM EDTA, 0.1% [v/v] bromophenol blue, and 0.1% [v/v] xylene cyanol) and were then separated using 4 or 6% PAGE. PAGE was conducted in 1x TAE running buffer at 200 V at 4°C, and the gels were dried prior to autoradiography.

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

The structural images were generated using PyMOL Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC).

Fluorescence and electron microscopy

B. subtilis cells bearing the indicated plasmid or expression cassette were grown overnight in MMS7 medium, in the presence of chloramphenicol or spectinomycin, at 30°C. The cultures were diluted in fresh medium to OD$_{560}$ ~0.05 and incubated until OD$_{560}$ ~0.4. Synthesis of the LacI:GFP fusion, from the BG1311 strain, was induced by addition of xylose (0.5%). Plasmid-borne $\omega:yfp$, $\delta:gfp$ or $\deltaD60A:gfp$ genes were expressed from their native promoters. When indicated IPTG (10 µM final concentration) was added to BG947 or BG1097 cells to induce synthesis of chromosomal-encoded $\delta:gfp$ or $\deltaD60A:gfp$ gene. In the absence of IPTG, cellular autofluorescence was not observed. For nucleoid visualization, the sample (1.5 ml) was incubated with DAPI (final concentration 5 µg/ml) on ice and in darkness for 10 min before slide preparation [27]. The cells were harvested (1.5 ml), centrifuged, and the pellet resuspended in pre-warmed MMS7 medium (50 µl). An aliquot was placed on a polyllysine-coated glass slide and covered with a coverslip, and incubated at 30°C as previously described [21]. Images were acquired using a Nikon Eclipse E-1000 fluorescence microscope equipped with a Nikon C-CU Universal condenser, a Smrock GFP-3035 bright-line zero band-pass filter cube, and a Hamamatsu Orca-ER c4742-95 charge-couple device (CCD) camera. Time-lapse photo-microscopy, with images gathered every 20 s over a 10 min period, was carried out with cells growing as micro-colonies on a slide, and analyzed with the Image Pro Plus 6.1 software using macro-directed cell recognition and measurement of the focus number and position as described [21].

Circular pCB30 harboring parS2 DNA (5 nM) was incubated with the indicated protein(s) for 15 min at 37°C in buffer C (50 mM TEA [pH 7.5], 50 mM NaCl, 10 mM MgCl$_2$, 1 mM ATP) as previously described [31]. After negative staining with 1% uranyl acetate or after fixation with 0.2% (v/v) glutaraldehyde for 10 min at room temperature, the DNA-protein complexes were visualized by electron microscopy (EM) [27,35]. The procedures for adsorption of the complexes to mica, rotational shadowing with platinum, and EM image evaluation have been previously described [27].

Results

Contribution of $\delta_2$ and $\omega_2$ to segregation stability

The functionality of the proteins analyzed in this work was tested using the plasmid stabilization assay described previously [27]. The $\delta$ and $\omega$ gene products and parS1 ($P_\delta$) and parS2 ($P_\omega$)
are necessary to stabilize an unstable and unrelated plasmid replicon (Fig 2, S1 Table). However, under certain conditions (i.e., when the δ gene is transcribed from a IPTG inducible promoter (P_{hsp}), see below) a single parS site is sufficient to stabilize plasmid segregation [28].

When δ2 was replaced by fused δ:Gfp, which is also a dimer in solution (£(δ:GFP)_{2}$), faithful plasmid segregation was observed (Fig 2). Similar results were observed when δ was replaced by the fused δ:cfp gene (data not shown). Previously it was shown that: i) ATP-bound δ binds nsDNA, but ADP-bound δ and apo-δ do not bind nsDNA (S2 Table) and ii) ATP-bound δ,D60A, which is unable to hydrolyze ATP, binds nsDNA with a 2-fold higher affinity (S2 Table) [27]. When δ2 was replaced by (£δ,D60A-GFP)_{2} plasmids were randomly segregated (Fig 2). These results suggest that the C-terminal fusion does not affect the activity of δ2, and that ATP hydrolysis is essential for plasmid segregation.

To gain insight into how δ binding affinity to nsDNA contributes to plasmid segregation a screening assay was performed. An exchange of a single negatively charged residue (e.g., δ,D211) to alanine in the DNA binding domain resulted in in vitro binding to nsDNA with > 6-fold higher affinity than wt δ (S2 Table). When δ2 was replaced by the δ,D211A variant plasmids were faithfully segregated (Fig 2). However, in the absence of ATP hydrolysis, increased affinity for nsDNA (i.e., in the δ,D60A D211A variant, S2 Table) was not sufficient to facilitate faithful plasmid segregation (Fig 2), suggesting that ATP hydrolysis rather than increased affinity for nsDNA is required for plasmid segregation.

<table>
<thead>
<tr>
<th>Genes</th>
<th>δ constructs</th>
<th>Stability (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- -</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ω -</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>- δ</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>δ_{AN/9}</td>
<td>£N_{2/4}</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ω : sfp</td>
<td>δ</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ω δ</td>
<td>δ</td>
<td>100</td>
</tr>
<tr>
<td>ω δ_{Gfp}</td>
<td>δ_{Gfp}</td>
<td>99.6</td>
</tr>
<tr>
<td>ω δ,D60A_{Gfp}</td>
<td>δ,D60A_{Gfp}</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ω δ,D211A_{Gfp}</td>
<td>δ,D211A_{Gfp}</td>
<td>97.2</td>
</tr>
<tr>
<td>ω δ,D60A_2D211A_{Gfp}</td>
<td>δ,D60A_2D211A_{Gfp}</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ω δ,K242A_{Gfp}</td>
<td>δ,K242A_{Gfp}</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Fig 2. Scheme of the different ω2 and δ2 variants used and their contribution to plasmid stability. Essential domains in the δ protein are highlighted and the asterisks indicate the position of the mutated residues. The Par pHPl4 vector and derivatives (~8 copies per cell) bearing the whole pSM19035 par locus or part of it were grown in LB medium at 30°C for at least 120 generations of growth, and plasmid stability was measured as described in the Materials and Methods section.

doi:10.1371/journal.pone.0131943.g002
Previous results showed that wt $\omega_2$ or $\omega_2\Delta N19$ binds and represses $P_\delta$ utilization both in vivo and in vitro (S3 Table) [30], but the latter lacks the region essential for $\omega_2$-$\delta_2$ interaction [32]. When $\omega_2$ was replaced by $\omega_2\Delta N19$ plasmid partitioning was impaired (Fig 2), suggesting that $\omega_2$-$\delta_2$ interaction is necessary for faithful plasmid segregation.

The $\omega$ gene was fused to the yfp gene at either the 5' - or 3' -end leading to yfp-$\omega$ and $\omega$:yfp genes, respectively. When the $\omega$ gene was replaced by the yfp-$\omega$ or the $\omega$:yfp gene, transcribed from its native $P_\omega$ (parS2), plasmid partition was impaired (Fig 2 and data not shown). In vivo experiments revealed that $\omega$:yfp repressed $P_\delta$ utilization with an efficiency comparable to that of wt $\omega_2$. In the presence of $\delta_2$, $\omega$:YFP further repressed $P_\delta$ utilization (S3 Table). Similar results were observed when $\omega$:YFP was replaced by $\omega_2$, but not when $\omega_2\Delta N19$, which fails to interact with the $\delta_2$, was used. It is likely therefore that: i) $\omega$:YFP interacts with $\delta_2$. Conversely, YFP-$\omega$ did not repress transcription from $P_\delta$, and the presence of $\delta_2$ did not overcome such defect (S3 Table). It is likely that: i) $\omega$:YFP is a dimer in solution [i.e, further denoted as (\omega:YFP)$_2$] because only the dimeric form of the ribbon-helix-helix $\omega$ protein binds parS DNA [29,31]; ii) (\omega:YFP)$_2$ binds parS1 DNA and represses $P_\delta$ utilization, and interacts with $\delta_2$ as wt $\omega_2$, but (\omega:YFP)$_2$ bound to parS DNA fails to stimulate the $\delta_2$ ATPase activity (data not shown); and iii) the (\omega:YFP)$_2$-parS interaction can be used as a marker to localize plasmid DNA and for $\delta_2$-$\omega_2$ interaction. Similar results were reported for GFP:ParB of P1 plasmid, which was also used as marker of in vivo plasmid location. P1-GFP:ParB fails to promote proper plasmid segregation, but in the presence of GFP:ParB, a plasmid bearing P1-ParB and P1-ParA is accurately segregated [20,21]. P1-ParB does not contribute to the regulation of the parAB operon, and the cognate sequence of the promoter that transcribes both parA and parB genes is different from the parS sequence [3]. In contrast, in pSM10935 the parS sites overlap with $P_\delta$ and $P_\omega$, and $\omega_2$-mediated repression of both promoters leads to plasmid incompatibility [30,33]. To discriminate whether $\omega_2$ stimulation of the $\delta_2$ ATPase is essential for faithful partitioning or (\omega:YFP)$_2$ is a dominant negative variant, the stability of pBC706 (plasmid-borne $P_\omega \omega$ and $P_\delta \delta$ genes) was studied (S4 Table). Plasmid pCB706 was introduced into B. subtilis BG1469 or BG1447 cells bearing the \omega:YFP or the $\omega\Delta N19$:yfp gene, respectively, integrated as a unique sequence at the host amyE locus of the B. subtilis genome, transcribed from the IPTG-inducible promoter ($P_{\text{hop}}$) (S1 Table). In parallel, as a control, we introduced pBC706 or pCB586 (plasmid-borne $P_\omega \omega$ gene) into the BG947 strain bearing the parAB gene (BG214 cells bearing pCB706,Fig 2) or when IPTG was omitted (data not shown).

At low transcription levels (10 $\mu$M IPTG), there were ~400 (\omega:YFP)$_2$/CFU (Table 1). Under this experimental condition, expression of (\omega:YFP)$_2$ partially reduced, by about 2-fold (47 ± 4%), pCB706 faithful partitioning (S4 Table) when compared to the absence of the $P_{\text{hop}}$ \omega:YFP gene (BG214 cells bearing pCB706, Fig 2) or when IPTG was omitted (data not shown). Similar results were observed when $P_{\text{hop}}$ \omega:YFP was replaced by BG1447-borne $P_{\text{hop}}$ \omega\Delta N19:yfp bearing pCB706. Here, faithful pBC706 partitioning was also reduced about 2-fold (52 ± 3%) (S4 Table). However, in the presence of 50 $\mu$M IPTG, plasmid partitioning was impaired (< 1% of cells retained the plasmid after 100 generations) by the expression of the $P_{\text{hop}}$ \omega:YFP or the $P_{\text{hop}}$ \omega\Delta N19:yfp gene in trans. As previously shown [27], at low transcription levels (10 $\mu$M IPTG), expression of (\delta:GFp)$_2$ did not affect faithful pBC706 segregation, and it enhanced the segregation of pCB586 (plasmid-borne $P_\omega \omega$ gene). However, the presence of 50 $\mu$M IPTG decreased the efficiency of pBC706 stable inheritance (S4 Table). It is likely that: i) (\omega:YFP)$_2$ is not dominant-negative over $\omega_2$; and ii) plasmid incompatibility and/or increased (\omega:YFP)$_2$- or (\omega\Delta N19:YFP)$_2$-mediated repression of the $\omega$ and $\delta$ genes might account for the decreased efficiency of plasmid inheritance.
Experimental setup used to study plasmid partitioning in vivo

To investigate plasmid localization in living cells, first a plasmid containing an array of lacO operators (to be tagged by the chromosomally expressed LacI:GFP repressor) was constructed (BG1311 strain, see S1 Table). Unfortunately, the array of lac operators apparently affected plasmid replication in our genetic background, leading to gross rearrangements of a sub-population of cells. We therefore aimed to measure plasmid positioning by localizing the (ω:YFP)2-parS DNA complex.

The number of plasmid copies per cell was determined by quantitative PCR and also by hybridization upon normalizing to two distinct chromosomal genes. Under the growth conditions used there were on average, ~8 ± 1 plasmid copies/cell. This is in perfect agreement with previous data using the same replicon [36]. To analyze the dynamic localization of ω2 and/or δ2 during plasmid segregation in vivo we used three different systems that were grown asynchronously under slow growth rate conditions (in MMS7 medium) with a generation time of ~60 ± 5 min and at 30°C. In the first system the expression of plasmid borne ω2 and δ2 genes (or their variants) was controlled by ω2,(ω2:YFP)2 or ω2ΔN19 (S3 Table).

To determine the number of δ2 and ω2 molecules per cell, we performed quantitative immunoblots using anti-δ and anti-ω antibodies and purified δ2 and ω2 proteins as a standard. Our analysis, from at least four independent experiments, revealed that BG214 cells bearing a plasmid-borne Pδ δ (or Pδ δ: GFP or Pδ δD60A: gfp) and Pω ω (or Pω ω: yfp or Pω ω2ΔN19), genes have ~1,400 ± 105 δ2 and ~1,300 ± 110 ω2 molecules/CFU (Table 1). Since the large majority of BG214 cells bearing plasmid were single- and two-cells clusters (with an average of 1.6 cells per CFU) (Figs3–5), it was considered that each cell under controlled conditions contained 875 δ2 molecules (1.2 ± 0.1 μM) and 812 ω2 molecules (~ 1.1 ± 0.1 μM) and that the constructed variants had similar levels (Table 1). In the second system, plasmid-borne Pδ δ (or Pδ δ: gfp, Pδ δ

### Table 1. Protein (ω:YFP)2 binds Pδ and represses transcription.

<table>
<thead>
<tr>
<th>Gene(s)a</th>
<th>Molecules/CFUb</th>
<th>Protein ω2</th>
<th>Protein δ2</th>
<th>Molar concentration/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pω ω (pCB586)</td>
<td>~1,300 (810)</td>
<td>NA</td>
<td>−1.1 μM</td>
<td>NA</td>
</tr>
<tr>
<td>Pω ωΔN19 (pCB742)</td>
<td>~1,400 (875)</td>
<td>NA</td>
<td>−1.9 μM</td>
<td>NA</td>
</tr>
<tr>
<td>Pω ω: yfp (pCB846)</td>
<td>~1,280 (800)</td>
<td>NA</td>
<td>−1.0 μM</td>
<td>NA</td>
</tr>
<tr>
<td>Pδ δ: gfp (pCB578)</td>
<td>NA</td>
<td>−7,500 (4,600)</td>
<td>NA</td>
<td>−6.0 μM</td>
</tr>
<tr>
<td>Pδ δD60A: gfp (pCB760)</td>
<td>NA</td>
<td>−7,700 (4,800)</td>
<td>NA</td>
<td>−6.2 μM</td>
</tr>
<tr>
<td>Pω ω Pδ δ (pCB706)</td>
<td>~1,300 (810)</td>
<td>−1,400 (875)</td>
<td>−1.1 μM</td>
<td>−1.2 μM</td>
</tr>
<tr>
<td>Pω ω: yfp Pδ δ (pCB843)</td>
<td>~1,320 (825)</td>
<td>−1,350 (850)</td>
<td>−1.1 μM</td>
<td>−1.1 μM</td>
</tr>
<tr>
<td>Pω ω Pδ δ60A (pCB761)</td>
<td>~1,180 (810)</td>
<td>−1,380 (870)</td>
<td>−1.1 μM</td>
<td>−1.2 μM</td>
</tr>
<tr>
<td>$P_{hap} \delta: gfp$ (BG947)c</td>
<td>-</td>
<td>−6,500 (4,000)</td>
<td>-</td>
<td>−5.6 μM</td>
</tr>
<tr>
<td>$P_{hap} \delta: gfp P_{ω} \omega$ (BG947)c (pCB586)</td>
<td>~1,200 (750)</td>
<td>−6,600 (4,100)</td>
<td>−1.0 μM</td>
<td>−5.7 μM</td>
</tr>
<tr>
<td>$P_{hap} \delta: D60A: gfp$ (BG1097)c (pCB846)</td>
<td>~1,300 (815)</td>
<td>−6,500 (4,000)</td>
<td>−1.1 μM</td>
<td>−5.6 μM</td>
</tr>
<tr>
<td>$P_{hap} \omega: yfp$ (BG1469)c</td>
<td>~400 (250)</td>
<td>NA</td>
<td>−0.3 μM</td>
<td>NA</td>
</tr>
</tbody>
</table>

aThe plasmid or the strain bearing the relevant promoter(s) and gene(s) are indicated between parentheses.
bThe molecules/CFU were estimated as described in Materials and methods. The estimated numbers of molecules/cell are denoted between parentheses.
cThe $P_{hap} \delta: gfp$, $P_{hap} \delta: D60A: gfp$ and $P_{hap} \omega: yfp$ genes integrated as unique copies into the amy locus in B. subtilis are under the control of the LacI expression cassette (LacI repressor-Hyper-Spank promoter, $P_{hap}$) (see S1 Table). The BG947, BG1097 and BG1469 strains were grown in the presence of 10 μM IPTG. NA, not applicable.

doi:10.1371/journal.pone.0131943.t001
δD60A or δD60A:
gfp, in the absence of ω2 repression, was constitutively expressed from its native promoter, with ~7,500 δ2 molecules/CFU or ~4,600 molecules (6 μM)/cell (Table 1). In the third system, chromosomally encoded δ:
gfp (or δD60A:
gfp) was under the control of the LacI repressor, and its expression was induced by IPTG addition, whereas plasmid-borne ω2 (or ω2ΔN19) was controlled by its own promoter (Pω). Addition of 10 μM IPTG did not significantly change the average number of cells/CFU, and our analysis revealed that under these conditions there were ~6,500 (δ:
gfp)2, or (δD60A:
gfp)2 molecules/CFU or ~4,000 δ2 and ~815 ω2 (or ω2ΔN19) molecules/cell (Table 1).

Protein ω2 binds parS DNA and slightly facilitates plasmid clustering

The fluorescence of YFP-tagged ω2 expressed from its native promoter in asynchronous cells bearing plasmids harboring a parS site was analyzed (Fig 3A). In the absence of δ2, i.e. in
plasmid-borne $\omega$-yfp, ($\omega$-YFP)$_2$ formed bright foci. The ($\omega$-YFP)$_2$ fluorescence was neither homogeneously distributed on the cell cytosol nor formed clouds of fluorescence on the nucleoid (Fig 3).

The ($\omega$-YFP)$_2$ fluorescence formed discrete foci that were broadly distributed without showing any specific pattern (see Fig 3A and 3D). The distribution of ($\omega$-YFP)$_2$ fluorescence signal did not significantly vary with cell lengths. A similarly broad distribution was reported when the same replicon bearing an array of lacO sites and LacI-GFP was used [36]. It is likely, therefore, that the ($\omega$-YFP)$_2$ fluorescence highlights the location of the plasmid-borne parS site.

In vitro, stoichiometric $\omega_2$ concentrations have a footprint of ~70 ± 7 bp on parS DNA [16,30,31], and EM and AFM measurements revealed that there are 7 ± 1 $\omega_2$/parS2 site, consisting of 7 contiguous iterons [8,27]. This is consistent with the observation that the volume of the ($\omega$-YFP)$_2$-parS foci is equivalent to one of the tau subunits of DNA polymerase (< 15 tau subunits/focus) [37]. The ($\omega$-YFP)$_2$ fluorescent foci never exceeded the number of plasmid copies (~8 ± 1/cell) (Fig 3A). As already mentioned, the total amount of ($\omega$-YFP)$_2$ in pCB846 bearing cells was ~800 ± 54 molecules/cell (Table 1). From these numbers and the number of ($\omega$-YFP)$_2$ fluorescent foci observed, we calculate that >85% of the ($\omega$-YFP)$_2$ molecules should be free in solution. Since the total fluorescence in the cells is provided by the ($\omega$-YFP)$_2$-parS foci, it was assumed that the free dimeric molecules do not assemble to give a quantitative fluorescence signal.

Further analysis of the fluorescence of ($\omega$-YFP)$_2$ revealed the presence of one to eight discrete ($\omega$-YFP)$_2$ foci/cell rather than patched structures (Fig 3A and 3D). More than 50% of the cells contained 4 or more foci per cell (Fig 3D). Since the number of plasmid origins (an indirect estimate of the number of plasmid copies) was not significantly altered during the experimental time and ($\omega$-YFP)$_2$ might not be a limiting factor, we assumed that ($\omega$-YFP)$_2$ might slightly facilitate plasmid clustering and that there were ~1.8 plasmid copies/focus. Alternatively, ($\omega$-YFP)$_2$ bound to parS may impair plasmid decatenation without altering the number

---

**Fig 4. Subcellular co-localization of ($\delta$:CFP)$_2$ and ($\omega$:YFP)$_2$.** Cells bearing plasmid-borne $\omega$-yfp and $\delta$-cfp genes were grown in MMS7 at 30°C. Images of the merged fluorescence from ($\delta$:CFP)$_2$ (in red) and ($\omega$:YFP)$_2$ (in yellow) are shown. Scale bar is 2 μm.

doi:10.1371/journal.pone.0131943.g004
of plasmid origins. We favor the former hypothesis, that individual protomers can contact sub-sites across parS sites. This is consistent with the observation that in vitro 8 to 10 ω2 molecules/plasmid facilitates plasmid bridging (pairing), albeit with low efficiency (~1% of total plasmid molecules) when analyzed by EM or by AFM [8,27]. Unlike the small ribbon-helix-helix (ω:YFP)_2 protein (see Fig 3A and 3D), the helix-turn-helix large ParB-like proteins (represented by P1-ParB, F-SopB and B. subtilis-Sop0J), upon binding to parS, spread over nsDNA many kb to promote bridging, looping and condensation of nsDNA [10–12,15].

Protein δ2 facilitates in vivo re-localization of the (ω:YFP)_2-parS foci

To determine if the localization of the fluorescent foci was modified upon interaction of (ω:YFP)_2 bound to parS with δ2, and to understand the role of ATP hydrolysis on this localization, the fate of the (ω:YFP)_2-parS fluorescent foci was studied in the presence of wt δ2 or δ2D60A. In the presence of plasmid-borne Pδδgfp and Pωωyfp genes, there was a significant reduction in the number of fluorescent foci. The fluorescent foci re-localized toward cell quarters in bilobed cells, and at mid-cell in cells with one nucleoid (Fig 3B and 3D), suggesting that in the presence
of δ₂, the (ω:YFP)_2-parS fluorescent foci might co-localize with the cell nucleoid. Similar results were observed when δ₂ was replaced by the δ₂D60A variant. This is consistent with the observation that: i) the (δ:GFP)_2 (or δ₂D60A) fluorescence co-localizes with the nucleoid (S1A and S1B Fig); and ii) (ω:YFP)_2-parS fluorescent foci co-localized with the Lacl-CFP bound to an the unstable array of plasmid-borne lacO sites (data not shown).

A quantification of >2,000 cells for each condition revealed that in the presence of wt δ₂ or δ₂D60A (which binds but does not hydrolyze ATP) there were <2 (ω:YFP)_2 foci/cell in ~41% or ~58% of total cells (Fig 3B, 3C and 3D). However, in the absence of δ₂ or δ₂D60A only ~17% of ~2,000 total cells contained <2 (ω:YFP)_2 foci/cell (Fig 3A and 3D). Since plasmid copy number was not significantly altered (~8/cell) in any of the three conditions, we concluded that δ₂ or δ₂D60A promoted plasmid pairing (Fig 3D). On the other hand, in the presence of δ₂D60A or δ₂ only ~16% and ~27% of ~2,000 total cells, respectively, contained 4 or more (ω:YFP)_2 fluorescent foci/cell, whereas in the presence of only (ω:YFP)_2 ~52% of total cells contained 4 or more (ω:YFP)_2 foci/cell (Fig 3D). These data are consistent with the in vitro observations that: i) δ₂ and δ₂D60A, upon interacting with ω₂-parS, increased plasmid pairing with different frequency, with ~20% of total complexes paired in the presence of wt δ₂, and ~60% of total complexes in the case of δ₂D60A; and ii) in the absence of ATP hydrolysis (δ₂D60A condition) the plasmids cannot unpair [8,27].

Distribution of δ₂ on the nucleoid

Previously we have shown that: i) δ₂ binds nsDNA and forms discrete blobs (~5 ± 1 δ₂/ blob) as seen by AFM, rather than bundles in the absence of any support, or filamentous structures on DNA [8]; ii) δ₂D60A binds nsDNA with higher apparent affinity than wt δ₂ (see S2 Table), because the δ₂D60A-nsDNA complexes have a longer half-life than the wt δ₂-nsDNA complexes [17]; and iii) (δK36A:GFP)_2, which does not bind ATP, shows a fluorescence signal distributed in the cytosol [27]. This is consistent with absence of binding to nsDNA of apo-δ₂ or ADP-bound δ₂ in vitro (S2 Table).

When the fluorescence of (δ:GFP)_2 was analyzed, it was found that it was regularly distributed over the nucleoid forming clouds of fluorescence, although low-density areas of fluorescence were observed (S1A and S2A Figs). Similar results were reported for P1-ParA and Caulobacter crescentus ParA in vivo [21,38].

When (δ:GFP)_2 was replaced by (δD60A:GFP)_2, it was found that, similar to the wt protein, the fluorescence was regularly distributed over the nucleoid, and low-density areas of fluorescence were also observed (S1B and S2B Figs). Since dynamic fluorescence on the nucleoid was observed with both proteins (S2 Fig), but (δD60A:GFP)_2 cannot hydrolyze ATP, we favor the hypothesis that δ₂ protein detachment from the nucleoid is independent of ATP hydrolysis.

In vitro limiting δ₂ concentrations have a footprint of ~30 ± 10 bp on nsDNA, and by AFM it was measured that a δ₂ blob occupies ~80 ± 20 bp of nsDNA, and that there are ~5 ± 1 δ₂/ blob [8,27]. In the absence of ω₂, there are ~4600 (δ-GFP)_2 or (δD60A:GFP)_2 molecules/cell, which drops to ~800 in the presence of ω₂. Under this protein concentration, we are assuming the protein should be in its dimeric form, because a monomer does not bind nsDNA (data not shown). According to these data, we propose that (δ:GFP)_2 or (δD60A:GFP)_2 fluorescence is regularly distributed over the nucleoid, with less than 5% of the fluorescence located elsewhere (S1 and S2 Figs). Since >95% of the observed cloud of fluorescence is located on the nucleoid it is likely that there are ~800 (δ-GFP)_2 or (δD60A:GFP)_2 blobs/nucleoid(s) in the absence of ω₂ and ~160 δ₂ blobs/nucleoid(s) in the presence of ω₂.
Nucleoid bound (δ:CFP)₂ captures and tethers (ω:YFP)₂-parS copies

It has been shown that the plasmid replication machinery is highly mobile and predominantly located at or near the cell pole in vivo [36]. In the previous sections we have shown that: i) (δ:GFP)₂ or (δD60A:GFP)₂ fluorescence was apparently regularly distributed on the nucleoid (S1A and S1B Fig); ii) in the presence of δ₂ or δD60A₂ the (ω:YFP)₂ fluorescent foci re-localized toward the middle of cells with one nucleoid, or toward cell quarters in bilobed cells; and iii) in the presence of δ₂ or δD60A₂ the number of (ω:YFP)₂ foci was reduced, although the fluorescence signal per focus increased (Fig 3B and 3C). To study whether δ₂ interaction with ω₂ bound to parS DNA leads to capture and tethering of plasmid copies to the nucleoid, Pδδgfp was replaced by Pδδcfp, so that the localization of the two proteins could be studied simultaneously (i.e., plasmid-borne Pδδcfp and Pωωyfp genes were used, S1 Table). At or near physiological concentrations of both proteins, the cloud of (δ:CFP)₂ fluorescence (denoted in red) formed on the nucleoid was not homogenously distributed, suggesting a certain dynamism (see below), and discrete (ω:YFP)₂ fluorescent foci in the cytosol were not observed (Fig 4). (ω:YFP)₂ formed 1 to 3 discrete foci at random positions on the nucleoid in ~80% of the cells (Fig 4). The increased brightness of the (ω:YFP)₂ fluorescent foci, as well as the reduced number of foci/cell observed suggested that many plasmids copies have been paired (Fig 4). Areas lacking the cloud of (δ:CFP)₂ fluorescence also lacked the (ω:YFP)₂ focus, suggesting that the ω₂-parS complex on the nucleoid is a δ₂-dependent reaction. To rationalize this observation, we propose that (δ:CFP)₂, upon interaction with (ω:YFP)₂, captures and tethers the plasmid molecules to (δ:CFP)₂ on the nucleoid, leading to plasmid-nucleoid bridging (or pairing) (Fig 4). We propose that (ω:YFP)₂, which fails to stimulate (δ:CFP)₂ ATPase activity, will lead to accumulation of these bridging intermediates.

parS-bound ω₂ stimulates δ₂ disassembly from the nucleoid

The dynamic change that the ω₂:δ₂ interaction may promote in (δ:GFP)₂ or (δD60A:GFP)₂ localization was analyzed at or near physiological concentrations of both proteins. In the presence of both ω₂ and (δ:GFP)₂, the (δ:GFP)₂ fluorescence was more irregularly distributed on the nucleoid when compared to the absence of ω₂ (Fig 5A vs S1A Fig), suggesting that after (δ:GFP)₂ detachment from the nucleoid, fluorescence in the cytosol was not observed (Fig 5A). Alternatively, the areas of low fluorescence observed here were simply due to the fact that ω₂ repressed δgfp expression and there was not sufficient protein to produce the cloudiness on the nucleoid. To test this hypothesis, the (δ:GFP)₂ concentration was artificially increased in the background, but the same outcome was observed (Fig 6A).

In the presence of ω₂, (δD60A:GFP)₂, which binds but cannot hydrolyze ATP, lost its regular distribution over the nucleoid, and it accumulated as discrete foci or patched regions on the nucleoid (Fig 5B). A comparison of Fig 5B and S1B Fig revealed that the (δD60A:GFP)₂ fluorescence detached from the nucleoid even in the absence of ATP hydrolysis, and that the foci or patched regions may correspond to non-disassembled plasmid-nucleoid bridging complexes. This is consistent with the observation that δ₂D60A and ω₂ lead to accumulation of bridging intermediates in vitro [8].

To confirm that the areas of low fluorescence observed here are not simply due to the lower (δD60A:GFP)₂ concentration we performed experiments in the presence of ω₂ΔN19, which also represses δD60A:γfp expression (S3 Table), but fails to interact with δ₂ [32]. In the presence of ω₂ΔN19, the fluorescence of (δD60A:GFP)₂ dramatically changed, and was indistinguishable from that observed in the presence of (δD60A:GFP)₂ alone (i.e, in the absence of ω₂ΔN19, Fig 5C vs S1B Fig). Similar results were observed when (δD60A:GFP)₂ was replaced
by δ₂ in the presence of ω₂ΔN19 (data not shown). It is likely that the interaction of ω₂ with (δ:GFP)₂ or (δD60A:GFP)₂ dramatically changed their pattern of fluorescence.

**Fig 6.** Time lapse of (δ:GFP)₂ or (δD60A:GFP)₂ at unbalanced levels in the presence of ω₂ or ω₂ΔN19. Cells had the ω₂ gfp (C and D) genes integrated into the B. subtilis chromosome, and plasmid-borne P₃ amyE (pCB586) (A and C) or P₃ ωΔN19 gene (pCB742) (B and D). Images of the same cells with GFP fluorescence from (δ:GFP)₂ or (δD60A:GFP)₂ are shown for the indicated times. Scale bar is 1 μm.

doi:10.1371/journal.pone.0131943.g006

**Dynamic redistribution of δ₂ on the nucleoid**

To further determine the type of complexes that (δ:GFP)₂ or (δD60A:GFP)₂ could form, the δ₂ concentration was artificially raised and uncoupled from ω₂ expression. The δgfp or δD60A-gfp gene was transcribed from the IPTG-inducible promoter (P₃ amyE), and integrated as a unique copy at the host amyE locus (S1 Table). In the presence of 10 μM IPTG, there were ~4,000 molecules (δ:GFP)₂ or (δD60A:GFP)₂/cell (Table 1). Under this experimental condition plasmid segregation was not significantly affected, but in the presence of 50 μM IPTG plasmid partitioning is
significantly impaired (S4 Table) [28]. Hence, the former condition was used for further analyses.

In the absence of ω2, the fluorescence of (δ-GFP)2 or (δD60A:GFP)2 was regularly distributed over the nucleoid (S3A and S3B Fig), and these clouds of fluorescence by (δ:GFP)2 or (δD60A:GFP)2 showed dynamic behavior in a time-dependent manner (S3A and S3B Fig), suggesting that protein disassembly from the nucleoid does not require hydrolysis of ATP. The presence of physiological ω2 concentrations significantly increased the dynamism of the cloud of fluorescence (Fig 6A and 6C). Time-lapse microscopy, with images gathered every 20 s over a 10 min period, were carried out in cells growing as micro-colonies on a slide. A re-organization and decrease in the level of (δ:GFP)2 fluorescence at a given location in the presence of physiological ω2 was taken as an indirect measure of (δ:GFP)2 disassembly from the nucleoid, rather than no assembly. This is consistent with the observation that in the absence of ω2 or in the presence of ω2ΔN19 the fluorescence was more regularly distributed over the nucleoid (Fig 6A vs Fig 6B or S3A Fig). It is likely that the interaction of (δ:GFP)2 bound to the nucleoid with a ω2-parS complex stimulates the (δ:GFP)2, ATPase, and (δ:GFP)2-ADP might lose affinity for DNA. This is consistent with the observation that δ2-ADP showed a very low affinity for nsDNA (S2 Table).

In the presence of ω2, (δD60A:GFP)2 formed discrete foci or patched regions on the nucleoid (Fig 6C). To explain this pattern of (δD60:GFP)2 fluorescence and its partitioning disability, we propose that δ2D60A failed to promote ATP hydrolysis-dependent plasmid-nucleoid disassembly, but still (δD60:GFP)2 redistributed on the nucleoid in the absence of ATP hydrolysis (Fig 6C). It is likely that ω2-mediated stimulation of (δD60:GFP)2 relocation is unlinked from plasmid movement, because discrete foci or patched regions were attributed to the accumulation of tethered plasmids. However, when ω2 was replaced by ω2ΔN19, the amount of (δD60:GFP)2 was not modified but the fluorescence was regularly distributed over the nucleoid (Fig 6D). This is consistent with the observation that ATP hydrolysis is necessary for disassembly of quaternary complexes (parS-ω2-δ2-nsDNA) or plasmid unpairing [8], but it is not essential to redistribute δ2 on the nucleoid. Similar results were reported for F-SopA (see [39]).

Protein δ2 variants impaired in nsDNA binding show a complex phenotype

Exchange of a single positively charged residue in δ2 (e.g., K242, Fig 1D) to alanine resulted in a 20-fold decrease in binding efficiency to nsDNA in vitro (Fig 7A, S2 Table) [17], but this mutation only reduced plasmid stability by to 2- to 3-fold (Fig 2). In contrast, exchange of a single negatively charged residue in δ2 (e.g., D211) to alanine resulted in a 6-fold increase in binding efficiency to nsDNA in vitro (Fig 7A, S2 Table) without affecting faithful plasmid segregation (Fig 2). These data are in apparent contradiction with the prevailing partitioning model, and negate the requirement for ParA binding to nsDNA for efficient partitioning [40–41]. To explain these results, the complexes formed by these δ2 variants upon binding to nsDNA were analyzed by EMSA and EM. Protein δ2K242A or δ2D211A binds and catalyzes the hydrolysis of ATP to an extent similar to wt δ2 (A.V., unpublished results). Protein δ2, in the ATP-bound form, cooperatively binds nsDNA with KDapp ~140 nM, but δ2K242A seemed to fail to form stable complexes with nsDNA even in the presence of 1200 nM (Fig 7A and S2 Table). However, δ2K242A formed protein-DNA complexes in the presence of a ~ 20-fold excess relative to the wt δ2 Kapp (Fig 7A, lanes 15–16 and S2 Table) [17]. Therefore, we analyzed whether the δ2K242A mutation was still able to form a complex with nsDNA in vivo. In the absence of ω2 (δK242A:GFP)2 formed a regular cloud of fluorescence over the nucleoid, indistinguishable from the one observed with (δ:GFP)2. However, when the cells were not fixed
with paraformaldehyde before visualization, a large fraction of cells contained the fluorescence distributed into the cytosol (data not shown). It is likely that \( \delta K242A:GFP \) forms transient complexes on nsDNA, but a cooperative interaction with \( \omega 2 \) might ameliorate this defect in vivo because plasmid segregation was only marginally affected (Fig 2). To test this hypothesis, EM experiments were performed. In the presence of limiting protein concentrations (\(*10\)-fold below \( K_{Dapp} \), 300 nM, S2 Table), \( \delta 2K242A \) assembled to form one discrete blob per DNA molecule in \(~37\%\) of the DNA molecules \((n = 530)\) (Fig 7Bc). When the DNA was linearized the \( \delta 2K242A \) blobs showed a random location, which indicated non-specific binding to DNA (data not shown). Similar types of complexes were observed in the presence of wt \( \delta 2 \) or the \( \delta 2D211A \) variant (Fig 7Ba and 7Be). As previously documented [8,17], intermolecular bridging of two plasmid molecules by \( \delta 2 \), \( \delta 2K242A \), or \( \delta 2D211A \) was not observed \((n = 530, 440 \text{ and } 460, \text{ respectively})\) (Fig 7B).

Previously it was shown by AFM that \( \omega 2-parS \) complexes \((220 \pm 56 \text{ nm}^3)\) are smaller in size than the \( \delta 2-\text{nsDNA} (430 \pm 42 \text{ nm}^3) \) complexes, and both complexes are smaller than the quaternary complex \( \text{parS-} \omega 2-\delta 2-\text{parS}, 800 \pm 100 \text{ nm}^3 \) [8,27]. In the presence of \( \omega 2, \delta 2-\text{mediated clusters larger than the protein alone were observed in } \sim 80\% \) of the \( \text{parS DNA molecules and plasmid pairing in the remaining } \sim 20\% \) \((n = 456)\) (Fig 7Bb). Similar results were observed when \( \delta 2 \) was replaced by \( \delta 2D211A \) (Fig 7Bf). In the presence of preformed \( \omega 2-parS \) complexes \( \delta 2K242A \) only co-localized with \( \omega 2 \) bound to \( \text{parS} \) and facilitated plasmid pairing formation in \( \sim 42\% \) \((n = 86)\) of the DNA molecules (Fig 7Bd). When the \( \omega 2: \delta 2K242A \) ratio was reduced, protein clusters were observed in \( \sim 62\% \) of the DNA molecules, and \( \sim 18\% \) of the \( \text{parS DNA} \)
molecules were paired with DNA molecules juxtaposed at their $\omega_2$-parS DNA ($n = 586$). Together these data suggest that $\delta_2$K242A at 300 nM formed transient complexes that cannot be detected by EMSA (Fig 7A), but can be visualized by EM (Fig 7B). To confirm that the presence of $\omega_2$ increases the stability of $\delta_2$K242A-nsDNA complexes by decreasing the off rate of $\delta_2$K242A binding to DNA, EMSA experiments were performed in the presence of various $\omega_2$ concentrations. The presence of $\omega_2$ and limiting $\delta_2$K242A (300 nM) facilitated the formation of a slow-mobility ternary $\omega_2$-parS DNA-$\delta_2$K242A complex (Fig 7C), but this ternary complex was not observed if ATP was omitted (S2 Table, data not shown). It is likely that: i) the $\omega_2$-parS DNA complexes stabilized the $\delta_2$-DNA complex to form ternary ($\text{parS-}\omega_2-\delta_2$K242A) and quaternary ($\text{parS-}\omega_2-\delta_2$K242A-$\omega_2$-parS) complexes; and ii) $\delta_2$ binding to the nucleoid is a crucial step in accurate plasmid partitioning.

Discussion

Plasmid pSM19035 partitioning, which uses to the non-filament-based mode, depends on the dynamic interaction among the $\delta_2$ ATPase bound to chromosomal DNA, the $\omega_2$ CBP and the parS sites. It is a multi-step process with discrete functional transitions. First, plasmid replication occurs mostly at nucleoid-free regions (e.g., at the cell poles) and occasionally moves out of them in the absence of ParAB [36]. In the presence of only the small ParB-like $\omega_2$ protein, binding to the parS region in the newly replicated plasmid leads to moderate plasmid clustering (Fig 3A). This finding is consistent with previous data showing that two $\omega_2$-parS complexes form transient cluster intermediates, and $\omega_2$-mediated clustering accounts only for $\sim 1\%$ of total protein-DNA complexes in vitro by EM or AFM analyses [8,17,27]. In contrast, the large helix-turn-helix ParB proteins (e.g., P1-ParB, F-SopB or chromosomal-encoded Spo0J), upon binding to parS, spread over nsDNA many kb and promote bridging (pairing), looping and condensation of nsDNA [10–12,15].

Second, in the presence of only $\delta_2$, this small ATPase binds dynamically to nsDNA (i.e., the nucleoid) in a process where ATP binding, but not hydrolysis, is essential; constitutive ($\delta$:GFP)$_2$ or ($\delta$D60A:GFP)$_2$ expression led to a dynamic cloud of fluorescence on the nucleoid (S3 Fig). Unlike pB171-ParA [42], we did not detect oscillation of ($\delta$:GFP)$_2$ or ($\delta$D60A:GFP)$_2$ from pole to pole. It was estimated that under constitutive expression there are $\sim 800$ ($\delta$:GFP)$_2$ or ($\delta$D60A:GFP)$_2$ blobs/cells (see Table 1). Since a fluorescence signal of free ($\delta$:GFP)$_2$ in solution was not detected (see S1–S3 Figs), it is likely that bundle structures were not formed in solution (S3 Fig). A dynamic cloud of fluorescence with slow detachment was observed in constitutively or Lacl-regulated ($\delta$:GFP)$_2$ or ($\delta$D60A:GFP)$_2$, e.g., in the presence or the absence of ATP hydrolysis, respectively (S3 Fig). Similarly, F-SopA relocation is not impeded by severely weakened ATP hydrolysis [39]. In the large ParA ATPases (e.g., P1-ParA or F-SopA) the reaction is more complex. Here, ParA binds and hydrolyses ATP and this enables ParA-ADP to bind specific DNA sequences required to regulate the expression of the ParAB locus; in addition, binding to ATP without hydrolysis produces a slow conformational transition in ParA that enables it to bind nsDNA and form a carpet on the DNA [4,6].

Third, the interaction of parS-$\omega_2$ complexes with $\delta_2$ at the nucleoid relocates the plasmid copies from a broad distribution towards the high concentration of $\delta_2$ bound to the nucleoid (plasmid-nucleoid pairing) (Fig 4). This plasmid capturing and tethering is consistent with the fact that the interaction of $\omega_2$ with $\delta_2$ enhances binding of the latter to nsDNA, and facilitates plasmid-nucleoid pairing (Fig 7). When $\omega_2$ was replaced by $\omega_2$AN19, the plasmids freely diffuse, leading to random segregation (Fig 2), suggesting that $\delta_2$ bound to the nucleoid captures, moves and tethers plasmid-borne $\omega_2$-parS by interaction with $\omega_2$. 
Fourth, the dynamic $\delta_2$-$\omega_2$ interactions at the paired complexes should alter the relative stoichiometry of both proteins. *In vitro*, the $\delta_2$ ATPase activity was maximal at $\sim 1.5:1 \omega_2:\delta_2$ ratios [27], and this may correspond to fluorescence depleted zones (Figs 5 and 6). This is consistent with the *in vitro* observation that $\delta_2$-ADP promotes plasmid unpairing and it enhances dissociation of $\delta_2$ from the nsDNA (the nucleoid) [27]. However, to explain the slow $\delta_2$ or ($\delta$D60A:GFP)$_2$ re-association with the nucleoid, we have to assume that $\omega_2$ may induce a conformational transition in $\delta_2$ or in ($\delta$D60A:GFP)$_2$ that weakens its re-assembly onto the nucleoid, as seen *in vitro* [23].

Fifth, the transient disassembly of $\delta_2$ from the plasmid-nucleoid complex should increase the relative concentration of $\omega_2$. This hypothesis is based on the observation that when both proteins are present at about stoichiometric concentrations, disassembly of $\delta_2$ increases, because its ATPase activity is activated. The individual $\omega_2$-parS complexes (i.e, the individual plasmids) should then ratchet along the newly formed cloud of $\delta_2$-nsDNA that could be seen as a cargo moving daughter plasmids away from each other over the surface of the nucleoid and re-pairing in a distant location on the nucleoid, following an oscillating wave of $\delta_2$ binding and release from the nucleoid. By this dynamic process, the $\omega_2$-parS complexes could actively move towards the newly separated nucleoids, so that at cell division, each daughter cell should receive at least one plasmid copy. Finally, the accumulation of discrete foci and patched structures observed with $\omega_2$-$\delta_2$D60A suggests that ATP hydrolysis is required for plasmid unpairing, and this defect contributes to the impairment in plasmid partitioning (Fig 2). This is consistent with the *in vivo* data that showed that ($\delta$D60A:GFP)$_2$ redistributed on the nucleoid in the presence of $\omega_2$ (Figs 5B and 6C), resulting in increased plasmid pairing (Fig 3), and with *in vitro* data showing that parS-$\omega_2$-$\delta_2$D60A-$\omega_2$-parS complexes cannot disassemble, but the parS-$\omega_2$-$\delta_2$-parS complexes can easily become unpaired [8]. This may effectively bias plasmid random diffusion toward the cell quarters, resulting in accurate plasmid segregation. The data presented in this work are supporting the non-filament-based modes of partitioning, which is the mode proposed for the large P1-ParAB-parS or F-SopAB-sopC [6,22,24] or the large/small *C. crescentus* ParBA-parS system [25].

Supporting Information

**S1 Fig. Subcellular localization of ($\delta$-GFP)$_2$ or ($\delta$D60A:GFP)$_2$.** Cells bearing plasmid-borne $P_\delta$ $\delta$-gfp (A) or $P_\delta$ $\delta$D60A-gfp gene (B) were grown in MMS7 at 30°C. Fluorescence images of cells, images of the same cells stained with DAPI to show DNA, and the merge of both images are shown. Scale bar is 5 $\mu$m.

(PDF)

**S2 Fig. Time lapse of ($\delta$-GFP)$_2$ or ($\delta$D60A:GFP)$_2$ fluorescence.** Cells bearing-plasmid borne $P_\delta$ $\delta$-gfp (A) or $P_\delta$ $\delta$D60A-gfp gene (B) were grown in MMS7 at 30°C. Images of the same cells with fluorescence from ($\delta$-GFP)$_2$ or ($\delta$D60A-GFP)$_2$ are shown for the indicated time. Scale bar is 5 $\mu$m.

(PDF)

**S3 Fig. Subcellular localization of ($\delta$-GFP)$_2$ or ($\delta$D60A:GFP)$_2$.** Illustration showing the structure of the $P_{his}$ $\delta$-gfp (A) or $P_{his}$ $\delta$D60A-gfp (B) expression cassettes integrated in the host chromosome, rendering strains BG947 and BG1097, respectively. Cells were grown in MMS7 at 30°C in the presence of 10 $\mu$M IPTG. GFP fluorescence images of cells, images of the same cells stained with DAPI to show DNA, and the merge of both images are shown. Scale bar is 5 $\mu$m.

(PDF)
S1 Table. Strains and plasmids.
(DOCX)

S2 Table. Relative binding of δ2 or its variants to nsDNA.
(DOCX)

S3 Table. Protein (ω:YFP)₂ binds Pδ and represses transcription.
(DOCX)

S4 Table. Effect of ω:yfp expression in faithful segregation or plasmid incompatibility.
(DOCX)

Acknowledgments
This work was supported in part by the Ministerio de Economía y Competitividad, Dirección General de Investigación grants BFU2012-39879-C02-01 to J.C.A. and BFU2012-39879-C02-02 to S.A., and by the Comunidad de Madrid grant S2009MAT-1507 to J.C.A., and BIO0260-2006 to S.A. A.V. thanks the Consejería de Educación de la Comunidad de Madrid for its fellowship (CPI/0266/2008) and the European Social Fund (ESF). We thank F. Pratto and Chiara Marchisone for the construction of some gene fusions. V.S.L. thanks S. Austin’s laboratory (Gene Regulation and Chromosome Biology Laboratory, Maryland) for technical help with the cytological analyses. The authors declare no conflicts of interest.

Author Contributions
Conceived and designed the experiments: VSL AV NES RL. Performed the experiments: VSL AV NES RL. Analyzed the data: VSL AV SA JCA. Contributed reagents/materials/analysis tools: VSL AV NES RL SA. Wrote the paper: VSL AV SA JCA.

References


