ParABS System in Chromosome Partitioning in the Bacterium *Myxococcus xanthus*

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**Abstract**

Chromosome segregation is an essential cellular function in eukaryotic and prokaryotic cells. The ParABS system is a fundamental player for a mitosis-like process in chromosome partitioning in many bacterial species. This work shows that the social bacterium *Myxococcus xanthus* also uses the ParABS system for chromosome segregation. Its large prokaryotic genome of 9.1 Mb contains 22 *parS* sequences near the origin of replication, and it is shown here that *M. xanthus* ParB binds preferentially to a consensus *parS* sequence in vitro. ParB and ParA are essential for cell viability in *M. xanthus* as in *Caulobacter crescentus*, but unlike in many other bacteria. Absence of ParB results in anucleate cells, chromosome segregation defects and loss of viability. Analysis of ParA subcellular localization shows that it clusters at the poles in all cells, and in some, in the DNA-free cell division plane between two chromosomal DNA masses. This ParA localization pattern depends on ParB but not on FtsZ. ParB inhibits the nonspecific interaction of ParA with DNA, and ParA colocalizes with chromosomal DNA only when ParB is depleted. The subcellular localization of ParB suggests a single ParB-*parS* complex localized at the edge of the nucleoid, next to a polar ParA cluster, with a second ParB-*parS* complex migrating after the replication of *parS* takes place to the opposite nucleoid edge, next to the other polar ParA cluster.

**Introduction**

Genetic information is written in long DNA molecules. A typical bacterial chromosome extends to a length over a thousand times greater than the cell in which it resides. Therefore, chromosomal DNA organization, its transcription, replication, and segregation must be highly organized in the cytoplasm and tightly coordinated in time [1,2]. Negative DNA supercoiling is the main mechanism for bacterial chromosome compaction, generating topological domains of about 10 kb, the interwound DNA loops and their boundaries being highly dynamic [3]. In *Escherichia coli*, a higher-order structure of chromosomal DNA (or macrodomain) has been described, with a length between 0.8 to 1 Mb, which is organized by MatP protein and multiple *matS* DNA sequences [4]. In *Bacillus subtilis*, the nucleoid adopts an organization where the origins of chromosomal replication (*oriC*) are located near opposite cell poles and termini (*ter*) at the mid-cell [5]. In *Pseudomonas aeruginosa*, the *oriC*-ter axis is oriented from the old pole of the cell to the cell division plane or to the incipient newborn pole [6]. And in a newly divided *Caulobacter crescentus* cell, loci occupy specific regions in the cytoplasmic space with respect to its linear genomic position, being *oriC* at the old cell pole and *ter* at the newborn pole [7]. Unlike eukaryotic cells, chromosome segregation is coupled to chromosome replication, and loci separate progressively just after being replicated [7–9]. Models for chromosome segregation without mitotic-like apparatus have been proposed [10–13]. However, it is assumed that a chromosomal ParABS system, originally described in plasmids, acts as a mitotic-like apparatus to segregate replicated chromosomes [1,2,14]. ParABS systems, which have been identified in over two hundred bacterial chromosomes, consist of three components. One is the *cis*-acting *parS* site that is highly conserved among bacterial species, and is located in the *oriC*-proximal region of the chromosome. The majority of bacterial species have between one and four repeats of putative *parS* sites, although several with five to eight repeats, and even a few with twenty or more, are known [15]. The second component of ParABS systems, protein ParB, binds to the *parS* sites to form a large nucleoprotein complex near *oriC* as well as to the third component of the system, ParA. The latter is an ATPase proposed as the element that provides the force for the segregation of the “centromeric” *parS* sites via dynamic polymerization-depolymerization events [2,14,16–18]. Genes encoding *parA* and *parB* also are usually found in the *oriC*-proximal regions of the chromosome, and they have been shown to participate in proper chromosome partitioning in numerous bacteria [15,19].

*Myxococcus xanthus* is a Gram-negative soil δ-proteobacterium used as a prokaryotic model for the investigation of several processes involved in multicellular development, coordinated cell movements, and cellular responses to external signals such as light [20–25]. *M. xanthus* has a single large circular chromosome of about 9.14 Mbp. It has been suggested that this enlarged genome (and those of related myxobacteria of the order myxococcales), is a consequence of extensive, but not random, gene duplications whose subsequent divergence enabled evolution of the signaling systems required for the striking multicellular lifestyle of myx-
bacteria [24]. For all these reasons, it is of particular interest to study the organization of the chromosome and its segregation in this bacterium. The main objective of this work was to ascertain if the DNA sequences that encode the predicted ParABS elements, taken from the \textit{M. xanthus} genome annotation, have a role in chromosome segregation in \textit{M. xanthus}. A second objective was to determine if these ParABS elements are physically or functionally interconnected. A conclusion of this work is that ParB binds preferentially to a \textit{parS} consensus sequence in \textit{vitro}. It is also shown here that ParB is essential for viability and its absence generates anucleate cells demonstrating the key role of ParB in chromosome segregation. ParA is also essential for viability and localizes in DNA-free zones such as at the cell poles and along the cell division plane, prior to cell division. However, this localization pattern is independent of FisZ. ParA subcellular positioning depends on ParB, the absence of which causes ParA to be delocalized from DNA-free zones and colocalizes with chromosomal DNA. Therefore, ParB appears to prevent colocalization of ParA and DNA by guiding ParA to DNA-free zones. In most cells, ParB localizes at the edge of the chromosomal DNA in subpolar positions to thereby limit ParA localization to polar clusters.

Results and Discussion

\textbf{parABS Loci in \textit{M. xanthus}}

MXAN\_7477 and MXAN\_7476 in the \textit{M. xanthus} genome have been annotated as encoding \textit{parA} and \textit{parB}, respectively [24] (Fig. 1A). These two genes are co-transcribed as an operon together with MXAN\_7475 (encoding the bacteroflin BacM) and MXAN\_7474 (encoding a putative lipoprotein of unknown function). Neither BacM nor MXAN\_7474 is necessary for chromosome segregation or optimal cell growth under standard conditions [25]. Chromosomal \textit{parAB} loci are usually found in the oriC-proximal region of bacterial chromosomes [15]. Consistent with this, the \textit{M. xanthus} \textit{parAB} locus indicated above is about 35 kb away from oriC. Bioinformatic analysis of 400 sequenced prokaryotic chromosomes indicated 1030 putative \textit{parS} sites which are located, mostly, within oriC-proximal regions of their respective chromosomes [15]. From these \textit{parS} sites, Livny and coworkers created a \textit{parS} consensus matrix (Fig. 1B), and found that \textit{M. xanthus} contains 12 putative \textit{parS} sites, about 4.4 kb upstream from \textit{parA}. To refine this analysis in \textit{M. xanthus}, I decided to repeat the search for \textit{parS} sites in the \textit{M. xanthus} genome modifying the \textit{parS} consensus matrix obtained previously. The search was performed using as query a putative \textit{parS} sequence (16 pair of bases) that retains the highly conserved nucleotides, and in which the less conserved ones are varied (Fig. 1C). This search uncovered a 3 kb cluster of 22 putative \textit{parS} sequence repeats (Fig. 1D) located about 4 kb upstream of \textit{parA}, between genome positions 9108251 to 9111304 (Fig. 1A). In addition, this search also pointed out another \textit{parS} site located at position 349383 to 349398, distant from the \textit{parAB} locus. Two additional \textit{parS} sites in the \textit{M. xanthus} genome, near each end of the \textit{parAB} cluster, were indicated in another report using as query the 12 \textit{parS} \textit{M. xanthus} sequences previously identified and allowing for one mismatch [26]. The first sequence (position 9105392-9105407) has a \textit{“G”} in position 7, and the second sequence (position 9111742-9111757) contains a \textit{“G”} in position 3. Only two examples of each of these case exists among the 1030 predicted \textit{parS} sites mentioned earlier. Positions 3 and 7 are otherwise highly conserved. The level of nucleotide conservation is important because the 1030 \textit{parS} sites, found in 276 of the 400 sequenced strains, were identified using as a reference only the 15 \textit{parS} sites from \textit{Streptomyces coelicolor} and the 10 \textit{parS} sites from \textit{B. subtilis} that have been shown to bind ParB \textit{in vivo} [27–29].

Taking the 22 putative \textit{parS} sites described here (Fig. 1D), the \textit{M. xanthus} \textit{parS} consensus sequence can be assigned to be TGTTCCACGTGGAACG (Fig. 1E).

\textbf{\textit{M. xanthus} ParB Binds to a Consensus \textit{parS} in \textit{vitro}}

In order to determine if \textit{M. xanthus} ParB is able to bind to the DNA fragment containing the 22 \textit{parS} cluster described in the above section, an agarose gel electrophoretic mobility shift assay was performed. For this, a 3.12 kb $^{32}$P-radiolabeled probe, corresponding to the DNA segment from positions 9108215 to 9111334 of the \textit{M. xanthus} genome and containing the 22-repeat \textit{parS} stretch was incubated with increasing amounts of purified ParB, and electrophoresed in a 0.7% agarose gel. Clear mobility shifts of the \textit{parS}-cluster probe appeared at ParB concentrations...
>2 μM (Fig. 2A). This indicates that ParB interacts with the DNA fragment containing the 22 M. xanthus parS sites. It can also be observed that higher amounts of ParB resulted in slower-migrating bands. It has been previously described elsewhere [29,30], that large DNA probe fragments show multiple slower-migrating bands as ParB concentration increase, indicating that several molecules of ParB are binding per DNA fragment, generating a large nucleoprotein complex near the origin of replication. These large structures could potentially demarcate, organize or localize the nucleoprotein complex near the origin of replication. These large DNA probe fragments show multiple slower-migrating bands observed that higher amounts of ParB resulted in slower-migrating bands.

To assess its preferential binding to parS sites, ParB binding was tested with another DNA probe similar in size and G+C content but without the parS-cluster (3005 bp and 65.7% G+C versus 3120 bp and 64.9% G+C). The DNA probe chosen contained the Mxβ phage attP site involved in phage integration into the M. xanthus chromosome at the attB site [31]. ParB could bind to the attP fragment but only at ParB concentrations far higher than with the parS probe (Fig. 2B), indicative of the significantly greater of ParB for the probe bearing the parS-cluster. In order to further establish the preferential binding of ParB to the sequence with the parS-cluster, a DNA binding competition assay was performed. Prior to the 30 minutes of ParB incubation with the labeled probe with the parS-cluster, ParB was incubated with unlabeled attP probe at more than two-hundred fold excess for 1 hour. As can be seen in Fig. 2C, ParB complexes with labeled parS probe appeared even with cold attP probe present at over a 227-fold excess. By contrast, with a similar excess of cold parS probe, ParB complexes with labeled attP probe could not be detected (Fig. 2D) confirming that ParB has a greater affinity for the probe with the parS-cluster.

Next, to determine if ParB binds specifically to a single parS site, a 50 bp DNA probe containing the M. xanthus parS consensus sequence TGTTCACAGTGGAACG (Fig. 1E), which spans positions 9109710 to 9109759 of the M. xanthus genome, was used in the gel mobility assay. ParB was found to bind to this single parS site (Fig. 2E) with a single retarded band observed even at the highest ParB concentration used, in contrast to the 3 kb probe with several parS repeats which yielded a large complex at high concentrations. However, when ParB was incubated with a similar DNA fragment but containing 11-point mutations (TaccCGCa-caAggtG) of the most conserved base pairs of the parS consensus sequence (Fig. 1B), only a faint band could be barely detected even at the highest ParB concentration used (Fig. 2F). These in vitro results are consistent with specific binding of ParB to a consensus parS sequence.

ParB is Essential for Viability in M. xanthus

ParB has been shown to be important for chromosome partitioning in numerous bacteria [14,32]. In order to determine if M. xanthus ParB participates in chromosome segregation, it was necessary to create a parB mutant. It was not possible to delete parB in M. xanthus, suggesting that ParB is essential for viability. In fact, the endogenous parB gene could be deleted only if an extra copy of the gene was also present (located at the 1.38-kb locus described in detail in reference 33). Moreover, conditional expression of parB by placing it under the control of a vanillate-inducible promoter, which is derepressed in the presence of vanillate [33,34], resulted in viable cell growth only under permissive conditions when vanillate was present (Fig. 3A, left panel), whereas there was no growth under restrictive conditions without vanillate (Fig. 3A, right panel). Furthermore, restricting parB expression results in cells (after a 48-hour growth with no vanillate to ensure complete growth arrest) in aberrant cellular morphology when examined under a microscope, and considerable amounts of cellular debris, indicating cellular death, could be observed (Fig. 3B, right panel).

Thus, these data clearly demonstrate that parB is essential for viability in M. xanthus. In most chromosomal par systems studied, mutations or deletions of the par genes did not produce lethality [14,32,35], a notable exception being C. crescentus [36]. For instance, in Deinococcus radiodurans and P. aeruginosa, the absence of ParB resulted in bacterial growth retardation [32,37]. Additionally in P. aeruginosa, parB mutants were affected in swimming and swimming motility [32], and in B. subtilis, the absence of ParB ortholog Spo0J caused a sporulation defect [38]. It has been proposed that the essentiality of the par system in C. crescentus is due to a cell division defect, indicating that ParAB are required for cytokinesis [39]. Although this may also be the reason why the par system is essential in M. xanthus, the dramatic filamentous cell morphology phenotype that C. crescentus cells present in the absence of parB is not observed with M. xanthus.

M. xanthus ParB is Involved in Chromosome Partitioning

Chromosomally encoded ParB or ParA proteins have been reported to have a role in chromosome partitioning in several bacteria. It is established that a lack of Par proteins or the presence of mutant forms of these proteins causes an increase in anucleate cells [6,28,36–38,40–42]. To ascertain if the absence of ParB in M. xanthus results in anucleate cells, a culture of parB conditional strain, P. van-parB ΔparB, was grown in the presence of vanillate. The cell culture was washed to remove vanillate from the media and the cells were examined under the microscope after 0, 24, 36, and 48 hours. Samples were incubated with DAPI 10 minutes before being placed on the agarose pad to observe chromosomal DNA by fluorescence microscopy. In the presence of vanillate, all cells had normal rod-shape morphology and contained chromosomal DNA, with dividing cells having DNA in both compartments (Table 1, Figs. 4A and B). It should be noted that the chromosomal DNA does not occupy the entire cytoplasmic space, leaving areas proximal to the cell poles free of DNA. After 24 hours of ParB depletion, anucleate cells start to appear, together with dividing cells bearing DNA only in one compartment instead of both, although the number of cells with such anomalies is small (1% of 400 cells observed; Table 1). After 36 and 48 hours of ParB depletion, the population of anucleate cells is quite significant (between 10.1 and 21.6%, n = 310 and n = 219, respectively), and the number of dividing cells with DNA in only one compartment also increases (between 14.4 and 9.4%, n = 310 and n = 219, respectively) (Table 1, Figs. 4C and D; these counts considered only cells that conserved the typical smooth rod-shape morphology). After 48 hours of ParB depletion several rounded cells (with or without DNA), significant amounts of cellular debris, and free chromosomal DNA, presumably released from dead cells into the media, can be observed (Fig. 4E). This suggests that M. xanthus ParB is involved in chromosome partitioning, and its absence provokes chromosome segregation anomalies and cellular death.

Subcellular Localization of M. xanthus ParA

All attempts to delete chromosomal parA from M. xanthus were unsuccessful. The chromosomal parA gene was only deleted when the strain harbored another copy of parA in trans under the control of the Pvan promoter, indicating that, as with parB, parA is essential for viability in M. xanthus (data not shown). In this case, basal levels of expression from the Pvan promoter without the addition of vanillate, sufficed to allow the cells to live. To determine the subcellular localization of ParA, a strain was created harboring Pvan-parA-YFP, bearing a copy of parA fused to yfp, whose expression was under the control of the inducible vanillate promoter and inserted at the 1.38-kb locus. After growing this strain, cells were examined under the microscope and as fluorescent ParA-YFP...
Figure 2. ParB binds preferentially to parS in vitro. (A) Agarose EMSA on after incubating different amounts of ParB with a $^{32}$P-labeled 3120 bp DNA fragment containing the parS-cluster, (B) or with a 3005 bp DNA containing the Mx8 phage attP sequence. (C) Agarose EMSA of the binding of $^{32}$P-labeled parS-cluster to ParB, or to ParB previously incubated with higher amounts of unlabeled attP DNA fragment, as indicated. (D) Agarose EMSA of the binding of $^{32}$P-labeled attP fragment to ParB, or to ParB previously incubated with higher amounts of unlabeled parS-cluster, as indicated. (E) EMSA using a 6% polyacrylamide gel after incubating a $^{32}$P-labeled 50 bp DNA fragment containing the M. xanthus parS consensus
could be observed even in the absence of vanillate, it was not included in these analyses. ParA-YFP fluorescence was found to cluster at both poles, and in some cells at the cell division plane (Fig. 5, upper panels). Merging the images obtained for ParA-YFP with that of the chromosomal DNA visualized using DAPI staining, it is clearly apparent that ParA-YFP localizes at the DNA-free regions. As mentioned in a previous section, the chromosome in M. xanthus does not occupy all regions of the cytoplasmic space, with DNA-free regions at the poles and, in some cells at the cell division plane (Fig. 5, bottom panels). Approximately 84% out of the 615 cells had two ParA-YFP clusters only at the two poles (Figs. 6A and B), and in the remaining cells an additional ParA-YFP cluster was observed in the cell division plane as well (Figs. 6C–E). Even after separation of the dividing cells, ParA remains localized at the newborn pole, which earlier was part of the cell division plane in the mother cell (Fig. 6E). Therefore, the polar localization of ParA appears to be a consequence of a prior location at the midcell and raises the question of how ParA is being recruited to the cell division site. In C. crescentus, ParA forms a cloud-like structure extending from the new pole towards the old one. The duplicated ParB-parS complex associates with the ParA-cloud and is pushed apart towards the new pole, by a ParB-dependent ParA-ATPase activity [16,43]. A similar mechanism has been proposed for V. cholerae chromosome I [44]. It has also been suggested that the nucleoid forms a structural matrix for the assembly of a track-like structure of ParA that guides the ParB-parS complex movement [16,45]. In the multigenomic aerial hyphae of S. coelicolor, ParA accumulates at the tip of the hyphae and it extends from the tip towards the rest of the hyphae as helical filaments providing a scaffold for a regular distribution of several ParB-parS complexes [46]. In B. subtilis, the ParA ortholog Soj localizes to the septa and as relatively faint punctuate foci.
within the cytoplasm [18]. In M. xanthus, the symmetrical ParA localization at DNA-free poles, observed in this study, seems to be incompatible with ParB-parS transport by ParA through a nucleoid structural matrix. This, however, should not be discarded since a faint ParA fluorescence can be detected throughout the cytoplasm even in the regions with DNA.

The Localization of ParA is not Dependent on FtsZ

To obtain more insight into ParA cellular localization, this was examined in the absence of FtsZ, the bacterial tubulin homolog that forms a ring in the midcell region whose constriction culminates in cell division [47–49]. In M. xanthus, FtsZ localizes at the cell division plane in most cells, and its absence results in filamentous morphology and, eventually, cell death [33]. Whether ParA-YFP localization at the midcell depends on FtsZ, was studied by inserting a copy of P

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**Figure 4. ParB is involved in chromosome partitioning in M. xanthus.** Merged DIC and fluorescence images of cells from a P

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ParB Controls ParA Localization

Since ParB has been shown to influence ParA localization in several bacteria [16,18,50,51], this was tested in *M. xanthus* by examining ParA-YFP in ParB-depleted cells. For this, a copy of parA-yfp under the control of the IPTG promoter was inserted at the *Mxan_18–19* locus to generate strain MR2530. Additionally, strain MR2526 also has the plasmid pMR3826 with the vanillate-inducible parA-yfp construct integrated at the *Mxan_18–19* locus. MR2526 was grown in CTT media to exponential phase. After a 3-hours of IPTG (1 mM) induction of mCherry-ParB expression, samples were taken and stained with DAPI for microscopy. In 12.7% of the cells observed (n = 550), a single focus of mCherry-ParB was seen just at the edge of the nucleoid (Fig. 9A). Most of the cells presented two mCherry-ParB foci at both edges of a single nucleoid (50.7% of the cells; Fig. 9C), or two foci at both subpolar edges of the two separated nucleoids (25.3% of the cells; Figs. 9D and E). The remaining cells had one focus localized at the edge of the nucleoid and another in an intermediate position (11.3% of the cells; Fig. 9B). The localization pattern of ParB in *M. xanthus* thus resembles those previously described in other bacteria, and where ParB localization was linked to its ability to bind parS [16,44,54]. Since, as shown in this study, *M. xanthus* ParB binds preferentially to a consensus parS sequence in vitro (Fig. 2E), the single mCherry-ParB focus seen in 12.7% of the cells may correspond to ParB bound to a not as yet replicated or segregated, parS (Fig. 9A). Then, the presence of cells having one focus at the edge of the nucleoid and other in an intermediate position (Fig. 9B) could indicate that parS has replicated and is being moved to the other edge of the nucleoid, resulting in cells with two ParB-parS clusters at both edges of the nucleoid (as seen in 50.7% of the cells). This final location of both ParB-parS complexes persists even after the two chromosomes have been segregated (Figs. 9D and E). Thereby, the division of the cell would provide two daughter cells with a single ParB-parS complex, completing the cell cycle.

Simultaneous observation of mCherry-ParB and ParA-YFP shows that ParB is in close proximity to polar ParA in 76% of the cells (Figs. 9C–E), which correspond to cells with the two ParB-parS complexes fully segregated. While ParB may inhibit the presence of ATP-bound ParA within the nucleoid, it may not affect ParA polymerization at the poles, where no DNA-ParA complex is present. When the two newly replicated chromosomes separate, a DNA-free space is created at the cell division plane where, presumably, the dispersed ParA could polymerize to create a new midcell ParA cluster. Therefore, the localization of ParB appears to be consistent with its role controlling ParA localization.
Figure 5. Subcellular localization of ParA in *M. xanthus*. Fluorescence microscope images of cells from the strain *P. van*parA-yfp (MR2504) grown without vanillate. DIC (top left), ParA-YFP fluorescence (top middle, in green), merged DIC with ParA-YFP fluorescence (top right), merged DIC with DAPI fluorescence (bottom left), DAPI fluorescence (bottom middle, in red), and merged DAPI (in red) with ParA-YFP (in green). Black scale bar represents 10 μm.
doi:10.1371/journal.pone.0086897.g005
Figure 6. Distribution of cells according to its ParA localization. DIC, ParA-YFP (in green), and DAPI (in red) microscope fluorescence images of cells from the strain P$_{mCherry}$-parA-yfp (MR2504) grown without vanillate. Black scale bar represents 5 µm. A total of 615 cells from three independent experiments were examined and the mean and the standard deviation are reported. (A) A representative cell with two polar clusters of ParA-YFP and one chromosomal mass (68.5±10.2%). (B) Cell having two polar clusters of ParA-YFP and two distinct chromosomal masses (15.4±8.7%). (C) Representative cell with two polar clusters of ParA-YFP, two distinct chromosomal masses, and an additional cluster of ParA-YFP in the cell division plane but with no pinch in its cellular morphology (10.4±6.8%). (D) Same as in (C) but with incipient constriction along the cell division plane (3.2±0.7%). (E) Cells recently divided, showing two polar clusters of ParA-YFP and one chromosomal mass, in each of the two cells (2.5±1.4%). doi:10.1371/journal.pone.0086897.g006

Materials and Methods

Bacterial Strains and Growth Conditions

_E. coli_ strain DH5α was used for plasmid constructions and was grown at 37°C in Luria broth medium (LB) supplemented with the appropriate antibiotics. _M. xanthus_ was grown at 33°C in rich Casitone-Tris (CTT) medium [55]. Media were supplemented with inducer (0.5 mM vanillate or 1 mM isopropyl β-D-thiogalactoside (IPTG)) or antibiotic (40 µg/ml kanamycin (KanR), 10 µg/ml oxytetracycline (TetR) for solid media, and 2.5 µg/ml oxytetracycline for liquid media), as required.

Construction of Strains and Plasmids

_M. xanthus_ strains and plasmids used in this study are listed in Table 2 and Table 3. Standard protocols and commercially available kits were used in the preparation and manipulation of chromosomal and plasmid DNA. All constructs were verified by DNA sequencing. Plasmids were introduced into _M. xanthus_ by electroporation, and integration of the plasmids by homologous recombination was selected on CTT plates containing the appropriate antibiotic and/or by negative selection via a _galK_ gene that confers sensitivity to galactose (GalS).

_M. xanthus_ parB coding sequence was PCR-amplified using genomic DNA from wild type DK1050, as DNA template, and primers 14_ParB(for (5'gtgtgagctactgatggagagagagagagaga-3’) and 15_ParB.rev (5’aaaaaagcttacctcctcagagaaggtc-3’). This PCR product and the plasmid pMR3553, which bears the 1.93-kb sequence for chromosome integration and the _ParA_ promoter [33], were digested with NdeI and EcoRI and ligated, obtaining the plasmid pMR3594. To generate the plasmid pMR3620 for deleting chromosomal _parB_, two PCR products were generated. The first PCR product contains about 0.92 kb of _parB_ upstream DNA sequence, and it was obtained using DK1050 genomic DNA as a template and the primers 26_UpParB.for (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’) and 27_UpParB.rev (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’). The second PCR product has around 0.94 kb of _parB_ downstream sequence, and it was obtained using the primers 28_DownParB.for (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’) and 29_DownParB.rev (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’), and DK1050 genomic DNA as a template. After NdeI-EcoRI digestion, _parA_ from this PCR product was cloned into a NdeI-EcoRI-digested pMR3653 plasmid [33], exchanging _fixε_ gene for _parA_. The plasmid pMR3826 was made cloning the _parA-yfp_ fragment, by digesting pMR3785 with NdeI and NheI, into the plasmid pMR3690 [33] previously digested with the same restriction enzymes. To make plasmid pMR3828, the _M. xanthus_ parB coding sequence was PCR-amplified using _M. xanthus_ DK1050 genomic DNA as a template and the primers 18_ParB.for (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’) and 15_ParB.rev (5’aaaaaagcttacctcctggtcagcagcagcagcaga-3’), and then digested by EcoRI and cloned into the EcoRI-digested vector pVCHYN-2 [34], resulting in the plasmid pMR3733 encoding an mCherry-parB fusion. Then, mCherry-parB fragment was amplified by PCR using plasmid pMR3733 as DNA template, and the primers 49_mCherry.for (5’aaaaaagcttacctcctggtcagcagcagcaga-3’) and 42_ParB.rev (5’aaaaaagcttacctcctggtcagcagcaga-3’). This PCR product was digested by XbaI and cloned into XbaI-digested pMR3690 to generate the plasmid pMR3785 encoding an mCherry-parB fusion. Then, mCherry-parB fragment was amplified by PCR using plasmid pMR3733 as DNA template, and the primers 49_mCherry.for (5’aaaaaagcttacctcctggtcagcagcagcaga-3’) and 42_ParB.rev (5’aaaaaagcttacctcctggtcagcagcaga-3’). This PCR product was digested by XbaI and cloned into XbaI-digested pMR3690 to generate the plasmid pMR3785 encoding an mCherry-parB fusion. Then, mCherry-parB fragment was amplified by PCR using plasmid pMR3733 as DNA template, and the primers 49_mCherry.for (5’aaaaaagcttacctcctggtcagcagcagcaga-3’) and 42_ParB.rev (5’aaaaaagcttacctcctggtcagcagcaga-3’). This PCR product was digested by XbaI and cloned into XbaI-digested pMR3690 to generate the plasmid pMR3785 encoding an mCherry-parB fusion. Then, mCherry-parB fragment was amplified by PCR using plasmid pMR3733 as DNA template, and the primers 49_mCherry.for (5’aaaaaagcttacctcctggtcagcagcagcaga-3’) and 42_ParB.rev (5’aaaaaagcttacctcctggtcagcagcaga-3’). This PCR product was digested by XbaI and cloned into XbaI-digested...
pMR3487 [33], resulting in the plasmid pMR3828 which has the mCherry-parB fusion under the control of a IPTG-inducible promoter, and the 1.38-kb sequence for chromosomal integration. Plasmid pMR3684 used for ParB purification was obtained by isolating the parB coding sequence fragment after the digestion of plasmid pMR3594 by NdeI and EcoRI, and cloning into these sites in pTYB12 (New England Biolabs). In order to create plasmid pMR4051, it was necessary to generate two precursor plasmids. First, a DNA fragment of 1.861 kb containing the IPTG inducible promoter, a multicloning site, and the lacI gene repressor was obtained by digesting pMR3487 [33] with PstI and NdeI. This fragment was cloned into a PstI-NdeI-digested pMR2700 plasmid [57], generating the plasmid pMR4046. The plasmid pMR4046 was digested with HindIII, releasing the M. xanthus 1.38-kb

Figure 7. ParA localization is not dependent on FtsZ. DIC, DAPI (in red), and ParA-YFP (in green) microscope fluorescence images of cells from the strain MR2536 (P_{iptg}-ftsZ ΔftsZ, P_{van}-parA-yfp), after 6 hours of IPTG removal (FtsZ depletion). Scale bar represents 10 μm. doi:10.1371/journal.pone.0086897.g007
sequence for chromosomal integration, and ligated with *M. xanthus* Mxan_18–19 sequence, used for a chromosomal integration in a previous work [33], obtaining the second precursor plasmid pMR4048. The 1.319 kb Mxan_18–19 sequence was isolated after HindIII digestion of plasmid pMR3691 [33]. Finally, a PCR-amplified parA-yfp sequence, obtained using pMR3785 as DNA template and the primers 97_parA.for (5'-aaaaaatctagaatggtg-cactgcatcacgcg-3') and 98_yfp.rev (5'-aaaaaaggtaccttacttgata-cagctcgtcca-3'), was digested with KpnI and XbaI, and cloned into a KpnI-XbaI-digested pMR4048, producing the plasmid pMR4051.

To generate the *M. xanthus* parB conditional mutant strain, MR2472, the wild type DK1050 strain was electroporated with plasmid pMR3595, obtaining the strain MR2461. This strain contains the P\_van-parB sequence integrated at the *M. xanthus* 1.38-kb locus. Then, MR2461 was electroporated with plasmid pMR3620, which contains sequences upstream and downstream of parB in the genome to generate a parB deletion and the galK gene, creating strain MR2462. MR2462 was grown for several generations with 0.5 mM of vanillate and no Kan and plated on CTT plates supplemented with 2% galactose and 0.5 mM of vanillate to select for the loss of the Gal\(^R\) marker. This evicts vector DNA bearing either wild type parB or the ΔparB allele by intramolecular recombination events. Gal\(^R\) Kan\(^S\) colonies were diagnosed by PCR to isolate a strain harboring the inducible P\_van-parB construct and the ΔparB allele (MR2472). The strain MR2504 was obtained electroporating plasmid pMR3785 into the wild type DK1050 strain. The strain MR2526 was obtained electroporating the strain MR2520 with plasmid pMR3826, and MR2520 by electroporating DK1050 with plasmid pMR3828. The strain MR2536 was generated by electroporating MR2916, the strain that conditionally expresses ftsZ from an IPTG-inducible promoter [33], with plasmid pMR3826 in presence of 1 mM IPTG. The strain MR2538 was obtained by electroporating the parB conditional mutant strain MR2472 with plasmid pMR4051, in presence of 0.5 mM vanillate.

**ParB Expression and Purification**

To overexpress intein-tagged *M. xanthus* ParB, 10 ml starter culture of freshly transformed *E. coli* BL21(DE3) containing plasmid pMR3684 was grown at 37°C in LB medium with 100 \(\mu\)g/ml of ampicillin (Amp) to an OD\(_{600}\) of 0.6. It was added to 1 l of fresh LB/Amp, grown at 37°C to an OD\(_{600}\) of 0.55, and after 30 min incubation at 18°C, overexpression of intein-tagged ParB was induced overnight at 18°C with 1 mM IPTG. After overnight induction with IPTG, cells were harvested by centrifugation (15 min at 5000 \(\times\)g) and the pellet was stored at -70°C until further use. Intein-tagged ParB was purified using chitin resin and the intein was removed by on-column intramolecular cleavage in the presence of 50 mM dithiothreitol using the IMPACT kit protocols (New England Biolabs). The cleaved protein was passed through a small amount of chitin resin a second time to remove residual intein and dialyzed extensively against 25 mM Tris pH 8, 50 mM NaCl, 5 mM MgCl\(_2\), 0.1 mM EDTA, 10% glycerol and 2 mM \(\beta\)-mercaptoethanol.

**Mobility Shift Assays**

Electrophoretic mobility shift assays (EMSA) in agarose gels: The 3120-bp DNA probe containing the 22-repeat parS-cluster was obtained by PCR using primers 58_parS.for (5'-...
ccgttcgctttcgtgacgggtccaggttcc-3') and 59_parS.rev (5'-agtaacgccgctcagcaccacttcgacgt-3') 32P end-labeled with [γ-32P]ATP and T4 polynucleotide kinase, and DK1050 genomic DNA as a template. For the 3005-bp attP probe, the primers used were 78_attP.for (5'-aaaaaaaagcttggggatggagccagacgg-3') and 79_attP.rev (5'-aaaaaaaagcttgggatgcggtggaccatg-3').

Table 2. Relevant strains.a

| strain | integrated plasmid(s) | relevant genotype or description | source |
|--------|------------------------|---------------------------------|--------|
| DK1050 |                        | M. xanthus wild type            | [61]   |
| DK1622 |                        | M. xanthus wild type            | [62]   |
| MR2461 | pMR3594                | M. xanthus DK1050 1.38-kb:Pvan-parB | This study |
| MR2472 | pMR3594                | M. xanthus DK1050 1.38-kb:Pvan-parB, ΔparB | This study |
| MR2504 | pMR3785                | M. xanthus DK1050 1.38-kb:Pvan-parA-yfp | This study |
| MR2526 | pMR3826, pMR3828       | M. xanthus DK1050 Mxan_18–19:Pvan-parA-yfp, 1.38-kb:Pvan-mCherry-parB | This study |
| MR2536 | pMR3636, pMR3826       | M. xanthus DK1622 1.38-kb:Pvan-mCherry-parB, ΔparB, Mxan_18–19:Pvan-parA-yfp | This study |
| MR2538 | pMR3594, pMR4051       | M. xanthus DK1050 1.38-kb:Pvan-parB, ΔparB, Mxan_18–19:Pvan-mCherry-parB | This study |

*aOther strains, precursors to those listed here, are described in the text.

doi:10.1371/journal.pone.0086897.t002
buffer (40 mM Na-phosphate pH 8, 20 mM NaCl, 7% glycerol, 20 μg/ml BSA, and 100 μg/ml sheared salmon sperm DNA), and loaded onto an 0.7% agarose gel and run at 100 V at 4°C in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). Gels were dried and analyzed by autoradiography. In competitive EMSA in polyacrylamide gels: A 50 bp DNA duplex that contains a parS site was generated diluting oligonucleotides 60_parS.hib (′5′-tgctcagcttcctcttcgcgagatgcctgccgagt-3′) and 61_parS.hib (′5′-actcaactgcgacttcgccgagtggagtgctagt-3′) to a final concentration of 5 μM each. A 50 bp DNA duplex that contains a mutated parS site was generated diluting oligonucleotides 89_parS.hib (′5′-tgctcagcttcctcttcgcgagatgcctgccgagt-3′) and 90_parS.hib (′5′-actcaactgcgacttcgccgagtggagtgctagt-3′) to a final concentration of 5 μM each. Each mixture was heated to 95°C for 10 min and slowly cooled to room temperature, and then 32P end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Labeled DNA sample was incubated for 1 hour at 30°C with M. xanthus ParB protein in binding buffer, and 15 μM ParB protein for 1 hour at 30°C.

Addendum in Proof

While this paper was under review, similar findings were reported by Harms et al. (2013) [59], who also showed that ParB and ParA are essential proteins, examined their subcellular localization patterns, and confirmed the in vitro binding of ParB to a consensus parS sequence and ParB participation in chromosome segregation. The present work suggests that, in addition ParB helps in correct chromosome segregation by inhibiting the non-specific interaction between ParA and DNA and thereby prevents ParA colocalization with chromosomal DNA. It is also shown here that the polar and mid-cell localization pattern of ParA does not depend on the presence of ParS, the critical element for bacterial cell division.

Acknowledgments

I especially thank Professors Monserratt Elias-Arnanz and Francisco Murillo for the use of fluorescence microscopy and other laboratory facilities. Dr. Francisco Garcia-Heras and Prof. Monserratt Elias-Arnanz for providing M. xanthus ParB protein, Drs. Esteban Toro, Monserratt Elias-Arnanz and S. Padmanabhan for critical reading of the manuscript, and J. A. Madrid for technical support.

Author Contributions

Conceived and designed the experiments: AAI. Performed the experiments: AAI. Analyzed the data: AAI. Contributed reagents/materials/ analysis tools: AAI. Wrote the paper: AAI.

References

1. Toro E, Shapiro I (2010) Bacterial Chromosome Organization and Segregation. Cold Spring Harbor Perspectives in Biology 2: a000349–a000349. doi:10.1101/cshperspect.a000349.
2. Wang X, Montero Llopis P, Rusher DZ (2013) Organization and segregation of bacterial chromosomes. Nat Rev Genet 14: 191–203. doi:10.1038/nrg3375.
3. Postow L, Hardy CD, Arusaga J, Coszarelli NR (2004) Topological domain structure of the Escherichia coli chromosome. Genes Dev 18: 1766–1779. doi:10.1101/gad.1297504.
4. Merzier R, Prat M-A, Schahb S, Robin S, Karoui El M, et al. (2008) The MatP/MatS Site-Specific System Organizes the Terminus Region of the E. coli Chromosome into a Macrodomain. Cell 135: 475–485. doi:10.1016/j.cell.2008.08.013.
5. Telemann AK, Graumann PL, Lin DC-H, Grossman AD, Losick R (1998) Chromosome arrangement within a bacterium. Current Biology 8: 1102–1109. doi:10.1016/S0960-9822(98)00464-6.
6. Vallen-Gely I, Boccard F (2013) Chromosomal Organization and Segregation in Pseudomonas aeruginosa. PLoS Genet 9: e1003492. doi:10.1371/journal.pgen.1003492.
7. Telemann AK, Graumann PL, Lin DC-H, Grossman AD, Losick R (1998) Chromosome arrangement within a bacterium. Current Biology 8: 1102–1109. doi:10.1016/S0960-9822(98)00464-6.
8. Nielsen HJ, Li Y, Youngren B, Hansen FG, Austin S (2006) Progressive segregation of the Escherichia coli chromosome. Mol Microbiol 61: 383–393. doi:10.1111/j.1365-2958.2006.05243.x.
9. Leserlin C, Gigaet E, Boccard F, Espéol O (2012) Sister chromatid interactions in bacteria revealed by a site-specific recombination assay. EMBO J 31: 3468–3479. doi:10.1038/emboj.2012.194.
10. Lemon KP, Grossman AD (2001) The extrusion-capture model for chromosome partitioning in bacteria. Genes Dev 15: 2031–2041. doi:10.1101/gad.913301.
11. Dworkin J, Losick R (2002) Does RNA polymerase help drive chromosome segregation in bacteria? Proc Natl Acad Sci USA 99: 14089–14094. doi:10.1073/pnas.102539899.
12. Wooldridge CL (2002) The role of co-transcriptional translation and protein translocation (transient) in bacterial chromosome segregation. Mol Microbiol 45: 17–29. doi:10.1046/j.1365-2958.2002.02993.x.
13. Jun S, Mulder B (2006) Entropy-driven spatial organization of highly confined polymers: lessons for the bacterial chromosome. Proc Natl Acad Sci USA 103: 12388–12393. doi:10.1073/pnas.0605305103.
14. Mierzejewska J, Jagura-Burzyk G (2012) Prokaryotic ParA/ParB-parS system links bacterial chromosome segregation with the cell cycle. Plasmid 67: 1–14. doi:10.1016/j.plasmid.2011.08.003.
15. Levy J, Yamachi Y, Waldor MK (2007) Distribution of Centromere-Like parS Sites in Bacteria: Insights from Comparative Genomics. J Bacteriol 189: 8693–8703. doi:10.1128/JB.01259-07.
17. Toro E, Hong S-H, McAdams HH, Shapiro L (2008) Caulobacter requires a dedicated mechanism to initiate chromosome segregation. Proc Natl Acad Sci USA 105: 15435–15440. doi:10.1073/pnas.0807461105.

18. Murray H, Errington J (2008) Dynamic control of the DNA replication initiation protein DnaA by Soj/ParA. Cell 135: 74–84. doi:10.1016/j.cell.2008.07.044.

19. Gerdes K, Howard M, Szardenings F (2010) Pushing and pulling in prokaryotic cell motility. Nat Rev Microbiol 8: 551–563. doi:10.1038/nrmicro2378.

20. Kaiser D, Robinson M, Kroos L (2010) Myxobacteria, polarity, and cellular morphogenesis. Cold Spring Harbor Perspectives in Biology 2: a003638. doi:10.1101/cshperspect.a003638.

21. Zhang Y, Ducret A, Shaevitz J, Mignot T (2012) From individual cell motility to multicellular morphogenesis. Cell 148: 1209–1222. doi:10.1016/j.cell.2012.04.048.

22. Goldman BS, Nierman WC, Kaiser D, Slater SC, Durkin AS, et al. (2006) The bacterial chromosome partitioning protein, ParB, is required for normal chromosome segregation as well as the initiation of sporulation in Bacillus subtilis. J Bacteriol 188: 5320–5329.

23. Mohl DA, Easter J, Gober JW (2001) The chromosome partitioning protein, ParB, is required for normal chromosome segregation in Caulobacter crescentus. Cell 105: 675–684. doi:10.1016/S0092-8674(01)00891-8.

24. Charaka VK, Mierzejewska J, Thomas CM, Jagura-Burczy G (2004) ParB of Pseudomonas aeruginosa: interactions with its partner ParA and its target parS and specific effects on bacterial growth. J Bacteriol 186: 6983–6988. doi:10.1128/JB.186.20.6983–6988.2004.

25. Harms A, Treuner-Lange A, Schumacher D, Søgaard-Andersen L (2013) Tracking of chromosome and replisome dynamics in Myxococcus xanthus reveals a novel chromosome arrangement. PLoS Genet 9:e1003802. doi:10.1371/journal.pgen.1003802.

26. Brachat A, Chamoun J-M, Brenner SE (2004) WebLogo: a sequence logo generator. Genome Res 14: 1189–1190. doi:10.1101/gr.694004.

27. Ruiz-Vázquez R, Murillo FJ (1984) Abnormal motility and fruiting behavior of Myxococcus xanthus bacteriophage-resistant strains induced by a clear-plaque mutant of bacteriophage Mx8. J Bacteriol 160: 819–825.

28. Köpke D (1979) Social gliding is correlated with the presence of pili in Myxococcus xanthus. Proc Natl Acad Sci USA 76: 5952–5956.