A dynamic, mitotic-like mechanism for bacterial chromosome segregation

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The mechanisms that mediate chromosome segregation in bacteria are poorly understood. Despite evidence of dynamic movement of chromosome regions, to date, mitotic-like mechanisms that act on the bacterial chromosome have not been demonstrated. Here we provide evidence that the *Vibrio cholerae* ParAI and ParBI proteins are components of an apparatus that pulls the origin region of the large *V. cholerae* chromosome to the cell pole and anchors it there. ParBI interacts with a conserved origin-proximal, centromere-like site (*parSI*) that, following chromosome replication, segregates asymmetrically from one pole to the other. While segregating, *parSI* stretches far away from neighboring chromosomal loci. ParAI forms a dynamic band that extends from the pole to the segregating ParBI/*parSI* complex. Movement of ParBI/*parSI* across the cell occurs in concert with ParAI retraction. Deletion of *parAI* disrupts proper origin localization and segregation dynamics, and *parSI* no longer separates from nearby regions. These data suggest that ParAI forms a dynamic structure that pulls the ParBI-bound chromosome to the pole in a process analogous to anaphase of eukaryotic mitosis.

Keywords: *Vibrio cholerae;* chromosome segregation; ParA; ParB

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All dividing cells must have mechanisms to ensure that their genomes are faithfully segregated to daughter cells. While the conserved mitotic apparatus used by eukaryotes to direct chromosome segregation is well defined, relatively little is certain regarding the mechanisms that mediate chromosome segregation in prokaryotes. In recent years, it has become clear that bacterial chromosomes are both highly organized and dynamically segregated. Particular chromosomal loci occupy specific subcellular locations (Gordon et al. 1997; Webb et al. 1997), and the relative spatial arrangement of most chromosomal loci correlates with their linear map positions (Telem et al. 1998; Niki et al. 2000; Viollier et al. 2004). Specific chromosomal regions, particularly those close to the origin of replication, are actively and rapidly positioned in the cell (Glaser et al. 1997; Webb et al. 1998; Jensen and Shapiro 1999). The observed movements are more rapid than could be accounted for by previous models of passive segregation mediated by chromosome attachment to the growing cell membrane (Jacob et al. 1963).

Several mechanisms may contribute to the dynamic movements of bacterial chromosomes [for reviews, see Errington et al. 2005; Leonard et al. 2005]. The observation in *Bacillus subtilis* that the DNA replication machinery appears to be localized as a stationary “factory” at the cell center (Lemon and Grossman 1998) led to the proposal that DNA polymerase could provide the bidirectional force for segregation (Lemon and Grossman 2000). Similarly, RNA polymerase, acting on directionally biased genes near the origin, has been hypothesized to impart both motive force and directionality to segregation (Dworkin and Losick 2002; Kruse et al. 2006). While both of these models could potentially explain the symmetric and bidirectional segregation of the *Escherichia coli* and *B. subtilis* chromosomes from the middle of the cell, it is difficult to directly apply them to the asymmetric segregation process that occurs in *Caulobacter crescentus* (Mohl and Gober 1997; Viollier et al. 2004) and in *Vibrio cholerae* (Fogel and Waldor 2005). In both of these organisms, the origin region is located close to one pole (the “old” pole) early in the cell cycle, and after replication, one copy remains at that pole while the other traverses the entire length of the cell to the opposite (“new”) pole. Consistent with an asymmetric segregation pattern from the pole, the replication machinery of *C. crescentus* localizes to the old pole, and then after replication initiation, migrates to the cell center (Jensen et al. 2001). The presence of a moving replisome suggested a modification of previous models in which the origin regions are positioned rapidly by an origin-specific mechanism, and then the bulk of the chromosome is...
segregated by a replication factory in directions established by the positioning of the origins at the poles (Jensen et al. 2001).

Most bacterial chromosomes encode orthologs of plasmid partitioning [Par] proteins near their origins (Gerdes et al. 2000). In plasmids, par loci consist of three components: a DNA-binding protein [often termed ParB], an ATPase [ParA], and a centromere-like site [parS]. ParB binds parS and spreads along the DNA, forming a large nucleoprotein complex. Formation of this complex and its interaction with ParA are required for efficient plasmid segregation (Ebersbach and Gerdes 2005; Leonard et al. 2005). The Par-family ATPases fall into two distinct phylogenetic groups; type I ParAs contain the conserved Walker-box ATP-binding motif, whereas type II ParAs are structurally related to eukaryotic actin (Gerdes et al. 2000). Both type I and type II ParAs form ATP-dependent filamentous polymers in vitro (Møller-Jensen et al. 2002; Barillà et al. 2005; Lim et al. 2005). Type II ParAs appear to mediate plasmid segregation by polymerizing between plasmid pairs and “pushing” them apart toward the poles (Møller-Jensen et al. 2003). The mechanism by which type I plasmid ParAs function is less clear. Type I ParAs from some plasmids appear to oscillate back and forth in the cell (Ebersbach and Gerdes 2001; Lim et al. 2005; Adachi et al. 2006), but it is unknown how oscillation positions plasmids. Recently, a plasmid ParA was shown to polymerize into radial filaments on ParB-bound DNA in vitro, and a model was proposed in which plasmids are positioned by ParA pushing in all directions in the cell (Lim et al. 2005).

While the essential role of par loci in plasmid partitioning has been long appreciated, their functions in bacterial chromosomal biology is less clear. The B. subtilis Par proteins Soj [ParA] and Spo0J [ParB] are nonessential but have effects on chromosome segregation (Ireton et al. 1994; Sharpe and Errington 1996; Lee et al. 2003; Wu and Errington 2003; Lee and Grossman 2006). Spo0J binds to at least 8 sites in a large region around the origin [Lin and Grossman 1998], and deletion of spo0J results in an increased frequency of anucleate cells (Ireton et al. 1994). Together, Spo0J and Soj appear to facilitate efficient separation of newly duplicated origins (Lee and Grossman 2006), but a mechanistic understanding of their role in chromosome segregation remains to be defined. In contrast to B. subtilis, the C. crescentus ParA and ParB are essential, and their overexpression or depletion results in defects in cell growth, division, and chromosome segregation [Mohl and Gober 1997; Mohl et al. 2001]. ParB of C. crescentus binds to sites near the origin of the chromosome (Mohl et al. 2001) and localizes as foci at the extreme poles [Mohl and Gober 1997]. While C. crescentus ParA and ParB affect both cell division and chromosome segregation, recent evidence suggests that the cell division defects are due to the effects of chromosome segregation on the localization of MipZ, an inhibitor of FtsZ ring formation (Thanbichler and Shapiro 2006).

Thus, while there is strong evidence in several bacteria for active movement of chromosomal DNA during segregation, specific molecular mechanisms that mediate such movements have not been clearly elucidated.

V. cholerae is a curved, Gram-negative rod that causes the severe diarrheal disease cholera. It contains two circular chromosomes, 2.96 and 1.07 Mb [chrI and chrII, respectively]. Despite having coordinated replication initiation (Egan et al. 2004), the segregation of the two chromosomes is likely governed by distinct mechanisms as the localization and segregation patterns of their origin regions are different (Fogel and Waldor 2005; Fiebig et al. 2006). The origin region of chrI is localized near the old pole, where an asymmetric segregation process is initiated in which the duplicated origin segregates across the cell to the new pole. In contrast, the origin of chrII is localized at the cell center, where after replication, and at a later point in the cell cycle, the duplicated origins move symmetrically to the quarter positions of the cell, which become the new cell centers.

The distinct localization and segregation dynamics of the origin regions of the two chromosomes suggests the existence of mechanisms capable of discriminating between and interacting with the two origin regions to mediate their cell cycle-dependent segregation dynamics. Both chromosomes contain unique origin-proximal parAB loci. Here we explored the role of the Par proteins in the segregation of chrI. Our findings suggest that the chrI Par proteins participate in a mitotic-like pulling mechanism to mediate the asymmetric polar segregation of the origin region of chrI in V. cholerae.

Results

A YFP-ParBI fusion protein forms discrete polar foci that segregate asymmetrically

Fusions of GFP to ParB proteins in several plasmid and chromosomal systems form punctate fluorescent foci, marking the location of the ParB–parS nucleoprotein complex in the cell [Glaser et al. 1997; Lin et al. 1997; Li and Austin 2002]. We constructed a fusion of YFP to the N terminus of the ParB protein encoded on chrI [ParBI] in order to investigate if its subcellular localization was suggestive of a role in chromosome segregation. YFP-ParBI formed well-defined fluorescent foci in wild-type V. cholerae cells [Fig. 1A]. In contrast, expression of this fusion protein in E. coli resulted in only diffuse fluorescence [Supplementary Fig. S1], suggesting that there are V. cholerae specific binding sites for ParBI. Almost all V. cholerae cells with YFP-ParBI foci contained at least one focus at the extreme pole of the cell [94%]. The majority of cells [67%] contained two foci, one at the extreme pole and the second either at the opposite pole or at an intermediate position between the poles [Fig. 1B]. The number of foci per cell and their localization was very similar to our observations of the origin of chrI [oriCIvC] detected with the lacO/tetO fluorescent repressor–operator systems [FROS] (Fogel and Waldor 2005), suggesting that ParBI likely binds near the origin of chrI.
Lin and Grossman (1998) defined the binding site for the *B. subtilis* ParB ortholog Spo0J and noted the widespread conservation of this sequence in many bacteria. In *V. cholerae*, there are three copies of this sequence on chrI and none on chrII. The three sites are clustered together ∼65 kb to the right of oriClvcr, with 3 kb separating them. We found that these three sites, and not closely related sequences elsewhere on chrI or chrII, formed foci with YFP-ParBI when tested in *E. coli* [Supplementary Fig. S1; data not shown]. As many newborn *V. cholerae* cells contained only a single focus [Fig. 1B, top], and the number and distribution of foci were qualitatively similar to those of origins labeled with other markers [Fogel and Waldor 2005; Fiebig et al. 2006], we concluded that there is likely one YFP-ParBI focus per copy of oriClvcr, marking the subcellular location of the origin-proximal parSI locus. Expression of YFP-ParBI for the time periods used in these experiments did not affect the growth rate of wild-type cells, nor does it alter the localization of other origin regions visualized by FROS [data not shown].

Analysis of foci in more than a thousand cells revealed that YFP-ParBI foci are often found at the extreme pole (Fig. 1C,D). In cells with two YFP-ParBI foci, the focus closest to a pole (Fig. 1C, blue circles) was on average only 4.3% of the cell length away, and many foci were

![Figure 1](https://genesdev.cshlp.org/3271/GENES & DEVELOPMENT/genesdev.cshlp.org)
lapped the edge of the cell. The other focus (Fig. 1C, orange squares) was found close to the opposite pole or at intermediate positions between the poles, presumably representing an actively segregating origin. As expected, time-lapse microscopy (Fig. 1E) revealed that the YFP-ParBI foci segregate with asymmetric dynamics that recapitulate the segregation pattern of the FROS-labeled origins [Fogel and Waldor 2005]. Late predivisional cells contain YFP-ParBI foci at both poles; these poles become the old poles of each daughter cell. Around the time of division, the polar foci duplicate, and one segregates across the cell to the new pole, the other remains at the old pole. The most significant difference between YFP-ParBI foci and the origin-proximal loci studied previously with FROS is the extreme polarity of YFP-ParBI; the FROS foci were rarely at the edge of the cell. The extreme polarity of the YFP-ParBI foci suggested the possibility that the ParBI-parSI nucleoprotein complex anchors the chromosome to the pole, as has been proposed for *C. crescentus* [Mohl and Gober 1997]. The origin, 65 kb away, would be expected to be near the pole, but not as close as the anchored site.

**ParAI is required for polar positioning and asymmetric segregation of ParBI**

We constructed a *V. cholerae* strain with a deletion of *parAI*. The Δ*parAI* strain had an increased frequency of filamentous cells, 1.8% versus 0.4% for wild type, suggesting a mild defect in cell division, but otherwise the strain appeared to grow normally. We examined the localization of YFP-ParBI in the Δ*parAI* mutant and found that the foci were dramatically mislocalized [Fig. 1F]. In Δ*parAI* cells, the YFP-ParBI foci were dissociated from the cell poles and instead were generally found near the cell center in cells with a single focus or close to the quarter positions in the cell with two foci [Fig. 1G]. In Δ*parAI* cells with two foci, the mean position of the closest-to-pole ParBI focus [Fig. 1H, blue circles] was 16.4% of the cell length [vs. 4.3% for wild type], and >98.5% of the foci were farther away from the pole than the mean value for wild type. Not only was the mean position of the closest-to-pole focus changed, but the distribution of the distances was significantly wider for the Δ*parAI* strain [Fig. 1, cf. D and I]. This suggests that YFP-ParBI foci in the Δ*parAI* strain have a greater degree of freedom in their localization, a behavior that is consistent with loss of an attachment between the ParBI-bound chromosome and the pole. Recently, mislocalization of an origin-proximal locus in a *V. cholerae* parAI deletion mutant was shown by FISH [Saint-Dic et al. 2006]. Together these observations suggest that ParAI affects the localization of a relatively large origin-proximal domain of chromosome I.

Remarkably, the asymmetric pattern that characterizes the segregation of oriCI<sub>vc</sub> was absent in the Δ*parAI* mutant. Time-lapse analysis of Δ*parAI* cells showed generally bidirectional movement of YFP-ParBI foci [Fig. 1J]. The presence of separated and symmetrically distributed YFP-ParBI foci in the Δ*parAI* strain [Fig. 1G,J] suggests that ParAI-independent mechanisms can still segregate duplicated origins, even though wild-type polar localization and asymmetric segregation are lost.

The defects in the Δ*parAI* cells are attributable to the deletion of *parAI*, as expression of either a His-tagged ParAI (ParAI-His) or a fluorescent fusion to ParAI (ParAI-CFP) restored the polar localization of YFP-ParBI foci [Fig. 2A,B, respectively]. Moreover, the ability of ParAI to localize ParBI to the pole likely requires ATP, as substitutions in the conserved ATP-binding motif of ParAI abolished the ability of ParAI-CFP to complement the *parAI* deletion [Fig. 2C,D]. In addition to the *parSI* site, other origin-proximal loci on both sides of oriCI<sub>vc</sub> were also mislocalized in the Δ*parAI* mutant [see below]. These observations suggest that ParAI is required to position the ParBI-parSI complex at the extreme pole, thereby affecting the localization of a relatively large origin-proximal region.

**ParAI promotes separation of the parSI region from neighboring loci during segregation**

In order to study the spatial relationship between parSI and another origin-proximal locus, we expressed CFP-ParBI and TetR-YFP in a strain containing a tetO array integrated 15 kb to the right of oriCI<sub>vc</sub> [Fig. 3A, tetO(1)]. Under the growth conditions used, the majority (~85%) of these dual-labeled cells had replicated the origin region of chrI and therefore had four foci, one CFP-ParBI and one TetR-YFP for each copy of the origin region [Fig. 3B]. As expected from their localization in single fluorescence experiments [Fig. 1; Fogel and Waldor 2005], most cells contained one CFP-ParBI and one TetR-YFP focus at the extrema of one pole, and a second pair either at the opposite pole or at intermediate positions [Fig. 3B]. In most cells, for the pair of foci closest to a pole (the old pole), the CFP-ParBI and TetR-YFP foci were adjacent or overlapping, as might be expected for two loci separated by only 50 kb [Fig. 3B, single white star]. Despite this close association, their orientation with respect to the pole was not random. In fact, for 95.2% of cells (*n* = 849), the CFP-ParBI foci were found to be closer to the pole than the TetR-YFP foci [Fig. 3B, single white star; Fig. 3C]. Analysis of parSI and a tetO-tagged locus on the other side of the origin [Fig. 3A, tetO(n)] similarly showed that *parSI* was consistently closest to the pole [data not shown]. The ordered arrangement of these foci both relative to each other and to a cellular structure (the pole) reveals an extremely fine level of spatial organization for this region of the chromosome.

From the time-lapse experiments with YFP-ParBI alone [Fig. 1D], we know that for cells containing both a polar and an intermediate ParBI focus, the intermediate focus is in the process of segregating across the cell to the opposite pole. In cells with a segregating pair of CFP-ParBI and TetR-YFP foci, we analyzed the spatial relationship of the two loci during segregation. For these segregating foci, the CFP-ParBI focus was closer to the...
new pole and thus appeared to be “leading” the TetR-YFP focus, in 99% of the cells [Fig. 3B, white double stars]. Similar results were obtained for the tetO(2) locus [data not shown]. The order of segregation, with CFP-ParBI foci leading TetR-YFP foci, was confirmed by time-lapse analysis [data not shown]. Thus, there is an ordered relationship between these loci both at the pole and during segregation. These observations also imply that DNA replication is unlikely to provide the motive force for segregation of this region, as ParBI foci segregate ahead of regions replicated earlier than parSI [tetO(2)].

In contrast to the close proximity of CFP-ParBI and TetR-YFP foci at the old pole, for the segregating foci, CFP-ParBI was often dramatically separated from its companion TetR-YFP focus (Fig. 3B, white double arrow). The interfocal distances were often >1 µm and in some cells as much as 1.6 µm [Fig. 3D orange bars], as if strong forces were stretching the two foci apart. To our knowledge, such remarkable dynamic variability in the distance between two close genetic loci has not been reported before in bacteria. Similar behavior has been observed in eukaryotic cells between sites in the centromere and nearby sites on the chromosome arms (Goshima and Yanagida 2000). In this case, the separation was attributed to the action of mitotic spindles pulling at the centromere. In addition, the segregating YFP-ParBI foci, but not those at the pole, were often significantly elongated (Fig. 3B, double white star) as if subject to a distorting force. This distortion, together with the ordered arrangement and dynamic separation of parSI relative to adjacent loci, is consistent with the parSI site serving as the target for active segregation machinery.

When parSI (CFP-ParBI foci) and tetO(2) (TetR-YFP foci) were visualized together in the ΔparAI background, as expected, both were significantly mislocalized [Fig. 3E,F]. In cells with two foci, the position of the closest-to-pole CFP-ParBI focus as a fraction of cell length was 19.7% versus only 2.9% in the wild-type background, and for the TetR-YFP foci, 19.2% versus 9.4% [Fig. 3, cf. C and F]. In addition to their mislocalization, the ordered arrangement (parSI always closer than tetO(2)) relative to the pole was not observed; instead, their orientation appeared random [Fig. 3, cf. B and E]. Furthermore, the interfocal distances between CFP-ParBI and TetR-YFP foci were markedly reduced in the absence of ParAI. In ΔparAI cells containing two pairs of foci, there were no cells in which CFP-ParBI and TetR-YFP foci were separated by >0.6 µm [Fig. 3D, blue bars] and the mean interfocal distance was only 0.19 µm compared with a mean of 0.65 µm in the wild-type strain. Thus, ParAI is required for a cell cycle-specific (i.e., during segregation) separation of the 50 kb of DNA between parSI and tetO(2). This requirement is consistent with a role for ParAI in applying force during segregation. The loss of the ordered arrangement relative to the pole suggests that ParAI continues to exert effects on the origin region both before and after segregation.

ParAI has a dynamic and complex subcellular distribution

To study how ParAI might act to affect origin dynamics, we examined the subcellular localization of fluorescent
ParAI fusions able to complement the parAI deletion (see above, Fig. 2B). The distribution of ParAI-CFP in ΔparAI cells generally conformed to one of three patterns roughly correlated with cell size (Fig. 4A). In smaller (younger) cells, there was invariably a distinct focus at one pole and a dense patch of fluorescence extending from the other pole (Fig. 4A, panels I). In early predivisional cells, the distribution of ParAI-CFP was more variable; many cells had foci, usually of different intensity, at both poles and a dense patch of fluorescence extending from the other pole (Fig. 4A, panels II). In the oldest cells, particularly those that had visibly initiated septation, the ParAI-CFP distribution was again highly consistent; dense bands or patches extended from the midcell or septum toward, but not reaching the poles, which were occupied by distinct foci (Fig. 4A, panels III). The localization of ParAI fluorescent fusions was essentially the same in wild-type cells; in contrast, YFP or CFP alone produced only diffuse fluorescence (data not shown).

In some cells, the dense bands of ParAI-CFP appear to be composed of single or double curved structures extending from the pole or septum (Fig. 4A). Deconvolution of z-sections of ParAI-CFP expressed in ΔparAI cells shows more clearly that ParAI-CFP does not fill the entire diameter of the cell (Fig. 4B) and suggests that it may form a cytoskeletal structure as fluorescence from two sections 0.1 µm apart do not completely colocalize (Fig. 4B, merged). The same deconvolution processing on a z-stack of cells expressing the nonfunctional ParAI-CFP[K16E] showed no such structures (Fig. 4B).

Time-lapse experiments revealed that the localization of ParAI-CFP was highly dynamic. Although there was some variability, analysis of a large number of time-lapse experiments revealed consistent patterns, the most common of which is represented in Figure 4C. When a predivisional cell (Fig. 4C, time = 0) divides (time = 3), it produces two “newborn” cells both with the ParAI-CFP distribution seen in small cells (Fig. 4A, panels I). Deconvolution of z-sections of ParAI-CFP expressed in ΔparAI cells shows more clearly that ParAI-CFP does not fill the entire diameter of the cell (Fig. 4B) and suggests that it may form a cytoskeletal structure as fluorescence from two sections 0.1 µm apart do not completely colocalize (Fig. 4B, merged). The same deconvolution processing on a z-stack of cells expressing the nonfunctional ParAI-CFP[K16E] showed no such structures (Fig. 4B).

Figure 3. Separation of parSI from a neighboring chromosomal locus. (A) Diagram of chrI of V. cholerae and the relative locations of tetO₁(−90 kb) and tetO₂(−15 kb) sites (red bars) as well as the parSI region (+65 kb, green bar), which contains the three ParBI-binding sites. The location of the parAI and parBI genes (purple arrows) and the origin region (orange box) are also represented. Strains YBB025 [wild-type, WT] and MF302 [ΔparAI] contain the tetO cassette integrated at tetO₂ and the plasmid pMF310 that expresses CFP-ParBI (pseudo-colored green) and TetR-YFP (red). (B,E) Representative YBB025 and MF302 cells of different sizes. The white arrowhead shows a cell with two CFP-ParBI foci and one TetR-YFP focus. The white double-headed arrow shows a cell with ~1 µm of space between CFP-ParBI and TetR-YFP foci. The closely associated foci at the (presumed) old pole are marked with a single white star, and segregating pairs of foci are marked with double white stars. (C,F) Automated analysis of the positions of the CFP-ParBI foci pairs (green circles and squares) and TetR-YFP foci pairs (red circles and squares) in YBB025 (n = 240) (B) and MF302 (n = 240) (E). (D) Histogram of the distribution of interfocal distances between segregating pairs of TetR-YFP and CFP-ParBI foci for YBB025 (orange bars), and for MF302 (blue bars); each set of distances was grouped into 10 equal-sized bins. Bar, 1.0 µm.
ersbach and Gerdes 2001; Lim et al. 2005). Interestingly, the ParA ortholog on chromosome II (ParAII), a type I ATPase more related to plasmid ParA proteins, oscillates rapidly (Supplementary Fig. S2). Our observations suggest that the *V. cholerae* ParAI has different behavior that involves cell cycle-coordinated retraction toward the new pole.

**YFP-ParBI and ParAI-CFP colocalize, and their dynamics are coordinated**

To explore the relationship between ParAI and ParBI, YFP-ParBI and ParAI-CFP were coexpressed in ΔparAI cells. These experiments revealed two different types of association: (1) Polar YFP-ParBI foci colocalized with polar ParAI-CFP foci (Fig. 5A, white arrowhead); and (2) in cells with two YFP-ParBI foci, the segregating focus was always at the edge of a band of ParAI-CFP fluorescence (Fig. 5A, white star). In small cells containing two close, presumably recently separated, YFP-ParBI foci (Fig. 5B, panels I,II), the band of ParAI-CFP extended across the cell from the new pole to the segregating YFP-ParBI focus. In older cells, in which the segregating YFP-ParBI focus was almost at the opposite pole, the band of ParAI-CFP was smaller, filling only the space between the segregating YFP-ParBI focus and the new pole (Fig. 5B, pan-
In late predivisional cells (Fig. 5B, panels V, VI), YFP-ParBI foci are at each pole, and ParAI-CFP was almost completely located at the cell center or septum from which it extended outward toward the two old poles. Thus, before division is complete, both ParAI and ParBI are positioned to repeat the cycle.

In order to analyze the relationship between YFP-ParBI and ParAI-CFP fluorescence in a large number of cells, 

**Figure 5.** YFP-ParBI colocalizes with ParAI-CFP at the pole and at the edge of ParAI-CFP bands. ΔparAI cells contained plasmids expressing YFP-ParBI and ParAI-CFP. (A) Phase, YFP, CFP, and merged images of a representative cell showing the two distinct types of colocalization: foci at the pole (white arrowhead) and YFP-ParBI-focus at the edge of ParAI-CFP bands (white star). (B) Representative cells of different lengths and at different points during chromosome segregation showing the relationship of ParAI-CFP localization with YFP-ParBI. (C) Graphical representation of the relationship between YFP-ParBI separation (green circles) and the localization of ParAI-CFP fluorescence along the cell’s length (red surface) in 314 cells. For each cell, width-averaged ParAI-CFP fluorescence (Z-axis) at each point along the midline (X-axis) is graphed versus the distance between the YFP-ParBI foci of that cell. The positions of the YFP-ParBI foci [green] are overlaid on the graph [at an arbitrary Z-axis height of 0.3 for visibility]. Dashed arrows point to images of cells (panels a–d) representative of data at the indicated position of the graph. The width-averaged ParAI-CFP fluorescence values were background-subtracted, then normalized as a fraction of total cellular fluorescence. The positions along the midline (X-axis) and the distance between YFP-ParBI foci (Y-axis) were normalized as a fraction of total cell length. Only cells with above-background signal for both fusions and wild-type localization of the YFP-ParBI foci were analyzed. (D) Time-lapse analysis of ΔparAI cells expressing ParAI-CFP and YFP-ParBI showing that ParAI-CFP retracts toward the new pole ahead of segregating YFP-ParBI foci. Bar, 1.0 μm.
we designed software to quantify the distribution of total cellular ParA-CFP fluorescence along the length of the cell (see Materials and Methods). Figure 5C shows this analysis for 314 cells that contained two YFP-ParBI foci. Cells with short distances between YFP-ParBI foci have ParA-CFP spread over a large fraction of the cell (Fig. 5C, panel a). In cells with greater separation between their YFP-ParBI foci, the highest intensities of ParA-CFP fluorescence are found closer to the distant pole (Fig. 5C, panels b,c). When the YFP-ParBI foci are completely segregated to opposite poles, the dramatic redistribution of ParA-CFP fluorescence from the poles to a large region centered at mid-cell is readily apparent (Fig. 5C, panel d).

At all cell sizes, ParA-CFP was present at the old pole where the nonsegregating YFP-ParBI focus resides, suggesting that ParA interacts with ParBI at the old pole throughout the cell cycle. Time-lapse analysis of cells coexpressing YFP-ParBI and ParA-CFP confirm the relationship between segregating YFP-ParBI foci and ParA-CFP (Fig. 5D). Segregating YFP-ParBI foci (Fig. 5D, green foci) appear to be pulled by retracting ParA-CFP (Fig. 5D, red fluorescence) to the new pole. At time 0, the cell in Figure 5D is about to divide. From time 0 to 12 min, ParA-CFP relocates such that it extends from the new pole of each daughter cell to the old pole, where the YFP-ParBI foci have duplicated. Between 12 and 16 min, the YFP-ParBI foci segregate across the cell, and ParA-CFP is positioned at the middle of the cell, where it will be positioned to repeat the cycle. The in vivo dynamics of the ParBI and ParA fluorescent fusions suggest that ParA both pulls the ParBI-bound DNA across the cell, and anchors it at the pole.

**Mutations in the ATP-binding motif of ParA abolish its dynamics**

As the activity of many Walker-type ParA proteins has been shown to require ATP hydrolysis (Leonard et al. 2005), we made two separate substitution mutations, K16E and K16Q, in the predicted ATP-binding pocket of the ParA-CFP fusion protein. In a plasmid ortholog of ParA, these substitutions have been reported to block ATP binding (K16E) and to allow binding but prevent hydrolysis (K16Q), respectively (Fung et al. 2001). As described above, neither construct was capable of complementing the mislocalization of YFP-ParBI foci in ΔparA background (Fig. 2C,D). We examined the subcellular distribution of these constructs to investigate the effects of these mutations on ParA dynamics. In both wild-type and ΔparA backgrounds, ParA-CFP[K16E] showed only homogeneous diffuse fluorescence (Fig. 6A) and had no detectable dynamics in time-lapse experiments (data not shown). The K16Q mutation similarly resulted in diffuse fluorescence and lack of wild-type localization and dynamics, however, it also formed faint foci (Fig. 6A). When YFP-ParBI was coexpressed with ParA-CFP[K16Q], their foci were always colocalized (Fig. 6B). The loss of ParA dynamics with both substitutions suggests that ATP binding and hydrolysis are required for the dynamic localization of ParA. The
colocalization of the K16Q mutant, but not the K16E mutant, with YFP-ParBI foci suggests that the ATP-bound state, but not the unbound state (presumably the case for the K16E substitution), may promote the interaction of ParAI with ParBI.

**Discussion**

Our findings show that ParAI, ParBI, and parSI [ParABS] are components of a mechanism that mediates the polar localization and asymmetric segregation of the origin region of chrI. ParBI interacts with parSI sites near oriCI, apparently forming a complex that localizes and asymmetrically segregates with the origin [Figs. 1B,D,F, 2A]. ParAI is required for the extreme polar localization of the ParBI–parSI complex and its pattern of segregation [Fig. 1C,E,G]. This type 1 ATPase is also required for dramatic cell cycle-dependent separation of parSI from neighboring loci, a phenomenon consistent with active DNA movement [Fig. 3]. Moreover, our observations suggest a functional relationship between segregating ParBI foci and ParAI dynamics, as ParAI-CFP retraction toward the new pole coincided with YFP-ParBI movement. Taking our data together, we propose the following model for ParABI-mediated segregation of the origin region of chrI via a pulling mechanism (Fig. 7): In late predivisional cells containing two fully replicated and segregated chromosomes, the origin of each chromosome is attached to the pole by an interaction between ParAI, anchored to an as-of-yet unknown polar protein/structure, and ParBI, bound to the chromosome at the origin-proximal parSI site [Fig. 7A]. At some point, perhaps related to the assembly of the cell division machinery, ParAI nucleates at the forming septum and polymerizes outward as bands or networks of polymers toward both poles. The next round of DNA replication yields sister copies of the origin region, each containing a ParBI–parSI complex. One complex is captured by the ParAI already present at the old pole, the other ParBI–parSI complex is captured by the ParAI extending from the closing septum that will become the new pole [Fig. 7B]. The completion of cytokinesis produces two daughter cells in which ParBI-bound DNA is pulled across the cell by the retracting ParAI polymers [Fig. 7C,D].

Our findings provide the first in vivo evidence for mitotic-like pulling forces mediating DNA movement in bacteria. Given the localization of ParAI ahead of segregating YFP-ParBI foci, ParAI pulling is strongly suggested by its requirement for both the distortion of YFP-ParBI foci [Fig. 3B] and their separation from nearby regions during segregation [Fig. 3B,D,E]. A pulling mechanism such as this provides an uncomplicated explanation for the asymmetric segregation pattern of the origin region of chrI in *V. cholerae*. While the dynamics of chromosome segregation have been described in only a few bacteria, given the widespread presence of chromosome-encoded Par proteins related to *V. cholerae* ParAI and ParBI in diverse bacteria [Gerdes et al. 2000], it is likely that the Par-directed pulling segregation mechanism described here is conserved in many other systems.

**ParBI localizes with parSI sites near the origin**

The formation of visible foci by fluorescent ParBI fusions suggests that, like other ParB proteins, ParBI forms a large nucleoprotein complex on chrI DNA. In comparison to the distribution of the eight or more SpolII-binding sites over a 800-kb region that spans the origin of the *B. subtilis* chromosome [Lin and Grossman 1998], the three parSI sites on *V. cholerae* chrI are much closer together. These conserved ParB-binding sites, originally identified in the *V. cholerae* genome by Lin and Grossman (1998), were recently shown to be functional for ParABI-mediated plasmid stabilization [Saint-Dic et al. 2006]. The greater number and distribution of parS sites in *B. subtilis* may reflect an adaptation of the Par origin-localizing system to facilitate large-scale compaction and localization of the chromosome during sporulation. As in *V. cholerae*, the four to five parS sites in *C. crescentus* are clustered together within an ∼10-kb region, close to the origin [Mohl et al. 2001]. The similarity of the distributions of the *V. cholerae* and *C. crescentus* parS sites along with the polar localization of their respective origins suggest that the *C. crescentus* and *V. cholerae* chrI par systems likely carry out the same function for origin localization, although they may have other roles that differ.
ParAI does not influence bulk nucleoid segregation

The ParABSI system primarily functions to position and segregate the origin region of chrI. Deletion of parAI did not dramatically disrupt partitioning of chrI to daughter cells, supporting the emerging view that bacterial chromosome segregation is mediated by multiple, likely overlapping or redundant mechanisms (Errington et al. 2005). Similarly, in C. crescentus, it was shown that MreB is required for proper positioning of the origin but not for other regions of the chromosome (Gitai et al. 2005). As C. crescentus origin localization is so similar to that of V. cholerae, it is possible that MreB plays similar roles in both bacteria, either as a separate mechanism or perhaps as a scaffolding for Par-mediated segregation, as proposed by Gitai et al. (2005). It seems likely that bulk chromosome segregation may be directed by conserved processes such as replication, transcription, and condensation; perhaps additional, species-specific, mechanisms might mediate the localization of specific chromosome regions such as the origin and terminus.

The parAI mutant has only a subtle growth defect despite the dramatic mislocalization of origin proximal regions in this background. The function of the extreme polar localization of chromosomal origins in bacteria such as V. cholerae and C. crescentus is unclear. Perhaps there are particular environmental conditions in which there is a strong advantage to having a more elongated and/or organized genome. If the chrI par genes do influence the segregation of origin-distal regions of the chromosome, then redundant mechanisms must be able to compensate in their absence. It will be interesting to examine if mutations in other genes implicated in chromosome segregation, such as mreB and mukB, have synthetic effects with parAI, as has been recently reported for soj in B. subtilis (Lee and Grossman 2006).

It is possible that there is cross-talk between the chrI and chrII par systems; the absence of a significant growth defect in the ΔparAI mutant may be attributable to the activity of ParAI, encoded in the par locus on chrII. While ParAI is phylogenetically grouped with plasmid ParA proteins, both are type II ATPases, and they are 45% similar. Consistent with this idea, the localization and segregation of the ParB-parSI complex in the ΔparAI background resembles the dynamics of oriCHvc. While the mechanism to properly localize the parSI site at the pole is clearly dependent on ParAI, it is possible that ParAI might substitute for ParAI in other important steps during segregation. One interesting possibility is that the ParABI–parSI system may have a function specifically in origin separation, as is the case for Soj in B. subtilis (Lee and Grossman 2006). If so, ParAI might promote the separation of duplicated ParBI–parSI complexes, thus partially suppressing the parAI deletion.

Pulling or pushing DNA

Polymerizing ParA proteins could localize DNA by providing either “pushing” or “pulling” motive force (Møller-Jensen et al. 2002, Barillà et al. 2005). To date, the only well-established mechanism for Par-mediated DNA segregation is the ParM–ParR system of plasmid R1. ParM [a type II ParA ATPase] polymerizes bidirectionally between ParR-bound [ParB-like] plasmids. In this system, the ParM–ParR interaction stabilizes the ParM filament, facilitating its continued polymerization, thereby pushing the plasmid clusters in opposite directions in the cell (Møller-Jensen et al. 2002, 2003, Garner et al. 2004). Recently, another elegant mechanism was proposed for SopA/SopB-mediated segregation of the F plasmid that also invokes a pushing force. In this case, radial asters of SopA filaments pushing in all directions were hypothesized to mediate F segregation (Lim et al. 2005). In both models, the polymerizing ParA-like protein is found between segregating ParB–DNA complexes. Therefore, the region of ParA fluorescence increases with the distance between ParB complexes. This is not the case with the V. cholerae ParAI and ParBI proteins; rather, ParAI is found between the segregating ParBI focus and the new pole; fluorescent ParAI “shrinks” toward the new pole with increased ParBI segregation, consistent with a pulling mechanism.

Several recent observations regarding the biochemistry of ParA proteins and of ParA–ParB interactions suggest how ParAI may pull the ParBI–parSI complex toward the new pole. In vitro, in the presence of ATP, ParA monomers can polymerize and form filamentous structures (Møller-Jensen et al. 2002, 2003, Barillà et al. 2005). The nature of the structure of ParAI in V. cholerae requires future investigation; many of our images raise the possibility that this structure is composed of many ParAI polymers, possibly even a network or lattice of small ParAI polymers. In some plasmid systems, ParA binding to ParB stimulates ParA hydrolysis of ATP, promoting its disassociation from ParB and depolymerization. Assuming that similar processes occur in vivo with the V. cholerae ParAI and ParBI proteins, then the interaction of ParAI filaments with the ParBI–parSI complex would result in their depolymerization, thereby pulling the origin region of chrI toward the new pole via a “Brownian ratchet” mechanism (Raj and Peskin 2006). In this type of mechanism, ParBI, present as part of a large ParBI/parSI complex, would interact with ATP-bound ParAI molecules at the ends of ParAI polymers [within a larger filamentous structure] and in so doing, stimulate their ATPase activity. ATP hydrolysis by the terminal subunit would result in its dissociation as a monomer of ParAI • ADP [i.e., depolymerization]. If the ParBI–parSI complex is only free to diffuse toward the polymer [it would be prevented from diffusing away by simultaneous contact with multiple ParAI polymers], it will come into contact with the new leading edge of the ParAI polymer, and again experience a positive interaction that can be propagated along the DNA, shifting the average position of nearby regions closer to the polymer edge. The observed retraction of ParAI-CFP as YFP-ParBI segregates [Fig. 6] is consistent with the idea that ParBI stimulates depolymerization of ParAI polymers. As ParAI-CFP fluorescence is consistently associated with the nonsegregating YFP-ParBI foci at the old pole, it is
possible that ParAI is undergoing a constant cycle of polymerization and depolymerization to anchor ParBI-parSI at the pole. This type of equilibrium might involve an unknown polar protein that stimulates ParAI polymerization, whereas ParBI-parSI antagonizes it. While the biochemistry of this interaction is still highly speculative, it is consistent with our observations regarding substitutions in ParAI, as a mutation likely to prevent ParAI ATP binding abolished ParAI–ParBI colocalization, whereas a different substitution predicted to allow ATP binding, but not ATP hydrolysis, colocalized with ParBI. Further studies of the biochemistry of these proteins will be important to understand the details of ParAI dynamics and how ParAI–ParBI interactions could generate force for chromosome movement.

Materials and methods

Strains and plasmids

A list of the strains and plasmids used in this work is available in Supplementary Table S1. All V. cholerae strains used in this study were derived from the sequenced clinical isolate N16961 (Heidelberg et al. 2000). The ΔparAI mutation was made by allelic exchange, as described (Fogel and Waldor 2005), using pMF158, a derivative of pCVD442 (Donnenberg and Kaper 1991) containing homology with regions flanking parAI. The deletion was confirmed by PCR. yfp-parBI was constructed by “splicing by overlap extension” PCR (Horton et al. 1989) and then inserted into pBAD33 and pBAD18 (Guzman et al. 1995) to make plasmids pMF302 and pMF341, respectively. parAI-yfp and parAI-cfp fusions were constructed by inserting parAI upstream of the yfp or cfp gene of plasmids p4414 and p4416, containing monomeric versions of yfp and cfp (generously provided by S. Bunnell, Tufts Medical School, Boston, MA). After determining that the sequences of the resulting fusions were correct, they were subcloned into pBAD33, yielding pMF320 and pMF321. ParAI K16E (pMF322) and K16Q (pMF323) substitutions were introduced into these plasmids by PCR mutagenesis using the QuickChange kit protocol (Stratagene) and verified by sequencing. MF310 was constructed by inserting the QuickChange kit protocol (Stratagene) and verified by sequencing. ParAI–parSI interactions could generate force for chromosome movement.

Microscopy

Cells were routinely prepared for microscopy by inoculating fresh single colonies taken from LB plates into M63 minimal media containing 0.1% casamino acids and 0.2% glucose and grown at 37°C to a density of 0.3–0.5 OD600 units. Expression of the fluorescent fusions from pBAD plasmids was induced by addition of 0.08% arabinose for 20–30 min. For experiments with strains containing the tetO scaffold, 80 nM Anhydrotetracycline was added to the culture media at the same time as arabinose, to reduce Tetr-YFP binding as described previously (Lau et al. 2003). Ten microliters to 20 µL of the broth culture was adsorbed onto thin agarose pads on microscope slides and allowed to settle for 3–4 min, then the remaining media was aspirated and a coverslip was placed on top. Slides were sealed with nail polish. Images were acquired with a Zeiss Axioimagine 2 microscope equipped with a 100× α-plan lens, filter sets for YFP and CFP fluorescence, and a cooled CCD Hamamatsu Orca camera. Openlab 3.0 software was used for image acquisition and processing.

Image analysis and measurements

Using MatLab software (MathWorks), we developed automated image analysis programs to facilitate localization of fluorescent foci in large numbers of cells as well as for analysis of other types of fluorescence signals. Briefly, during data collection, sets of phase-contrast and fluorescence images were collected for each field of cells. Segmentation of the phase-contrast images into individual cells was done by binary thresholding followed by region detection and shape filters. For each cell body, morphological shrinking operations were performed on the binary image to create a central skeleton that perfectly represented the individual curvature of the cell. The poles were calculated as the two points on each side of the long axis of the cell that were most distant from the midpoint of the skeleton (similar to an approach for identifying poles in Voilhier et al. 2004). The gap between the poles and the skeleton was filled in by linear interpolation to create a complete curved midline. For each cell body, the corresponding region from the fluorescence image was analyzed and foci were detected by determining points of local maxima in fluorescence intensity. The position of each focus in the cell was determined by finding the closest point on the curved midline and measuring along the curve to each pole. In Figure 5C, the average ParAI-CFP intensity along the curved midline was calculated for each cell. For each pixel along the midline axis, the fluorescence intensity of all of the pixels in the cell lying along the perpendicular line from that point were summed and then averaged. This information is represented as an average intensity along the long axis of the cell and plotted against the position of YFP-ParBI foci in the same cell. Deconvolution of ParAI-CFP fluorescence was performed with Velocity software [Improvision] using a calculated point-spread function on an image-stack of 0.1-µm sections.

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References


A mitotic-like mechanism in Vibrio cholerae


A dynamic, mitotic-like mechanism for bacterial chromosome segregation

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