The chromosome partitioning protein, ParB, is required for cytokinesis in *Caulobacter crescentus*

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Summary

In *Caulobacter crescentus*, the genes encoding the chromosome partitioning proteins, ParA and ParB, are essential. Depletion of ParB resulted in smooth filamentous cells in which DNA replication continued. Immunofluorescence microscopy revealed that the formation of FtsZ rings at the mid-cell, the earliest molecular event in the initiation of bacterial cell division, was blocked in cells lacking ParB. ParB binds sequences near the *C. crescentus* origin of replication. Cell cycle experiments show that the formation of biparally localized ParB foci, and presumably localization of the origin of replication to the cell poles, preceded the formation of FtsZ rings at the mid-cell by 20 min. These results suggest that one major function of ParA and ParB may be to regulate the initiation of cytokinesis in *C. crescentus*.

Introduction

The bacterial cell cycle is a deceptively simple process, which, for most organisms, culminates in cell division when the pre-divisional cell reaches a critical size of twice the unit mass. Therefore, newly divided rod-shaped bacteria, such as *Escherichia coli*, are remarkably similar in size, usually varying no more than 10 per cent in length. As in eukaryotic cells, the bacterial cell cycle has been divided into periods of DNA synthesis, chromosome partitioning or segregation and, finally, cell division. The timing of each of these processes must be highly coordinated to ensure that each daughter cell inherits a full complement of genetic material. However, in contrast to eukaryotic cells, the mechanisms in bacteria that serve to couple DNA replication, partitioning and cell division are largely unknown.

For most bacteria, replication initiation follows the binding of the replication initiator protein, DnaA to the replication origin (reviewed in Kornberg and Baker, 1992). Although the regulation of initiation varies widely in different bacteria, one aspect typically involves a refractory period in which DnaA is prevented from binding to the origin. For example, in *E. coli*, hemi-methylated sequences in the origin promote the binding of SeqA that antagonizes DnaA binding (Lu et al., 1994; Slater et al., 1995). In *Caulobacter crescentus*, the response regulator CtrA fulfils a similar function (Quon et al., 1998). Upon completion of DNA synthesis, the circular daughter chromosomes are separated through the action of topoisomerases and terminus-specific recombinases, and then partitioned toward the poles of the pre-divisional cell (reviewed in Rothfield, 1994; Wake and Errington, 1995). Partitioning has been hypothesized to be a necessary prerequisite for cytokinesis, which, in *E. coli* cells, requires a period of post-replication protein synthesis (Donachie and Begg, 1989). It is not known whether this period of protein synthesis supplies proteins for chromosome partitioning or cell division. The earliest known cytological event in bacterial cytokinesis is the assembly of FtsZ in a mid-cell ring around the circumference of the cell. FtsZ is a bacterial homologue of tubulin and is required for the formation of a constriction at the site of cell division. The assembly of the FtsZ ring results in the recruitment of other cell division proteins, such as FtsA, FtsQ, FtsI and FtsK, to the mid-cell location, a process that eventually culminates in cell division (reviewed in Bramhill, 1997).

How is chromosome partitioning linked to cell division? Until recently, few details of the chromosome-partitioning process were known. Unlike eukaryotic cells, bacteria lack a defined mitotic spindle or other cytoskeletal structures that might be involved in directing the newly replicated chromosomes toward the poles of the cell. It is envisioned that the partitioning apparatus must consist of elements that are analogous to those involved in chromosome segregation in eukaryotic cells: a centromere binding protein, a mechanism to move the chromosomes towards the cell poles and, possibly, a mechanism of condensing the nascent chromosomes. Recent experiments have shown that ParA and ParB, the cellular homologues of...
partitioning proteins found in some phage and unit-copy plasmids, may have a critical role in partitioning bacterial chromosomes (Ireton et al., 1994; Sharpe and Errington, 1996; Glaser et al., 1997; Lin et al., 1997; Mohl and Gober, 1997).

Although the genes encoding these proteins are found in virtually all bacterial genomes sequenced thus far (the notable exception being enteric bacteria and Haemophilus influenzae), most of our information regarding their function comes from elegant work on the E. coli bacteriophage P1, which exists as a circular episome when a prophage, and the F-factor plasmid. Both of these genetic elements exist as single-copy episomes and are inherited by each daughter cell with remarkable efficiency, even in the absence of selection. It has been demonstrated that mutations at three loci, parA, parB and a cis-acting site, parS (sopABC respectively, in F-factor), result in a plasmid-partitioning defect (Austin and Abeles, 1983; Mori et al., 1986). parB has been shown to encode a DNA-binding protein that specifically recognizes the parS sequence that functions as the plasmid equivalent of a centromere (Davis and Austin, 1988; Mori et al., 1989). ParA is an ATPase, whose activity is stimulated by the presence of ParB bound to DNA (Davis and Austin, 1988). These proteins are hypothesized to interact with host factors, resulting in plasmid segregation. This idea is supported by the cytological observation that newly replicated F-factor plasmids move towards the poles of the cell only in the presence of wild-type copies of sopA and sopB (Niki and Hiraga, 1999).

An analogous role has been formulated for the cellular homologues of ParA and ParB. In the sporulating bacterium, Bacillus subtilis, the parB homologue, spo0J, is required for entry into the sporulation pathway. Strains containing mutations in spo0J accumulate anucleate cells (up to 3%) under vegetative growth conditions, indicative of a possible role in chromosome partitioning (Ireton et al., 1994). The block in sporulation in spo0J mutants is attributable to a lack of Spo0A-regulated transcriptional activation (Ireton et al., 1994; Cervin et al., 1998; Quisel et al., 1999). Spo0A is a global regulator of early sporulation genes whose activity is controlled by phosphorylation in response to several internal and external stimuli. Interestingly, mutations in the parA homologue, soj (spo0JA) relieve the sporulation requirement for Spo0J, suggesting that these two proteins function as components of a switch that regulates Spo0A phosphorylation in response to chromosome partitioning (Ireton et al., 1994).

In the dimorphic bacterium, Caulobacter crescentus, parA and parB are located in an operon that maps near the origin of replication (Cori). In contrast to B. subtilis, both parA and parB are essential for viability (Mohl and Gober, 1997). Cell cycle expression experiments have shown that although the transcription of parAB varies during the cell cycle, the cellular levels of each protein remain constant throughout the cell cycle, suggesting that the synthesis of stoichiometric amounts of each protein is critical for function. In support of this idea, overexpression of either parA or parB leads to a significant defect in chromosome partitioning (Mohl and Gober, 1997). Cell cycle immunolocalization experiments have shown that the subcellular distribution of ParB parallels that of chromosome movement; initially, there exists a single focus of ParB, localized to one pole of the cell, and as the cell cycle progresses, another focus is localized to the opposite pole. Presumably, the bipolar localization of ParB reflects binding to the origin of replication region, as fluorescent in situ hybridization (FISH) experiments have shown that the C. crescentus origin region is localized to the poles of the pre-divisional cell (Jensen and Shapiro, 1999). In unsynchronized cultures, ParA was also shown to be bipolarly localized in pre-divisional cells (Mohl and Gober, 1997). Similar results have been obtained for Spo0J localization in B. subtilis (Glaser et al., 1997; Lin et al., 1997). Caulobacter crescentus and B. subtilis ParB homologues have been shown to bind to DNA sequences adjacent to the origin of replication (Mohl and Gober, 1997; Lin and Grossman, 1998). Furthermore, the origin of replication in B. subtilis has been visualized during the cell cycle and shown to segregate rapidly towards the poles of the cell (Webb et al., 1997; 1998). These observations in C. crescentus and B. subtilis, as well as experiments that monitored sporangium-specific gene expression in spo0J mutants (Sharp and Errington, 1996), have led to the hypothesis that ParB (Spo0J), and possibly ParA (Soj), function to position the origin of replication region of the chromosome towards the poles of the pre-divisional cell as the initial event in chromosome partitioning.

In this report, we investigate the molecular nature of the lethality of parB null mutations. We demonstrate that strains that are depleted of ParB undergo a block in cell division with the accumulation of smooth filamentous cells in culture. Furthermore, we demonstrate that this block in cell division is attributable to a defect in FtsZ ring assembly, the earliest known event in bacterial cytokinesis. Cell cycle immunolocalization experiments show that the formation of bipolar foci of ParB precedes FtsZ ring formation. As previous experiments have demonstrated that ParA and ParB are synthesized in stoichiometric amounts during the cell cycle, an increase in the ratio of ParA to ParB may be resulting in a cell division block. We tested whether overexpression of ParA would also result in a cell division defect. Overexpression of ParA led to the production of filamentous that lacked FtsZ rings, however, simultaneous overexpression of both proteins had no such cell division defect.

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Results

Depletion of ParB blocks cell division and FtsZ ring formation

To investigate the lethality of parB null mutations, we constructed a strain in which the only copy of parB was under control of a promoter inducible by xylose (xylAp). This strain (UC9031) was unable to form colonies on complex media lacking xylose, confirming previous results indicating that parB is an essential gene. These cells were grown overnight in media containing 8 mM xylose and then inoculated into fresh medium lacking xylose. Then, we assayed the relative levels of ParA and ParB, as well as monitoring the phenotype, over time, using phase contrast microscopy and immunofluorescent microscopy (Fig. 1). The levels of ParB decreased to an almost undetectable concentration by 8 h following the removal of xylose from the medium, whereas the ParA concentration remained

**Fig. 1.** Depletion of ParB results in a cell division block that produces long smooth filaments. *Caulobacter crescentus* strain UC9031 possesses a frame-shifted copy of *parB* at the par operon locus, and a wild-type copy of the *parB* gene in the xylose-inducible *xylA* operon. To eliminate *parB* expression, log phase cultures were washed and then suspended in PYE medium without xylose. Samples were removed every 2 h for immunoblot (A and B), DAPI staining and FtsZ immunofluorescence (C).

A. Immunoblot showing the relative levels of ParA and ParB under conditions of ParB depletion. The time in hours (hrs) following depletion is indicated. ParA and ParB were detected using specific antiserum.

B. Quantitative representation of the immunoblot data shown in A. ParA levels are represented by circles. ParB levels are represented by squares.

C. DAPI staining (left and right panels) and FtsZ immunofluorescence (right panels) in ParB-depleted cells. All panels are combined phase and fluorescence micrographs. Between 0 and 4 h, most cells exhibit normal morphology and DNA staining. By 6 h, cells are continually elongating but not dividing. By 8 h, depletion of ParB produces long filaments: very few cells possess constrictions associated with division sites and most filaments are smooth. No defect in DNA staining is apparent, suggesting that replication is not adversely effected by ParB depletion or cell filamentation. FtsZ ring formation was visualized with polyclonal anti-FtsZ antibody (right panels) and goat anti-rabbit IgG antibody conjugated to Cy3. FtsZ rings appear as bright orange bands at unpinched division sites, and then as spots at pinched sites. Between 0 and 4 h, ParB-depleted cells demonstrated normal patterns of division and FtsZ ring formation. White arrows identify FtsZ rings near the mid-cell. After 6 h, cells began to divide less frequently but some were still competent for FtsZ ring formation. After 8 h, ParB-depleted cells were elongated and the frequency of FtsZ ring formation is decreasing dramatically. In addition, some polar divisions resulted in FtsZ ring formation near the terminus of the cell. Almost all cells are elongated filaments, and FtsZ rings are absent following 10 h of depletion. See Fig. 2A for a quantitative representation of this experiment.

relatively constant (Fig. 1A and B). Following 4–6 h of ParB depletion, a slight defect in cell growth was readily observed (Figs 1C and 2A). The first notable effect was an aberrant placement of the site of cell division, with the formation of anucleate minicells and larger cells containing DNA (data not shown). Over time, there was an accumulation of long, smooth filamentous cells in culture (Figs 1C and 2A). This suggests that the depletion of ParB from these cells results in a cell division block. Wild-type cells, when shifted from xylose-containing medium to a medium that contained only glucose, showed no such cell division block (data not shown). Interestingly, many of the cell division events that did occur took place at non-mid-cell locations, very often near the pole. The presence of cell division events at non-mid-cell or polar locations has also been observed when C. crescentus cells are depleted of the replication initiation protein, DnaA (Gorbatyuk and Marczynski, 2001). The simplest interpretation of the experiment presented here is that ParB is required for cell division, and thus provides an explanation for the lethality of parB null mutations. This is in stark contrast to mutations that result in partitioning defects in other organisms such as mukB mutations in E. coli (Hiraga et al., 1989) and spo0J mutations in B. subtilis that are not lethal (Ireton et al., 1994).

The accumulation of smooth filaments, when the cells were depleted of ParB, indicated that cell division was blocked at an early stage. As conditional ftsZ mutants in E. coli form smooth filaments, we tested whether there was a defect in FtsZ localization under conditions of ParB depletion. The assembly of a ring of FtsZ, that girdles the circumference of the cell at a mid-cell location, is the earliest known cytological event in the initiation of cell division (reviewed in Bramhill, 1997). When strain UC9031 was grown in media containing xylose, and then shifted to medium without xylose for 2 h, there was a fraction of cells that exhibited a band of FtsZ localization at the mid-cell, as assayed by immunofluorescence microscopy (Fig. 1C). This result is consistent with previous observations showing that FtsZ rings are present in only a fraction of pre-divisional cells, as localization occurs at a specific time in the cell cycle (Quardokus et al., 2001). As ParB was depleted from these cells, there was a gradual accumulation of cells that did not possess FtsZ rings. In those cells that did have rings, they were often located near the poles, an expected finding as some of the cell division events under these conditions produce minicells. The simplest interpretation of this result is that a lack of ParB blocks FtsZ ring formation.

The smooth morphology of ParB-depleted cells suggested that they were blocked at an early stage of cell division. To determine if depletion of ParB affects a step in cell division, before or following formation of FtsZ rings, we monitored the rate of FtsZ ring formation and the rate of cell growth during the course of ParB depletion. If ParB acts at a step in cell division that follows FtsZ ring assembly, the fraction of elongated cells with intact FtsZ rings should remain relatively constant during the initial stages of filamentation; if ParB is necessary for the assembly of new FtsZ rings, then we should observe a

Fig. 2. Depletion of ParB from Caulobacter crescentus blocks assembly of new FtsZ rings and cell division.

A. The frequency of FtsZ ring staining between 0 and 10 h after ParB depletion. Multiple fields of each time-point were counted. At time 0 h, 38% of cells possess FtsZ rings (total of 382 cells counted). After 2 and 4 h, 52% and 43% of cells possessed rings (197 and 246 cells counted respectively). A sharp decline in FtsZ ring formation occurred between 4 and 6 h. The ratios of FtsZ ring staining at 6, 8 and 10 h, were 15% (244 cells counted), 10% (81 cells counted) and 7% (113 cells counted) respectively.

B. Cell perimeter length was estimated from phase micrographs of multiple fields. Images were captured and analysed with NIH IMAGE software. Values are relative units of perimeter length. After ParB depletion for 4–6 h, Caulobacter cells began to elongate. The lack of FtsZ ring formation points towards a block early in the cell division process. Growth rate is not adversely effected by ParB depletion as cell mass rather than cell number continues to double. However, viability decreased to 0.072%, following 10 h of ParB depletion.
decrease in the frequency of FtsZ rings that is concurrent to cell elongation. Our results indicate that the latter model is correct, as FtsZ ring-containing cells decreased as a fraction of the population at the same time-points that filamentation appeared to begin (Fig. 2B). Therefore, ParB is required for either the synthesis of FtsZ or the assembly of FtsZ rings, but not for division at previously existing sites.

**Mechanism of the inhibition of FtsZ ring formation**

What mechanism is operating under these conditions to block FtsZ ring formation? One formal possibility is that ParB depletion activates an inhibitor of cell division. Several inhibitors of FtsZ ring formation have been described in *E. coli*. The most relevant amongst these is the SOS-inducible protein, SulA. The SOS response is characterized by the induction of several genes involved in DNA repair and an inhibition of cell division in response to either DNA damage or an inhibition of DNA replication (reviewed in Walker, 1996). Under these conditions, SulA prevents the polymerization of FtsZ. Although *C. crescentus* undergoes a typical SOS response when *sulA* (reviewed in Walker, 1996). Under these conditions, SulA prevents the polymerization of FtsZ. Although *C. crescentus* undergoes a typical SOS response when *sulA* is depleted of ParB. The identity of the SOS-regulated cell division inhibitor is unknown, we used an indirect assay for an SOS response. We reasoned that the most probable pathway for induction of an SOS response under these conditions would be an inhibition of DNA replication. To test this possibility, we assayed replication by measuring the incorporation of [α-32P]-dGTP into DNA (Marczynski et al., 1990) during the course of ParB depletion. We found that the rate of DNA replication in ParB-depleted cells, even after 10 h of depletion, was not significantly less than control cells in which ParB was not depleted (Table 1). Furthermore, flow cytometry experiments demonstrated that ParB-depleted cells contained a range of six to 14 chromosomes (data not shown). Although this is a indirect test, we conclude from this experiment that the block in FtsZ ring formation in ParB-depleted cells is probably not attributable to an SOS response. To provide an additional test for SOS induction, we assayed the rate of transcription of the *C. crescentus* *uvrA* promoter that is induced by an SOS response (J. C. Draper and J. W. Gober, unpublished observation). Depletion of ParB resulted in less than a twofold increase in *uvrA* transcription, a result consistent with a lack of SOS induction (data not shown).

In *E. coli*, both FtsZ and FtsA are required for the formation of a stable ring structure. One possibility is that the synthesis of these two components is inhibited in ParB-depleted *Caulobacter* cells. To test this possibility, we measured the expression of *lacZ* from both the *ftsZ* and *ftsQ* promoters in normal and ParB-depleted cultures (Fig. 3). Interestingly, depletion did not block transcription from either reporter. Instead, we observed a small increase in expression from both the *ftsZ* and *ftsQ* promoter constructs. To ensure that the transcription of the *ftsZ* promoter resulted in the synthesis of FtsZ, we used polyclonal antibodies directed towards FtsZ to investigate the relative levels of protein in wild-type and ParB-depleted cells. We detected an approximate 50% decrease in FtsZ protein levels by Western blot analysis of ParB-depleted UC9031 grown in medium without xylose. In light of the

### Table 1. The effect of ParB depletion on the rate of DNA replication

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<th>Time (h)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
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<td>cpm/OD</td>
<td>83 147</td>
<td>59 058</td>
<td>97 645</td>
<td>78 524</td>
<td>89 088</td>
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* Key: a. The rate of DNA replication was determined by pulse labelling cells with [α-32P]-dGTP (Marczynski et al., 1990) and is expressed as scintillation counts per minute [cpm/OD (A660nm)].

![Fig. 3.](image)
increased transcription from the *ftsZ* and *ftsQA* promoters, the observed decrease in protein is possibly because of the instability of unpolymerized FtsZ. In *Caulobacter*, FtsZ protein also undergoes cell cycle proteolytic degradation (Kelly et al., 1998). Overall, these results suggest that the expression of *ftsZ* and *ftsQA* is not significantly affected by ParB depletion. These experiments do not rule out the possibility that either ParA or ParB regulate other, as yet unidentified, cell division promoters, although it may be that they interact directly with the division apparatus.

We have shown previously that a change in the relative levels of ParA or ParB results in a cell division defect. In the depletion experiments presented here, the ratio of ParA to ParB would also be greatly increased. Therefore, we tested whether overexpression of ParA would result in the same defect in FtsZ ring formation and growth as cells that were depleted of ParB (Fig. 4). In this experiment, an extra copy of either *parA*, or both *parA* and *parB*, was placed under control of the *xylA* promoter in wild-type cells. The phenotype of the cells was monitored microscopically over time. Overexpression of *parA* resulted in a phenotype that was virtually indistinguishable from ParB-depleted cells (Fig. 4, A–F). There was a relatively rapid inhibition of normal cell division and FtsZ ring formation, with filamentous cells and some minicells appearing in culture as little as 6 h after the addition of xylose. These results indicate that overexpression of *parA* inhibits FtsZ ring formation and division. To test whether raising ParB levels can suppress the effect of ParA overexpression, we placed both *parA* and *parB* under control of the *xylA* promoter. In this case, cell division was restored, but the majority of cells in culture possessed abnormal morphology. Elongated and shortened cells may result from aberrantly placed division events. This could explain our previous observation that co-overexpression results in anucleate cells. Altogether, these results suggest that ParA may inhibit FtsZ ring formation, and ParB might serve to regulate ParA activity, such that partitioning and cell division are coupled.

The formation of a partitioning complex precedes FtsZ ring formation

The experiments presented above show that ParB is required for recruitment of FtsZ to the site of cell division. Furthermore, altering the ratio of ParA:ParB also results in a cell division defect. In previous experiments, we have demonstrated that overexpression of ParA results in a loss of ParB foci and that overexpression of ParB causes the formation of mislocalized ParB foci, often at non-polar locations (Mohl and Gober, 1997). These results may indicate that the mislocalization of either ParA or ParB is responsible for the cell division defect. For wild-type cells, we propose that the cell cycle localization of ParB and ParA and, by implication, the origin of replication region to the poles of the cell, is a required event for FtsZ ring formation and cell division. To test this idea, we assayed the cell cycle-dependent localization of ParB and FtsZ in synchronized populations of cells. Swarmer cells were isolated (>95% pure) by density centrifugation, suspended in fresh medium and allowed to progress through the cell cycle. At various time-points, a portion of the culture was removed, the cells were fixed and processed for immunofluorescent microscopy using antibodies directed against ParB and FtsZ. These experiments indicated that arrival of ParB foci, to both poles of the cell, occurs at about 60 min into the cell cycle, shortly after the swarmer to stalked cell transition, and concurrent with chromosome replication (Fig. 5). Notably, the arrival of ParB foci at poles preceded FtsZ ring formation. To demonstrate better the close relationship between ParB localization and cell division, we graphed the fraction of cells with bipolar ParB foci and mid-cell FtsZ ring structures over time (Fig. 6). The sigmoidal nature of both curves is indicative of a sharp change in the pattern of localization during the cell cycle. The data shows that bipolar ParB localization occurred approximately 20 min before the formation of FtsZ rings.

ParB stimulates the initiation of FtsZ ring formation and cell division

Next, we tested whether reintroduction of ParB should stimulate FtsZ ring formation in ParB-depleted cultures. To accomplish this, we grew strain UC9031 in medium without xylose for 6 h, and then added xylose, inducing expression of *parB* from the *xylA* promoter. Cells were fixed and FtsZ rings were stained by immunofluorescence.
Between 100 and 500 cells were counted at each time-point. A quantitative representation of this experiment is depicted as Fig. 7.

ParB was localized to both poles, and in 63% of late stalked cells FtsZ rings have formed. After 100 min, greater than 94% of cells possessed bipolar ParB and approximately 24% of cells had FtsZ rings. Arrows indicate ParB staining and FtsZ ring formation. In 88% of late stalked cells (80 min), division cycle cue.

Division cycle timing of polar ParB localization and FtsZ ring formation suggests that newly replicated origins are partitioned before FtsZ rings assemble at the mid-cell. In swarmer and early stalked cells (0–40 min), ParB is localized to only one pole (89% at 0 min; 73% at 20 min; 70% at 40 min), and FtsZ rings have not yet formed (98.5% to 97.2% without rings at these time-points). White arrows indicate staining at one pole. After 60 min, 61% of stalked cells demonstrated bipolar localization of ParB and approximately 24% of cells had FtsZ rings. Arrows indicate ParB staining and FtsZ ring formation. In 88% of late stalked cells (80 min), ParB was localized to both poles, and in 63% of late stalked cells FtsZ rings have formed. After 100 min, greater than 94% of cells possessed bipolar localization of ParB and 86% had FtsZ rings. The pre-divisional cells began to divide and new ParB foci appeared between 120 and 140 min. In these cells, two foci of ParB sometimes appeared in the stalked cell compartment of the pre-divisional cell (shown by arrows; ParB column, 140 min). Between 100 and 500 cells were counted at each time-point. A quantitative representation of this experiment is depicted as Fig. 7.

Discussion

A long-standing problem in prokaryotic biology has been understanding how bacterial cells regulate progression through the cell cycle. Newly replicated chromosomes must be partitioned to opposite poles of the pre-divisional cell before the completion of division, implying that these two processes are temporally coupled. Here we present evidence that ParA and ParB, the cellular homologues of plasmid-partitioning proteins, are required for a specific step in the cell division cycle: the onset of cytokinesis. In previous work, it has been demonstrated that both parA and parB are required for viability in Caulobacter (Mohl and Gober, 1997). To investigate the nature of the lethality of parB mutations, we constructed a Caulobacter mutant in which the sole copy of parB was placed under the control of a xylose-inducible promoter. When cultures were removed from xylose and depleted of ParB, a marked cell division phenotype appeared. After 4–6h, the cells elongated, forming long, smooth filaments. The lack of constrictions or invaginations of the cell wall suggested that ParB depletion inhibited an early step in the division process. Formation of the FtsZ ring, which provides the scaffolding required for septal constriction at the division site, is the earliest known molecular event in the initiation of cell division (reviewed in Bramhill, 1997). To test whether the cell division defect observed in ParB depletion mutants was attributable to a block in the initiation of division, we assayed the formation of FtsZ rings in wild-type and ParB-depleted cultures. Using immunofluorescence microscopy, we found that ParB is essential for the formation of new FtsZ rings but not necessarily required during depletion and then after ParB induction (Fig. 7). As expected, depletion of ParB resulted in a dramatic decrease in FtsZ ring formation and cell division. After 6h, cells became long filaments with fewer than 10% exhibiting FtsZ rings. The induction of ParB expression caused a dramatic increase in the number of cells with FtsZ rings, and subsequently the frequency of division. One hour after inducing ParB expression, over 30% of cells possessed FtsZ rings. After 2h, more than 70% of the culture possessed FtsZ rings. The relatively rapid recruitment of the cell division apparatus supports the idea that ParB is acting as a positive regulator of division. Interestingly, the placement of rings in some filaments did not follow a pattern that would probably result in cells with normal length (Fig. 7D). In addition, a number of cells (10%) contained more than one FtsZ ring. As these cells possess multiple chromosomes, this suggests that the number of chromosomes in the cell, and, by implication the number of ParB foci, may influence the frequency of cell division events in Caulobacter.

Fig. 6. Bipolar localization of ParB foci precedes FtsZ ring formation. Time course images of ParB localization and FtsZ ring formation in wild-type Caulobacter cells. The time, in minutes, is indicated to the left of the micrographs. In the left hand column, orange foci of Cy3 fluorescence are localized ParB. In the right hand column, FtsZ immunofluorescence and phase images were merged. Orange bars across the middle of the cell are Cy3-stained FtsZ rings. These experiments demonstrate that ParB and, therefore, the two newly replicated origins are localized to both poles of stalked cells before FtsZ rings assemble at the mid-cell. In swarmer and early stalked cells (0–40 min), ParB is localized to only one pole (89% at 0 min; 73% at 20 min; 70% at 40 min), and FtsZ rings have not yet formed (98.5% to 97.2% without rings at these time-points). White arrows indicate staining at one pole. After 60 min, 61% of stalked cells demonstrated bipolar localization of ParB and approximately 24% of cells had FtsZ rings. Arrows indicate ParB staining and FtsZ ring formation. In 88% of late stalked cells (80 min), ParB was localized to both poles, and in 63% of late stalked cells FtsZ rings have formed. After 100 min, greater than 94% of cells possessed bipolar localization of ParB and 86% had FtsZ rings. The pre-divisional cells began to divide and new ParB foci appeared between 120 and 140 min. In these cells, two foci of ParB sometimes appeared in the stalked cell compartment of the pre-divisional cell (shown by arrows; ParB column, 140 min). Between 100 and 500 cells were counted at each time-point. A quantitative representation of this experiment is depicted as Fig. 7.
Fig. 7. Induction of parB in depleted cells stimulates FtsZ ring formation and cell division. Depletion of ParB from an otherwise wild-type culture resulted in the production of long filaments that lack FtsZ rings. To demonstrate the reversible nature of the ParB division block, parB expression was induced using the xylA promoter in cells after 6 h of depletion. Immunofluorescence was performed on fixed cultures after either depletion or reintroduction of ParB to assay the reappearance of FtsZ rings.

A. and B. 0 (A) and 2 h (B) after removing strain UC9031 from the presence of xylose, cells still divided normally as ParB levels have not dropped below a critical threshold concentration. White arrows indicate FtsZ rings at the mid-cell.

C. After 4 h, the cells began to elongate and fewer FtsZ rings were present.

D. After 6 h of depletion, fewer than 10% of cells had FtsZ rings and greater than 90% had become elongated filaments.

E. One hour after inducing ParB expression in the previously depleted culture, approximately 30% of cells possessed one or more FtsZ rings. White arrows indicate Z rings. Newly formed rings appeared to be randomly distributed along the length of the cell filament.

F. Two hours after induction of ParB, greater than 70% of the culture had formed FtsZ rings. White arrows indicate rings that have formed beside one another.

Fig. 8. ParA and ParB activities are required for cytokinesis. Light grey spheres represent ParA, dark grey spheres are ParB and black line drawings represent the chromosomes. We hypothesize that ParA functions as a cell division inhibitor and ParB regulates its activity with respect to the completion of DNA partitioning. ParB bound to the chromosome, is initially localized to one pole of the swarmer and early stalk cells. Replication of the origin region results in the duplication of ParB foci. One ParB/origin DNA complex remains at the stalked pole whereas the other is partitioned to the new swarmer pole. Before replication, ParA is capable of inhibiting FtsZ ring formation and is distributed throughout the cell. Localization of ParB to both poles may sequester ParA activity away from the mid-cell, resulting in FtsZ ring assembly and then division.

for the completion of division from previously existing structures. In addition, induction of ParB synthesis in depleted cells resulted in the reappearance of FtsZ rings. We hypothesize that the inhibition of cell division, observed in these cells, is responsible for the lethality of null mutations in parB. These results are consistent with the view that ParB is required for the initiation of cell division.

Fluorescence microscopy experiments have revealed a simple cycle in which the origin and terminus regions of bacterial chromosomes become localized to the pole and mid-cell in a cell cycle-dependent fashion. For example, in *B. subtilis* the origins of newly replicated chromosomes are abruptly moved towards opposite poles during the cell cycle (Webb et al., 1997; 1998). The observed discontinuous and rapid rate of movement supports so-called ‘active’ models of partitioning. A similar distribution of origin and terminus DNA in synchronized populations of *C. crescentus* has also been observed (Jensen and Shapiro, 1999). Our hypothesis is that the initiation of cell division is not only dependent on the synthesis but also upon the polar localization of ParB bound to parS sequences near to the origin of replication. Approximately six ParB DNA-binding sites, almost identical in sequence to those found in *B. subtilis* (Lin and Grossman, 1998), are distributed adjacent to the par operon and the origin of replication in *Caulobacter* (J. Easter, D. A. Mohl and J. W. Gober, unpublished). Arrival of ParB, bound to these DNA sites, probably signals the duplication and successful partitioning of the origin of replication, and, thereby, releases the cell division block. Therefore, ParB recognizing its DNA binding sites could be part of a chromosome-counting mechanism, using the two poles as a means to distinguish between identical DNA molecules. If polar localization of ParB is required for division, then arrival of ParB foci to the poles of the cell should precede the formation of FtsZ rings. In support of this, we have shown that the arrival of ParB at the cell poles precedes FtsZ ring formation by approximately 20 min (Fig. 6). In previous work, we have determined that ParA also is localized to the poles of the cell at the pre-divisional stage (Mohl and Gober, 1997). We envisage that cell division initiates when ParA interacts with the ParB–DNA complex at the poles of the cell (Fig. 8). ParA may also be inhibiting cell division in ParB-depleted cells of *C. crescentus*. In this regard, overexpression of ParA results in a cell division phenotype that is identical to ParB-depleted cells, whereas overexpression of both proteins results in a partitioning defect, but no evident cell division defect. Therefore, these proteins must be expressed in stoichiometric amounts for normal cell division to occur. In this case, overexpression of ParA, and depletion of ParB, both would lead to an increase in the ratio of ParA to ParB in the cell. We have determined that depletion of ParB results in an increase in ParA levels (D. A. Mohl and J. W. Gober, not published). This is a consequence of increased expression of the par operon that ParB autoregulates. Therefore, it is possible that under conditions of ParB depletion, ParA may be inhibiting cell division (Fig. 8).

The deduced amino acid sequence of parA shows that it is homologous to a large distinct family of ATPases (Motellebi-Veshareh et al., 1990; Koonin, 1993). Members in this family have been shown, in many cases, to interact with a protein that modulates ATPase activity, such as ParB. We propose that the modulation of ParA ATPase activity is a molecular switch that responds to spatial events in the bacterial cell cycle. The plasmid and phage-encoded parA and parB gene products have been the most intensely studied in this regard. The parA gene products of plasmid and phage have been shown to bind to DNA sequences within their own promoter and function to regulate expression of the parAB operon (Friedman and Austin, 1988; Hayes et al., 1994; Hirano et al., 1998). This autoregulation is critical for proper partitioning, as overexpression of parA or parB leads to partitioning defects (Abeles et al., 1985; Funnell, 1988). Purified ParA possesses a weak ATPase activity *in vitro* (Davis et al., 1992; Watanabe et al., 1992; Davey and Funnell, 1994). Experiments have shown that both ATP and ADP can be bound to ParA (Davey and Funnell, 1997). The ATP-bound form interacts with the partitioning complex (i.e. ParB bound to parS), whereas the ADP-bound form preferentially stimulates the binding of ParA to its operator sequences (Bouet and Funnell, 1999). It has been proposed that ATP and ADP function to switch the activities of ParA within the cell. ParB regulates the switch in ParA activity as it can stimulate ATP hydrolysis when bound to parS.

An analogous switch in ParA activities is probably a critical aspect in regulating cellular processes in bacteria. For example, partitioning of the origin-proximal third of the chromosome must occur before placement of the polar septum to ensure segregation of DNA into the forespore during sporulation in *B. subtilis*. Soj (ParA) and SpoOJ (ParB) are thought to operate this developmental checkpoint by directly regulating the transcription of key sporulation genes. Soj has been shown to oscillate from pole to pole, with this dynamic localization being dependent on SpoOJ (Marston and Errington, 1999; Quisel et al., 1999) in the absence of SpoOJ, Soj moves from the poles and associates, primarily, with the nucleoid in which it inhibits the transcription of early sporulation promoters. This switch in activities is a consequence of interaction with SpoOJ at the pole and is probably attributable to a change in the nucleotide-bound form of Soj (Quisel et al., 1999).

Evidence presented here suggests that the interaction of ParA and ParB in *Caulobacter* modulates their influence
on the initiation of cell division. We do not know the molecular nature of the inhibition of cell division in Caulobacter, however, there exist two equally plausible possibilities. To determine whether Caulobacter ParA and ParB may be acting at the level of transcription, we assayed the expression of ftsZ and ftsQA promoters in the ParB depletion strain. Both appeared to be transcribed at slightly higher levels when ParB was depleted. Despite the increase in ftsZ and ftsQA promoter activity, FtsZ protein levels appear to decrease somewhat (Fig. 4). Given that the initiation of division and selection of the division site is not yet fully understood, it is difficult to rule out transcriptional repression as a mode of ParA inhibition. Alternatively, ParA may directly inhibit the assembly of the cell division machinery.

The data presented here raise the question of whether ParA and ParB function both as partitioning proteins as well as regulators of cell division. In B. subtilis, direct experiments that visualized the subcellular localization of the replication origin indicated no evidence of mislocalization in spoQJ mutants (Webb et al., 1997b). This result suggests that other proteins are involved in orienting the chromosome. In E. coli, which does not possess Par protein homologues, the origin of replication is also oriented towards the cell poles (Gordon et al., 1997; Niki and Hiraga, 1998). The uniform distribution of DNA in ParB-depleted filamentous cells indicates that partitioning is uninterrupted in the absence of ParB in Caulobacter, a finding that is in agreement with origin-labelling experiments in B. subtilis (Webb et al., 1997). Based on these observations, we hypothesize that ParA and ParB in Caulobacter probably function as regulators of cell division and are not directly involved in the mechanics of partitioning. The partitioning defects observed when these proteins are overexpressed (Mohl and Gober, 1997) are probably attributable to perturbations in coordinating cell cycle events.

**Experimental procedures**

**Strains and plasmids**

All strains were derived from Caulobacter crescentus LS107 that is an ampicillin-sensitive, synchronizable strain (Stephens et al., 1997). Cultures were grown in 0.2% peptone and 0.1% yeast extract (PYE) (Poindexter, 1964) and supplemented with antibiotics as indicated. ParB depletion strain UC9031 was derived from LS107. To create a xylose-inducible parB expression strain, we cloned parB into plasmid pSNX228–1 (M. R. K. Alley, unpublished) and integrated a copy into the chromosome at the xyA locus. Using a two-step gene replacement strategy, we swapped the wild-type parB gene at the par locus for a frame-shifted mutant. To create a frame-shift in parB, we used site-directed mutagenesis (Kunkel and Roberts, 1987) to insert two basepairs (bp) after the seventh codon, resulting in a translation stop at codon 131. Then, this was subcloned into plasmid pDELI3 using endogenous HindIII and EcoRI sites, upstream of parA and downstream of parB, to create pPB91D1, integrated into LS107 through homologous recombination, and then transduced into the xyAparB expression strain using bacteriophage λCR30. We plated the recipient Caulobacter cells onto PYE medium containing 2% sucrose and 8 mM xylose to select for cells that lost the pDELI3-encoded sacB. Sucrose-insensitive mutants were screened for growth on media lacking xylose. Those colonies which required xylose for growth proved to have a frame-shifted copy of parB at the par locus, resulting in a strain in which the only copy of parB was under control of the inducible xyA promoter (UC9031). ParA and ParA/ParB overexpressing strains were described in an earlier report (Mohl and Gober, 1997). Reporter strains were created by mating pMSP8LC (ftsQA-lacZ) (Sackett et al., 1998) and plac290HB2.0BP (ftsZ-lacZ) (Kelly et al., 1998) into UC9031, using E. coli S17-1 (Simon et al., 1983).

**Immunofluorescence microscopy**

The preparation of anti-ParB antibodies was described previously (Mohl and Gober, 1997). To prepare anti-FtsZ antibodies, the Caulobacter ftsZ gene was amplified by polymerase chain reaction (PCR) using a primer that introduced a BamH1 restriction site, just before the start codon. This was subcloned into the overexpression vector, pET15b, and histidine-tagged-FtsZ protein was purified using a nickel-sepharose column under denaturing conditions according to the manufacturer (Novagen). Antibodies were prepared by a commercial source (Cocalico). For immunofluorescence microscopy, Caulobacter cultures were grown to mid-log phase and then fixed with formaldehyde and glutaraldehyde at final concentrations of 2.5% formaldehyde, 30 mM NaPO4, pH 7.5 and between 0 and 0.03% glutaraldehyde. Cultures were fixed for 15 min at 30°C in a rotary shaker and for an additional 45–60 min on ice. Cells were then washed three times with phosphate-buffered saline (PBS) containing 140 mM NaCl, 3 mM KCl, 8 mM Na2HPO4 and 1.5 mM KH2PO4 and resuspended in 50 mM glucose, 10 mM EDTA and 20 mM Tris-HCl at pH 7.5. To the fixed cells, lysozyme was added to a final concentration of 300 μg ml⁻¹; 20 μl of cells was placed onto poly-L-lysine-treated slides for 30 s, aspirated until dry, and then rinsed with approximately 20 ml of distilled water. Slides were then blocked with PBS containing 0.05% TritonX-100 (PBST) with 2% bovine serum albumin (BSA) for 15 min and incubated with either polyclonal rabbit anti-ParB, anti-ParA or anti-FtsZ antibody. Slides were washed for 20 min in PBST, then blocked with PBST + 2% BSA, incubated for 1 h with Cy3 conjugated mouse anti-rabbit IgG antibody and diluted 1:1000 in PBST + 2% BSA (Jackson Biological). Slides were washed for 20 min in PBST, rinsed with distilled water, air-dried and then covered with 50% glycerol containing 0.1 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) and a coverslip. Fluorescent images were captured with an Optronics cooled CCD camera attached to a Zeiss Axioplan microscope at 1000× magnification. All images were captured with a PHOTOSHOP AV capture plug in on a Macintosh Power PC AV computer.
Microscopy and cell measurements

Cells stained with DAPI were isolated from log-phase cultures grown in either PYE or M2 minimal media (MM) (Johnson and Ely, 1977). Before staining, cells were fixed with 2.5% formaldehyde, 30 mM NaPO₄, pH 7.5 and 0.03% glutaraldehyde for 15 min at 30°C and then 1 h on ice. For DAPI fluorescence, cells were placed onto poly-L-lysine-treated slides aspirated dry and rinsed with distilled water. Slides were then incubated in ice cold methanol for 4 min and ice cold acetone for 30 s. Slides were allowed to air dry and were then covered with 50% glycerol containing 0.1 μg ml⁻¹ DAPI (Sigma). All DAPI and phase images were captured by an Optronics cooled CCD camera attached to a Zeiss Axioplan microscope at 1000x magnification using the PHOTOSHOP AV capture plug in and a Macintosh Power PC. ADOBE PHOTOSHOP 4.0 was used to overlay fluorescence and phase images. Cell size measurements were based on relative pixel units calculated by NIH IMAGE 1.62b software.

Cell synchronization and depletion experiments

Caulobacter crescentus cells were synchronized essentially as described previously (Evinger and Agabian, 1977). A 500 ml culture was grown to an OD₆₀₀nm = 0.8–1.0 in M2 minimal media. Cells were isolated and resuspended in cold 50% Percoll (Sigma) in M2 salts. Pure swarmer cells were isolated from a high-mobility band in the Percoll gradient and then washed three times with cold M2 salts. Pellets were suspended into pre-warmed M2 media to an OD₆₀₀nm = 0.5. Time-points were removed every 20 min, starting at time 0 min. Portions of the cultures were fixed immediately for FtsZ and ParB immunofluorescence.

Caulobacter strain UC9031 was depleted of ParB by washing a late-log phase culture grown in PYE containing 0.0625% xylose. Cells were washed three times with PYE, and then suspended in fresh PYE. Cultures were allowed to grow for up to 10 h. Cultures were diluted at the start of depletion such that the OD₆₀₀nm at each time-point would be between 0.5 and 1.0.

The rate of DNA replication was assayed by pulse-labelling cultures with [α-³²P]-dGTP as described previously (Marczynski et al., 1990). Flow cytometry was performed at the Stanford University FACS facility as described in Quon et al. (1998).

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ParB is required for cell division in Caulobacter 753


