Novel Divisome-Associated Protein Spatially Coupling the Z-Ring with the Chromosomal Replication Terminus in Caulobacter crescentus

Shogo Ozaki,a Urs Jenal,b Tsutomu Katayamaa

aDepartment of Molecular Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
bBiozentrum, University of Basel, Basel, Switzerland

ABSTRACT Cell division requires proper spatial coordination with the chromosome, which undergoes dynamic changes during chromosome replication and segregation. FtsZ is a bacterial cytoskeletal protein that assembles into the Z-ring, providing a platform to build the cell division apparatus. In the model bacterium Caulobacter crescentus, the cellular localization of the Z-ring is controlled during the cell cycle in a chromosome replication-coupled manner. Although dynamic localization of the Z-ring at midcell is driven primarily by the replication origin-associated FtsZ inhibitor MipZ, the mechanism ensuring accurate positioning of the Z-ring remains unclear. In this study, we showed that the Z-ring colocalizes with the replication terminus region, located opposite the origin, throughout most of the C. crescentus cell cycle. Spatial organization of the two is mediated by ZapT, a previously uncharacterized protein that interacts with the terminus region and associates with ZapA and ZauP, both of which are part of the incipient division apparatus. While the Z-ring and the terminus region coincided with the presence of ZapT, colocalization of the two was perturbed in cells lacking zapT, which is accompanied by delayed midcellular positioning of the Z-ring. Moreover, cells overexpressing ZapT showed compromised positioning of the Z-ring and MipZ. These findings underscore the important role of ZapT in controlling cell division processes. We propose that ZapT acts as a molecular bridge that physically links the terminus region to the Z-ring, thereby ensuring accurate site selection for the Z-ring. Because ZapT is conserved in proteobacteria, these findings may define a general mechanism coordinating cell division with chromosome organization.

IMPORTANCE Growing bacteria require careful tuning of cell division processes with dynamic organization of replicating chromosomes. In enteric bacteria, ZapA associates with the cytoskeletal Z-ring and establishes a physical linkage to the chromosomal replication terminus through its interaction with ZapB-MatP-DNA complexes. However, because ZapB and MatP are found only in enteric bacteria, it remains unclear how the Z-ring and the terminus are coordinated in the vast majority of bacteria. Here, we provide evidence that a novel conserved protein, termed ZapT, mediates colocalization of the Z-ring with the terminus in Caulobacter crescentus, a model organism that is phylogenetically distant from enteric bacteria. Given that ZapT facilitates cell division processes in C. crescentus, this study highlights the universal importance of the physical linkage between the Z-ring and the terminus in maintaining cell integrity.

KEYWORDS Caulobacter crescentus, cell division, chromosome organization, chromosome segregation, subcellular localization
distinct cell cycle phases to ensure proper execution and completion of the events in time and space. In contrast, bacteria have the capacity to execute chromosome replication and segregation alongside cell division, thereby compressing cell cycle processes to achieve rapid growth (1). Therefore, to successfully transmit the chromosome to daughter cells over generations, bacteria require regulatory tuning of cell division processes with the chromosomal architecture, which undergoes dynamic changes during chromosome replication and segregation (2–5). However, chromosomal organization characteristics differ among various bacteria and it remains unclear how the system that coordinates cell division with dynamic chromosome architecture is conserved in the bacterial kingdom.

Bacterial cell division is initiated by the ubiquitous GTPase FtsZ (6–10), a tubulin homolog that polymerizes into bundled filaments termed Z-rings (11–13). The formation of the Z-ring at incipient division sites provides a scaffold for assembly of the divisome, a dynamic protein complex responsible for cell division (6, 14). Moreover, Z-rings can act as treadmilling platforms for the peptidoglycan synthesis machinery to stimulate septum formation (15, 16). To prevent the production of abnormal cells with no chromosomes or guillotined chromosomes, the position and timing of Z-ring formation must be tightly regulated in concert with the dynamic chromosomal architecture throughout the cell cycle. In the gammaproteobacterium Escherichia coli, at least two inhibitory systems operate to ensure Z-ring formation at the midcell region: Min and nucleoid occlusion. In the Min system, the FtsZ antagonist MinC shuttles dynamically between the two cell poles with the aid of MinD and MinE, thereby inhibiting Z-ring formation at the poles (17–21). In contrast, the nucleoid occlusion system relies on the nucleoid-associated FtsZ inhibitor SlmA to prevent Z-ring formation over the chromosomes (22–24). While neither minCDE nor slmA is essential for growth, inactivation of both leads to a synthetic lethal phenotype (25). Thus, the Min and nucleoid occlusion systems complementarily regulate Z-ring formation.

FtsZ-associated protein Z-ring-associated protein A (ZapA) is also implicated in spatial coordination of the Z-ring in E. coli (26). ZapA binds directly to FtsZ to stabilize the Z-ring at midcell (27). ZapA homologs are found in most bacterial species, suggesting that it plays an evolutionarily conserved role in Z-ring formation (14, 28, 29). In addition, ZapA forms a complex with the coiled-coil protein ZapB, which has a unique affinity for the nucleoid-associated MatP protein (27, 30, 31). MatP, a prerequisite for compaction of the replication terminus of the E. coli chromosome, binds to the 13-mer matS sites that are distributed within an 800-kb stretch around the replication terminus, a region opposite the origin of chromosome replication (32–34). Because the MatP-matS complexes can interact, bridging DNA between distal matS sites, MatP-mediated DNA clustering helps organize the replication terminus region into a compacted DNA called the Ter macrodomain (33, 35, 36). Thus, the interaction network comprising matS, MatP, ZapB, ZapA, and FtsZ, termed the Ter linkage, is proposed to connect the chromosome replication terminus with the Z-ring for regulatory tuning of Z-ring formation in time and space (9). Mutants lacking the zapB or matP gene are moderately compromised for chromosome segregation and cell division, highlighting the role of the Ter linkage in these cell cycle processes (30). Intriguingly, ZapB and MatP homologs are found only in enteric bacteria. Thus, the general relevance of the physical linkage between the chromosomal replication termini and the Z-ring outside the enteric bacterial species remains unknown.

In this report, we provide evidence that the aquatic alphaproteobacterium Caulobacter crescentus, a model organism that is phylogenetically distant from enteric bacteria, physically links the Z-ring to the replication terminus region in vivo. This bacterium grows by asymmetric binary fission, producing two genetically identical but physiologically distinct progeny cells (37–41) (Fig. 1A). Whereas the stalked progeny is sessile and able to initiate chromosome replication and cell division (entry into the S phase), the motile swarmer progeny blocks replication initiation, thereby experiencing an extended nonproliferating period termed the G1 phase. Localization of C. crescentus FtsZ is subjected to cell cycle regulation primarily by the ParA-type ATPase MipZ, which
directly binds FtsZ to destabilize its polymerization (42, 43). In the G1 phase, MipZ is sequestered to the old cell pole, located opposite the new cell pole that inherits FtsZ from the previous cell division. As the cell enters the S phase, MipZ adopts a bipolar localization, thereby forcing FtsZ to relocate to the midcell region, where the MipZ

![Diagram](image_url)

**FIG 1** The replication terminus region of the *C. crescentus* chromosome colocalizes with the divisome in a ZapT-dependent manner. (A) The cell cycle of *C. crescentus*. Localizations of the origin of replication (Cori, orange), the terminus (ter, cyan), FtsZ (yellow), and MipZ (green) at distinct cell cycle stages (G1, S, G2/M) are shown schematically. (B) Schematic representation of FROS-ter, which consisted of three modules: LacI-CFP expressed from the xylose-dependent promoter (Pxyl), \((lacO)\)n repeats integrated near the terminus, and a zapA-mCherry fusion replacing the native zapA gene. The relative positions of the individual modules are shown schematically on the circular *C. crescentus* genome. (C to I) Analyses of FROS-ter in wild-type and ΔzapT mutant strains. SHQ63 (wild-type [WT]) cells (C to E) and SHQ75 (ΔzapT) cells (G to I) grown in M2G medium were analyzed using fluorescence microscopy. Representative differential interference contrast (DIC) images and fluorescent images of ZapA-mCherry and LacI-CFP are shown. Scale bar (C and G), 5 μm. Demographics of ZapA-mCherry (D and H) and LacI-CFP (E and I) expression were generated using Oufti software. For cells with a unipolar ZapA-mCherry focus, the ZapA-marked cell pole was defined as a new pole. “S” and “L” indicate the shortest and longest cells, respectively. (F) The percentages of cells in which the distance between the LacI-CFP focus and ZapA-mCherry focus was ≤5 pixels were plotted as “colocalized” (n > 200). ***, confidence level of >99.9% by a z-test.
concentration is low. Here, we show that FtsZ colocalizes with the replication terminus region throughout most of the *C. crescentus* cell cycle. Moreover, we identified a Z-ring-associated protein that controls Z-ring colocalization with the terminus region (ZapT). Although ZapT was annotated as a putative transcriptional regulator belonging to the MerR protein family (44), potential ZapT binding sites are preferentially distributed within the open reading frames near the terminus-proximal regions. Moreover, we found that ZapT interacts with ZapA and ZauP, the counterpart of *E. coli* ZapA and the functional homolog of *E. coli* ZapB, respectively. Furthermore, we provide evidence that ZapT is an important component controlling cell division and chromosome segregation in *C. crescentus*. Because putative ZapT homologs are found in diverse proteobacterial species, the regulatory mechanism mediated by ZapT might be widely conserved.

**RESULTS**

The *C. crescentus* divisome colocalizes with the chromosome terminus.** To examine whether the terminus colocalizes with the divisome in *C. crescentus*, we used fluorescence-reporter-operator system-based terminus visualization (FROS-ter), in which a \( \text{(lacO)}_n \) array integrated near the terminus (ter) region is visualized in living cells through its specific interaction with LacI-CFP (LacI-cyan fluorescent protein) (45). In addition, to monitor the position of the divisome simultaneously, a C-terminal mCherry fusion of ZapA (ZapA-mCherry), which colocalizes with the Z-ring throughout the cell cycle (14), was expressed from the native locus in the FROS-ter background (Fig. 1B).

In exponentially growing cell cultures, ZapA-mCherry and LacI-CFP appeared as a single focus in most cells (Fig. 1C). Demographic representations revealed that the ZapA-mCherry focus preferentially localizes at one cell polar region in shorter G1 cells and relocates to the midcell region as the cells grow (Fig. 1D). This is consistent with previous reports describing the subcellular dynamics of FtsZ and ZapA (14, 42). Moreover, we found that the demographic representation of ZapA-mCherry largely coincided with that of LacI-CFP (Fig. 1D and E), indicating that the ter region also exhibits a dynamic behavior akin to that of the divisome during the cell cycle. Further investigation of individual cells revealed that in a large proportion of cells (75%), the pixel distances between ZapA-mCherry and LacI-CFP foci were \( \leq 340 \text{ nm} \) (5 pixels) (Fig. 1D to F; see also Fig. S1 in the supplemental material). Together, these observations indicate that the divisome colocalizes with the ter region through most of the Caulobacter cell cycle. It should be noted that the ZapA-mCherry and LacI-CFP foci did not colocalize in a subpopulation (25%) of cells (Fig. 1F), implying that the distance between the divisome and the ter region can change dynamically within a certain timescale, possibly through association/dissociation cycles.

**Identification of ZapT.** To explore the molecular link between the divisome and the ter region, we set out to identify novel divisome components that interact directly with the ter region in *C. crescentus*. Guided by previous studies of the interaction between the divisome and the chromosome terminus in *E. coli* (9), we focused our attention on ZapA and its interaction partner ZauP (29). Their counterparts in *E. coli*, ZapA and ZapB, respectively, form a complex with MatP, a nucleoid-associated protein that binds to DNA loci near the chromosome terminus. Although MatP is conserved exclusively in Enterobacteriaceae and Vibrionaceae species (33), we reasoned that a functional homolog of MatP could operate in *C. crescentus* through a direct interaction with ZapA and/or ZauP.

To identify potential interacting partners of ZapA and ZauP, we used coimmunoprecipitation coupled to mass spectrometry (CoIP-MS) to examine a strain expressing either ZapA with a C-terminal 3xFLAG tag (ZapA-3F) or ZauP with an N-terminal 3xFLAG tag (3F-ZauP). The FLAG-tagged proteins in the cell lysate were recovered using anti-FLAG antibody-conjugated magnetic beads, and the materials retained on the beads were analyzed by mass spectrometry. To eliminate proteins that interacted nonspecifically with the anti-FLAG antibody or the beads, we performed similar experiments using the wild-type NA1000 strain as a negative control. Significant differences between the levels of protein enrichment in the FLAG-tagged samples and control
samples were visualized using a volcano plot (Fig. 2A and B). The profiles revealed that ZauP, the putative DNA binding protein ZapT (CCNA_01434; annotated as a MerR-type transcriptional regulator) (Fig. S2A), a uridylate kinase (CCNA_01998), the RecQ DNA helicase (CCNA_03578), and a putative zinc metalloprotease (CCNA_03619) were highly enriched in the elution fraction of the strain expressing ZapA-3F (Fig. 2A). In addition, ZapA and ZapT were enriched in the elution fraction of the strain expressing 3F-ZauP (Fig. 2B), exposing ZapT as a likely candidate DNA binding protein associated with ZapA and ZauP.

To corroborate this idea, we performed an additional CoIP-MS analysis using a strain expressing ZapT with a C-terminal 3xFLAG tag (ZapT-3F) from the native locus and found that both ZapA and ZauP were highly enriched in the elution fraction (Fig. 2C). These findings indicate that ZapT resides in close proximity to the Z-ring as a consequence of a direct or indirect interaction with ZapA and ZauP. It should be mentioned that our CoIP experiments failed to enrich FtsZ or other divisome components in the elution fraction, possibly because the protein interactions within the divisome are not very stable and thus were lost during the washing step in our assay. In contrast, ZapA, ZauP, and ZapT appeared to form a stable protein complex.

To investigate if ZapT is conserved in other bacteria, we performed the analysis of gene clusters in combination with gene cooccurrence. Because the C. crescentus genome encodes five MerR-family proteins (including ZapT), a general sequence-homology search failed to distinguish between ZapT homologs and homologs of other four MerR-family proteins. However, the zapT gene is arranged in an operon, with the upstream ihfA gene encoding integration host factor A (46). Thus, we reasoned that a
MerR family protein that is encoded by a gene flanking the \textit{ihfA} gene in a possible operon should be a putative ZapT homolog. Moreover, given that ZapT operates together with ZapA and ZauP, the putative \textit{zapT} gene must coexist with \textit{zapA} and \textit{zauP} genes in the same genome. On the basis of these ideas, we surveyed the genomic sequences of the 22 organisms that were previously reported to possess putative \textit{zapA} and \textit{zauP} genes (29). Of these, four organisms do not carry an annotated \textit{ihfA} gene. In contrast, all of the other organisms conserve the possible operon comprising the cognate \textit{ihfA} gene and a putative \textit{zapT} gene belonging to the MerR family (Fig. S2B). Those organisms include the alphaproteobacterium \textit{Brucella abortus}, the betaproteobacterium \textit{Burkholderia rhizoxinica}, and the gammaproteobacterium \textit{Pseudomonas aeruginosa}. This observation is in line with the idea that ZapT homologs are widespread in diverse proteobacterial species.

\textbf{ZapT facilitates positioning of the Z-ring.} To determine if ZapT contributes to Z-ring formation, we analyzed a \textit{C. crescentus} mutant strain lacking ZapT. To minimize the risk of a polar effect upon gene disruption, an in-frame deletion (\textit{ΔzapT}) was introduced into the \textit{zapT} gene. As shown previously for \textit{ΔzapA} and \textit{ΔzauP} mutants (29), the resulting \textit{ΔzapT} mutant was viable in peptone-yeast extract (PYE) medium and the synthetic M2G medium (minimal medium supplemented with 0.2\% glucose), displaying a doubling time comparable with that of the wild-type NA1000 strain. Consistently, the size distribution of the \textit{ΔzapT} mutant cells grown in PYE medium was comparable to that of wild-type cells (with mean lengths of 2.4 ± 0.67 μm for the wild type and 2.4 ± 0.77 μm for \textit{ΔzapT} mutant). Moreover, a flow cytometry analysis showed that cellular DNA contents were indistinguishable between wild-type and \textit{ΔzapT} mutant cells (Fig. 3A).

To visualize the Z-ring, an \textit{FtsZ-YFP} (FtsZ-yellow fluorescent protein) fusion was ectopically expressed from the vanillate-dependent promoter in exponentially growing cells (42). As expected, a discrete YFP focus was formed in most wild-type cells (Fig. 3B). A demographic representation revealed that the cell cycle dynamics of \textit{FtsZ} localization coincide well with that of ZapA-mCherry, namely, unipolar localization in shorter G1-phase cells and midcell localization in larger S-phase and G2/M-phase cells (Fig. 1; see also Fig. 3B and C). Similarly, the \textit{ΔzapT} mutant strain produced shorter cells with unipolar Z-ring and longer cells with midcell Z-ring. However, analysis of the size distributions of those shorter cells with unipolar \textit{FtsZ} revealed that the \textit{ΔzapT} mutant cells were more elongated than the wild-type cells (Fig. 3E), indicating that the timing of \textit{FtsZ} relocation from the cell pole to midcell was slightly delayed in the \textit{ΔzapT} mutant strain. The observed phenotype of the \textit{ΔzapT} mutant was suppressed by the plasmid carrying \textit{zapT} (Fig. 3E). Taken together with a previous report of the finding that a mutant \textit{ΔzapA} mutant or \textit{ΔzauP} mutant displayed moderately impaired midcell localization of the Z-ring (29), our findings indicate that, like ZapA and ZauP, ZapT is important for the timely formation of the Z-ring at midcell \textit{in vivo}.

\textbf{ZapT ensures colocalization of the divisome with the ter region.} To further investigate whether ZapT facilitates colocalization of the divisome with the \textit{ter} region, the \textit{zapA-mCherry} allele and FROS-ter were introduced into the \textit{ΔzapT} mutant strain. As seen in the wild-type \textit{zapT} strain, ZapA-mCherry and LacI-CFP formed single foci in most cells (Fig. 1C and G). However, a demographic analysis revealed that the ZapA-mCherry signals were less concentrated at the cell poles in the shorter \textit{ΔzapT} mutant cells than in the wild-type cells (Fig. 1D and H). A more erratic polar localization of ZapA-mCherry in the absence of ZapT is consistent with the observation that Z-ring formation at midcell was delayed in the \textit{ΔzapT} mutant strain. Furthermore, colocalization of ZapA-mCherry and LacI-CFP was significantly reduced in \textit{ΔzapT} cells (Fig. 1F; see also Fig. S1), suggesting that ZapT is required for robust colocalization of the \textit{ter} region with the divisome. In a subpopulation of \textit{ΔzapT} mutant cells, the position of ZapA-mCherry coincided with that of LacI-CFP, which might have been due to an accidental overlap of the two foci during random motion. Alternatively, an additional, as-yet-
unidentified complementary pathway(s) might exist to ensure colocalization of the ter region with the divisome.

**Subcellular dynamics of ZapT.** To examine subcellular localization of ZapT, we generated a strain expressing a C-terminal mNeonGreen fusion of ZapT (ZapT-mNeonGreen) from the native zapT locus. Fluorescence microscopy of the resulting strain revealed that the localization pattern of ZapT-mNeonGreen parallels that of ZapA-mCherry: i.e., the cells formed a single discrete focus of ZapT-mNeonGreen at one cell polar region in shorter cells and at the midcell region in larger cells (Fig. 4A). Indeed, when ZapT-mNeonGreen and ZapA-mCherry were simultaneously expressed, the two positions coincided in most cells (Fig. 4B), supporting the idea that ZapT forms a complex with ZapA in vivo.

Next, we analyzed a possible interaction between ZapT and the ter region using a strain with the zapT-mNeonGreen allele in the FROS-ter background. As observed for ZapA (Fig. 1), ZapT-mNeonGreen formed a discrete focus at a position close to the ter region marked by the LacI-mCherry (Fig. 4C). Moreover, the time-lapse analysis demonstrated that ZapT-mNeonGreen undergoes dynamic changes in its location during the cell cycle, following the position of the ter region (Fig. 4D). These observations are consistent with the idea that the colocalization between divisome and the ter region is mediated by their interaction with ZapT.

**ZapT preferentially associates with the ter region.** Next, we analyzed the distribution of ZapT binding sites throughout the *C. crescentus* chromosome. We reasoned that ZapT binding sites are enriched within the ter-proximal region if ZapT plays a direct role in spatial coordination of the divisome and the chromosome terminus. To test this
idea, we performed chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) using the ZapT-3F-expressing strain used for CoIP-MS. To eliminate false-positive signals, similar experiments were performed using the wild-type NA1000 strain as a negative control. On the basis of the ChIP-seq experiments, we identified 10 genomic positions that displayed discrete peaks of sequencing coverage in a ZapT-3F-dependent manner, a hallmark of ZapT binding sites (Fig. 5A). Although we did not find a consensus DNA sequence motif, these potential binding sites were mostly found within open reading frame regions (Fig. 5A). Moreover, the distribution of the ZapT binding sites was strongly biased toward the ter-proximal region. With one exception, the other binding sites were located at genome positions ranging from 1.3 to 2.2 MB, which corresponds to the chromosome terminus and its flanking regions. This finding suggests that ZapT-DNA complexes are preferentially generated at the ter region.

To probe for ZapT-DNA interactions in vitro, we performed an electrophoretic mobility shift assay using a C-terminally hexahistidine-tagged ZapT-3F protein (ZapT-His) and a 0.3-kb DNA fragment containing the highest ChIP peak, “g” (ligand E1); the intermediate peak “e” (ligand E2); or no distinct peak (ligand E3) (Fig. 5A; see also Fig. 4).
FIG 5 Genome-wide identification of ZapT binding sites. (A) ChIP-seq was performed using the SHQ10 (ZapT-3F) strain. The coverage of every 50-bp window was normalized to the total number of reads and plotted against the NA1000 genomic position. Asterisks indicate false-positive peaks that appeared in both the SHQ10 and control NA1000 samples. The horizontal red line indicates the threshold. The genomic positions that displayed distinct ChIP peaks in SHQ10 cells but not NA1000 cells are indicated by arrows a to j. Close-up representations of the individual ChIP peaks are also shown with gene arrangements. For simplicity, the names of the genes have been omitted. The x axis indicates the genomic position of the reference NA1000 genome. The origin (ori), terminus (ter), and the podJ locus used for the FroSter assay are indicated. (B) EMSA. The ligand DNA (E1, E2, or E3) (10 nM) was incubated with the indicated amounts of ZapT-His, followed by polyacrylamide gel electrophoresis. DNA was visualized by GelStar staining. The band signals of the protein-free DNA were analyzed using Image J, and the results are plotted as percentages.
Fig. S3). In our experimental setup, ZapT-His retains the avidity to bind DNA, as the levels of the signals of the protein-free DNA decreased in a ZapT-His-dependent manner (Fig. 5B). The resulting DNA-ZapT-His complexes were not stable enough to form a discrete band. Also, the concentrations of ZapT-His required for a 50% decrease of the protein-free DNA were largely comparable for different DNA molecules tested, arguing for low sequence specificity of ZapT-His in DNA binding under in vitro conditions. Given that DNA binding proteins of the MerR family often require coeffectors to coordinate their DNA binding activities (44), it is conceivable that ZapT also relies on an unidentified factors mediating its interaction with the terminus region. Moreover, DNA topology such as DNA bending, looping, or superhelicity might be required for the specific interaction of ZapT with the terminus region.

**Overproduction of ZapT blocks cell division.** To gain insight into the function of ZapT, we analyzed cell morphology and DNA content following overexpression of ZapT. To this end, we transformed the *Caulobacter* wild type with a low-copy-number plasmid expressing ZapT-3F from a cumate-dependent promoter or its vector control. After a 6-h induction with cumate, approximately 10-fold-excess amounts of ZapT-3F were accumulated (Fig. S4). Under these conditions, the wild-type *Caulobacter* cells carrying the ZapT-3F plasmid were more elongated than those carrying the empty vector (Fig. 6A). Indeed, the size distribution of ZapT-expressing cells was significantly differentiated from that of control (Fig. 6B and C). A flow cytometry analysis revealed that the fractions of cells harboring two chromosomes were markedly increased upon ZapT-3F induction (25% for vector control and 43% for the ZapT-3F plasmid) (Fig. 6A), arguing for a cell cycle delay in the G2/M phase. As a control, we performed similar experiments using overexpression of green fluorescent protein (GFP), which resulted in no obvious cell division delay (data not shown). These findings suggest that overexpression of ZapT interferes with the cell division process.

To investigate whether the observed cell division block was dependent on *zapA* or *zauP*, we performed a similar cell morphology analysis using a mutant strain harboring the Δ*zapA* or Δ*zauP* allele. The size distributions of cells expressing the empty vector were comparable for the wild-type, Δ*zapA*, and Δ*zauP* strains (Fig. 6B and C). In contrast, when ZapT-3F was overexpressed, the Δ*zapA* and Δ*zauP* strains produced elongated cells such as were observed in the wild-type strain background (Fig. 6B and C). Thus, the interference of ZapT overexpression with the cell division process occurs independently of ZapA or ZauP. It should be mentioned that a *zapA* mutant strain with an in-frame deletion of the residues between positions 13 and 97 was reported to produce mildly elongated cells (the mean length of 3.10 ± 1.90 μm), indicative of a cell division defect (29). Because the Δ*zapA* allele used in this study lacks the residues between positions 4 and 105, different 3′ ends of the *zauP* mRNA may explain differences in the cell division phenotype of these *zapA* mutants.

**Excess ZapT impairs midcell repositioning of the Z-ring.** To examine if the compromised cell division caused by excess ZapT-3F resulted from impaired positioning or assembly of the Z-ring, we analyzed the localization of an FtsZ-YFP fusion in cells overexpressing ZapT-3F (Fig. 7; see also Fig. S5). When ZapT-3F was overexpressed from a low-copy-number plasmid, the cells were elongated but retained the ability to form discrete YFP foci (Fig. 7A, lower panel), indicating that the presence of excess ZapT does not lead to general destabilization of FtsZ polymers. However, a large (50%) fraction of cells overproducing ZapT-3F displayed a unipolar FtsZ-YFP focus (Fig. 7A, lower panel). In contrast, in cells carrying the control vector, FtsZ-YFP localized mainly to midcell and only a small (10%) fraction of cells showed unipolar localization (Fig. 7A, upper panel). FtsZ localizes at midcell during most of the cell cycle to initiate cell division and transiently remains at the new cell pole after completion of division until relocating to midcell for the next round of cell division. Thus, the observed increase in unipolar localization of FtsZ-YFP in cells overexpressing ZapT-3F could be explained by debilitated or delayed relocation of FtsZ from the pole to midcell.
To examine if excess ZapT-3F also perturbs midcell repositioning of the ter region, FROS-ter experiments were performed in exponentially growing cells overexpressing ZapT-3F. Both the control strain carrying the empty vector (pQF) or its derivative harboring zapT-3F (pQF::zapT) was grown in PYE medium supplemented with 1 μM cumate for 6 h. Cell morphology and DNA content were analyzed using phase-contrast microscopy and flow cytometry, respectively. The number of cells containing two chromosome equivalents is shown as a percentage of the total number of cells. (B and C) The pQF plasmid or the pQF::zapT plasmid was introduced into the wild-type, ΔzapA, or ΔzauP strain. After a 6-h induction performed with 1 μM cumate, cells were analyzed using phase-contrast microscopy. (B) The distributions of cell lengths are shown using a box plot. The P value was calculated using the Mann-Whitney-Wilcoxon test. (C) The number of cells analyzed (N) and mean cell length with standard deviation (SD) are indicated.

To examine if excess ZapT-3F also perturbs midcell repositioning of the ter region, FROS-ter experiments were performed in exponentially growing cells overexpressing ZapT-3F. Both the control strain carrying the empty vector and the strain overexpressing ZapT-3F produced cells with either one or two LacI-CFP foci (Fig. 7B). For the control vector strain, the localization profile of LacI-CFP was essentially the same as that observed earlier using wild-type cells (Fig. 7B, upper panel; see also Fig. 1): cells with a single focus near midcell (82%) or the cell pole (10%) and cells with two foci (8%) at midcell. In contrast, in a strain overexpressing ZapT, only 22% of the cells had a single CFP focus at midcell, while an increased number of cells showed two or three LacI-CFP foci (56% and 12%, respectively) (Fig. 7B, lower panels). Cells with two LacI-CFP foci were substantially elongated, and the foci were generally positioned slightly off midcell, where the ter region is sequestered after constriction in unperturbed cells. Moreover, we observed a small (1%) proportion of cells displaying unipolar localization of LacI-CFP in the ZapT-overexpressing strain, which was comparable to that observed for the control strain (8%). These observations are consistent with the idea that relocation...
of the terminus from the cell pole is largely maintained under conditions in which ZapT-3F is overexpressed although the positioning of the terminus at midcell is disturbed. Therefore, we suggest that excess ZapT specifically blocks midcell localization of the divisome without affecting the dynamics of the terminus.

To further interrogate the cause of the impaired positioning of FtsZ under conditions of excess ZapT, we turned our attention to MipZ, a ParA-type ATPase that inhibits FtsZ polymerization. Newborn *Caulobacter* cells sequester MipZ to the old cell pole, opposite the FtsZ-occupied new pole. Conversely, cells that are replicating their chromosomes impose bipolar localization on MipZ, which drives FtsZ relocation to midcell, where MipZ concentrations are low (42, 43). Because relocation of FtsZ from the cell pole to midcell is compromised in cells overexpressing ZapT-3F, we reasoned that excess ZapT might interfere with the bipolar localization of MipZ. To test this hypothesis, we analyzed localization of a C-terminal YFP fusion of MipZ (42). As expected, MipZ-YFP was localized at either one or both cell poles in the vector control strain (Fig. 7C). The fraction of cells with bipolar localization of MipZ-YFP (86%) was

**FIG 7** Overproduction of ZapT causes aberrant localization of FtsZ and MipZ. The images show localization of FtsZ, the terminus region, MipZ, and the nucleoid in the presence of excess ZapT. The pQF or pQF::zapT vector was introduced into a strain expressing FtsZ-YFP (SHQ67) (A), FROS-ter (SHQ54) (B), MipZ-YFP (SHQ66) (C), or none of those products (NA1000) (D). Log-phase cells that were incubated for 6 h in PYE medium supplemented with 1 μM cumate were analyzed by fluorescence microscopy. (D) For visualization of nucleoid, cells fixed in 70% ethanol were incubated for >10 min in 2 μg/ml DAPI solution before imaging. Arrowheads indicate polar MipZ-free zones.
comparable with the fraction with midcell localization of FtsZ-YFP (90%) (Fig. 7A and C), which is consistent with the idea that the bipolar MipZ gradient restricts Z-ring formation near the midcell region. Next, we performed a similar experiment using cells overexpressing ZapT-3F. Overall, 96% of the cells overexpressing ZapT-3F formed two or more discrete MipZ foci and 4% formed a single focus, indicating that MipZ assembly was not affected by excess ZapT. Notably, in 50% of the cells harboring multiple MipZ-YFP foci, at least one of the foci was positioned distal to the cell pole, leaving polar zones devoid of MipZ. This finding is consistent with the observation that relocation of FtsZ is perturbed under conditions of excess ZapT. Because the entire cytosolic space was occupied by the nucleoid in a manner irrespective of ZapT overexpression (Fig. 7D) and overexpression of ZapT-mNeonGreen yielded cells with a single discrete fluorescent focus (Fig. S4C), mislocalization of MipZ is unlikely to result from the presence of a DNA-free zone at the cell pole or from the irregular condensation of the nucleoids. Also, a ΔzapT mutant grown in PYE complex medium showed normal MipZ localization (Fig. S6). Thus, an excess of ZapT changes the spatial arrangement of MipZ, either directly or indirectly.

**DISCUSSION**

In this study, we found that the replication terminus of the *C. crescentus* chromosome is physically linked to the divisome during most of the cell cycle. We propose that ZapT, a previously uncharacterized MerR family protein, contributes to spatial coordination of the terminus and divisome via its interaction with the Z-ring-associated proteins ZapA and ZauP. ZapT coincides with both ZapA and the terminus DNA in living cells. Notably, mutants lacking the zapT gene displayed misplacement of the divisome and the terminus, arguing that ZapT plays a prominent role in the physical link between these structures. The divisome and terminus are centrally colocalized in stalked and predivisional cells and, after division, remain localized at the new cell poles for a defined period (47–49). Thus, the physical linkage of the divisome and terminus appears spatially coordinated while cells progress through the cell cycle.

The observation that Z-ring formation at midcell is delayed in ΔzapT cells strongly argues for functional importance of ZapT in the process of cell division. In the absence of ZapT, G1 cells often failed to properly localize ZapA to the cell pole. Because ZapA was previously shown to facilitate Z-ring formation *in vivo* (29) and to interact with ZapT, the observed delay of formation of the Z-ring in the ΔzapT mutant cells likely resulted from irregular positioning of ZapA. The idea of a physiological role of ZapT in the process of cell division is further supported by the observation that excess ZapT levels caused a division arrest. Although the division-arrested cells retained the ability to recruit the chromosome terminus near midcell, the terminus had lost the physical connection to the divisome, leaving inert FtsZ polymers at the cell pole. In agreement with this, the FtsZ inhibitor MipZ was often mislocalized under these conditions, creating MipZ-free cell poles where FtsZ is able to polymerize. Therefore, it is possible that excess ZapT molecules might directly or indirectly interfere with MipZ to reduce its accessibility to the cell pole, thereby stabilizing polar FtsZ polymers. The polar localization of MipZ is driven by the essential DNA partitioning ATPase ParA that localizes to the FtsZ-marked new cell pole (50, 51). Because ZapT and ParA are sequestered to the same cell pole in the G1 phase, it is possible that the mislocalization of MipZ under ZapT overexpression results from perturbed functions of ParA. Coincidently, similar mislocalization of MipZ was observed in *Caulobacter* cells expressing the dominant-negative ParA K20R variant (52). The ParA Lys20 residue comprises the Walker A-type ATP binding motif. Thus, it is conceivable that overexpressed ZapT might directly or indirectly perturb the ATPase cycle of ParA, thereby inhibiting the process of MipZ localization. Under laboratory conditions, the mRNA levels of the *Caulobacter ihfA-zapT* operon peak in G1 phase and remain relatively low during the S phase where MipZ dynamically relocates to both poles (53, 54). Therefore, it is also possible that the observed impact of excess ZapT on MipZ localization might have been due to the overruling of physiological fluctuations of ZapT during the cell cycle, where differences
in ZapT concentrations may facilitate spatial distribution of factions involved in cell cycle progression and cell division.

We propose that DNA-ZapT complexes play a direct role in the spatial coordination of the terminus and divisome. In E. coli, the nucleoid-associated MatP protein and the divisome component ZapB have been proposed to play a central role in maintaining a physical linkage between the terminus and divisome (33). Several lines of evidence indicate that MatP oligomers facilitate condensation of the replication terminus region of the E. coli chromosome through binding to multiple recognition sites distributed within an 800-kb stretch around the terminus (32–34). On the other hand, ZapB associates with FtsZ via ZapA (29). Thus, a physical connection of the terminus region to the Z-ring can be mediated by the physical interaction between MatP and ZapB. By analogy, it is possible that ZapT can form a nucleoprotein complex consisting of the terminus region of the C. crescentus chromosome, ZauP, and ZapA to recruit the Z-ring. This idea is fully consistent with our genome-wide ChIP-seq results indicating the proximal region preferential DNA binding of ZapT, although E. coli MatP shares little or no sequence similarity with C. crescentus ZapT.

In the colocalization analysis performed for ZapT and the terminus DNA, some cells form a ZapT-mNeonGreen focus at a position slightly distant from the ter-proximal podJ locus marked by LacI-mCherry (Fig. 4C and D). Because the ZapT binding sites determined by ChIP-seq are 90 kb away from the podJ locus (Fig. 5A), a spatial lag between ZapT-mNeonGreen and LacI-mCherry foci could be explained by the spacing between their binding sites. Also, it is possible that ZapT might primarily bind to the Z-ring rather than DNA. We infer that Z-ring-associated ZapT dynamically follows the movement of the terminus DNA with a certain lag time, which could reflect the distance between ZapT-mNeonGreen and LacI-mCherry foci.

Coordinating cell cycle progression with dynamic chromosome architecture is a sophisticated strategy to ensure the faithful inheritance of genetic materials. We suggest that the ZapT-ZapA-ZauP complex forms a physical linkage between the Z-ring and the chromosomal replication terminus. As ZapT, ZapA, and ZauP are conserved in various organisms, including members of the alpha-, beta-, and gammaproteobacterial groups, our findings expand our understanding of how cell division is coupled to chromosome replication and segregation in the bacterial kingdom. FtsZ and ZapA homologs are found even in the eukaryotic red alga Cyanidioschyzon merolae (FtsZ1 and ZED, respectively) and play an essential role in the cognate mitochondrial division (55–57). It is possible that a ZapT-like DNA binding protein could exist in this case, coordinating mitochondrial DNA with division.

MATERIALS AND METHODS

Bacterial strains and DNAs. The strains, plasmids, and oligonucleotides used in this study are listed in Tables 1, 2, and 3, respectively. Caulobacter strains were grown at 30°C in PYE or minimal medium supplemented with 0.2% glucose (M2G). When necessary, the culture medium was supplemented with xylose (0.03%), cumate (1 μM), or vanillate (0.5 mM) as indicated. Detailed procedures for construction of the strains and plasmids are described in Text S1 in the supplemental material.

Microscopy. Differential interference contrast, phase-contrast, and fluorescence microscopy analyses were performed using a Nikon Eclipse 80i microscope and an Andor Zyla 4.2 scientific complementary metal oxide semiconductor (sCMOS) camera. Quantitative image analyses of cells and fluorescent signals were performed using the Oufti and MicrobeJ software packages (58, 59).

For visualization of the nucleoid, cells were fixed in 70% ethanol, harvested by centrifugation, and resuspended in phosphate-buffered saline containing 2 μg/ml DAPI (4’,6-diamidino-2-phenylindole).

FACS. The fluorescence-activated cell sorter (FACS) assay was performed essentially as described previously (60–62). In brief, exponentially growing cells (100 μl) were fixed in ice-cold 70% ethanol and then harvested by centrifugation and washed in 1 ml of FACS buffer (10 mM Tris-HCl [pH 7.5] and 1 mM magnesium chloride). The DNA was stained in 1 ml of FACS buffer supplemented with 2 μM Sytox Green (Thermo Fisher Scientific) at room temperature for 0.5 h. The fluorescent intensity and light scattering were analyzed using a FACSCalibur system (BD Biosciences).

ColIP-MS. To perform immunoprecipitation, an overnight culture (100 ml) was diluted in fresh PYE medium (1 liter) and cultivated at 30°C for 3 h. The cells were harvested by centrifugation (5,000 rpm, 20 min, 4°C) and resuspended in IP wash buffer (10 mM HEPES KOH [pH 7.4] and 75 mM sodium chloride) to adjust the optical density at 600 nm (OD_{600}) to 100 (9 ml cell suspension). After the addition of Complete protease inhibitor (Roche) and 25 μg/ml DNase I, the cells were lysed through two passages in a French press. The sample was then incubated at 4°C for a further 15 min in the presence of 0.1%
TABLE 1 Bacterial strains used in this study

| Species and strain | Genotype | Reference or source |
|--------------------|-----------|---------------------|
| **Caulobacter crescentus** | | |
| NA1000 | A wild-type Caulobacter crescentus strain | 61 |
| PV2665 | CB15N cc0006Δ(tetO54) xyG : pHP472 : pHP489 | This study |
| SHQ10 | NA1000 CCNA::3xFLAG-zapT | This study |
| SHQ48 | NA1000 zapA::mCherry | This study |
| SHQ56 | NA1000 zapA::mCherry | This study |
| SHQ63 | SHQ56 xyG : pHP472 : pHP489 | This study |
| SHQ66 | NA1000 mipZ-pMT151 (ZapP::mipZ, Pxy::mipZ) | This study |
| SHQ67 | NA1000 vanK::pMT383 (ftsZ:Gfp) | This study |
| SHQ68 | NA1000 ΔCCNA::3xFLAG (zapA::mCherry) | This study |
| SHQ69 | NA1000 ΔCCNA::3xFLAG (zapA::mCherry) | This study |
| SHQ75 | SHQ48 zapA::mCherry xyG : pHP472 : pHP489 | This study |
| SHQ136 | SHQ48 vanK::pMT383 (ftsZ:Gfp) | This study |
| SHQ143 | NA1000 zapA::mCherry | This study |
| SHQ173 | SHQ143 zapA::mCherry | This study |
| SHQ177 | SHQ143 lacA::Pcumate-lacImCherry | This study |
| SHQ178 | SHQ177 Pxy::pHP489 | This study |
| UJ9492 | NA1000 3xFLAG-zapA | This study |
| UJ9812 | NA1000 zapA::3xFLAG | This study |
| **Escherichia coli** | | |
| DH5a | A general cloning strain | Invitrogen |
| Top10 | A general cloning strain | Invitrogen |
| UJ5190 | S17-1 pMT151 | This study |
| UJ5195 | S17-1 pMT383 | This study |
| UJ9399 | DH10B pNPTS-ZapA-mCh | This study |
| UJ9486 | DH10B pNPTS-3FzapB | This study |
| UJ9524 | Top10 pQF | 65 |
| UJ9727 | DH10B pNPTS138-01434_3F | This study |
| UJ9811 | Top10 pNPTS-zapA3F | This study |
| UJ10015 | Top10 pNPTS-zapA-CKO | This study |
| UJ10016 | Top10 pNPTS-zapB-CKO | This study |
| UJ10032 | Top10 pQFO1434-3F | This study |

IGEPAL, followed by ultracentrifugation (30,000 rpm, 1 h, 4°C). The supernatant was mixed with a 30-μl slurry of anti-FLAG M2 magnetic beads (Sigma-Aldrich) equilibrated with buffer (10 mM HEPES KOH [pH 7.5], 75 mM NaCl, and 0.1% IGEPAL) and was incubated for 1 h at 4°C with gentle agitation. The beads were harvested using a magnetic stand and washed three times in 700 μl of elution buffer 1 (0.1 M ammonium bicarbonate. Bead-bound materials were eluted in two steps using (i) 100 μl of elution buffer 1 (0.1 M ammonium bicarbonate, 1.6 M urea, 0.5 μg trypsin) and (ii) 80 μl of elution buffer 2 (0.1 M ammonium bicarbonate, 1.6 M urea, 0.2 M Tris(2-carboxyethyl)phosphine). Liquid chromatography-tandem mass spectrometry was performed as described previously (61).

**ChIP-seq.** ChIP was performed essentially as described previously (61). Briefly, exponentially growing cells were fixed for 10 min using a 1% formaldehyde solution, washed thoroughly, resuspended in buffer,

TABLE 2 Plasmids used in this study

| Plasmid | Description | Reference or source |
|--------|-------------|---------------------|
| mNG-sTFq2 | A plasmid with the mNeonGreen gene | Addgene |
| pET21a01434_3F6H | A pET21a (Novagen) derivative to purify ZapT with C-terminal 3xFLAG and 6xHis | This study |
| pLaCQF | A pLaCQF derivative with the lacImCherry gene | This study |
| pMT151 | A kanamycin resistance-integrating plasmid with the MipZ-YFP ORF under the control of the lacI promoter | 42 |
| pMT383 | A kanamycin resistance-integrating plasmid with the FtsZ-YFP ORF under the control of the vanillate-dependent promoter | 42 |
| pNPTS-3FzapB | A pNPTS138 derivative with 3F-zapB | This study |
| pNPTS-ZapA-mCh | A pNPTS138 derivative with zapA-mCherry | This study |
| pNPTS01434-CKO | A pNPTS138 derivative to introduce an in-frame deletion of zapT | This study |
| pNPTS01434-mNG | A pNPTS138 derivative with zapT-mNeonGreen | This study |
| pNPTS138 | A kanamycin-resistant suicide vector | 66 |
| pNPTS138-01434_3F | A pNPTS138 derivative with ZapT-3F | This study |
| pNPTS-zapA-CKO | A pNPTS138 derivative to introduce an in-frame deletion of zapA | This study |
| pNPTS-zapA3F | A pNPTS138 derivative with ZapA-3F | This study |
| pNPTS-zapB-CKO | A pNPTS138 derivative to introduce an in-frame deletion of zapB | This study |
| pQF | A low-copy-number tetracycline-resistance-conferring vector with the cumate-dependent promoter | 65 |
| pQF01434-3F | A pQF derivative with ZapT-3F | This study |
| pQF1434-3F | A pQF derivative with zapT-mNeonGreen | This study |
| pRVCHYC-2 | A kanamycin-resistance-conferring plasmid with the mCherry ORF | 47 |

*aORF, open reading frame.*
and lysed through two passages in a French press. After DNA shearing with sonication was performed, the cell debris was removed by ultracentrifugation and the cleared cell lysate was incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich). After washing of the beads was performed, the bound materials were incubated at 65°C overnight to reverse cross-linking. The resulting DNA samples were purified using a DNA cleanup kit (Macherey-Nagel) and then analyzed by deep sequencing (single-end NextSeq).

The sequence data were analyzed using Galaxy (https://usegalaxy.eu). Briefly, Bowtie 2 was used to map the reads onto the NA1000 reference genome. After read extension to 300 nucleotides (nt), the coverage of every 50-bp window was deduced. The values normalized to the total number of reads were used to identify the ChIP peaks. Two independent ChIP-seq experiments were performed to confirm the reproducibility of the results.

**TABLE 3 Oligonucleotides used in this study**

| Oligonucleotide | Sequence (5’–3’) |
|-----------------|------------------|
| 1               | TGTGGTCACCTCGATGTCGG |
| 2               | TGACGGAGCTCTCGAGGAAA |
| 3               | CGAGGTGATCATCGGAGG |
| 4               | CAACTATGTCAGCCCGCTCT |
| 5               | TATGTAGAGGCGACCCCAAA |
| 6               | GGTGTCAGCTCAATCGG |
| 7               | AGTTAGGATCACGTCGAGGG |
| 8               | GATACAGCTGCTGAGGAGAG |
| 9               | GCTGCTGAGGAGAGCCGACG |
| 10              | TCAGTACGGTACAGTCGAGG |
| 11              | AGGAGCTGAGAGGGAG |
| 12              | CATCAAGAATAAAAGCAACT |
| 13              | AATTTTCTAGAACGCCCTCAC |
| 14              | ATTTGTGGAGGAGGAGCT |
| 15              | CCGGCCGAGTACAGGTCGAC |
| 16              | GTGCGGATCGAGAAGCTCGCG |
| 17              | GCTGCGGTCACCTCAGG |
| 18              | GCTGCGGACAGTGAGGAGG |
| 19              | GCTGCGGTCACCTCAGG |
| 20              | GCTGCGGACAGTGAGGAGG |
| 21              | GCTGCGGACAGTGAGGAGG |
| 22              | GCTGCGGACAGTGAGGAGG |
| 23              | GCTGCGGACAGTGAGGAGG |
| 24              | GCTGCGGACAGTGAGGAGG |
| 25              | GCTGCGGACAGTGAGGAGG |
| 26              | GCTGCGGACAGTGAGGAGG |
| 27              | GCTGCGGACAGTGAGGAGG |
| 28              | GCTGCGGACAGTGAGGAGG |
| 29              | GCTGCGGACAGTGAGGAGG |
| 30              | GCTGCGGACAGTGAGGAGG |
| 31              | GCTGCGGACAGTGAGGAGG |
| 32              | GCTGCGGACAGTGAGGAGG |
| 33              | GCTGCGGACAGTGAGGAGG |
| 34              | GCTGCGGACAGTGAGGAGG |
| 35              | GCTGCGGACAGTGAGGAGG |
| 36              | GCTGCGGACAGTGAGGAGG |
| 37              | GCTGCGGACAGTGAGGAGG |
| 38              | GCTGCGGACAGTGAGGAGG |
| 39              | GCTGCGGACAGTGAGGAGG |
| 40              | GCTGCGGACAGTGAGGAGG |
| 41              | GCTGCGGACAGTGAGGAGG |
| 42              | GCTGCGGACAGTGAGGAGG |
| 43              | GCTGCGGACAGTGAGGAGG |
| 44              | GCTGCGGACAGTGAGGAGG |
| 45              | GCTGCGGACAGTGAGGAGG |
| 46              | GCTGCGGACAGTGAGGAGG |
| 47              | GCTGCGGACAGTGAGGAGG |
| 48              | GCTGCGGACAGTGAGGAGG |
| 49              | GCTGCGGACAGTGAGGAGG |
| 50              | GCTGCGGACAGTGAGGAGG |
| 51              | GCTGCGGACAGTGAGGAGG |
| 52              | GCTGCGGACAGTGAGGAGG |
| 53              | GCTGCGGACAGTGAGGAGG |
| 54              | GCTGCGGACAGTGAGGAGG |
| 55              | GCTGCGGACAGTGAGGAGG |
| 56              | GCTGCGGACAGTGAGGAGG |
| 57              | GCTGCGGACAGTGAGGAGG |
| 58              | GCTGCGGACAGTGAGGAGG |
| 59              | GCTGCGGACAGTGAGGAGG |

Ozaki et al. March/April 2020 Volume 11 Issue 2 e00487-20 mBio.asm.org

and lysed through two passages in a French press. After DNA shearing with sonication was performed, the cell debris was removed by ultracentrifugation and the cleared cell lysate was incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich). After washing of the beads was performed, the bound materials were incubated at 65°C overnight to reverse cross-linking. The resulting DNA samples were purified using a DNA cleanup kit (Macherey-Nagel) and then analyzed by deep sequencing (single-end NextSeq).

The sequence data were analyzed using Galaxy (https://usegalaxy.eu). Briefly, Bowtie 2 was used to map the reads onto the NA1000 reference genome. After read extension to 300 nucleotides (nt), the coverage of every 50-bp window was deduced. The values normalized to the total number of reads were used to identify the ChIP peaks. Two independent ChIP-seq experiments were performed to confirm the reproducibility of the results.

**Purification of ZapT-His.** Rosetta 2(DE3) cells (Novagen) harboring pET21a01434_3F6H were grown exponentially in LB medium (1 liter) supplemented with ampicillin and chloramphenicol, and ZapT-His

---

and lysed through two passages in a French press. After DNA shearing with sonication was performed, the cell debris was removed by ultracentrifugation and the cleared cell lysate was incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich). After washing of the beads was performed, the bound materials were incubated at 65°C overnight to reverse cross-linking. The resulting DNA samples were purified using a DNA cleanup kit (Macherey-Nagel) and then analyzed by deep sequencing (single-end NextSeq).

The sequence data were analyzed using Galaxy (https://usegalaxy.eu). Briefly, Bowtie 2 was used to map the reads onto the NA1000 reference genome. After read extension to 300 nucleotides (nt), the coverage of every 50-bp window was deduced. The values normalized to the total number of reads were used to identify the ChIP peaks. Two independent ChIP-seq experiments were performed to confirm the reproducibility of the results.

**Purification of ZapT-His.** Rosetta 2(DE3) cells (Novagen) harboring pET21a01434_3F6H were grown exponentially in LB medium (1 liter) supplemented with ampicillin and chloramphenicol, and ZapT-His
was induced for 3 h by addition of 1 mM isopropyl-β-D-thiogalactoside. The resulting cells were harvested by centrifugation and resuspended in buffer A (25 mM Tris HCl [pH 7.5], 300 mM sodium chloride, 5% glycerol) supplemented with 5 mM imidazole and 0.2 mg/ml lysozyme. The cell suspension was incubated on ice for 30 min, frozen in liquid nitrogen, and thawed to lyse cells. After ultracentrifugation (48,000 rpm, 20 min), the supernatant was loaded onto a nickel-Sepharose 6 Fast Flow column (0.5 ml) equilibrated with buffer A containing 5 mM imidazole. After washing was performed with buffer A containing 20 mM imidazole, the ZapT-His proteins retained on the column were eluted in buffer A containing 250 mM imidazole. ZapT-His was further purified using HiTrap heparin (1 ml): ZapT-His was loaded in buffer B (25 mM Tris HCl [pH 7.5], 5% glycerol) containing 100 mM NaCl, washed in buffer B (plus 200 mM sodium chloride) and in buffer B (plus 500 mM sodium chloride), and eluted in buffer B (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 200 mM sodium chloride) and in buffer B (plus 500 mM sodium chloride), and eluted in buffer B (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 200 mM sodium chloride) and in buffer B (plus 500 mM sodium chloride), and eluted in buffer B (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 200 mM sodium chloride) and in buffer B (plus 500 mM sodium chloride), and eluted in buffer B (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 2,000 mM sodium chloride). After dialysis against buffer B (plus 100 mM sodium chloride), (plus 200 mM sodium chloride) and in buffer B (plus 500 mM sodium chloride), and eluted in buffer B (plus 2,000 mM sodium chloride).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.
FIG S1, PDF file, 0.8 MB.
FIG S2, PDF file, 1.5 MB.
FIG S3, PDF file, 0.3 MB.
FIG S4, TIFF file, 1.9 MB.
FIG S5, PDF file, 2.8 MB.
FIG S6, PDF file, 2.9 MB.

ACKNOWLEDGMENTS

We thank A. Schmidt and E. Lezan for the MS analysis, C. Beisel for sequencing, A. Kaczmarczyk for plasmid construction and critical review of the manuscript, and F. Hamburger and K. Ozaki for plasmid construction.

This work was supported by an ERC grant and MEXT/JSPS KAKENHI (grant number JP18H02377).

REFERENCES

1. Marczynski GT, Petit K, Patel P. 2019. Crosstalk regulation between bacterial chromosome replication and front chromosome partitioning. Front Microbiol 10:279. https://doi.org/10.3389/fmicb.2019.00279.
2. Surzvitsev IV, Jacobs-Wagner C. 2018. Subcellular organization: a critical feature of bacterial cell replication. Cell 172:1271–1293. https://doi.org/10.1016/j.cell.2018.01.014.
3. Reyes-Lamothe R, Sherratt DJ. 2019. The bacterial cell cycle, chromosome inheritance and cell growth. Nat Rev Microbiol 17:467–478. https://doi.org/10.1038/s41579-019-0212-7.
4. Katayama T, Kasho K, Kawakami H. 2017. The DnaA cycle in Escherichia coli: activation, function and inactivation of the initiator protein. Front Microbiol 8:2496. https://doi.org/10.3389/fmicb.2017.02496.
5. Taniguchi S, Kasho K, Ozaki S, Katayama T. 2019. Escherichia coli CrfC protein, a nucleoid partition factor, localizes to nucleoid poles via the activities of specific nucleoid-associated proteins. Front Microbiol 10:72. https://doi.org/10.3389/fmicb.2019.00072.
6. Haeusser DP, Margolin W. 2016. Splitsville: structural and functional insights into the dynamic bacterial Z ring. Nat Rev Microbiol 14:305–319. https://doi.org/10.1038/nrmicro.2016.26.
7. Badrinarayanan A, Le TBK, Laba MT. 2015. Bacterial chromosome organization and segregation. Annu Rev Cell Dev Biol 31:171–199. https://doi.org/10.1146/annurev-cellbio-100814-125211.
8. den Blaauwen T, Hamoen LW, Levin PA. 2017. The divisome at 25: the road ahead. Curr Opin Microbiol 36:65–84. https://doi.org/10.1016/j.mib.2017.01.007.
9. Männik J, Bailey MW. 2015. Spatial coordination between chromosomes and cell division proteins in Escherichia coli. Front Microbiol 6:306. https://doi.org/10.3389/fmicb.2015.00306.
10. Adams DW, Errington J. 2009. Bacterial cell division: assembly, maintenance and disassembly of the Z ring. Nat Rev Microbiol 7:642–653. https://doi.org/10.1038/nrmicro2198.
11. Löwe J, Amos LA. 1998. Crystal structure of the bacterial cell-division protein FtsZ. Nature 391:203–206. https://doi.org/10.1038/34472.
12. Bi E, Lutkenhaus J. 1991. FtsZ ring structure associated with division in Escherichia coli. Nature 354:161–164. https://doi.org/10.1038/354161a0.
13. Erickson HP, Taylor DW, Taylor KA, Bramhill D. 1996. Bacterial cell division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. Proc Natl Acad Sci U S A 93:519–523. https://doi.org/10.1073/pnas.93.1.519.
14. Goley ED, Yeh Y-C, Hong S-H, Fero MJ, Abeluk E, McAdams HH, Shapiro L. 2011. Assembly of the Caulobacter cell division machine. Mol Microbiol 80:1680–1698. https://doi.org/10.1111/j.1365-2958.2011.07677.x.
15. Yang X, Lyu Z, Miguel A, McQuillen R, Huang KC, Xiao J. 2017. GTPase activity-coupled treading of the bacterial tubulin FtsZ organizes septal cell wall synthesis. Science 355:744–747. https://doi.org/10.1126/science.aak9995.
16. Birson-Filho AW, Hsu Y-P, Squyres GR, Kuru E, Wu F, Jukes C, Sun Y, Dekker C, Holden S, VanNieuwenhze MS, Brun YY, Garner EC. 2017. Treading by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science 355:739–743. https://doi.org/10.1126/science.aak9973.
17. de Boer PAJ, Crossley RE, Rothfield LI. 1989. A division inhibitor and a division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. Proc Natl Acad Sci U S A 93:519–523. https://doi.org/10.1073/pnas.93.1.519.
60. Ozaki S, Matsuda Y, Keyamura K, Kawakami H, Noguchi Y, Kaso K, Nagata K, Masuda T, Sakaiyama Y, Katayama T. 2013. A replicase clamp-binding dynamin-like protein promotes colocalization of nascent DNA strands and equipartitioning of chromosomes in E. coli. Cell Rep 4:985–995. https://doi.org/10.1016/j.celrep.2013.07.040.

61. Lori C, Ozaki S, Steiner S, Böhm R, Abel S, Dubey BN, Schirmer T, Hiller S, Jenal U. 2015. Cyclic di-GMP acts as a cell cycle oscillator to drive chromosome replication. Nature 523:236–239. https://doi.org/10.1038/nature14473.

62. Dubey BN, Lori C, Ozaki S, Fucile G, Plaza-Menacho I, Jenal U, Schirmer T. 2016. Cyclic di-GMP mediates a histidine kinase/phosphatase switch by noncovalent domain cross-linking. Sci Adv 2:e1600823. https://doi.org/10.1126/sciadv.1600823.

63. Ozaki S, Kawakami H, Nakamura K, Fujikawa N, Kagawa W, Park SY, Yokoyama S, Kurumizaka H, Katayama T. 2008. A common mechanism for the ATP-DnaA-dependent formation of open complexes at the replication origin. J Biol Chem 283:8351–8362. https://doi.org/10.1074/jbc.M708684200.

64. Reinders A, Hee C-S, Ozaki S, Mazur A, Boehm A, Schirmer T, Jenal U. 2016. Expression and genetic activation of cyclic di-GMP-specific phosphodiesterases in Escherichia coli. J Bacteriol 198:448–462. https://doi.org/10.1128/JB.00604-15.

65. Kaczmarszyk A, Vorholt JA, Francez-Charlot A. 2013. Cumate-inducible gene expression system for sphingomonads and other Alphaproteobacteria. Appl Environ Microbiol 79:6795–6802. https://doi.org/10.1128/AEM.02296-13.

66. Ozaki S, Schalch-Moser A, Zumthor L, Manfredi P, Ebbensgaard A, Schirmer T, Jenal U. 2014. Activation and polar sequestration of PopA, a c-di-GMP effector protein involved in Caulobacter crescentus cell cycle control. Mol Microbiol 94:580–594. https://doi.org/10.1111/mmi.12777.