Filament Formation of the FtsZ/Tubulin-like Protein TubZ from the Bacillus cereus pXO1 Plasmid

Shota Hoshino and Ikuko Hayashi

From the Department of Supramolecular Biology, Graduate School of Nanobioscience, Yokohama City University, 1-7-29 Suehiro, Tsurumi, Yokohama 230-0045, Japan

Background: FtsZ/tubulin-like protein TubZ is a type III plasmid partitioning factor from Bacillus virulence plasmids.

Results: The crystal structure shows that Bacillus cereus TubZ forms a protofilament-like dimer.

Conclusion: Despite the sequence divergence of TubZ proteins, their structures and biochemical activities are very similar.

Significance: The results provide insights into the protofilament formation mechanism of the TubZ family proteins.

Stable maintenance of low-copy-number plasmids requires partition (par) systems that consist of a nucleotide hydrolase, a DNA-binding protein, and a cis-acting DNA-binding site. The FtsZ/tubulin-like GTPase TubZ was identified as a partitioning factor of the virulence plasmids pBtToxix and pXO1 in Bacillus thuringiensis and Bacillus anthracis, respectively. TubZ exhibits high GTPase activity and assembles into polymers both in vivo and in vitro, and its “treadmilling” movement is required for plasmid stability in the cell. To investigate the molecular mechanism of pXO1 plasmid segregation by TubZ filaments, we determined the crystal structures of Bacillus cereus TubZ in apo-, GDP-, and guanosine 5’-3’-(thio)triphosphate (GTP\(\gamma\)S)-bound forms at resolutions of 2.1, 1.9, and 3.3 Å, respectively. Interestingly, the slowly hydrolyzable GTP analog GTP\(\gamma\)S was hydrolyzed to GDP in the crystal. In the post-GTP hydrolysis state, GDP-bound B. cereus TubZ forms a dimer by the head-to-tail association of individual subunits in the asymmetric unit, which is similar to the protofilament formation of FtsZ and B. thuringiensis TubZ. However, the M loop interacts with the nucleotide-binding site of the adjacent subunit and stabilizes the filament structure in a different manner, which indicates that the molecular assembly of the TubZ-related par systems is not stringently conserved. Furthermore, we show that the C-terminal tail of TubZ is required for association with the DNA-binding protein TubR. Using a combination of crystallography, site-directed mutagenesis, and biochemical analysis, our results provide the structural basis of the TubZ polymer that may drive DNA segregation.

The cytoskeleton is a cellular scaffold made up of protein filaments. The dynamic network of the filaments is essential for many biological phenomena, such as cell division, chromosomal segregation, and cell movement. Until recently, it was thought that the cytoskeleton was a feature unique to eukaryotes. Analyses of cells using immunofluorescence and live-cell fluorescence microscopy have shown that filamentous structures indeed exist in prokaryotic cells, although their dynamics differ greatly (reviewed in Refs. 1–3). FtsZ, the bacterial homolog of tubulin, is highly conserved in eubacteria and archaea (4). Whereas tubulin is a major component of the mitotic spindle and plays a vital role in chromosomal segregation, FtsZ forms a contractile Z-ring at midcell and drives cytokinesis, which is functionally analogous to the actin contractile ring (5). Although FtsZ is evolutionally distant from tubulin, they both assemble into polymers in a GTP-dependent manner (6, 7). The similarity of their three-dimensional structures further provides evidence that they evolved from a common ancestor (8, 9).

Bacterial low-copy-number plasmids generally encode par genes to achieve proper DNA segregation into daughter cells. The par system consists of three components: a centromeric DNA site, a centromere-binding protein, and a nucleotide hydrolase, which acts as a linear motor (10). By classifying the hydrolase components, the systems fall into three categories because the hydrolases are more distinct than the centromere-binding proteins. Type I partition systems use ParA ATPase proteins with a Walker-type motif, whereas type II systems use actin-like ATPases called ParM. Recently, a new partition system has been identified in virulent Bacillus species (11, 12). The system involves an FtsZ/tubulin-like protein called TubZ or RepX, which is essential for the maintenance of the pBtToxix plasmid from Bacillus thuringiensis (Bt)\(^2\) and the pXO1 plasmid from Bacillus anthracis (Ba) and Bacillus cereus (Bc) (hereafter named Bt-TubZ, Ba-TubZ, and Bc-TubZ, respectively). TubZ polymerizes in a GTP-dependent manner in vitro and treadmills in vivo, i.e. the plus-end of a filament grows in length, whereas the minus-end shrinks, resulting in directional polymerization (13–15). A point mutation that eliminates TubZ GTP hydrolysis negatively affects plasmid stability, which suggests that TubZ is one of the tubulin-based cytoskeletal proteins whose polymer acts as a “cytomotive” filament in the force-
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The generation system to accomplish plasmid partitioning (16). The tubZ gene is co-transcribed with tubR, a gene that encodes a DNA-binding protein in the tubRZ operon. Genetic analyses of the minimal replicon of the pBtoxis and pXO1 plasmids showed that the tubRZ operon is essential for maintaining the plasmids in host cells (11, 13). In B. thuringiensis, TubR (Bt-TubR) binds to the promoter region of the tubRZ operon, negatively regulates its transcription, and presumably recruits TubZ to the partition site (13, 15, 16). Although the gene organization of the tubRZ operon is similar among virulent bacilli, Ba-TubZ and Bc-TubZ, which are 98% identical, are quite divergent from Bt-TubR, sharing only 21% amino acid identity (4, 14). Recently, the crystal structures of Bt-TubZ were determined, revealing that it belongs to the FtsZ/tubulin superfamily of proteins (16, 17). Electron microscopy studies showed that, unlike FtsZ or tubulin, Bt-TubZ forms a double helical filament similar to the polymer structure of the actin-like ATPase ParM (14, 17). To elucidate the detailed mechanism of Bc-TubZ filament formation, we determined the crystal structures of Bc-TubZ in the apo-, GDP-, and GTPγS-bound forms. Together with biochemical analysis, we found that the M loop mediates filament stabilization in Bc-TubZ, which suggests that diverse mechanisms facilitate molecular recognition in filament formation for plasmid segregation.

EXPERIMENTAL PROCEDURES

Cloning, Protein Expression, and Purification—The tubZ gene from B. cereus ATCC 10987 (Bacillus Genetic Stock Center) was amplified by PCR from genomic DNA and cloned into pET21d with or without a stop codon for the wild-type protein and the protein containing a C-terminal histidine tag, respectively. All proteins were expressed in E. coli strain BL21 (DE3). Wild-type protein was purified from the bacterial extract by 40% saturated ammonium sulfate precipitation, followed by HiTrap Q ion exchange chromatography (GE Healthcare). Histidine-tagged proteins were purified with HisTrap HP, followed by ion exchange and size exclusion chromatographies using Resource Q and Superdex 200 (GE Healthcare), respectively.

The Bc-TubZ protein with a C-terminal truncation (Bc-TubZΔ, residues 1–389) was characterized by limited proteolysis of wild-type Bc-TubZ with the thermolysin protease. The protein fragment was analyzed by N-terminal sequencing and mass spectroscopy. The Bc-tubZΔ and Bc-tubR genes were cloned into pET28a using Ndel and NotI in-frame with an N-terminal histidine tag and an additional tobacco etch virus protease recognition sequence. After cleavage of the tag by the histidine-tagged tobacco etch virus protease, the protein was loaded onto a Resource Q column (18). Fractions containing Bc-TubZΔ were pooled and concentrated to 0.5 mM for crystallization. Selenomethionine-substituted Bc-TubZ was expressed at high levels, crystallization trials of wild-type Bc-TubZ failed. Because Bc-TubZ was susceptible to proteolysis during purification, we proteolytically digested Bc-TubZ and identified the stable fragment. The truncated fragment Bc-TubZΔ (residues 1–389) was expressed as a fusion protein with an N-terminal histidine tag, purified to homogeneity, and crystallized with or without GTPγS. The apo-Bc-TubZ structure was determined by multilength anomalous diffraction. Although crystallization screening for the
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GTP-bound form of Bc-TubZΔ was performed in the presence of the slowly hydrolyzable GTP analog GTPγS, we were only able to obtain crystals of Bc-TubZΔ in complex with GDP, as judged by the \( F_o - F_c \) omit electron density map (supplemental Fig. S1). The structure of GTPγS-Bc-TubZΔ was only obtained by soaking GTPγS into GDP-Bc-TubZΔ crystals. The structures of both GTPγS-Bc-TubZΔ and GDP-Bc-TubZΔ were solved by molecular replacement using the apo-Bc-TubZΔ structure as a search model. The model of apo-Bc-TubZΔ consists of residues 3–60, 62–63, 68–79, and 89–376 (Fig. 1A). The asymmetric unit contains a monomer that has two functional domains, an N-terminal GTP-binding domain and a C-terminal GTPase-activating domain, as seen in the FtsZ structure (25, 26). Crystallographic packing and gel filtration analyses showed that apo-Bc-TubZΔ is monomeric (supplemental Fig. S2). Three regions adjacent to the guanine nucleotide-binding pocket (residues 61 and 64–67 in the T2 loop and residues 80–88 in the T3 loop) (supplemental Fig. S3) are disordered and exhibit no clear electron density. The base recognition loop T5 is positioned above the guanine base is tightly recognized by the T5 loop. When the protein binds to the nucleotide, the corresponding regions become well ordered and form hydrogen bonds to the nucleotide (Fig. 1, B and C). Furthermore, the conformational change occurs within the C-terminal portion of Bc-TubZΔ and its neighboring helix H5 (Fig. 1D). In the apo-form, the H5 helix region is partially unfolded and makes a kink at Ala-163, thus contacting H11 in a parallel manner by forming hydrophobic interactions among Leu-157 and Leu-160 in H5 and Val-366 and Ile-369 in H11 (supplemental Fig. S2C). On the other hand, H11 in the nucleotide-bound form pivots around Leu-362 by 50°, which leads H11 to contact H5 in an antiparallel manner (Fig. 1E). Lys-377 in H11 forms a hydrogen bond with Glu-161 in H5. The surrounding residues, Leu-157, Leu-160, Ile-369, Ile-373, and Ile-376, create a hydrophobic core that stabilizes the interaction. We presume that this conformational change is caused by the structural instability of apo-Bc-TubZΔ because the structures of apo-Bt-TubZΔ and GDP-Bc-TubZΔ are identical, despite a lack of primary sequence similarity (16). Although the C terminus of Bc-TubZ is proteolytically sensitive and is suggested to be locally unfolded, the intramolecular interaction of the tail seems to stabilize Bc-TubZ because the melting points of Bc-TubZ and Bc-TubZΔ are 59.0 and 54.8 °C, respectively (supplemental Fig. S4). The GDP-Bc-TubZΔ crystal structure reveals two molecules in the asymmetric unit, which is semisymmetric in the crystal lattice (see below). H11 of GDP-Bc-TubZΔ is directed toward the nearest longitudinal subunit. Nucleotide binding induces the conformational change of H5, by which H11 may convey an activating signal of Bc-TubZ polymerization to the adjacent subunit. In the GTPγS-bound structure, one of the GDP molecules is replaced with GTPγS in the same nucleotide-binding pocket, which induces no conformational change in the vicinity of GTPγS (supplemental Fig. S1).

Structural Comparison of TubZ and FtsZ Proteins—To date, the crystal structures of Bt-TubZ and Clostridium botulinum phage C strain Stockholm TubZ (Cb-TubZ) have been determined (16, 17, 27). Superposition of GTPγS-Bc-TubZΔ with GTPγS-Bt-TubZΔ and apo-Cb-TubZΔ shows that their structures resemble one another (root mean square deviation of 2.3 Å in 326 Ca positions and 2.5 Å in 291 Ca positions, respectively) (Fig. 2A). The N-terminal domain is structurally well conserved among the FtsZ/tubulin family proteins and is related to typical
GTPases with a Rossmann fold, such as p21\textsuperscript{ras} (8). The GTPase domain of Bc-TubZ (residues 1–200) shares relatively low sequence homology (25%) with the N terminus of \textit{Methanococcus jannaschii} FtsZ but displays significant structural similarity, with a root mean square deviation of 2.5 Å over 170 C\textsubscript{α} atoms (Fig. 2B). Notably, although \textit{B. cereus} and \textit{B. thuringiensis} are closely related species that share a similar genetic background, the GTPase domains from Bc-TubZ and Bt-TubZ show weak sequence conservation (23%), indicating that Bc-TubZ is evolutionarily divergent from Bt-TubZ to a similar extent as FtsZ (4, 28). The GTPase domain of Cb-TubZ shares only 21% sequence identity with that of Bc-TubZ and has a remarkably truncated H6 helix. Furthermore, Cb-TubZ lacks H0, which locates between the N- and C-terminal domains of both Bc-TubZ and Bt-TubZ. The C-terminal domain of the FtsZ/tubulin family proteins is less conserved but has an essential role in the activation of GTP hydrolysis (26). The superposition between the GTPase-activating domains of Bc-TubZ, Bt-TubZ, and Cb-TubZ reveals that no structural reorganization occurs in their C-terminal domains. However, H9 and H10 are structurally divergent among the three TubZ proteins. Because the N-terminal portion of H10 is predicted to participate in the protofilament contacts in Bt-TubZ, the subtle conformational change may affect the subunit-subunit interactions in protofilament formation (17).

Although the subunit-subunit interactions are partly stabilized by H0 in \textit{M. jannaschii} FtsZ, H0 in Bc-TubZ bridges the gap between the N- and C-terminal domains mainly by hydrophobic interactions (supplemental Fig. S5) (8). Bc-TubZΔH0 is attached to H7 in a parallel manner, which is similar to Bt-TubZ H0, although the N terminus of the two \textit{Bacillus} TubZ proteins shows weak sequence homology (16, 17). Furthermore, our limited proteolytic analyses showed that the N terminus of Bc-TubZ is resistant to proteases (data not shown), indicating that H0 possesses an ordered structure that forms the extensive hydrophobic core of TubZ.

\textbf{Longitudinal Contacts between GDP-Bc-TubZΔ Molecules—}

The presence of GDP in Bc-TubZΔ stabilizes the residues

\begin{figure}
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\caption{Crystal structure of Bc-TubZ. A and B, schematic representations of apo-Bc-TubZ and GDP-Bc-TubZ, respectively. α-Helices are colored green, and β-strands are colored orange. Secondary structural elements are labeled in A. The active site loops (T1–T7) and loops on the other side of the molecule (M, T0, and T7) are labeled in B. GDP is shown as a space-filling model. C, close-up view of the active site in the GDP-bound form. The nucleotide recognition loops are colored cyan. Interacting residues are shown as sticks. D, conformational change of H11. The apo-bound form is colored yellow, and the nucleotide-bound form is colored green. The hinge residue Leu-362 is labeled (red arrowhead). E, stereo view of the interaction between H5 and H11. Residues involved in the interaction are labeled and shown as sticks (Glu-161 in magenta, Lys-377 in blue, and hydrophobic residues in yellow). This figure was made using PyMOL (38).}
\end{figure}
around the nucleotide-binding site. The GTPase domain of GDP-Bc-TubZ interacts with the C-terminal domain of the adjacent subunit, which makes a semicontinuous single-stranded protofilament-like arrangement in the crystals (Fig. 3A). This head-to-tail association of GDP-Bc-TubZ is consistent with the crystal structures of FtsZ and Bt-TubZ, in which the proteins form a longitudinal subunit repeat (17, 26). The contact of GDP-Bc-TubZ with two longitudinally neighboring molecules is 1246 Å², covering 26% of the subunit surface. The C-terminal domain of chain B buries 976 Å² of the upper surface of chain A. The interface contains a number of hydrogen bonds and hydrophobic interactions with a molecular spacing of 45 Å. This 45-Å molecular spacing is very similar to that in a previous study examining the structure of Bt-TubZ (17). Together with the evidence that GTPγS was hydrolyzed in the crystal structure of GDP-Bc-TubZ, this observation suggests that the subunit-subunit interaction with the 45-Å spacing reflects the protofilament contact. On the other hand, the subunit contact on the opposite side of chain A is 270 Å² with a molecular spacing of 52 Å, which is too far for the protein-protein interactions. The arrangement of the molecules of the 52-Å contact is considered to be a crystallographic artifact because the molecular interaction with the 45-Å spacing gives a slight bend to the dimer formation, and hence, a molecular gap is required for crystallization.

Soaking the GDP-Bc-TubZ crystals with GTPγS was successful only for the chain B molecule. The conformation of the loops surrounding the nucleotide is essentially the same between the GDP-Bc-TubZ and GTPγS-Bc-TubZ structures, except that the oxygen atoms of the γ-phosphate group in GTPγS make hydrogen bonds with the backbone nitrogen atoms of Gly-124 and Thr-125 in the T4 loop (supplemental Fig. S1B). This observation is consistent with the previous structures of Bt-TubZ and FtsZ showing that the protein fold itself is the same in the apo-, GDP-, and GTP-bound forms. The GDP molecule in chain A is buried in the GTP-binding pocket and covered by the T7 loop of chain B, which makes nucleotide exchange difficult. This explains why the nucleotide in Ba-TubZ polymers is GDP (14). The subunit-subunit interface of the 976-Å² contact is robust. There are three unique contacts that resemble the subunit-subunit interactions found in FtsZ and Bt-TubZ. The T7 loop, which mediates GTP hydrolysis but not nucleotide binding, covers the vicinity of GDP in the lower subunit and enables the flanking H8 helix to interact with the T3 loop by forming a salt bridge between Glu-238 and Arg-85 (Fig. 3B). In the crystal structure of GTPγS-Bt-TubZ, Arg-87 (Arg-85 in B. cereus) forms a hydrogen bond with the γ-phosphate group of GTPγS and is suggested to be a key residue in the nucleotide switch mechanism (17). Glu-238 is one of the conserved acidic residues in the FtsZ/tubulin superfamily proteins that catalyzes GTP hydrolysis. The subunit-subunit interaction of GDP-Bc-TubZ in the post-GTP hydrolysis state is somewhat stabilized by the hydrogen bonds not only between Arg-85 and Glu-238 but also between Arg-85 and Glu-332 in the S9 strand. Although Arg-85 and Glu-238 are conserved in Bt-TubZ (Asp-269 in B. thuringiensis corresponding to Glu-238), Glu-332 is replaced with Gly-358 in B. thuringiensis. In the GTPγS-Bt-TubZ structure, the flanking residue Lys-359
forms a hydrogen bond with the γ-phosphate of the nucleotide. This basic residue presumably stabilizes the transition state of GTP hydrolysis, which is also seen in the RhoA-RhoGAP system (29). In GDP-Bc-TubZΔ, Arg-333 is positioned similarly to Lys-359 in B. thuringiensis but does not form any intra- or intermolecular contacts with any other residues. Moreover, few basic residues are capable of catalyzing the phosphoryl transfer reaction in the vicinity of the phosphate groups, which indicates that further structural analysis of the GTP-bound form is required to elucidate the GTP hydrolysis mechanism of Bc-TubZ. The H10-S9 loop is called the M loop, which is involved in subunit-subunit interactions and has the most divergent

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**Figure 3.** Protofilament interaction in Bc-TubZ. A, semicontinuous GDP-Bc-TubZΔ protofilament structure in the crystal lattice. The N-terminal GTPase domain is shown in green, the H7 helix in yellow, and C-terminal domain in magenta. The molecular spacing of 45 Å is shown in the middle, whereas the subunit interfaces with less interaction are shown at the top and bottom (52 Å spacing). Note that the 45 Å spacing is similar to that observed in the Bt-TubZ crystal structure. The regions containing the subunit-subunit interactions with the 45 Å spacing are boxed. B–D, stereo views of the GDP-binding site, the vicinity of Phe-313, and the C-terminal tail region, respectively. Residues described under "Results" are shown as sticks. In D, Arg-333 is exposed to solvent and makes few contacts with residues in H11. E and F, structural comparison of the GTPγS-Bt-TubZ and FtsZ dimers, respectively. The dimers of GTPγS-Bt-TubZ (chains D and E) (E) and FtsZ (F) observed in the crystal structures are shown. The orientation of the molecules and the color scheme are the same as for GDP-Bc-TubZΔ in A. The lower subunits are superimposed. The GTPγS-Bt-TubZ dimer is twisted, whereas GDP-Bc-TubZΔ and FtsZ are not.
sequence among the FtsZ/tubulin family proteins. Our finding shows that the assembly mechanism is not strictly conserved between the TubZ proteins.

The N-terminal portion of S9 makes contacts with the sugar-binding loop T5 in the lower subunit. T5 is further stabilized by H10 in the upper subunit, with a hydrogen bond between Arg-152 and Glu-320 (Fig. 3B). H10 creates a hydrophobic environment together with the H6-H7 loop in the lower subunit. Phe-313 is inserted into the hydrophobic groove formed by Tyr-189, Leu-200, and Tyr-204 and makes non-polar contacts with the aliphatic atoms of Arg-380 make hydrophobic contacts with Tyr-313 in the H10-S9 loop of the upper subunit (Fig. 3C). Leu-200 bridges the gap between the T5 and T7 loops by contacting Phe-229, suggesting that T5 contributes to the subunit-subunit interactions in a cooperative manner with T7. The third contact exists at the C-terminal domain of the adjacent subunit, where the aliphatic atoms of Arg-380 make hydrophobic contacts with Tyrs surrounding the upper subunit is highly acidic and is possibly involved in the association with the basic C-terminal tail, which was trimmed for the crystallographic analyses (supplemental Figs. S3 and S6). The tail of Bc-TubZ likely stabilizes the protofilament as well as the monomer structure and provides the binding site for DNA that may interact with Bc-TubR encoded in the tubRZ operon (30).

It should be noted that, although the monomer structures of Bc-TubZΔ and Bt-TubZ are very similar to each other, the filamentous structure of GTPγS-Bt-TubZ shows a right-handed twist, whereas the dimer structure of GDP-Bc-TubZΔ has no twist (Fig. 3, A and E). The subunit orientation of GDP-Bc-TubZΔ resembles more closely that of the FtsZ dimer (Fig. 3F). There are some possible reasons for the difference of the twists between the two TubZ proteins. One may consider that, because GDP-Bc-TubZΔ is in the post-GTP hydrolysis state, Bc-TubZ may change the subunit arrangement when it hydrolyzes GTP. Another possibility is that the Bc-TubZΔ dimer may be distorted because of crystal packing. The biochemical examination of the subunit interface is required for Bc-TubZ.

**GTPase Activation Switch**—To characterize Bc-TubZ polymerization activity, we first determined the critical concentration of Bc-TubZ needed for GTP hydrolysis (supplemental Fig. S7). Bc-TubZ at different concentrations was incubated in the polymerizing buffer, and the change in NADH concentration was measured by its absorbance at 340 nm. The critical concentration was 0.18 μM, which is very similar to that previously reported for Bc-TubZ (0.2–0.4 μM) (14). To test the effect of an artificial tag, we next measured the GTP hydrolysis and self-assembly levels of Bc-TubZ with a C-terminal histidine tag (Bc-TubZ-His) (Fig. 4A). Bc-TubZ-His showed a similar level of activity as the wild-type protein, consistent with the previous electron microscopy study of Bt-TubZ (17). On the other hand, the Bc-TubZΔ construct showed little activity at 2 μM, indicating that the C terminus is critical for GTP hydrolysis. In solution, Bc-TubZΔ is unstabilized because of the lack of the C-terminal tail and is deficient in forming polymers. In the GDP-Bc-TubZΔ crystal lattice, despite the fact that the C-terminal tail is deleted, the subunit-subunit contact is tightly arranged and results in GTP hydrolysis. Our data imply that the C terminus of Bc-TubZ plays a role in fine-tuning TubZ assembly. Structural analysis of Bt-TubZ also confirms this observation, where Bt-TubZ lacking the C-terminal tail forms polymers at high concentrations (10–50 μM) but tends to aggregate readily (16).

Because the polymerization activity of FtsZ is coupled to the GTP hydrolysis rate, we examined whether the polymerization is related to the GTP hydrolysis activity (31). We performed light scattering assays for Bc-TubZ and its mutants in the presence of 0.5 mM GTP and 5 μM GTPγS (Fig. 4B). The wild-type protein showed a rapid increase in turbidity up to ~60 s, followed by a gradual decrease. Consistent with the GTPase activity measurements, the C-terminal histidine tag did not affect assembly, whereas Bc-TubZΔ completely abolished polymerization, which indicates that GTP hydrolysis of Bc-TubZ is coupled to self-assembly.

Our GDP-Bc-TubZΔ structure suggests that the hydrogen bonds between Arg-85 and two glutamate residues (Glu-238 and Glu-332) are crucial for both the subunit-subunit interactions and GTP hydrolysis. To inspect the protofilament model of Bc-TubZ, we constructed three point mutants (R85A, E238A, and E332A) of Bc-TubZ-His and examined their GTPase activities (2 μM). B, the kinetics of Bc-TubZ polymerization were monitored by measuring the increase in light scattering. A protein concentration of 2 μM was used.

**FIGURE 5.** GTP hydrolysis and self-assembly of Bc-TubZ mutants. A, the GTPase activities of 2 μM Bc-TubZ-His and its mutants were measured. The R85A and E238A mutations abolished the GTPase activity, whereas the activity of Bc-TubZ-His(E332A) was 40% of that of the wild type. The R333A mutation had no effect on GTPase activity. B, light scattering analyses of Bc-TubZ-His mutants. The relative activity of each reaction was determined by comparing the turbidity after a 60-s incubation. The polymerization activity was consistent with the GTPase activity, except for Bc-TubZ-His(E238A).
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FIGURE 6. Effect of Bc-TubR and DNA in Bc-TubZ polymerization. A, Bc-TubR binding to Bc-TubZ polymers. Sedimentations of Bc-TubZ and Bc-TubZ-His were performed in the presence or absence of Bc-TubR. BSA binding was also examined as a negative control. B, GTPase activity of 2 μM Bc-TubZ in the presence or absence of 2 μM Bc-TubR and 3 nM pBS_Bc-tubRZ.

reports showing that the T3 loop makes contacts with the γ-phosphate group and acts as a GTP hydrolysis switch (32, 33). The E238A mutation in T7 abrogated the GTPase activity as well. However, this mutant assembled into polymers two times more effectively than the wild type (Fig. 5B), which indicates that E238A forms stable polymers but is deficient in GTP hydrolysis. This result is in agreement with the previous analysis of Bt-TubZ in vivo, demonstrating that Bt-TubZ(D269A), equivalent to Bc-TubZ(E238A), assembles into stable filaments in the cell but results in severe plasmid segregation defects (15). The E332A mutant retained 40% of the original activity in both the GTPase and polymerization analyses. Indeed, the critical concentration of the E332A mutant was increased by 3-fold to 0.52 μM. On the other hand, a mutation in the flanking residue Arg-333 (R333A-His) led to similar levels compared with the wild type, indicating that Glu-332, but not Arg-333, is involved in the GTP hydrolysis reaction. Thus, our data show that the M loop participates in protofilament formation, stabilizes the filament structure, and supports GTP hydrolysis, although the residues involved in the process differ between the two species.

Plasmid Partitioning—Although there are still arguments as to whether TubZ contributes to replication or segregation of the Bacillus virulence plasmids, studies have shown that the polymerization activity of TubZ is essential for plasmid stability (11, 12, 15). Because TubZ displays a high level of GTPase activity, polymerizes into a double helical filament, and treadmills in vivo, it has been suggested that TubZ works as a cytomotive filament, such as the type II plasmid partitioning ATPase ParM, i.e. TubZ assembles into filaments to produce a driving force and achieves partitioning by pushing plasmids attached to the growing ends of filaments (17). In the parMRC system, ParM forms a stable polymer in the presence of the ParR-parC complex in vivo (34). By binding to parC, ParR plays a dual role in transcriptional repression and segregosome organization of the R1 plasmid, which provides a nucleation site for the assembly of ParM (35, 36). In contrast, TubZ polymerization is not dependent on TubR but rather on TubZ protein concentration in vivo (15, 37). Although the function of TubR in the type III plasmid segregation system is not yet clear, given that Bt-TubR represses the transcription of the tubRZ operon and forms a ternary complex with the centromeric DNA and Bt-TubZ, one may speculate that Bt-TubR forms a pBtoxis segregosome and recruits Bt-TubZ to segregate the plasmid into the daughter cells (16). To test the interaction between Bc-TubR and Bc-TubZ polymers, we carried out sedimentation of polymerized Bc-TubZ with or without Bc-TubR (Fig. 6A). Bc-TubR was co-sedimented with Bc-TubZ polymers in vitro but did not enhance Bc-TubZ sedimentation. This result was confirmed by the GTPase activity analyses of Bc-TubZ with Bc-TubR, which showed that Bc-TubR did not promote the GTPase activity of Bc-TubZ (Fig. 6B). Consistent with this, negative-stain EM images showed that, in the presence of Bc-TubR, Bc-TubZ formed similar polymers compared with Bc-TubZ alone (supplemental Fig. S8). Co-sedimentation of Bc-TubR with Bc-TubZ-His polymers was also examined because the C-terminal tail of Bc-TubZ is critical for ternary complex formation between Bc-TubZ, Bc-TubR, and DNA. The additional tag at the C terminus of Bc-TubZ abolished the interaction with Bc-TubR. These results indicate that Bc-TubR interacts with Bc-TubZ polymers and that the Bc-TubZ tail is required for Bc-TubR association. We further examined the GTPase activity of Bc-TubZ in the presence of Bc-TubR and the plasmid carrying the tubRZ operon. Unlike the parMRC system, the complex of Bc-TubR and the plasmid did not stimulate the GTPase activity of Bc-TubZ, which suggests either that the GTPase activity is not essential or that other cellular components are required for the activation of the cytoskeletal function of Bc-TubZ.

DISCUSSION

Although our data show that the residues involved in the GTP hydrolysis mechanism are not strictly conserved between Bc-TubZ and Bt-TubZ, their overall structural properties resemble each other. A significant feature of TubZ lies in the C-terminal tail, which plays a critical role in both the structural stability of TubZ and TubR association. The previous electron microscopy study of the Bt-TubZ filament showed that the C-terminal tail is exposed to solvent, which is consistent with our proteolytic analysis of the Bc-TubZ polymer, demonstrating that the C-terminal tail is sensitive to thermolysin (data not shown). The tail of TubZ comprises several basic residues and likely binds both TubR and DNA. Moreover, our Bc-TubZ sedimentation analysis suggests that Bc-TubR is capable of associ-
ating with the Bc-TubZ filament in the absence of DNA, raising the question of whether Bc-TubR recognizes the specific site of Bc-TubZ filaments. One possibility is that centromeric DNA may define the higher order structure of the segrosome by Bc-TubR association, and thus, the segrosome can recognize distinct sites of the Bc-TubZ filament at a specific location in the cell. With regard to the TubR recognition sequence, although the binding sequence of Bt-TubR has been identified as four 12-bp imperfect direct repeats located upstream of the tubR gene, no similar sequence has been found in the corresponding region in pXO1 (13). Instead, a 24-bp inverted repeat down-stream of the tubZ gene has been predicted to be the putative centromeric DNA in B. anthracis (11). Because there are certain varieties of the centromere-binding proteins in the plasmid partition systems, the molecular mechanisms may not be simple, even among virulent Bacillus species. Finally, the segregation model has been proposed for plasmid transport by TubZ; the TubZ filament works as a tram and gives the plasmid a ride to its destination by interacting with TubR (16). If this is the case, how does the complex composed of TubR and the plasmid recognize the destination and detach from the tram? Mecha-nistic details of the interaction between the segrosome and the TubZ filaments will provide insights into the new plasmid partition system and the novel function of the cytoskeletal proteins.

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Shota Hoshino and Ikuko Hayashi

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