In vitro assembly and GTP hydrolysis by bacterial tubulins BtubA and BtubB

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A recent study identified genuine tubulin proteins, BtubA and BtubB, in the bacterial genus *Prosthecobacter*. We have expressed BtubA and BtubB in *Escherichia coli* and studied their in vitro assembly. BtubB by itself formed rings with an outer diameter of 35–36 nm in the presence of GTP or GDP. Mixtures of BtubB and BtubA formed long protofilament bundles, 4–7 protofilaments wide (20–30 protofilaments in the three-dimensional bundle). Regardless of the starting stoichiometry, the polymers always contained equal concentrations of BtubA and BtubB, suggesting that BtubA and B alternate along the protofilament. BtubA showed negligible GTP hydrolysis, whereas BtubB hydrolyzed 0.40 mol GTP per min per mol BtubB. This GTPase activity increased to 1.37 per min when mixed 1:1 with BtubA. A critical concentration of 0.4–1.0 μM was indicated by light scattering experiments and extrapolation of GTPase versus concentration, thus suggesting a cooperative assembly mechanism.

Introduction

The tubulin family includes α- and β-tubulin, which are the main subunits of eukaryotic microtubules; γ-tubulin, which nucleates these microtubules and regulates their dynamics at the minus end; and a number of other tubulins involved in the structures of basal bodies and centrioles (McKean et al., 2001). FtsZ, a prokaryotic homologue of tubulin, is the major cytoskeletal protein in bacterial cell division and is found in nearly all bacteria and archaea. FtsZ is much more distant in terms of sequence than any eukaryotic tubulins. Until recently, recognizable members of the tubulin family were found exclusively in eukaryotes. However, Jenkins et al. (2002) reported the discovery of two new tubulin sequences, *btuba* and *btubb*, found in the genome of the bacterium *Prosthecobacter dejongeii* (division *Verrucomicrobia*). The corresponding proteins BtubA and BtubB were shown by BLAST analyses to share 31–35% and 34–37% sequence identity with eukaryotic α- and β-tubulin, respectively, and only 8–11% identity with FtsZ sequences. They are therefore true tubulins and not FtsZ. Interestingly, based on the 95% complete sequence of the genome, *Prosthecobacter* does not contain FtsZ, a trait only shared by the phyla *Chlamydiae*, *Planctomycetes*, and *Crenarchaeota*, and the species *Ureaplasma urealyticum* (Erickson, 2000; Margolin, 2000; She et al., 2001; Glockner et al., 2003; Vaughan et al., 2004).

The presence of *btuba* and *btubb* genes in *Prosthecobacter* likely originated from a horizontal gene transfer from a eukaryote. However, the significant sequence differences between *btuba/b* and eukaryotic tubulin mean that they have evolved considerably since the transfer event. Furthermore, BtubA and BtubB apparently play a beneficial role in *Prosthecobacter* because their corresponding genes were not lost over evolutionary time, and very similar genes are found in three other *Prosthecobacter* species.

Our efforts have been directed toward characterizing BtubA and BtubB in vitro. Because these proteins share similarity with tubulin, we examined two main characteristics of tubulin—self-assembly and GTPase activity. BtubA and BtubB share several assembly characteristics with FtsZ and tubulin, and also have some important differences. Therefore, these proteins offer a potentially powerful genetic system, like FtsZ, that can help unravel the mysteries of tubulin assembly dynamics.

Results and discussion

The studies described here were done in either HMK (pH 7.7) or MM (pH 6.5) buffers. HMK is closer to physiological conditions in *Escherichia coli* cytoplasm (Cayley et al., 1991; Record et al., 1998), but MM has been used for many assembly studies of FtsZ and tubulin. BtubA alone did not form any recognizable polymer structures in the presence of either GDP or GTP. However, BtubB did undergo self-association, forming rings that appeared to be one subunit thick (Fig. 1 a). The outside diameter of these rings, 35–36 nm, is significantly larger

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than the 23- and 25-nm diameter of FtsZ and γ-tubulin rings (Zheng et al., 1995; Erickson and Stoffler, 1996; Erickson et al., 1996; Oegema et al., 1999), but is actually very close to the 38-nm diameter of αβ-tubulin single rings (Voter and Erickson, 1979). Assuming that the BtubB subunits are 4 nm apart, a ring one subunit thick would contain ~24 subunits with a 14.8° bend at each interface. Also, these rings sometimes formed what appears to be a double ring or short spiral (unpublished data). Rings were only formed with BtubB that had been exposed to GXP. GDP-induced rings persisted for at least 30 min after treatment with saturating amounts of GTP. The BtubB rings thus seem stable in either GTP or GDP, in contrast to tubulin (Melki et al., 1989) and FtsZ (Lu et al., 2000), which are stabilized preferentially by GDP.

Although BtubB formed only rings by itself, and BtubA failed to assemble under various conditions, mixtures of the two formed bundles of linear protofilaments in the presence of GTP (Fig. 1 b). The bundles were typically 4–7 protofilaments wide and appeared to have irregular packing. Measurement of these bundles under various conditions gave an average width of 24 nm. They lack the regular lateral packing of protofilaments in the microtubule wall and more closely resembled the bundles of FtsZ filaments induced by calcium (Lu et al., 2000). These BtubA/B bundles were reproducibly formed at various protein concentrations, as well as in both the HMK (Fig. 1 b) and MM buffers (unpublished data). Some rings persisted along with the protofilament bundles, although they were much less abundant than the bundles.

Time-course EM experiments demonstrated a complicated pattern of assembly. To observe these early stages of assembly it was necessary to look at BtubA/B protein mixtures in the 1–2-μM range, just slightly above the critical concentration (see below). At higher concentrations the assembly was too fast to make specimens of early assembly forms. The earliest polymers, formed in less than 1 min, appeared to be short pairs of protofilaments (Fig. 1 c). After several minutes the paired protofilaments were longer, and in places added a third protofilament (Fig. 1 d). These two- and three-protofilament bundles appeared to have a twist, as segments showing clearly separated protofilaments alternated with segments where the separation was obscured. Where the separation was clearest, the protofilaments were separated (center to center) by ~0.7 nm. Finally, after 15 min numerous bundles 4–7 protofilaments wide were observed (Fig. 1 e). These are similar to the protofilament bundles formed at higher protein concentrations after two min of assembly (Fig. 1 d). FtsZ assembles primarily into single protofilaments, and assembly is much faster, reaching a plateau in only a few seconds (Chen et al., 2005). FtsZ protofilaments can form bundles under certain conditions (Gonzalez et al., 2003; Oliva et al., 2003), but the single protofilament is a stable product of assembly in many conditions. Tubulin also forms protofilaments, but these are never seen isolated. Rather they associate by lateral bonds to make a two-dimensional sheet of protofilaments that will become the microtubule wall (Erickson, 1974). The microtubule protofilament sheet is only one layer thick, and much more regular than the three-dimensional bundles of BtubA/B protofilaments.

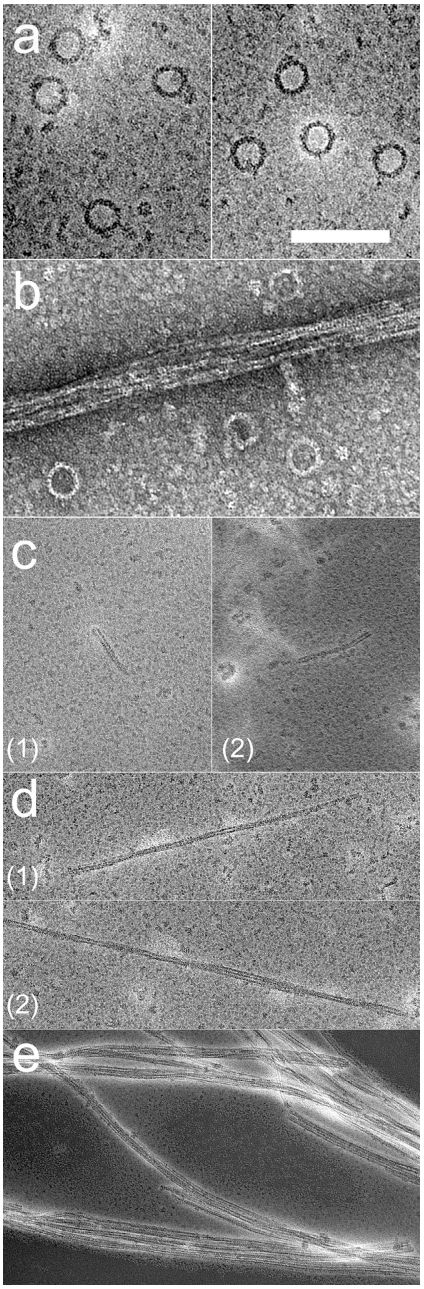

Figure 1. **Negatively stained EM.** (a) Rings formed by BtubB alone (5 μM). (b) A mixture of rings and protofilament bundles formed by BtubA + BtubB (5 μM each). Bar, 100 nm (for a and b). (c–e) Time-course EM for the polymerization of BtubA/B (1.2 μM each in HMK buffer) after addition of 200 μM GTP. (c) Short two-protofilament-wide bundles appear early in the reaction, 45 s. (d) At 2.5 min protofilament bundles are longer and add a third protofilament. (e) At 15 min protofilament bundles are longer and wider. Bar, 200 nm (for c–e).

Thin-section EM was used to further explore the structure of the protofilament bundles. Longitudinal sections showed slightly irregular bundles of parallel protofilaments similar to those seen in negative stain (Fig. 2 a). In cross section, most of the bundles showed an irregular profile with a diameter averaging 43 nm. This is similar to the width in negative stain, but the three-dimensional structure suggests that the bundles actually contain 20–30 protofilaments. In some instances the bundles

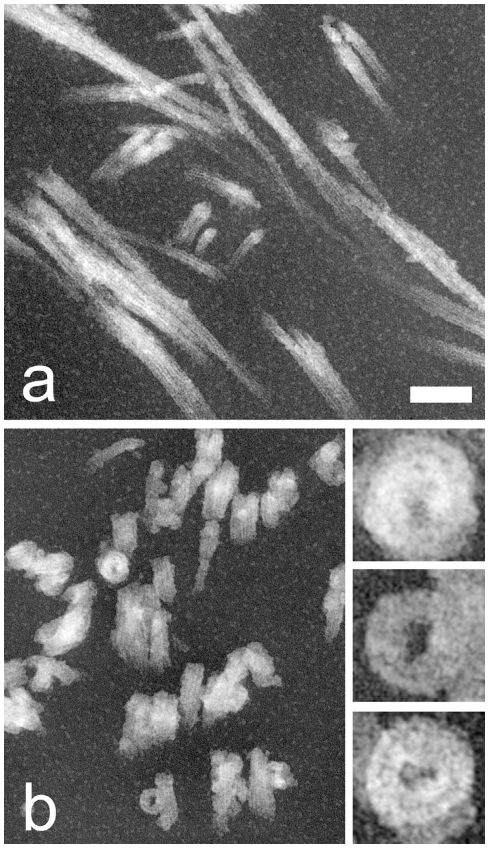

Figure 2. **Thin sections of BtubA/B polymers assembled in HMK buffer with 1 mM GTP.** (a) A longitudinal section of protofilament bundles. (b) A cross section of protofilament bundles. Three bundles with an apparent lumen are shown in the three panels on the right. The bar represents 200 nm in a and b, and 25 nm in the three panels to the right of b.

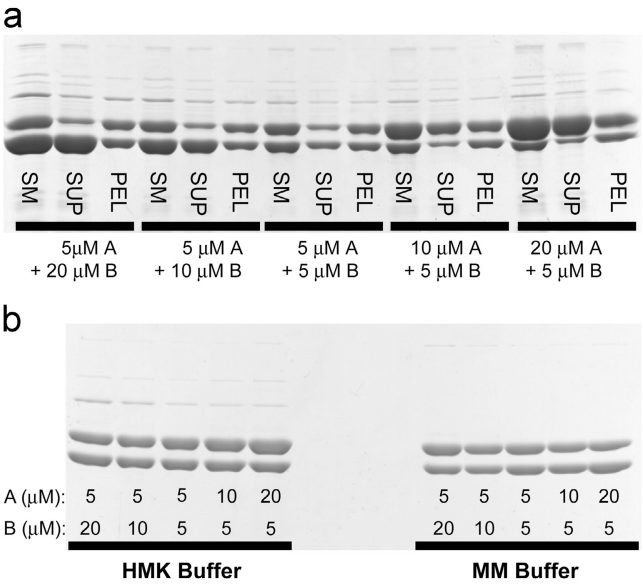

Figure 3. **Assembly of BtubA/B at various molar ratios in HMK buffer with 1 mM GTP, assayed by pelleting polymers.** (a) Starting material (SM), supernatants (SUP), and resuspended pellets (PEL) were analyzed by SDS-PAGE. (b) The same reactions (as shown in panel a) plus reactions in MM buffer, but showing only the resuspended pellets for clarity. BtubA and BtubB are at a 1:1 molar ratio in all polymerized (pelleted) fractions.

showed a hollow tubular profile ~40 nm in outside diameter (Fig. 2 b). These tubular structures may be more numerous than it appears from the section, because the very small lumen can only be resolved when the tubule is precisely oriented in cross section. Thus, a significant fraction of the bundles may actually be tubular. The 40-nm diameter of the tubules is substantially larger than the 25-nm diameter of a microtubule, but the wall is much thicker. The wall occasionally appears to be two-layered (Fig. 2 b, right insets), but overall even the tubular bundles appear to have an irregular packing of protofilaments rather than the regularity of a microtubule wall.

A pelleting assay was performed to investigate the stoichiometry of BtubA and BtubB in the polymers (Fig. 3). Regardless of the initial ratio of the two proteins, from 4:1 to 1:4, they were always equimolar in the pellet. We conclude that the two proteins alternate in the protofilament, like α- and β-tubulin in the microtubule protofilament. It may be that they form a heterodimer before polymerization, but this will need experimental verification.

The amount of guanine nucleotide bound to BtubA and BtubB was measured. Initial studies of purified protein equilibrated in HMK buffer indicated that the ratio of GXP per BtubA was 0.71, whereas for BtubB it was 0.31. Because the purification buffers did not contain any guanine nucleotide and no guanine nucleotide was added to the stored protein in these early preparations, it seemed likely that some of the originally bound GXP had been lost during the purification process. Therefore, these proteins were incubated with an excess of GDP (200 μM) and then free nucleotide was removed and the protein equilibrated into the HMK buffer via a quick-spin G-25 Sephadex resin column. In this case the measured ratio of guanine nucleotide bound per BtubA and BtubB was 1.01 and 1.10, respectively. So, similar to tubulin (Weisenberg et al., 1968) and FtsZ (Lu et al., 1998), BtubA and BtubB each bind one molecule of GXP.

Both tubulin and FtsZ hydrolyze GTP as a part of their assembly reaction, so we next checked to see if BtubA/B assembly involved GTP hydrolysis. When tested individually, BtubB had a significant GTPase activity of 0.40 mol GTP per min per mol BtubB (Fig. 4 a). BtubA hydrolyzed only 0.13 mol GTP per min per mol BtubA, which is a background level that we have observed with inactive mutants of FtsZ (Redick et al., 2005). When BtubB was mixed with an equal amount of BtubA the GTPase increased to 1.37 mol GTP per min per mol BtubB. This is intermediate between the rate of 5–15 per min for FtsZ (Lu et al., 1998) and 0.06 per min for tubulin at steady state (O'Brien et al., 1987). Importantly, the line for the hydrolysis of BtubA/B did not extrapolate to zero, but intersected the x-axis at ~1 μM. This suggests a critical concentration effect, where hydrolysis is negligible below 1 μM, and all BtubA/B above 1 μM generates a constant rate of hydrolysis.

In eukaryotic tubulins the GTP bound to β-tubulin is exchangeable and is hydrolyzed, whereas the GTP on α-tubulin is nonexchangeable and is not hydrolyzed (David-Pfeuty et al., 1977; MacNeal and Purich, 1978). The basis for this difference

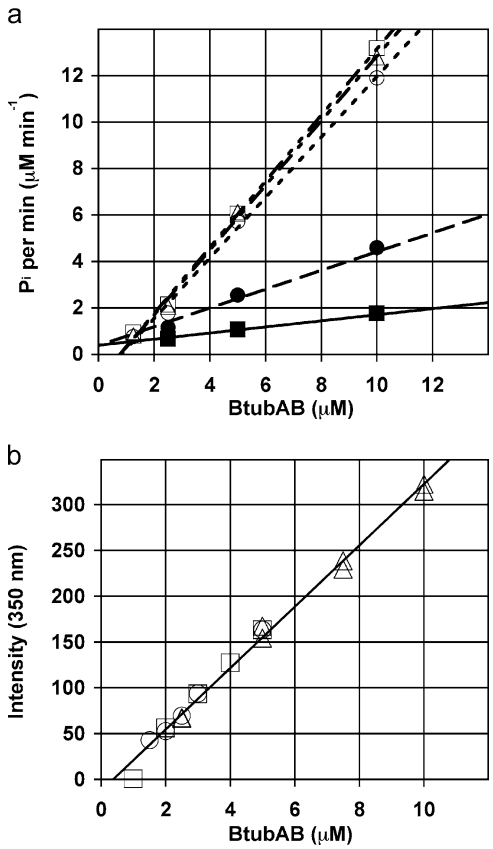

Figure 4. **GTPase activity and light-scattering studies of BtubA/B.** (a) GTPase activity of BtubA/B in HMK buffer. The filled squares are BtubA; filled circles are BtubB; open squares, open circles, and open triangles represent three separate experiments with a 1:1 molar ratio of BtubA and BtubB. (b) BtubA and BtubB were mixed at 1:1 molar ratios in solution followed by the addition of 1 mM GTP at RT. Light-scattering data were collected at 350 nm and the plateau values were plotted versus the corresponding protein concentration. This plot represents the results from three separate experiments that were globally fit to a straight line. The line extrapolates to a critical concentration of 0.4 μM.

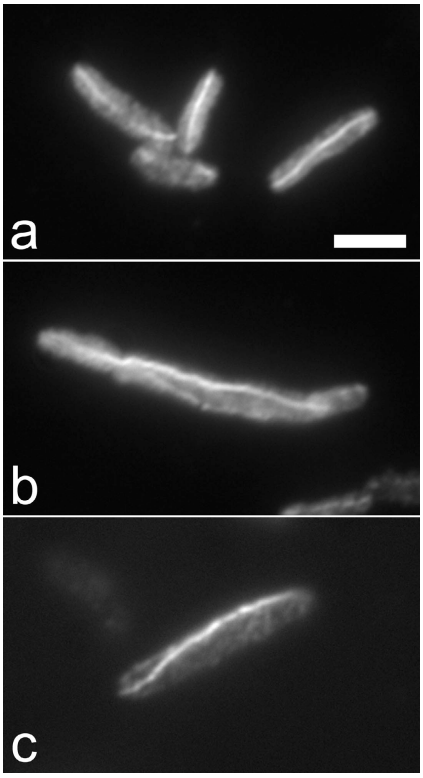

Figure 5. **Immunofluorescent staining of BtubA/B structures in vivo.** *E. coli* expressing both BtubA and BtubB were grown and fixed with a mixture of PFA and glutaraldehyde. Images are from cells that were stained with anti-BtubB antibody. Cells were grown at 37°C with 0.01% arabinose to mid-log phase. Bar, 2 μm.

is largely attributed to the T7 or "synergy" loop on the minus end of the subunit (Erickson, 1998; Nogales et al., 1998). In α-tubulin, a completely conserved E254 contacts the GTP of the β-tubulin below it in the protofilament and contributes to the hydrolysis mechanism. The equivalent D212 in FtsZ is also completely conserved and essential for GTP hydrolysis. In β-tubulin this residue is a lysine, substituting a positive charge for the negative. This is thought to be the reason that there is no hydrolysis at the interdimer interface (α-plus end and β-minus end). Notably, the equivalent residue is glutamate in both BtubA and BtubB, so all interfaces in these protofilaments carry the negative charge and may be competent for GTP hydrolysis. We should note, however, that the synergy loop of BtubA deviates substantially from that of FtsZ and tubulin. This may be the reason why BtubA alone had no GTPase activity.

We have assessed the assembly of BtubA/B via light-scattering experiments (Fig. 4 b). If the polymers are of uniform size and shape, light scattering can be used as a measure of total polymer. The EM results showed the protofilament bundles to be approximately the same diameter after the assembly plateau regardless of the starting protein concentration, so we decided to determine how the light scattering varied with the total protein concentration. As shown in Fig. 5 b, the light scattering was remarkably linear. This suggests that the polymers at the plateau are the same size and shape regardless of the total protein concentration.

As with the GTP hydrolysis, the line did not extrapolate to zero, but intercepted the x-axis at 0.4 μM. This is somewhat less than the 1-μM intercept for the GTPase, but the two separate experiments both suggest a critical concentration of 0.4–1 μM. A critical concentration is a hallmark of cooperative assembly (Oosawa and Kasai, 1962). A more complete understanding of the basis for the cooperativity will require tools for accurately measuring kinetics of assembly.

We wanted to study the structures formed by BtubA/B in *Prosthecobacter*, but we have so far not been able to grow a culture suitable for immunofluorescence. As an alternative we coexpressed BtubA and BtubB in *E. coli*, and examined whether they could form a structure in the *E. coli* cytoplasm. Western blotting confirmed that both proteins were expressed, and that BtubA was several-fold more abundant than BtubB. Immunostaining with the affinity-purified BtubB antibody showed prominent rods running the length of most cells (Fig. 5). Frequently the rods were in the center of the cell (Fig. 5 a), but in other cells they seemed to form a loose spiral at the membrane. This appearance could implicate membrane binding, but it

could also be produced by a slight buckling of the rod as it is pushing on each end of the cell.

The cytoplasm of *Prosthecobacter* is probably similar to that of *E. coli*, so we think it is likely that BtubA/B forms similar rods. *Prosthecobacter* has an elongated spindle shape with a prosthecate (stalk) projecting from one end. The rod of BtubA/B may contribute to both the overall elongated shape and the prosthecate. It is notable that a previous study of *Prosthecobacter* by EM (Jenkins et al., 2002) failed to find anything resembling microtubules. Our present results explain this failure because BtubA/B is seen to form protofilament bundles rather than microtubules. The EM also did not show any structure that would correspond to protofilament bundles. It is possible that they are only a few protofilaments wide and therefore difficult to see in the granular bacterial cytoplasm. We are hopeful that we will be able to visualize *Prosthecobacter* by immunofluorescence in the future.

BtubA/B are probably not essential for the elongated shape of *Prosthecobacter*. The shape of all nonspherical bacteria is produced by the actin homologue MreB, which forms spiral filament bundles under the cell membrane (Jones et al., 2001). The genome of *Prosthecobacter dejongeii* is 95% complete, and three *MreB* genes have been annotated (Bouzek, H., personal communication). These MreB proteins are probably the primary determinants of the elongated shape. The bacterial tubulins may be providing a supplementary structural role.

Mutational analysis is a powerful approach to study assembly and function, and eukaryotic tubulins have been studied extensively (Reijo et al., 1994; Richards et al., 2000; Anders and Botstein, 2001). However, these studies were limited to determining the phenotype in vivo, because of the difficulty of purifying mutant tubulins from yeast. The discovery of BtubA/B provides the first system for readily preparing mutant tubulins for characterization in vitro.

Materials and methods

Growth and induction

The coding sequences of BtubA and BtubB were put into the pET15 expression vector (Novagen) and expressed as NH_2-terminally his-tagged proteins in *E. coli* strain BL21(DE3). Cultures for both proteins were grown in Luria broth medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter of water) supplemented with 100 μg/ml ampicillin at 37°C to A_{600} ~1.0 and induced by adding 1.0 mM IPTG and lowering the temperature to 20°C. After a 3-h induction, bacteria from a 1-liter culture were centrifuged and resuspended in 20 ml of 50 mM Hepes and 300 mM NaCl, pH 7.0 (buffer A). Lysozyme was added to 0.75 mg/ml and mixed at 4°C for 30 min and the culture was frozen overnight at −80°C. The bacteria were thawed and PMSF was added to 1 mM. The bacteria were refrozen and upon thawing 1 mM fresh PMSF was added. Next, DNAasel was added to 10 μg/ml and $MgCl_2$ to 0.01 M, and the bacteria were sonicated three times for 30 s and a fourth time for 1 min. The sonicated bacteria were mixed on a rotator (Cole-Parmer) at RT for 25 min. Cell walls and insoluble debris were removed by centrifuging at 32,000 rpm for 20 min at 4°C in a rotor (Type 42; Beckman Coulter). The supernatant from the spin was collected and fresh PMSF was added to 2 mM. This protein supernatant was placed over a prepared Talon Metal Affinity column (CLONTECH Laboratories, Inc.). The column was washed with buffer A and then bound protein was eluted with buffer A containing 150 mM imidazole. The fractions containing protein were identified by SDS-PAGE and pooled. Finally, the imidazole was removed by dialysis or equilibration over a resin column (PD-10; Amersham Biosciences) into buffer A. The protein was aliquoted and stored in this buffer at −80°C.

Before polymerization experiments, BtubA and BtubB were thawed and incubated with 200 μM GDP for ~30 min on ice (this procedure was later modified by adding GTP at a concentration equal to that of the protein before freezing and storage). The thawed proteins were then equilibrated into either HMK (50 mM Hepes, 5 mM MgAc, 350 mM KAc, and 1 mM EGTA, pH 7.7) or MM (50 mM MES, 2.5 mM MgAc, and 1 mM EGTA, pH 6.5) buffer using Quick Spin columns (Roche). The equilibrated proteins were clarified of any precipitate formed and their concentrations were determined from their A_{280} values. The extinction coefficients were 55,502 M^{-1} cm^{-1} for BtubA and 45,962 M^{-1} cm^{-1} for BtubB, which are based on amino acid composition plus the contribution from 1 mol guanine nucleotide (GXP) per mol of protein.

Construction of dual BtubA/B expression

BtubA and BtubB sequences were Klenow-blunted, and were then put into SmaI-cut pJSB5 (an arabinose-inducible pBAD based vector [Redick et al., 2005]) to make pJSB5-BtubA and pJSB5-BtubB. The BtubB sequence was then removed from pJSB5-BtubB by cutting with SalI and HindIII restriction sites, and was introduced into pJSB5-BtubA downstream of the BtubA sequence by inserting it into SalI- and SphI-cut pJSB5-BtubA, thus forming a BtubAB "operon." The expression plasmid will be called pJSB5-BubA-B.

Electron microscopy

Negatively stained samples were prepared by applying ~10 μl of the assembled BtubA/B to a carbon-coated grid and washing off with 3–4 drops of 2% aqueous uranyl acetate. Electron micrographs were taken at 50,000×.

To prepare samples for cross sectioning, BtubA/B was assembled in a 200-μl volume at equimolar concentrations of 5 or 25 μM with 1 mM GTP at RT in HMK buffer. After ~15 min polymers were fixed with glutaraldehyde, pelleted, and processed for thin sectioning as previously described for FtsZ (Lu et al., 2000).

Guanine nucleotide concentration and hydrolysis

BtubA or BtubB was mixed with a twofold excess of GTP and separated from free nucleotide using a Quick Spin column (Roche) equilibrated in HMK buffer. The concentration of protein was determined by A_{280}, 1/3 volume of perchloric acid was added, and samples were placed on ice for 20 min. In parallel, controls were prepared by using HMK buffer in place of the protein. During the 20-min incubation the samples were periodically mixed. The tubes were centrifuged at 4°C for 5 min at 20,000 *g*. Supernatants were removed and absorbance spectra were read for the BtubA/B samples using the control samples as reference blanks. The concentration of GXP in the supernatants was determined from the A_{256} using an extinction coefficient of 1.24×10^4 M^{-1} cm^{-1} (Bock et al., 1956).

GTPase activity was determined by measuring the concentration of evolved phosphate, using an improved sensitivity malachite green assay (Geladopoulos et al., 1991). Proteins were diluted to the desired concentrations (ranging from 0.5 to 20 μM) in HMK buffer and prewarmed to 37°C. The reaction was initiated by adding GTP to 1 mM. Aliquots were taken at 0-, 1-, 2-, 5-, 10-, and 20-min intervals, and transferred to tubes containing 2.3 volumes water and 3.3 volumes 0.6 M perchloric acid and placed on ice to halt the GTPase reaction. After taking all time points, samples and monobasic potassium phosphate standards were aliquoted in triplicate to microtiter plates and assayed as described in detail elsewhere (Redick et al., 2005).

Assaying polymer by pelleting

GTP was added to a final concentration of 1 mM to 150 μl of various molar ratios of BtubA and BtubB, and 50 μl was removed for a starting sample. The remaining 100 μl (in polycarbonate ultracentrifuge tubes) was centrifuged in the TLA100 rotor (Beckman Coulter) for 20 min at 250,000 *g* at 20°C. The supernatants were removed and the pellets were resuspended in 100 μl of the buffer. Samples were prepared from the starting material, supernatant, and resuspended pellets, and were analyzed by SDS-PAGE.

Assaying polymer by 90° light scattering

Varying concentrations of a 1:1 molar ratio of BtubA and BtubB in HMK buffer (total volume of 100 μL) were loaded in a quartz cuvette with a 1-cm path length. The cuvette was placed in a Shimadzu fluorimeter that had both the excitation and emission wavelengths set at 350 nm and a slit width of 3 nm. A baseline of scattering for the protein mixture without added GTP was established for 20 s and then polymerization was initiated by the addition of 0.2 mM GTP. The nucleotide was introduced into the solution with a pipette followed by mixing. The elapsed time for the nu-

cleotide addition step was typically 15 s. The net change in light scattering after nucleotide addition was recorded until a plateau was established. These plateau values were plotted versus the protein concentration to determine the critical concentration for protofilament bundle assembly.

Immunofluorescence microscopy

pAbs were prepared in rabbits against BtubA and BtubB purified as described above. The antibodies were affinity purified on a column of BtubA or BtubB coupled to CNBr-Sepharose.

The fixation method used was adapted from Stricker and Erickson (2003). An overnight 3-ml culture of the dual *E. coli* strain BW27783 transformed with pJSB5-FtsA-B was diluted 1:200 and allowed to grow for 1 h at 37°C. The culture was then induced with 0.01% arabinose and grown for an additional 2 h. These samples were fixed as described by Addinall et al. (1996), washed three times with PBS plus 0.05% Tween 20 (PBST), and resuspended in 25 mM Tris-HCl, 50 mM glucose, and 10 mM EDTA, pH 8.0 (GTE). 20 μl of cell suspension was placed on a polylysine-coated slide. These cells were allowed to adhere to the slide for 10 min and were permeabilized with 100 μl of 2 mg/ml lysozyme (freshly dissolved in GTE) for ~4 min at RT. The slide was then washed three times with PBST.

Samples were blocked and stained as described by Addinall et al. (1996) using various dilutions of affinity-purified rabbit anti-BtubA (2.15 mg/ml) or B (1.56 mg/ml) as the primary antibody and 20 μg of goat anti–rabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc.)/ml as the secondary antibody. The slide was then washed three times in PBST, mounted in SlowFade Light (Molecular Probes, Inc.) according to the manufacturer's instructions, and sealed with nail polish. Samples were viewed on a microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) using Plan-Neofluar oil immersion lenses (NA = 1.30) at 40 and 100×. Images were captured using a camera (CoolSNAP HQ; Roper Scientific) and manipulated using Adobe Photoshop.

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