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Guanine Nucleotide-Dependent Assembly of FtsZ into Filaments

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FtsZ is an essential cell division protein that is localized to the leading edge of the bacterial septum in a cytokinetic ring. It contains the tubulin signature motif and is a GTP binding protein with a GTPase activity. Further comparison of FtsZ with eukaryotic tubulins revealed some additional sequence similarities, perhaps indicating a similar GTP binding site. Examination of FtsZ incubated in vitro by electron microscopy revealed a guanine nucleotide-dependent assembly into protein filaments, supporting the hypothesis that the FtsZ ring is formed through self-assembly. FtsZ3, which is unable to bind GTP, does not polymerize, whereas FtsZ2, which binds GTP but is deficient in GTP hydrolysis, is capable of polymerization.

FtsZ is an essential cell division protein in *Escherichia coli* (11, 31, 37) and *Bacillus subtilis* (2) and is conserved in other eubacteria (10). In both *E. coli* and *B. subtilis* FtsZ is localized in a ring at the division site (5, 39). In *E. coli*, certain mutant FtsZs lead to an altered ring geometry and abnormal polar morphology, suggesting that FtsZ determines septal morphogenesis (6). The FtsZ ring is formed at midcell before there is visible invagination, is located at the leading edge of the septum throughout division, and is dispersed upon completion of division. One possible explanation for the dynamic behavior of FtsZ is that it undergoes a reversible polymerization reaction (24). Interestingly, FtsZ contains a glycine-rich sequence motif (GGGTGTG) that is important for GTP binding (13, 28, 32). A similar sequence, (A/G)GGTG(S/A)G, is present in eukaryotic tubulins and has been designated the tubulin signature motif (42). In addition, FtsZ has a GTPase activity that shows a dependence upon the protein concentration, suggesting that the GTPase activity is dependent upon protein oligomerization (13, 28, 39). Therefore, we have examined the effect of GTP upon FtsZ oligomerization.

E. coli FtsZ isolated from an overproducing strain migrates as a single species in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) but yields multiple species upon native gel electrophoresis (Fig. 1) (12). The median molecular weight of these species obtained from size exclusion chromatography is 500,000, equivalent to approximately 12 monomers (28). This result indicates that FtsZ self-associates, which has also been inferred from the genetics of *ftsZ* (4, 40).

To determine if the oligomerization state of FtsZ is affected by GTP, purified FtsZ was incubated with GTP at 37°C and examined by freeze-etch electron microscopy (20). Examination of FtsZ incubated in the absence of GTP revealed the presence of particles that were larger (diameters ranging from 5 to 15 nm; Fig. 2A) than expected for FtsZ monomers (4 to 6 nm, assuming a spherical shape) but consistent with the range of sizes estimated from size exclusion chromatography. In contrast, examination of FtsZ after incubation with GTP revealed the presence of numerous fibers of variable length with a calculated diameter of 7 nm (Fig. 2B). This same pattern was observed with FtsZ incubated in the presence of GTPγS, indicating that hydrolysis of GTP was not required for the assembly of FtsZ into filaments. The purified *B. subtilis* FtsZ also produced protein filaments in the presence of GTP that appeared identical to those produced by the *E. coli* FtsZ (data not shown).

Initial attempts to examine filament formation by FtsZ with negative staining were not readily reproducible. This failure suggested that the negative staining disrupted the filaments or that conditions utilized during freeze-etch electron microscopy promoted polymerization. To determine what conditions might favor polymerization, we examined the literature on the in vitro assembly of tubulin into microtubules, which has been well characterized (reviewed in reference 36). Polymerization occurs readily in the presence of microtubule-associated proteins, which lowers the concentration of tubulin dimer necessary to initiate polymerization. In the absence of microtubule-associated proteins polymerization of purified tubulin can be observed in the presence of glycerol and a high Mg^{2+} concentration or with polycations such as DEAE dextran (16). Recently, purified yeast tubulin was shown to assemble in the presence of DEAE dextran (3). To determine if DEAE dextran could promote FtsZ assembly, purified FtsZ was incubated with DEAE dextran with and without GTP and then observed by negative staining. When FtsZ was incubated in the presence of GTP, protein filaments that were 18 nm in diameter and of various lengths were observed (Fig. 3). These filaments tended to bundle, forming aggregates. Occasionally filaments with smaller diameters were observed, although the 18-nm-diameter filament was the predominant species. In the absence of GTP no protein filaments were observed. If the FtsZ was incubated at 4°C in the presence of DEAE dextran and GTP, no filaments were observed, indicating that the assembly was temperature dependent. Attempts to produce assembly of FtsZ in the presence of glycerol and a high Mg^{2+} concentration were unsuccessful; however, a high Mg^{2+} concentration (>15 mM) induced an amorphous aggregation, which was enhanced in the presence of glycerol.

It was unlikely that GTP was hydrolyzed during FtsZ assembly in the presence of DEAE dextran since the assembly buffer lacked K^+, previously shown to be required for GTP hydrolysis (28). Monitoring of GTP hydrolysis confirmed that GTP was not hydrolyzed during the standard 1-h incubation (data not shown). To determine if filament formation by FtsZ was specific for GTP, FtsZ was incubated with a variety of

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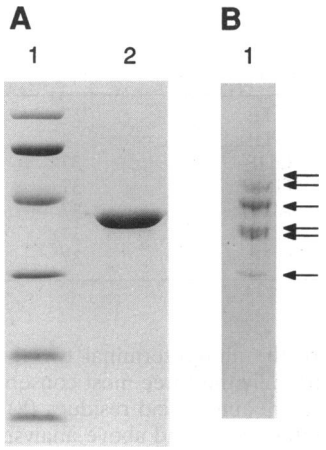

FIG. 1. Gel analysis of purified FtsZ. FtsZ was purified from W3110 containing the expression vector pKD126 as described previously (12, 28). (A) SDS-PAGE of purified FtsZ (lane 2) and molecular weight markers (from the top, phosphorylase *b*, 97.4K; bovine serum albumin, 66K; ovalbumin 45K; carbonic anhydrase, 31K; trypsin inhibitor, 21.5K; and lysozyme, 14.4K) (lane 1). (B) Native gel electrophoresis of purified FtsZ. Electrophoresis was performed on a Pharmacia Phast electrophoretic system using precast, continuous 8 to 25% gradient polyacrylamide Phast gels. The arrows indicate the positions of the most prominent bands.

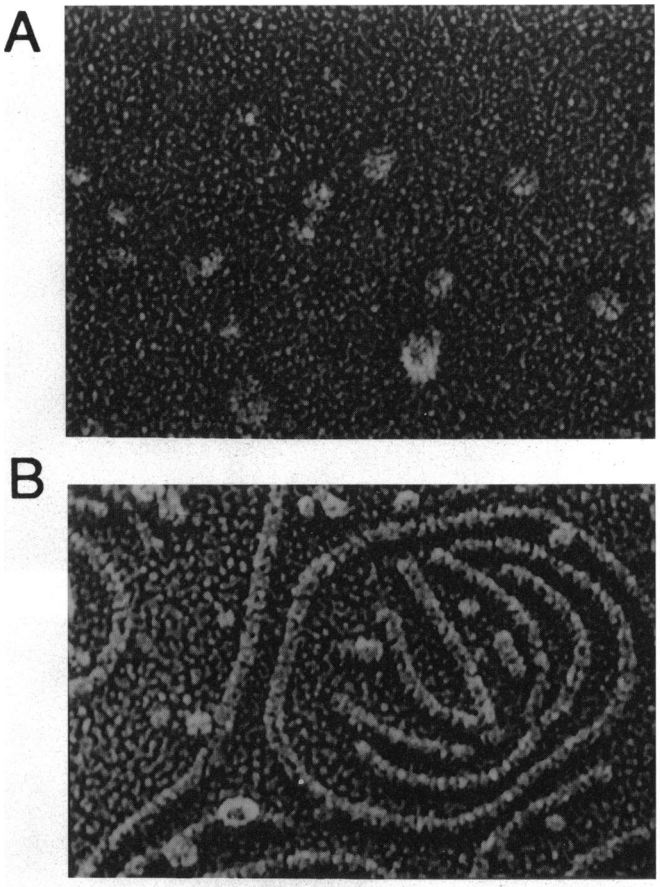

FIG. 2. GTP-induced filament formation of FtsZ observed by freeze-etch electron microscopy. Magnification is ×300,000. Purified FtsZ (purified as described in reference 12) at 300 to 350 μg/ml was incubated in assembly buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.2], 10 mM $MgCl_2$) with (B) or without (A) 1 mM GTP. After 1 h of incubation at 37°C the sample was analyzed by freeze-etch electron microscopy as described elsewhere (20). The incubated FtsZ was diluted 10-fold in buffer [70 mM KCl, 30 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)] and mixed with a slurry of finely ground mica flakes. The mica flakes were washed with the same buffer and quick-frozen by impact against a liquid helium-cooled copper block. The frozen slurry was then freeze fractured and deep etched for 4 min at −100°C in a Balzer freeze-etch unit. The sample was replicated with 2 nm of platinum rotary deposited at 10° above the horizontal and backed with 10 nm of carbon rotary deposited at 75°. The mica was removed by floating the sample on hydrofluoric acid for 18 h at room temperature. The replica was rinsed several times in distilled water and picked up on 400-mesh, Formvar-coated grids. Replicas were examined with a JEOL 100 CM transmission electron microscope.

nucleotides and examined by negative staining. Filaments identical to those seen in the presence of GTP were observed following incubation with GDP but were not observed following incubation with GMP or ATP (Table 1). Since FtsZ binds both GTP and GDP, but not GMP or ATP (28), our results suggest that it is the nucleotide-bound form of FtsZ that is a substrate for polymerization. This conclusion is also supported by analysis of two mutant FtsZ proteins, FtsZ2 and FtsZ3 (4). FtsZ3 contains an amino acid change, T to A at position 108 (T108A), in the glycine-rich cluster and displays dramatically reduced GTP binding and GTPase activity (12, 28). FtsZ2 contains an amino acid change (D212G) in another highly conserved region of FtsZ and displays nearly normal GTP binding but no GTPase activity (12). When examined by negative staining following incubation with GTP and DEAE dextran, FtsZ2 was observed to form typical filaments whereas FtsZ3 did not produce filaments (Table 1).

The similarities between FtsZ and tubulins, the glycine-rich cluster, GTPase activity, and the ability to assemble into filaments in vitro, caused us to further compare the primary sequences of the two proteins to see if additional similarities existed. To compare FtsZ and tubulin, three members (yeast, filamentous fungus, and human) of each of the three families of eukaryotic tubulins (α, β, and γ) were compared with three FtsZs. The alignment shown in Fig. 4 reveals a few additional similarities beyond the glycine-rich cluster. The longest region of extended similarity exists between residues 200 and 260 in tubulin and residues 158 and 217 in FtsZ. Within this region 4 amino acids are invariant in these proteins, possibly defining two additional motifs (A and B in Fig. 4) (25). The first motif (designated A) contains two invariant residues and has the following consensus sequence: DXXZZZXNXXZ (where Z is a large hydrophobic amino acid [L, M, I, V, or F] and X is any amino acid). The second motif (designated B) also contains two invariant residues and has the consensus sequence GXZNXDZXXZ (the N is an H in the *Aspergillus nidulans* γ-tubulin but is an N in all other sequenced tubulins). In addition, only large hydrophobic amino acids are found at five positions between these two motifs. This conservation of large hydrophobic amino acid residues throughout this region suggests that the structures of FtsZ and tubulins in this region are similar.

In addition to the glycine-rich cluster and the region identified above, other regions that contain both invariant and conserved residues can be aligned. To align these regions, gaps have to be introduced in the FtsZ sequences. Significantly, two of these gaps occur where gaps exist among the three tubulin families (positions 40 and 122 in α-tubulin from *Saccharomyces*

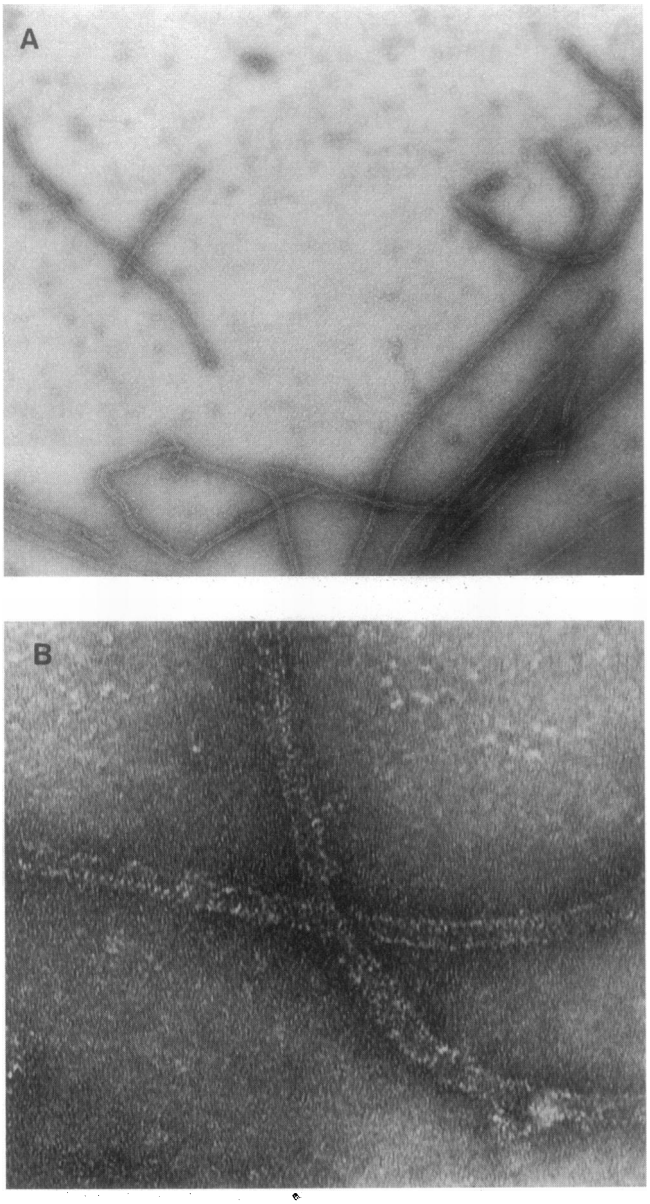

FIG. 3. FtsZ filaments observed by negative staining in the presence of GTP and DEAE dextran. The final magnifications were ×40,000 (A) and ×200,000 (B). FtsZ at 200 μg/ml was incubated in assembly buffer (50 mM HEPES [pH 7.2], 10 mM $MgCl_2$) containing 1 mM EGTA and DEAE dextran (20 μg/ml) and with 1 mM GTP. After 1 h of incubation at 37°C, 5 μl of the sample was spotted directly on a carbon grid, blotted dry, and immediately stained with 1% uranyl acetate. The excess uranyl acetate solution was blotted, and the grids were examined in a JEOL 100-CXII transmission electron microscope.

cerevisiae). Once these gaps are introduced, other invariant and conserved residues, including a D at position 70 in α-tubulin (designated motif D) and V(V/T)XP(Y/F) (motif C) at positions 183 to 187 in α-tubulin, are indicated. The alignment, however, reveals little similarity between FtsZ and tubulins beyond residue 220 in FtsZ (260 in tubulin) and no similarities beyond residue 315 in FtsZ (353 in tubulin). Even among the FtsZs from these divergent bacteria, however, there is very limited homology beyond residue 315 (1, 26). Significantly, the most conserved regions of both the tubulin and

TABLE 1. Filament formation by FtsZ observed by negative staining

Protein	Nucleotide	Temp (°C)	Filaments
FtsZ	GTP	37	+
FtsZ	GTP	4	−
FtsZ	GDP	37	+
FtsZ	GMP	37	−
FtsZ	ATP	37	−
FtsZ2	GTP	37	+
FtsZ3	GTP	37	−

FtsZ families are the amino-terminal two-thirds, and within the tubulin superfamily the three most conserved peptides are found in the first 200 amino acid residues (8).

The experimental results and above analysis of the primary sequence alignments between FtsZs and tubulins may indicate a similar GTP binding site. It is known that α- and β-tubulins and FtsZ interact with guanine nucleotides but do not contain the sequence elements conserved in the GTPase superfamily (7). There is still uncertainty in the composition of the GTP binding site in tubulin, as GTP has been photo-cross-linked to a number of different peptides located in the amino-terminal half of the protein (17, 19, 23, 34). One of these peptides includes the invariant D at position 70 (within motif D) in the yeast α-tubulin sequence (Fig. 4) (23). It is tempting to speculate that the GTP binding sites in tubulin and FtsZ are similar and consist of the glycine-rich cluster (G box), the long homologous region identified above (residues 158 to 217 in *E. coli* FtsZ), and possibly other invariant residues identified in this comparison. Consistent with this suggestion, we have observed that the amino-terminal half of FtsZ, which includes the sequence similarities in Fig. 4, is sufficient for GTP binding (38).

The GTPase activity of *E. coli* FtsZ displays a lag that is dependent upon the protein concentration (13, 28), suggesting that FtsZ must oligomerize to express the GTPase activity. With *B. subtilis* FtsZ no lag is observed in the GTPase activity; however, the specific activity is dependent upon the protein concentration, again indicating that the FtsZ might oligomerize to express the GTPase activity (39). It is possible, therefore, that tubulin is a paradigm for the dynamic behavior of FtsZ, as suggested previously (24). The dynamics of tubulin assembly into microtubules have been intensely studied (reviewed in reference 21). The assembly of the tubulin dimer into microtubules requires bound GTP; however, GTP hydrolysis is not required for the assembly but lags behind (9). With FtsZ we have shown that FtsZ assembly into fibers does not require GTP hydrolysis, since assembly occurs in the presence of a nonhydrolyzable analog of GTP and under buffer conditions (absence of K^+) in which GTP hydrolysis does not occur (28). In addition, we observed assembly in the presence of GDP. Tubulin polymerization usually requires GTP; however, under some conditions polymerization can be observed in the presence of GDP, for example, in the presence of taxol (14). It may be that in the presence of DEAE dextran the equilibrium for FtsZ assembly is shifted towards polymerization and that GDP is sufficient but that other, more physiological conditions may have a strict requirement for GTP.

In this study we have observed the guanine nucleotide-dependent assembly of FtsZ into filaments with diameters of 7 and 18 nm. It is possible that these two different sizes of filaments indicate polymorphism of assembly or that the thin filaments are protofilaments that constitute the larger filaments. The requirement of GTP or GDP for polymerization is

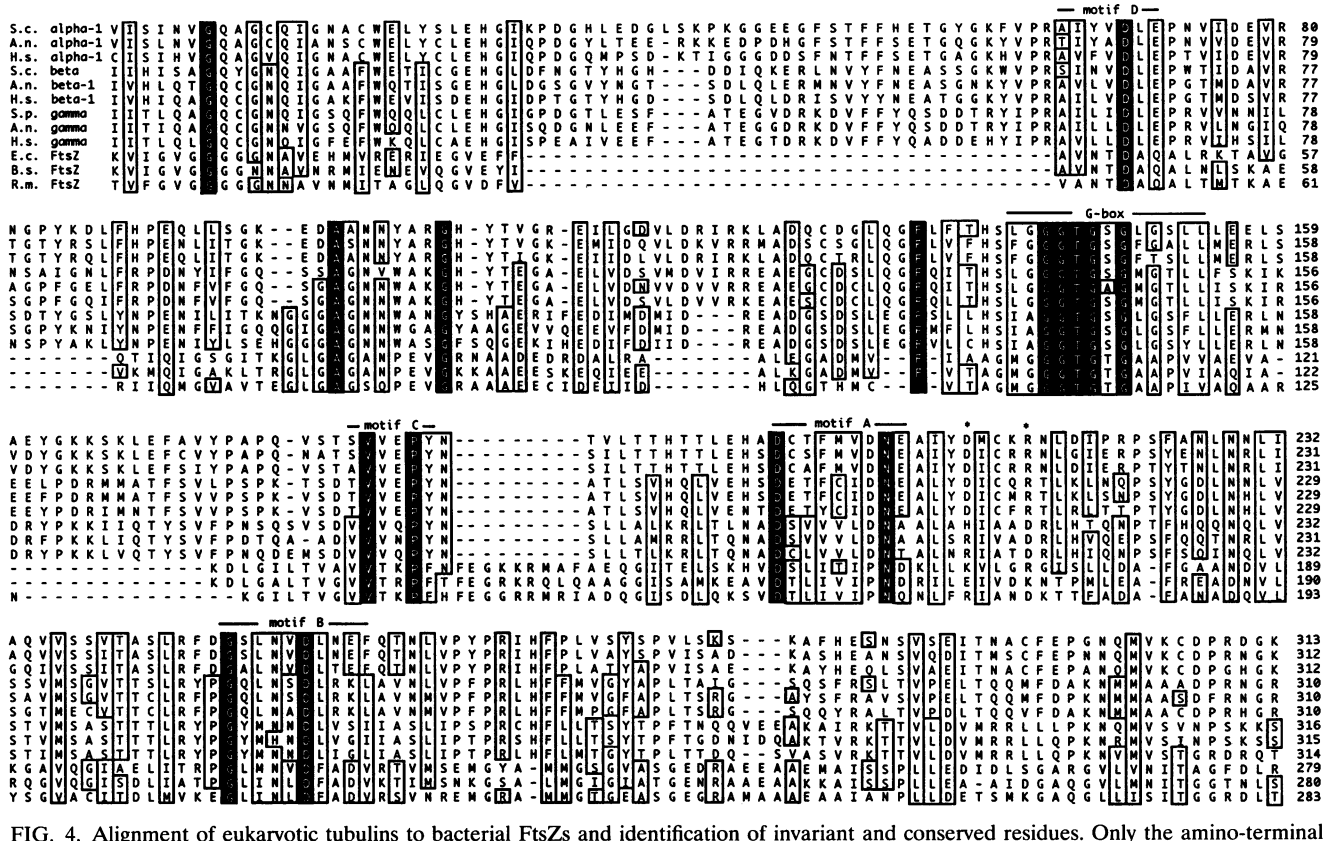

FIG. 4. Alignment of eukaryotic tubulins to bacterial FtsZs and identification of invariant and conserved residues. Only the amino-terminal portions (except for the extreme N termini) of the proteins (approximately 75%), which display homology, are shown. The primary sequences of displayed proteins were aligned by using the Megalign program (DNAStar) with some adjustments made manually following inspection of the initial alignment. For the alignment shown here three members of each tubulin family were chosen from yeasts, filamentous fungi, and humans. They include α-tubulins from *S. cerevisiae* (S.c.) (33), *A. nidulans* (A.n.) (15), and humans (H.s.) (18); β-tubulins from *S. cerevisiae* (29), *A. nidulans* (27), and humans (22); and γ-tubulins from *Schizosaccharomyces pombe* (S.p.) (35), *A. nidulans* (30), and humans (42). The primary sequences of the three bacterial FtsZs are from *E. coli* (E.c.) (41), *B. subtilis* (B.s.) (1), and *Rhizobium meliloti* (R.m.) (26). Invariant residues in all of the proteins in this analysis are indicated by filled-in boxes, and conserved residues are indicated by open boxes. Additional instances of similar residues not conserved in all proteins are boxed only if two of the three FtsZs or 10 of the 12 sequences contain conserved residues. Similar residues were as follows: L, V, I, M, and F; T and S; D, E, Q, and N; and Y and F.

supported by our study of two mutant proteins, FtsZ3 and FtsZ2, for their ability to polymerize (Table 1). FtsZ3 is encoded by a lethal allele (4), deficient in GTP binding (28), and unable to form filaments. In contrast, FtsZ2, which is capable of binding GTP but deficient in GTP hydrolysis (8), is able to polymerize. Interestingly the *ftsZ2* allele can support cell growth but results in a complex phenotype including cell filamentation, minicell formation, resistance to cell division inhibitors *minCD* and *sulA*, and temperature-dependent cell lysis (4). It will be of interest to determine to what extent these phenotypes are due to a direct effect of the *ftsZ2* mutation on the dynamics of polymerization of FtsZ. Also, further work will be required to determine the structure of the filaments that we have observed and to determine if the FtsZ polymerization that we have observed in vitro occurs in vivo. This possibility is feasible, as the concentration at which we observed polymerization of FtsZ in vitro (6 μM) is lower than the estimated in vivo concentration of FtsZ (10 μM).

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