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Published in:
Biochemistry

DOI:
[10.1021/bi011370i](https://doi.org/10.1021/bi011370i)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

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Citation for published version (APA):

Scheffers, D-J., de Wit, J. G., Blaauwen, T. D., & Driessen, A. J. M. (2002). GTP hydrolysis of cell division protein FtsZ: Evidence that the active site is formed by the association of monomers: Evidence that the Active Site Is Formed by the Association of Monomers. *Biochemistry*, 41(2), 521 - 529.
<https://doi.org/10.1021/bi011370i>

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GTP Hydrolysis of Cell Division Protein FtsZ: Evidence that the Active Site Is Formed by the Association of Monomers[†]

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Received June 29, 2001; Revised Manuscript Received October 30, 2001

ABSTRACT: The essential prokaryotic cell division protein FtsZ is a tubulin homologue that forms a ring at the division site. FtsZ forms polymers in a GTP-dependent manner. Recent biochemical evidence has shown that FtsZ forms multimeric structures in vitro and in vivo and functions as a self-activating GTPase. Structural analysis of FtsZ points to an important role for the highly conserved tubulin-like loop 7 (T7-loop) in the self-activation of GTP hydrolysis. The T7-loop was postulated to form the active site together with the nucleotide-binding site on an adjacent FtsZ monomer. To characterize the role of the T7-loop of *Escherichia coli* FtsZ, we have mutagenized residues M206, N207, D209, D212, and R214. All the mutant proteins, except the R214 mutant, are severely affected in polymerization and GTP hydrolysis. Charged residues D209 and D212 cannot be substituted with a glutamate residue. All mutants interact with wild-type FtsZ in vitro, indicating that the T7-loop mutations do not abolish FtsZ self-association. Strikingly, in mixtures of wild-type and mutant proteins, most mutants are capable of inhibiting wild-type GTP hydrolysis. We conclude that the T7-loop is part of the active site for GTP hydrolysis, formed by the association of two FtsZ monomers.

FtsZ is a key prokaryotic cell division protein, forming a structural element known as the Z-ring at the site of cell division (for reviews, see refs 1–3). FtsZ has been identified in most prokaryotic species studied to date (reviewed in ref 4) and is conserved across the evolutionary divide, being essential for division of chloroplasts and mitochondria in some eukaryotes (5, 6). In *Escherichia coli*, the Z-ring is critical for the localization of all other protein components of the cell division machinery (4). The Z-ring is likely to consist of polymers similar to the FtsZ polymers that can be formed in vitro in a GTP-dependent manner (7–9). In *E. coli*, the Z-ring is thought to be tethered to the cytoplasmic membrane by ZipA (10).

FtsZ is a 40.3 kDa protein, that binds and hydrolyzes GTP (11–13). It shows a striking structural similarity to α - and β -tubulins, both as a monomer and when modeled onto protofilaments (Figure 1A) (14–17). It shares with tubulins a unique GTP-binding motif, the G-box [GGGTG(ST)G] (12). Recent in vivo data suggest that FtsZ is present in the cytosol as multimeric structures (18). Analytical ultracentrifugation has shown that FtsZ associates into multimeric structures and that Mg²⁺ induces indefinite linear oligomerization of FtsZ in the presence of GDP, which is enhanced in the presence of crowding proteins (19–21). Although the

formation of FtsZ polymer protofilaments may be non-cooperative (20–22), various reports have indicated that the GTPase activity of FtsZ occurs only at FtsZ concentrations that allow the formation of large FtsZ polymers (19, 23–25). The latter evidence indicates that the GTPase activity of FtsZ is self-activated, i.e., that association of FtsZ monomers triggers the GTPase activity of FtsZ. This is in line with our previous finding that FtsZ polymers consist mainly of GDP bound FtsZ (26).

Structural analysis of the organization of FtsZ subunits in polymers, as well as described mutants, point to an important role for the T7-loop region (“tubulin-loop” no. 7) (27) of FtsZ in self-association of monomers. The loop is also known as the HC1-loop, since it includes the first short α -helical structure of the C-terminal part of the protein, H8 (14). The T7-loop is highly conserved among FtsZ proteins from different species (Figure 1B) and shows homology to the Mg²⁺-binding loop of the Walker B consensus sequence for ATPases (28, 29). Mutations in *E. coli* FtsZ affecting GTP hydrolysis occur either in the T7-loop or in domains involved in nucleotide binding as judged from the crystal structure (14). Modeling of the crystal structure of *Methanococcus jannaschii* FtsZ1 onto electron microscopy images of protofilaments of FtsZ1 suggests that the active site for GTP hydrolysis may be shared by two FtsZ subunits, with the GTP-binding domain located on one monomer and modulation of hydrolysis by the T7-loop of the other domain (Figure 1A) (15). The model closely resembles the organization of tubulin in filaments (17). A strictly conserved Glu254 in the α -tubulin T7-loop is thought to reach into the active site of β -tubulin to activate the GTPase activity of β -tubulin, whereas the strictly conserved Lys254 of β -tubulin renders

[†] This work was supported by Grant 805-33-222 from the Division for Earth and Life Sciences (ALW) of the Dutch Organization for the Advancement of Scientific Research (NWO).

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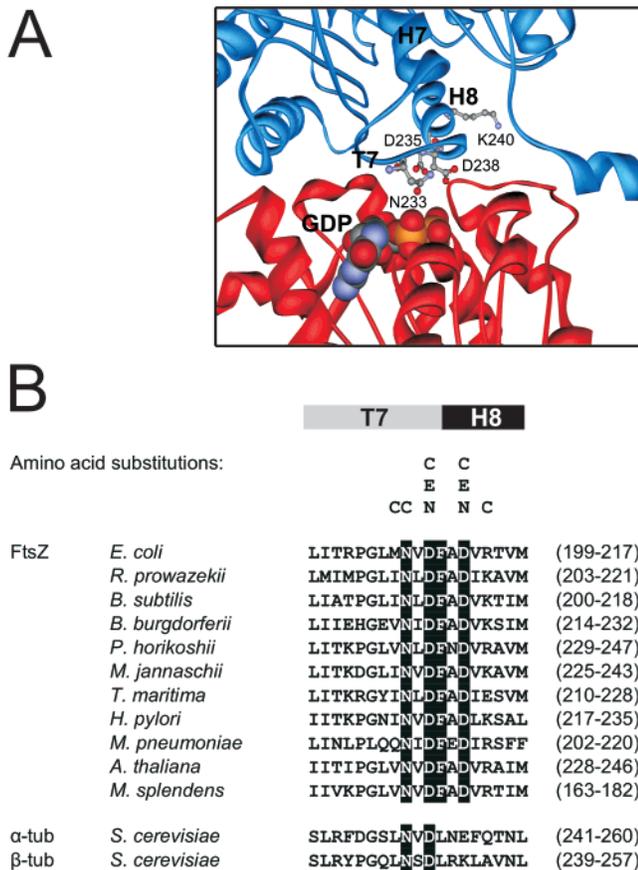


FIGURE 1: (A) Model of the nucleotide binding site of FtsZ in a protofilament and alignment of the T7-loop region of FtsZ. (A) The crystal structure of *M. jannaschii* FtsZ1 [PDB accession code 1FSZ, (14)] was oriented according to the α , β -tubulin heterodimer structure [PDB accession code 1TUB, (16)] as described by Löwe and Amos (15). Residues N233, D235, D238, and K240 of *M. jannaschii* FtsZ1 are the equivalent of *E. coli* FtsZ N207, D209, D212, and R214, respectively. (B) Alignment of FtsZ from different bacterial and eukaryotic species, and yeast α - and β -tubulin. The T7-loop region and the following helix 8 are indicated in gray and black at the top. Amino acid changes described in this paper are indicated at the corresponding positions above the *E. coli* FtsZ sequence. Fully conserved residues are highlighted; other conserved groups are indicated using ClustalX terminology (44). A BLAST search was performed with *E. coli* FtsZ, and an evolutionary tree was drawn with proteins with less than 60% positives. FtsZs from representative branches were selected and aligned using ClustalX 1.8 (44). *E. coli* FtsZ (SWISSPROT: FTSZ_ECOLI), *Rickettsia prowazekii* FtsZ (SWISSPROT: FTSZ_RICPR), *Bacillus subtilis* FtsZ (SWISSPROT: FTSZ_BACSU), *Borrelia burgdorferii* FtsZ (GENBANK: C70137), *Pyrococcus horikoshii* FtsZ1 (SWISSPROT: FTZ1_PYRHO), *Methanococcus jannaschii* FtsZ1 (SWISSPROT: FTZ1_METJA), *Thermotoga maritima* FtsZ (GENBANK: AAC24604), *Helicobacter pylori* FtsZ (GENBANK: D71873), *Mycoplasma pneumoniae* FtsZ (SWISSPROT: FTSZ_MYCPN), *Arabidopsis thaliana* chloroplast FtsZ (GENBANK: AAC35987), *Mallomonas splendens* mitochondrial FtsZ (GENBANK: AAF35433), *Saccharomyces cerevisiae* α - and β -tubulin (GENBANK: AAA35181 and CAA24603).

α -tubulin inactive as a GTPase (residue numbers correspond to bovine brain tubulin) (27). The Glu254 residue of α -tubulin corresponds to Asp212 in *E. coli* FtsZ. The *E. coli* SulA resistant *ftsZ2* mutant carries a D212G mutation, which abolishes the GTPase activity of FtsZ but not GTP binding (30, 31). Engineered N207D and D209N mutations bind GTP, but display a severely reduced GTPase activity (32), while R214A and R214K mutations do not alter GTPase

activity (19). Recently, we have shown that replacement of the D212 residue with cysteine or asparagine, but not with glutamate, allows purification of GTP-containing mutant protein. The D212C and D212N mutants could be polymerized by the addition of cations, indicating that the nucleotide binding site in the polymer contains a cation that is coordinated by D212 (33).

Although the T7-loop seems to be involved in GTP hydrolysis, it is unlikely that it is critical in the formation of FtsZ dimers. A study of 10 randomly generated mutations that abolished the FtsZ–FtsZ interaction in a λ -hybrid assay revealed only one mutant with two amino acid substitutions of which one mapped in the T7-loop, F210A (34).

In this paper, we present a mutational analysis of the T7-loop. Various T7-loop residues were mutated on the basis of their position in the interaction domain (Figure 1A) and their possible role in GTP hydrolysis. Cysteine mutants of residues M206, N207, D209, D212, and R214 were tested for polymerization, GTP binding, and hydrolysis. Polymerization was studied with and without Ca^{2+} , which induces FtsZ–polymer bundling and stabilizes the polymers (35, 36). D209 and D212 were also replaced with asparagine and glutamate to evaluate the role of the negative charges. All mutants except R214C displayed different polymerization and GTP hydrolysis behavior as compared to wild-type FtsZ. Interaction studies of the mutant proteins with wild-type FtsZ revealed that most mutants suppress the GTP hydrolysis activity of the wild-type FtsZ. These data provide the first conclusive biochemical evidence that the T7-loop is part of the active site, formed by two FtsZ monomers, for GTP hydrolysis of FtsZ.

EXPERIMENTAL PROCEDURES

FtsZ Mutagenesis and Protein Purification. FtsZ mutagenesis was carried out using the Stratagene Quick-Change mutagenesis kit with the *ftsZ* expression plasmid pRRE6 (26) as a template. Mutations were confirmed by DNA sequencing (BioMedical Technology Center, Groningen University Hospital, NL). Wild-type and mutant proteins were expressed in *E. coli* BL21 (DE3), except for mutant FtsZD212E, which was expressed in *E. coli* BL21 (DE3, pLysS) (Promega). Purification of wild-type and mutant proteins was as described (26). Proteins were concentrated using Centrprep-30 concentrators (Amicon).

GTP Hydrolysis Assay. GTPase activity was monitored by measuring the release of phosphate using a colorimetric phosphate assay (37). A phosphorus stock solution (Sigma Diagnostics) was used for calibration. FtsZ (at concentrations indicated in the text) was incubated at 30 °C in buffer A (50 mM Mes/NaOH, pH 6.5, and 50 mM KCl) with MgCl_2 and CaCl_2 as indicated. The reaction was started by the addition of GTP to 1 mM. At different time points during a 30-min interval, 30 μL samples were withdrawn and transferred to 200 μL of a malachite green solution on ice. After 5 min, 30 μL of 34% w/v citric acid was added, and the absorption at 660 nm was read immediately with a Spectramax 340 plate reader (Molecular Devices Corp).

Thin-Layer Chromatographic Analysis of GTP Binding and Hydrolysis. Thin-layer chromatography (TLC) was used to assay for binding and hydrolysis of GTP by FtsZ and mutant proteins. Protein (0.2 mg/mL) in buffer A with MgCl_2 as indicated was equilibrated at 30 °C. 20 μM of [α - ^{32}P]-

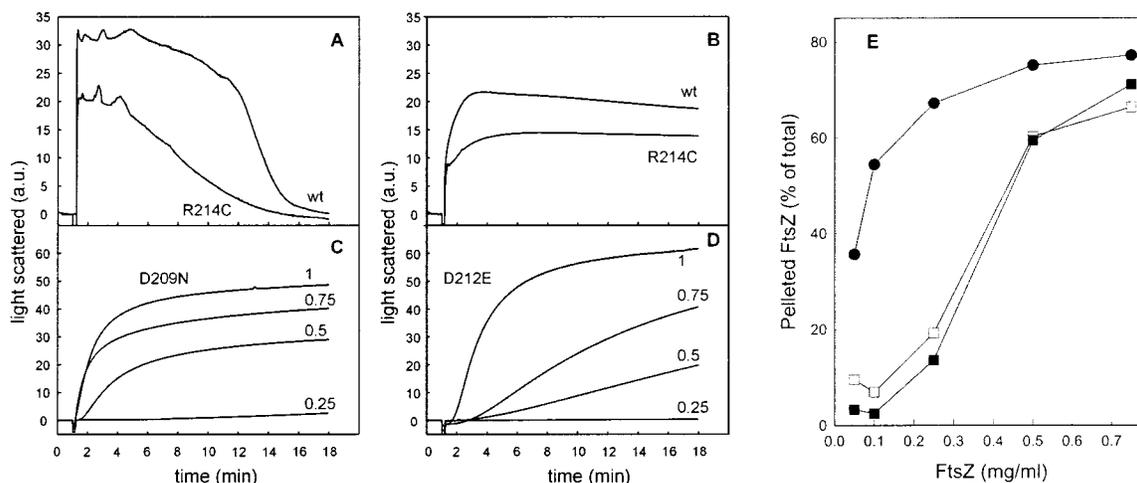


FIGURE 2: Polymerization of wild-type and mutant FtsZ. Polymerization of wt FtsZ and R214C in the absence (A) and presence (B) of calcium, and of D209N (C) and D212E (D) in the presence of calcium and at a protein concentration of 0.25, 0.5, 0.75, and 1 mg/mL. Protein (0.5 mg/mL, unless indicated otherwise) was incubated at 30 °C in buffer A supplemented with 10 mM MgCl₂ (A) or 5 mM MgCl₂ and 10 mM CaCl₂ (B–D) for 1 min before the addition of 0.5 mM GTP. Polymer formation was monitored by 90° light scattering. For panel A, the photomultiplier was set to 350 V; for panels B, C, and D it was set to 275 V; therefore, the signal amplitude cannot be compared between panel A and panels B, C, and D. (E) Concentration dependence of the polymerization of FtsZ (●), D209N (□) and D212E (■). Varying concentrations of the proteins were polymerized with 1 mM GTP for 20 min at 30 °C in buffer A supplemented with 5 mM MgCl₂ and 10 mM CaCl₂. Polymers were recovered by sedimentation (10 min, 20 °C, 280000g), and the amount of polymerized protein was determined by CBB-stained SDS–PAGE.

GTP (33 mCi/mmol, Amersham) was added to the protein mixtures, after 5-min aliquots were withdrawn. Protein was precipitated using ammonium sulfate, and the nucleotide content was determined using TLC as described (26). The relative amounts of bound nucleotide were determined by densitometric scanning.

Light Scattering. Light scattering experiments were performed using an Aminco Bowman Series 2 spectrometer (SLM Instruments) as described (26). Excitation and emission wavelengths were set to 350 nm, with slit widths of 4 nm. Protein (concentrations indicated in the text) was incubated in 200 μ L of buffer A (with MgCl₂ and CaCl₂ as indicated), in a fluorescence cuvette with a 1-cm path length. The sample was maintained at 30 °C. After 1 min of data collection, GTP was added to 0.5 mM, with a sample volume increase of 1 μ L.

Electron Microscopy. Protein (concentrations indicated in the text) was polymerized with 0.5 mM GTP at 30 °C in buffer A with MgCl₂ and CaCl₂ as indicated. Samples were prepared for electron microscopy as described (26) and viewed in a Philips 400T transmission electron microscope (a gift of A. Knoester, Shell Research and Technology Center, Amsterdam, NL).

Sedimentation of FtsZ Polymers. Polymerization of FtsZ was determined by sedimentation essentially as described (24). FtsZ, FtsZD209N, and FtsZD212E (concentrations indicated in the text) were incubated in buffer A with 5 mM MgCl₂ and 10 mM CaCl₂ at 30 °C. Polymerization was induced with 1 mM GTP, and after 20 min, samples were centrifuged for 10 min at 20 °C in a Beckman TLA 100 ultracentrifuge at 280000g. Supernatant and pellet fractions were recovered, run on SDS–polyacrylamide gels, and stained with Coomassie Brilliant Blue. The relative amount of protein present in the pellet fractions was determined by densitometric scanning.

Sedimentation of Polymer Mixtures. FtsZ (0.2 mg/mL) was mixed with mutant protein (concentrations indicated in the

text) in buffer A with 5 mM MgCl₂ and 10 mM CaCl₂ at room temperature (RT). Polymerization was induced with 1 mM GTP, and after 5 min, samples were centrifuged in a Beckman airfuge as described (26). To identify the cysteine mutants, the pellet fractions were resuspended and labeled with 3-(*N*-maleimidopropinyl)biotin (biotin–maleimide) (Molecular probes) for 10 min at RT. The samples were run on SDS–polyacrylamide gels followed by Western blotting onto PVDF membrane (Roche). Labeled protein was detected using a streptavidin–alkaline phosphatase conjugate (Boehringer Mannheim).

Chemical Modification of Cysteine Residues. Sulfhydryl-specific methanethiosulfonate (MTS)¹ reagents and *N*-ethyl-maleimide (NEM) were used to modify D209C, D212C, and R214C. Protein was incubated with MTS-ethylammonium (MTSEA), MTS-ethylsulfonate (MTSES) (both from Anatrache), and *N*-ethyl-maleimide (NEM) (Sigma) at 2.5, 10, and 0.5 mM respectively, and labeled for 10 min at RT (38). Labeled proteins were used immediately.

RESULTS

Construction of T7 Loop Mutants. To investigate the role of the T7-loop in FtsZ function, we used site-directed mutagenesis to replace methionine 206, asparagine 207, aspartate 209, aspartate 212, and arginine 214 with a cysteine residue (Figure 1). Aspartate residues 209 and 212 were also replaced with asparagine and glutamate residues. All mutant proteins, except D212E, were overexpressed to levels similar to wild-type (wt) FtsZ and purified (not shown). For expression of the D212E mutant protein *E. coli* strain BL21(DE3)pLysS was used since the expression plasmid was toxic to strain BL21(DE3), probably due to promoter leakage.

Mutant Polymerization Activities. To characterize the in vitro properties of the T7-loop mutants, we first analyzed

¹ Abbreviations: MTSEA, methanethiosulfonate-ethylammonium; MTSES, methanethiosulfonate-ethylsulfonate; NEM, *N*-ethyl-maleimide; DTT, 1,4-dithio-DL-threitol.

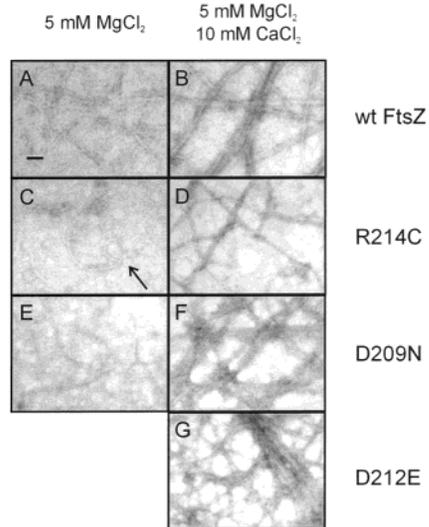


FIGURE 3: Electron microscopy of negatively stained FtsZ and mutant FtsZ polymers. (A and B) wt FtsZ (0.2 mg/mL); (C and D) R214C (0.2 mg/mL); (E and F) D209N (0.4 mg/mL); and (G) D212E (0.4 mg/mL). The arrow indicates the extreme curvature of the R214C filament. Polymerization was performed with 0.5 mM GTP for 10 min at 30 °C in buffer A with 5 mM MgCl₂ (panels A, C, and E) or 5 mM MgCl₂ and 10 mM CaCl₂ (panels B, D, F, and G). Bar: 100 nm.

Table 1: Summary of the Most Important Characteristics of T7-Loop Mutants

mutant	GTP binding ^a (% of wt FtsZ)	GTPase activity ^b (% of wt FtsZ)	polymerization ^c		copolymer with wt FtsZ ^e	inhibition of wt FtsZ GTPase activity
			without Ca ²⁺	with Ca ²⁺		
M206C	15	31	–	–	+/-	–
N207C	27	7	–	–	–	+
D209C	177	18	–	–	+/-	+
D209E	10	23	–	–	+/-	+
D209N	203	17	+/-	+	+	+
D212C	87	17	+ ^d	+ ^d	N/A ^f	+
D212E	37	16	–	+	+	+
D212N	93	37	+ ^d	+ ^d	N/A ^f	+
R214C	21	64	+	+	N/D ^g	–

^a GTP binding as a percentage of [α -³²P]-GTP bound to wt FtsZ (see Figure 4A). Binding was corrected for background in the absence of added FtsZ. ^b GTPase activity as a percentage of wt FtsZ activity determined in polymerization buffer with 5 mM MgCl₂. ^c Polymerization was determined using light scattering and electron microscopy. +, polymers; –, no polymers; +/-, a low amount of polymers was detected with electron microscopy. ^d Electron microscopy revealed nucleotide independent cation-induced polymerization (33). ^e Copolymerization was monitored by light scattering. +, copolymerization; –, no copolymerization; +/-, slow incorporation into polymers. ^f N/A, not applicable: mutants could not be used for light scattering assay. ^g N/D, not detectable: light scattering signals from wt FtsZ and R214C could not be separated.

whether the mutants were capable of polymerization with GTP using light scattering and electron microscopy. Mutant R214C polymerizes to a lesser extent as wt protein, but acts similar to the wild type with instant polymerization upon GTP addition independent of Ca²⁺ (Figure 2A and Figure 3C) and increased bundling in the presence of 10 mM Ca²⁺ (Figure 2B and Figure 3D). M206C, N207C, D209E, and D209C were incapable of polymerization as studied by light scattering and electron microscopy both with and without Ca²⁺ (not shown, Table 1). D209N and D212E showed polymerization in the presence of Ca²⁺ (Figure 2C,D and

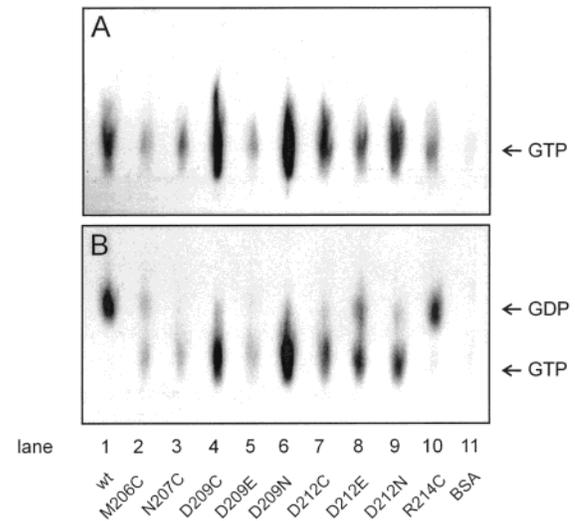


FIGURE 4: GTP binding and hydrolysis of the mutant FtsZ proteins. (A) FtsZ and mutant proteins (0.2 mg/mL) were incubated in buffer A with 20 μ M of [α -³²P]-GTP (33 mCi/mmol) at 30 °C. BSA (0.2 mg/mL) was used as a negative control. After 5 min, the protein nucleotide content was analyzed by precipitation with ammonium sulfate followed by nucleotide extraction. (B) As in panel A, with 5 mM MgCl₂ added to buffer A. Lanes: (1) wt FtsZ; (2) M206C; (3) N207C; (4) D209C; (5) D209E; (6) D209N; (7) D212C; (8) D212E; (9) D212N; (10) R214C; and (11) BSA.

Figure 3F,G) in a protein concentration-dependent fashion (Figure 2E). The gradual increase observed in the light scattering represents slow polymer bundling as evidenced by electron microscopy (not shown). Polymerization of D209N in the absence of calcium is revealed by electron microscopy. Although the amount of filaments was very low (Figure 3E), an increase in time was observed (not shown). The D212N and D212C mutants showed polymerization with divalent cations in the absence of added nucleotide (33). A sedimentation experiment to compare wt protein to D209N and D212E showed that D209N and D212E have a higher protein concentration threshold for the calcium-dependent polymerization (Figure 2E).

GTPase Activities. Since the polymerization activity of FtsZ is coupled to the GTP hydrolysis rate (19, 24, 25), we tested whether the polymerization behavior of the mutants was coupled to the GTP hydrolysis activity. First, binding of GTP was assessed using TLC. All T7-loop mutants bound GTP, although to various extents indicating differences in affinity (Figure 4A, Table 1). M206C, N207C, and D209E bound less GTP than wt protein, whereas D209C and D209N bound more GTP than wt protein. This shows that GTP-binding is affected by alterations in the T7-loop. The inclusion of MgCl₂ converts the assay to a “single turnover” GTPase assay. Only wt protein, M206C, D212E, and R214C showed significant hydrolysis as evidenced by the presence of GDP (Figure 4B). “Multiple turnover” GTPase measurements revealed that all mutants exhibited, albeit reduced, Mg²⁺-dependent GTPase activity (Figure 5A, Table 1). Out of the mutants capable of polymerization, R214C retained about half of the wt GTPase activity, while D209N and D212E were dramatically impaired in the GTPase activity. Even at concentrations up to 0.8 mg/mL, D209N and D212E showed no self-activation of the GTPase activity (not shown). The reduced activity of D209E and D212E indicates that the aspartate residues in the T7-loop cannot be substituted

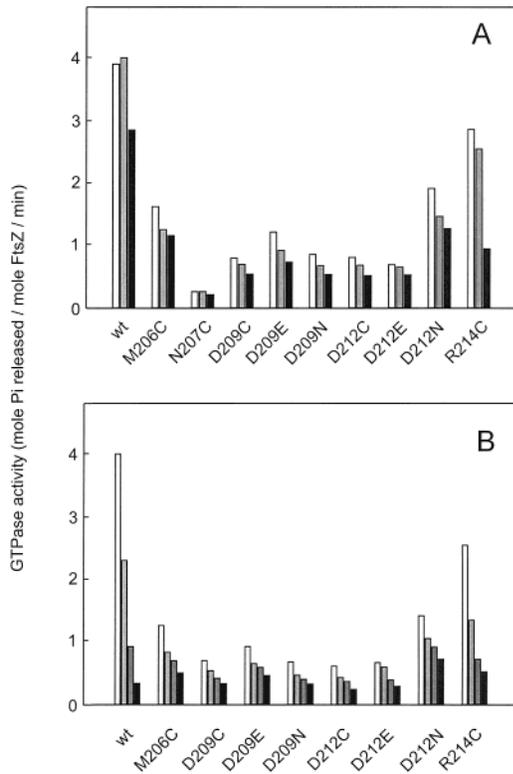


FIGURE 5: GTPase activities of the mutant FtsZ proteins at varying Mg^{2+} and Ca^{2+} concentrations. GTPase activity was determined over a 30-min time interval. (A) FtsZ and mutant proteins (0.1 mg/mL) were incubated in buffer A at 30 °C with increasing $MgCl_2$ concentration: 1 mM, white bars; 5 mM, gray bars; and 10 mM, black bars. (B) FtsZ and mutant proteins (0.1 mg/mL) were incubated in buffer A at 30 °C with 5 mM $MgCl_2$, in the absence (white bars) and presence of $CaCl_2$: 2.5 mM, gray bars; 5 mM, dark gray bars; and 10 mM, black bars.

by another negatively charged residue. As with wt protein, the GTPase activity of the mutants increased linearly with protein concentration (above the critical concentration for FtsZ GTP hydrolysis). A concentration of 0.1 mg/mL FtsZ was chosen for further characterization of the mutant proteins.

The T7-loop harbors a Walker B-like amino acid sequence motif (28) and is presumably located in the vicinity of the GTP binding site on a contacting FtsZ monomer (15). Our results with the D212 mutants show that D212 is implicated in cation coordination (33). To further characterize cation coordination by the T7-loop region, we studied the GTPase activity of the mutants at various concentrations of Mg^{2+} and Ca^{2+} . R214C responded similar as wt protein to increased concentrations of these divalent cations (Figure 5A,B). In the presence of Ca^{2+} , the activity of N207C was almost undetectable (not shown). The GTPase activity of the other mutants was barely affected by the presence of Ca^{2+} or increased Mg^{2+} .

Copolymerization Assays. Next, we tested whether the mutant FtsZ proteins could be incorporated into polymers formed with wt protein. FtsZ was mixed with excess mutant protein, and GTP-dependent polymerization was followed by light scattering (Figure 6, Table 1). A signal bigger than observed with wt protein alone is interpreted as copolymerization. From Figure 6A, it is evident that D209N forms stable copolymers with wt protein. This experiment was performed at conditions that give rise to polymers of D209N that can be detected with electron microscopy (Figure 3E)

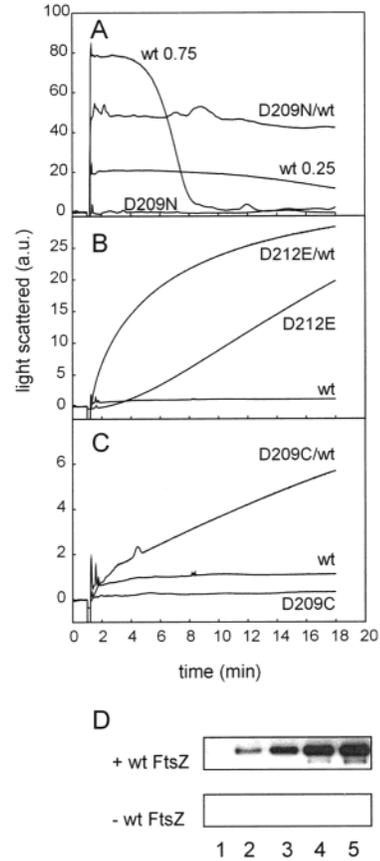


FIGURE 6: Copolymerization of FtsZ with FtsZ T7-loop mutants. Polymer formation was monitored by 90° light scattering as described in Experimental Procedures. (A) Copolymerization of wt FtsZ (0.25 mg/mL) with wt FtsZ (control, wt 0.75) or FtsZ D209N (at 0.5 mg/mL) in the absence of calcium. FtsZ alone (0.25 mg/mL, wt 0.25) and D209N alone (0.5 mg/mL) are shown as references. FtsZ M206C, N207C, D209C, D209E, and D212E did not form copolymers. Addition of wt FtsZ to FtsZ R214C did not alter the light scattering signal as compared to FtsZR214C alone. Protein mixtures were incubated at 30 °C in buffer A with 10 mM $MgCl_2$ for 1 min before induction of polymerization with 0.5 mM GTP. (B) Copolymerization of wt FtsZ (0.1 mg/mL) with FtsZ D212E (0.4 mg/mL) in the presence of calcium. FtsZ alone (0.1 mg/mL) and D212E alone (0.5 mg/mL) are shown as a reference (wt). Addition of wt FtsZ to FtsZ D209N or FtsZ R214C did not alter polymerization. Protein mixtures were incubated at 30 °C in buffer A with 5 mM $MgCl_2$ and 10 mM $CaCl_2$ for 1 min before induction of polymerization with 0.5 mM GTP. (C) Copolymerization of wt FtsZ (0.1 mg/mL) with FtsZ D209C (0.4 mg/mL) in the presence of calcium. FtsZ alone (0.1 mg/mL) is shown as a reference (wt). FtsZ M206C and FtsZ D209E showed a similar gradual increase in light scattering. Polymerization was as in panel B. Because of the weak light scattering of 0.1 mg/mL FtsZ in the absence of Ca^{2+} , higher protein concentrations were used in the experiment depicted in panel A. For panel A, the photomultiplier was set to 350 V; for panels B and C it was set to 275 V; therefore, the signal amplitude cannot be compared between panel A and panels B and C. (D) Control. FtsZ D209C, at various concentrations, was polymerized as described, either with wt FtsZ at 0.2 mg/mL (+ wt FtsZ), or without wt FtsZ (- wt FtsZ). Pellet fractions were analyzed for the presence of polymerized FtsZ D209C using biotin-maleimide. D209C concentrations: no D209C (lane 1); 0.05 mg/mL (lane 2); 0.1 mg/mL (lane 3); 0.2 mg/mL (lane 4); 0.3 mg/mL (lane 5).

but not by light scattering (not shown). In the presence of Ca^{2+} , D212E shows rapid copolymerization with wt protein (Figure 6B). Clearly, the polymerization of the D209N and D212E mutants is stimulated by the addition of wt protein.

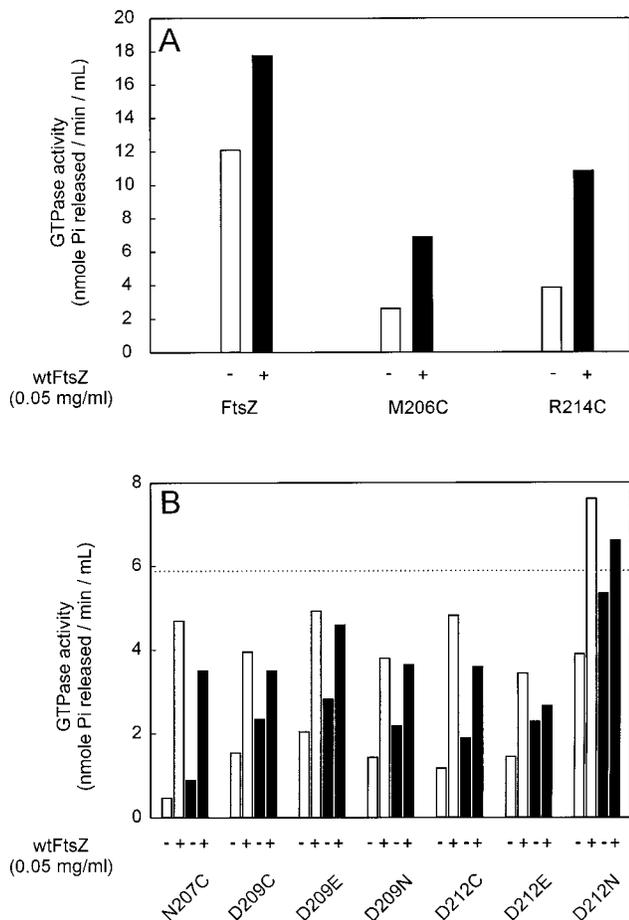


FIGURE 7: GTP hydrolysis by mixtures of wt FtsZ with T7-loop mutants. (A) GTPase activities of FtsZ, M206C, and R214C at 0.1 mg/mL (open bars) and supplemented with wt FtsZ at 0.05 mg/mL (closed bars). (B) GTPase activities of FtsZ mutants N207C, D209C, D209E, D209N, D212N, D212E, D212C alone and supplemented with wt FtsZ at 0.05 mg/mL. Mutant protein was used at 0.1 mg/mL (open bars) or 0.2 mg/mL (closed bars); the addition of wt FtsZ is depicted below the graph. The activity of wt FtsZ alone at 0.05 mg/mL (5.87 nmol of Pi released/min) is depicted as a dashed line.

R214C behaved similar alone as mixed with wt protein, as did D209N in the presence of Ca^{2+} (not shown). The mutants M206C, D209C, and D209E are slowly incorporated into polymers or add onto the wt polymers at a slow rate in the presence of Ca^{2+} (shown for D209C which displayed the most pronounced increase in Figure 6C). N207C is completely incapable of co-assembly with wt protein (not shown). To verify that the increase in light scattering reflected copolymerization, we made use of the fact that wild-type FtsZ does not contain cysteine. Mixtures of wt FtsZ and mutant proteins that contain a unique cysteine residue were copolymerized, and the polymers were sedimented. The pellet fractions were resuspended, labeled with biotin-maleimide (all cysteines were accessible for labeling), and analyzed using immunoblotting and streptavidin-conjugated antibodies. This showed that D209C (Figure 6D) and M206C (not shown) sedimented only when wt protein was present. R214C sedimentation was found irrespective of the presence of wt protein (not shown).

These results indicate that the presence of FtsZ facilitates the polymerization of several mutants that do not polymerize individually or that polymerize only at increased protein

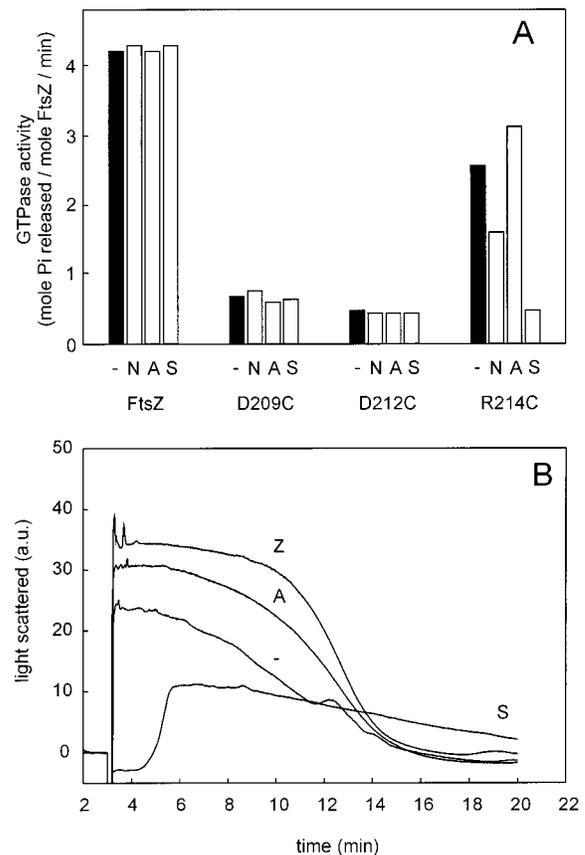


FIGURE 8: GTP hydrolysis and polymerization of modified cysteine mutants. (A) GTPase activities of wt FtsZ, D209C, D212C, and R214C at 0.1 mg/mL [-, black bars] and after labeling or with neutral NEM [N]; positively charged MTSEA [A]; or negatively charged MTSES [S] (open bars). (B) Polymerization of untreated FtsZ R214C (trace -) or after labeling with MTSEA (trace A) and MTSES (trace S). Labeled protein (0.5 mg/mL) was incubated at 30 °C in buffer A supplemented with 10 mM MgCl_2 for 1 min before the addition of 0.5 mM GTP. Polymer formation was monitored by 90° light scattering (photomultiplier at 350 V). Polymerization of wt FtsZ is shown as a reference (trace Z); labeling had no effect on polymerization of wt FtsZ.

concentrations (D209N and D212E) as compared to wt protein. The most likely explanation is that wt protein forms nucleation seeds onto which mutant proteins can add.

Inhibition of FtsZ GTPase Activity by Mutant Proteins. The interaction between wt protein and mutant proteins was examined further by means of their GTPase activity. Using FtsZ at 0.05 mg/mL as a reference, the influence of the presence of mutant proteins when mixed with wt protein at 2- or 4-fold excess was determined. As shown in Figure 7B, the N207C, D209C/E/N, and D212C/E/N mutants suppressed the GTPase activity of the wt protein. Except for D212N, a 2-fold excess of the mutants reduced the GTPase levels below that of FtsZ alone, while inhibition was even stronger when the mutant proteins were present at a 4-fold excess. The M206C and R214C mutants did not display this GTPase suppressing effect (Figure 7A). These results clearly show that association of mutant proteins, containing a dysfunctional T7-loop, with wt protein leads to inhibition of the GTP hydrolysis of FtsZ. We conclude that the active site for GTP hydrolysis is formed by association of two FtsZ monomers.

Chemical Modification of Cysteine Mutants of Charged Residues. Replacement of the charged residues D209, D212, and R214 with a unique cysteine allows the use of specific

sulfhydryl reagents to probe the function of the charged residues. FtsZ and the mutants were labeled with the neutral, positively, and negatively charged reagents, NEM, MTSEA, and MTSES, respectively. These reagents did not affect the GTPase activity of wt protein and D209C and D212C (Figure 8A). The GTPase activity of R214C was increased by the labeling with the positively charged MTSEA and reduced by the neutral NEM and the negatively charged MTSES (Figure 8A). The stimulation in GTPase activity by MTSEA labeling was reflected in improved polymerization behavior of R214C as revealed by light scattering (Figure 8B) and polymer sedimentation (not shown). MTSES-labeled R214C displayed an unusual scattering behavior: after a small drop in the signal, a transient polymerization at a low level was observed. The sulfhydryl reagents had no effect on FtsZ polymerization (not shown). These labeling experiments indicate that the positive charge at position 214 is not essential but optimizes the *in vitro* GTPase and polymerization activities.

DISCUSSION

In this report, we present a study of the role of the T7-loop in FtsZ GTP hydrolysis and polymerization. The work was prompted by structural (15) and mutagenesis data (30) on FtsZ that suggested a role for the T7-loop in hydrolysis of the GTP on an adjacent FtsZ monomer, explaining the observed self-activation of GTP-hydrolysis (25). To characterize the role of the T7-loop in FtsZ functioning in more detail, we constructed and characterized a series of mutations in this region. Our results show that mutations in the T7-loop have a dramatic effect on the GTP binding, hydrolysis, and polymerization activities of FtsZ (see Table 1). *In vitro*, several T7-loop mutants strongly influence FtsZ GTP-hydrolysis, showing that wt and mutant proteins interact. In combination with our finding that D212 of FtsZ is involved in the coordination of the Mg²⁺ ion (33), these data provide conclusive evidence that the active site for GTP hydrolysis is formed by association of two FtsZ monomers.

R214C – A Benign Mutation. R214C is the only mutant that readily polymerizes (with and without calcium) with a relatively high GTP hydrolysis activity. R214C responds similar to wt protein to increasing cation concentrations, i.e., by polymer bundling and a reduction in the GTP hydrolysis. Since R214C is the only mutant in this study that shows a decrease in GTP hydrolysis activity linked to bundling, we conclude that the reduction of GTP hydrolysis of FtsZ at increased cation concentrations is caused by polymer bundling and not vice versa.

The positive charge at the end of the T7-loop region is highly conserved: most FtsZ species contain an Arg or a Lys residue at this position (R214 in *E. coli* FtsZ). In R214C, GTP hydrolysis was more reduced than in the previously described R214A and R214K replacements (19). The reduction in GTPase and polymerization activity was partly restored by modification of the cysteine with a positively charged sulfhydryl reagent. This implies that the positive charge at position 214 has a structural function. We propose that R214 functions in the coordination/retention of the bound P_i that results from GTP hydrolysis. The extreme curvature observed in some R214C polymer images (see Figure 3C)

provides support for this hypothesis. FtsZ polymers assembled with GDP display more curvature than polymers assembled with GTP (39), while GTP incorporated into FtsZ polymers is hydrolyzed rapidly (26). Experiments in which FtsZ polymerization was performed with [γ -³²P]-GTP showed that P_i remains bound to FtsZ after hydrolysis (D.-J. S. and A.J.M.D., unpublished observations). A role for R214 in P_i coordination would explain both the observed polymer curvature and the relatively small effects of R214 mutations on GTP hydrolysis activity (this work, 19).

M206C and N207C Render FtsZ Polymerization Incompetent. Both M206C and N207C display a strong reduction of the GTP binding and hydrolysis activity and do not form polymers. M206C is however still able to interact with wt protein, as evidenced by the copolymerization experiments, which in the case of M206C does not affect wt protein GTPase activity. N207C has also lost the capability to copolymerize with wt protein, but since it reduces the wt protein GTPase activity in co-GTPase experiments, we conclude that N207C does associate with wt protein.

D209 and D212 Cannot Be Replaced by Glutamate. The conserved aspartate residues D209 and D212 were replaced by cysteine as well as by asparagine and glutamate to study the effect of conservative substitutions. All replacements inhibited the FtsZ GTPase activity in a more or less severe manner but did not prevent GTP binding. Therefore, substitution of the negative charge of D209 or D212 does not restore wt FtsZ GTP hydrolysis activity. A recent publication describes severely reduced GTP hydrolysis for D209A and D212A mutations (40). This indicates that both size and charge of the D209/212 residues are critical for FtsZ functioning.

Polymerization of the D209/D212 Mutants. Only the D209N and D212E substitutions rendered the protein polymerization competent at increased critical FtsZ concentrations (apart from D212C and D212N, which show cation-induced polymerization (33)). Of all mutants studied, D209N is the only one that copolymerizes with wt protein both in the absence and presence of calcium, while D212E copolymerizes with wt protein only in the presence of calcium. The polymers formed by D209N and D212E, with or without wt protein, are stable over long time periods. This can be explained as follows: the presence of a nonfunctional T7-loop at the FtsZ–FtsZ longitudinal interface inhibits GTP hydrolysis. In copolymers, a mixed population is formed where many monomer interfaces contain a nonfunctional T7-loop. At these interaction sites, GTP hydrolysis does not occur. This leads to (i) polymers that are stable since there is non-hydrolyzed GTP present throughout the polymers, and (ii) a prolonged presence of GTP in the reaction mixtures. We cannot explain why D209N polymerizes poorly, and D212E not at all in the absence of calcium. The increase in the threshold concentration observed with both proteins is an indication for suboptimal polymerization (Figure 2E). The presence of calcium may provide an extra stimulant for polymerization of the negatively charged FtsZ, which facilitates polymerization of D209N and D212E (41). Similar results were reported recently for a D209A mutant, which was capable of assembly without calcium and a D212A mutant that required either calcium or DEAE-dextran for polymerization (40). These latter two mutants were tested

at 1 mg/mL, and concentration dependence was not studied (40).

The Cooperativity of FtsZ Assembly. This report deals with the assembly of FtsZ at conditions known to induce lateral association of protofilaments (9, 35, 36, 39). Lateral assembly of FtsZ implies cooperativity, as pointed out recently (22) and is evidenced by the concentration dependence of the D209N and D212E mutants. Linear assembly of FtsZ in a noncooperative fashion was recently described (20–22). Using analytical ultracentrifugation, Mg²⁺-induced self-association of FtsZ into linear oligomers has been observed that did not require GTP (20, 21). The necessity of Mg²⁺ for the formation of these oligomers is in line with our finding that a coordinated cation is required for FtsZ polymerization (33). Romberg and co-workers described the formation of short single protofilaments with GTP in a noncooperative, i.e., isodesmic way, as well as partially cooperative assembly with the slowly hydrolyzable GTP analogue guanylyl-(α,β)-methylene-diphosphonate (GMP-CPP) (22). The noncooperative linear assembly may precede cooperative assembly of laterally associated FtsZ protofilaments. The cooperativity described here and elsewhere (19, 24, 25, 36, 42) does not preclude an initial noncooperative assembly that is not detected in our assays. Further work is required to understand the kinetics of FtsZ polymerization in terms of a unifying model.

Concluding Remarks. Using a set of mutants in the T7-loop region, we have been able to show, by interaction studies with wt protein, that the T7-loop is critical for FtsZ GTP hydrolysis, as part of the active site formed by two FtsZ monomers. Mutations in the T7-loop either disrupt the loop structure or replace a critical residue. The T7-loop most likely coordinates cation and GTP binding and GTP hydrolysis at the adjacent FtsZ monomer. The importance of the nucleotide interface was also stated in a recent molecular dynamics simulation that identified a conformational change of the T3-loop induced by the γ -phosphate of GTP (43). This study pointed out that GTP hydrolysis may induce a structural change at the nucleotide interface by pushing against the T7-loop region of the adjacent monomer. The high turnover of GTP that FtsZ displays in solution seems not to be critical for functioning, since this work and previous reports describe FtsZ mutants that are capable of *in vivo* and/or *in vitro* assembly with severely inhibited GTP-hydrolysis activity (11, 12, 30, 40). Although most of the T7-loop mutants interact with wt protein, they do not readily assemble into polymers, indicating that the interface may be critical for the formation of FtsZ polymers. This indicates that *in vivo*, control of the Z-ring stability most likely lies at another level than GTP turnover. The regulation of FtsZ polymerization in the presence of FtsA and ZipA presents future experimental challenges.

ACKNOWLEDGMENT

We thank Prof. Dr. Marileen Dogterom (FOM Institute AMOLF, Amsterdam) and Prof. Dr. Nanne Nanninga (Swammerdam Institute for Life Sciences (SILS), University of Amsterdam) for valuable discussions, Wijnand Takkenberg (SILS) for help with the electron microscopy, and Dr. Dirk Jan Slotboom for help with the sequence alignments and sulfhydryl reagents.

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BI011370I