Mapping Flexibility and the Assembly Switch of Cell Division Protein FtsZ by Computational and Mutational Approaches*§*

Received for publication, March 16, 2010, and in revised form, May 5, 2010. Published, JBC Papers in Press, May 13, 2010, DOI 10.1074/jbc.M110.117127

Antonio J. Martín-Galiano†1, Rubén M. Buey†5, Marta Cabezas‡2, and José M. Andreu‡13
From the †Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu, 9, 28040 Madrid, Spain and ‡Biomolecular Research Biology and Chemistry, the Paul Scherrer Institut, CH-5232 Villigen PSI, Switzerland

The molecular switch for nucleotide-regulated assembly and disassembly of the main prokaryotic cell division protein FtsZ is unknown despite the numerous crystal structures that are available. We have characterized the functional motions in FtsZ with a computational consensus of essential dynamics, structural comparisons, sequence conservation, and networks of co-evolving residues. Employing this information, we have constructed 17 mutants, which alter the FtsZ functional cycle at different stages, to modify FtsZ flexibility. The mutant phenotypes ranged from benign to total inactivation and included increased stages, to modify FtsZ flexibility. The mutant phenotypes and core helix H7 deviated FtsZ assembly into curved filaments with inhibited GTPase, which still polymerize cooperatively. These mutations may perturb the predicted closure of the C-terminal domain onto H7 required for switching between curved and straight association modes and for GTPase activation. By mapping the FtsZ assembly switch, this work also gives insight into FtsZ druggability because the curved mutations delineate the putative binding site of the promising antibacterial FtsZ inhibitor PC190723.

Cell division protein FtsZ and its eukaryotic structural homolog tubulin are cytoskeletal GTPases, which assemble into different polymers that play crucial roles in living cells. FtsZ is the main component of the Z-ring that drives bacterial cell division (Ref.1; reviewed in Ref. 2), whereas tubulin is the building block of eukaryotic microtubules, involved in chromosome segregation, among other tasks. Tubulin is a major target of antitumor drugs, including microtubule-stabilizing agents such as taxol (3). FtsZ is an attractive antimicrobial target, due to its essential role in bacterial cell division, which is a process completely different from eukaryotic cytokinesis. Several small molecules inhibit FtsZ assembly or bacterial cell division (reviewed in Refs. 4–6). GTP analogs substituted at C8 selectively inhibit purified FtsZ while supporting tubulin assembly (7). FtsZ has been validated as the target of an effective bactericidal compound PC190723 (8), which is an FtsZ polymer-stabilizing agent (9).

FtsZ and tubulin require GTP to assemble and disassemble as a result of hydrolysis to GDP. This indicates the existence of molecular switches in their assembly-disassembly cycle triggered by the presence/absence of the nucleotide γ-phosphate, which permits polymer regulation. FtsZ polymerizes into tubulin-like protofilaments (10, 11) that can be straight or curved in relation to the GTP- or GDP-bound nucleotide (12–14). GTP binding displaces the balance to the straight filaments and sheet and bundle condensates, whereas GDP-FtsZ filaments tend to be curved and eventually depolymerize. These observations suggest that curved and straight forms of FtsZ exist as interchanging structural entities. Additional evidence that FtsZ has a built-in assembly switch is provided by a GTP-FtsZ monomer isomerization equilibrium that is required to explain the cooperative polymerization of single-stranded FtsZ protofilaments (15–18). GDP actively triggers the disassembly switch because GDP addition disassembled apoFtsZ polymers (14) and dissolved the crystals of protofilament-like apoFtsZ dimers (19). FtsZ filament disassembly, recycling, condensation, and curvature are important for the constriction of the Z-ring (20).

FtsZ consists of an N-terminal GTP binding domain with a modified Rossmann fold and a C-terminal GTPase-activating domain with a chorismate mutase fold (21). Both domains are linked by the long core helix H7 and loop T7, which probably arose only once during evolution (22). Upon filament formation, the C-domain from the upper subunit contacts the N-domain and the top of H7 of the lower subunit, whereas the T7 loop from the upper subunit acts synergistically by providing the co-catalytic aspartate residues that complete the GTPase site (19, 22–24). This engages cooperative polymerization and GTP binding/hydrolysis, which requires the fine coupling of both domains, probably by the evolutionary development of a distinctive combination of elements including hinges, moving parts, and energetically coupled residue networks providing signal transmission across monomers.

In G-proteins such as p21ras, up to three switch regions are evident from structural comparison between active and inactive structures, induced by GTP and GDP, respectively (25, 26). Insight into the structural switch of tubulin was provided by the comparison of the curved and straight structures (27, 28) and by...
an antibody that recognizes GTP-tubulin conformation remnants in microtubules (29). It has been proposed from structural comparisons and biochemical properties that unassembled tubulin, instead of undergoing nucleotide-induced structural changes (30), exists predominantly in a ground curved state that isomerizes into the active state by polymerization-driven structural changes. GTP binding only reduces the activation free energy for the change (31). Tubulin straightening by the microtubule lattice contacts has been supported by three subsequent studies (32–34). In the case of FtsZ, structural comparisons initially suggested a molecular switch involving a tubulin-like “curved to straight” displacement of the H7 helix (31), but this has not been supported by superposition of the appropriate crystal structures (35). The structural comparison of 14 different FtsZ structures has shown a certain conformational flexibility in FtsZ. This analysis has further supported a lack of nucleotide-induced structural changes in FtsZ monomers and revealed that the small structural differences observed are attributable to interspecies differences rather than to interconvertible conformations (35). This high similarity between FtsZ structures suggests the existence of a bias toward a given protein state or that the dynamic nature of the FtsZ switch may be beyond the scope of crystallographic studies with FtsZ monomers or dimers. Determination of the potential structural changes coupled to FtsZ assembly would therefore require determining the crystal structure of an FtsZ protofilament.

A structural change at FtsZ loop T3 was predicted by molecular dynamics of GTP/GDP forms and validated after introducing an alternative tryptophan as a fluorescence probe in the W319Y-Y92W mutant of Mj-FtsZ (36). In a related approach, tryptophan mutants affected assembly by forming an aromatic stack at the C-terminal domain of Ec-FtsZ (37). However, the large amount of information available in the numerous FtsZ sequences and structures has not been systematically analyzed to reveal the FtsZ assembly switch. Given the current limitations of FtsZ crystallography, we have addressed this problem by predicting flexible moving parts and connecting allosteric networks of the FtsZ molecule and by mutating key residues, which modify FtsZ assembly at different stages of its functional cycle. Among other movements, we have found that a closure movement of part of the C-terminal domain is required for FtsZ to assemble into straight filaments, which is blocked in several curved FtsZ mutants. Mutants with a gain or loss of assembly capacity have also been generated. Mapping the FtsZ switch gives insight into the mechanism of action of new antibacterial compounds targeting FtsZ assembly.

**EXPERIMENTAL PROCEDURES**

_FtsZ Sequence Dataset and Analyses_—Cell division protein FtsZ sequences from 399 complete prokaryotic genomes (ftp.ncbi.nih.gov/genomes/Bacteria) were detected by running the TIGR00065 model from the TIGRfam database (38), using the hmmpfam program of the HMMER suite (39). Only one strain per species and the best hit per genome were collected. This strategy improves the functional homogeneity of the hits by selecting the canonical cell division FtsZ1 ortholog and avoiding the presence of other FtsZ orthologs whose properties may be slightly different. The taxonomical bias was palliated by removing redundant sequences at the 80% identity level using CD-HIT (40). The 223 surviving sequences provide a functionally solid but sufficiently variable dataset for various further analyses. Sequences were aligned by Muscle (41) using default parameters (alignment file available upon request). Residue conservation was assessed by calculating relative entropy as described (42) using the relative amino acid occurrences in the non-redundant FtsZ dataset as background frequencies. Conservation was estimated by averaging over a 7-residue window the relative entropy calculated for individual positions, which reduced the impact of isolated conserved residues. Correlated mutations were analyzed by statistical coupling analysis (SCA) (43), applying hierarchical clustering on those with an SCA score >1.6. Trivial interactions (&lt;4 positions in sequence) were discarded.

_Structural Analyses_—Structural analyses including comparisons and several calculations were carried out using GROMACS v3.3 (44) on a set of structures (35): Mj-FtsZ (Protein Data Bank (PDB) ID 1FSZ (GDP); 1W58 (GMPCPP), 1W59/AB (no nucleotide, dimer), 1W5B/AB (GTP, dimer), 1W5A/AB (GTP-Mg2+/GTP dimer), 2VAP (GDP), Aa-FtsZ (2R6R, GDP), Pa-FtsZ (2VAV (GDP), 1OFU/AB (GDP)), and Bs-FtsZ (2VAM, sulfate-bound). Moving tendencies between structures were evaluated through difference distance plots (45) considering Cα residue atoms. Distances between residues were calculated by an in-house PERL script that calls the _g mindset_ GROMACS module. Salt bridges were detected between charged groups at ≤5.5Å distance. Hydrogen bonds between suitable donor and acceptor atoms were detected using default GROMACS parameters. Protein flexibility was analyzed as described (46) with modifications. To improve the quality of the predictions, hydrogen atoms were added to the structures, and energy minimization was carried out prior to structure comparison and essential dynamics simulations. To properly cover the structural space of the protein, dynamic trajectories from a total of 2000 Monte Carlo sample structures were generated by CONCOORD v2.0 (47) on all atoms of FtsZ from four species (Mj-FtsZ, 1FSZ; Aa-FtsZ, 2R6R; Bs-FtsZ, 2VAM; Pa-FtsZ, 2VAV) using yamber2 van der Waals parameters and Engh-Huber bond parameters for _dist_ and 2500 _disco_ iterations before sample structure rejection. The covariance matrix of atomic displacements was calculated, and eigenvectors were extracted and analyzed as described (48). The movement vectors corresponding to the extreme projections were inspected, and the four most relevant eigenvectors on the trajectory were analyzed by DynDom v1.5 (49), which finds relative fixed and motion domains linked by bending regions and hinge residues in the protein. To calculate displacements between structures of the four species analyzed, the only coordinates taken were those of the backbone atoms in the section of the protein conserved in essentially all FtsZ1 homologs.
Mapping the FtsZ Activation Switch

which ranges from S1 to S10 β-strands (50). DynDom parameters applied were 5 residues as window length, ≥20 residues as domain length, and internal/external displacement ratio of ≥1.

**FtsZ Mutant Construction and Purification**—Full-length, untagged Mj-FtsZ point mutants were constructed with the QuikChange site-directed mutagenesis kit II (Stratagene) using the pHis17-MJ0370 plasmid (21) with appropriate pairs of complementary oligonucleotides carrying the mutation. Mutated ftsZ genes were confirmed by complete open reading frame sequencing.

The FtsZ mutants were overproduced in *Escherichia coli* BL21(DE3)pLys, and purified by fast protein liquid chromatography using anionic exchange and subsequent hydrophobic and desalting chromatographies as described (11, 13) with yields of 3–15 mg of protein/liter of culture. Protein and FtsZ-bound nucleotide were spectrophotometrically quantified (13). All FtsZs contained bound nucleotide (mutants, 0.22–0.88 guanine per FtsZ; wild type, 0.26–0.44). Mutant proteins did not show any significant alterations in their folding as examined by their circular dichroism spectra (not shown), acquired as described (51).

**Biochemical Methods and Electron Microscopy**—Mj-FtsZ polymerization at 55 °C was monitored by 90° angle light scattering maximum were adsorbed to carbon-coated grids for microscopy, 20000/50,000 magnification.

Analyses of FtsZ Structures—Following the sequence analyses, 10 crystal structures were selected, containing 14 FtsZ molecules with different combinations of nucleotide, association state (monomer or dimer), and species (see “Experimental Procedures”) as a reasonable sample of potential conformational

**RESULTS**

The Evolution of FtsZ Sequences—The first steps to map the FtsZ assembly switch consisted of extracting information con-
states to obtain a comparative three-dimensional picture. The collective B-factor profile (averaged and normalized, $B_f$) of the FtsZ structures (Fig. 2A, red line) presented generally higher peaks at the C-domain than at the N-domain. The more flexible zones of the N-domain correspond to positions 57–61 (at $H_1–S_2$ loop) and 197–205 (at $H_6–H_7$ loop /top $H_7$).

The C-terminal domain contained sharp valleys, indicating rigid zones centered at positions matching $\beta$-strands $S_7–S_10$. Strikingly, $B_f$ and sequence conservation showed a clear inverted correlation (Fig. 2A). This suggested that structural rigidity and evolutionary conservation are tightly correlated properties in the FtsZ family.

Analyzing the residue contacts in Mj-FtsZ structure showed that the core helix $H_7$ is by far the element forming the most long range residue interactions (defined as contacting residues located $\geq$20 positions away in sequence) (Fig. 2B). This is typical of important regions for folding and stability (54). However, both domains can fold independently in FtsZ from *Thermotoga* (19), which suggests that these $H_7$ contacts are actually involved in interdomain communication. For instance, $H_7$ makes contact with the $H_1$ helix and the $H_1–S_2$ loop, with all the (roughly parallel) $\beta$-strands in the N-domain except $S_3$ and with all the (roughly perpendicular) $\beta$-strands in the C-domain (Fig. 2B). Thus, the high level of sequence conservation observed in the $\beta$-sheets might also relate to selective pressure to keep interactions with $H_7$.

To map potentially moving elements, the maximal inter-residue displacements between all 14 FtsZ structures were plotted in a difference distance map (Fig. 2C). The N-terminal domain showed very small interdomain displacements except for residues 54–62 (H1 + loop H1–S2) versus 196–207 (H6 + loop H6–H7 + H7) (Fig. 2C), which was the largest collective displacement observed in the protein ($\geq$3.5 Å, 9 versus 12 contiguous residues). These residues have high $B_f$ values (see above),
and they overlap with two of the predicted moving regions (58–64 and 191–207) in molecular dynamics simulations comparing GTP and GDP-bound FtsZ (36). These two sections are joined by a series of predicted salt bridges along the H1 and H7 helices: Arg55–Asp212, Arg55–Glu213, and Glu59–Lys220 (Fig. 2D). In addition, residue Lys228 (T7 loop) is at bonding distance to Glu62 (H1–S2 loop) and to Asp324 at the base of the C-domain, thus bridging the C- and N-terminal domains. In the four homologs examined (Mj-FtsZ, Pa-FtsZ, Aa-FtsZ, and Bs-FtsZ), an ion pair equivalent to Arg55–Asp212 is fully conserved, whereas several of the other charged residues are substituted by amide or hydroxyl groups, possibly making hydrogen bonds instead of salt bridges. Of note, the distances between residues equivalent to Glu62–Lys228 in Pa-FtsZ, Aa-FtsZ, and Bs-FtsZ structures are quite variable, suggesting the possibility of forming or breaking this bond through small displacements of H7–T7 with respect to the N-domain. Regarding the C-domain, although the location of loops and helices was quite variable with respect to the rest of the molecule, the strands were quite rigid, in concordance with the 𝐵𝑖 𝐹 analyse above.

FIGURE 2. Residue flexibility and contacts in FtsZ structures. Bf analysis, displacements, residue contacts, and charge pairs in FtsZ structures. A, collective Bf values (red line) for equivalent residues of 14 structures (between residues 40–335 in Mj-FtsZ) + r – 5.D. (rose lines). To counteract the effect of the crystallographic resolution, the B-factors of C atoms were normalized by dividing by the average B-factor value of each structure and averaging, rendering Bf. The gray line shows the inverse relative entropy (maximal relative entropy value minus the relative entropy value, Fig. 1A). Positions with Bf < 0.87 were considered rigid. The coefficient of determination, r², between the red and gray profiles is 0.39 (p value 4 × 10⁻¹⁴) for the whole protein and 0.63 (p value 3 × 10⁻³⁴) for beta-strands. Color codes for secondary structure elements are as in Fig. 1A. B, contact map of Mj-FtsZ structure. Secondary structure elements contacting helix H7 (≤5 Å, any atom) through ≤2 residues are circled. C, difference distance plot between the structures of 14 FtsZ. Maximal distance differences observed considering C atoms from all structure pair comparisons were plotted. D, clusters of residues in helices H1 and H7 and adjacent zones (brown) that show displacements ≥3Å between Mj-FtsZ and the other structures. Acidic (red) and basic residues (blue) at saline/hydrogen-bond distance in these zones of Mj-FtsZ are labeled.

By comparing the Mj-FtsZ dimer and monomer structures, we observed a bending of the H6–H7 loop and the top of H7 in the lower subunit of FtsZ dimers (empty or GTP-bound, PDB IDs: 1W59, 1W5A, 1W5B), with respect to the corresponding position in the upper subunit of the dimer or in monomers. Displacements between 2 and 4 Å are measured at residues 201–NMPLK205 (supplemental Fig. S2). We did not observe this movement in other FtsZ structures, suggesting that it is induced by subunit association.
FtsZ Flexibility Characterized by Essential Dynamics—Because structural comparisons revealed marginally flexible regions, the FtsZ flexibility was explored by essential dynamics (ED). ED focuses on regions accumulating the most dynamic variability, whereas ignoring those progressively contributing less to the complex movements in proteins (48). Extreme projections were taken from trajectories generated by CONCOORD, which generates structures constrained by interatomic distances and provides a less precise but wider conformational sampling than molecular dynamics simulations (47). Up to 66–75% motion variance was accumulated by the top four eigenvectors in representative structures from four species (Mj-FtsZ, Pa-FtsZ, Aa-FtsZ, Bs-FtsZ), which were further scrutinized. The positions of the pseudorigid domains and flanking bending points provide a mechanical view of a structure. These patterns were delineated by DynDom (49) on the Mj-FtsZ top four extreme projections (Fig. 3; see supplemental movies S1–S4), three of which were reproduced in the other species with typical 1–2 residue shifts. Many of these bending hotspots contained a small conserved residue (e.g. Gly^{63}, Ala^{156}, Gly^{221}) surrounded by polar and charged residues, a typical composition of hinges (55). The most relevant ED movements detected were angular in nature rather than translations, which were smaller than 0.5 Å (although a 1.2 Å displacement in H7 had been observed by comparing Mj-FtsZ and Pa-FtsZ structures (31)).

The first consensus movement (Fig. 3A) implies a twisted bending (perpendicular to the axis joining the mass centers of the domains) of the large section of the N-domain that interacts with the nucleotide (S1–S6) with respect to the rest of the molecule. Three of the four hinges involve β-strands: ^{61}I{E}G{A}{K}^{66} (H1–S2/S2), ^{155}V{A}^{156} (S5), and ^{196}T{L}^{187} (S6), whereas the other one (^{173}A{M}^{176}) falls on H5. Some of these bending points have conserved sequences, present low βf, and/or flank the major concerted displacement among FtsZ crystal structures described above (Fig. 2, A and C). In this movement, the core of the N-domain containing the nucleotide binding cup behaves as a solid block, whereas helix H6, loop H6–H7, and the top of H7 (up to ^{214}L{L}^{215}) are more flexible. A second Mj-FtsZ movement (Fig. 3B) was not among the top four movements in the other species and was not further considered. The second consensus movement (third Mj-FtsZ movement, Fig. 3C) corre-

![FIGURE 3. Essential dynamics summary of Mj-FtsZ structure. A–D, superimposition of backbone surfaces from the two extreme projections, in lighter and darker colors, for the first (A), second (B), third (C), and fourth (D) main ED eigenvectors (see "Experimental Procedures"). Motion domains are in blue, red, green, and brown. Predicted bending residues are labeled in yellow. The predicted angle and percentage of twist/closure are given in labels colored as the reference motion domain.](image-url)
Mapping the FtsZ Activation Switch

FIGURE 4. FtsZ mutant light-scattering screen. A, FtsZ mutants mapped onto FtsZ structure. B, bidimensional representation of mutant polymerization behavior through $t_{\text{max}}$ and $t_{1/2}$ values. The map represents average ± S.D. from at least two replicates. Fully detailed light scattering curves from a second-stage mutant are provided in supplemental Fig. S3. Mutants are colored according to their classification in both panel A and panel B. wt, wild type.

sponds to a closure bending (parallel to the axis joining the mass centers of the motion domains) between the nucleotide binding domain and the C-domain with a single hinge $^{222}$LVE$^{224}$ at the bottom third of the H7 helix. Finally, the third conserved movement (Fig. 3D) fractionates the molecule into four motion subdomains. Two of them are 3–4-residue shifted versions of the former consensus movements involving the N-terminal domain, and the other two correspond to a partition of the C-domain. These C-terminal subdomains bend with respect to each other by a closure rotation through a series of piled bend domains. These C-terminal subdomains bend with respect to each other by a closure rotation through a series of piled bend C-domain. These C-terminal subdomains bend with respect to each other by a closure rotation through a series of piled bend -strands (T7–H8 junction, S7, H9–S8, S9, S10) and separate a front and a back section in our FtsZ view, which are discontinuous in the sequence. The front section (bottom H7, T7, S7–H9, H9, S8–H10, H10, S9–S10) has more motional freedom, whereas the back section (H8, H9–S8, H10–S9, S10–S11) is more static and makes multiple contacts with the N-domain.

**Designing FtsZ Flexibility Mutants**—To recapitulate, the ED predictions fractionate the FtsZ molecule into four motion subdomains, two per domain, linked by consistent bending zones. The N-terminal domain contains the core nucleotide binding subdomain (N1) and the H6/H6–H7/top H7 subdomain (N2), which is probably more flexible because it is not trapped in the dense interaction network between H7 and the $\beta$-strands. The C-terminal domain contains a front motional subdomain (C1) and a back subdomain (C2), rigid and more engaged to the N-terminal domain. ED and the rest of the computational approaches employed agreed on a consensus of zones, namely H1 + H1–S2, H7 + T7, and the S7–S10 C-terminal $\beta$-sheet as relevant for the FtsZ flexibility. This consensus approach was expected to be more robust than approximations based on a single criterion for revealing these hotspots and was employed to refine the selection of particular residues with additional structural considerations (see supplemental Table S1).

To experimentally validate the relevance of predicted residues on the FtsZ activation switch, 17 FtsZ mutants were constructed, aiming to shift the polymerization equilibrium toward alterations that resemble the assembly-active (made possible by GTP binding) or -inactive (favored by GDP binding) states of FtsZ. Ideal switch-disturbing mutations should involve relatively conserved residues, sequence-correlated with other parts of the molecule, bridging motional parts in the structure or acting as hinges, or with some reported phenotype in the literature. In 13 cases, charged, polar, or large residues were substituted by alanine to remove their interactions. Three conserved glycines were also selected, given the occurrence of glycine in protein cavities, and replaced by the bulkier residue valine. Finally, in one mutant, threonine was introduced in a potential ED hinge residue (A156T), which reproduces the equivalent mutation A129T that causes thermosensitivity in *E. coli* (56). Replacing residues were very rare or unseen at the equivalent position in natural FtsZ sequences. Thus, a limited but comprehensive set of representative mutants was generated, covering both domains and the H7 helix (Fig. 4). Previous studies had selected positions to generate altered FtsZ forms based on topological criteria such as association surfaces and the nucleotide binding site (12, 24, 57–59). In contrast, the design of mutants in our study was based on insights into the functional dynamics independently of their location of the protein.

**Assembly Screen of FtsZ Mutants**—The 17 purified FtsZ mutants (Fig. 4A) were folded and had bound nucleotide (see “Experimental Procedures”). The effects of mutations were first screened by monitoring polymerization in the presence of GTP $^{2+}$ using 90° light-scattering assays (Fig. 4B). The reference behavior of 12.5 mM wild-type FtsZ with 1 mM GTP consisted of a maximum scattering of $-8 \times 10^5$ counts/s with a rise time ($t_{\text{max}}$) of 1.9 ± 0.6 min after GTP addition followed by a half-decay time ($t_{1/2}$) of 11.1 ± 0.9 min due to nucleotide consumption (supplemental Fig. S3). These simple parameters permitted classification of the mutant FtsZ into six groups, named A–F, according to their dynamic behavior archetypes (Fig. 4B). A first set of mutations (group A, including R55A, G63V, L131A, K220A, and D229A) was benign and clustered around the wild-type behavior. Group B (A156T, D212A) underwent...
faster depolymerization ($t_{1/2}<7.5$ min) than the wild type. Type C mutant E62A showed a polymerization delay but normal depolymerization within the experimental error. Group D mutants (K228A, S272A) were distinctive in sharing a noticeably slower polymerization and depolymerization (2-fold longer $t_{\text{max}}$ and $t_{1/2}$). Mutants in groups A–D showed maximal scattering values within 80–200% of the wild type. In contrast, mutants in group E (D185A, G221V, I251A, H288A, G320V, D324A) had maximal scattering peak values $\leq$6-fold smaller than the wild type ($<1.5 \times 10^5$ arbitrary units), indicating reduced polymerization or a very different polymer morphology. Interestingly, this lower scattering value was reached immediately after GTP addition, whereas depolymerization was not observed in any of these mutants after 1 h, which indicated severe functional modification. Unspecific aggregation was discarded because the scattering was abolished in the range of minutes by either a temperature downshift from 55 to 25 °C or the addition of an excess of GDP. Finally, the type F mutant, M164A, did not react at all after GTP addition. Notably, when mapped on the structure, a clear correlation between residue topology and mutation effects was found (Fig. 4A), indicating the importance of different zones on FtsZ function.

Biochemical Properties of Mutant FtsZ Proteins—Selected mutants representing the six groups were further examined with a battery of biochemical tests, consistent in the measurement of GTPase activity (Fig. 5A), sedimentation in different conditions (Fig. 5B and 5D), critical concentration (Cr) for polymerization with potassium (Fig. 5C) and sodium (Fig. 5D), and nucleotide content (Fig. 5E) (data are summarized in supplemental Table S2). These assays were complemented with electron microscopy (Fig. 6).

The group A ("wild-type-like") member R55A had a slightly higher (26%) GTPase activity than the wild-type FtsZ as its most notorious effect but a similar pelleting pattern, Cr value, and polymer shape. These weak variations justify considering this group A mutant as negative control of mutation effect.

The short $t_{1/2}$ of group B ("increased GTPase") mutant A156T was associated with an increment of 62% in the GTPase activity and a lower GTP content of its polymers. On the other hand, the pelleting pattern and Cr were not substantially modified. Polymers were straight but shorter than the ones from the wild type.

The long $t_{\text{max}}$ of the group C mutant E62A ("slow assembly") was accompanied by a low sedimenting fraction (28%, Fig. 5B).
and a Cr 3-fold higher than the wild type (Fig. 5D). The bulk GTPase activity changed as a consequence of a lower amount of polymerized FtsZ. Polymers of E62A were indistinguishable from the wild-type (not shown).

The polymers of the two group D (“gain of assembly”) mutants were more stable than the wild type as evidenced by the high pelleting fraction with GTP, close to 100% (Fig. 5B), and their Cr-K⁺ of only 0.2–0.3 μM (Fig. 5C). Despite the ∼2-fold increase in t₁/₂, S272A presented GTPase activity comparable with wild-type, whereas K228A showed only a moderate reduction of ∼25% (Fig. 5A). The Cr-Na⁺ of both mutants was also markedly lower than the wild type. Given that when sodium replaces potassium, more than 99% the wild-type Mj-FtsZ GTPase activity is inhibited (11, 13), this suggests that the increased stability of K228A and especially S272A is not due to a lower GTPase activity. K228A polymerized into straight filaments with some enhanced tendency to form regular bundles and did show evidence of sedimentation with only Mg²⁺ or with GDP. On the other hand, S272A is enriched in polymers that circularized into “C” shapes and mono- and multilayer large rings (toroids). Toroids had a typical 100–120-nm outer diameter, roughly corresponding to 70–90 FtsZ subunits.

Group E (“curved”) mutants H288A and G320V showed a 3.5-fold reduced and inactivated GTPase activity, respectively. Surprisingly, Cr-K⁺ was equivalent to the wild-type, indicating an analogous elongation affinity. Moreover, the Cr value was indifferent to substitution of K⁺ by Na⁺. Both mutants sedimented with GTP/Mg²⁺ more than wild-type FtsZ, which indicates that the patent light-scattering reduction is due to the polymer shape rather than to a diminished polymerizing activity. In fact, all mutants in this group polymerized into thin spirals except I251A, which made short curved polymers. H288A formed short spirals, whereas G320V assembled into longer, more regular spirals, suggesting an enhanced curved phenotype. The spiral dimensions of G320V polymers (44 nm pitch, ∼30 nm width) indicate a subunit-to-subunit curvature of ∼17°, comparable in absolute value with the relative rotation of 11° observed between subunits in a FtsZ crystal dimer (19)). In both group E mutants, the ∼4 nm thickness of polymers is compatible with one FtsZ molecule-wide filament. These two mutants retained a polymerized fraction with Mg²⁺ alone without the addition of exogenous GTP. In the case of G320V, a polymerized fraction was detectable up to at least 6 min after the addition of GDP (Fig. 5B), compatible with slow dissociation of GTP. Finally, type F (“loss of func-

FIGURE 6. Electron micrographs of FtsZ polymers. Samples were taken at scattering maxima (see “Experimental Procedures”). Scale bar represents 200 nm.

FIGURE 7. FtsZ switch model based on essential motion domains, and FtsZ assembly-disassembly cycle. A, structural view. Contact nomenclature is according to supplemental Table 1 in Ref. 19. The inset is an upward transversal view of intersubunit contacts. B, sketch view of the proposed FtsZ functional cycle. The step proposed to be modified by each mutant is indicated (see Supplemental Discussion).
Mapping the FtsZ Activation Switch

FtsZ Monomer Flexibility and Predicted Subdomain Movements Modify the Association Interfaces in the FtsZ Filament—The ability to assemble cooperatively is built into the FtsZ structure by a sophisticated system of motion subdomains, allosteric networks, and interacting surfaces. ED analysis has revealed the fractionation of FtsZ into four motion subdomains that, for clarity, we term N1, N2, C1, and C2 (Fig. 7A). The motion subdomains bend through sets of concerted hinges, in a manner compatible with the displacements among FtsZ structures and collective Bf. The N-terminal subdomain N1 consists of the nucleotide binding loops plus their supporting β-strands and helices (triads STH1–5 + ST6, residues Ile40–Pro191). Subdomain N2 consists of helix H6, loop H6–H7, and the top of H7 (residues Pro191–Val214) and is not packed between the rigid β-strands of the N- and C-domains. C1 and C2 are non-sequential subdomains. C1 is at the front (according to our viewpoint) with more freedom to rotate, whereas C2 is at the back and is more rigid due to extensive connections to subdomain N1 and H7. Sequence conservation, low Bf, and ED initially suggested that the β-strands are bending zones through which C1 can rotate with respect to N1. In addition, the extensive contacts to β-strands (Fig. 2B) and allosteric network 3 (Fig. 1, D and E) point to a central role for H7 in the motion of the subdomains of FtsZ. Two bending points fractionate H7 into three-thirds: the top H7 third in N2, the central third in N1, and the bottom third in C1.

Given the correlation found among the structural comparisons and the ED analyses, we speculate that the small differences observed among the homologous FtsZ structures recapitulate essential modes of motion of the protein, which may reflect different adaptations to similar concerted movements. In this regard, the interspecies displacements observed between sections of H1 and H7 reflect the relative rotation between both N-terminal subdomains. A statistically significant correlation between the sequence variability and collective Bf has been observed for the FtsZ family (Fig. 2A). It will be interesting to analyze whether rigidity is also related with selective pressure in other protein families.

Interestingly, each of the four predicted motion subdomains of FtsZ participates in at least one of the four longitudinal contacts in a protofilament-like FtsZ dimer (Fig. 7A) (supplemental table in Ref. 19). This suggests that a concerted realignment of the four subdomains is required for filament assembly and, subsequently, for disassembly triggered by GTP hydrolysis. Hence, we propose that there is a permanent dialog between the positioning of subdomains, the bound nucleotide, and the association surfaces. This FtsZ machinery allows the progressive changes of association affinity required to overcome the energetic barriers of the binding/dissociation conformational changes. Likewise, the active and allosteric sites of several GTPases communicate through conserved residue networks (60). The interacting surfaces of FtsZ may function as allosteric sites, through the three allosteric networks detected, gradually favoring relative subdomain movements associated with the ordered events of nucleotide binding, nucleation, elongation, GTP hydrolysis, and depolymerization (Fig. 7B). However, the precise structural changes in the FtsZ functional cycle are difficult to predict. The FtsZ machinery permits it to respond to the cytokinetic demands in vivo through remodeling of the Z-ring. The acquisition of the straight shape may also imply lateral interactions relevant for the stabilization of the Z-ring (61). It should be noted that although FtsZ cooperative assembly is induced by GTP in vitro, it is also regulated by the balance of promoting (FtsA, ZapA, ZipA) and inhibiting factors (EzrA, MinCDE, SulA) in vivo (where the GTP level is maintained). GTP hydrolysis triggers disassembly in both cases.

Insights from Mutations, Inactive and Straight Mutants—Mutants in group A, although initially designed to remove charges or to change the volume of side chains at several strategic positions in FtsZ, behaved similarly to the wild-type protein (Fig. 4). These benign mutations constituted empirical negative controls of mutation effect.

The inactivating mutation M164A (group F) may be explained by the disruption of a conserved van der Waals contact at the top of the FtsZ molecule between Met164 in loop T5 (N-domain) and Leu204 in helix H7, corresponding to two motion subdomains in ED (Fig. 7A). Met164 is part of the SCA network 1, and its backbone is involved in the top contact A1 with the subunit above (Fig. 7A). It may be hypothesized that this mutation either disrupts the transmission of the signal from the GTP γ-phosphate to H7 or releases the N-domain to-H7 connection. The mutation E62A (type C), at a residue that is also one of the ED-predicted hinges in the nucleotide binding domain (Fig. 3A), resulted in slow assembly into normal polymers, possibly by hindering assembly cooperativity.

The increased disassembly of mutant A156T (group B), at β-strand S5, may be explained by its enhanced GTPase. Ala156 is an ED-predicted hinge between the core N-domain and the rest of the molecule (Fig. 3A). The equivalent temperature-sensitive E. coli FtsZ mutant A129T (56) might assemble into unstable polymers unable to form a functional Z-ring at the non-permissive temperatures.

The gain of assembly mutants K228A and S272A have slow polymerization and depolymerization with an enhanced assembly phenotype (class D). The polymer stabilization of these mutants is apparently due to the reduced disassembly rate, with a nearly normal GTPase, GTP content, and polymer shape. This suggests that reversible disassembly steps are partially blocked. The K228A mutation stabilized straight polymers, suggesting a reduction in a post-hydrolysis conformational change from straight to curved polymers. Mutating the adjacent residue Asp229, which is not engaged in salt bridges, to alanine had insignificant effects (group A). S272A stabilized toroidal polymers, suggesting a reduction in the disassembly of curved GDP polymers. These class D phenotypes may be related to perturbations in the contacts between the mobile subdomain C1 with respect to either subdomain N1, in the case of K228A, or subdomain N2, in the case of S272A.

On the other hand, analysis of the FtsZ sequences from Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium avium, and Mycobacterium bovis, in comparison with...
Mapping the FtsZ Activation Switch

those from *Methanococcus jannaschii*, *E. coli*, *Bacillus subtilis*, and *Streptococcus pneumoniae*, has indicated substitutions other than at our mutated switch residues, which are predominately conserved in mycobacterial FtsZ (not shown). Other local differences should be responsible for the characteristically inantly conserved in mycobacterial FtsZ (not shown). Other than at our mutated switch residues, which are predom-

Mutations at the C-terminal γ-Sheet Block FtsZ in Curved Assemblies—The mutations H288A (at β-strand S8) and G320V (at S9), involving predicted ED hinge points, resulted in GTP-containing yet curved FtsZ polymers, which disassembled with GDP (group E). Similar results were obtained by mutating the also predicted hinges D185A at loop H5–S6, G221V at the C-domain face of H7, I251A (S7), or D324A (loop S9–S10), made to remove the ion pair with Lys^{228}. The spiral morphology of group E mutant polymers is reminiscent of Mj-FtsZ polymers assembled with GDP and macromolecular crowding (13, 14). The spiral polymers formed by the group E mutants were apparently single-stranded, which requires an isomerization step for the polymerization to have cooperative behavior (15). Mutants in this curved phenotype significantly cluster at or facing the C-terminal β-sheet. These results strongly support the predicted bending of the conserved C-terminal β-sheet in switching between curved and straight FtsZs (see “Results”). The inhibition of the GTPase activity in these curved polymers may be due to an anomalous positioning of the co-catalytic aspartate residues Asp^{215} and Asp^{238} and the “arginine finger” Arg^{169} (19) with respect to the nucleotide (Fig. 7A, contacts B1 and B2). Mutations in group E block FtsZ in a curved structure that propagates into the spiral polymers observed, possibly by removing bonds or introducing wedges that hamper C-domain movement. We think that in the case of G221V and G320V, the most plausible explanation is that the larger side chain prevents the C1 subdomain from rotating and closing the cleft between H7 and the C-terminal domain due to sterical clashes with large hydrophobic residues located in front of these glycines, namely Leu^{225} for G320V and Met^{290} for G221V. These very conserved glycines could have been evolutionarily selected to generate cavities that allow for the C1 subdomain rotation. We expect that due to their severely altered assembly, this type of FtsZ mutant may be lethal for bacteria, as suggested by others, who did not observe spontaneous mutations in the β-sheet of the C-domain in Bs-FtsZ (64).

For a FtsZ protofilament to assemble cooperatively, both the top and the bottom interfaces of each monomer have to switch between low and high affinity states (15, 17). Considering the information gained from group E mutants, we propose that simultaneous top and bottom contacts favor a change of the GTP-bound monomer geometry from curved to straight, easing the 11° intradimer tilt and settling the GTPase activity residues in the active position. The conformational transitions at this stage may be transmitted through allostERIC network 2 across subdomains N1, C1, and C2. This suggests that a trimer may be enough to produce the first straight subunit.

The Assembly and Disassembly Cycle of a FtsZ Monomer—Our set of mutations affects the FtsZ functional cycle at several different stages. We propose a minimal reaction scheme for the FtsZ cycle of assembly and disassembly consisting of seven discrete states (Fig. 7B), based on the known properties of the FtsZ system. This is an extension of simplified schemes employed in previous FtsZ isomerization-polymerization models (15, 17, 18). The scheme refers to the conformational state and nucleotide bound to a single monomer when unassembled, in oligomeric nuclei, or in single-stranded filaments, whereas ignoring the exact degree of protein self-association in the latter two. FtsZ bound to GDP (D) is assumed in an inactive curved (C) state (named DC), which upon binding of GTP (T) retains a closely related curved conformation (TC'). GTP-bound FtsZ associates isodesmically into an inactive nucleus conformation (TC")n, which can switch into an active high affinity straight (S) nucleus conformation (TS)n. This active nucleus cooperatively elongates into longer polymers in which the same active monomer conformation is transmitted (TS)_{m}. The nucleotide γ-phosphate is eventually hydrolyzed in the polymers, leading to (DS')_{m} which relaxes to a curved conformation (DC")_{m} that disassembles back to the ground state DC. Enhancing the protein association may force TC' to assemble, in a side dead-end reaction, into curved polymers (TC")_{m}. Although a number of other reaction mechanisms are possible, we believe that the choice of this particular reaction pathway is a reasonable account of the assembly properties of wild-type Mj-FtsZ (11, 13), necessarily simplified here to the formation of single protofilaments (as observed for Ec-FtsZ (13)). Based on the properties of each class of FtsZ mutant, the steps more probably affected in the model functional cycle of FtsZ, whose hypothetical transduction mechanisms can be outlined, are proposed (Fig. 7B) (Supplemen
tal Discussion).

Mutational Insight into Drugging the FtsZ Switch—In this work, we have characterized the flexibility of the FtsZ molecule and mapped FtsZ residues involved in its assembly switch. We employed a robust consensus of computational approaches and 17 mutations at predicted key residues that modulate FtsZ assembly, acting at different stages of its functional cycle. Among these, group E mutants prevent the FtsZ curved-to-straight transition, which probably involves a rotation of the C-terminal domain closing onto core helix H7. Structural and kinetic studies with each of these mutant classes might give further insight into the precise nature of the sequential mechanism of the FtsZ assembly switch. Another interesting question is whether the FtsZ activation switch can be modulated with small molecules with antibacterial activity. Interestingly, two group E mutations, G221V and H288A, which block Mj-FtsZ in curved polymers with inhibited GTPase, map in positions equivalent to residues Gly^{196} and Asn^{263} of Sa-FtsZ, whose mutations confer resistance to the antibacterial compound PC190723 (8). Both sets of mutants (Mj-group E and Sa-resistant), although not completely coincident, delineate the long cleft between the C-domain and H7, which is in a position equivalent to the taxol binding site of tubulin. PC190723 is a polymer-stabilizing agent for Bs-FtsZ and Sa-FtsZ (9). It is to be investigated whether this compound, by binding to said cleft, interferes with the curved-straight switching in Bs-FtsZ and Sa-FtsZ, similarly to what taxanes do for tubulin by favoring its assembled straight form (65, 66). This brings attention to this cleft between the C-terminal domain and H7 as a druggable site for the rational design of compounds whose binding may mimic or oppose the mutations described here, thus targeting FtsZ polymer dynamics in bacterial pathogens.
