Investigation of Regulation of FtsZ Assembly by Sula and Development of a Model for FtsZ Polymerization^{∇†}

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In *Escherichia coli* FtsZ organizes into a cytoskeletal ring structure, the Z ring, which effects cell division. FtsZ is a GTPase, but the free energy of GTP hydrolysis does not appear to be used for generation of the constriction force, leaving open the question of the function of the GTPase activity of FtsZ. Here we study the mechanism by which Sula, an inhibitor of FtsZ induced during the SOS response, inhibits FtsZ function. We studied the effects of Sula on the in vitro activities of FtsZ, on Z rings in vivo, and on a kinetic model for FtsZ polymerization in silico. We found that the binding of Sula to FtsZ is necessary but not sufficient for inhibition of polymerization, since the assembly of FtsZ polymers in the absence of the GTPase activity was not inhibited by Sula. We developed a new model for FtsZ polymerization that accounts for the cooperativity of FtsZ and could account for cooperativity observed in other linear polymers. When Sula was included in the kinetic scheme, simulations revealed that Sula with strong affinity for FtsZ delayed, but did not prevent, the assembly of polymers when they were not hydrolyzing GTP. Furthermore, the simulations indicated that Sula controls the assembly of FtsZ by binding to a polymerization-competent form of the FtsZ molecule and preventing it from participating in assembly. In vivo stoichiometry of the disruption of Z rings by Sula suggests that FtsZ may undergo two cooperative transitions in forming the Z ring.

Cytokinesis in bacteria is accomplished by a septal apparatus which becomes organized at the site of division. The earliest cytologically detectable event in establishment of this apparatus is the formation of the Z ring at midcell (8, 9, 17, 21). The Z ring is composed of polymers of FtsZ which are attached to the membrane through membrane-associated proteins FtsA and ZipA (55, 56). All other cell division proteins are recruited to the division site by linkage to the Z ring and depend upon it for persistence there.

The Z ring is a very dynamic structure through which subunits of FtsZ cycle with a half time of about 10 seconds (2). This dynamism is correlated with the rate of the GTPase activity of FtsZ. The physiological function of the GTPase activity of FtsZ and the corresponding dynamism of the Z ring is not clear. The free energy of GTP hydrolysis does not appear to be used to generate the constriction force, since FtsZ mutants compromised in GTPase activity are able to constrict (7, 17, 50).

In vitro, FtsZ undergoes GTP-dependent polymerization (18, 45, 48, 57). Because the enzymatic site for GTP hydrolysis is formed at the interface of polymerizing subunits of FtsZ, the

GTPase activity is coupled to polymerization (63, 64). Both the polymerization and the GTPase display critical behavior and are therefore cooperative. Above the critical concentration (~1 μ M), FtsZ monomers assemble to form enzymatically active linear polymers that are dynamic due to the GTPase activity (12, 47). Currently, it is not understood how single-stranded linear polymers can be cooperative, i.e., show a critical concentration for assembly. At slightly higher protein concentrations (2 to 3 μ M), the linear polymers interconnect further to form an elastic network (22). These results reveal that, in the presence of GTP, FtsZ has an intrinsic tendency to undergo transitions between states of organization as a function of concentration.

In *Escherichia coli* and *Bacillus subtilis*, the intracellular concentration of FtsZ varies little during the cell cycle (71), so the regulation of the Z ring is accomplished by control over FtsZ assembly. During the uninterrupted cell cycle, spatial coordination of cell division involves inhibition of Z-ring formation over the nucleoid by Noc (4, 74) and inhibition at the cell poles by Min (19, 32). These mechanisms are not completely understood (for a review, see reference 17). The cell also coordinates cell division in response to damage to DNA. In *E. coli*, the exposure of cells to DNA damage leads to the induction of the SOS response. Among the induced genes is *sulA* (69), a labile inhibitor of cell division (34) that targets FtsZ (5, 33). Sula is degraded by the Lon protease and has a half-life of ~1 min (43).

Insights into the mechanism by which Sula controls FtsZ have been gained in previous studies. Sula has been purified as a fusion protein to MalE and *Staphylococcus* protein A (30, 44, 68). Both fusions inhibit the polymerization and GTPase activity of FtsZ (44, 68), but the studies led to two different models for the inhibition of FtsZ by Sula. Trusca et al. (68)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant genotype	Reference or source
<i>E. coli</i> strains		
JKD7-1	W3110 <i>ftsZ::kan recA56</i>	15
W3110	Wild-type strain	
AND101	W3110 $\Delta(\lambda attL-lon)::bla lacI^q P_{208-ftsZ-gfp}$	This study
Plasmids		
pKD4	<i>ftsZ</i> on pGB2	5
pGAD424	Yeast two-hybrid vector	Clontech
pZ-F268E	pKD4 with F268 of <i>ftsZ</i> mutated to E	This study
pZ-F268A	pKD4 with F268 of <i>ftsZ</i> mutated to A	This study
pZ-F269A	pKD4 with F269 of <i>ftsZ</i> mutated to A	This study
pZ-P300A	pKD4 with P300 of <i>ftsZ</i> mutated to A	This study
pZ-C149A	pKD4 with C149 of <i>ftsZ</i> mutated to A	This study
pZ-R31A	pKD4 with R31 of <i>ftsZ</i> mutated to A	This study
pAND110	<i>ftsZ(F268A)</i> cloned into pGAD424	This study
pAND111	<i>ftsZ(F269A)</i> cloned into pGAD424	This study
pAND112	<i>ftsZ(P300A)</i> cloned into pGAD424	This study
pACT3	Expression vector	20
pA3	Chromosomal <i>sulA</i> cloned into pACT3 cut with XbaI and HindIII	This study
pKD126	<i>ftsZ</i> cloned in pJF118EH	44
pJC93	<i>malE-sulA</i> fusion cloned into pBAD18	44
pJC94	<i>malE-sulA10</i> fusion cloned into pBAD18	44
pZAO	pBR322 derivative carrying <i>ftsQ</i> , <i>ftsA</i> , and <i>ftsZ</i>	5
pBR322		
pBEF0	pGB2 derivative carrying <i>ftsA</i> , <i>ftsZ</i> , and <i>envA</i>	5
pGB2	Low-copy-number vector with pSC101 origin of replication	13
pSEB160	<i>ftsZ</i> gene cloned downstream of the arabinose promoter on pBAD18	S. Pichoff and J. Lutkenhaus, unpublished data
pAND403	<i>GFP-sulA</i> cloned into pBAD18 cut with HindIII and XbaI	This study

proposed capping as a possible mechanism, while Mukherjee et al. (44) proposed sequestration. Existing data cannot distinguish between these possibilities.

In contrast to the wild-type FtsZ protein, SulA had no discernible effect on FtsZ mutants selected as resistant to SulA. Mukherjee et al. (44) studied FtsZ-F268C (FtsZ114), a mutant protein that does not bind SulA in the yeast two-hybrid system. Both the GTPase activity and polymerization were resistant to MalE-SulA (44). Trusca et al. (68) studied FtsZ-D212G (FtsZ2), a mutant protein that still binds SulA in the yeast-two-hybrid system (33, 68). Polymerization of D212G, which occurs only in the presence of DEAE dextran, was found to be insensitive to protein A-SulA (68).

SulA from *Pseudomonas aeruginosa* has been crystallized in a complex with FtsZ. SulA binds the T7 loop involved in polymerization and hydrolysis of GTP (14). The crystal structure and the in vitro studies described above demonstrate that SulA interacts directly with FtsZ but that this interaction is not sufficient for control of cytokinesis. What are the necessary and sufficient conditions for regulation of FtsZ by SulA? Does SulA inhibit FtsZ assembly by sequestration or capping? What is the basis for SulA-resistant *ftsZ* mutations that still bind SulA?

Here, we studied the effects of SulA on the cooperative transitions that FtsZ undergoes in vitro. We find that SulA inhibits FtsZ polymerization only in the presence of energy flux provided by the GTPase activity. We developed a kinetic model for FtsZ polymerization that accounts for the cooperativity of assembly. By employing this model, we were able to simulate the polymerization reaction and recapitulate the ef-

fects of SulA. This leads to a model for FtsZ polymerization that explains why the GTPase activity is necessary for SulA inhibition. We also examine the stoichiometry of SulA inhibition of Z-ring assembly in vivo.

MATERIALS AND METHODS

Strains and growth conditions. Table 1 lists the bacterial strains and plasmids used in this study. Bacterial strains were grown in LB at 37°C, unless otherwise indicated. Antibiotic selection was maintained for all markers at the following concentrations unless specified otherwise: ampicillin, 100 µg/ml; chloramphenicol, 20 µg/ml; tetracycline, 12.5 µg/ml; and spectinomycin, 50 µg/ml. Medium was supplemented with 0.2% D-glucose or with the specified amount of isopropyl-β-D-thiogalactopyranoside (IPTG) or arabinose to repress or induce transcription from the *tac* and *lac* promoters or *ara* promoter present on expression vectors. Induction of arabinose has no effect on the level of SulA (3).

E. coli strain AND101 was constructed by transduction of strain W3110 with P1vir grown on *E. coli* EC448 (*ftsZ-gfp/Amp^r*). In addition to *ftsZ* at the normal locus, strain EC448 expresses FtsZ-green fluorescent protein (GFP) expressed from another chromosomal locus under the control of a modified *lac* promoter (73). The transductants were selected for ampicillin resistance on 25 µg/ml, and the expression of FtsZ-GFP was confirmed by induction with IPTG (50 µM for 2 h) and observation of Z rings by fluorescence microscopy. Plasmids containing *malE-sulA* were introduced into strain AND101 by selecting for ampicillin resistance at 250 µg/ml. Note that strain AND101 is sensitive to ampicillin at this concentration.

Plasmid construction. For construction of mutant FtsZ proteins, plasmid pKD4 (5) was mutagenized utilizing the QuikChange site-directed mutagenesis kit from Stratagene according to the technical manual provided. The sequences of primers used for this purpose are available upon request. Mutant constructs were verified by sequencing. To test for interaction with SulA in the yeast two-hybrid system, the mutant alleles of *ftsZ* were amplified by PCR using the following primers: 5'-AGTCTGCAGTTAATCAGCTTG CTTACG3' and 5'-ACCGGATCCCTATGTTTGAACCAATGG3'. The PCR products were cut with PstI and BamHI (restriction sequence underlined in the primers) and cloned into

pGAD424 (Clontech). The pA3 plasmid was constructed by amplifying the chromosomal copy of *sulA* using the following primers: 5'ATATTCTAGACTG GATTGATTATGTACACTTCAGGC3' and 5'ATATAAGCTTTTAATGATACAAATT CAAATTAGAGTGAATTTTA3'. The PCR products were digested with HindIII and XbaI (restriction sequences underlined in the primers) and cloned into the corresponding sites of the expression vector pACT3 (20).

For construction of pAND403, the *sulA* gene from *E. coli* was PCR amplified using the following primers: 5'GTCAAAGCTTCTTAATGATACAAATT AGAG 3' and 5'CAGTTCTAGATACACTTCAGGCTATGCACATCG3'. The PCR product was cut with XbaI and HindIII (restriction sequences underlined in the primers) and cloned into the corresponding sites on pJC106.

Plasmids pZAO, pGB2, pBEF0, and pBR322 have been described previously (5, 13).

Complementation and SulA resistance. Strain JKD7-1 (*ftsZ::kan*) carrying the temperature-resistant plasmid pKD3A was used for complementation. This strain has *ftsZ* at the chromosomal locus disrupted with a *kan* insertion (15). Plasmids carrying the *ftsZ* mutations were transformed into the strain and tested for growth at the nonpermissive temperature after checking that pKD3A had been displaced. To test for SulA resistance, plasmids carrying the *ftsZ* mutations were transformed into strain W3110 carrying pA3. Transformants were resuspended in 1 ml of LB, grown for 1 h, and serially diluted 10-fold, and 5- μ l portions of the dilutions were spotted on plates with the appropriate antibiotic supplemented with either glucose or IPTG (10 μ M, 100 μ M, or 1 μ M IPTG). Resistance to SulA was scored against the plasmids carrying the wild-type copy of *ftsZ*.

Overexpression and purification of proteins. The pKD126 plasmid was used for overexpression of FtsZ (16), and FtsZ was purified as described previously (49). pJC93 and pJC94 were used for overexpression and purification of MalE-SulA and MalE-SulA10, respectively (44). Mutant FtsZ proteins were purified by the same protocol as for the wild-type proteins using derivatives of pKD126 carrying the mutation.

FtsZ polymerization and GTPase activity assays. Assays for FtsZ polymerization by sedimentation and electron microscopy were performed as described previously (47). Briefly, FtsZ at various concentrations was incubated in the polymerization buffer (Pol buffer) (50 mM morpholineethanesulfonic acid [pH 6.5], 10 mM MgCl₂, 200 mM KCl) for 10 min at room temperature, and polymerization was initiated by the addition of GTP. The reaction mixtures were centrifuged, and the pellets were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained by Coomassie blue. Thereafter, the images of gels were captured by a charge-coupled-device camera, and the relative amount of FtsZ in the bands was determined densitometrically using the Alpha Innotech software. To calibrate the image-capturing equipment and to establish the linear range of measurement, standard known amounts of FtsZ protein were first run and measured. Thereafter, protein samples from sedimentation assays were diluted to fall within this linear range. We found the variation between replicate experiments to be $\sim 10\%$ under our conditions.

GTPase activity assays were performed as described previously (44, 47), except that the specified concentrations of MalE-SulA were included in the reactions. Our measurements of the concentration-dependent activation of GTPase activity are congruent with previous studies under similar conditions (10, 38, 47, 70).

For aluminum fluoride polymerization experiments, instead of GTP, GDP was added along with AlCl₃ and NaF (at concentrations of 100 μ M and 10 mM, respectively) to initiate polymerization reactions. GMPCPP was obtained from Jena Biosciences (Jena, Germany). Polymerization reactions were done as with GTP, except that GMPCPP was used.

Pull-down assays. Freshly purified MalE-SulA was bound to amylose resin. FtsZ (at 10 μ M in Pol buffer) was incubated with 100 μ l of bed volume of MalE-SulA-amylose resin. The reactions were centrifuged in a tabletop centrifuge and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) to detect bound proteins. For a negative control, amylose resin with no MalE-SulA bound was incubated with FtsZ under the same conditions and centrifuged. This confirmed that FtsZ did not bind to amylose and that FtsZ does not sediment on its own at the centrifugation speeds used in this experiment.

Nucleotide exchange kinetics. 2'- (or 3'-)-O-(*N*-methylanthraniloyl)guanosine 5'-diphosphate (mant-GDP) and 2'- (or 3'-)-O-(*N*-methylanthraniloyl)guanosine 5'-triphosphate (mant-GTP) nucleotides were obtained from Molecular Probes. mant-GDP and mant-GTP bound FtsZ specifically and reversibly with affinities similar to the unlabeled nucleotide (see Fig. S2 in the supplemental material). Nucleotide exchange was measured in a π^* -180 spectrometer from Applied Photophysics (Surrey, United Kingdom) where the excitation wavelength was set at 360 nm and emission wavelength was set at 448 nm. Both the excitation and emission slit openings were set at 5 nm.

Theory and simulations. Let $[Z]$, $[S]$, and $[ZS]$ denote the concentrations of free FtsZ, free SulA, and the FtsZ-SulA complex, respectively. Then we can write:

$$K_d = [Z][S]/[ZS] \quad (1)$$

where K_d is the dissociation constant.

By conservation, we can write:

$$[S] = [S_{\text{total}}] - [ZS] \quad (2)$$

where S_{total} is the total SulA.

By substituting equation 2 in equation 1, we obtain:

$$K_d = [Z]([S_{\text{total}}] - [ZS])/[ZS] \quad (3)$$

At steady state, this becomes:

$$K_d = C_c([S_{\text{total}}] - [ZS])/[ZS] \quad (4)$$

where C_c is the critical concentration for polymerization of FtsZ.

To calculate the K_d for FtsZ-SulA interaction, we assumed that the FtsZ inactive in our sedimentation and GTPase activity assays is complexed with SulA; in other words: $[FtsZ_{\text{active}}] = [FtsZ_{\text{total}}] - [MalE-SulA-FtsZ]$ where $FtsZ_{\text{active}}$ is active FtsZ and $FtsZ_{\text{total}}$ is total FtsZ. Taking into account the 10% variability in our measurements, we calculate that the binding of SulA to FtsZ is 90 to 99%. Using equation 4 above gives us a K_d in the range 10^{-8} to 10^{-7} . We eliminated lower values for K_d (10^{-9} and lower) because the *Escherichia coli* FtsZ-SulA complex is not tight enough to be detected by affinity chromatography or size exclusion chromatography (A. Dajkovic and J. Lutkenhaus, unpublished data). This is in contrast to the *Pseudomonas* protein (14) which seems to form a more stable complex. For our simulations, we used parameter values for on and off rates for the steady-state polymers similar to a previously published study (11). The values for the forward (α_+) and reverse rate (α_-) constants for the internal equilibrium of FtsZ were as follows: $\alpha_+ = 1 \text{ s}^{-1}$ and $\alpha_- = 3^{-1}$. The value for the on rate for the equilibrium polymer (β_+) was $5e6 \text{ M}^{-1}\text{s}^{-1}$, and the value for the off rate for the equilibrium polymer (β_-) was 5.6 s^{-1} . We implemented the model by including seven intermediate species (dimers, trimers, tetramers, etc.) between the activated monomer and the polymer in the reaction mechanism as described previously (11, 12). For equilibrium polymers, β_+ was the same, but the β_- was scanned until it agreed with experiments. The model was simulated with CellDesigner (36, 53), with Chemical Kinetics Simulator from IBM, and with Berkeley Madonna.

Microscopic observation of live cells after induction of MalE-SulA. Cells were grown and induced as described below. For microscopic observation, slides were prepared by pipetting 100 μ l of 7.5% low-melting-point agarose on a precleaned microscope slide (Fisher) and flattening it with a coverslip. After the agarose hardened, the coverslip was removed, and 3 μ l of cells was spotted on the surface and covered with another coverslip. The cells were brought into focus in phase, and pictures of GFP fluorescence were taken every 30 seconds starting at 4.5 min after induction and every 15 seconds starting at 5 min after induction. The exposure times were 1 second. Pictures were taken with CoolSnap ES charge-coupled-device camera, and the images were captured by MetaMorph software (Universal Imaging, Inc.). The rings were considered completely gone when the fluorescence in the medial region of the cell reached the fluorescence of the cytoplasm.

Quantitative immunoblotting of FtsZ and MalE-SulA. Strain AND101 was transformed with pJC93 and grown on LB plates containing 0.4% glucose and 250 μ g/ml ampicillin. Transformants from plates incubated overnight at 37°C were inoculated and grown for 2 h at 37°C in LB supplemented with 0.4% glucose, 250 μ g/ml ampicillin, and 50 μ M IPTG. The cultures were then shifted to 25°C where the growth was continued until mid-log phase as measured by optical density ($0.2 > OD_{600} > 0.4$ where OD_{600} stands for optical density at 600 nm). The cells were washed and resuspended into medium consisting of LB supplemented with 250 μ g/ml ampicillin and 50 μ M IPTG. The cultures were induced with 0.4% arabinose, and samples were taken for immunoblotting and for microscopy every 2.5 min. For chloramphenicol experiments, 250 μ g/ml of chloramphenicol was added to cultures at 4 min. The cultures were allowed to grow, and samples were collected from cultures with and without chloramphenicol at 15 min.

Cells for microscopic examination were immediately fixed in glutaraldehyde and paraformaldehyde as described previously (1). Samples for immunoblotting were centrifuged, and the cell pellet was resuspended in SDS-PAGE sample buffer. Microscopic examination was carried out, and the percentage of cells with Z rings at various time points was determined ($n > 500$). We conducted the

experiment 10 times, and ~90% of cells lost their rings by 7.5 min each time. To determine the amount of MalE-SulA sufficient to inhibit ring formation, samples from 5 min and 7.5 min were run along with standard amounts of MalE-SulA on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. On separate gels, samples were run with standards of known amounts of FtsZ protein. The first sets of immunoblots were run to determine the approximate amount of protein in the sample. The samples were then run again with a difference of no more than twofold between the highest standard and the lowest standard. This way, a more accurate determination of the protein level was possible. The FtsZ samples were incubated with anti-FtsZ antibody diluted 1:3,000 and then with the alkaline phosphatase-conjugated goat anti-mouse secondary antibody. The samples were developed using the Bio-Rad kit. The MalE-SulA samples were incubated with anti-MalE antibody diluted 1:5,000 and then with the horseradish peroxidase-conjugated secondary antibody. The samples were developed using the ECL kit from Amersham Biosciences. All quantifications were repeated five times to establish reproducibility.

RESULTS

MalE-SulA increases the observed critical concentration for polymerization of FtsZ. To distinguish between two proposed mechanisms for the activity of SulA, we studied the effect of changing the concentration of MalE-SulA on the critical concentration of FtsZ. We incubated increasing concentrations of FtsZ with fixed concentrations of MalE-SulA. If SulA inhibits FtsZ by sequestration, SulA would bind FtsZ monomers, resulting in an increase in the observed critical concentration for polymerization of FtsZ. Increasing concentrations of FtsZ in reaction mixtures with a fixed amount of SulA would result in a linear increase in the amount of polymers formed. On the other hand, if SulA inhibited FtsZ by capping, then increasing the concentration of FtsZ at a given SulA concentration would result in a nonlinear increase in the amount of polymer (62). We used the MalE-SulA fusion protein in sedimentation assays for polymerization of FtsZ as previously described (47). This assay measures the steady-state level of FtsZ polymers which is achieved rapidly upon the addition of GTP. In the absence of MalE-SulA, FtsZ polymerized with a critical concentration of $0.9 \mu\text{M}$ ($\pm 10\%$) (Fig. 1A), congruent with other published reports (11, 47, 59, 75).

When MalE-SulA was included in the polymerization reactions, the observed critical concentration for polymerization of FtsZ increased and higher concentrations of FtsZ were required to obtain the same quantity of polymers. The observed effect was linearly dependent on the amount of MalE-SulA added. The addition of $3.5 \mu\text{M}$ MalE-SulA to the polymerization reaction mixture raised the critical concentration to ca. $4.3 \mu\text{M}$. The addition of $5 \mu\text{M}$ MalE-SulA raised the critical concentration to $5.9 \mu\text{M}$ (Fig. 1A). In repeated experiments with various concentrations of MalE-SulA, the increase in the observed critical concentration corresponded to the concentration of MalE-SulA in the polymerization reaction mixture. The behavior conformed to $[\text{FtsZ}_{\text{active}}] = [\text{FtsZ}_{\text{total}}] - [\text{MalE-SulA}]$ (where $\text{FtsZ}_{\text{active}}$ is active FtsZ and $\text{FtsZ}_{\text{total}}$ is total FtsZ) within experimental error, consistent with a sequestration mechanism.

The effect on the amount of observed polymers of FtsZ was the same whether MalE-SulA was added before or after polymerization was initiated by the addition of GTP (data not shown). This indicates that MalE-SulA was able to establish a rapid equilibrium with FtsZ and reverse the polymerization reaction on the time scale of seconds or minutes. These results

FIG. 1. SulA increases the observed critical concentration for polymerization of FtsZ. Various concentrations of FtsZ were incubated in Pol buffer for 10 min, and GTP was added to initiate polymerization. The reaction mixtures were centrifuged at $200,000 \times g$ for 15 min, and the pellets were analyzed by SDS-PAGE. The amount of protein (densitometric units) in the bands on the gels was quantified and plotted against FtsZ concentration. (A) FtsZ critical concentration is determined as the concentration of FtsZ at the intercept on the x axis [FtsZ]. The addition of $3.5 \mu\text{M}$ MalE-SulA leads to an increase of the critical concentration to $4.3 \mu\text{M}$. The addition of $5 \mu\text{M}$ MalE-SulA leads to a shift to $5.9 \mu\text{M}$. (B) The critical concentration for FtsZ114 is unaffected by the presence of MalE-SulA. (C) MalE-SulA10 has no effect on the critical concentration of FtsZ.

are consistent with SulA binding to FtsZ monomers and removing them from the pool available for polymerization.

The same stoichiometry of inhibition was observed when the polymerization reactions were assayed by electron microscopy (see Fig. S1 in the supplemental material). When MalE-SulA was in excess of FtsZ, no discernible polymers were observed; however, when FtsZ was in excess, polymers were observed. This is also consistent with a sequestration mechanism, since capping proteins do not prevent polymerization (51).

TABLE 2. Summary of *ftsZ* mutations^a

Mutation	SulA resistance	Complementation	GTPase	Interaction with SulA
<i>ftsZ</i> (F268C) (<i>ftsZ</i> 114)	+	+	+	—
<i>ftsZ</i> (F268G)	+	+	+	—
<i>ftsZ</i> (F268A)	+	+	+	—
<i>ftsZ</i> (D269A)	+	+	ND	—
<i>ftsZ</i> (D212G) (<i>ftsZ</i> 2)	+	—	—	+
<i>ftsZ</i> (D209N)	+	—	—	+
<i>ftsZ</i> (G105S)	—	At 30°C	—	+
[<i>ftsZ</i> 84(Ts)]				

^a +, positive; —, negative; ND, not done.

We further studied an allele of *ftsZ* [*ftsZ*114(F268C)] resistant to SulA. The FtsZ-F268C protein polymerized with a critical concentration very similar to that of the wild-type protein. Polymerization reactions with FtsZ-F268C were unaffected by the addition of MalE-SulA (Fig. 1B), and the observed critical concentration was the same with or without the addition of MalE-SulA.

ftsZ(F268A) and *ftsZ*(D269A) are two additional mutations isolated by site-directed mutagenesis. Both FtsZ-F268A and FtsZ-D269A proteins are resistant to SulA in vivo and do not bind FtsZ in the yeast two-hybrid system (Table 2) (see supplemental material also). The FtsZ-F268A protein was purified. It polymerized and displayed GTPase activity which was also unaffected by the presence of MalE-SulA. The critical concentration for polymerization was within 15% of that observed for the wild-type and FtsZ-F268C proteins (data not shown).

To verify that the observed effect of MalE-SulA on the critical concentration for polymerization of FtsZ was specific, we performed sedimentation assays in the presence of MalE-SulA10. *sulA10* is an allele of *sulA* whose gene product is unable to inhibit cell division or inhibit the GTPase activity of FtsZ (33, 44). This allele also shows no interaction with FtsZ in the yeast two-hybrid system (33). The addition of purified MalE-SulA10 protein to the polymerization reaction mixture had no effect on the observed critical concentration of FtsZ polymerization even when added at 6 μ M (Fig. 1C). These data indicate that the effect of MalE-SulA on the critical concentration of FtsZ was specific.

MalE-SulA controls the transition between steady states of FtsZ GTPase activity. FtsZ GTPase activity undergoes a concentration-dependent sigmoidal transition between the steady state of no activity and the steady state of full activity (68). Because the GTPase activity is coupled to polymerization, this transition clearly reflects the cooperativity involved in FtsZ assembly. We therefore examined the effect of SulA on the GTPase activity of FtsZ.

We measured the GTPase activity over a range of concentrations spanning from 0.5 to 9 μ M and plotted the specific activity against the FtsZ concentration. The data were fitted to the Hill function (see Materials and Methods), and two representative curves are shown in Fig. 2A. The addition of MalE-SulA leads to a shift in the midpoint of the transition and has no effect on the slope of the curve regardless of the concentration used (1 to 4 μ M). This result indicates that SulA sequesters FtsZ away from the assembly reaction but has no

effect on the cooperativity. The observed shift corresponds to the increased concentration of MalE-SulA in the reaction mixture so that higher levels of FtsZ are required to attain the same activity. The stoichiometry of the effect is one to one (Fig. 2A), consistent with a sequestration mechanism. Figure 2B contains representative data for the concentration of FtsZ at the midpoints of transitions for reaction mixtures with the indicated concentrations of MalE-SulA.

MalE-SulA does not control the nucleotide exchange rate of FtsZ. The data presented thus far are consistent with varied interpretations of the possible mechanism by which SulA inactivates FtsZ. Since the GDP-bound form of FtsZ is inactive for assembly, it is possible that SulA inhibits the release of bound GDP and regulates polymerization of FtsZ through control over nucleotide exchange.

To test the possibility that SulA affects nucleotide release, we chose fluorescent nucleotides mant-GDP and mant-GTP. These nucleotides bind FtsZ reversibly with affinities similar to those of unlabeled nucleotides (see Fig. S2 in the supplemental material). We used a pull-down assay (Fig. 3A) to verify that FtsZ bound SulA in the presence of these nucleotides (see Materials and Methods). Briefly, freshly purified MalE-SulA was prebound to amylose resin and incubated with FtsZ in the presence or absence of mant-GDP and mant-GTP. The reac-

FIG. 2. MalE-SulA increases the concentration of FtsZ required to see activation of the FtsZ GTPase activity. Various concentrations of FtsZ were tested for GTPase activity in the presence or absence of MalE-SulA, and the specific activity was plotted against the FtsZ concentration and fitted to the Hill function. (A) Sigmoidal transition between the steady states of FtsZ GTPase activity in the absence (filled circles) or presence of 4 μ M MalE-SulA (open circles). (B) Concentration of FtsZ (y axis) at the midpoint of the transition with the indicated concentrations of MalE-SulA.

FIG. 3. MalE-SulA does not affect the nucleotide exchange rate of FtsZ. (A) MalE-SulA binds to FtsZ in the presence of mant nucleotides as assayed by a pull-down assay. Freshly purified MalE-SulA bound to amylose resin (100 μ l) was incubated with FtsZ (10 μ M in a total reaction mixture volume of 500 μ l in Pol buffer) either in buffer alone (lanes 1 and 2) or in the presence of mant-GDP (lanes 3 and 4) or mant-GTP (lanes 5 and 6). For a negative control, amylose resin without any MalE-SulA was incubated with FtsZ under the same conditions (lanes 1, 3, and 5). The reaction mixtures were centrifuged in a tabletop centrifuge, and the pellets were analyzed by SDS-PAGE. (B) The nucleotide exchange rate of FtsZ bound with fluorescent nucleotide mant-GDP was measured by the rapid addition of unlabeled nucleotide in a stopped-flow apparatus. The curves obtained from averaging 10 measurements show the decrease in fluorescence as a function of time as the fluorescent mant-GDP bound to FtsZ is replaced by GDP. The nucleotide exchange rate of FtsZ (5 μ M) was determined to be 13 s^{-1} . The bottom graph shows residuals. (C) The nucleotide exchange rate was determined to be 13 s^{-1} in the presence of equimolar amounts of MalE-SulA (5 μ M). The bottom graph shows residuals.

tion mixtures were centrifuged to separate the resin and bound proteins from the rest of the reaction mixture, and the pellets were analyzed by SDS-PAGE (Fig. 3A).

We then measured the nucleotide exchange rate of FtsZ and examined the effect of MalE-SulA. FtsZ was prebound with the fluorescent nucleotide mant-GDP or mant-GTP, and the rate at which the mant nucleotide was released was measured following the rapid addition of unlabeled nucleotide in a stopped-flow apparatus. Figure 3B shows a typical curve obtained by averaging 10 measurements where FtsZ was prebound with mant-GDP and unlabeled GTP was added. The data obtained were fitted to a single exponential curve, and the exchange rate constant (k_{off} for mant-GDP) was determined to be 13 s^{-1} . The addition of equimolar amounts of MalE-SulA had no effect on the nucleotide release rate of FtsZ, and the k_{off} for mant-GDP was measured at 13 s^{-1} (Fig. 3C shows an average of 10 measurements). An equimolar amount of MalE-SulA is sufficient to inhibit the GTPase activity and polymerization of FtsZ.

GTPase activity of FtsZ is necessary for regulation of FtsZ polymerization by SulA. Analysis of *ftsZ* mutations conferring resistance to SulA revealed at least two classes: class A, such as *ftsZ*(F268C) (*ftsZ*114), *ftsZ*(F268A), and *ftsZ*(D269A), which prevent SulA binding, and class B, such as *ftsZ*(D212G) (*ftsZ*22), that still bind SulA. *ftsZ*(D209N) is another mutation we isolated that is similar to *ftsZ*(D212G) (Table 2; also see supplemental material). Inspection of the FtsZ-SulA protein structure reveals that the residues altered by class A mutations lie at the SulA-FtsZ interface, consistent with their being involved in this interaction (Fig. 4, residues corresponding to F268 and D269 are yellow and brown, respectively). The residues altered in the class B mutants are not at the FtsZ-SulA interface, which is consistent with these mutations not affecting SulA binding (Fig. 4, D212 is white).

The FtsZ-D212G protein has been shown to copolymerize with FtsZ and reduce the GTPase activity (50). Also, its polymerization is resistant to SulA when polymerized in the presence of DEAE dextran (68). This led us to investigate whether the GTPase activity of FtsZ was necessary for sensitivity to SulA. FtsZ polymers can be assembled in the absence of GTP hydrolysis in several ways. They can be assembled in the presence of the nonhydrolyzable GTP analogue GMPCPP, in the presence of GDP and aluminum fluoride, in buffer without magnesium, or in buffers with a pH of <6 (37, 42, 46). Following Hill and Kirschner (31), we refer to these polymers as equilibrium polymers, since they reach a true thermodynamic equilibrium. This is in contrast to the situation where FtsZ is hydrolyzing GTP and the polymers are dynamic. Using the same thermodynamic logic, we will refer to the enzymatically active polymers as steady-state polymers because polymerization never reaches an equilibrium, but a dissipative steady state with a defined energy flow (31).

First we verified that SulA bound FtsZ in the presence of GMPCPP and in the presence of GDP and aluminum fluoride using a pull-down assay (Fig. 5A). Next we used the sedimentation assay to test the effects of MalE-SulA on the critical concentration of polymers formed in the presence of GMPCPP. In contrast to its effect on steady-state polymers, MalE-SulA did not influence the critical concentration or the total amount of equilibrium polymers formed whether it was

FIG. 4. Locations of residues in FtsZ altered by SulA-resistant mutations. Chain A from the crystal structure of the FtsZ dimer from *Methanococcus jannaschii* (Protein Data Bank accession number 1FSZ) was aligned with chain A from the FtsZ-SulA complex from *Pseudomonas aeruginosa* (Protein Data Bank accession number 1OFU). Chain A of FtsZ from *P. aeruginosa* is shown in green, and chain B of the FtsZ dimer from *M. jannaschii* is shown in cyan. GDP molecules are shown in magenta. SulA is shown in red. SulA binds at the edge of the interface of polymerizing FtsZ subunits. The amino acid at position 268 is shown in yellow, and the amino acid at position 269 is shown in orange. Both are at the FtsZ-SulA interface. The residue altered by the FtsZ2 mutation (D212G) is shown in white. It is not at the FtsZ-SulA interface but is on the interface of FtsZ subunits formed during polymerization.

added to the reaction mixture before or after initiation of polymerization (Fig. 5B; in the experiment shown here, MalE-SulA was incubated with FtsZ before the addition of nucleotides). This demonstrates that MalE-SulA did not prevent the reaction from going to completion and that it did not perturb polymers once they were formed. The FtsZ detected by sedimentation was polymeric as verified by electron microscopy (Fig. 5E).

We next assembled FtsZ in the presence of GDP and aluminum fluoride. The formation of polymers under these conditions was also insensitive to the activity of SulA (Fig. 5C; in the experiment shown here, MalE-SulA was incubated with FtsZ before the addition of nucleotides). This insensitivity was likewise independent of whether MalE-SulA was added to the polymerization reaction before or after the initiation of assembly by the addition of nucleotide. Examination of these reaction mixtures by electron microscopy verified that FtsZ was indeed forming polymers (Fig. 5D; in the experiment shown here, MalE-SulA was incubated with FtsZ before the addition of nucleotides). Similar results were obtained when FtsZ polymerization was carried out in the absence of magnesium (Fig.

FIG. 5. GTPase activity of FtsZ is required for inhibition by SulA. (A) MalE-SulA binds to FtsZ in the presence of GMPCPP or GDP and aluminum fluoride as assayed by a pull-down assay. Amylose resin (100 μ l) containing bound MalE-SulA was incubated with FtsZ (10 μ M in a total reaction mixture volume of 500 μ l in Pol buffer) either in buffer alone (lanes 1 and 2) or in the presence of GMPCPP (lane 3 and 4) or GDP and aluminum fluoride (lanes 5 and 6). For a negative control, amylose resin without any MalE-SulA was incubated with FtsZ under the same conditions (lanes 1, 3, and 5). The reactions were centrifuged in a tabletop centrifuge, and the pellets were analyzed by SDS-PAGE. (B) Effect of MalE-SulA (3.5 μ M) on the critical concentration of FtsZ polymerized with GMPCPP. The number of FtsZ molecules in the cell pellet is shown on the y axis. (C) FtsZ at 5 μ M was incubated with or without 6 μ M MalE-SulA with GDP or GTP or with GDP plus AIF₄. The reaction mixtures were centrifuged to pellet the polymers, and the pellets were analyzed by SDS-PAGE. (D to F) Electron microscopic examination of the products of polymerization reactions with GDP plus AIF₄ (D), GMPCPP (E), and in buffer without Mg²⁺ (F). Bar, 60 nm. In all experiments shown in this figure, MalE-SulA was incubated with FtsZ before the addition of nucleotide to allow for binding to unpolymerized FtsZ.

5F; in the experiment shown here, MalE-SulA was incubated with FtsZ before the addition of nucleotides). We have also found that lowering the pH of the polymerization reaction below pH 6 makes FtsZ polymers insensitive to SulA. The acidic pH markedly reduces the GTPase activity (data not shown).

Under all conditions examined, the assembly of equilibrium polymers was insensitive to SulA. This insensitivity was independent of the initial conditions of the polymerization reaction, i.e., whether SulA was present before or after the polymerization reaction was initiated.

Model for cooperative assembly of FtsZ and the effects of SulA. The data presented thus far are consistent with a mech-

FIG. 6. Model for cooperative assembly of FtsZ and the effects of SulA. (A) Kinetic and thermodynamic schemes for the assembly mechanism of FtsZ. The first step involves an internal equilibrium of the FtsZ molecule between a polymerization-incompetent form (Z) and a polymerization-competent form (Z^*) at a forward rate of α_+ and a reverse rate α_- . The following steps are elongation with the rate constant β_+ . (B) Simplified kinetics scheme showing the equilibrium between FtsZ and SulA. (C) Results of simulations for the formation of steady-state polymers of FtsZ. The FtsZ concentration was set at 5 μM . The solid line with no symbols shows the appearance of polymers in the absence of SulA. The line with solid triangles shows the appearance of polymers in the presence of 5 μM SulA when K_d for FtsZ-SulA was set at 10^{-8} M. The line with solid circles shows the appearance of FtsZ polymers in the presence of 5 μM SulA when the K_d was set at 10^{-7} M. (D) Results of simulations for the formation of equilibrium polymers of FtsZ. The FtsZ concentration was set at 5 μM . The solid line represents the formation of equilibrium polymers of FtsZ in the absence of SulA. The broken line shows the formation of equilibrium FtsZ polymers in the presence of 5 μM SulA when the K_d for FtsZ-SulA was set at 10^{-8} M and SulA was allowed to interact with Z^* .

anism in which SulA acts to inhibit the assembly of FtsZ by sequestering monomers. A seeming inconsistency is the resistance of equilibrium polymers to the action of SulA. If SulA binds FtsZ monomers, why does it not inhibit FtsZ assembly in the absence of GTPase activity?

To investigate the effect of SulA quantitatively and to gain additional insight into the mechanism of the polymerization reaction, we developed a new model for FtsZ assembly (24). Existing kinetic models for FtsZ assembly are based on actin and explain cooperativity of assembly by assuming that FtsZ filaments are double stranded (11, 12). However, FtsZ filaments are routinely observed by electron microscopy to be single stranded (48, 60). Furthermore, quantitative electron microscopy methods, such as scanning transmission electron microscopy, are consistent only with single-stranded filaments (60).

Thermodynamically, cooperativity requires that the energetics of the nucleation step is different from those of subsequent elongation steps (54). We have proposed that an isomerization step in the monomer of FtsZ can explain cooperativity of FtsZ assembly (17). Here we present a kinetic scheme based on this idea (Fig. 6A) that fits the kinetics of FtsZ assembly (see Fig. S3 in the supplemental material [data used were from reference 11]). Thermodynamic analysis shows that an activation step in the monomer provides an energy difference (ΔG of activation [ΔG_{activ}]) that can account for the cooperativity of assembly (Fig. 6A). The nucleation in this scheme occurs at the

level of the dimer, since the free energy change for this step consists of a favorable bond energy (ΔG of the bond [ΔG_{bond}]) and two unfavorable activation energies for FtsZ monomers, since two activated monomers have to react: $\Delta G_{\text{nucl}} = 2\Delta G_{\text{activ}} + \Delta G_{\text{bond}}$, where ΔG_{nucl} is the ΔG of nucleation (Fig. 6A). The free energy for all subsequent steps involves only the bonding energy and one unfavorable activation energy for FtsZ monomer: $\Delta G_{\text{elong}} = \Delta G_{\text{activ}} + \Delta G_{\text{bond}}$. Because of this, the dimerization (nucleation) step and all the subsequent steps provides the thermodynamic basis of cooperative polymerization of FtsZ and can be termed $\Delta\Delta G_{\text{cooperativity}}$, the free energy of cooperativity.

Previous studies found a concentration-independent step in the polymerization mechanism, indicating either an isomerization step in the FtsZ monomer or an equilibrium with a molecular species which is not limiting (such as nucleotides). All previous models assumed that nucleotide exchange was the concentration-independent step in the polymerization reaction that accounts for the lag in polymerization kinetics, but nucleotide exchange was not directly measured in those studies (11, 12). Our measured nucleotide exchange rate is 1 order of magnitude higher than the previously proposed concentration-independent step. Since this rapid nucleotide exchange rate cannot make an appreciable contribution to the assembly kinetics, we omit the nucleotide exchange step for simplicity.

However, a concentration-independent step in the assembly mechanism remains. This concentration-independent step is consistent with an isomerization reaction in the FtsZ molecule that converts it from an inactive, polymerization-incompetent state (species Z in Fig. 6A) to an active, polymerization-competent state (species Z* in Fig. 6A). In addition to the relative energies, these two states differ with respect to the affinities that FtsZ subunits have for each other. The inactive state has negligible affinity for other FtsZ subunits, while the active state has a significant affinity for other FtsZ subunits and produces FtsZ assembly above the critical concentration. A simplified scheme of this polymerization mechanism is shown in Fig. 6B.

We then proceeded to study the effects of SulA on the polymerization of FtsZ, by incorporating an FtsZ-SulA binding step in the kinetic mechanism (Fig. 6B). The dissociation constant for SulA-FtsZ interaction was estimated to be between 10^{-7} M and 10^{-8} M (see Materials and Methods). We assumed that the on rate (σ_+ in Fig. 6B) for FtsZ-SulA complex formation was diffusion limited (in the range 2×10^6 to 5×10^6 M $^{-1}$ s $^{-1}$) (52). Changing this parameter 10-fold or greater has no effect on the conclusions drawn from the simulations. The dissociation rate for the complex is then constrained by the K_d ($K_d = k_{\text{off}}/k_{\text{on}}$ [where k_{on} is rate of association]; in our case $K_d = \sigma_-/\sigma_+$, in Fig. 6B).

We simulated the mechanism to determine how SulA affects the polymerization of equilibrium and steady-state FtsZ polymers. Since the on rate is diffusion limited, we scanned the parameter space for the dissociation constant of the FtsZ-SulA interaction in the calculated range. We found that all values lead to inhibition of steady-state polymers as observed in our sedimentation assays. Figure 6C shows that 5 μ M SulA prevented the polymerization of 5 μ M FtsZ for the minimum and maximum calculated values of K_d in our model. This was true if SulA was set to interact with the polymerization-competent form (Z* in Fig. 6A and B) or polymerization-incompetent form (Z in Fig. 6A and B) of FtsZ.

Next, we simulated the formation of equilibrium polymers. We asked two questions concerning the effects of SulA on equilibrium polymers. (i) Can SulA interact with either form of the FtsZ monomer (Z* and Z) and lead to the experimentally observed effects, i.e., lack of inhibition in the absence of GTPase activity? (ii) How low must the rate of dissociation of FtsZ subunits from the polymers be in order to be congruent with the lack of inhibition of equilibrium polymers by SulA?

Simulating the formation of equilibrium polymers in the presence of SulA revealed two different behaviors in this region of phase space. Polymers were not formed in the simulations if SulA was allowed to bind to the polymerization-incompetent state of FtsZ, i.e., the FtsZ species that is the substrate in the isomerization step in the polymerization mechanism (species Z in Fig. 6A and B). This is at odds with our experimental results above where we find that SulA does not prevent the polymerization of FtsZ in the absence of GTPase activity. On the basis of this, we reject the scenario where SulA interacts with the polymerization-incompetent state of the FtsZ molecule. On the other hand, polymers were formed in the simulations if SulA was allowed to bind only to the polymerization-competent state of FtsZ, which is the product of the isomerization step in the polymerization mechanism (species Z* in Fig. 6A). This is in agreement with experimental results,

since we observed that polymers formed in the presence of MalE-SulA when there was no GTPase activity. On the basis of this agreement between the simulations and experiments, we conclude that SulA must bind the polymerization-competent form of the FtsZ molecule. In our simulations under these conditions, the amount of polymer at equilibrium was unaffected by SulA as long as β_- (the intrinsic rate of disassembly of FtsZ subunits from the polymers in the absence of GTPase activity) remained very low compared to the dynamics of the SulA-FtsZ complex. This would be the case if the polymers were stabilized in the absence of GTPase activity. The only effect of SulA was to introduce a lag in the appearance of the plateau in the polymerization reaction. Figure 6D shows the results of the effect of SulA on the formation of equilibrium polymers of FtsZ for the tightest K_d (10^{-8} M) in the calculated range. We also simulated reactions in which SulA bound to FtsZ even tighter ($K_d < 10^{-9}$ M). Again, this did not affect the total amount of equilibrium polymers formed, only the time it took to reach the plateau. The delay (100 seconds for a K_d of 10^{-8} M) is too short to be detected in our sedimentation assays and is also unlikely to present a barrier for Z-ring assembly in vivo. In all cases, the simulations reproduce the in vitro results only when β_- was kept at least 3 orders of magnitude lower than the frequency of oscillations of the FtsZ-SulA complex.

Increasing intracellular FtsZ concentration reverses inhibition by SulA. The model that emerges from the data and simulations suggests that SulA binds the polymerization-competent form of the FtsZ molecule and prevents it from assembling. The amount of inactive FtsZ in the reaction mixtures is directly proportional to the concentration of SulA, and it conforms to the equation $[\text{FtsZ}_{\text{active}}] = [\text{FtsZ}_{\text{total}}] - [\text{MalE-SulA}]$. Because this model was elaborated on the basis of in vitro observations and computer simulations, we wanted to test it for in vivo conditions. If our model is correct, then increasing the level of FtsZ in the cell would be able to reverse the inhibitory effect of SulA simply by increasing the pool of FtsZ available for polymerization. Increasing the levels of FtsZ, FtsA, and FtsQ in the cell has previously been shown to overcome the division inhibition due to increased SulA in *lon* mutants following SOS induction (41). Increasing this combination of cell division proteins also suppresses other cell division phenotypes (25), so the effect cannot be attributed specifically to FtsZ.

To test the protective effect of increasing just the concentration of FtsZ, we utilized the pSEB160 plasmid which carries the *ftsZ* gene downstream of the arabinose promoter on pBAD18 (27). We put this plasmid into strain W3110 carrying pA3 ($P_{\text{lac-sulA}}$) and spotted serial 10-fold dilutions of a late-exponential-phase culture on plates with different concentrations of arabinose and IPTG to induce the expression of *ftsZ* and *sulA*, respectively. Induction of SulA with 100 μ M IPTG inhibited colony formation by $>10^4$ (Fig. 7). However, the addition of 0.05% arabinose to induce FtsZ expression completely restored colony formation. This result demonstrates that increasing intracellular [FtsZ] suppresses the inhibitory effect of SulA and is consistent with the model we are proposing. We note that this experiment alone does not rule out other models.

Quantitative immunoblotting of FtsZ and MalE-SulA. Expression of SulA leads to the disappearance of Z rings (6, 44). To determine the in vivo stoichiometry at which SulA effects

FIG. 7. Increasing the level of FtsZ in the cell reverses the inhibitory effect on growth caused by the expression of Sula. Plasmid pSEB160 which carries *ftsZ* downstream of the arabinose promoter on pBAD18 was put into strain W3110 carrying the plasmid pA3. A late exponential culture was serially diluted 10-fold. Five microliters from each dilution was spotted on LB plates containing 100 μ M IPTG and 0 and 0.05% arabinose (ara).

this function, we utilized quantitative immunoblotting. Strain AND101, which has a copy of FtsZ-GFP expressed from an IPTG-inducible promoter on the chromosome at an ectopic site (Table 1), was transformed with pJC93, which contains MalE-SulA under the control of the arabinose promoter. Since the level of expression from the arabinose promoter at sub-saturating levels of the inducer varies between cells (65), we decided to fully induce the promoter. This approach leads to a uniform increase in the protein level within the cell population, allowing us to monitor kinetically the increase of MalE-SulA and the corresponding disappearance of Z rings.

Preliminary observation of live cells after induction of MalE-SulA revealed that the disappearance of Z rings occurred in a synchronous wave 6 to 7 min following induction. The facts that the rings disappear rapidly and that the MalE-SulA level increases linearly (data not shown) suggest that relatively small changes in the level of MalE-SulA led to the disassembly of Z rings. Also, Z rings in unconstricted cells and constricting cells disappeared, indicating that all Z rings in the population are sensitive to the action of Sula. This result is consistent with the observation that Sula can lead to a rapid cessation of division (41).

To quantitate these results, cells were fixed at various time points following induction, and the fraction of cells with Z rings was determined. Samples were taken at the same time for the measurement of cellular MalE-SulA and FtsZ levels. In multiple experiments, $\sim 90\%$ of cells had lost their Z rings by 7.5 min after induction of MalE-SulA (Fig. 8A). By contrast, the percentage of cells with rings at 2.5 and 5 min after arabinose addition was the same as before induction ($\sim 90\%$). Samples collected for immunoblotting were used to determine the levels of MalE-SulA, FtsZ, and FtsZ-GFP at various time points. The number of FtsZ molecules per cell was determined to be 5,500 ($\pm 15\%$) at each time point (Fig. 8D). FtsZ-GFP represented less than 20% of the total FtsZ (Fig. 8D, lane 1). We also determined the amount of FtsZ present in strain AND101 without the pJC93 plasmid and when the plasmid was uninduced. The levels in all experiments were the same (data not shown), confirming that MalE-SulA disrupted Z rings without affecting the level of FtsZ. Assuming an average cell volume of 50 pl, the level of FtsZ corresponds to an intracellular [FtsZ] of 6 to 7 μ M. At 5.0 min, the amount of MalE-SulA was measured to be 2,000 ($\pm 15\%$) molecules per cell, and at 7.5

FIG. 8. Quantitative immunoblotting of FtsZ and Sula. (A) Effect of induction of MalE-SulA on the number of Z rings. *E. coli* AND101 cells containing pJC93 (P_{ara} -malE-sulA) were grown in the presence of 0.2% glucose (to repress expression of MalE-SulA), washed, and resuspended in medium containing arabinose (0.4%). Samples were taken at 0, 2.5, 5, and 7.5 min, and the cells were fixed. The percentage of cells with Z rings was determined by fluorescence microscopy of FtsZ-GFP. (B) Immunoblot of MalE-SulA from the experiment in panel A was developed by using the ECL kit (Amersham Biosciences) and antibodies to MalE. Lanes 1 and 2 are cell extracts taken at 5 and 7.5 min, respectively. Lanes 3, 4, 5, and 6 were loaded with 100, 50, 20, and 10 ng of MalE-SulA, respectively. (C) Band intensity in panel D was measured densitometrically and plotted against the amount of MalE-SulA loaded to generate a standard curve (circles). The amount of MalE-SulA in cell extracts (triangles) was interpolated from the standard curve. The amount of MalE-SulA was determined to be 20 and 27 ng per lane for 5 and 7.5 min, respectively. This is calculated to be 2,000 and 2,600 molecules per cell, respectively. (D) Immunoblot of FtsZ from the experiment in panel A was developed with the Bio-Rad kit using antibodies to FtsZ. Lane 1 was loaded with the cell extract taken at 0 min, and standards were loaded in lanes 2, 3, 4, 5, and 6 with 25 ng, 10 ng, 7.5 ng, 5 ng, and 2.5 ng of purified FtsZ, respectively. The amount of FtsZ in lane 1 was determined densitometrically to be ~ 10 ng, which translates into $\sim 5,500$ molecules of FtsZ per cell. The top band in lane 1 is FtsZ-GFP.

min, it was determined to be 2,600 ($\pm 15\%$) molecules per cell (Fig. 8B and C).

In the above experiments, the level of MalE-SulA is continuously increasing following induction. However, it is possible that a lesser amount of MalE-SulA acting over a longer time leads to the disassembly of Z rings. To test this, chloramphen-

icol was added at 4 min after induction when rings were still present and checked 11 minutes later. The percentage of cells with rings did not change and was still $\sim 90\%$. At this time, the level of MalE-SulA from the treated culture was ~ 700 molecules per cell (data not shown). This level of MalE-SulA is insufficient to disrupt Z rings even when present over a considerable period of time, reinforcing the idea that the disassembly of Z rings occurred when the cellular concentration of SulA reached a critical inhibitory value.

DISCUSSION

FtsZ is a stable protein, and the intracellular concentration changes little during the cell cycle or in response to changes in the environment. Therefore, rapid responses required for regulation of the Z ring occur through control over FtsZ assembly. The activity of FtsZ in response to DNA damage is controlled by the product of the *sulA* gene. In this study, we quantitatively examined the effects of purified MalE-SulA on the known biochemical activities of FtsZ. MalE-SulA shifted the observed critical concentration for polymerization of FtsZ in proportion to the concentration of MalE-SulA present in the reaction mixture. In other words, it affected the total amount of polymer formed, but it did not affect the cooperativity of polymerization. This suggests that SulA does not change the nature of self-association of FtsZ subunits. The same behavior was observed with the concentration-dependent activation of FtsZ GTPase activity: there was no change in cooperativity. This means that SulA establishes an equilibrium with a species of FtsZ incapable of enzymatic activity. (Note that this function of SulA is in sharp contrast with MinC, which does not affect the GTPase activity and must therefore be engaged in equilibrium with a higher-order structure.) The species of FtsZ that lacks GTPase activity is the monomer, as the enzymatic site is formed only between associating subunits. If SulA were capping FtsZ as has been proposed (14, 68), then the GTPase activity should be affected with a different stoichiometric ratio, depending on the oligomeric species that SulA was capping. If capping of polymers were to occur, it would represent a minor part of the cycle of SulA in the cell.

One important finding of our study is that SulA has no effect on FtsZ polymers when they are not hydrolyzing GTP (equilibrium polymers). The function of the FtsZ GTPase activity has remained unclear. The free energy of GTP hydrolysis does not seem to be used for constriction, since mutant proteins with lesions in the GTPase domain that make GTPase activity undetectable in vitro, such as FtsZ-D212G, are able to constrict (7, 17, 50). Our results suggest that the GTPase activity has a regulatory function.

To further investigate the effect of SulA on FtsZ in the absence of the GTPase activity, we computationally implemented a new kinetic model for FtsZ polymerization. We found a good fit with the results of our in vitro experiments only when the intrinsic rate of disassembly of FtsZ molecules from the polymer (β_- in Fig. 6A) was vanishingly low, namely, at least 3 orders of magnitude smaller than the off rate for the FtsZ-SulA complex. This indicates that the energy barriers for dissociation of subunits from the polymer are too high to be spontaneously overcome on the time scales required for proper regulation of the Z ring. The correct kinetic cycle that results

FIG. 9. (A) Diagrammatic representation of the model for FtsZ polymerization and SulA action. Under normal conditions, monomers of FtsZ are fluxing through the polymers (cycle 1) at a rate determined by the GTPase activity (γ_{diss}) which controls the rate of dissociation (β_-) of FtsZ subunits from the polymers. When present, SulA establishes a rapid equilibrium with the polymerization-competent form of the FtsZ molecule (Z^*) and causes it to cycle through a SulA-bound state (cycle 2). This makes a fraction of FtsZ unavailable for polymerization. (B) Structuring equilibria of FtsZ. Polymerization-competent FtsZ monomers (Z^*) form polymers with a critical concentration of $1 \mu\text{M}$. Polymers are engaged in an equilibrium that involves the formation of higher-order structures, such as networks of FtsZ polymers. Because these equilibria are linked, the effect of SulA on the polymerization impinges upon the formation of FtsZ polymers. (C) Model for SulA action in vivo. Under normal conditions, the subunits of FtsZ in the Z ring are in a dynamic equilibrium with the subunits located in the cytoplasm or on the membrane. DNA damaging conditions lead to the derepression of the SOS regulon, resulting in the expression of SulA. SulA forms complexes with FtsZ in the cytoplasm, resulting in the sequestration of FtsZ away from the pool available for participation in functional structures. When the molar ratio of SulA to FtsZ is between 0.1 and 0.5, the Z rings disassemble although polymers are still present. Blue, FtsZ; red, SulA.

from the simulations and experiments is shown in Fig. 9A. Polymerization-competent species of FtsZ (Z^*) form polymers with a diffusion-limited β_+ rate. The GTPase activity with the catalytic rate of the GTPase activity (γ_{diss}) converts the polymer into a labile species which disassembles into constituent units at the rate of β_- . This rate is much faster than γ_{diss} because FtsZ subunits are converted to a polymerization-incompetent state by the GTPase activity. γ_{diss} is the catalytic

rate of the GTPase activity (and the rate of energy dissipation of the polymers) that drives the entire cycle. The GTPase activity provides the thermodynamic force to destabilize the FtsZ-FtsZ bonds in the polymer, thereby making the polymerization reaction reversible on physiological time scales. But why is there reversibility?

Through reversible associations of FtsZ subunits with polymers, the Z ring can rapidly exchange FtsZ subunits with the cytoplasm. This exchange also permits the exchange of information about the internal state of the cell. This occurs when FtsZ monomers become engaged in other equilibria which regulate the state of organization of the cytokinetic apparatus. One such equilibrium involves Sula. Our simulations suggest that the flux of FtsZ through polymers is necessary to sensitize FtsZ polymers to physiological regulation by Sula. Because polymers are engaged in all equilibria that involve the formation of higher-order structures (such as the Z ring), their total amount will impinge upon those equilibria. Through Sula, the cell reduces the amount of FtsZ in polymers and thus disrupts the Z ring.

Another result suggested by our simulations is that Sula must be interacting with the product of the monomer isomerization step in the polymerization reaction. We think that this is consistent with it interacting with a polymerization-competent state of the FtsZ monomer, i.e., a species of FtsZ that has a significant affinity for itself and therefore dominates the kinetic cycle. What is this state? It cannot simply be the result of nucleotide exchange (i.e., just a GTP-bound form of FtsZ), as we have found that this step is too rapid. One possibility is that this state is due to different conformations of the GTP-bound FtsZ. Another possibility is that the polymerization-competent form of the FtsZ molecule represents a specific dynamic mode of the FtsZ molecule. Detailed thermodynamic and structural studies will be required to resolve these possibilities.

The Z ring, like many other cytoskeletal structures, is very dynamic. Reducing the dynamics of the ring by using FtsZ mutants with decreased GTPase activity does not dramatically affect its ability to participate in cell division (5, 67). However, rings composed of polymers that are less dynamic due to decreased GTPase activity are unable to respond to physiological regulation by Sula and perhaps other regulators of the Z ring. Interestingly, the dynamics of FtsZ₈₄ is reduced ninefold (67), but it remains sensitive to Sula (unpublished data). It is not clear how much the GTPase activity has to be slowed down to obtain resistance. However, the *ftsZ(D212G)* mutant studied here retains very little GTPase activity, reduced about 200-fold. It will require additional modeling to determine the decrease that is necessary because the full kinetic cycle would include a step after polymerization but before GTPase activity is activated whose kinetics is not yet known. Therefore, the flux of chemical energy and FtsZ through the Z ring, while not necessary for the coherence of the ring and its function in cytokinesis, is necessary for maintaining it in a quasistable state that allows the cell to control its function on physiological time scales (58).

Cooperativity in the structuring equilibria of FtsZ and Z-ring disassembly. The model that emerges out of the in vitro work led us to determine the amount of Sula required to inhibit Z-ring formation in vivo. Sula inhibited Z-ring forma-

tion in vivo with somewhat lower stoichiometry compared to what we observed in vitro. Quantitative immunoblotting revealed that MalE-SulA resulted in Z-ring disassembly when it reached $\leq 50\%$ of the total cellular level of FtsZ. A previous study likewise found that a reduction in FtsZ levels by as little as 30 to 40% was sufficient to block cell division in *E. coli* (15). Why do Z rings disappear when the level of FtsZ decreases by only 30 to 50%?

It has been estimated that 30% of cellular FtsZ in *E. coli* is actually present in the Z ring (2). The estimates for intracellular concentration of FtsZ vary between strains but are generally 6 to 7 μM as we determined here (38, 61). This means that 2 μM of FtsZ is present in the ring with another 0.9 μM free in the cytoplasm as required by the critical concentration. Thus, at 3 μM Sula, there should still be enough free FtsZ (at least 3 μM) so that it should be above the critical concentration for polymerization ($\sim 1 \mu\text{M}$). Since rings are absent at this concentration, polymerization of FtsZ is not sufficient for Z-ring formation, i.e., a threshold concentration of polymers is necessary for the coherence of the Z ring. To explain the absence of rings and to stay congruent with the known in vitro behavior of FtsZ, we suggest that FtsZ in the cell exists in at least three states of organization, monomers, polymers, and higher-order structures, and that transitions between these states are cooperative (Fig. 9B).

The cooperative behavior between monomers and polymers has been well established experimentally, although there has been some confusion in how to explain this cooperativity since no conceptual model existed that could account for cooperativity in single-stranded filaments (11, 17, 60). The new model for FtsZ polymerization we presented here accounts for the cooperativity of FtsZ and could be used to explain the cooperativity in other single-stranded polymers. The cooperativity results from the differences in the free energy changes between the nucleation step and the elongation steps. This difference arises from the free energy change that is associated with isomerization of the monomer. This isomerization is consistent with converting the monomer from a polymerization-incompetent state to a polymerization-competent state. If all monomers were in the polymerization-competent state at all times, then there would be no critical concentration. Critical concentration, according to this model, is the concentration at which polymerization-competent forms of FtsZ interact productively. This leads to a rapid shift in the equilibrium in the monomer, as most polymerization-competent FtsZ molecules become engaged in polymers. It is interesting to note that an ensemble of FtsZ molecules is poised between the disordered regimen and the ordered regimen at around 1 μM , which is very near the physiological concentration of the protein. This allows the cell to use this transition between a disordered regimen of dispersed molecules and an ordered regimen of polymers to modulate cell division in response to changes in physiology.

The transition between polymers and higher-order structures is also cooperative requiring a threshold concentration of polymers, achieved at or around 3 μM of FtsZ. Theoretical models for cooperative association of polymers in higher-order structures exist, and we think they will prove relevant to FtsZ (35, 40).

The higher-order structures which form the basis of the Z ring likely represent a physically distinct regimen of organiza-

tion of FtsZ. One such regimen is detected with reconstituted FtsZ networks in vitro. At FtsZ concentrations of 2 to 3 μM and under physiological buffer conditions, FtsZ polymers associate laterally and bundles interconnect. At this point, FtsZ polymer networks acquire new properties and become elastic, i.e., they stiffen and become solid-like (22). Note that the presence of another cooperative transition in the organization of FtsZ is likely a target of control by factors that affect the equilibrium constant for this transition and shift it to higher or lower concentrations of FtsZ. ZapA and ZipA are good candidates for such factors given that they promote the bundling of FtsZ polymers in vitro (26, 28, 29). Also, negative regulators like MinC might affect this step.

Support for this model comes from various studies. Thanedar and Margolin (67a) observed dynamic FtsZ helices in addition to the Z ring. These helices likely correspond to polymerized FtsZ outside of the ring. Interestingly, induction of SulA in their study prevented Z-ring formation, but the FtsZ helices were still present. We suggest that the SulA level achieved in their study was sufficient to prevent Z-ring formation but insufficient to lead to disassembly of polymers (helices).

Chen and Erickson (12) observed two critical concentrations in monitoring FtsZ polymerization in vitro by fluorescence resonance energy transfer. One critical concentration was at 0.7 μM , similar to the 0.9 μM observed here, and a second was at 3 μM (12). This second critical concentration probably corresponds to the second transition we are suggesting here and must be due to lateral interactions between FtsZ polymers. In addition to the longitudinal contacts that FtsZ molecules make to form polymers, an interaction involving lateral contacts may be required for the coherence of higher-order structures like the Z ring. Consistent with this, *ftsZ* mutations that are likely to affect residues involved in lateral interactions do not prevent FtsZ polymerization but do prevent formation of Z rings (39, 66). Also, FtsZ networks show rheological properties indicating significant interfilament interactions (22), and electron microscopy of FtsZ polymers show interconnected bundles of FtsZ polymers (45).

Another explanation for the observed stoichiometry could be that under normal physiological conditions, some of the cellular FtsZ is sequestered by another cellular protein. There are examples of such sequestering proteins for eukaryotic cytoskeletal proteins actin and tubulin. A recent paper reported negative regulation of FtsZ by ClpX chaperone in *Bacillus subtilis* and proposes a model in which this occurs by a sequestration mechanism (72). ClpX has been shown to interact with FtsZ in *E. coli*, raising the possibility that it is a universal factor involved in regulating FtsZ (23). ClpX could therefore act as a sequestering factor for FtsZ in *E. coli*, effectively eliminating a portion of FtsZ from the pool available for participation in functional structures. If this were the case, the mechanism of sequestration would have to be very different from that of SulA, since ClpX, unlike SulA, does not inhibit the GTPase activity of FtsZ.

The model described here is represented diagrammatically in Fig. 9C. Production of SulA after DNA damage leads to formation of SulA-FtsZ complexes. Once enough SulA is produced to lower the concentration of uncomplexed FtsZ below the threshold concentration required for ring formation, dis-

assembly of the Z ring will occur and cell division will cease. At this stage, polymers of FtsZ would still be present in the cell. If the concentration of SulA increases further, more FtsZ is sequestered until less than 0.9 μM FtsZ is left free to polymerize. When DNA damage is repaired, the SOS regulon is repressed and SulA is quickly degraded by the action of the Lon protease and FtsZ again becomes free to polymerize. The Z ring reforms, and cell division resumes.

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ADDENDUM IN PROOF

While this work was under review, a similar model for FtsZ polymerization was described (S. Huecas, O. Llorca, J. Boskovic, J. Martín-Benito, J. M. Valpuesta, and J. M. Andreu, *Biophys. J.* **94**:1796–1806, 2008).

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