Background: Kil peptide from bacteriophage λ targets FtsZ to prevent host cell division.

Results: Kil disrupts FtsZ protofilaments producing shorter oligomers of variable size with reduced GTPase activity.

Conclusion: At high concentrations, Kil likely inhibits FtsZ assembly via a subunit sequestration mechanism.

Significance: This is the first biophysical study of how a bacteriophage disruptor of bacterial division inhibits FtsZ assembly.

The effects of Kil peptide from bacteriophage λ on the assembly of Escherichia coli FtsZ into one subunit thick protofilaments were studied using combined biophysical and biochemical methods. Kil peptide has recently been identified as the factor from bacteriophage λ responsible for the inhibition of bacterial cell division during lytic cycle, targeting FtsZ polymerization. Here, we show that this antagonist blocks FtsZ assembly into GTP-dependent protofilaments, producing a wide distribution of smaller oligomers compared with the average size of the intact protofilaments. The shortening of FtsZ protofilaments by Kil is detectable at concentrations of the peptide in the low micromolar range, the midpoint of the inhibition being close to its apparent affinity for GDP-bound FtsZ. This antagonist does not only interfere with FtsZ assembly but also reverses the polymerization reaction. The negative regulation by Kil significantly reduces the GTPase activity of FtsZ protofilaments, and FtsZ polymers assembled in guanosine-5′-[(α,β)-methylene]triphosphate are considerably less sensitive to Kil. Our results suggest that, at high concentrations, Kil may use an inhibition mechanism involving the sequestration of FtsZ subunits, similar to that described for other inhibitors like the SOS response protein SulA or the moonlighting enzyme OpgH. This mechanism is different from those employed by the division site selection antagonists MinC and SlmA. This work provides new insight into the inhibition of FtsZ assembly by phases, considered potential tools against bacterial infection.

In most bacteria, the tubulin homologue FtsZ is the first cell division protein to position at the division site, leading to the assembly of a multiprotein complex called the divisome, which coordinates membrane constriction with septum formation (1, 2). Polymerization of FtsZ into filaments is essential for its function, as it is thought to be the main force carrying out membrane constriction during the division process (3, 4). The association of FtsZ into single-stranded polymers, also called protofilaments, has been comprehensively investigated in vitro (3–5). Protofilaments form upon addition of GTP, are continuously recycled with a half-time of ~10 s, and eventually disassemble in vitro when the GTP is consumed. Using enzymatic GTP-regeneration systems (RS)4 to maintain a high GTP/GDP ratio, FtsZ polymers can be stabilized for longer periods of time while preserving their dynamic properties (6–8). Alternatively, the lifetime of the polymers can be prolonged by adding slowly hydrolysable GTP analogues (3). FtsZ assembles cooperatively, giving rise to protofilaments narrowly distributed in size under a variety of conditions at neutral pH, at a protein concentration range of 0.4–1.5 g/liter (8, 9). Although the average number of FtsZ subunits per protofilament varies with buffer composition (9), protofilaments are always too short to encompass the entire 3–4-μm circumference of a rod-shaped bacterium. Hence, these basic structural units must further assemble into higher order structures to form the observed Z-ring in vivo (10, 11). Indeed, protofilaments can be arranged in vitro into several higher order structures involving lateral interactions in the presence of crowding agents (7, 12), calcium ions (13), or certain buffer conditions (14).

In Escherichia coli, the assembly of the Z-ring at mid-cell is achieved by the concerted action of two site-selection systems, namely the Min system and nucleoid occlusion, which prevent aberrant division at inappropriate cellular sites. The Min system is a widely conserved mechanism in bacteria that blocks assembly of the division machinery at the cell poles (15). Although MinDE components oscillate from pole to pole

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The abbreviations used are: RS, enzymatic GTP regeneration system; FtsZ-GTP, GTP bound FtsZ; FtsZ-GDP, GDP bound FtsZ; GMPCPP, guanosine-5′-[(α,β)-methylene]triphosphate; FCS, fluorescence correlation spectroscopy.
attached to the inner membrane (16), MinC is the component of the system that disrupts FtsZ polymers to prevent Z-ring assembly (17). The molecular mechanism underlying FtsZ polymerization inhibition by MinC has been widely studied using biochemical, biophysical, and structural approaches (18–21). This antagonist reduces the size of FtsZ protofilaments maintaining a narrow distribution of sizes, likely affecting subunit turnover (21). All published studies agree that MinC does not modify FtsZ GTPase activity (17, 21). Nucleoc exclusion is mediated by SlmA, a sequence-specific DNA-binding protein that interferes with FtsZ assembly preventing Z-ring formation in the vicinity of the nucleoid (22). This protein forms an active dimer of dimers complex with the DNA (23) that blocks FtsZ assembly (24, 25). Similar to MinC, the GTPase activity of FtsZ subunits arranged into single-stranded protofilaments is insensitive to the action of SlmA (23).

Other negative regulators of FtsZ assembly in E. coli include the SOS response factor SulA (26); YeeV and the membrane protein CptA (YgX), both considered part of toxin-antitoxin systems (27, 28); and OpgH, a moonlighting enzyme that delays division increasing cell size (29). In contrast to MinC and SlmA, most of these proteins antagonize FtsZ polymerization through a sequestration mechanism entailing significant reduction of the rate at which GTP is hydrolyzed by FtsZ (28–30).

Bacteria can be infected by bacteriophages that eventually block division during the infection process. These viruses constitute a potential tool to fight against pathogenic bacteria, but to date the molecular details of the action of their inhibitory factors on division ring assembly remain largely unknown. It has been recently reported that the kil gene from E. coli bacteriophage λ encodes a 47-amino acid protein that antagonizes FtsZ polymerization in vivo and in vitro, blocking cell division at the latest stages of the viral lytic phase (31). According to this study, the inhibitor requires the presence of ZipA for its activity in vitro, although this requirement is overcome when Kil is overproduced. In vitro, Kil interacts with FtsZ in pulldown experiments and precludes the sedimentation of FtsZ polymer bundles elicited by GTP plus Ca\(^{2+}\) or truncated ZipA (31). When present, this inhibitor produces a slight increase in the GTPase activity of FtsZ, under conditions promoting bundle formation through lateral interaction of protofilaments (31). Although these results clearly identify Kil as an FtsZ polymerization inhibitor, new research is still required to explore the molecular mechanisms by which this protein counteracts FtsZ assembly and particularly to address the influence of this new antagonist on the one subunit thick protofilaments that constitute the basic unit of the Z-ring.

With the goal of gaining some understanding about the inhibitory mechanism of Kil, we have studied the effect of this protein on FtsZ assembly under conditions favoring the formation of unbundled protofilaments. The influence of the inhibitor on the size distribution of the protofilaments elicited by GTP and GMPCPP was addressed by analytical ultracentrifugation and fluorescence correlation spectroscopy (FCS). Moreover, using fluorescence anisotropy, we have quantitatively determined the affinity of the interaction of the antagonist with FtsZ. The response of the GTPase activity of FtsZ to the action of Kil was also investigated. Our study provides new information that sheds light on the inhibitory mechanism of Kil, the features of which we discuss and compare with data available for other negative regulators of FtsZ assembly.

### Experimental Procedures

#### Construction of DPH677 Strain—To overproduce an N-terminally hexahistidine-tagged Kil peptide (His\(_6\)-Kil), the kil gene from CC4506 genomic DNA was amplified using primers DPH170 and DPH211 (31) and cloned into pET28a (Stratagene) between the NdeI and HindIII sites, creating pDPH100. The His\(_6\)-kil-containing sequence was then cleaved from pDH100 using XbaI and Xhol and cloned into pET15b to produce pDH148. pDH148 was then introduced into E. coli strain DPH673 (31), to create the BL21(DE3) expression strain DPH677. To reduce the toxicity of expressed kil, this strain also carries plasmid pBS58, which expresses extra ftsA and ftsZ, as well as a deletion of the zipA gene and an ftsA* mutation to bypass zipA.

#### Protein Expression, Purification, and Labeling—E. coli FtsZ was purified by the calcium-induced precipitation method as described elsewhere (32).

His\(_6\)-Kil was overproduced in DPH677, as described above. Expression and purification were carried out as described previously for the His-FLAG-Kil construction (31) with some modifications. Briefly, after elution from the affinity column, samples were pooled, aliquoted, and frozen at −80 °C in elution buffer (100 mM sodium phosphate, pH 4.5, 10 mM Tris-HCl, 8 M urea). Kil was renatured prior to every experiment by extensive dialysis (three buffer changes with a final overnight incubation at 4 °C) and centrifuged at 470,000 \(\times\) g at 4 °C for 1 h to remove aggregates.

FtsZ was covalently labeled at amine groups with Alexa 488 carboxylic acid succinimidyl ester dye (Molecular Probes/Invitrogen) in the GTP-assembled form as described elsewhere (7, 33). The degree of labeling, estimated from the molar absorbance coefficients of the protein and the dye, was typically 40–80%.

#### Circular dichroism—CD was employed to ascertain the amount of secondary structure present in refolded Kil and to check for the presence of a cooperative temperature unfolding transition, which are indicative of a folded protein. Experiments were performed in a Jasco J-810 spectropolarimeter equipped with a Peltier PTC-423S system. Isothermal wavelength spectra were acquired at a scan speed of 50 nm/min with a response time of 2 s and averaged over five scans at 15 °C. The cuvette path length was 0.1 cm. The concentration of Kil was 0.2 g/liter (26 \(\mu\)M) in 20 mM sodium phosphate, pH 7.5, 100 mM KCl. Ellipticities ([\(\theta\)]) are expressed in units of degrees cm\(^2\) dmol\(^{-1}\), using the residue concentration of the protein (34). For CD-monitored temperature-scanning denaturation experiments, the sample was layered with mineral oil to avoid evaporation, and the heating rate was 60 °C/h. Because transitions were not reversible, a thermodynamic analysis could not be carried out, and curves were only fitted to sigmoidal transitions to calculate their temperature midpoint (\(T_m\)).

#### Fluorescence Spectroscopy and Anisotropy—Emission spectra of Kil were acquired to gain some insight into the environment
of the only tryptophan of Kil under different conditions. Anisotropy measurements were conducted to monitor the binding of Kil to FtsZ. Emission spectra were acquired in a FluoroLog FL-3 spectrophotometer (Jobin Yvon-Spex), using a 3 × 3-mm path length cuvette (Hellma Hispania) and a protein concentration of 5 μM. The sample was excited at 280 nm, and the temperature was kept constant at 20 °C. Kil was refolded and measured in Z300 buffer (50 mM Tris-HCl, pH 7.5, 300 mM KCl, 5 mM MgCl₂, and 1 mM EDTA). To acquire the emission spectra of the unfolded protein, Kil was diluted in buffer containing urea (100 mM sodium phosphate, 10 mM urea, pH 6.3), with samples having a final urea concentration of 7 M.

Anisotropy measurements were performed in a BMG PolarStar Galaxy plate reader using 96-well black plates from Corning. The temperature was regulated at 26 °C, and the 485- and 520-nm excitation and emission filters were employed. The concentration of FtsZ-Alexa 488 in the titrations was 210 nM. Anisotropy measurements were performed in buffer containing urea (100 mM sodium phosphate, 10 mM Tris-HCl, 8 mM urea, pH 6.3), with samples having a final urea concentration of 7 M.

The GTP hydrolysis rate was determined by measuring released inorganic phosphate using the malachite green-molybdate reagent (37, 38) in Z300 buffer. The two proteins were incubated together for 10–20 min on ice prior to GTP addition. FtsZ was kept at 5 μM for the measurements at fixed FtsZ concentrations, and the specific GTPase activity values obtained at different Kil concentrations were normalized to those in the absence of inhibitor. Data were fit with the following Hill equation:

\[ GTPase_{norm} = \frac{GTPase_{max}}{1 + \left(\frac{C_{1/2}}{C}\right)^n} \]

where \(GTPase_{max}\) represents the concentration of Kil (c) with half of the maximal effect on FtsZ GTPase activity. MATLAB (version 7.10; MathWorks, Natick, MA) was used with the cooperativity coefficient \(n\) constrained to 1.

Fluorescence Correlation Spectroscopy—FCS was used to monitor titrations of FtsZ-GTP polymers with Kil to obtain a \(C_{1/2}\) value for the inhibition. Experiments were conducted in a Fluoview1200 confocal laser scanning microscope (Olympus) equipped with an FCS module from PicoQuant with a single photon avalanche diode for the detection. Excitation at 488 nm was achieved by using an argon ion laser beam focused into the sample through a water immersion objective (UPLSAPO ×60, numerical aperture 1.2). To avoid photobleaching and excitation saturation effects, the excitation power dependence of the autocorrelation profiles was checked, and a threshold excitation power was determined to be that at which the profiles became excitation power-independent. The emitted fluorescence was collected through the same objective and passed through a 505-nm dichroic mirror and a 580/70 bandpass filter. A pinhole of 90 μm diameter was placed in the emission path to reject out of focus signals. Nonspecific absorption of the proteins was prevented by using coverslips treated with Vectabond reagent (Vector Laboratories) and polyethylene glycol-succinimidyl ester as described elsewhere (41, 42). In the samples, FtsZ was used at 12.5 μM with 25 nM FtsZ-Alexa 488 as a tracer. As in the sedimentation velocity experiments, a GTP-regeneration system was used to increase the lifetime of the FtsZ protofilaments for their study. Measurements were performed in Z300 buffer at 22 °C.

For each sample, five autocorrelation traces were acquired for 1 min each and globally analyzed using FFS Data Processor software (version 2.4 extended, Scientific Software Technologies Center, Belarus) (43), essentially as described previously for equivalent traces of FtsZ-GDP and FtsZ-GTP obtained under two photon excitation (8, 21, 33). In addition to the diffusion components, a term for the time-dependent population and depopulation of the tracer triplet state was also included in this case as shown in Equation 1,

\[ G(\tau) = 1 + \left(\frac{1}{N} \right) \left( \frac{1 + \frac{f_{\text{trap}}}{1 - f_{\text{trap}}} e^{-\frac{\tau}{\tau_{\text{trap}}}}}{1 + \frac{f_{\text{trap}}}{1 - f_{\text{trap}}} e^{-\frac{\tau}{\tau_{\text{trap}}}}} \right) \left( \sum_{i} \left( \frac{1 + \frac{\tau}{\tau_{\text{trip}_i}^2}}{1 + \frac{\tau}{\frac{\sigma}{\tau_{\text{trip}_i}^2}} \left( 1 + \frac{\tau}{\tau_{\text{trip}_i}^2} \right)^{i/2} } \right) \right) \]

(Eq. 1)
where \( N \) is the average number of fluorescent molecules in the effective volume; \( F_{triph} \) and \( \tau_{triph} \) are the fractional population and the relaxation time of the triplet state, respectively; and \( S \) is a structure parameter that characterizes the shape of the detection volume \( (S = Z_{a}/w_{o} \) where \( w_{o} \) and \( Z_{a} \) are the lateral and axial dimensions of the effective volume, respectively). Parameter \( S \) was obtained by calibration with a 10–25 nm solution of Atto 488-carboxylic acid (ATTO-TEC, GmbH) measured under the same illumination conditions as the samples and varied from 5 to 7. From this same calibration, the \( w_{o} \) value was calculated assuming a \( D \) of 400 \( \mu m^{2} \) \( s^{-1} \) for Atto 488 dye in aqueous solution at 25 °C. \( F_{i} \) is the fractional contribution of species \( i \) to the autorecorrelation function, and \( \tau_{i} \) is its translational diffusion time \( (D_{i} = w_{o}^{2}/4\pi\tau_{i} \) where \( D_{i} \) is the translational diffusion coefficient of species \( i \)).

As in previous studies (8, 21, 33), a model involving two diffusion components assigned to free dye (\( \tau_{i} \) independently determined and fixed and \( F_{i} < 0.2 \)) and to unassembled FtsZ protein \( (D_{i} = 55 \pm 5 \mu m^{2} \) \( s^{-1} \) was used for FtsZ-GDP samples. For the FtsZ-GTP samples, a three-component model was used, with components assigned to free dye (\( F_{i} \) fixed to that determined from analysis of the FtsZ-GDP traces), unassembled FtsZ (\( \tau_{i} \) fixed to that determined for FtsZ-GDP), and protofilaments (\( \tau_{i} \) and \( F_{i} \) were free parameters in the fits). Parallel analysis in which the diffusion time and contribution of the free dye were allowed to flow did not significantly affect the relative contributions and diffusion times of the other components in the fit. Traces acquired for samples containing unlabeled Kil peptide in addition to FtsZ-GTP were found to be well described by this three-component model, allowing calculation of an average diffusion coefficient \( (D) \) as a weighted mean of the \( D_{i} \) corresponding to the FtsZ species, as described previously for samples containing other antagonists (21). This \( (D) \) was normalized by the difference between values corresponding to FtsZ-GDP samples and FtsZ-GTP samples lacking Kil peptide, thus obtaining a \( D_{norm} \) value (21). From the variation of this \( D_{norm} \) with Kil concentration, \( c \), a \( C_{50} \) value for the inhibition of FtsZ polymerization was obtained, using the empirical Hill equation, as shown previously (21): \( D_{norm} = (c/C_{50})^{n}/(1 + (c/C_{50})^{n}) \). Fits were performed using MATLAB (version 7.10; MathWorks, Natick, MA), with the cooperativity coefficient \( n \) constrained to 1 (21).

**Results**

**Kil Peptide Displays Secondary and Tertiary Structure Elements and Self-assembles in Solution**—Purified Kil peptide presents a significant amount of secondary structure and tertiary contacts, suggesting that it is properly folded. To determine the effect of Kil on FtsZ polymerization, we purified the peptide following a previously described protocol (31) rendering Kil peptide with a \( >95\% \) purity, according to SDS-PAGE. Characterization of the isolated peptide by far-UV CD, which provides information about the secondary structure in proteins (34), indicated the presence of significant \( \alpha \)-helix structure (Fig. 1A). Deconvolution of the CD spectrum of Kil using the CDNN program (44) rendered a 47% helical content, in good accordance with predictions based on the primary structure of Kil by the Predict Protein program (45). Thermal unfolding of the protein monitored by far-UV CD showed two steep transitions (at 30 – 40 and at 60 – 70 °C), pointing at a shift from a native to an unfolded state via an unfolding intermediate (Fig. 1B). The far-UV CD spectrum at 48 °C (Fig. 1A), when the first transition was complete, showed the presence of some secondary structure (20% of helical content according to deconvolution with CDNN). The fluorescence emission spectra showed a blue shift of the emission maximum from 357 to 341 nm upon refolding, compatible with the only tryptophan harbored by Kil being in a
Kil Peptide Disrupts FtsZ Protofilaments Triggered by GTP Leading to Shorter Oligomers of Variable Size—To characterize the effect of Kil on single-stranded FtsZ protofilaments, both sedimentation velocity and fluorescence correlation spectroscopy methods were employed. These techniques in combination provide information about the size of FtsZ protofilaments (8) and have been used previously to study other FtsZ inhibitors (21). For these experiments, a stable high GTP/GDP ratio was achieved by the use of an enzymatic GTP regeneration system (6).

Sedimentation velocity analysis of the effect of Kil on FtsZ polymers was compatible with the disruption of FtsZ polymers, resulting in multiple oligomeric species of variable size that were always smaller than the size corresponding to the 15 S narrow distribution of FtsZ-GTP protofilaments favored in the absence of Kil. Sedimentation velocity profiles of FtsZ-GTP polymers were obtained in the absence and presence of increasing concentrations of Kil peptide in Z300 buffer (Fig. 3). In the absence of Kil, the sedimentation coefficient distribution of FtsZ displayed a narrow peak corresponding to the polymer with an $s$ value of $\sim 15$ S, which indicated the adoption of a narrow distribution of preferred fibril sizes (Fig. 3), as described previously for FtsZ-GTP protofilaments under our working conditions (9). At the lowest concentration of Kil assayed (0.3 $\mu$M, 42:1 FtsZ/Kil ratio), the effect on the FtsZ polymers was almost negligible, with only a minor shift of the main peak corresponding to the polymers toward lower $s$ values. The decrease was more marked at 0.7 $\mu$M Kil, and at 1.5 $\mu$M antagonist (8:1 FtsZ:Kil ratio) an ensemble of peaks at lower $s$ values was also detected. At $\sim 3$ $\mu$M, the 15 S narrow peak was lost with the concomitant appearance of a number of wider peaks at lower $s$ values (Fig. 3). Above this concentration of the modulator, the sedimentation coefficient profiles consistently showed several peaks, and the average $s$ value of these peaks diminished with increasing Kil concentrations. Some loss of material was observed at the higher concentrations of Kil, suggesting that aggregates are formed.

To further characterize the inhibition of FtsZ assembly into protofilaments by Kil, FCS experiments were undertaken in which the motion of FtsZ species, related to their size, was monitored by the use of a trace amount of fluorescently labeled FtsZ in the absence and presence of increasing concentrations of unlabeled Kil. FCS profiles are sensitive to the formation of FtsZ protofilaments triggered by GTP because of their considerably slower translational mobility compared with that of the unassembled protein, due to their larger size (Fig. 4A). The autocorrelation curves obtained with GTP in the absence of Kil in Z300 buffer were compatible with the formation of protofilaments diffusing at $\sim 4.5 \mu$m$^2$/s, in good agreement with previously reported values for FtsZ-GTP polymers under our working conditions (300 mM KCl (9)). Addition of Kil peptide to FtsZ before triggering polymerization with GTP resulted in profiles falling between those obtained for the assembled and unassembled FtsZ as the concentration of Kil in the solution was raised. Spikes corresponding to aggregates of FtsZ eventually crossed the observation volume at the higher concentrations.

**FIGURE 2.** Analytical ultracentrifugation analysis of Kil peptide. A, sedimentation velocity profile of 12 $\mu$M Kil peptide. B, sedimentation equilibrium analysis of 12 $\mu$M Kil peptide. The gradient shown corresponds to 37,000 rpm. Similar results were obtained at 22,000, 30,000, and 37,000 rpm. The dotted line corresponds to the fit of a one-component model giving an $M$, of 16,200 ± 200. Simulations of the theoretical gradients of the monomer, dimer, and trimer are shown for comparison. Experiments were performed in Z300 buffer.

more hydrophobic environment, pointing at a gain of folded structure (Fig. 1C).

We determined the oligomeric state of Kil peptide in solution using analytical ultracentrifugation. The homogeneity of the solutions of this peptide was first evaluated by sedimentation velocity. The sedimentation distribution profile showed a main peak (1.9 ± 0.1 S, 40–60% of total signal) and a variety of less abundant bigger species (3–15 S, Fig. 2A), suggesting that the peptide tends to self-associate. The major peak (at $\sim 2$ S) was compatible, according to molecular weight estimates, with species from spherical dimers to moderately elongated tetramers but not with the Kil monomer. The average molecular weight obtained by sedimentation equilibrium (at 3, 6, and 12 $\mu$M Kil) was 16,500 ± 800 (Fig. 2B), a value slightly higher than the expected Kil dimer size ($\sim 15,400$). Therefore, we conclude that the main peak observed in the sedimentation coefficient distributions should correspond to a Kil dimer.
of Kil, in good agreement with the observations by sedimentation velocity. The faster diffusion of FtsZ species together with their slower sedimentation (see above) clearly indicate that the Kil peptide antagonizes FtsZ assembly into protofilaments leading to the formation of smaller oligomers.

The inhibitory action of Kil on the protofilaments was detectable by FCS at low concentrations of the antagonist (~1 μM), in good agreement with sedimentation velocity data (see above). Analysis of the variation of the average translational diffusion coefficient of FtsZ species with Kil concentration rendered a $C_{1/2}$ value of $3.2 \pm 0.7 \mu M$, representing the concentration of the peptide at which half of the maximum depolymerization effect was observed (Fig. 4B). No difference was detected in the level of inhibition of polymerization obtained for FtsZ-GTP irrespective of the addition of Kil peptide before or after triggering polymerization with GTP (Fig. 4B).

To sum up, the orthogonal sedimentation velocity and FCS analysis showed that, in our conditions, Kil was able to efficiently counteract FtsZ assembly into protofilaments with a narrow size distribution giving rise to smaller heterogeneous species. Moreover, the negative modulation of FtsZ polymerization by this peptide is apparent even at substoichiometric levels.
FtsZ Protofilament Disruption by Bacteriophage λ Kil Peptide

Effect of Kil on the GTPase activity of FtsZ and on the sedimentation of FtsZ-GMPCPP protofilaments. A, dependence of the GTPase activity, normalized to the values determined in the absence of inhibitor, on Kil peptide concentration. The concentration of FtsZ was 5 μM. Solid line corresponds to the fit of the Hill equation to the experimental data as indicated in the text. B, GTP hydrolyzed per min at variable concentrations of FtsZ in the absence (circles) and presence of 5 μM Kil (triangles) and 10 μM Kil (squares). Measurements were performed in Z300 buffer. C, sedimentation velocity profiles of 12.5 μM FtsZ-GMPCPP in the absence (solid line) and presence (dashed line) of 24.4 μM Kil obtained using interference optics. Samples were equilibrated in Z300 buffer, and 0.4 mM GMPCPP was added prior to measurement.

As Kil had a notable influence on the GTPase activity of FtsZ protofilaments, we wondered whether FtsZ polymers induced by the slowly hydrolysable GTP analogue GMPCPP were also sensitive to the presence of Kil. Sedimentation velocity analysis of FtsZ-GMPCPP polymers showed a single sharp peak at a larger s value (18 S) than GTP polymers (15 S), as reported previously (Fig. 5C) (9). The addition of a high concentration of Kil produced a broadening of the polymer peak together with a modest reduction in the s value (from 18 S to 15 S, Fig. 5C). A small decrease in the overall signal corresponding to the polymer was also detected, which might be explained by partial release of FtsZ subunits, the signal of which would be masked by that of the highly concentrated Kil species. This result contrasts with the dramatic decrease in the average s value of the polymers and the highly heterogeneous profiles observed for the GTP protofilaments at the same concentration of Kil, and even with the behavior of these polymers at 10-fold lower concentration of the inhibitor (Fig. 3). The minor effect of Kil on the FtsZ-GMPCPP polymers was below the resolution of FCS measurements, and the profiles obtained in the absence and presence of 15 μM Kil overlapped (data not shown), in contrast with the apparent shift of the FtsZ-GTP profiles at this concentration of the peptide. Therefore, the negative effect of Kil on FtsZ-GMPCPP polymers was milder than on FtsZ-GTP polymers, and it was only detected at high concentrations of the inhibitor peptide.

Kil Peptide Binds to FtsZ-GDP with an Apparent Affinity Similar to the Concentration of Kil That Exerts Half-maximum Effect on FtsZ-GTP Polymers—To evaluate the interaction of Kil peptide with FtsZ, we turned to fluorescence anisotropy measurements. This method was previously used to monitor binding of FtsZ to MinC (21). Titration of Kil into samples containing FtsZ-Alexa 488 produced a concentration-dependent increase in the anisotropy of the labeled protein, rendering isotherms through which the binding was quantitatively analyzed (Fig. 6). Addition of unlabeled FtsZ to samples containing FtsZ-Alexa 488 and Kil reduced the anisotropy value compared with that measured in the absence of unlabeled FtsZ, and the level of competition was compatible with a Kil-FtsZ interaction of similar affinity as the binding of Kil to FtsZ-Alexa 488 (data not shown). A simple 1:1 model compatible with the data was fit to the binding isotherms determined in Z300 buffer, rendering an apparent dissociation constant of 2.7 ± 0.2 μM (ΔG =...
FtsZ Protofilament Disruption by Bacteriophage λ Kil Peptide

![Graph](image)

**FIGURE 6.** Binding of Kil to FtsZ monitored by fluorescence anisotropy. Anisotropy binding titrations of FtsZ-GDP with Kil in Z300 buffer (solid squares) or in this buffer but with 500 mM KCl (open inverted triangles). The concentration of FtsZ-Alexa 488 was 0.21 μM. Solid line indicates best fit of a simple 1:1 binding model using BIOEQS software as described in the text. Dotted line is for guiding the eye. Data are the average of at least three independent experiments. Error bars represent S.D.

−7.6 ± 0.1 kcal/mol). This value is very close to the concentration of Kil at which half of the maximum inhibition effect on FtsZ assembly was achieved, under these conditions, as determined by FCS (see above). Anisotropy data pointed toward a decrease in the affinity of Kil for FtsZ with ionic strength, suggesting that electrostatic interactions participate in the recognition between the two proteins. Increasing the ionic strength up to 500 mM KCl resulted in a binding curve that did not reach saturation at the maximum concentration of Kil tested (10 μM, Fig. 6). At 100 mM KCl, the binding curve was biphasic, suggesting the presence of additional binding events and the first transition occurring at lower Kil concentrations than the midpoint of the binding curve at 300 mM KCl (data not shown).

**Discussion**

In this work, we have analyzed the effect of Kil, a small protein from bacteriophage λ that mediates the inhibition of E. coli division during infection by this phage, on the assembly of FtsZ into single-stranded protofilaments using a combined biophysical and biochemical approach. One of the key findings of this work is that the presence of Kil peptide dramatically alters the GTP-induced polymerization of FtsZ interfering with the normal formation of polymers in a narrow distribution of favored sizes (8, 9, 46). At relatively low concentrations, this antagonist gives rise to a wide distribution of polymers of smaller sizes compared with the average of ~60 FtsZ subunits per protofilament under the experimental conditions of this study (Fig. 7) (9). This is in contrast with the mode of action of other inhibitors like MinC, which act at higher concentrations than Kil and preserve the narrow distribution of FtsZ protofilament lengths (21). The different features of the inhibition by these two proteins may be related to the actual purpose of their inhibitory function. For example, Kil effectively blocks Z-ring formation throughout the E. coli cell with the goal of delaying cell lysis upon induction of lytic phase by bacteriophage λ (31). In contrast, the seemingly more conservative inhibition by MinC might stem from its role in blocking Z-ring polymerization only in polar regions of the cell but not near the midpoint. FtsZ polymers closer to the cell midpoint might reach their optimal size once released from inhibition by MinC. In addition, activity of MinC in vivo is significantly enhanced by MinD protein, which recruits MinC to the membrane and forms copolymers (47).

According to our results, Kil peptide has a dose-dependent highly negative impact on the GTPase activity of FtsZ under conditions leading to the formation of protofilaments. Previous analysis of the effect of Kil on the GTP hydrolysis rate of FtsZ showed a modest but measurable enhancement in the GTPase activity of the division protein under buffer conditions significantly different from the ones used in this work (31). Consistent with our results in the buffer conditions employed throughout this work, when we measured the GTPase activity under the same buffer conditions employed by Haesuer et al. (31), we still observed a decrease in the GTPase activity of FtsZ upon addition of Kil (data not shown). We cannot offer an explanation of these contradictory results, apart from the fact that we used a version of Kil with a shorter epitope tag, and we followed a different methodology to perform the GTPase activity assay and to purify FtsZ.

As observed here for the Kil peptide, there are other antagonists of FtsZ polymerization that severely reduce the GTPase activity of the protein, including the SOS response factor SulA, the nutrient-dependent regulator of the cell cycle OpgH and toxins like YeeV (28–30). The inhibition exerted by some of these proteins likely occurs by direct binding to FtsZ and sequester the pool of FtsZ available to polymerize (30). We propose that to delay cell lysis, Kil may use a similar sequestration mechanism when it is present at high concentrations. In addition to the Kil concentration-dependent decrease of the specific
GTPase activity of FtsZ, the increase of the apparent critical concentration of FtsZ for GTP hydrolysis observed in increasing concentrations of Kil would further support a sequestration-like mechanism. Such an increase in the critical concentration is a hallmark of sequestration and was previously described for other inhibitors like SulA and OpgH (29, 30). Interestingly, calculation of an apparent $K_d$ value for the binding of Kil to FtsZ from the shift in the apparent critical concentration, according to a model in which it is assumed that the inhibition occurs through sequestration (see Equation 6 in Ref. 30), renders a value of $2.3 \mu M$ consistent with the affinity obtained by anisotropy. The increase in the apparent critical concentration by Kil is lower than its concentration, likely because the strength of the binding of the antagonist to FtsZ is a bit lower than the affinity of FtsZ filament formation (determined by the critical concentration of polymerization). A sequestration mechanism is not used by the cell division site selection antagonists SlmA and MinC, which do not alter the GTPase activity of FtsZ, at least when it is assembled into protofilaments (17, 21, 23). The spatial restriction of these two regulators to certain regions of the cytoplasm, where they break the polymers leaving entirely functional FtsZ units able to polymerize at mid-cell, seems less compatible with a sequestration mechanism.

Kil peptide effectively counteracts FtsZ assembly when at sub-stoichiometric levels. Such behavior has also been observed for other FtsZ polymerization antagonists, including SulA (26), a hypermorphic form of the essential cell division protein FtsA (48), and MciZ, a peptide expressed during sporulation in B. subtilis that blocks FtsZ polymerization (49, 50). In the case of SulA, the inhibition of FtsZ assembly was demonstrated to occur through a sequestration mechanism (30, 51). In contrast, a recent report has shown that MciZ antagonizes FtsZ assembly by using a polymer capping-based regulatory mechanism (49). MciZ binds to the C-terminal polymerization interface of FtsZ with high affinity in the nanomolar range and inhibits FtsZ polymerization by steric hindrance, shortening the filaments and raising the specific GTPase activity. The MciZ-mediated increase of the apparent critical concentration of FtsZ for GTP hydrolysis suggests that a sequestration mechanism may operate at high concentrations of this antagonist (49). The combination of capping and sequestration effects has been also described for other cytoskeletal modulatory proteins (52). It remains to be determined whether a capping-like mechanism might explain the inhibition of FtsZ polymerization by substoichiometric levels of antagonists such as Kil.

Despite the evidence that Kil sequesters FtsZ from the assembly pool, it is notable that FtsZ polymers formed in the presence of GMPCPP are more resistant to the negative influence of Kil than protofilaments assembled in GTP. Mild depolymerization effects on FtsZ-GMPCPP polymers have been previously reported for other FtsZ antagonists, including SulA and MinC, despite their distinct modes of action (19, 21, 30). A remarkably different behavior has been recently reported in the presence of the nucleoprotein complexes of SlmA, which induce bundling of these polymers by a still unclear mechanism (25). In the case of SulA, the resistance of the FtsZ-GMPCPP polymers to SulA-mediated inhibition appears to be related to a 3–4-fold lower affinity of SulA for GMPCPP-bound FtsZ (30). The mild effect of MinC, however, may be related to the reduced assembly dynamics of these polymeric structures (19), although the less efficient binding of MinC to FtsZ-GMPCPP (21) may also contribute to the overall result. Our preliminary fluorescence anisotropy experiments indicate that Kil binds to FtsZ-GMPCPP similarly to FtsZ-GTP but less tightly compared with FtsZ-GDP (data not shown), which suggests that the interaction with FtsZ-GDP is important for the inhibition by Kil (see below). Even in the case that the antagonist interacted with FtsZ-GMPCP and FtsZ-GDP with similar strengths, the slow hydrolysis of GMPCPP would reduce the amount of FtsZ-GMPCP, and this might explain the resistance of these polymers (53). The lower cycling of FtsZ units in the FtsZ-GMPCPP polymers may also diminish their sensitivity to the action of Kil.

Kil peptide binds to FtsZ-GDP with an apparent affinity in the low micromolar range (~3 μM), coincident with the concentration of peptide required to achieve half-maximum depolymerization. This suggests that the recognition of the GDP form of FtsZ plays a key role in the inhibition mechanism. Interaction of Kil with GTP-bound FtsZ was also detected, although it seems to be less favored than the recognition of FtsZ-GDP. This interaction might, nevertheless, enhance the efficiency of Kil as a disruptor of preformed FtsZ-GTP polymers. The apparent affinity of Kil for FtsZ is lower than that reported for SulA (3 versus 0.8 μM, respectively), although the different ionic strengths used in both studies (300 mM salt for Kil versus 100 mM for SulA) (30) might account for the observed difference. The interaction of MciZ with FtsZ is at an even higher affinity with a $K_d$ ~0.1 μM (49). It seems that the stronger binding of MciZ, Kil, and SulA to FtsZ correlates with stronger inhibition of FtsZ assembly compared with MinC and SlmA, as suggested by the $K_{1/2}$ values of ~3 μM at 300 mM KCl for Kil and ~10 μM at 100 mM KCl for MinC. SlmA by itself does not block FtsZ assembly. However, MinC and SlmA are each strongly activated by cofactors, which include the membrane protein MinD and specific SlmA-binding sites on DNA, respectively (24, 54, 55). These cofactors greatly enhance MinC and SlmA activity on FtsZ. In contrast, no cofactors are needed for the strong inhibition of FtsZ assembly by SulA, MciZ, and Kil in vitro, although there is evidence that ZipA enhances Kil activity in vivo (31).

In conclusion, here we have demonstrated that the Kil peptide is able to disrupt FtsZ protofilaments into a range of shorter oligomers by interacting with FtsZ-GDP and inhibiting overall GTPase activity (Fig. 7). These results point to a SulA-like sequestration mechanism (at least at high concentrations of inhibitor), which would be in line with the functional need of both SulA and Kil to effectively disrupt Z-ring formation throughout the bacterial cell, in contrast to antagonists involved in spatial regulation of FtsZ polymerization such as MinC and SlmA. The study of natural FtsZ inhibitors is not only important for understanding the regulation of cell division as well as FtsZ polymerization itself, but it may also provide some clues toward the rational design of potential antibiotics targeting this essential protein.
FtsZ Protofilament Disruption by Bacteriophage λ Kil Peptide

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