Suprastructures and Dynamic Properties of Mycobacterium tuberculosis FtsZ

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Tuberculosis causes the most death in humans by any bacterium. Drug targeting of bacterial cytoskeletal proteins requires detailed knowledge of the various filamentous suprastructure and dynamic properties. Here, we have investigated by high resolution electron microscopy the assembly of cell division protein and microtubule homolog FtsZ from Mycobacterium tuberculosis (MtbFtsZ) in vitro in the presence of various monovalent salts, crowding agents and polycations. Supramolecular structures, including two-dimensional rings, three-dimensional toroids, and multistranded helices formed in the presence of molecular crowding, were similar to those observed by fluorescence microscopy in bacteria in vivo. Dynamic properties of MtbFtsZ filaments were visualized by light scattering and real time total internal reflection fluorescence microscopy. Interestingly, MtbFtsZ revealed a form of dynamic instability at steady state. Cation-induced condensation phenomena of bacterial cytomotive polymers have not been investigated in any detail, although it is known that many bacteria can contain high amounts of polycations, which may modulate the prokaryotic cytoskeleton. We find that above a threshold concentration of polycations which varied with the valence of the cation, ionic strength, and pH, MtbFtsZ mainly formed sheets. The general features of these cation-induced condensation phenomena could be explained in the framework of the Manning condensation theory. Chirality and packing defects limited the dimensions of sheets and toroids at steady state as predicted by theoretical models. In first approximation simple physical principles seem to govern the formation of MtbFtsZ suprastructures.

Bacterial cell division protein FtsZ, a homolog of eukaryotic tubulin, assembles at midcell into a ring called the Z-ring. The ring forms on the inside of the cytoplasmic membrane and mediates membrane constriction, resulting in the production of two newborn cells.

In addition to forming a Z-ring, FtsZ also formed dynamic transient helical assemblies. Depending on distinct stages of the cell cycle, three different localization patterns of FtsZ leading to cytokinesis were identified. In newborn cells, FtsZ moved in helical patterns over the whole length of the cell, and as time progressed this helix became shorter and redistributed into a ring structure (1, 2). Recently, we have shown by electron microscopy that crowding agents could induce Escherichia coli FtsZ to form rings and toroids about 200 nm in diameter or three-dimensional helical structures with pitches of several hundred nm in vitro (3).

Tuberculosis causes the most death in humans by any bacterium. More detailed knowledge of Mycobacterium tuberculosis FtsZ (MtbFtsZ) suprastructures and their formation may lead to efficient drug design directly targeting the cell division machinery. MtbFtsZ has been studied previously and was reported to differ from E. coli FtsZ in several aspects (4). In the presence of K⁺ ions, E. coli FtsZ formed short filaments about 180 nm long independent of pH, whereas MtbFtsZ formed similar short filaments at pH 7.7 but several μm long mostly double-stranded filaments (4) at pH 6.5. The overall polymerization rate of MtbFtsZ and subunit turnover at steady state was about 10 times slower than E. coli FtsZ (4). We have extended these polymerization experiments using sodium or rubidium salts, various crowding agents, pH values, and multivalent cations. We found MtbFtsZ to be a polymorphic protein, forming various suprafilamentous structures depending on ionic conditions, crowding agents, polycations, and pH. We have characterized their formation by high resolution electron microscopy and compared the results with the previously studied E. coli FtsZ suprastructures (3). Long linear filament bundles formed by MtbFtsZ under some conditions allowed us to study both the polymerization rate and the steady-state dynamics by time-lapse TIRF microscopy for the first time. Condensation of double-stranded helical filaments of DNA and F-actin by multivalent cations has been extensively studied experimentally (5, 6) and successfully treated by Manning’s theory of linear polyelectrolytes (7). Up until now, cation-induced condensation phenomena on bacterial cytomotive polymers have not been investigated in any detail, although it is known that many bacteria, including E. coli, can contain high amounts of polycations (8–10) and thus may be important modulators of the bacterial...
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cytoskeleton. Here, we have probed the Manning condensation theory on the linear protofilaments of MtbFtsZ.

EXPERIMENTAL PROCEDURES

Chemsicals—Methyl cellulose (MC) was from WAKO, polyvinyl alcohol was from Kanto Chemical, and fluorophores were from Invitrogen. All other chemicals used were from Sigma.

Protein Preparation—MtbFtsZ was expressed and purified similarly as described (4), yet additional gel filtration chromatography on a HiLoad 26/60 Superdex pg column (GE Healthcare) was employed. The protein was either used fresh or was frozen in small aliquots in Eppendorf tubes cooled in liquid nitrogen and stored at −80°C until use. Buffers used were KMg buffers at pH 6.6 and pH 7.7 (30 mM Mes, pH 6.6, or 30 mM Hepes, pH 7.7, 0.2 mM EGTA, 3 mM magnesium acetate, 300 mM potassium acetate, 1 mM dithiothreitol). NaMg buffers contained 300 mM sodium acetate instead of potassium acetate. We found no difference replacing magnesium acetate by MgCl₂, potassium acetate by KCl, or sodium acetate by NaCl. Adjustment of pH was done with KOH for KMg buffers and NaOH for NaMg buffers. The buffers were chosen for the following rationales. pH 6.6 is closer to the physiological pH of the cytoplasm of M. tuberculosis (4), but pH around 7.7 has been used for many in vitro assembly studies of E. coli FtsZ in the past (11).

Before an experiment, the protein was quickly thawed and dialyzed against one of the buffers with several changes of buffer using an oscillatory microdialysis system (BioTech). Dialyzed MtbFtsZ was subjected to a short high speed centrifugation step just before the experiment to remove any possible aggregates. The protein was stored on ice and used within the same day. The protein concentration was determined as described (4).

Preparation of FtsZ Suprastructures and Electron Microscopy—Monomeric MtbFtsZ was mixed with the appropriate amount of crowding agent at 24°C (final FtsZ concentration was mostly 12.5 μM), and polymerization was induced by addition of nucleotide to a final concentration of 2 mM. Sometimes a GTP regeneration system was added as described (12). This did not change the results. After several minutes, a drop of protein solution was applied to carbon-coated, glow-discharged copper grids, blotted, stained with 2% uranyl acetate, air-dried, and visualized under a Jeol JEM-2010 HC microscope operated at 100 keV and a nominal magnification between 8,000 and 60,000. Experiments with multivalent cations were done as follows. MtbFtsZ in appropriate buffer was polymerized by the addition of nucleotide. After about 15 min, when steady state was reached, polyvalent cations were added and left at room temperature between 30 min and several hours before visualizing under the electron microscope. Sometimes samples from light-scattering experiments were directly employed to the electron microscopy grids. Films were digitized with PhotoScan2000 (Z/I Imaging) at 7-μm steps, and Fourier transforms and averaged filtered images were calculated using the electron microscopy computing package described recently (13). Otherwise, electron micrographs were scanned at 1,200 dpi on an Epson scanner and displayed by ImageJ and Photoshop. Analysis of toroid sizes and parameters of helical suprastructures like pitch and amplitude was done by hand using ImageJ.

Results

Suprastructures Induced by Molecular Crowding—First, we studied the suprastructures formed by MtbFtsZ in the presence of the crowding agents MC or polyvinyl alcohol by electron microscopy. The suprastructures formed differed depending on the monovalent salts present (KCl, NaCl, rubidium chloride) and the pH. We probed pH 6.6 and 7.7 because these two pH values have been used in previous studies (3, 4, 11). The cytoplasm of M. tuberculosis contains more than 300 mM KCl (16, 17), and its pH is between 6.5 and 6.9 (11). The bacterial cytoplasm is a crowded environment, with up to 40% of the volume filled with various macromolecules (18).

In the presence of 300 mM potassium ions MtbFtsZ mainly formed two-dimensional rings or three-dimensional toroids. Rings varied in diameter, the average diameter was about 170 nm (averaged over 50 rings), whereas fully condensed toroids had an average inner diameter of approximately 125 nm and an outer diameter of approximately 250 nm (averaged over 50 toroids) (Fig. 1, A and B). This indicates that MtbFtsZ toroids grow approximately equally from a protofilament loop onto which more MtbFtsZ condenses both to the outside and inside, similar to DNA toroid formation (5) but different from E. coli FtsZ toroids, which formed protofilament loops about 200 nm in diameter from which the toroid grew only to the outside (3).
In the presence of 300 mM rubidium chloride, mostly rings and toroids were observed at pH 7.7, whereas a mixture of rings, toroids, and straight bundles formed at pH 6.6 (Fig. 1, C and D). The dimensions of the rings and the toroid were similar to those formed in KCl. The toroid morphology suggested that condensed MtbFtsZ is circumferentially wound around the toroid, much like one would coil a length of rope. This differed from E. coli FtsZ rings and toroids, which did not consist of only one long annealed FtsZ filament wrapped into circles, but of many individual single FtsZ filaments, which made lateral contacts between their neighbors (3).

The packing within these MtbFtsZ toroid appeared less regular than in E. coli FtsZ toroids, where liquid-like packing was generally observed (3). In optical transforms from MtbFtsZ toroids, no clear equatorial reflections were observed, and direct inspection clearly showed that the filaments spooling was irregular with many packing defects (Fig. 2B). In the presence of sodium ions (300 mM NaCl), rings and toroids were never observed. Instead, either straight linear bundles formed at pH 6.6, or a mixture of straight bundles and helical structures with a pitch of about 270 nm and an amplitude of about 230 nm were observed (Fig. 1, E and F). The coexistence of different morphological states implies that the total free energies of the various suprastructures formed are similar. Bundles showed a higher degree of ordering than toroids, and usually a weak off meridional reflection at about 40 Å and a broad equatorial reflection centered at about 42 Å were seen (Fig. 1G).

**Filament Dynamics**—Because the suprastructures formed in the presence of crowding agents depended largely on the specific monovalent ions used, we repeated and extended previous polymerization experiments (4) using highly purified MtbFtsZ. In the presence of 300 mM KCl at pH 6.6, MtbFtsZ after adding GTP formed mostly several micrometer long double-stranded filaments confirming the results from a previous study (4) (Fig. 3A). The equilibrium was shifted almost entirely to short single-stranded filaments (~200 nm) at pH 7.7 as reported previously (4) (Fig. 3B). When replacing 300 mM KCl by 300 mM rubidium chloride, MtbFtsZ formed almost entirely long multistranded filaments, consisting of two to four individual filaments at pH 6.6 (Fig. 3C) and mainly short single-stranded filaments at pH 7.7 (Fig. 3D). Yet, in the presence of 300 mM NaCl MtbFtsZ polymerized predominantly into several micrometer long multistranded filaments irrespective of the pH (Fig. 3, E and F).

Filament length was dependent on salt concentration and pH. For example, lowering the NaCl concentration to 150 mM at pH 7.7 shifted the equilibrium of MtbFtsZ filaments to predom-
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Lowering the KCl concentration to 0 at pH 6.6 led to the formation of few, rather short (~200 nm) and less structured filaments (supplemental Fig. S1A).

We followed the polymerization kinetics under physiological salt concentrations by light scattering. Assembly kinetics in the presence of K⁺ ions at pH 6.6 was similar to published results (4). Time courses in the presence of rubidium at pH 6.6 were similar to those with potassium, yet polymerization was faster in the presence of NaCl, pH 6.6. D, 300 mM rubidium chloride, pH 6.6. D, 300 mM rubidium chloride, pH 7.7. E, 300 mM NaCl, pH 6.6. F, 300 mM NaCl, pH 7.7. Scale bars, 200 nm.

FIGURE 3. Typical electron micrographs of MtbFtsZ filaments formed in aqueous solutions under various conditions. A, 300 mM potassium acetate, pH 6.6, B, 300 mM potassium acetate, pH 7.7. C, 300 mM rubidium chloride, pH 6.6. D, 300 mM rubidium chloride, pH 7.7. E, 300 mM NaCl, pH 6.6. F, 300 mM NaCl, pH 7.7. Scale bars, 200 nm.

For most filaments, the observed length decreased overall slowly over time (~100 nm/min); in other cases, filament length increased again for some time before continuing to depolymerize; and in other cases, filaments continued to polymerize (Fig. 4F and supplemental Movies S2 and S3). Interpretation of length fluctuations of bundles as we observed here in the presence of crowding agents is more complicated than for single filaments, especially because we did not know the polarity of filaments within bundles. Nevertheless, our results suggest that MtbFtsZ may display a mild form of dynamic microtubule-like instability.

Cation-induced Suprastructures—The formation of MtbFtsZ bundles induced by polycations was conveniently detected by changes in light scattering (Fig. 5A). Figs. 6 and 7 show the bundle formation of MtbFtsZ filaments induced by a number of cations. All of the polyvalent cations were added in the form of concentrated chloride salts to ensure that the variations among these data were not attributable to anion species. The concentration of cations needed for the onset of bundling increased with decreasing valence, indicating weakening ability to bundle (Fig. 5A). To confirm that an increase in light scattering corresponded to bundling, MtbFtsZ samples at different light-scattering levels were examined by electron microscopy (Fig. 6, A–D). Most of our experiments on cation-induced bundling of MtbFtsZ were done at neutral pH and at an ionic strength of 150 mM NaCl. Under these conditions, mostly long single or double-stranded filaments formed (Fig. 6A). Light scattering indicated that the onset of bundling by hexamine cobalt occurred at about 4 mM hexamine cobalt (Fig. 5A). This was in agreement with our electron microscopy observations that at 2 mM hexamine cobalt, no significant bundling was observed (Fig. 6B), whereas at 6 mM hexamine cobalt sheets, about 50–100 nm wide were predominant (Fig. 6C). At saturating light-scattering values (~14 mM hexamine cobalt), sheets were mostly larger in width than 200 nm (Fig. 6D).

The onset cation concentration for bundle formation was defined as described for F-actin (6), either as the point where the light intensity signal rose sharply (for cations with valence ≥3) or when light scattering increased more slowly and steadily (for some cations with valence 2), as the midpoint between the no cation value and the first saturation point of the light scattering signal. The onset concentration of cations with valence four was ~4 mM for both hexamine cobalt and spermine, but about 12 mM for spermidine, which has a valence of 3 (Fig. 5A). The bundling efficiency of different divalent cations was about 25–30 mM for MgCl₂, CaCl₂, and MnCl₂, 15 mM for BaCl₂, but only 5 mM for ZnCl₂ (Fig. 5A). Opposed to all other polycations studied, ZnCl₂ formed toroids at pH >7.3 with dimensions similar to those observed with crowding agents (Fig. 6F), whereas sheets similar to those induced by the other cations studied were observed at pH ~7.0 or lower (Fig. 6E).

In general, the behavior in Fig. 5A can be explained qualitatively by the predictions of the linear polyelectrolyte theory. For highly charged polymers the linear charge spacing b is much less than the Bjerrum length λ_b, the distance between elementary charges at which the electrostatic interaction energy equals the thermal energy kT. For double-stranded DNA at neutral pH, the linear charge spacing b equals 1.7 Å, with the Bjerrum length λ_b being 7.1 Å in water at 20 °C (6). According to the Manning counterion condensation theory (7), a consequence of the DNA high charge density is that a certain fraction of its charge is neutralized due to the territorial binding of counterions in the immediate environment. These condensed counterions are free to diffuse along the polymer axis, but inhibited from diffusing away. The fraction of polyelectrolyte charge...
compensated by the condensed counterions is given by the following equation (6),

\[
\theta = (1 - 1/N\delta)
\]  
(Eq. 1)

where \(N\) is the valence of the counterion and \(\delta = \lambda/b\).

From the FtsZ crystal structure (19) the linear charge density of MtbFtsZ can be estimated to be about 4.6 e/nm and \(b \approx 2.2 \text{ Å}\). This value has two implications: the average charge spacing along the filament axis is sufficiently small compared with the Bjerrum length to make the counterion condensation theory relevant (\(\delta_{\text{MtbFtsZ}} = \lambda/b = 3.2 > 1\)), but \(\delta\) is less than that of DNA (\(\delta_{\text{DNA}} = 4.2\)), suggesting that a smaller percentage of charge needs to be neutralized for condensation to occur. For MtbFtsZ according to Equation 1, the charge compensation \(\theta\) is calculated to be \(\sim 69\%\) for monovalent electrolytes and may reach \(\sim 84\%\) in the presence of sufficient divalent cations and up to \(\sim 90\%\) for trivalent cations. Hence, the predicted delocalized binding is stronger for counterions of higher valence, in which case the charged polymer is neutralized to a higher degree. The residual electrostatic repulsion

FIGURE 4. Polymerization and steady-state kinetics. A, light-scattering time courses of MtbFtsZ polymerization after adding GTP at 24 °C: blue, 300 mM KCl, pH 6.6; green, 300 mM rubidium chloride, pH 6.6; red, 300 mM NaCl, pH 6.6; black, 300 mM NaCl, pH 7.7; pink, 300 mM KCl, pH 7.7; light blue, 300 mM rubidium chloride, pH 7.7. B, critical concentration of MtbFtsZ: red, 300 mM NaCl, pH 6.6; green, 300 mM NaCl, pH 7.7; blue, 300 mM KCl, pH 6.6; black, 300 mM rubidium chloride, pH 6.6. C, TIRF microscopy image of polymerizing MtbFtsZ bundle 50 s after adding GTP. D, same bundle as in C about 300 s later. E, time courses of individual polymerizing MtbFtsZ bundles. F, behavior of individual bundles at steady state.
between polyelectrolytes of like charge tends to keep them apart. This repulsive force decreases with the presence of polyvalent counterions, due to the enhanced charge compensation. In addition to the weakened electrostatic repulsion between charged polymers due to counterions, an attractive interaction can also be induced by two polymers sharing counterions. The fluctuation and lateral redistribution of counterions (20) have each been shown theoretically to cause an attractive interaction between polyelectrolytes. At appropriate ionic conditions, a balance between attractive and repulsive forces occurs so that the filaments in the suspension form aggregates. It has been estimated that DNA condensation occurs as θ reaches 90%, which requires a valence of 3 or higher. MtbFtsZ formed stable sheets at 150 mM KCl with orders of ~20 mM divalent cations, suggesting that ~84% neutralization of the surface charge is required for sheet formation. This condition is intermediate between the 80% charge neutralization required for F-actin and the stringent 90% for DNA to form bundles (6).

Bundling efficiency of polyvalent cations was greatly dependent on ionic strength. Both the onset of bundle formation of MtbFtsZ as well as the bundle size as judged from the peak height of the light scattering signal were a function of the ionic strength (Fig. 5B).

**FIGURE 5.** Light-scattering signal of MtbFtsZ as a function of concentration of various cations. Each sample contained initially 0.5 mg/ml MtbFtsZ followed by sequential addition of concentrated cations: red, hexamine cobalt; orange, polylysine; blue, spermidine; light blue, spermine; yellow, barium chloride; pink, manganese chloride; green, MgCl₂; black, CaCl₂. B, effect of ionic strength on the bundle formation of barium chloride at four different NaCl concentrations: red, 75 mM; blue, 150 mM; green, 300 mM; black, 500 mM. C, variation of the bundling onset of hexamine cobalt (blue), spermine (green), and barium chloride (red) versus the NaCl concentration. Data points represent the average over three individual experiments. D, millimolar concentrations of nucleotides reverse the formation of MtbFtsZ bundles. Light scattering increases after adding spermine up to 10 mM (red). Then additional nucleotide was added (blue), and light scattering started to decrease after adding ~8 mM additional GTP.
This is a direct consequence of the Manning theory, namely that the association constant, $K$, is a function of the solutions ionic strength $c$,

$$\log K = \text{constant} - N \log c$$  \hspace{1cm} (Eq. 2)

where $N$ is the valence of the cation. The Manning one-variable approach predicts a power law dependence with a slope varying between 1 and $N$ in the log-log plot, which approaches 1 with increased concentration of the polyvalent counterion. Such dependence is expected to hold only at $c < 100$ mM. In practice, testing the ionic strength dependence for MtbFtsZ sheets is limited to high ionic strength, $\text{MgCl}_2$, and GTP (several millimolar) to keep MtbFtsZ filaments polymerized prior to adding polycations. For hexamine cobalt, we find the slope between $\log[\text{Co}(\text{NH}_3)_6^{3+}]$ and $\log[\text{KCl}]$ to be $\sim 1.2$ (Fig. 5C), in agreement with the Manning theory. Spermine also gave values of $\sim 1.2$, whereas similar experiment using $\text{BaCl}_2$ yielded a slope $\sim 0.4$. Values of less than 1 may be attributed to the complications of ionic conditions especially with divalent polycations as addressed above and to oversimplifications of the Manning approach.

Addition of millimolar amount of nucleotides have been reported to dissolve cation-induced F-actin bundles. A similar behavior was observed when additional GTP was added to cation-induced MtbFtsZ sheets (Fig. 5D). This dissociation of MtbFtsZ sheets is consistent with competitive binding of polyanionic nucleotides and the polycations and does not imply a specific binding of GTP to MtbFtsZ filaments.

The formation of supramolecular aggregates by molecular crowding and multivalent cations has been extensively studied both for F-actin and DNA (5, 21, 22). In all experiments there was a generic feature: the F-actin bundles or DNA toroids at steady state were always of finite size. The observation that stable F-actin bundle aggregates have a restricted width poses an interesting puzzle and raises a fundamental biophysical question. Theoretical equilibrium solutions of charged rods of fixed length are predicted to form networks at low linker concentrations (23). Beyond a critical linker concentration, cylindrical aggregates should grow in size until the supply of free rods is depleted. Explanations for the finite size of F-actin bundles have focused on the possibility of long range interactions (24), on kinetic limitations to the bundle size (25), on packing defects (26) and chirality (27). For MtbFtsZ, we find that increasing the cation concentration leads to the formation of larger sheets (Fig. 6, A–D). After several hours, sheets were found to have associated into a meshwork of much larger aggregates, but not into a single large structure (Fig. 7). Sheets did not disintegrate, and the strong cation linkages prevented filament dynamics opposed to the MtbFtsZ suprastructures induced by molecular crowding.

First, we should emphasize that MtbFtsZ forms sheets opposed to bundles formed by F-actin in the presence of multivalent cations (6). Whereas the width $W$ and heights $H$ of a bundle are similar, as a bundle has an approximately circular cross-section, a sheet is a structure where the width $W \gg H$ and $H$ can still consist of multiple filament layers. In a sheet,
filaments have a more preferred orientation relative to each other, whereas the interfilament interface is less well defined in a three-dimensional bundle. Therefore, the association of sheets into larger aggregates must be more restricted than that of bundles. MtbFtsZ sheets induced by various cations usually seemed to try to associate in a side-by-side manner over time (Fig. 7). Yet this association was limited due to the obvious tendency of MtbFtsZ sheets to twist (Fig. 7). Our results therefore clearly favor chirality (27) as the main reason for the limited aggregate size of MtbFtsZ at high cation-linker concentrations at steady state. Optical diffraction patterns from cation-induced sheets were similar to bundles formed in the presence of crowding agents (Figs. 6H and 1G).

**DISCUSSION**

Recently, we have shown for *E. coli* FtsZ that both ring-like and helical structures of FtsZ similar to those observed by low resolution fluorescence microscopy *in vivo* (2) can be induced *in vitro* by crowding agents and that their molecular structures, suprastructures, and interfilament interactions can be visualized by high resolution electron microscopy (3). Whereas *E. coli* FtsZ in aqueous solutions formed short single protofilaments about 180 nm in length, MtbFtsZ could assemble into single or doublet filaments several micrometers long. In the presence of crowding agents, both *E. coli* FtsZ and MtbFtsZ formed mostly rings and toroids in the presence of physiological KCl concentrations (~300 mM). Ring dimensions were on average smaller for MtbFtsZ. Nevertheless, ring or toroid formation did not seem to be affected by filament length. The architecture of MtbFtsZ toroids appeared to consist of filaments circumferentially wound around the toroid, much like one would coil a length of rope. Condensation of MtbFtsZ toroids can be explained in analogy with DNA condensation. The first step in DNA toroid formation is the spontaneous formation of a nucleation loop, which acts as a nucleation site for condensation on which the remainder of the DNA polymer condenses to form a prototoroid. This prototoroid then grows equally inward and outward by the addition of free DNA polymers from the solution (5). MtbFtsZ toroids also appear to grow equally inward and outward, which differs from *E. coli* FtsZ toroids which only grew outward, indicating that the 200-nm inner toroid dimension was an energy-minimized state in this system (3). In the presence of sodium ions crowding agents shifted the equilibrium of MtbFtsZ mostly to straight bundles (with some helical bundles depending on the pH), whereas *E. coli* FtsZ formed helical spirals under these conditions (3). We probed whether the difference between sodium and chloride ions was dependent on the ion size, by observing the suprastructures formed in the presence of rubidium chloride and crowding agents. Yet, in the presence of rubidium chloride also mostly rings and toroids formed similar to those in the presence of KCl. Additionally, the polymerization kinetics and filament length of MtbFtsZ in aqueous solutions were similar for both KCl and rubidium chloride. The elongation rate was faster in the presence of NaCl and may be responsible for the formation of mostly straight filaments rather than toroids. Why exactly sodium and potassium ions induce different polymorphic structures of FtsZ remains elusive at present.

The polymorphism we observed with MtbFtsZ has remarkable similarities to bacterial flagella, which can adopt different conformations classified as straight filaments, helical filaments with different pitches and wave height, as well as circular shapes with a very small pitch similar to a toroid, depending on pH and ionic conditions (28). The polymorphism of bacterial flagella implied that flagella molecules adopted at least two different structural conformations. A similar interpretation may be applied for the different suprastructures of MtbFtsZ found in the presence of molecular crowding.

Addition of cations to MtbFtsZ filaments led to the formation of sheets, and in one case rings and toroids were observed. The formation of these aggregates could be explained within the general framework of the Manning counterion condensation theory. The most direct implication is that proteins with sufficient numbers of positive charges, exposed appropriately on their surface, will inevitably bind to the negatively charged FtsZ filaments even in solutions of high physiological ionic strength. Several FtsZ-binding proteins (FtsA, ZipA, ZapA,B, SepF, MinC, SulA, EzrA, and ClpX) have been identified (29). The strong electrostatic effects between polyelectrolytes and their counterions suggests that the binding of some of these proteins to FtsZ may not be an interaction between specific amino acid residues but rather due to a long range electrostatic interaction involving larger areas of the various protein surface.

*In vivo*, a high amount of polycations exists in various bacteria. Polyamines are widely distributed in biological materials and probably have fundamental physiological roles in many cells. They are thought to influence fidelity in biosynthesis of nucleic acids and proteins as well as maintenance of cell integrity (30).

In *E. coli*, the concentration for the polyamine spermidine was found to be almost as high as the intracellular Mg$^{2+}$ concentration of ~24 mM (8). In the moderately halophilic bacteria of *Vibrio costicola* the intracellular Mg$^{2+}$ concentration was about 50 mM (9). In exponentially growing *Neurospora cassa*, both the Mg$^{2+}$ and spermidine values reached concentrations of about 16 mM (10). Although ribosomal RNA is known to bind substantial amounts of polyvalent cations, the amount of free Mg$^{2+}$ or spermidine may modulate the formation of supramolecular structures in various bacterial species.

Condensation phenomena induced by molecular crowding or multivalent cations have been observed for F-actin and DNA in the past, and in all experiments there was a generic feature, namely that the bundles or toroids formed at steady state were always of finite size. Theoretically, the bundles or toroids should form one single aggregate over time. Numerous explanations for the observed finite sizes of F-actin or DNA suprastructures have been put forward, but none of them could be clearly verified experimentally. Our results show that MtbFtsZ toroids formed by molecular crowding display packing defects, thus favoring the theory that topological defects (26, 31) limit the toroid size under these conditions, whereas in the case of cation-induced MtbFtsZ sheets, size was limited by chirality (27). Our investigation shows that the formation of supramolecular structures by the linear cell division protein FtsZ of *M. tuberculosis* is governed in first approximation by simple physical principles.
In vivo, the situation may be more complex. In addition to molecular crowding, a number of accessory proteins act in concert to regulate cell division at various levels of FtsZ assembly (32). Although individually these proteins are not required for Z-ring assembly, their various roles in modulating the polymerization of FtsZ mean that the combined loss of certain regulatory proteins produces a synthetic lethal division phenotype. For example, a special regulatory system, the Min-system, is crucial for the precise positioning of the Z-ring. Several other protein factors, including ZipA, ZapA and probably also FtsA as well as the nucleoid occlusion protein SimA, additionally cross-link the FtsZ filaments in the Z-ring and anchor them to the cytoplasmic membrane. An unknown signal triggers ring contraction and possibly disassembly. The presence of high levels of inhibitor proteins such as SulA, MinC, or ClpX might help to antagonize the ring-stabilization proteins and tip the balance toward disassembly (32).

Time-lapse TIRF microscopy of MtbFtsZ bundles at steady state revealed surprising evidence of a form of dynamic instability, rather than a treadmilling process, which may have been expected from the fluorescence recovery after photobleaching experiments which show a turnover of an entire MtbFtsZ filament within about 80 s (4). At present, there are to many unknowns to come up with a model for dynamic instability of MtbFtsZ. First of all, processes within bundles are more difficult to interpret as in single filaments. Second, the site of GTP hydrolysis in the polymers carry unhydrolyzed GTP (33), so there must be a considerable lag between the time a subunit enters a proto-filament and when it hydrolyzes its GTP. What triggers hydrolysis is unknown. Our data support a model where spontaneous hydrolysis of GTP occurs rarely, but when it does happen, additional hydrolysis is accelerated vectorially on the adjacent subunits on the same or neighboring filaments. This type of coupled or vectorial hydrolysis has been proposed to explain how the GTP cap generates microtubule dynamic instability (34). Inside the cell, steady-state dynamics of the Z-ring may could differ as it may be fine tuned by a large number of accessory proteins.

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