Mycobacterium tuberculosis FtsZ requires at least one arginine residue at the C-terminal end for polymerization in vitro

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We examined whether C-terminal residues of soluble recombinant FtsZ of Mycobacterium tuberculosis (MtFtsZ) have any role in MtFtsZ polymerization in vitro. MtFtsZ-ΔC1, which lacks C-terminal extreme Arg residue (underlined in the C-terminal extreme stretch of 13 residues, DDVDDVVPFFMR), but retaining the penultimate Arg residue (DDVDVVPFFMR), polymerizes like full-length MtFtsZ in vitro. However, MtFtsZ-ΔC2 that lacks both the Arg residues at the C-terminus (DDDDVDVPPFM), neither polymerizes at pH 6.5 nor forms even single- or double-stranded filaments at pH 7.7 in the presence of 10 mM CaCl2. Neither replacement of the penultimate Arg residue, in the C-terminal Arg deletion mutant DDDDDVDVPPFMK/H/A/D, enabled polymerization. Although MtFtsZ-ΔC2 showed secondary and tertiary structural changes, which might have affected polymerization, GTPase activity of MtFtsZ-ΔC2 was comparable to that of MtFtsZ. These data suggest that MtFtsZ requires an Arg residue as the extreme C-terminal residue for polymerization in vitro. The polypeptide segment containing C-terminal 67 residues, whose coordinates were absent from MtFtsZ crystal structure, was modeled on tubulin and MtFtsZ dimers. Possibilities for the influence of the C-terminal Arg residues on the stability of the dimer and thereby on MtFtsZ polymerization have been discussed.

Keywords FtsZ; Mycobacterium tuberculosis; polymerization; GTPase; cell division

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Introduction

FtsZ, the principal essential bacterial cell division protein, polymerizes in GTP-dependent manner at the mid-site of bacterial cells and forms Z-ring in vivo [1,2] and in vitro [3–5]. Escherichia coli FtsZ (EcFtsZ) deletion mutant, which lacks the stretch of residues (316–383) from the C-terminus, was found to polymerize both in vitro [6] and in vivo [7], although it could not participate in cell division [7]. Equivalent stretch of C-terminal residues of Mycobacterium tuberculosis FtsZ (MtFtsZ) (aa 313–379), and of FtsZ of Methanococcus jannaschii (MjFtsZ), Bacillus subtilis (BsFtsZ), Pseudomonas aeruginosa (PaFtsZ), and Aquifex aeolicus (AaFtsZ), whose crystal structures have been solved so far [8–10], was found to form an unstructured tail end of C-terminal domain [8–10]. Biochemical role of the unstructured end of C-terminal domain, encompassing aa 313–379 that are not seen in the crystal structure of MtFtsZ [8], or of the C-terminal residues in this stretch, in MtFtsZ polymerization has not been investigated till date. A point mutation study on the FtsZ of Mycobacterium smegmatis (MsFtsZ) has shown that change of three D residues (also conserved in mycobacterial FtsZ), which are present in tandem (underlined) at the C-terminal end of MsFtsZ peptide sequence (aa 371–385) (GIA DDVDVPPFMK), to A does not affect polymerization in vitro and in vivo [11]. In the present study, using MtFtsZ mutants carrying substitutions and progressive deletions of C-terminal residues, the role of C-terminal residues in MtFtsZ polymerization in vitro was investigated.

Materials and Methods

Cloning of MtftsZ gene and construction of MtftsZ mutants

The ftsZ gene of M. tuberculosis (MtftsZ) was cloned in pET15b+ as described [12]. The oligonucleotide primers and the constructs generated and/or used in this study are listed, respectively, in Supplementary Tables S1 and S2. MtftsZ C-terminal deletion mutants (MtftsZ-ΔC1 and
MtftsZ-ΔC2) were generated using PCR amplification from cosmid MTCY270 with MtZf1 as forward primer and the appropriate mutation-containing reverse primer, MtZ-delC1r or MtZ-delC2r, using *Pfu* DNA polymerase (Fermentas), following the manufacturer’s protocol. MtftsZ C-terminal substitution constructs, in which the C-terminal R residue was deleted and the penultimate R residue was changed to K (MtFtsZ-ΔC1K), H (MtFtsZ-ΔC1H), A (MtFtsZ-ΔC1A), and D (MtFtsZ-ΔC1D), were generated through PCR amplification using MtZf1 as the forward primer and the specific mutation-containing primers, MtZ-delC1R-Kr, MtZ-delC1R-Hr, MtZ-delC1R-Ar, and MtZ-delC1R-Dr, respectively, as the reverse primers. All the deletion constructs were cloned first in pBS (KS) between *Bam*HI and *Xba*I sites and sequence verified. These pBS (KS) constructs were digested with *Bam*HI and *Not*I, the released inserts were end-filled using Klenow DNA polymerase (NEB) according to the manufacturer’s protocol, and subcloned into end-filled *Bam*HI site of pET15b+. The constructs, MtftsZ-Stop and MtftsZ-ΔC2-Stop, which are devoid of nucleotide sequence from the pET15b+ vector backbone, were generated by re-amplifying MtftsZ and MtftsZ-ΔC2 with MtZf1 as the forward primer and MtZr1-stop and MtZ-delC2r-stop, respectively, as the reverse primers. These constructs have one stop codon inserted at the 3’ end of the desired full length or the deletion sequence to avoid amino acids of pET15b+ backbone from getting added at the C-terminus of the respective protein. These constructs were identically subcloned into pET15b+. The sequence of the clones was verified on both the strands. The desired mutation expected in each clone alone was found. EcftsZ gene was cloned as described [13].

### Overexpression and purification of proteins

Overexpression and purification of soluble wild-type and mutant 6×His-tagged MtFtsZ proteins were carried out as described [14], with modifications [12]. Protein quantitations were all SDS–PAGE based, by making comparisons of Coomassie-stained FtsZ bands with those from known amounts of bovine serum albumin. The 6×His-tag-free ΔC2 mutant protein was prepared as described [12]. The MtftsZ-Stop and MtftsZ-ΔC2-Stop proteins, which are devoid of the 20 residues from the pET15b+ vector backbone, were also overexpressed and purified in an identical manner [12]. EcFtsZ was overexpressed and purified as described for MtFtsZ.

### Light scattering assay

In order to monitor polymerization of various FtsZ preparations, light scattering (LS) assay [15] was carried out as described [14], with modifications [12]. Reactions were carried out at 30°C using FluoroMax-4 spectrofluorimeter. Protein samples were taken at 8.6 μM, in 2-(N-morpholino)ethanesulfonic acid–NaOH (pH 6.5) buffer containing 50 mM KCl and 10 mM MgCl₂. LS assays of protein samples were monitored in a 250-μl cuvette initially for 200 s to get a baseline and then for an additional 600 s after the addition of 1 mM GTP. Experiments were repeated thrice, at least with three independent protein preparations. The results were consistent.

### GTPase activity assay

GTPase activity [16,17] of FtsZ preparations was assayed using malachite green reagent-based colorimetric method [18], as described [19,20]. In brief, MtFtsZ-Stop or ΔC2-Stop (10 μM) was incubated in 400 μl of 50 mM HEPES–NaOH, pH 7.7, buffer containing 350 mM KOAc and 5 mM Mg(OAc)₂, and 1 mM GTP for 1, 2, 5, 10, and 20 min at 37°C. At the above time points, 60 μl of the reaction mixture was withdrawn and added to tubes containing 2.3 volumes of water and 3.3 volumes of 0.6 M perchloric acid and kept on ice until such samples from all the time points have been collected. Equal volume of malachite green reagent prepared as described previously [19] was added, kept the tubes at room temperature for 30 min, and optical density was measured at 655 nm. Monobasic potassium phosphate in the range of concentrations from 5 to 40 μM was used to prepare the standard curve. The optical density value of the FtsZ sample in the linear range of the standard curve was used to calculate specific activity. The GTPase activity assays were repeated thrice with different MtFtsZ-Stop and ΔC2-Stop preparations.

### Transmission electron microscopy

Polymerization of FtsZ preparations was determined using transmission electron microscopy (TEM), as described [21], with modifications [12]. Polymerization reactions were carried out in the same buffer used in LS assay, at 37°C for 10 min, spotted on carbon-coated copper grids, and visualized under JEOL JEM 100 CX II transmission electron microscope at an acceleration voltage of 80 kV. All TEM pictures were taken at ×14,000 or ×20,000 magnification, with further digital magnification to ×50,000. Polymerization assays for MtFtsZ, MtFtsZ-Stop, ΔC2, ΔC2-Stop (at 10 or 13 μM), at pH 6.5 and 7.7, and EcFtsZ preparations (10 μM) at pH 7.7, with and without 10 mM CaCl₂, were carried out in 50 mM HEPES–NaOH, containing 100 mM KCl and 10 mM MgCl₂, and visualized under TEM as described previously [20]. All the assays were performed in triplicate and repeated at least six times using independent protein preparations.

### FtsZ pelleting assay

Polymerization capability of MtFtsZ and deletion mutants was examined using polymerized FtsZ pelleting assay also,
as described [3], with minor modifications. One hundred micrograms each of MtFtsZ, ΔC1, and ΔC2 or MtFtsZ-Stop and ΔC2-Stop were taken in 200 μl of polymerization buffer used in LS assay and TEM experiments. Before the addition of GTP, 20 μl aliquot each was withdrawn and used as the loading or protein input control. Remaining 180 μl was allowed to polymerize for 10 min at 30°C, by adding GTP at 5 mM final concentration. Immediately after the polymerization reaction, the samples were centrifuged at 80,000 rpm (247000 g) at 4°C in Beckman TLA100 rotor (180 μl in 200 μl ultracentrifuge tube; with maximum force of 436,000 g and minimum force of 336 000 g for 100,000 rpm), in Optima™ TLX ultracentrifuge, for 10 min. Twenty microliters of supernatants were withdrawn from each tube and resolved separately on SDS–PAGE. Remaining supernatants were completely and carefully removed. Pellet fraction from each tube was resuspended in 100 μl of 1× SDS–PAGE loading buffer and 1/10th fraction was loaded on another SDS–PAGE. The FtsZ pelleting assay experiment was carried out in triplicate and repeated thrice with independent MtFtsZ wild-type and mutant protein preparations.

1-Anilinonaphthalene-8-sulfonic acid fluorescence assay

In comparison with wild-type MtFtsZ preparations, mutant FtsZ preparations were examined for primary structural alterations using 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence assay as described previously [12,22] in freshly prepared 20 mM Tris–HCl (pH 8) buffer. A total of 1 μM MtFtsZ or mutant proteins and 50 μM ANS were incubated separately or in mixture in the same cuvette. Excitation wavelength of 360 nm was used and emission spectra were measured from 400 to 600 nm. Each spectrum was measured by an average of three scans.

Circular dichroism spectroscopy

Mutant FtsZ preparations were examined for secondary structural alterations using circular dichroism (CD) spectroscopy in JASCO J-715 spectropolarimeter as described [23], but with minor modifications [12]. A 2 mm path-length cuvette was used and CD values were measured over 200–250 nm wavelength range at every 0.5 nm interval. About 100 μg protein was used in 1 ml of 5 mM potassium phosphate buffer (at pH 7.2 and 7.7), per assay at room temperature. Each spectrum was recorded using an average of three scans and were smoothened using a sliding window of five. CD values were converted to mean residue ellipticity [θ] for plotting.

Modeling of MtFtsZ C-terminal tail region

1RLU, with a resolution of 2.08 Å bound to GTPγS, was used as the starting molecule for model building and as a reference molecule to analyze various structural aspects of MtFtsZ. The amino acid sequence of the entire polypeptide chain of FtsZ protein from M. tuberculosis (MtFtsZ, accession number: GI: 54041007, 379 aa) was obtained from the NCBI Entrez database. A database search to identify similar sequences in the PDB structure database was carried out using the standard BLAST tool [24], using default parameters and the BLOSUM62 substitution matrix. A similar search was carried out against PDB [24,25], to identify suitable structural templates for the tail region of MtFtsZ. The alignment with the best template thus obtained was carefully verified by examining additional pairwise [26] and multiple alignments [27] with other closely related sequences. The crystal structure of MtFtsZ that contained coordinates for the first 312 residues was obtained from PDB [8].

The model of the C-terminal tail region of 67 residues was built using Modeller [28] using standard homology modeling protocols and added to the rest of the polypeptide residues 1–312 determined by X-ray crystallography, to make up the entire 379 residue polypeptide of a subunit. Two alternative dimers, illustrative of protein–protein associations leading to the protofilament formation, were then built as described in the Results section. The models of the subunit and those of the dimer were regularized by conjugate gradient energy minimization for about 150 steps using CNS [29], using default parameters. The quality of the model was assessed by using Procheck [30] and Verify3D [31]. Structural superpositions were carried out using DALI, and structure-guided sequence alignment was carried out using Clustal W. Visualization and structural analyses were carried out using PyMol [32].

Results

Experimental strategy

The experimental strategy to find out the role of C-terminal residues of MtFtsZ in polymerization involved generation of progressive C-terminal deletion mutants, which are devoid of C-terminal extreme residues Arg379, or Arg379 and Arg378 (Fig. 1), and determination of polymerization using 90° LS assay, TEM, and sedimentation (pelleting) of polymerized FtsZ. Structural changes in the mutants, if any, were determined using ANS fluorescence assay and CD spectroscopy. In addition to polymerization assay, GTPase activities of the proteins were also determined. We had earlier shown that the presence of 6×His tag at the N-terminus does not interfere with polymerization capability of MtFtsZ or its mutants [12]. Therefore, MtFtsZ and the MtFtsZ mutants used in the present study were N-terminal 6×His-tagged proteins and will be referred to without 6×His prefix. Further, all the experiments were carried out using two sets of wild-type and mutant MtFtsZ proteins. One set consisted of wild-type and mutant
MtFtsZ proteins that carried 20 additional amino acids from pET15b+ vector backbone at the C-terminal end of the protein. The second set consisted of wild-type MtFtsZ and relevant mutants, which were devoid of 20 additional amino acids from pET15b+ vector backbone at the C-terminal end, and which were referred to with the word ‘Stop’ as suffix to their name (Fig. 1).

**The C-terminal end Arg378 is essential for polymerization**

The polymerization capability of soluble, recombinant MtFtsZ, and the deletion mutants ΔC1 and ΔC2 (Fig. 1) was first determined at 8.6 μM each, using 90° LS assay. MtFtsZ showed polymerization in the presence of 10 mM Mg^{2+} and 1 mM GTP at pH 6.5 [Fig. 2(A)]. The C-terminal deletion mutant ΔC1, which lacked the C-terminal extreme Arg379 (bold and underlined) of the 13 residue-containing C-terminal peptide sequence DDDDVPPFMR (Fig. 1) to become DDDDVPPFMR, also showed polymerization under identical conditions [Fig. 2(A)]. But, the deletion mutant ΔC2, which lacked both the residues (Arg379 and Arg378) of MtFtsZ sequence at the C-terminal (Fig. 1) to become DDDDVPPFM, did not show rise in LS assay under identical conditions at pH 6.5 [Fig. 2(A)]. When these experiments were repeated under identical conditions with MtFtsZ-Stop and ΔC2-Stop, similar to ΔC2, ΔC2-Stop also did not polymerize, while MtFtsZ-Stop showed polymerization like MtFtsZ [Fig. 2(B)]. The 6× His-free preparation of ΔC2 also did not show polymerization (data not shown). These observations indicated that the presence of at least one R residue at the C-terminus seems to be essential for MtFtsZ polymerization in vitro.

However, it is possible that ΔC2 or ΔC2-Stop might not form polymers, but might form filaments, which might be undetectable in LS assay, as reported for MtFtsZ [20].

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**Figure 1** Schematic representation of the extents of C-terminal progressive deletion mutants The wild-type or mutant proteins without the suffix ‘Stop’ in their names contain the 20 additional residues at the C-terminus from the vector backbone. The wild-type or mutant proteins with the suffix ‘Stop’ to their names do not contain any extra residues from the vector. P, polymerization; NP, no polymerization.

**Figure 2** Polymerization assay using 90° LS (A) MtFtsZ (closed diamond) and deletion mutants ΔC1 (open diamond) and ΔC2 (closed triangle) (8.6 μM each). (B) MtFtsZ (closed diamond), MtFtsZ-Stop (closed circle), ΔC2 (closed triangle), and ΔC2-Stop (open circle) (8.6 μM each).
Therefore, TEM was used to examine polymerization reaction samples of the two sets of proteins MtFtsZ, ΔC1, and ΔC2 (one set) and of MtFtsZ-Stop and ΔC2-Stop (second set). TEM experiments carried out under conditions used for LS assay at pH 6.5 revealed that only MtFtsZ and ΔC1 formed polymers (Fig. 3, middle left and middle panels). ΔC2 did not form polymers or filaments (Fig. 3, middle right panel). Similarly, at pH 6.5, while MtFtsZ-Stop formed polymers, ΔC2-Stop did not form polymers or filaments (Fig. 3, lower left and right panels). The 6×His-free preparations of ΔC2 also did not form polymers, whereas 6×His-free preparations of MtFtsZ formed polymers (data not shown). Thus, TEM experiments could not detect polymers or filaments of ΔC2 and ΔC2-Stop, and the data support observations made in LS assay.

Recently, it was shown that MtFtsZ forms long two-stranded filaments at pH 6.5, but forms short single-stranded filaments at pH 7.7, and predominantly double-stranded filaments and small bundles along with some single-stranded filaments in the presence of 10 mM CaCl2 at pH 7.7 [20]. They found that the double-stranded filaments gave strong LS signal, whereas the shorter single-stranded filaments gave LS signal too weak to be measured. Thus, the short single-stranded filaments could not be detected using LS assay, but could be visualized using TEM [20]. Keeping in view of this possibility, although ΔC2 and ΔC2-Stop did not polymerize at pH 6.5, it was possible that they might form single-stranded and/or double-stranded filaments at pH 7.7 or in the presence of 10 mM CaCl2 at pH 7.7. We found that MtFtsZ and MtFtsZ-Stop could form filaments at pH 7.7 [Fig. 4(A), left two panels], but ΔC2 and ΔC2-Stop did not form filaments at pH 7.7 [Fig. 4(A), right two panels]. MtFtsZ and MtFtsZ-Stop were found to form double-stranded filaments at pH 7.7/10 mM CaCl2 [Fig. 4(B), middle panels], as reported for MtFtsZ [20]. On the contrary, different preparations of ΔC2 and ΔC2-Stop consistently and repeatedly failed to form even single- or double-stranded filaments at pH 7.7 in the presence of 10 mM CaCl2 [Fig. 4(C), left two panels]. In comparison, EcFtsZ formed short filaments of expected morphology at pH 7.7 and filament bundles at pH 7.7 in the presence of 10 mM CaCl2 [Fig. 4(C), right two panels], as reported [33]. These observations indicated that the obligatory requirement of MtFtsZ for Arg as the C-terminal end residue for polymerization is independent of any polymerization conditions, and therefore, it is the inherent structural feature of MtFtsZ in vitro.

We wanted to further verify the polymerization inability of ΔC2 and ΔC2-Stop using FtsZ polymer pelleting assay as described [3]. Polymeric form of FtsZ will get into pellet fraction, whereas polymerization-incompetent FtsZ will stay in the supernatant, when centrifuged at 80,000 rpm at 4°C in Beckman TLA100 rotor with maximum force of 436 000 g and minimum force of 336 000 g for 100,000 rpm. FtsZ protein pelleting assay for the two sets of proteins, MtFtsZ, ΔC1, and ΔC2 (first set), and MtFtsZ-Stop and ΔC2-Stop

Figure 3 Transmission electron micrographs of polymerized samples of MtFtsZ, ΔC1, ΔC2, MtFtsZ-Stop, and ΔC2-Stop Polymerization of the proteins (10 μM each) was carried out in the presence of 1 mM GTP at pH 6.5. Upper row: (–) GTP control samples. Middle row: MtFtsZ or ΔC1 or ΔC2, (+) 1 mM GTP. Lower row: MtFtsZ-Stop or ΔC2-Stop, (+) 1 mM GTP.
MtFtsZ requires C-terminal arginine for polymerization in vitro

(second set), was carried out under polymerization conditions identical to those used in LS assay. In addition to the polymerization at pH 6.5 used in LS assay, since we found that MtFtsZ formed filaments at pH 7.7 and double-stranded filaments at pH 7.7/10 mM CaCl₂, as reported by others [20], polymerization for pelleting assay was also carried out under these conditions. In the pelleting assay at pH 6.5, ΔC2 remained mostly in the supernatant fraction after polymerization reaction, whereas ΔC1 and MtFtsZ went into pellet fraction due to polymerization [Fig. 5(A), main gel panels].

Figure 4 Transmission electron micrographs of polymerized samples of MtFtsZ, MtFtsZ-Stop, ΔC2, ΔC2-Stop, and EcFtsZ. Polymerization of the proteins (10 μM each) was carried out in the presence of 1 mM GTP at pH 7.7 (A) and in the presence of 10 mM CaCl₂ at pH 7.7 (B and C). The magnified inset frames show double-stranded filaments.

Figure 5 Pelleting assay of polymers of MtFtsZ, ΔC1, and ΔC2 at pH 6.5 in three independent samples (A) SDS–PAGE profile. The rightmost panel shows a (−) GTP control for pelleting of ΔC1. (B) Quantitation of relative protein amount was based on (A).
As the negative control, we repeated pelleting assay of ΔC1 without GTP and found negligible amount of protein in the pellet [Fig. 5(A), extreme right gel panel]. Quantitation showed >80% of ΔC2 in the supernatant fraction, whereas more than equivalent amount of ΔC1 and MtFtsZ stayed in the pellet fraction [Fig. 5(B), middle and right bar graphs]. Similarly, in the pelleting assay at pH 6.5, while MtFtsZ-Stop could be recovered in the polymerized form from the pellet fraction, ΔC2-Stop stayed in the supernatant fraction [Fig. 6(A), gel panels]. Quantitation showed about 80% of ΔC2-Stop staying in the supernatant fraction, whereas an equivalent fraction of MtFtsZ-Stop was found in the pellet fraction [Fig. 6(A), bar graphs]. Pelleting assay at pH 7.7 in the presence of 10 mM CaCl₂ for MtFtsZ-Stop and ΔC2-Stop also showed ΔC2-Stop in the supernatant fraction, whereas MtFtsZ-Stop went into pellet fraction [Fig. 6(B), gel panels]. Quantitation showed about 90% of ΔC2-Stop in the supernatant fraction, whereas an equivalent quantity of MtFtsZ-Stop was found in the pellet fraction [Fig. 6(B), bar graph panels]. Thus, data from polymerized FtsZ protein pelleting assay for the two sets of proteins at pH 6.5 and 7.7, and in the presence of 10 mM CaCl₂ at pH 7.7, also showed that while MtFtsZ and MtFtsZ-Stop formed polymers, ΔC2 and ΔC2-Stop did not form polymers, which is in concurrence with the observations from LS assay and TEM experiments. Thus, the observations from LS assay, TEM of protein polymers formed under different conditions, and polymerized MtFtsZ protein pelleting assay confirmed that deletion of more than one C-terminal extreme Arg residue abolishes polymerization capability of MtFtsZ in vitro.

Arg 378 substitution mutants also do not polymerize in vitro

Experiments described above showed that although the C-terminal extreme Arg379 (R in DDDDVDPFPFMRR) is not required for polymerization, the presence of at least one Arg residue at the extreme terminus of MtFtsZ seemed necessary for polymerization. In order to investigate whether there is an ‘obligatory’ requirement for the C-terminal extreme residue to be Arg for polymerization, a series of substitution mutations (with Lys, His, Ala, and Asp) were introduced in lieu of Arg378 residue (bold and underlined) in ΔC1(R) (as DDDVDVPFPFM) to generate ΔC1(K), ΔC1(H), ΔC1(A), and ΔC1(D) (Fig. 1). None of these four substitution mutants showed polymerization in LS assay, whereas the positive control MtFtsZ elicited polymerization (Fig. 7). Therefore, the presence of at least one Arg residue as the C-terminal extreme residue seems to be mandatory for the polymerization of MtFtsZ in vitro.

Figure 6 SDS–PAGE profile of pelleting assay of polymers of MtFtsZ-Stop and ΔC2-Stop Samples analyzed from polymerization reaction: (A) at pH 6.5, with samples from supernatant on the left panel and samples from pellet on the right panel; lane 1, MtFtsZ-Stop (−) GTP; lane 2, MtFtsZ-Stop (+) 1 mM GTP; lane 3, ΔC2-Stop (−) GTP; lane 4, ΔC2-Stop (+) 1 mM GTP. (B) At pH 7.7, with/without 10 mM CaCl₂, with samples from supernatant on the left panel and samples from pellet on the right panel; lane 1, MtFtsZ-Stop (−) GTP; lane 2, MtFtsZ-Stop (+) 1 mM GTP; lane 3, MtFtsZ-Stop (+) 1 mM GTP (+) 10 mM CaCl₂; lane 4, ΔC2-Stop (−) GTP; lane 5, ΔC2-Stop (+) 1 mM GTP; lane 6, ΔC2-Stop (+) 1 mM GTP (+) 10 mM CaCl₂. (C) Input of protein in the pelleting assay at pH 7.7: SDS–PAGE profile (top panel) and quantitation of input protein (bottom panel); lane 1, MtFtsZ-Stop (−) GTP; lane 2, MtFtsZ-Stop (+) GTP; lane 3, MtFtsZ-Stop (+) GTP (+) 10 mM CaCl₂; lane 4, ΔC2-Stop (−) GTP; lane 5, ΔC2-Stop (+) GTP; lane 6, ΔC2-Stop (+) GTP (+) 10 mM CaCl₂.
ΔC2 and ΔC2-Stop do not polymerize even under forced conditions

Since ΔC2 and ΔC2-Stop did not polymerize under standard conditions, we wanted to find out whether they would polymerize under forced conditions, in comparison to that of MtFtsZ-Stop. The forced conditions used included the presence of 50 μg/ml DEAE–dextran, which could force polymerization on polymerization-defective mutant E. coli FtsZ2 [34,35] and polymerization-lethargic Mycobacterium leprae FtsZ (MtFtsZ) in vitro [12], and the presence of higher concentrations of the protein, GTP, or Mg"⁺. Even in the presence of 50 μg/ml DEAE–dextran, ΔC2 did not form polymers or filaments (Supplementary Fig. S1, lower right panel), as examined using TEM. As expected, in the presence of DEAE–dextran (50 μg/ml), MtFtsZ and ΔC1 formed extensive polymer bundles (Supplementary Fig. S1, lower left and middle panels). Similarly, even under different higher concentrations of the protein, GTP, or Mg"⁺, ΔC2-Stop did not show polymerization, whereas MtFtsZ-Stop showed polymerization, when analyzed using LS assay [Supplementary Fig. S2(A–C), respectively].

Secondary and tertiary structures of ΔC2 and GTPase activity

We wanted to verify whether there has been any gross structural alteration in the ΔC2 mutant protein, which might have resulted in the loss of polymerization activity. For this purpose, ANS fluorescence emission and CD spectroscopy profiles of MtFtsZ and ΔC2 were compared. ANS fluorescence emission assay (at pH 8) of MtFtsZ and ΔC2 showed that ΔC2 elicited marginally more blue shift and hyper-shift in the fluorescence, as compared with that by MtFtsZ [Supplementary Fig. S3(A)]. It indicated the existence of some tertiary structural difference between MtFtsZ and ΔC2. Further, the secondary structures of MtFtsZ and ΔC2 or of MtFtsZ-Stop and ΔC2-Stop were compared at pH 7.7 and 7.2. Far UV CD spectra of MtFtsZ-Stop and ΔC2-Stop at pH 7.7 showed only minor difference in mean residue ellipticity at 222 nm [θ₂₂₂], which is suggestive of minor difference in the helical content between MtFtsZ-Stop and ΔC2-Stop [Supplementary Fig. S3(B)]. However, the helical content of ΔC2 was found to be lower than that of MtFtsZ at pH 7.2 [Supplementary Fig. S3(C)]. This was indicative of secondary structural changes in ΔC2, which were pronounced at pH 7.2. The deletion of Arg379 and Arg378 did not affect GTPase activity of ΔC2-Stop in comparison with that of MtFtsZ-Stop. The specific activity of GTPase of ΔC2-Stop was almost comparable to that of MtFtsZ-Stop at pH 7.7. The specific activity values of GTPase of MtFtsZ-Stop at pH 6.5 and 7.7 were also comparable to the values reported by others [14,20,36] (Supplementary Table S3).

Structure model of MtFtsZ C-terminal tail segment

The crystal structures of MtFtsZ available in PDB (1RLU, 1RQ2, 1RQ7, 1Q1X, and 1Q1Y) all exhibit ordered structures only for residues 1–312 of the 379 residues in the polypeptide chain but do not contain coordinates for the 67 residues in the C-terminal end (the latter referred to as the ‘tail’ region hereafter). In order to find out the manner in which the C-terminal Arg residues influence filament formation, it was necessary to model the tail region in the context of MtFtsZ dimer on tubulin dimer and examine the placement of the Arg residues. It is well known that FtsZ structures (including MtFtsZ structure [8]) exhibit a high degree of structural similarity to tubulin [37]. Both α and β subunits of tubulin contained a region in their C-terminal ends that align well with the tail region of MtFtsZ. The coordinates of 2BTQ (Prosthetobacter dejongei) tubulin dimer was used for this purpose. A structure-based alignment obtained with DALI for the α subunits of tubulin, along with the crystal structure of MtFtsZ (Supplementary Fig. S4), was used as the seed to align the entire sequence of MtFtsZ. Residues 377–443 of tubulin from 2BTQ was used as the template to model the tail region of MtFtsZ. RMS deviation of the final modeled structure with that of tubulin is 2.7 Å, whereas that with residues 1–312 of MtFtsZ (1RLU) is 0.9 Å. The tail region contains four α-helices connected by loop regions, the entire region packing well against the rest of the MtFtsZ molecule [Fig. 8(A)]. A secondary structure prediction carried out by 11 different methods through the NPSA server at http://npsa-pbil.ibcp.fr/cgi-bin/, though not conclusive by itself, indicates a significant propensity for the formation of at least one to two helices in this region. A non-redundant set of 16 tubulin

Figure 7 Polymerization assay using 90° LS for MtFtsZ (closed diamond) and ΔC1 substitution mutants MtFtsZ-ΔC1(K) (open square), MtFtsZ-ΔC1(H) (×), MtFtsZ-ΔC1(A) (+), and MtFtsZ-ΔC1(D) (closed square) at 8.6 μM each
structures from different sources including that of 2BTQ, when superposed indicates that the C-terminal region in all these molecules adopt the same four helical architectures and also superpose well with each other (Supplementary Fig. S5). This indicates that structural conservation in this region is very high, increasing the confidence in our choice of using one of them as the template in modeling MtFtsZ tail region.

The subunit thus built was then used to build two types of protein–protein associations: (i) a dimeric or polymeric stretch based on tubulin 2BTQ/3DU7 structures [Fig. 8(B)] and (ii) a dimer based on the arrangement seen in 1RLU, 1RQ2, and other MtFtsZ crystal structures [Fig. 8(C)], respectively. The two types of arrangements will be referred to as longitudinal and horizontal associations, respectively, hereafter. In the horizontal association, the modeled ‘tail’ regions lie on the opposite surfaces of the subunits and do not exhibit any possibility of interaction with each other. In the longitudinal association, on the other hand, the tail regions of the two subunits in the filament interact with each other (through residues Asp370 and Arg331 that form a salt bridge, residues Asp210, Asn288, and Leu8 forming hydrogen bonds with residues Arg139, Arg140, and Gln45, respectively, of the other subunit), significantly strengthening the filament [Fig. 8(D)]. Incidentally, Arg139 and Arg140 are conserved across many bacterial genera, whereas Leu8, Gln45,
Asp210, and Asn288 are conserved in mycobacterial species. Moreover, it has been proposed that repositioning of the absolutely conserved Arg140 (and helix H8), to bring Arg140 closer to the γ phosphate of GTP, is required to achieve a fully polymerization-competent conformation [8]. Besides these features, the tail region is also in close proximity to the T7 loop and helix H11 immediately succeeding to it, of the neighboring subunit (residues 201–207 making up T7 loop and 208–215 constituting H11 helix), indicating a possibility of the influence the tail region can have on filament formation.

Discussion

All mycobacterial FtsZ proteins sequenced so far possess either Arg-Arg or Arg-His at the C-terminus (Supplementary Fig. S6). Like the positively charged C-terminal Arg residue in MtFtsZ, *Streptomyces coelicolor*, a member of *Actinomycetes* family to which mycobacteria belong, also possesses a positively charged residue, Lys, as the C-terminal extreme residue in its FtsZ (ScFtsZ). On the contrary, the nature of the extreme C-terminal residue shows divergence in EcFtsZ and BsFtsZ. Whereas chargeless Gly is present in BsFtsZ, negatively charged Asp residue is present in EcFtsZ (Supplementary Fig. S6). These changes are probably commensurate with the large sequence divergence at other parts of EcFtsZ and BsFtsZ, in comparison to mycobacterial FtsZ. However, replacement of Arg378 in DDDDVDPFPMR in ΔC1 with another positively charged residue Lys (ΔC1K) as in ScFtsZ, or with a negatively charged residue Asp (ΔC1D) as in EcFtsZ, or with a chargeless residue Ala (ΔC1A) similar to that in BsFtsZ, or with a neutral charge residue His (ΔC1H) (Supplementary Fig. S6) did not restore polymerization capability of MtFtsZ. Although deletion of even two residues from C-terminus abolished filament formation by MtFtsZ, an entire stretch of 63 residues in EcFtsZ [6,7], the C-terminal 16 residues in BsFtsZ [38], and 76 C-terminal residues in PaFtsZ [39] were dispensable for polymerization activity. The mycobacterial Arg 378 residue shows conservation in BsFtsZ (R in ...TFLRNRRN....) and EcFtsZ (R in ...AFLRKKQA....) (Supplementary Fig. S6). However, the stretch of C-terminal 16 residues of BsFtsZ [38] and 63 residues of EcFtsZ [6], which contain the conserved Arg residue, were shown to be redundant for polymerization. Thus, the requirements for the presence of C-terminal residues and particularly for an Arg residue as the C-terminal end residue for polymerization are deviations found for MtFtsZ from those of EcFtsZ, BsFtsZ, and PaFtsZ. This deviation might probably be due to the divergence in the primary structure, influencing the overall structure of MtFtsZ [8], besides the uniqueness of the C-terminal conserved stretch of residues (Supplementary Fig. S6).

The fact that identically prepared proteins, full-length MtFtsZ, and MtFtsZ-Stop formed single-stranded filaments, but ΔC2 and ΔC2-Stop did not form even single-stranded filaments at pH 7.7 or double-stranded filaments in the presence of 10 mM CaCl2 at pH 7.7, demonstrates that the polymerization incapability found in ΔC2 and ΔC2-Stop is intrinsic to the structural feature of the mutant proteins and is not due to any purification artifact. We did not attempt overexpression of these deletion mutants in a mycobacterial host, as our previous study has shown that in vitro non-polymerization status of mycobacterial FtsZ cannot be reverted to a polymerization-capable status by changing the expression host from *E. coli* to *M. smegmatis* [12]. The changes in the secondary and tertiary structures of the protein, which were brought about by the absence of an Arg residue as the extreme C-terminal residue, when compared with that in full-length MtFtsZ, probably might have prevented growth of oligomers into filaments. This possibility is supported by the experimental observation that ΔC2/ΔC2-Stop retained GTPase activity (and by the model as well—see below), and the GTPase activity might be due to the formation of oligomers, as FtsZ oligomers are known to elicit GTPase activity [35]. The modeled structure of the C-terminal tail region suggests that deletion of the two Arg residues at the C-terminus could result in the destabilization of the helical tail segment leading to disorder in the region. The CD profile, in which ΔC2 shows some loss of helical content at pH 7.2, and to a far lesser extent at pH 7.7, also supports this possibility.

Subunit structure with the helices in the tail region seems fairly confident given the conservation of the structure among all tubulin subunits and the close evolutionary relationship that FtsZ shares with tubulin. Modeling two possibilities of the filament indicates that the tail region of the whole FtsZ molecule, though compatible with both types of associations, makes a significant contribution to the stabilization of the filament in the longitudinal association model, wherein the modeling of the tail region was based on tubulin C-terminal end as the template (Fig. 8(A)). Several hydrogen bonds formed between the residues on the interface of the subunits illustrates this aspect (Fig. 8(D)). In the longitudinal association model, the orientation of the subunits of FtsZ has been assumed to be similar to that in tubulin. The possibility of minor changes in the precise orientation between MtFtsZ subunits cannot be ruled out, in which case differences in terms of details of the interactions involved at the interface can be anticipated. Nevertheless, the model of the filament reported here serves as a framework to understand the gross mode of filament formation and the role of the C-terminal tail region in the process. The model in fact is...
consistent with our experimental studies reported here, which indicate that deletion of amino acid residues from the C-terminal end segment leads to failure of polymerization of the molecule. Nucleotide binding does not appear to get significantly perturbed upon deletion of the segment, an observation that can also be explained by the model, which clearly shows that the tail region and the nucleotide binding sites are spatially distant from each other and hence the nucleotide binding can be independent of the tail region. This prediction based on the model is supported by the observation that ΔC2/ΔC2-Stop proteins retain GTPase activity, at levels that are comparable to that of wild-type protein.

The conformation of the fourth short helix seen in tubulin happens to be interrupted in the MtFtsZ model due to two consecutive prolines. The last four C-terminal residues adopt a helical turn, the stretch packing against the preceding helix through several Van der Waals interactions. Further, mutation of the penultimate Arg to Lys was also found to result in the loss of polymerization. It is possible that such a mutation can change the propensity of the segment to adopt a helical conformation, leading to disorder in the region, which in turn may destabilize the filaments. Since Arg378 and Arg379 residues are found to be flexible in the model, we cannot comment on their exact position in the model in terms of their possible contact residues, in order to determine their direct role, if any, other than stabilization of the helices in the tail region, in polymerization. Detailed mutagenesis studies on the residues in the tail region and on the residues on the subunit interface in the model might give more insights into the identification and role of other residues in MtFtsZ polymerization.

Supplementary Data
Supplementary data are available at ABBS online.

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