Crystal structures of the cell-division protein FtsZ from *Klebsiella pneumoniae* and *Escherichia coli*

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FtsZ, a tubulin-like GTPase, is essential for bacterial cell division. In the presence of GTP, FtsZ polymerizes into filamentous structures, which are key to generating force in cell division. However, the structural basis for the molecular mechanism underlying FtsZ function remains to be elucidated. In this study, crystal structures of the enzymatic domains of FtsZ from *Klebsiella pneumoniae* (KpFtsZ) and *Escherichia coli* (EcFtsZ) were determined at 1.75 and 2.50 Å resolution, respectively. Both FtsZs form straight protofilaments in the crystals, and the two structures adopted relaxed (R) conformations. The T3 loop, which is involved in GTP/GDP binding and FtsZ assembly/disassembly, adopted a unique open conformation in KpFtsZ, while the T3 loop of EcFtsZ was partially disordered. The crystal structure of EcFtsZ can explain the results from previous functional analyses using EcFtsZ mutants.

1. Introduction

FtsZ, a widely conserved tubulin-like GTPase, is a key player in bacterial cell division (Adams & Errington, 2009). In the presence of GTP, FtsZ polymerizes into filamentous structures called protofilaments, which further gather to form a ring-shaped structure (Z-ring) in cells (Bi & Lutkenhaus, 1991; Bramhill & Thompson, 1994). The Z-ring locates at the middle of the cell and constricts during cell division (Bi & Lutkenhaus, 1991). This constriction had been considered to provide the force for dividing the cell to generate two daughter cells, but recent studies have shown that cell-wall synthesis is a more plausible candidate to produce this force, which is regulated by FtsZ treadmilling coupled with its GTPase activity (Bisson-Filho *et al.*, 2017; Yang *et al.*, 2017). FtsZ has also been thought to be an attractive target for drug development because of its essentiality for cell viability and the lack of a functional homologue of FtsZ in humans (Fujita, Maeda *et al.*, 2017; Kaul *et al.*, 2015, 2016; Tan *et al.*, 2012).

To elucidate the structural basis for the molecular mechanism underlying FtsZ function, a number of FtsZ crystal structures from bacteria such as *Methanocaldococcus jannaschii* (Löwe & Amos, 1998), *Mycobacterium tuberculosis* (Leung *et al.*, 2004; Li *et al.*, 2013), *Thermotoga maritima* (Oliva *et al.*, 2004), *Thermobifida fusca* (PDB entry 4e6e; Joint Center for Structural Genomics, unpublished work), *Pseudomonas aeruginosa* (Oliva *et al.*, 2007), *Bacillus subtilis* (Oliva *et al.*, 2007), *Aquifex aeolicus* (Oliva *et al.*, 2007), *Staphylococcus epidermidis* (PDB entry 4m8i; Center for Structural Genomics of Infectious Diseases, unpublished work) and *Staphylococcus aureus* (Matsui *et al.*, 2014; Fujita, Harada *et al.*, 2017) have been determined. Most of the crystal structures of FtsZ represent a similar relaxed (R) conformation; the N- and

C-terminal domains are located close to each other. On the other hand, FtsZ from *S. aureus* (SaFtsZ) and FtsZ from *S. epidermidis* show a tense (T) conformation in which a cleft is formed between the N- and C-terminal domains, and the cleft can accommodate FtsZ inhibitors (Matsui *et al.*, 2012; Tan *et al.*, 2012; Fujita, Maeda *et al.*, 2017). Subsequently, crystal structures of SaFtsZ were determined in both the T and R conformations, both of which were GDP-bound (Fujita, Harada *et al.*, 2017). Thus, these structural analyses showed that the T/R conformation is not dependent on whether GTP or GDP is bound. Although the two conformations of FtsZ must be significant for its assembly/disassembly and treadmilling, it is still not clear whether these conformational changes are regulated by the nucleotide (GTP or GDP) state (Mukherjee & Lutkenhaus, 1994; Lu *et al.*, 2000), interactions between monomers (Wagstaff *et al.*, 2017) or other factors.

Although most functional studies on FtsZ have been carried out by observing the phenotypes of *Escherichia coli* cells expressing FtsZ mutants (Adams & Errington, 2009; Stricker & Erickson, 2003; Addinall *et al.*, 2005; Ma & Margolin, 1999), no structural information is available for *E. coli* FtsZ (EcFtsZ). Therefore, the results from these studies have been discussed using homology models generated from other FtsZ structures. *Klebsiella pneumoniae* is a Gram-negative enteric bacterium and a common human pathogen that is closely related to *E. coli* (98.7% sequence identity for *E. coli* and *K. pneumoniae* FtsZ). FtsZ from *K. pneumoniae* (KpFtsZ) is also an attractive target for drug development. Thus, the crystal structures of EcFtsZ and KpFtsZ are of value for the design of antibacterial agents as well as for elucidating their molecular mechanism.

Here, we report the crystal structures of the enzymatic domains of KpFtsZ (residues 11–316) and EcFtsZ (residues 11–316) at 1.75 and 2.50 Å resolution, respectively. Both FtsZs formed straight protofilaments in their crystals, and the two structures adopted R conformations. The T3 loop, which is involved in GTP/GDP binding and FtsZ assembly/disassembly, adopted a unique open conformation in KpFtsZ, while the T3 loop of EcFtsZ was partially disordered. The crystal structure of EcFtsZ can explain the results of previous functional analyses using EcFtsZ mutants. Thus, this study provides a structural basis for EcFtsZ, the structure of which has been unknown for many decades, and also sheds light on the role of the T3 loop in a structural assembly–disassembly cycle of FtsZ.

2. Materials and methods

2.1. Cloning, protein overexpression and purification

A *K. pneumoniae* FtsZ (residues 11–316)-coding gene codon-optimized for *E. coli* was synthesized (Integrated DNA Technologies) and subcloned into pColdI-TEV vector (pColdI with a TEV cleavage site). The EcFtsZ (residues 11–316) expression vector was generated by site-directed mutation of the pColdI-TEV-KpFtsZ vector. The *E. coli* JM109 strain was transformed with the plasmids and cultured in LB medium. The expression of His-tagged proteins was induced with

0.5 mM IPTG for 24 h at 288 K. The cells were harvested, resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl) and disrupted by sonication. The supernatants after centrifugation (20 000 rev min^{−1}, 45 min, 277 K) were loaded onto a 5 ml HisTrap HP column (GE Healthcare). The proteins were eluted using a 100–220 mM imidazole gradient. Fractions containing the desired protein were dialyzed against dialysis buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl) for 3 h at 277 K. TEV protease digestion to remove the His tag was performed by the addition of 1 mg His-tagged TEV protease and 1 mM DTT and standing overnight at 277 K. The mixture was loaded onto the HisTrap HP column again, and the flowthrough fraction was collected and diluted tenfold with dialysis buffer. The dilution was loaded onto a 5 ml HiTrap Q HP column (GE Healthcare) and the protein was eluted using a 150–550 mM NaCl gradient. The FtsZ fractions were further purified using a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare) equilibrated with gel-filtration buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl). The purity of the protein was confirmed by SDS–PAGE. The purified protein was concentrated to 10 mg ml^{−1} with Vivaspin 20 (10K molecular-weight cutoff; GE Healthcare), flash-frozen in liquid nitrogen and immediately stored at 193 K.

2.2. Crystallization, data collection and structure determination

KpFtsZ (residues 11–316) crystals were obtained by hanging-drop vapour diffusion at 293 K (1 µl protein solution + 1 µl reservoir solution) with reservoir solution consisting of 0.1 M PCB Buffer (sodium propionate, sodium cacodylate and bis-Tris propane in a 2:1:2 molar ratio) pH 4.5, 25% PEG 1500. Crystals of EcFtsZ (residues 11–316) were obtained by hanging-drop vapour diffusion at 293 K (1 µl protein solution + 1 µl reservoir solution) with reservoir solution consisting of 0.1 M PCB Buffer pH 5.0, 25% PEG 1500. Crystallization information is given in Table 1.

2.3. Data collection and processing

Crystals were flash-cooled in a stream of nitrogen at 100 K without cryoprotectants after mounting in a loop. X-ray diffraction data were collected at a wavelength of 1.0 Å on the micro-focus beamline BL32XU at SPring-8, Hyogo, Japan using an EIGER X 9M detector (Dectris). The data sets for both KpFtsZ and EcFtsZ were collected using a 0.1 s exposure per oscillation in 10° wedges from each crystal at a dose of 10 MGy. Because the sizes of most of the KpFtsZ and EcFtsZ crystals were less than 10 µm, 38 data sets for KpFtsZ and 70 data sets for EcFtsZ were collected using ZOO (Hirata *et al.*, 2019), which is an automated data-collection system developed at SPring-8. All of the data sets were merged, integrated and scaled using the KAMO system (Yamashita *et al.*, 2018) which runs BLEND (Foadi *et al.*, 2013), XDS and XSCALE (Kabsch, 2010) automatically. The KAMO system enables high-throughput data processing of multiple small-wedge data sets from microcrystals by clustering and outlier rejection. Data-collection and processing statistics are shown in Table 2.

Table 1

Crystallization information for KpFtsZ and EcFtsZ.

Protein	KpFtsZ (residues 11–316)	EcFtsZ (residues 11–316)
Method	Hanging-drop vapour diffusion	Hanging-drop vapour diffusion
Plate type	24-well plates	24-well plates
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	10	10
Buffer composition of protein solution	20 mM Tris–HCl pH 7.5, 150 mM NaCl	20 mM Tris–HCl pH 7.5, 150 mM NaCl
Composition of reservoir solution	0.1 M PCB Buffer pH 4.5, 25% PEG 1500	0.1 M PCB Buffer pH 5.0, 25% PEG 1500
Volume and ratio of drop	2 µl; 1:1 ratio	2 µl; 1:1 ratio
Volume of reservoir (µl)	200	200

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

	KpFtsZ (residues 11–316)	EcFtsZ (residues 11–316)
Diffraction source	BL32XU, SPring-8	BL32XU, SPring-8
Wavelength (Å)	1.0	1.0
Temperature (K)	100	100
Detector	EIGER X 9M	EIGER X 9M
Crystal-to-detector distance (mm)	120	180
Rotation range per image (°)	0.1	0.1
Total rotation range (°)	380	700
Exposure time per image (s)	0.1	0.1
Space group	<i>P</i> 1	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	37.12, 40.93, 44.20	37.12, 40.93, 44.20
α , β , γ (°)	89.22, 72.86, 73.44	89.22, 72.86, 73.44
Resolution range (Å)	42.1–1.75 (1.81–1.75)	50.0–2.50 (2.56–2.50)
Total No. of reflections	76494 (7633)	54805 (5618)
No. of unique reflections	22348 (1826)	8681 (869)
Completeness (%)	93.2 (93.7)	99.6 (99.8)
Multiplicity	3.4	6.3
$\langle I/\sigma(I) \rangle$	5.41 (2.11)	5.64 (2.00)
<i>R</i> _{int} (%)	19.8 (60.1)	22.7 (48.1)

Table 3

Structure solution and refinement.

Values in parentheses are for the outer shell.

	KpFtsZ (residues 11–316)	EcFtsZ (residues 11–316)
Resolution range (Å)	42.11–1.75 (1.81–1.75)	42.71–2.50 (2.59–2.50)
Completeness (%)	93.2 (93.7)	99.8 (99.8)
No. of reflections, working set	22343 (2258)	8678 (869)
No. of reflections, test set	1117 (113)	443 (43)
Final <i>R</i> _{cryst}	0.179 (0.220)	0.183 (0.225)
Final <i>R</i> _{free}	0.221 (0.260)	0.240 (0.273)
No. of non-H atoms		
Total	2416	2206
Protein	2235	2167
Ligand	34	28
Water	147	11
R.m.s. deviations		
Bonds (Å)	0.007	0.008
Angles (°)	0.95	1.01
Average <i>B</i> factors (Å ²)		
Overall	18.02	44.21
Protein	17.76	44.37
Ramachandran plot		
Most favoured (%)	98.03	97.31
Allowed (%)	1.64	2.36
Outliers (%)	0.33	0.33

2.4. Structure solution and refinement

The structure of KpFtsZ (residues 11–316) was determined by molecular replacement with *Phaser* using the structure of SaFtsZ (residues 12–316; chain *B* of PDB entry 5h5g; Fujita, Harada *et al.*, 2017) as a search model. The structure of EcFtsZ (residues 11–316) was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the structure of KpFtsZ as a search model. Several rounds of refinement were performed using *REFMAC5* (Murshudov *et al.*, 2011) and *phenix.refine* (Adams *et al.*, 2010) with manual model building using *Coot* (Emsley *et al.*, 2010). The final models of KpFtsZ and EcFtsZ showed excellent stereochemical parameters based on *MolProbity* (Chen *et al.*, 2010). All statistics are summarized in Table 3. Illustrations were prepared with *PyMOL* (<https://pymol.org/2/>) and *ESPrpt* (Robert & Gouet, 2014).

3. Results and discussion

3.1. Overall structures of KpFtsZ and EcFtsZ

The enzymatic domains of KpFtsZ (residues 11–316) and EcFtsZ (residues 11–316) were purified from an *E. coli*-based expression system, and the purified proteins were crystallized by hanging-drop vapour diffusion at 293 K. Their structures

were solved by molecular replacement using the structure of an R form of *S. aureus* FtsZ (chain *B* of PDB entry 5h5g) as a search model (Fujita, Harada *et al.*, 2017). The structures of KpFtsZ and EcFtsZ were determined at resolutions of 1.75 and 2.50 Å, respectively (Figs. 1*a* and 1*b* and Table 3). Their crystals belonged to space group *P*1, with one monomer in the asymmetric unit. The unit-cell parameters and crystal packing of KpFtsZ and EcFtsZ are mostly the same. In these crystals, the FtsZ molecules form a straight protofilament, and the repeat distances between monomers in the KpFtsZ and EcFtsZ crystals are 46.8 and 47.3 Å, respectively (Figs. 1*c* and 1*d*). The areas of interaction are 674 and 522 Å² in the KpFtsZ and EcFtsZ protofilaments (Supplementary Fig. S1), respectively, while they are 1168 and 798 Å² in the T and R conformations of SaFtsZ, respectively (Fujita, Harada *et al.*, 2017). These observations indicate that KpFtsZ and EcFtsZ form rather loose protofilaments in their crystals.

The structures of KpFtsZ and EcFtsZ share a canonical tubulin fold bound by a GDP molecule (Figs. 1*a* and 1*b*). Because we did not add GDP in the purification and crystallization steps, GDP must be provided from cellular pools in *E. coli* during the cultivation step. The final model of KpFtsZ

includes residues 11–316 (no residues are disordered), one GDP, one glycerol and 147 solvent molecules, while that of EcFtsZ includes residues 11–316 (residues 63–67 are disordered), one GDP and 11 solvent molecules (Table 3).

Because only two amino-acid differences are observed between the enzymatic domains in the KpFtsZ and EcFtsZ sequences (Fig. 2), the structures of KpFtsZ and EcFtsZ are very similar. Superimposition of the KpFtsZ and EcFtsZ molecules yielded a small root-mean-square deviation (r.m.s.d.) value of 0.519 Å for 277 C α atoms. The structures of KpFtsZ and EcFtsZ show the R (or closed) conformations, most similar to the FtsZs from *P. aeruginosa* (chain B of PDB entry 2vaw; r.m.s.d.s of 0.82 and 0.514 Å for 272 and 267 C α atoms, respectively; Supplementary Fig. S2; Oliva *et al.*, 2007) and *M. tuberculosis* (chain B from PDB entry 4kwe; r.m.s.d.s of 1.15 and 0.82 Å for 273 and 267 C α atoms, respectively; Li *et al.*, 2013), compared with the other FtsZ coordinates deposited in the Protein Data Bank.

Glu86 and Asp93 in KpFtsZ are substituted by Asp86 and Glu93 in EcFtsZ, respectively (Figs. 1c, 1d and 2). In the KpFtsZ crystal, the side chain of Glu86 forms a hydrogen bond to Asn303 from another FtsZ molecule in the neighbouring protofilament (Fig. 1c), but the side chain of Asp86 in EcFtsZ does not because of its shorter side chain (Fig. 1d). However, we cannot conclude that the inter-protofilament (lateral) interaction represents a real interaction *in vivo*. Because the hydrogen bond in the KpFtsZ crystals has almost no neighbouring amino acids in contact, the interaction would have minimal buried surface area.

3.2. Structural comparison of the T3 loop with other FtsZs

In *P. aeruginosa* FtsZ (PDB entry 2vaw), the T3 loop (residues 60–73 connecting β 3 and α 3; Fig. 2) in the R conformation interacts weakly with the β -phosphate of GDP (Fig. 3a; Oliva *et al.*, 2007), but stronger interactions would be observed between the γ -phosphate of GTP and the T3 loop

Figure 1
(a) Overall structure of KpFtsZ (residues 11–316). (b) Overall structure of EcFtsZ (residues 11–316). (c) Protofilaments of KpFtsZ in the crystal shown in light green, green and dark green. The neighbouring protofilament is shown in grey. Glu86, Asp93 and Asn303 are shown as stick models. The close-up view shows the interaction between Glu86 and Asn303, with a distance of 3.3 Å. (d) Protofilaments of EcFtsZ in the crystal shown in light cyan, cyan and dark cyan. The neighbouring protofilament is shown in grey. Asp86, Glu93 and Asn303 are shown as stick models. The close-up view shows the same area as shown in (c).

Figure 2
Multiple sequence alignment of FtsZ across species: *K. pneumoniae* FtsZ (KpFtsZ; UniProt ID A6T4N8), *E. coli* FtsZ (EcFtsZ; UniProt ID P0A9A6), *P. aeruginosa* FtsZ (PaFtsZ; UniProt ID P47204), *M. tuberculosis* FtsZ (MtbFtsZ; UniProt ID P9WN95), *B. subtilis* FtsZ (BsFtsZ; UniProt ID P17865), *S. aureus* FtsZ (SaFtsZ; UniProt ID Q6GHP9) and *S. epidermidis* FtsZ (SeFtsZ; UniProt ID Q8CPK4). Secondary-structural elements of KpFtsZ and EcFtsZ are indicated above the sequences. The circles indicate the positions of the differing amino acids between the enzymatic domains of KpFtsZ and EcFtsZ.

when GTP is modelled. On the other hand, the T3 loop in the open conformation interacts with another FtsZ molecule within a protofilament (which we call a 'longitudinal' interaction) in the *M. tuberculosis* FtsZ structure (chain A of PDB entry 4kwe; Li *et al.*, 2013). Thus, the T3 loop is responsible for GTP/GDP binding and assembly–disassembly regulation of FtsZ. In KpFtsZ the T3 loop adopts a different open conformation, while the middle part (residues 63–67) of the T3 loop was disordered in EcFtsZ (Fig. 3*a*). The T3 loops of KpFtsZ and EcFtsZ shows higher temperature factors (38.3 and

57.9 Å² for the T3 loop, and 17.8 and 44.4 Å² for the whole KpFtsZ and EcFtsZ chains on average, respectively), but weak electron density for this region is still traceable in KpFtsZ (Fig. 3*b*). In KpFtsZ, the side chain of Leu68 on the T3 loop makes hydrophobic contacts with Asp96 and Phe210 in the upper molecule within the same protofilament (longitudinal interactions), and Ile64 interacts with Glu237, Thr281 and Phe285 of the molecule of the neighbouring protofilament (lateral interactions). To our knowledge, such lateral interactions have never been observed in previous structural analyses of FtsZ. These observations suggest that the conformation of the T3 loop is regulated not only by binding of the nucleotide (GTP or GDP) but also by longitudinal and lateral interactions. The distinct open conformations of the T3 loop observed in KpFtsZ are likely to represent distinct states of FtsZ protofilaments during the assembly–disassembly cycle.

3.3. Structure–function relationship of EcFtsZ

The crystal structure of EcFtsZ can explain the results of some previous functional analyses using EcFtsZ mutants. For example, no Z-ring was observed in *E. coli* cells on the replacement of Asp96 by Ala in a previous functional analysis (Stricker & Erickson, 2003). In the EcFtsZ structure, the side chain of Asp96 makes van der Waals contact with Leu68 on the T3 loop (Fig. 4*a*), and a similar interaction was observed in KpFtsZ. As described, the T3 loop is involved in GTP/GDP binding and assembly–disassembly of FtsZ. As it can be assumed that the substitution of Asp96 by Ala weakens this van der Waals contact and increases the flexibility of the T3

Figure 3
(*a*) Superimposed T3 loops from KpFtsZ (green), EcFtsZ (cyan), PaFtsZ (magenta) and MtbFtsZ (yellow). (*b*) $F_o - F_c$ electron-density OMIT map (2.0σ) for the T3 loop of KpFtsZ. The T3 loop is shown as stick models. The neighbouring molecule within the same protofilament and the molecule in the neighbouring protofilament are coloured light green and grey, respectively. The residues interacting with the T3 loop are shown as stick models.

Figure 4
(*a*) Interaction between Asp96 and Leu68 in the same protofilament of EcFtsZ. (*b*) Intramolecular interaction between Arg89 and Glu93 of EcFtsZ. Glu250 and Asp251 from the neighbouring protofilament are represented.

loop, this mutation may cause severe effects on the stability of the protofilaments, resulting in the disappearance of the Z-ring.

Another example is the E93R substitution (Jaiswal *et al.*, 2010). The E93R mutant forms more stable and 15-fold thicker protofilaments than wild-type FtsZ. The GTPase activity of the E93R mutant is lower than that of wild-type FtsZ, and overexpression of the E93R mutant inhibits cell division. In the EcFtsZ structure, Glu93 electrostatically interacts with Arg89 within the molecule (Fig. 4*b*). Judging from the structure, the substitution of Glu93 by Arg will disrupt the intramolecular interaction; instead, the substitution will generate new lateral interactions between Arg93 and Glu250 or Asp251 of the neighbouring protofilament, assuming that the protofilament arrangement in the crystal is equivalent *in vivo*. Such stronger lateral interactions may cause the thicker bundle of protofilaments as observed in the previous study (Jaiswal *et al.*, 2010).

As described, KpFtsZ and EcFtsZ form rather loose protofilaments in their crystals. Recently, Wagstaff and coworkers determined the protofilament of EcFtsZ using electron cryomicroscopy (cryoEM; Wagstaff *et al.*, 2017). Note that the T conformers fitted into the cryoEM map of EcFtsZ well, rather than the R conformers. However, our EcFtsZ structure shows the R conformers aligned into a protofilament. Whereas the cryoEM experiments were conducted in the presence of 0.1 mM GMPCPP, we did not add GDP/GTP analogues to the crystallization solution. Because the experimental conditions are totally different, our EcFtsZ structure shows the R protofilaments in the crystal, differing from the previous cryoEM observation. Still, we cannot completely rule out the possibility that the structural difference in the protofilaments is a crystal artefact, because the lateral interactions support the formation of protofilaments in the crystals. On the other hand, the catalytically essential T7 loop is located close to the GTP/GDP nucleotide bound to the next subunit in the KpFtsZ and EcFtsZ crystals, similar to those of the normal longitudinal interactions in other FtsZs (for example SaFtsZ). Therefore, our EcFtsZ and KpFtsZ structures may represent a 'snapshot' of the dynamic events of the assembly/disassembly of protofilaments *in vivo*. Further biochemical and structural studies would be needed to address this issue.

In summary, the crystal structure of KpFtsZ (and probably also EcFtsZ) shows a unique open conformation of the T3 loop, and KpFtsZ and EcFtsZ form straight protofilaments in their crystals. Because the T3 loop is also involved in longitudinal and lateral interactions of protofilaments in our structures (Fig. 4*a*), the functional importance of the open/closed movement of T3 is highlighted here. Our structures can also explain the results from previous EcFtsZ mutational studies: some mutations disrupt the longitudinal and lateral interactions found in the crystals. Considering these facts, such interactions may also exist *in vivo*. We expect that the structural information that we present here will be useful not only for elucidating the molecular mechanism underlying the functions of FtsZ but also for drug design using molecular docking and molecular dynamics.

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