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The N-terminal domain of EzrA binds to the C terminus of FtsZ to inhibit *Staphylococcus aureus* FtsZ polymerization

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ABSTRACT

Bacterial cytokinesis is accompanied by a macro-molecular complex called the "divisome." The divisome consists of two major components involving positive regulators and negative regulators that regulate the polymerization of an essential cytoskeleton protein FtsZ, which plays a key role in bacterial cell division by assembling the Z-ring, and therefore has been identified as a target for antibiotics. The negative regulators prevent the Z-ring assembly by inhibiting FtsZ polymerization. In *Staphylococcus aureus*, a pandemic human pathogen, one of the negative regulators, EzrA, contains a trans-membrane anchor region at the N-terminus and has five predicted coiled-coils. Recent reports indicate that the polymerization of FtsZ can be inhibited by forming a complex with EzrA. In this study, we attempted to locate the binding site for the interaction between EzrA and FtsZ in *S. aureus* (SaEzrA and SaFtsZ, respectively), by generating various constructs of SaEzrA and SaFtsZ proteins based on limited proteolysis. Various constructs of SaEzrA and SaFtsZ proteins were expressed and homogeneously purified. A GST pull-down assay indicated that the N-terminal domain of SaEzrA interacts with the C-terminal tail of SaFtsZ, and the elongated shape of EzrA was predicted based on the Stokes radius of each construct.

1. Introduction

In bacterial cytokinesis, FtsZ, an essential cytoskeleton protein, is localized along the midline of cells, which is supported by a macromolecular complex called the "divisome" [1–3]. The assembly of the divisome is accomplished by polymerization of the tubulin homolog FtsZ into a Z-ring and the constriction of the Z-ring is responsible for dividing the parent cell into two equal daughter cells [3]. The polymerization of FtsZ serves as a scaffold for the localization of the divisome complex and provides a driving force to constrict the plasma membrane in the final stage of cytokinesis [3,4]. The Z-ring formation is modulated by the divisome involving two major components—the positive and negative regulators [3]. Positive regulators, such as SepF, promote the Z-ring formation of FtsZ [5,6]. SepF is considered to promote the polymerization of FtsZ and Z-ring formation by interacting directly with FtsZ [6,7], and consequently stabilizes the pre-formed Z-ring [5]. SepF is also known to bind to the C-terminal tail of FtsZ in *Bacillus subtilis*[5,8]. Negative regulators, such as EzrA, assist in destabilizing the Z-ring, which prevents aberrant cell division and controls cell division to maintain correct cell size [9].

FtsZ consists of two domains and a conserved GTPase domain at the N terminus, which enables polymerization on binding of guanidine tri-phosphate (GTP) to the site [10,11]. The C-terminal domain of FtsZ contains a β-sheet and a long helix (H7 helix) that penetrates between the N-terminal and C-terminal domains [10]. The polymerization of FtsZ is initiated by the binding of GTP in the presence of magnesium ions and is tightly regulated by GTP hydrolysis, which disassembles FtsZ polymers [12]. Recently, the crystal structure of FtsZ from *Staphylococcus aureus* containing a calcium ion bound to the T7 loop was reported [13], which plays an important role in regulating polymerization and GTPase activities [13].

EzrA, a negative regulator of FtsZ, consists of five predicted coiled-coil regions and one transmembrane anchor at the N terminus. It has been shown that EzrA inhibits the polymerization of FtsZ *in vitro*, which is associated with an aberrant cell division in low GC-content gram-positive bacteria [14,15]. SaEzrA is also considered to be an important component of the bacterial division complex [3] and plays multi-functional roles in the assembly of the divisome complex and peptidoglycan synthesis [16]. In the absence of SaEzrA, a significant increase in the average size of *S. aureus* cells was found, which suggests that EzrA contributes to the regulation of precise cell size in bacteria [9].

S. aureus, a pandemic human pathogen, causes staphylococcal diseases, including pneumonia, skin infections, and purulent

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disease [17]. Methicillin has been developed to treat staphylococcal disease; however, the abuse of antibiotics has led to problems of drug resistance [18–20]. FtsZ has been shown to be an attractive drug target for developing new antibiotics to treat staphylococcal diseases, as bacterial cytokinesis can be inhibited by suppressing the polymerization of FtsZ [21]. PC190723, 3-[(6-chloro[1,3]thiazolo[5,4-*b*]pyridin-2-yl)methoxy]-2,6-difluorobenzamide, was found to have a potential inhibitory activity toward methicillin-resistant *S. aureus* (MRSA) and a multi-drug-resistant *S. aureus* (MDRSA) [21]. The inhibitory mechanism of PC190723 was shown to arise from modulation of the disassembly of the FtsZ polymer [22]. The crystal structure of SaFtsZ in complex with PC190723 shows that the inhibitor binds to the cleft formed by the two domains [13]. In this study, we expressed and purified recombinant EzrA proteins from *S. aureus* (strain COL) for structural and biochemical studies. Binding studies using a GST pull-down assay were conducted to characterize the SaEzrA protein, and SaEzrA and SaFtsZ were co-expressed. It was also determined that SaEzrA forms an elongated shape, which gives a large Stokes radius value based on analytical gel filtration.

2. Materials and methods

2.1. Cloning

DNA constructs (Fig. 1A) were amplified using the polymerase chain reaction (PCR) with selected primers (Table 1) obtained from the genomic DNA of *S. aureus* (strain COL). The amplified DNAs were digested with *Bam*HI and *Xho*I restriction enzymes and introduced into the plasmids pGST-parallel2 and pHis-parallel2 [23], which had also been digested with the same restriction enzymes (Table 2). All the constructs except $SaEzrA_{164–564}$ were fused to an N-terminal GST tag followed by a tobacco etch virus (TEV) protease cleavage site (ENLYFQG). The plasmids were transformed into *Escherichia coli* BL21(DE3) star (Invitrogen) cells, and the cells harboring the plasmids were grown at 37 °C until the optical density (at 600 nm) reached 0.7–1.0 in Luria Bertani (LB) broth medium containing 100 μg/ml ampicillin. Further, 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was used to induce the cells for protein expression after which the cells were incubated for 16–18 h at 25 °C. The C-terminal of FtsZ (GST-SaFtsZ peptides) from *S. aureus* (strain COL) was cloned into pGST-parallel2 harboring residues 351–390 and 370–390. The plasmids harboring GST-SaFtsZ peptides were transformed into *E. coli* BL21(DE3) (Invitrogen) cells, and the subsequent purification protocol involved steps similar to those used for SaEzrA.

2.2. Protein expression and purification

$SaEzrA_{25–564}$ protein was isolated by harvesting the cells by centrifugation at 5000 rpm at 4 °C for 20 min, and resuspending the pellet in ice-cooled lysis buffer (20 mM Tris–HCl pH 8.0, 200 mM NaCl, and 1 mM phenylmethanesulfonylfluoride (PMSF)). The cells were lysed using a sonicator or microfluidizer (Microfluidics, USA) and the lysed cells were centrifuged at 12,000 rpm (Vision V506CA rotor) at 4 °C for 30 min to separate the supernatant and cell debris. The supernatant was applied to a glutathione-Sepharose column (GE Healthcare) pre-equilibrated with the lysis buffer. Initially, the column was washed extensively with the lysis buffer after which the protein was eluted with the elution buffer (150 mM Tris–HCl pH 8.0 and 15 mM reduced glutathione) and digested with TEV protease (1.3 mg/ml) in the presence of β-mercaptoethanol (5 mM) for 16–18 h at 4 °C. Further purification was performed by gel filtration using ÄKTA FPLC (GE Healthcare). The eluted fractions were pooled and then applied to a GST column to remove uncleaved $SaEzrA_{25–564}$ and the fusion tag was digested with TEV protease. The flow through was concentrated by ultrafiltration (Amicon) and the concentration of $SaEzrA_{25–564}$ protein was measured spectrophotometrically by recording the absorbance at

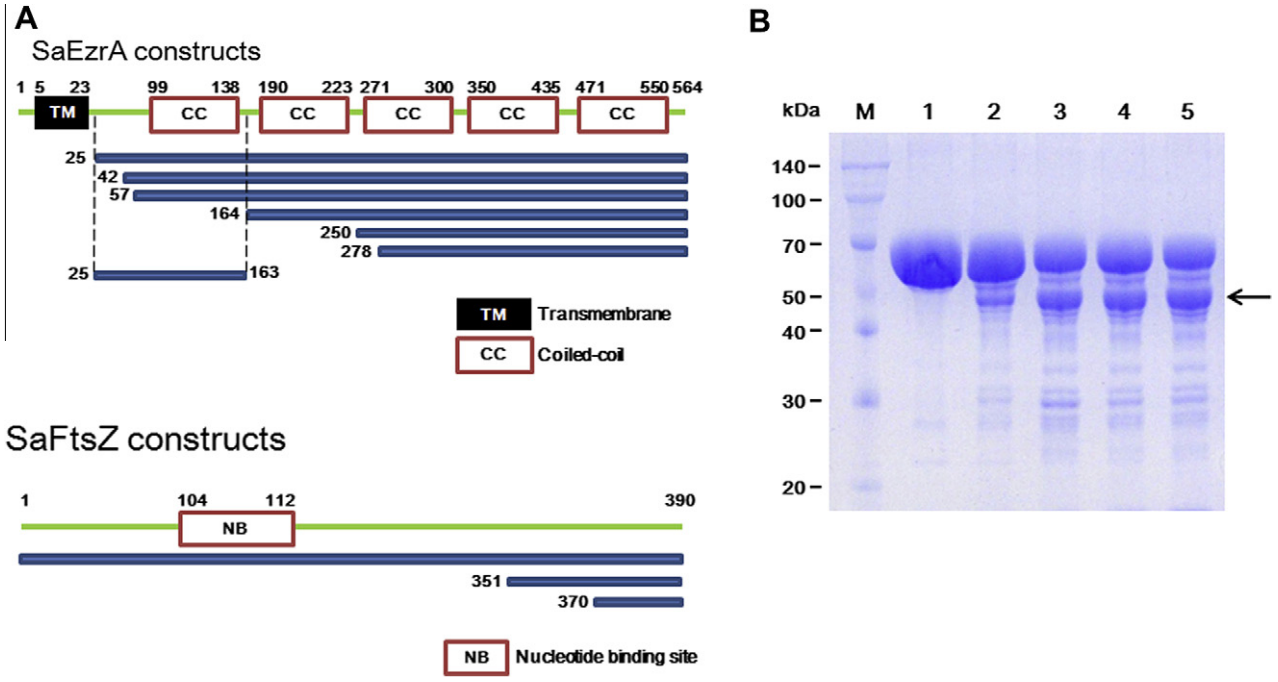


Fig. 1. The domain architecture of SaEzrA and SaFtsZ proteins and limited proteolysis of SaEzrA. (A) The constructs of full-length SaEzrA ($SaEzrA_{1–564}$) and full-length SaFtsZ ($SaFtsZ_{1–390}$) are shown in light-green. Other constructs are depicted in blue. (B) $SaEzrA_{57–564}$ was digested by proteinase K and the SDS–PAGE was analyzed by staining with Coomassie blue (lanes 1–7). The arrow indicates the predicted size of $SaEzrA_{164–564}$. M, protein marker; lane 1, no incubation with proteinase K (control); lanes 2–7, incubation for 2, 3, 5, and 10 min with proteinase K, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Primer sequence list using in this study. Primer sequences are listed from 5′ to 3′. The underlined nucleotides indicate the restriction enzyme sites; *Bam*HI (GGATCC), *Xho*I (CTCGAG).

Primer	Primer sequence (5′ → 3′)	Orientation
SaEzrA_25F	GCTATATGGATCCTCAAATAAACGACAAATTATTGAAAAAGC	Forward
SaEzrA_42F	GCTATATGGATCCGAGACGTTACCTTTTGATCAAAACCTT	Forward
SaEzrA_57F	GCTATATGGATCCAATTTAAAAGGTGAAACAAAAACGAAATAC	Forward
SaEzrA_164F	GCTATATGGATCCGGTGAGGCAGCAAGTCTACTTG	Forward
SaEzrA_250F	GCTATATGGATCCGATCACGTGAAAGTAGACAGTAC	Forward
SaEzrA_278F	GCTATATGGATCCGAAGAAGCTAATGATAAACTAGCTAATAT	Forward
SaEzrA_163R	GCTAATTCTCGAGTCAAAATTGATGACGATTTGCTAAAACATC	Reverse
SaEzrA_564R	GCTAATTCTCGAGTCATTGCTTAATAACTTCTTCTTCAATATG	Reverse
SaFtsZ_1F	GCTATATGGATCCGAAAACCTGTATTTTCAGGGCATGTTAGAATTTGAACAAGGATTTAATCATT	Forward
SaFtsZ_351F	GCTATATGGATCCAATTCATCAAATGCACAAGCAACTG	Forward
SaFtsZ_370F	GCTATATGGATCCGAAGATGATATTCCTAGCTTCATTAG	Forward
SaFtsZ_390R	GCTAATTCTCGAGTCAACGTCTTGTTCTTCTTGAACGTCTTTC	Reverse

Table 2
The list of constructs used in this study.

Host genome Gene construct	*S. aureus* (strain COL) Host vector
$SaEzrA_{25-163}$	pGST-parallel2
$SaEzrA_{25-564}$	pGST-parallel2
$SaEzrA_{42-564}$	pGST-parallel2
$SaEzrA_{57-564}$	pGST-parallel2
$SaEzrA_{250-564}$	pGST-parallel2
$SaEzrA_{278-564}$	pGST-parallel2
$SaFtsZ_{351-390}$	pGST-parallel2
$SaFtsZ_{370-390}$	pGST-parallel2
$SaEzrA_{164-564}$	pHis-parallel2
$SaFtsZ_{1-390}$	pRSFDuet-1

280 nm. The molar extinction was calculated to be 40,335 M^{-1} cm^{-1} from "ProtParam" (http://web.expasy.org/protparam/). The cloning and purification of the constructs $SaEzrA_{42-564}$, $SaEzrA_{57-564}$, $SaEzrA_{250-564}$, and $SaEzrA_{278-564}$ were performed as described above. To purify $SaEzrA_{164-564}$ protein, the cells were collected by centrifugation at 5000 rpm at 4 °C for 20 min and resuspended in ice-cooled binding buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, and 1 mM PMSF). The cells were lysed using a microfluidizer (Microfluidics, USA) and the lysed cells were centrifuged at 12,000 rpm (Vision V506CA rotor) at 4 °C for 30 min to separate the supernatant and cell debris. The supernatant was applied to a Ni-column pre-equilibrated with the binding buffer (20 mM Tris–HCl pH 7.9 and 500 mM NaCl). The column was washed extensively with the washing buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, and 60 mM imidazole) followed by step-wise elution of the $SaEzrA_{164-564}$ protein by varying the imidazole concentration between 100 and 500 mM. After removing the imidazole, the pooled soluble fractions were digested with TEV protease (1.3 mg/ml) in the presence of 5 mM β-mercaptoethanol. Further purification was performed by gel filtration using ÄKTA FPLC (GE Healthcare). Thereafter, the soluble fractions were collected and re-applied to a Ni-column to remove the uncleaved $SaEzrA_{164-564}$, which was followed by digestion of the fusion tag using TEV protease. The pure protein solution was concentrated by ultrafiltration (Amicon) and the concentration of $SaEzrA_{164-564}$ was estimated spectrophotometrically at 280 nm. All the constructs of SaEzrA proteins cleaved by the TEV protease contain a residual 5-amino acid sequence at the N terminus (GAMGS). All the protein solutions were stored as aliquots at −80 °C until further use. The purification of SaFtsZ constructs was performed in a similar manner to that described previously except without elution after GST-affinity chromatography.

2.3. Limited proteolysis

$SaEzrA_{57-564}$ protein (0.8 mg/ml) was digested with proteinase K (20 mg/ml, New England Biolabs) in 20 mM Tris–HCl (pH 8.0) containing 200 mM NaCl at a volume ratio of 4:1. The reaction mixtures were incubated in a time-dependent manner for 2, 3, 5, and 10 min. The control mixture contained no enzyme. The mixtures were treated with 5× loading buffer and boiled at 100 °C for 5 min to quench the reaction.

2.4. GST pull-down assay

GST-$SaFtsZ_{351-390}$ and GST-$SaFtsZ_{370-390}$ (GST-SaFtsZ peptides) were purified by GST affinity chromatography without elution. As a control, TEV protease (1.3 mg/ml) was added to the tube containing SaFtsZ peptides in order to remove them. SaEzrA constructs were incubated for 1 h at 4 °C with the GST-bearing resin containing GST-SaFtsZ peptides. The beads were washed thoroughly with 20 mM Tris–HCl (pH 8.0) containing 200 mM NaCl to remove unbound SaEzrA constructs and TEV protease. After extensive washing, the bound proteins were eluted with 50 mM reduced glutathione and loaded on SDS–PAGE. The gel was stained with Coomassie blue.

2.5. Co-expression and co-purification in affinity chromatography

$SaFtsZ_{1-390}$ (N-terminal poly-histidine tag) was co-transformed with $SaEzrA_{25-564}$ (N-terminal GST tag) or $SaEzrA_{25-163}$ (N-terminal GST tag) using *E. coli* BL21(DE3) star cells, and the cells were incubated at 37 °C in 1 L LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Each cell culture was incubated until an OD_{600} of 0.7–0.8 was reached and then induced with 0.5 mM IPTG. After incubating at 25 °C overnight, the cells were harvested by centrifugation at 5000 rpm at 4 °C and re-suspended in ice-cooled lysis buffer containing 1 mM PMSF. The cells were lysed using a microfluidizer (Microfluidics, USA) and spun down at 12,000 rpm at 4 °C for 30 min. The supernatants of $SaFtsZ_{1-390}$ and $SaEzrA_{25-564}$ were applied to a GST column pre-equilibrated with lysis buffer (20 mM Tris–HCl pH 8.0 and 200 mM NaCl). The column was extensively washed with the lysis buffer and the proteins were eluted with an elution buffer (150 mM Tris–HCl pH 8.0 and 15 mM reduced glutathione). The supernatants from $SaFtsZ_{1-390}$ and $SaEzrA_{25-163}$ were each loaded on a Ni-column pre-equilibrated with lysis buffer, and each Ni-column was washed with elution buffer (20 mM Tris–HCl pH 8.0, 200 mM NaCl, and 50 mM imidazole). The elution was performed stepwise using a gradient concentration of imidazole (100, 200, 300, 400, and 500 mM).

2.6. Analytical gel filtration

The purified SaEzrA constructs harboring residues 57–564, 164–564, 278–564, and 25–163 were loaded on a Superdex 200 (10/300) GL column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl pH 8.0 and 200 mM NaCl (flow rate: 0.5 ml/min). A standard curve was obtained using molecular weight markers (Sigma, MWGF200-1KT). The crystal structures (PDB IDs: 1FA2, 2HCY, 1V9E, 1HRC, respectively) of β-amylase, alcohol dehydrogenase, carbonic anhydrase, and cytochrome C were used to calculate the Stokes radii of each protein using the HYDROPRO program [24].

3. Results

3.1. The purification of recombinant SaEzrA proteins

In an effort to examine soluble variants of SaEzrA (Fig. 1A), various constructs truncating N-terminal or C-terminal regions were designed and cloned on the basis of limited proteolysis. On digesting $SaEzrA_{57-564}$ protein (0.8 mg/ml) with proteinase K (20 mg/ml, New England Biolabs) at a volume ratio of 4:1 on ice, prominent bands were observed on staining with Coomassie blue (Fig. 1B). One of the major products, approximately 50 kDa, appeared to be resistant to proteinase K (Fig. 1B). This was assumed to be the core domain of SaEzrA and was found to be similar to $SaEzrA_{164-564}$ (calculated size is 47.5 kDa) (Fig. 2D). Since C-terminal deletion constructs of *B. subtilis* EzrA (residues 25–549 and 25–531) was determined to be very unstable in our previous study (data not shown), we attempted to generate various constructs of SaEzrA at the N terminus (residues 25–564, 42–564, 57–564, 164–564, 250–564, and 278–564).

All of the constructs except $SaEzrA_{164-564}$ were fused to an N-terminal GST tag followed by a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) to facilitate the removal of the GST tag after affinity chromatography. The recombinant SaEzrA proteins were over-expressed in *E. coli* BL21(DE3) star cells in LB medium and the $SaEzrA_{25-564}$ purification was carried out using a GST resin. The TEV treatment was performed after the elution of GST affinity chromatography, so that the TEV protease has easy access to its recognition site on the SaEzrA proteins. On the basis of the SDS–PAGE results, most of the GST-fused EzrA proteins (GST-SaEzrA) were cleaved successfully by the TEV treatment (Fig. 2). $SaEzrA_{164-564}$, which contains an N-terminal polyhistidine tag, also showed an appropriate molecular weight after TEV cleavage (Fig. 2D). Following the TEV treatment, all the TEV-cleaved products were applied to a gel filtration column. Most of the SaEzrA constructs were eluted in a homogenous form in the gel filtration except $SaEzrA_{250-564}$ and $SaEzrA_{278-564}$ (Figs. 1 and 2), which showed a tendency toward partial degradation (Fig. 2E and F).

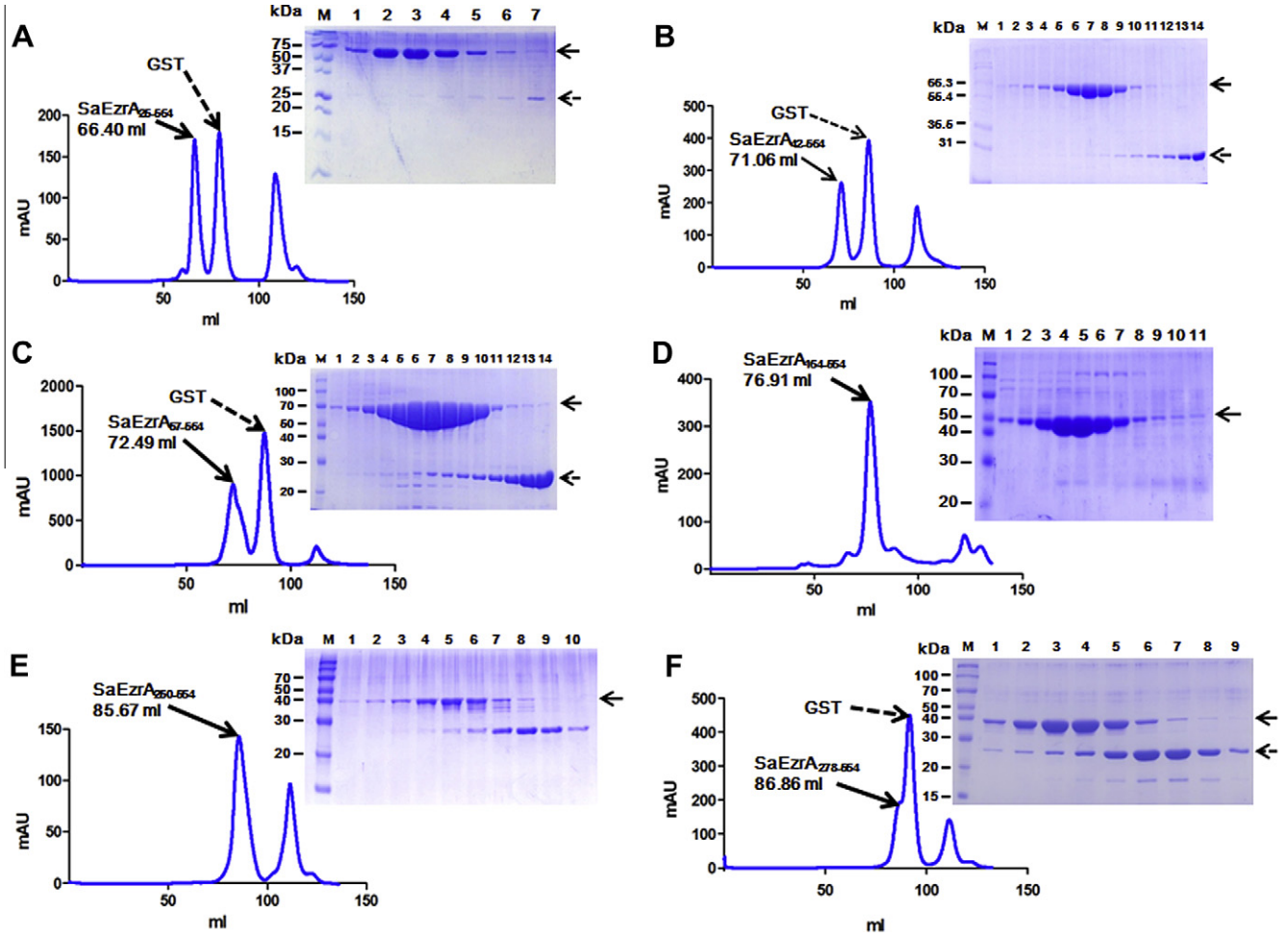


Fig. 2. The purification of recombinant SaEzrA proteins. The chromatogram of gel filtration with the corresponding SDS–PAGE placed above. All SDS–PAGE were visualized using Coomassie blue. The solid arrow indicates the position of SaEzrA constructs and the dashed arrow the GST proteins. (A) $SaEzrA_{25-564}$. (B) $SaEzrA_{42-564}$. (C) $SaEzrA_{57-564}$. (D) $SaEzrA_{164-564}$. (E) $SaEzrA_{250-564}$. (F) $SaEzrA_{278-564}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. GST pull-down assay

An earlier report concerning the interaction between FtsZ and EzrA proteins from *B. subtilis* indicated that EzrA is able to bind to the C terminus of FtsZ and that the C-terminal peptide of FtsZ can compete with the full-length FtsZ [25]. Therefore, in this study, we attempted to clarify the binding regions between FtsZ and EzrA from *S. aureus* using the GST pull-down method. GST-SaFtsZ proteins involving GST-SaFtsZ$_{351-390}$ and GST-SaFtsZ$_{370-390}$ were purified using a GST column without elution and were separated by SDS–PAGE (Fig. 3). To ensure that the SaEzrA proteins did not bind to the GST protein or resin but to the SaFtsZ peptides (residues 351–390 and 370–390) specifically, a control tube was treated with TEV protease before the binding assay to remove SaFtsZ peptides from the GST resin. Moreover, to attach SaEzrA constructs (residues 25–564, 42–564, 57–564, 164–564, 250–564, and 278–564) to the GST-SaFtsZ-bound GST resins, the SaEzrA proteins were introduced directly into the GST-SaFtsZ-bound resins and incubated for 1 h at 4 °C. As a result, SaEzrA$_{25-564}$, SaEzrA$_{42-564}$, and SaEzrA$_{57-564}$ bound tightly to the GST-SaFtsZ$_{351-390}$ and GST-SaFtsZ$_{370-390}$ but not to the TEV-treated tube (Fig. 3A and B), which indicated that the interaction between SaEzrA and SaFtsZ is specific. However, SaEzrA$_{164-564}$, SaEzrA$_{250-564}$, and SaEzrA$_{250-564}$ did not bind to GST-SaFtsZ$_{351-390}$ and GST-SaFtsZ$_{370-390}$ (Fig. 3A and B), which suggests that the N terminus of SaEzrA contributes largely to its binding to the C terminus of SaFtsZ *in vitro*.

3.3. Co-expression of EzrA and FtsZ

The possibility of co-expressing SaFtsZ and SaEzrA was investigated by co-transforming the clones of SaFtsZ and SaEzrA into *E. coli* BL21(DE3) or BL21(DE3) star cells. After several trials, involving various combinations of SaEzrA and SaFtsZ constructs, we identified the interaction between SaFtsZ$_{1-390}$ and SaEzrA$_{25-564}$ (Fig. 4A). Both SaFtsZ$_{1-390}$ and SaEzrA$_{25-564}$ proteins were expressed in a soluble form, and were co-eluted in a GST affinity chromatography fraction (Fig. 4A). Further, to clarify whether the N terminus of SaEzrA binds to the SaFtsZ, the N terminus-truncated SaEzrA$_{25-163}$ and SaFtsZ$_{1-390}$ clones were introduced into *E. coli* BL21(DE3) cells. However, upon cell lysis, SaEzrA$_{25-163}$ in complex with SaFtsZ$_{1-390}$ could not be eluted from a GST column. However, on loading the cell lysate onto a Ni–NTA column, the elution fractions contained SaFtsZ$_{1-390}$ in complex with SaEzrA$_{25-163}$ (Fig. 4B). In size-exclusion chromatography, both SaFtsZ$_{1-390}$/SaEzrA$_{25-564}$ and SaEzrA$_{25-163}$/SaFtsZ$_{1-390}$ complexes were not co-eluted, which suggests that the binding between SaEzrA and SaFtsZ is weak or transient.

3.4. Analytical gel filtration

On the basis of sequence analysis, five coiled-coil regions (residues 99–138, 190–223, 271–300, 350–435, and 471–550) of SaEzrA were predicted to form a coiled-coil connected by several gaps (30–50 amino acids each) (Fig. 1A) and several groups have established that EzrA can self-interact [14,15]. Thus, to investigate the overall shape of SaEzrA in solution, we measured the Stokes radii (R_H) of various SaEzrA constructs using analytical gel filtration calibrated with standards of structurally known marker proteins (Fig. 4C). The Stokes radius values of SaEzrA$_{57-564}$, SaEzrA$_{164-564}$, SaEzrA$_{278-564}$, and SaEzrA$_{25-163}$ were determined to be 5.78, 5.23, 4.69, and 3.74 nm, respectively (Fig. 4C). We also attempted to generate a model of SaEzrA to gain insight into the overall shape of SaEzrA. The calculated R_H values of the five constructs of SaEzrA agreed well with an elongated structure. As a reference, the overall structure of dimeric CheA from *Thermotoga maritima* (residues 225–529, 32.9 kDa; PDB code 2CH7) was compared with SaEzrA

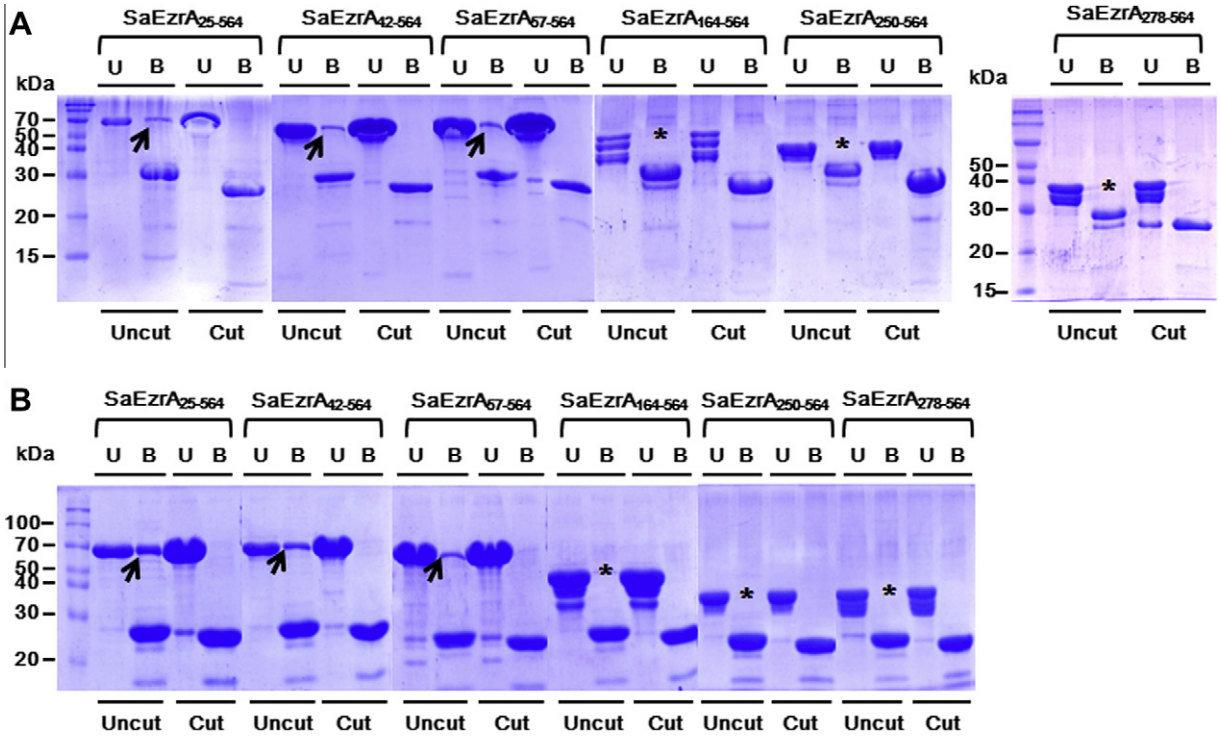
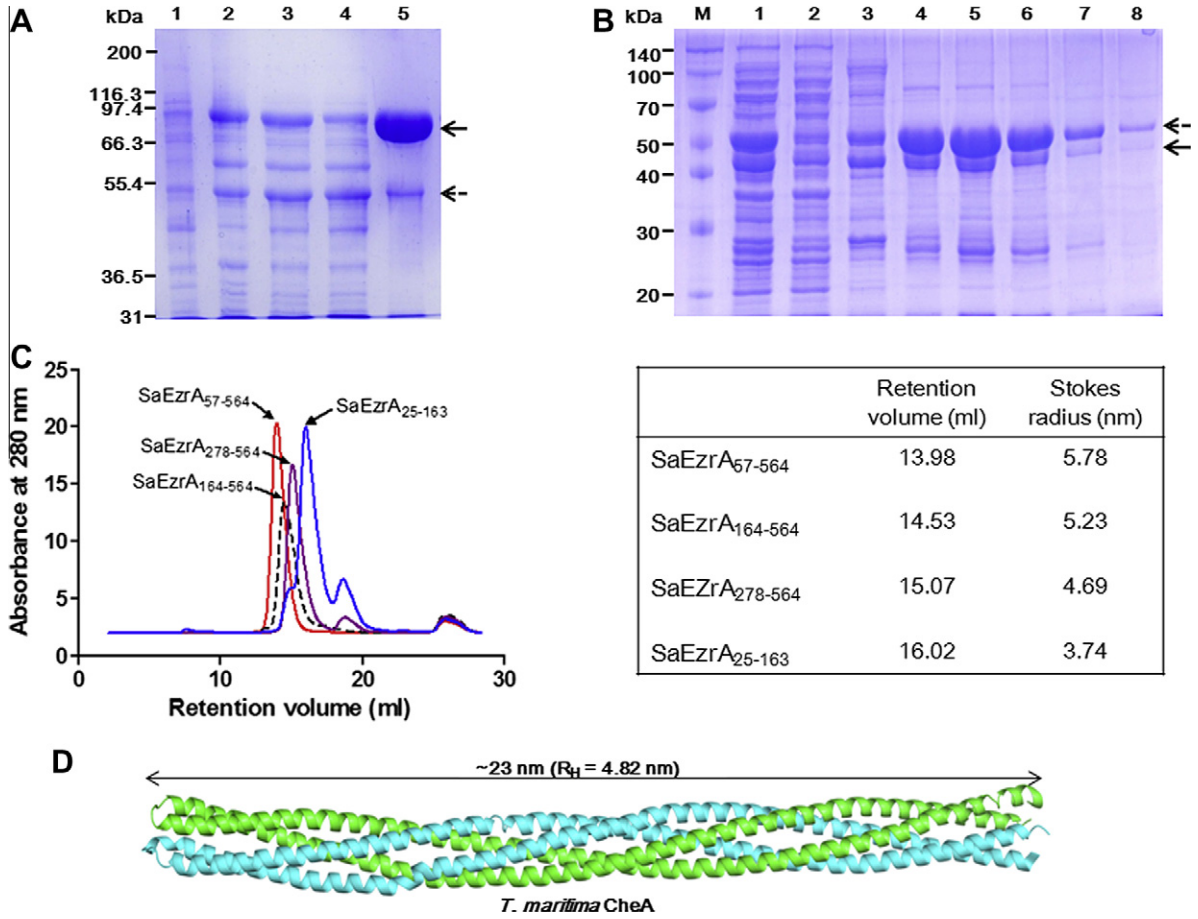


Fig. 3. GST pull-down analysis for the interaction between SaEzrA and SaFtsZ proteins (A). Each SDS–PAGE was visualized by Coomassie blue staining. Each construct is described above the relevant SDS–PAGE and solid arrows indicate the bound SaEzrA to the GST resin containing GST-SaFtsZ peptides. The expected positions of the bound SaEzrZ proteins are indicated by a star (∗). U, unbound SaEzrA constructs (supernatants from the GST pull-down assay); B, bound SaEzrA to GST-SaFtsZ peptides (elutes from the GST pull-down assay); Uncut, no TEV-treated tube (contains SaFtsZ peptides); Cut, TEV-treated tubes (no SaFtsZ peptides). (A) GST pull-down assay of SaEzrA constructs with GST-SaFtsZ$_{351-390}$. (B) GST pull-down assay of SaEzrA constructs with GST-SaFtsZ$_{370-390}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



	Retention volume (ml)	Stokes radius (nm)
$SaEzrA_{57-564}$	13.98	5.78
$SaEzrA_{164-564}$	14.53	5.23
$SaEZrA_{278-564}$	15.07	4.69
$SaEzrA_{25-163}$	16.02	3.74

Fig. 4. Co-expressed SaEzrA/SaFtsZ complexes purified by affinity chromatography and analytical gel filtration. (A) Co-expression and GST affinity chromatography of $SaFtsZ_{1-390}$ and $SaEzrA_{25-564}$. The solid arrow indicates GST-$SaEzrA_{25-564}$ and the dashed arrow, $SaFtsZ_{1-390}$. (B) Co-expression and Ni–NTA chromatography of $SaFtsZ_{1-390}$ and $SaEzrA_{25-163}$. The solid arrow indicates GST-$SaEzrA_{25-163}$ and the dashed arrow $SaFtsZ_{1-390}$. (C) Analytical chromatograms of $SaEzrA_{25-163}$, $SaEzrA_{57-564}$, $SaEzrA_{164-564}$, and $SaEzrA_{278-564}$. Retention volume and calculated Stokes radius are described. (D) The overall structure of dimeric CheA from *T. maritima* (PDB code 2CH7). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[26]. The calculated Stokes radius of the dimeric CheA was found to be 4.82 nm (PDB code 2CH7), which was similar to the experimental Stokes radius of $SaEzrA_{278-564}$ (34.3 kDa). This is due to the assumption that when the $SaEzrA_{278-564}$ construct dimerizes, an elongated coiled-coil structure similar to that of dimeric CheA is predicted (Fig. 4D).

4. Discussion

In this study, we expressed and purified various soluble constructs of SaFtsZ and SaEzrA proteins, and conducted binding assays based on GST pull-down assay and co-expression in *E. coli* cells. The binding assays indicated that the N terminus of SaEzrA binds to the C terminus of SaFtsZ. In the case of *B. subtilis*, it has been reported that EzrA has two distinct binding regions for interacting with FtsZ—one at its N terminus, which is required for the interaction with free monomeric FtsZ in monomeric or multimeric state, and the other at its C terminus, including the "QNR" patch, which is known to promote interaction with single-stranded polymers and bundled forms of FtsZ [27]. We could not rule out the possibility of the interaction of the C terminus of SaEzrA including the QNR patch with SaFtsZ, as weak interactions cannot be detected by Coomassie staining. To investigate the overall shape of SaEzrA in solution, we measured the Stokes radii of various EzrA constructs. The Stokes radii of all the measured constructs ($SaEzrA_{57-564}$, $SaEzrA_{164-564}$, $SaEzrA_{278-564}$, and $SaEzrA_{25-163}$) showed a large value, which indicated that the overall shape of SaEzrA is elongated coiled-coils if it is assumed that SaEzrA forms a dimer.

Acknowledgments

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References

[1] E. Harry, L. Monahan, L. Thompson, Bacterial cell division: the mechanism and its precision, Int. Rev. Cytol. 253 (2006) 27–94.

[2] N.W. Goehring, J. Beckwith, Diverse paths to midcell: assembly of the bacterial cell division machinery, Curr. Biol. 15 (2005) R514–R526.

[3] D.W. Adams, J. Errington, Bacterial cell division: assembly, maintenance and disassembly of the Z ring, Nat. Rev. Microbiol. 7 (2009) 642–653.

[4] S.G. Addinall, J. Lutkenhaus, FtsZ-spirals and -arcs determine the shape of the invaginating septa in some mutants of *Escherichia coli*, Mol. Microbiol. 22 (1996) 231–237.

[5] J.K. Singh, R.D. Makde, V. Kumar, D. Panda, SepF increases the assembly and bundling of FtsZ polymers and stabilizes FtsZ protofilaments by binding along its length, J. Biol. Chem. 283 (2008) 31116–31124.

[6] M.E. Gündoğdu, Y. Kawai, N. Pavlendova, N. Ogasawara, J. Errington, D.J. Scheffers, L.W. Hamoen, Large ring polymers align FtsZ polymers for normal septum formation, EMBO J. 30 (2011) 617–626.

[7] L.W. Hamoen, J.C. Meile, W. de Jong, P. Noirot, J. Errington, SepF, a novel FtsZ-interacting protein required for a late step in cell division, Mol. Microbiol. 59 (2006) 989–999.

[8] E. Król, S.P. van Kessel, L.S. van Bezouwen, N. Kumar, E.J. Boekema, D.J. Scheffers, *Bacillus subtilis* SepF binds to the C-terminus of FtsZ, PLoS One 7 (2012) e43293.
[9] A.M. Jorge, E. Hoiczyk, J.P. Gomes, M.G. Pinho, EzrA contributes to the regulation of cell size in *Staphylococcus aureus*, PLoS One 6 (2011) e27542.
[10] J. Löwe, L.A. Amos, Crystal structure of the bacterial cell-division protein FtsZ, Nature 391 (1998) 203–206.
[11] J. Löwe, Crystal structure determination of FtsZ from *Methanococcus jannaschii*, J. Struct. Biol. 124 (1998) 235–243.
[12] A. Mukherjee, J. Lutkenhaus, Dynamic assembly of FtsZ regulated by GTP hydrolysis, EMBO J. 17 (1998) 462–469.
[13] T. Matsui, J. Yamane, N. Mogi, H. Yamaguchi, H. Takemoto, M. Yao, I. Tanaka, Structural reorganization of the bacterial cell-division protein FtsZ from *Staphylococcus aureus*, Acta Crystallogr. D Biol. Microbiol. 68 (2012) 1175–1188.
[14] K.M. Chung, H.H. Hsu, H.Y. Yeh, B.Y. Chang, Mechanism of regulation of prokaryotic tubulin-like GTPase FtsZ by membrane protein EzrA, J. Biol. Chem. 282 (2007) 14891–14897.
[15] D.P. Haeusser, R.L. Schwartz, A.M. Smith, M.E. Oates, P.A. Levin, EzrA prevents aberrant cell division by modulating assembly of the cytoskeletal protein FtsZ, Mol. Microbiol. 52 (2004) 801–814.
[16] V.R. Steele, A.L. Bottomley, J. Garcia-Lara, J. Kasturiarachchi, S.J. Foster, Multiple essential roles for EzrA in cell division of *Staphylococcus aureus*, Mol. Microbiol. 80 (2011) 542–555.
[17] H.J. Jeong, W.J. Kim, M.J. Kim, S.C. Park, Nosocomial infection surveillance in the intensive care unit, Kor. J. Infect. Dis. 27 (1995) 105–117.
[18] R. Knox, J.T. Smith, The nature of penicillin resistance in *Staphylococci*, Lancet 278 (1961) 520–522.
[19] Y.-I. Kwon, T.-W. Kim, H.-Y. Kim, Y.H. Chang, H.-S. Kwak, G.-J. Woo, Y.-H. Chung, Monitoring of methicillin resistant *Staphylococcus aureus* from medical environment in Korea, Kor. J. Microbiol. Biotechnol. 35 (2007) 158–162.
[20] W.C. Noble, Z. Virani, R.G. Cree, Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*, FEMS Microbiol. Lett. 72 (1992) 195–198.
[21] D.J. Haydon, N.R. Stokes, R. Ure, G. Galbraith, J.M. Bennett, D.R. Brown, P.J. Baker, V.V. Barynin, D.W. Rice, S.E. Sedelnikova, J.R. Heal, J.M. Sheridan, S.T. Aiwale, P.K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, L.G. Czaplewski, An inhibitor of FtsZ with potent and selective anti-staphylococcal activity, Science 321 (2008) 1673–1675.
[22] N.L. Elsen, J. Lu, G. Parthasarathy, J.C. Reid, S. Sharma, S.M. Soisson, K.J. Lumb, Mechanism of action of the cell-division inhibitor PC190723: modulation of FtsZ assembly cooperativity, J. Am. Chem. Soc. 134 (2012) 12342–12345.
[23] P. Sheffield, S. Garrard, Z. Derewenda, Overcoming expression and purification problems of RhoGDI using a family of "Parallel"expression vectors, Protein Expr. Purif. 15 (1999) 34–39.
[24] J. Garcia De La Torre, M.L. Huertas, B. Carrasco, Calculation of hydrodynamic properties of globular proteins from their atomic-level structure, Biophys. J. 78 (2000) 719–730.
[25] J.K. Singh, R.D. Makde, V. Kumar, D. Panda, A membrane protein, EzrA, regulates assembly dynamics of FtsZ by interacting with the C-terminal tail of FtsZ, Biochemistry 46 (2007) 11013–11022.
[26] S.Y. Park, P.P. Borbat, G. Gonzalez-Bonet, J. Bhatnagar, A.M. Pollard, J.H. Freed, A.M. Bilwes, B.R. Crane, Reconstruction of the chemotaxis receptor-kinase assembly, Nat. Struct. Mol. Biol. 13 (2006) 400–407.
[27] D.P. Haeusser, A.C. Garza, A.Z. Buscher, P.A. Levin, The division inhibitor EzrA contains a seven-residue patch required for maintaining the dynamic nature of the medial FtsZ ring, J. Bacteriol. 189 (2007) 9001–9010.