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Cytological and biochemical characterization of the FtsA cell division protein of *Bacillus subtilis*

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Summary

The actin-like protein FtsA is present in many eubacteria, and genetic experiments have shown that it plays an important, sometimes essential, role in cell division. Here, we show that *Bacillus subtilis* FtsA is targeted to division sites in both vegetative and sporulating cells. As in other organisms FtsA is probably recruited immediately after FtsZ. In sporulating cells of *B. subtilis* FtsZ is recruited to potential division sites at both poles of the cell, but asymmetric division occurs at only one pole. We have now found that FtsA is recruited to only one cell pole, suggesting that it may play an important role in the generation of asymmetry in this system. FtsA is present in much higher quantities in *B. subtilis* than in *Escherichia coli*, with approximately one molecule of FtsA for five of FtsZ. This means that there is sufficient FtsA to form a complete circumferential ring at the division site. Therefore, FtsA may have a direct structural role in cell division. We have purified FtsA and shown that it behaves as a dimer and that it has both ATP-binding and ATP-hydrolysis activities. This suggests that ATP hydrolysis by FtsA is required, together with GTP hydrolysis by FtsZ, for cell division in *B. subtilis* (and possibly in most eubacteria).

Introduction

A fundamental question in cell biology is how cells divide to create two daughter cells of equal size. In nearly all bacteria, the process of cytokinesis begins with the polymerization of the essential GTPase, FtsZ, into a ring structure at the nascent division site (reviewed by Bramhill, 1997; Lutkenhaus and Addinall, 1997; Rothfield *et al*., 1999). The crystal structure of FtsZ closely resembles that of eukaryotic α- and β-tubulin (Löwe and Amos, 1998; Nogales *et al*., 1998). Like tubulin, FtsZ undergoes GTP/GDP-dependent polymerization, forming protofilaments, sheets and minirings *in vitro* (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994, 1998; Erickson *et al*., 1996; Yu and Margolin, 1997). In vegetative cells of *Bacillus subtilis* division septa form exclusively at mid-cell, but at the onset of sporulation the division machinery is switched to polar sites, resulting in asymmetric division. The switch to asymmetric division initially involves the assembly of FtsZ rings near both cell poles (Levin and Losick, 1996), but only one polar site is used for septum formation, indicating that FtsZ ring formation is not sufficient to drive septation, at least in sporulating cells.

In addition to FtsZ, at least another five gene products, FtsA, DivIB, FtsL, DivIC and PBP 2B, have been identified as part of the division machinery of *B. subtilis*. Recently, the sporulation-specific protein SpoIIE has also been shown to play a role in assembly of the asymmetric septum by interacting directly with FtsZ (Levin *et al*., 1997; Lucet *et al*., 2000). Assembly of these proteins into the cytokinetic ring appears to occur in a hierarchical order beginning with FtsZ. Localization studies showed that DivIB, DivIC and FtsL localize after FtsZ and that the absence of any one of these three proteins prevents localization of the other two, suggesting that their localization is co-dependent (Daniel and Errington, 2000; Katis *et al*., 2000). PBP 2B belongs to a family of high molecular weight penicillin-binding proteins, which catalyse the final stages of peptidoglycan synthesis (Ghuysen, 1991). It is consistent with its late function in septation that its localization depends on all other division proteins (Daniel *et al*., 2000).

Deletion of the *ftsA* gene of *B. subtilis* leads to impaired cell division and a defect in sporulation (Beall and Lutkenhaus, 1992). The sequence of FtsA indicates a cytoplasmic protein that is related to the actin, Hsp70 and sugar kinase family of ATP-binding proteins (Bork *et al*., 1992). The crystal structure of FtsA from the thermophilic eubacterium *Thermotoga maritima* has recently been solved, showing that the structure of FtsA is most similar to actin (van den Ent and Löwe, 2000). In *Escherichia coli*, some FtsA protein is phosphorylated *in vivo* (Sanchez *et al*., 1994), but ATPase or autokinase activity has not been demonstrated. There is some evidence that FtsA and FtsZ interact *in vivo*. A direct interaction between

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FtsA and FtsZ from different organisms, e.g. *B. subtilis*, *E. coli*, *Rhizobium meliloti*, *Agrobacterium tumefaciens* and *Staphylococcus aureus*, has been demonstrated using the yeast two-hybrid system (Ma *et al*., 1997; Wang *et al*., 1997; Yan *et al*., 2000). Moreover, localization studies in *E. coli* by means of immunofluorescence microscopy and FtsA–GFP fusions show that FtsA localization requires only FtsZ (Addinall and Lutkenhaus, 1996; Ma *et al*., 1996). The ratio of FtsA to FtsZ has been shown to be critical for cell division (Dewar *et al*., 1992; Dai and Lutkenhaus, 1995; Begg *et al*., 1998). In *E. coli* the average number of FtsA and FtsZ molecules per cell were estimated to be 50–200 and 5000–20000, respectively, a ratio of approximately 1:100 (Bi and Lutkenhaus, 1991; Pla *et al*., 1991; Wang and Gayda, 1992). Overexpression of either FtsA or FtsZ leads to a block in cell division that can be suppressed by increasing the level of the other protein.

In this study, we have examined the localization pattern and the biochemical activity of FtsA in *B. subtilis*. Using immunofluorescence microscopy, we have shown that FtsA localizes in a FtsZ-dependent manner to the vegetative division site. In sporulating cells, FtsA preferentially localized to only one of the two potential polar division sites, providing a possible mechanism for generating asymmetry. We have also determined the concentration of FtsA and FtsZ in vegetative and sporulating cells by quantitative immunoblotting. Finally, studies with purified FtsA showed that the native protein is mainly a dimer and that it binds and hydrolyses ATP.

Results

FtsA localizes to the division septum

To examine the subcellular localization of FtsA, anti-FtsA antibodies were affinity purified from FtsA antiserum. The specificity of the purified antibodies was checked by Western blot analysis (see below). Then the purified antibodies were used in immunofluorescence microscopy (IFM) experiments to label cells of the wild-type strain SG38, collected during exponential growth in CH medium. The majority of cells showed a fluorescent signal as a narrow transverse band or a pair of dots located at mid-

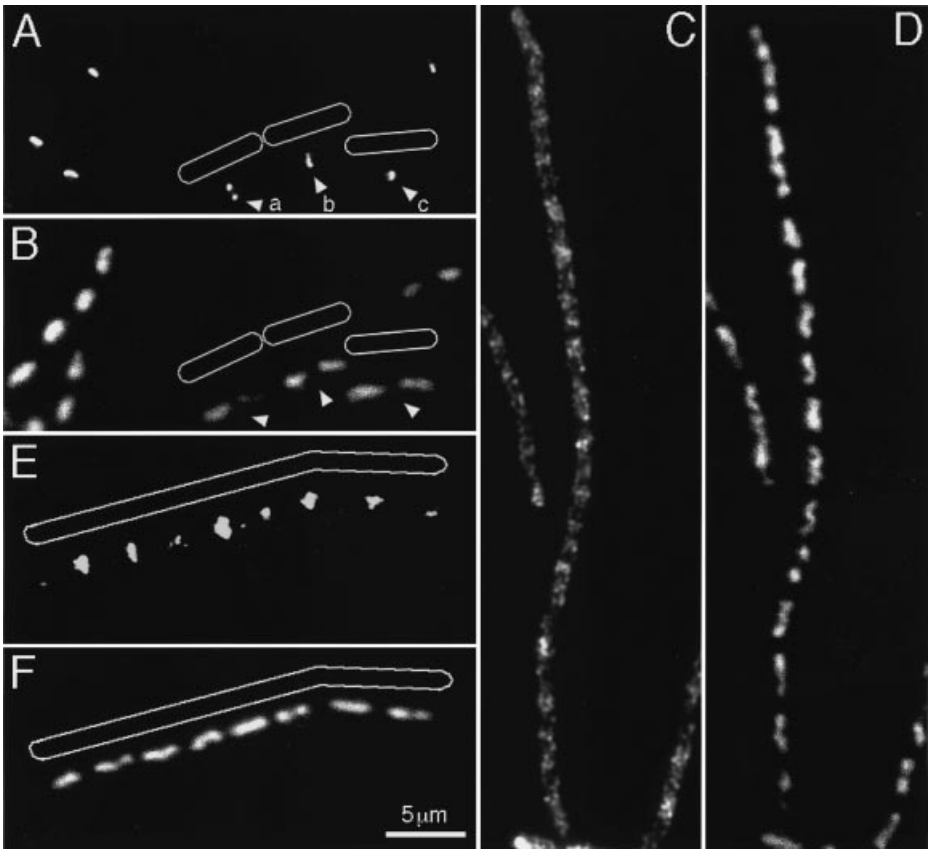

Fig. 1. Immunolocalization of FtsA in wild type and division mutants. Fluorescence micrographs of cells stained for DNA with DAPI (B, D and F) and immunostained with affinity-purified antibodies against the *B. subtilis* FtsA protein (A, C and E). Cells of vegetatively growing wild-type (SG38) (A and B), FtsZ-repressed (strain 1308 grown in the absence of IPTG) (C and D) and *divIB* mutant cells (strain 1306 grown at 37°C) are shown. Arrows in A and B point to cells with different FtsA localization patterns: (a) pair of dots (b) transverse band and (c) single, central dot. Cartoons have been placed near some of the cells illustrating their outlines.

cell (Fig. 1A, cells a and b). [In *B. subtilis*, sister cells tend to remain connected together in chains for some time after the division septum has been formed (Paulton, 1971).] Staining of the nucleoids with 4,6-diamidino-2-phenylindole (DAPI) revealed that the bands/dots were always localized between two well-separated nucleoids (Fig. 1B). In some cells a single, central dot was observed at mid-cell (e.g. cell c). Similar localization patterns were described previously for DivIC, DivIB and FtsL, where bands/pairs of dots apparently correspond to non-septated cells and central dots represent cells that had completed or almost completed septation (Harry and Wake, 1997; Katis *et al*., 1997; Daniel and Errington, 2000; Sievers and Errington, 2000). All of these observations are consistent with FtsA assembling into a ring structure at the site of division and constricting along with membrane invagination during division.

In *E. coli*, the order of assembly of division proteins after FtsZ seems to be linear (Ghigo *et al*., 1999), whereas in *B. subtilis* the later recruited division proteins FtsL, DivIC and DivIB seem to assemble in a concerted manner (Daniel and Errington, 2000; Katis *et al*., 2000). To determine the position of FtsA assembly in the hierarchy, we first examined the dependence of FtsA localization on FtsZ. To do this we studied the localization of FtsA in cells of a strain in which expression of *ftsZ* is dependent on the IPTG-inducible promoter P_{spac} (Beall and Lutkenhaus, 1991). As shown in Fig. 1C, depletion of IPTG prevented cell division and the cells formed long filaments. Under such conditions, FtsA was dispersed throughout the cytoplasm and no localization in the form of bands could be detected.

We then tested the localization of FtsA in a *divIB* mutant strain, in which septation is substantially inhibited at 37°C (Beall and Lutkenhaus, 1989). Here FtsA was found to localize in regularly spaced bands along the length of the filament, suggesting that FtsA localization is independent of DivIB (Fig. 1E). Staining of the nucleoids with DAPI revealed that the bands were localized between the nucleoids (Fig. 1F). FtsA localization probably also does not require DivIC and FtsL, as neither of these proteins localize properly in the absence of DivIB (Daniel and Errington, 2000; Katis *et al*., 2000). Thus, assembly of FtsA into the cytokinetic ring requires FtsZ but not the later acting division proteins.

FtsA preferentially localizes to one potential asymmetric division site during sporulation

It has been shown previously that, early in sporulation, FtsZ and the sporulation-specific protein SpoIIE are able to localize in a bipolar pattern, forming ring-like structures near each pole (Arigoni *et al*., 1995; Levin and Losick, 1996). To analyse the localization pattern of FtsA, wild-type cells were induced to sporulate and labelled with anti-FtsA antibodies for immunofluorescence microscopy. As summarized in Fig. 2, the majority of cells (75%) in stage I (as judged by the nucleoid staining) showed a fluorescent signal only at one potential asymmetric division site (Fig. 3A and B, cell a). A bipolar pattern was observed in 25% of sporulating cells, but generally one of the FtsA bands in each cell was much weaker than the other (Fig. 3A, cell b). By stage II when the asymmetric septum has been formed, most cells (69%) no longer showed any localized fluorescent signal and 31% had only one band of FtsA, which always lay at the pole opposite the asymmetric septum (Figs 2 and 3C and D). These data indicate that FtsA has a strong preference for localization at only one potential division site, suggesting that the two potential division sites near the cell poles usually differ greatly in their state of maturation.

FtsA and SpoIIE localize independently of each other

During sporulation, a modified septum is formed, due to the recruitment of SpoIIE into the septum. It has been shown that both SpoIIE and FtsA interact directly with FtsZ (Wang *et al*., 1997; Lucet *et al*., 2000; Yan *et al*., 2000). It was, therefore, interesting to test whether FtsA localization is affected by the presence or absence of SpoIIE. To visualize both proteins, we used a strain (1305) containing a functional SpoIIE–GFP fusion, and labelled sporulating cells simultaneously with mouse anti-GFP antibodies and rabbit anti-FtsA antibodies. The quantitative analysis of samples taken 80 min after initiation of sporulation is shown in Fig. 4. A few cells (5%, classes 1 and 2) showed localization of SpoIIE–GFP, at one or both poles, with no localized FtsA, but cells with localization of FtsA only were extremely rare (0.2%, class 6). In all of the remaining cells stained for FtsA, there was always co-localization with SpoIIE whether at

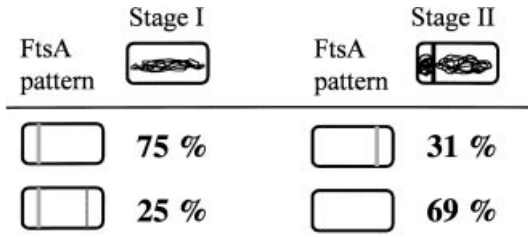

Fig. 2. Schematic illustration and percentage of the localization pattern of FtsA in preseptational (stage I) and cells with an asymmetric septum (stage II). Wild-type cells (SG38) were examined in samples taken 70 and 80 min after initiation of sporulation. The developmental stage of the cell was judged by the nucleoid staining. In stage I cells (346 cells counted), the nucleoid forms an axial filament, whereas in stage II cells (157 cells counted) the prespore nucleoid is fully condensed. The black line in the upper schematic cell shows the position of the asymmetric septum and the grey lines represent FtsA bands.

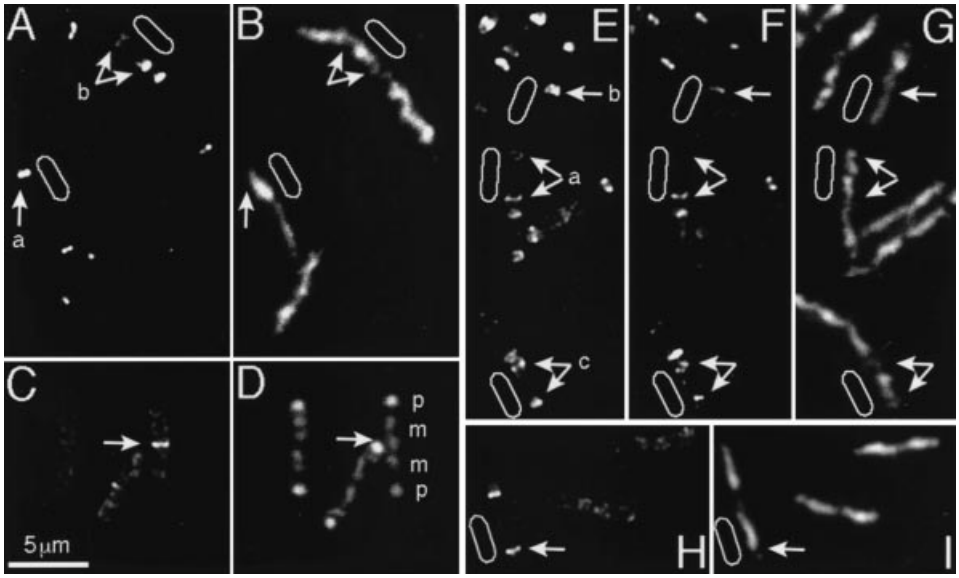

Fig. 3. Immunolocalization of FtsA in sporulating cells. Fluorescence micrographs of cells stained for DNA with DAPI (B, D, G and I) and immunostained with antibodies against the FtsA protein (A, C, F and H) or SpoIIE-GFP (E). Cartoons have been placed near some of the cells illustrating their outlines.
A–D. Localization of FtsA in sporulating wild-type cells (SG38), taken 70 min (A and B) or 80 min (C and D) after initiation of sporulation. In preseptational cells (A and B), FtsA localizes in a monopolar (cell a) or bipolar (cell b) pattern. After formation of the asymmetric septum, FtsA (arrow in C and D) localizes to the polar site opposite of the prespore (p), in the mother cell (m) compartment.
E–G. Localization of FtsA (F) and SpoIIE-GFP (E) in sporulating cells (strain 1305) taken 80 min after initiation of sporulation. Arrows point to a cell in which SpoIIE-GFP was localized to both potential asymmetric division sites, whereas FtsA was localized to a single site (a) and cells in which both SpoIIE-GFP and FtsA colocalize either in a monopolar (b) or a bipolar (c) pattern.
H and I. Localization of FtsA in sporulating cells of a *spoIIE* mutant (strain 1317), taken 90 min after initiation of sporulation. Only some cells show a localized band of FtsA (arrow), whereas in most cells (cell pair on the right) FtsA is dispersed throughout the cytoplasm.

one or both poles (classes 3–5; Fig. 3E–G, cells a, b, c). In contrast, about half of these cells (43.4%) had SpoIIE at both poles with only one pole staining for FtsA (class 3; Fig. 3E–G, cell a). We conclude that SpoIIE is recruited to the second pole of the sporangium much more efficiently than FtsA, which mainly localizes to one pole. It is also evident from the abundance of classes 1, 2 and 3 that recruitment of SpoIIE to polar sites can occur independently of significant amounts of localized FtsA.

Next, FtsA localization was examined in the absence of SpoIIE by labelling sporulating cells of strain 1317, which expresses an unstable deletion protein of SpoIIE (A. F., unpublished data). In most cells, FtsA was dispersed throughout the cytoplasm (Fig. 3H and I). A few cells ($< 20\%$) showed a clear localization of FtsA to one potential asymmetric division site. These results were very similar to those obtained for FtsZ in *spoIIE* null mutants (Feucht *et al*., 1996; Khvorova *et al*., 1998), suggesting that SpoIIE has no additional effect on targeting of FtsA during sporulation.

The ratio of FtsA to FtsZ in the cell is one to five

Wang and Gayda (1992) reported that the average number of FtsA molecules per cell was only 50–200 in

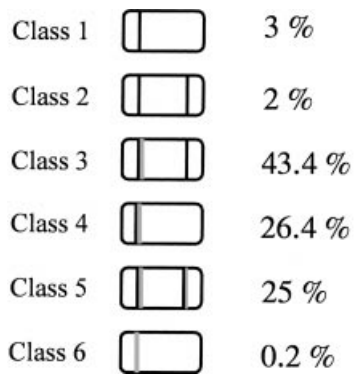

Fig. 4. Schematic illustration and percentage of the localization pattern of FtsA and SpoIIE-GFP in sporulating cells. Cells (406 cells counted) were scored 80 min after initiation of sporulation for localization of SpoIIE-GFP and FtsA. SpoIIE-GFP and FtsA are indicated by black and grey lines respectively. Classes 1 and 2 represent cells in which only SpoIIE-GFP is localized in a monopolar or bipolar pattern respectively. Class 3 are those cells in which SpoIIE-GFP is localized in a bipolar pattern, whereas FtsA is localized only to one site. In class 4 and 5 cells SpoIIE-GFP and FtsA colocalize in either a monopolar or bipolar pattern respectively. Class 6 cells did not have any SpoIIE-GFP localization.

E. coli, compared with 5000–20000 molecules of FtsZ (Bi and Lutkenhaus, 1991; Pla *et al*., 1991). The fact that FtsA was easily detected in our experiments by Western Blot analysis as well as in IFM suggested that the amount of FtsA per cell in *B. subtilis* might be higher than in *E. coli*. We used quantitative Western blot analysis to measure the amount of FtsA in cell extracts. In parallel, we also quantified the amount of FtsZ, so that the ratio of the two protein concentrations could be determined. Samples of exponentially growing cells of wild-type *B. subtilis* (strain SG38) in CH medium were analysed for FtsA and FtsZ proteins as described in *Experimental procedures* (Fig. 5). The average numbers of FtsA and FtsZ molecules per cell in CH medium were estimated to be 1000 and 5000, respectively, which gives a ratio of 1:5 (Table 1). To determine how the levels of the two proteins change during sporulation, strain SG38 was induced to sporulate and samples were taken at various time points and analysed as above. Surprisingly, the number of FtsZ molecules doubled during the early stages of sporulation whereas that of FtsA changed little (Table 1). Later in sporulation the concentration of both proteins decreased. The transient increase in FtsZ relative to FtsA early in sporulation could reflect the presence of two FtsZ rings but only one major FtsA ring.

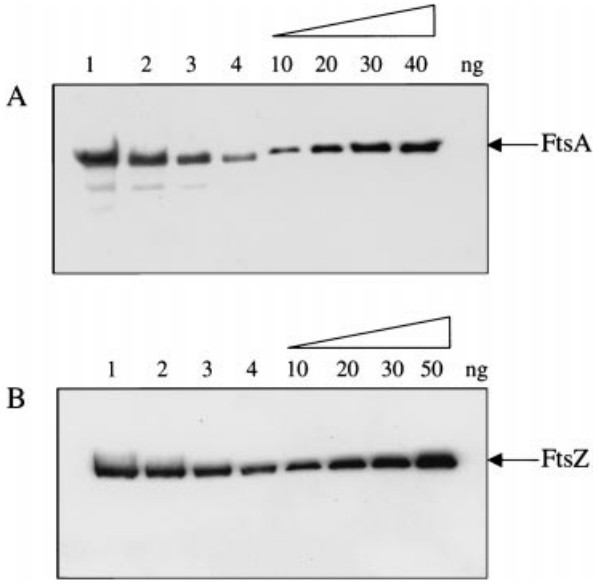

Fig. 5. Quantitative determination of FtsA and FtsZ proteins in *B. subtilis*. Extracts of cells (strain SG38) grown to an A_{600} of 0.7 in CH medium were treated as described in *Experimental procedures*. Different amounts of cell extract (5, 10, 17.5 and 25 μl, lanes 1–4 respectively) were loaded on 12% SDS–PAGE and the density of both FtsA (A) and FtsZ (B) bands were detected by Western blotting analysis and compared with the density observed over a 10–40 ng or 10–50 ng concentration range of purified FtsA and FtsZ proteins respectively. The quantities of both FtsA and FtsZ were calculated by taking into account the dilution of the sample and the number of cells.

Table 1. Amount of FtsA and FtsZ protein in vegetative and sporulating cells.

Media		Molecules of FtsA/cell[a]	Molecules of FtsZ/cell[a]	Ratio FtsZ–FtsA
CH[b]		1000	5000	5
SM[b]	t_0	900	4500	5
	t_1	830	7920	9.5
	t_2	600	5550	9.2
	t_3	650	3000	4.6

a. The number of cells was calculated as described in *Experimental procedures*.
b. Average numbers of two and three experiments in CH and SM respectively.

Purified FtsA protein behaves as a dimer

The amino acid sequence and the recently solved structure of FtsA indicate that it is related to the actin/hsp70/sugar kinase family of ATP-binding proteins (Bork *et al*., 1992; van den Ent and Löwe, 2000), but so far ATPase activity has not been demonstrated. To investigate the biochemical properties of FtsA, the wild-type *ftsA* gene from *B. subtilis* was cloned and the protein was overexpressed in *E. coli* (C43). Sequencing of the cloned *ftsA* gene revealed several differences from the published sequence (see *Experimental procedures*). The overproduction of *B. subtilis* FtsA in *E. coli* resulted in filamentous cells (data not shown). The protein was solubilized from the pellet fraction with sarcosyl and then fractionated by the successive use of anion exchange, affinity chromatography on HiTrap Blue and gel-filtration chromatography on Superdex S200. This yielded an electrophoretically pure preparation of FtsA. As shown in Fig. 6A, most of the purified FtsA eluted from Superdex 200 with an apparent M_r of around 105 KDa, strongly suggesting that it is predominantly dimeric. A smaller fraction of the protein eluted with a higher apparent M_r, suggesting the presence of multimeric forms.

To examine the oligomerization state of FtsA *in vivo*, we analysed the running position of FtsA from cell extracts of vegetative *B. subtilis* on non-denaturing PAGE followed by Western blotting. As shown in Fig. 6B, two bands of FtsA, of about equal strength, were detected (lane 1), the upper one running at the same position as the purified FtsA dimer (lane 2), suggesting that the dimer form exists *in vivo*.

Purified FtsA has ATP-binding and ATPase activities

As described in the previous section, the third step of purification of FtsA involved binding to a HiTrap blue column and elution with a gradient of ATP. Because HiTrap blue column specifically retains proteins requiring adenyl-containing cofactors, the success of this

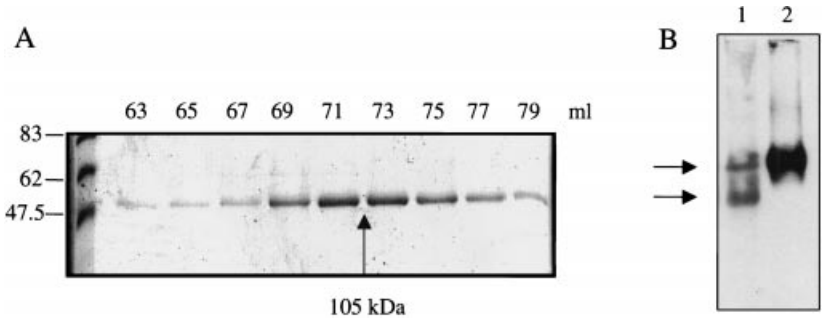

Fig. 6. FtsA exists as a dimer *in vitro* and *in vivo*.
A. FtsA after separation on a Superdex 200 gel filtration column analysed on SDS–PAGE stained with Coomassie brilliant blue. The majority of FtsA was eluted between 67 and 77 ml corresponding to an apparent M_r of 105 KDa. The sizes of the marker protein (KDa) are indicated to the left.
B. Proteins from cell extracts of vegetative grown SG38 to an A_{600} of 0.7 in CH medium (lane 1) and purified FtsA dimer (lane 2) were subjected to non-denaturing PAGE and Western blotted with anti-FtsA antibodies. FtsA from the cell extract runs at two positions (indicated by arrows) and the upper one corresponds to the dimer form of FtsA.

procedure suggested that the purified protein binds to ATP. We used UV cross-linking to confirm this and analyse the specificity of nucleotide binding. As shown in Fig. 7A, the electrophoretically pure preparation of FtsA, eluted as a dimer from the superdex 200 column after four steps of purification, was cross-linked to [α-^{32}P]-ATP. Only a single band was detected with mobility expected for FtsA (lane 2), which demonstrated that the FtsA preparation did not contain any other ATP-binding proteins. Cross-linking of FtsA to [α-^{32}P]-ATP did not occur when ethylenediaminetetra-acetic acid (EDTA) was added to the reaction mixture (lane 1), nor when excess unlabelled ATP was added (lane 3). Addition of excess unlabelled ADP or GTP significantly reduced the amount of bound [α-^{32}P]-ATP (lane 4 and 5), suggesting that the protein has a weak affinity for these other nucleotides.

To test whether FtsA has ATPase activity, we measured the hydrolysis of ATP by monitoring the release of ^{32}Pi from [γ-^{32}P]-ATP. The activity was readily detected, and analysis of the reaction revealed Michaelis–Menten kinetics (Fig. 7B) with a K_m of 0.7 mM and a V_{max} of 0.125 mol of ATP hydrolysed per second per mol of FtsA dimer.

Discussion

Cell division in prokaryotes is a complex process, involving the ordered assembly of several different kinds of protein into an active division apparatus followed by annular invagination of the cell wall and membrane layers, which generates two daughter cells. FtsA is part of the division apparatus, and to gain a better understanding of its function we studied its subcellular localization in *B. subtilis* as well as its biochemical properties.

Use of purified antibodies showed that FtsA is localized at septal sites (Fig. 1) similar to the localization pattern described previously for FtsZ (Wang and Lutkenhaus, 1993; Levin and Losick, 1996). A similar localization pattern was also seen with a GFP–FtsA fusion protein (A. F., unpublished data). FtsA is recruited to the division site after FtsZ but independently of DivIB (and presumably also of FtsL and DivIC), as FtsA was dispersed throughout the cytoplasm in FtsZ-depleted cells but localized

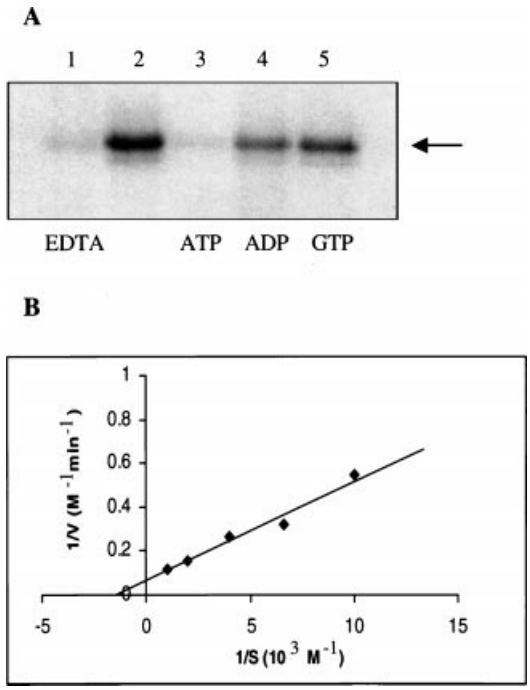

Fig. 7. FtsA binds and hydrolyses ATP.
A. [α-^{32}P]-ATP photoaffinity cross-linking of FtsA. FtsA was incubated with 0.5 μM [α-^{32}P]-ATP (lane 2), in presence of 10 mM EDTA (lane 1) or with competing unlabelled nucleotides (50 μM) (lane 3, ATP; lane 4, ADP; lane 5, GTP) and was subjected to UV cross-linking as described in *Experimental procedures*. The position of FtsA is indicated to the right.
B. Lineweaver–Burk plot of FtsA ATPase activity. Activity was measured as release of ^{32}Pi from [γ-^{32}P]-ATP.

normally in a temperature-sensitive *divIB* mutant at the non-permissive temperature (Fig. 1). This is consistent with the finding that FtsA interacts directly with FtsZ, as shown by yeast two-hybrid system analysis, and that FtsA of *E. coli* also requires only FtsZ for its localization (Ma *et al*., 1997; Wang *et al*., 1997; Yan *et al*. 2000). From this and other studies the hierarchical order of assembly in *B. subtilis* emerges as follows: FtsZ, FtsA, DivIB/DivIC/FtsL and then PBP 2B.

FtsA also localized at the potential polar division sites during sporulation. In contrast to FtsZ and SpoIIE, which show a bipolar localization pattern (Arigoni *et al*., 1995; Levin and Losick, 1996), FtsA seems mainly to target to one polar site (Figs 2 and 3). This is presumably the site at which the asymmetric septum subsequently forms. At the opposite pole the assembly of FtsA might be blocked, because no FtsA, or only a faint band could be detected. Recruitment of the complete set of division proteins to only one polar site could be an important mechanism for the regulation of septum formation in sporulation. It has previously been suggested that the rapid turnover of FtsL prevents immediate assembly of the second septum (Daniel and Errington, 2000). Now it seems that septum formation is controlled earlier, at the level of FtsA assembly. The preference of FtsA for one polar site strongly suggests that the two polar sites are different. This, and the data shown in Figs 2 and 3, lend support to the idea that the two poles also differ in the assembly of both SpoIIE and FtsZ (Wu *et al*., 1998), although these earlier effects are much less marked than that of FtsA. Differential maturation of the division machinery at the two potential division sites could play a crucial role in generating the asymmetry of this system and therefore the determination of cell fate (Lewis *et al*., 1994; Wu *et al*., 1998).

Measurement of the physiological levels of the FtsA and FtsZ proteins in vegetative and sporulating cells showed that the amount of FtsA remains constant (around 1000 molecules per cell), whereas the amount of FtsZ doubles transiently during the early stage of sporulation (around 5000 and 10000 per vegetative and sporulating cell respectively). These data, together with the IFM data showing that predominantly one ring of FtsA but two of FtsZ are formed after initiation of sporulation (Figs 2 and 3; Levin and Losick, 1996), suggest that the ratio of FtsA and FtsZ in one division complex is constant, at about 1:5. The localization pattern and the presence of around 1000 molecules of FtsA in *B. subtilis* also suggest that FtsA assembles into a ring structure (Figs 1 and 5). By contrast, it has been suggested that *E. coli* FtsA, which is estimated to be present at approximately 50–200 molecules per cell, is unlikely to form a complete ring structure (Wang and Gayda, 1992; Addinall and Lutkenhaus, 1996). The 5–20 times higher amount of FtsA in *B. subtilis* might compensate for the apparent absence of a ZipA homologue in *B. subtilis.* It is also possible that more ATP hydrolysis is required for cell division in Gram-positive bacteria because of the much thicker peptidoglycan layer or the higher internal osmotic pressure.

FtsA is structurally related to members of the actin family and it is therefore predicted to bind and hydrolyse ATP and possibly to polymerize (Korn *et al*., 1987; van den Ent and Löwe, 2000). Interestingly, during the purification of FtsA, we found that the protein exists mainly as a dimer, with a small proportion of multimers (Fig. 6). This is in agreement with yeast two-hybrid data, which demonstrated that FtsA interacts with itself in *E. coli* (Yan *et al*., 2000). Furthermore, *in vivo*, FtsA was detected in two distinct forms, as shown by non-denaturing PAGE (Fig. 6), with one form probably corresponding to the dimer. In other experiments we excluded the possibility that the lower band was a degradation product of the upper band or that the additional band might be associated with FtsZ (data not shown). Sanchez *et al*. (1994) reported that some FtsA protein is phosphorylated in *E. coli*. So far we could not detect a phosphorylated form of *B. subtilis* FtsA by analysing samples on isoelectric focusing gels (data not shown). Most likely, the faster migrating band corresponds to a monomer. This is supported by the finding that his-tagged FtsA of *T. maritima* was expressed in *E. coli* and purified as a monomer (van den Ent and Löwe, 2000). Another possibility is that the forms differ in terms of bound nucleotide (e.g. ADP vs. ATP).

We have shown that *B. subtilis* FtsA is capable of binding and hydrolysing ATP (Fig. 7), but it still remains to be elucidated whether nucleotide hydrolysis is associated with assembly or disassembly of filaments, as for eukaryotic actins (Korn *et al*., 1987). *ftsA* null mutants of *B. subtilis* are extremely filamentous, implying that this protein is important for division (Beall and Lutkenhaus, 1992). It is now possible that the ATPase activity of FtsA is required to enhance the dynamics of FtsZ rings. One possible role for this activity would be to provide additional energy to switch FtsZ between straight and curved conformations (Erickson *et al*., 1996), helping to bring about membrane constriction. A major challenge now is to find out how the biochemical activity of FtsA is coupled to its biological function in cell division.

Experimental procedures

Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 2. pFtsA-2 was constructed by amplifying the *ftsA* gene by PCR from chromosomal DNA of *B. subtilis* SG38. The following oligonucleotides were used 5′-GGAATTCCATATG AACAACAATGAACTTTACG-3′ and 5′-GAATGAGGATCCA TCTATTCCCAA-3′ introducing *Nde*I and *Bam*HI restriction

Table 2. Strains and plasmids.

Strain/plasmid	Relevant genotype[a]	Construction, source or reference
B. subtilis		
SG38	*trpC2 amyE*	Errington and Mandelstam (1986)
1305	*trpC2 amyE* Ω(*spoIIE*::pSG1902 *spoIIE-gfpF64L S65T cat*)	Wu *et al.* (1998)
1306	*trpC2* Ω(*amyE*::*gpr'-'lacZ cat*) Ω(*divIB*::*spec*)	Feucht *et al.* (1999)
1308	*trpC2* Ω(*amyE*::*gpr'-'lacZ cat*) Ω(*ftsZ*::pJSIZΔpble *ble* P_{spac}-*ftsZ*)	Feucht *et al.* (1999)
1317	*trpC2 amyE* Ω(*gpr*::pPS1326 *gpr'-'lacZ cat*) Ω(*aphA-3 spoIIE1705–2481*)	This work
E. coli		
DH5α	*F⁻ endA1 hsdR17 supE44* λ⁻ *thi-1 recA1 gyrA96 relA1* Δ*(lacZYA-argF) U169* ϕ80 d*lac* Δ*(lacZ)M15*	Gibco BRL
C43 (DE3)	BL21 (DE3) derivative	Miroux and Walker (1996)
Plasmids		
pET-11a	T7 expression vector	Novagen
pFtsA-2	pET-11a containing *ftsA*	This work

a. Numbers in parentheses after *spoIIE* refer to the first and last nucleotides of the *spoIIE* coding sequence.

sites respectively. The PCR product was digested with *Nde*I and *Bam*HI and subcloned into *Nde*I–*Bam*HI digested pET-11a. The cloned *ftsA* gene and also the 1.3 kbp PCR product were sequenced and compared with the published sequence (Beall *et al.*, 1988). Thereby, several discrepancies from the published sequence were found: codon 12 (ATC instead of CTC), codon 17 (ACA instead of ACC) codon 26 (GAT instead of GGT), codon 112 (GAG instead of GCG), codon 253 (GAA instead of GAG), codon 349 (GCA instead of CAA) and codon 350 (AGC instead of GGC). In several other independent PCR reactions and clonings, we invariably found the same sequence as for the expressed construct.

Strain 1317, which contains a deletion of the first 1704 bp of the *spoIIE* gene, was generated by ligation of the following four fragments and transformation into SG38 with selection for kanamycin resistance. A 1715 bp PCR fragment containing the sequence upstream of the *spoIIE* promoter was digested with *Not*I, the *aph-A3* cassette of pBEST501 was released by *Not*I–*Xba*I digest and gel purified, a 155 bp PCR fragment containing the promoter region of *spoIIE* was digested with *Xba*I–*Nde*I and a 1664 bp PCR fragment containing the 3′ end of *spoIIE* was digested with *Nde*I. The following oligonucleotides were used: 5′-AGCGGATCCAGC GGTTCTGCTGGGCG-3′; 5′-ATAGTTTAGCGGCCGCTGT CAAAAGAAGGTAAC-3′ (generating a *Not*I restriction site at the 3′ end of the fragment); 5′-CTAGTCTAGAAGACAACAT TCCGGAAATTC-3′; 5′-GCTTAAGCATATGTCTCATCTCCC ACCTG-3′ (generating a *Xba*I *and* a *Nde*I restriction site at the 5′ and 3′ end of the fragment respectively); and 5′-CGA ATTCCATATGAAAGCCGAACAGCACTCTC-3′; 5′-ACCGG TACCCCTTTACCAAGCTGTTTACG-3′ (generating a *Nde*I restriction site at the 5′ end of the fragment). *Bacillus subtilis* cells were made competent for transformation with DNA using the method of Anagnostopoulos and Spizizen (1961), as modified by Jenkinson (1983). DNA manipulations and *E. coli* transformations were carried out using standard methods (Sambrook *et al.*, 1989).

Media and growth conditions

Strain 1306 was grown at 30°C. Strain 1308, which contains *ftsZ* under the control of the IPTG-inducible P_{spac} promoter (Beall and Lutkenhaus, 1991), was grown in the presence of 0.5 mM IPTG. *Bacillus subtilis* cultures were grown in hydrolysed casein (CH) medium (Sterlini and Mandelstam, 1969) and sporulation was induced by growth in CH, followed by resuspension in a starvation medium (SM) (Sterlini and Mandelstam, 1969; Partridge and Errington, 1993). Times (min) after resuspension of cells in the starvation medium were denoted t_0, t_{60}, t_{90} and so forth. Nutrient agar (Oxoid) was used as a solid medium for growing *B. subtilis*. Chloramphenicol (5 μg ml^{-1}), spectinomycin (50 μg ml^{-1}) or kanamycin sulphate (5 μg ml^{-1}) were added as required. Media used for growing *E. coli* were 2xTY (Sambrook *et al.*, 1989) and nutrient agar (Oxoid) supplemented with ampicillin (100 μg ml^{-1}).

Division protein depletion

To inactivate DivIB protein, strain 1306 was grown first in CH at 30°C to an A_{600} of 0.2. The culture was shifted to 37°C to induce filamentation and samples for examination by immunofluorescence microscopy were taken at an A_{600} of 0.6. For FtsZ depletion, strain 1308 was grown overnight at 30°C in CH supplemented with 0.5 mM IPTG. The culture was pelleted by centrifugation and IPTG was removed by washing the cells with warm CH medium. The culture was diluted back to an A_{600} of 0.05 and grown at 37°C. Samples for immunofluorescence microscopy were taken at an A_{600} of 1.3.

Immunofluorescence microscopy

Anti-FtsA antibodies were purified from FtsA antiserum by a FtsA affinity column (CNBr activated sepharose, Sigma). Fixation, permeabilization and staining of the cells for immunofluorescence microscopy were performed as described previously (Pogliano *et al.*, 1995; Lewis *et al.*, 1996; Reznekov *et al.*, 1996) except that glutaraldehyde concentration was reduced to 0.005%. Affinity-purified anti-FtsA antibodies and monoclonal anti-GFP antibody (Clontech) were used at dilutions of 1:30 and 1:40 respectively. Images were grabbed, processed and assembled as described by Lewis and Errington (1997), except that MetaMorph version 3.6 software (Universal Imaging) was used.

Purification of FtsA protein

Plasmid pFtsA-2 was transformed into *E. coli* C43 (DE3) and the protein was overexpressed for 3 h at 37°C after induction with 1 mM IPTG. Cell pellets were frozen in dry ice and stored at −70°C until use. The cells from 1 l culture were resuspended in 35 ml of cold buffer A [100 mM Tris-HCl (pH 8), 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1% Triton X-100] and disrupted by sonication (three times for 10 s). The homogenate was centrifuged for 1 h at 29000 *g*. The pellet was resuspended in buffer A supplemented with 0.5% Triton X-100 and centrifuged again as before. This washing step was repeated twice. Proteins were extracted from the final pellet by treatment O/N at 4°C with 20 ml of buffer B [50 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5% Triton X-100 and 0.5% sarcosyl]. After centrifugation for 1 h at 29000 *g*, the supernatant was loaded onto a DEAE-sepharose column equilibrated in buffer B. The column was washed with 30 ml of buffer B and then developed with an 80 ml linear gradient (50 mM−1 M NaCl). The peak fractions of FtsA, eluted between 350 and 650 mM, were pooled and dialysed against a solution containing 50 mM Tris-HCl (pH 8), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100 and 1 mM $MgCl_2$ at 4°C for 48 h with one change of buffer (the second buffer containing 30% glycerol). A 2 ml sample of this preparation was diluted five times in buffer C [50 mM Tris-HCl (pH 8), 200 mM potassium acetate, 1 mM DTT, 0.5% Triton X-100 and 10 mM $MgCl_2$] and applied onto a 1 ml Hitrap blue column (Pharmacia) that had been equilibrated in buffer C. The column was washed with 10 ml of buffer C and then developed with a step gradient (20 ml of 0–5 mM ATP, 10 ml of 5–10 mM ATP). FtsA was eluted in a broad peak between 1 and 5 mM ATP. Peak fractions (18 ml) were pooled and concentrated on a Centriprep 10 (Amicon) device to 2 ml and loaded onto a Superdex S200 (Pharmacia) equilibrated in buffer D [50 mM Tris-HCl (pH 8), 1 M NaCl, 50 mM potassium acetate, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Triton X-100, 2 mM $MgCl_2$]. Elution was performed with the same buffer at 0.5 ml min^{-1} and fractions of 1 ml were collected. Each fraction (20 μl) was loaded on a 12% SDS–PAGE and the gel was stained with Coomassie brilliant blue. The fractions containing purified FtsA, eluted between 24 and 32 ml, were dialysed against buffer D and stored in 50% glycerol at −70°C. Calibration of the gel filtration Superdex 200 column was performed as described in Lucet *et al*. (2000).

Cross-linking of ATP to FtsA

UV-induced cross-linking of [α-^{32}P]-ATP to FtsA was carried out as described by de Boer *et al*. (1991) with some modifications. Purified FtsA (3 μg) was incubated in 50 μl of buffer E [50 mM Tris-HCl (pH 7.2), 50 mM potassium acetate, 1 mM DTT, 1 mM $MgCl_2$], containing 1 μg BSA and 0.5 μM [α-^{32}P]-ATP (3000 Ci $mmol^{-1}$, Amersham). Unlabelled nucleotides (50 μM) were added to the suspension when required. The samples were incubated for 30 min at 0°C and transferred to wells of a microtitre dish on top of a chilled lead brick, which was standing in ice water. The samples were irradiated at 254 nm at maximum energy for 10 min and then transferred to a tube containing 50 μl of 3 mM ATP in H_2O. The wells were washed with 100 μl of buffer E and the wash was combined with the corresponding sample before addition of 0.75 ml of cold 10% TCA. After incubation O/N at 4°C, the samples were centrifuged and the precipitates were washed first with 10% TCA and then with acetone prior to analysis by 12% SDS–PAGE. Gels were dried and exposed to Kodak Biomax MR film to visualize ^{32}P-labelled bands by autoradiography.

ATPase assay

ATPase activity was assayed by following the formation of $^{32}P_i$ from [γ-^{32}P]-ATP. The reaction mixture (300 μl) containing buffer F [50 mM Tris-HCl (pH 7.2), 50 mM potassium acetate, 1 mM DTT, 10 mM $MgCl_2$] and different concentrations of [γ-^{32}P]-ATP (0.1–1 mM) was incubated for 5 min at 37°C. The reaction was started by adding 50 nM purified FtsA. Samples (50 μl) were taken at different times and assayed as described previously (Najafi *et al*., 1996).

Quantification of FtsA and FtsZ

The total amount of FtsA and FtsZ proteins in cells was determined by quantitative immunoblot analysis. Cell extracts (10 ml) from both vegetative and sporulating cells were resuspended in 500 μl solubilization buffer [2% SDS, 10% β-mercaptoethanol, 8 M urea, 0.03% bromophenol blue, 1 M Tris-HCl (pH 7), 10 mM EDTA], sonicated (twice for 30 s) and boiled for 5 min. The proteins were separated on 12% SDS–PAGE and transferred (160 mA, 2 h) to polyvinylidene difluoride (PVDF) membranes (Amersham). The membranes were subjected to immunoblotting with either purified anti-FtsZ or anti-FtsA antibodies. Then, alkaline phosphatase-conjugated antibodies were used and blots were developed by exposure to ECF substrate (Amersham). Each blot was scanned using the fluorimager Storm (Molecular Dynamics). The ImageQuant package was employed to calculate the quantities of FtsA and FtsZ in cell extracts. Each blot also included standard solutions of purified FtsA or FtsZ proteins that covered the range between 10 and 40 ng for FtsA and 10–50 ng for FtsZ. *Bacillus subtilis* FtsZ was purified according to the method published previously (Wang and Lutkenhaus, 1993). The number of cells was determined by directly counting ethanol-fixed cells in a Helber bacteria counting chamber (average of six estimations). Each estimation was based on the average number of cells in 20 ruled squares, each with a volume of 50 pL. The number of cells ml^{-1} were 3.7×10^8 in a culture grown in CH media and taken at an A_{600} of 0.7. The number of cells ml^{-1} of a sporulating culture taken at t_0, t_1, t_2 and t_3 with an A_{600} adjusted to 0.7 were 3.9×10^8, 4.8×10^8, 7.3×10^8 and 8.2×10^8 respectively. (A sporulating cell, containing prespore and mother cell, was counted as one cell.)

Analysis of whole cell extracts on non-denaturing PAGE

Vegetatively growing cells (10 ml, A_{600} of 0.7) were centrifuged and the pellet was resuspended in 1 ml PBS

containing 5 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.1 mg ml^{-1} lysozyme. After incubation at 37°C for 5 min, the cells were broken by sonication on ice (two times for 30 s) and centrifuged for 30 min at 4°C. The supernatant (30 μl) and purified FtsA were subjected to non-denaturing 10% PAGE. Proteins were detected by immunoblotting with purified anti-FtsA antibodies.

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