

## Interaction between Cell Division Proteins FtsE and FtsZ<sup>∇</sup>

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**FtsE and FtsX, which are widely conserved homologs of ABC transporters and interact with each other, have important but unknown functions in bacterial cell division. Coimmunoprecipitation of *Escherichia coli* cell extracts revealed that a functional FLAG-tagged version of FtsE, the putative ATP-binding component, interacts with FtsZ, the bacterial tubulin homolog required to assemble the cytokinetic Z ring and recruit the components of the divisome. This interaction is independent of FtsX, the predicted membrane component of the ABC transporter, which has been shown previously to interact with FtsE. The interaction also occurred independently of FtsA or ZipA, two other *E. coli* cell division proteins that interact with FtsZ. In addition, FtsZ copurified with FLAG-FtsE. Surprisingly, the conserved C-terminal tail of FtsZ, which interacts with other cell division proteins, such as FtsA and ZipA, was dispensable for interaction with FtsE. In support of a direct interaction with FtsZ, targeting of a green fluorescent protein (GFP)-FtsE fusion to Z rings required FtsZ, but not FtsA. Although GFP-FtsE failed to target Z rings in the absence of ZipA, its localization was restored in the presence of the *ftsA*<sup>\*</sup> bypass suppressor, indicating that the requirement for ZipA is indirect. Coexpression of FLAG-FtsE and FtsX under certain conditions resulted in efficient formation of minicells, also consistent with an FtsE-FtsZ interaction and with the idea that FtsE and FtsX regulate the activity of the divisome.**

To ensure their survival, bacteria must successfully duplicate their genetic material and partition it equally to each daughter cell just prior to cytokinesis. In *Escherichia coli*, at least 14 proteins are directly linked to the process of cell division. FtsZ, the prokaryotic homolog of tubulin, is the first known component of the cell division apparatus to localize to the future division site (1, 5). Once localized, FtsZ polymerizes into the Z ring and is proposed to provide a scaffold upon which FtsA, ZipA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, FtsN, and AmiC are recruited (17, 34, 48). The resulting putative protein complex, or divisome, is required for the synthesis of the division septum and subsequent formation of new cell poles. Removal or inactivation of any of these proteins causes an arrest in cell division, yet cell growth continues, resulting in cell filamentation, lysis, and subsequent death. Although most components of the cell division machinery have likely been identified, how this multisubunit complex is assembled within the membrane and what the precise role of each protein is remain unknown.

In *E. coli*, cell division proteins localize to the future division site via a linear order of dependency (17). Because downstream cell division proteins fail to localize upon deletion or inactivation of an immediately upstream component, this suggests that each cell division protein interacts with the cell division component localizing immediately upstream. Nevertheless, evidence for a more complex system of interactions is mounting. For example, ZipA is necessary for localization of FtsK and downstream cell division proteins (24, 40), but it can

be deleted from cells carrying a gain-of-function mutation in *ftsA* (14). This result indicates that ZipA does not recruit downstream cell division proteins directly but rather enhances the ability of the Z ring to recruit these proteins. Likewise, the same mutation in *ftsA*, or increased levels of FtsZ, FtsA, and/or FtsQ, can partially bypass the loss of FtsK; in suppressed strains lacking FtsK, downstream cell division proteins, such as FtsI, are still recruited to the septum, indicating that FtsK is not required for their recruitment (15).

Recent evidence also supports the idea that cell division proteins in *E. coli* can exist in independent subassemblies. For example, a subassembly of FtsA, FtsI, and FtsN can be forced to localize to the cell poles independently of the rest of the cell division machinery (8). FtsQ can be artificially recruited to the Z ring at an early step, and this results in the recruitment of some late cell division proteins to a subassembly independently of some earlier proteins (18, 19). FtsL and FtsB can be isolated in a subcomplex with FtsQ (6). Finally, bacterial two-hybrid analyses indicate that cell division proteins interact with multiple partners (13, 28), consistent with the idea that the divisome assembles as a web of protein-protein interactions instead of a chain of binary interactions. However, bacterial two-hybrid studies can be problematic because of potential bridging proteins mediating protein-protein interactions. Moreover, when potential interactions among cell division proteins were tested with either of the bacterial two-hybrid systems, one cell division protein not tested was FtsE.

FtsE and FtsX show high homology to ATP-binding cassette (ABC) transporters, with FtsE being the ABC component and FtsX the component in the cytoplasmic membrane (11, 16). FtsE and FtsX localize to the Z ring, and the localization of FtsX is dependent on FtsZ, ZipA, and FtsA but not downstream proteins, FtsK, FtsQ, FtsW, and FtsI (43). Depletion of FtsE results in inhibition of cell division and growth, but this effect is mainly observed in growth medium lacking salt. The cell viability defect could be suppressed by addition of 1%

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strain		
TX3772	MG1655 $\Delta lacU169$	Laboratory collection
Top10	F <sup>-</sup> <i>mcrA</i> ( <i>mrr-hsdRMS-mcrBC</i> ) $\Phi 80lacZ\Delta M15 lacX74 recA1 ara139$ ( <i>ara-leu</i> )7697 <i>galU galK rpsL endA1 nupG recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F $\Delta proAB lacI^q \Delta M15$ Tn10)	Invitrogen
XL1-Blue	Wild-type strain	Stratagene
W3110	W3110 $\Delta lacU169 gal490 \lambda cI857 \Delta(cro-bioA)$	Laboratory collection
DY330	WX7/pCX41, <i>leu::Tn10 ftsZ<sup>o</sup> (repA(Ts) ftsZ<sup>+</sup>)</i>	50
WM746	pET11a- <i>ftsZ</i> in BL21(DE3); FtsZ overproducer	46
WM971	WM746 with <i>leu::Tn10</i> replaced by <i>leu::Tn5</i>	H. Erickson
WM1099	TX3772 <i>leu::Tn10</i>	33
WM1109	TX3772 <i>leu::Tn10 ftsA12</i> (Ts)	52
WM1115	TX3772 <i>ftsZ84</i> (Ts)	14
WM1125	CH2 ( <i>recA::Tn10, ftsA<sup>0</sup></i> )/pDB280 ( <i>repA</i> (Ts) <i>ftsA<sup>+</sup></i> )	52
WM1281	CH5 ( <i>recA::Tn10 \Delta zipA::kan</i> )/pCH32 ( <i>repA</i> (Ts) <i>zipA<sup>+</sup> ftsZ<sup>+</sup></i> )	23
WM1282	TX3772 $\Delta zipA::kan, ftsA^*$ (R286W)	22
WM1657	TX3772 <i>ftsA<sup>*</sup></i> (R286W)	14
WM1659	MG1655 <i>ftsE::kan</i> (FB21223, Tn5- <i>Sce-I</i> at position 284)	14
WM2497	DY330 $\Delta ftsX::kan$	F. Blattner
WM2650	W3110 $\Delta ftsX::kan$	This study
WM2712	W3110 + pWM2270	This study
WM2933		This study
Plasmids		
pWM176	Tet <sup>r</sup> ; IncP plasmid containing the <i>tac</i> promoter	35
pMK4	Tet <sup>r</sup> ; <i>E. coli</i> wild type <i>ftsZ</i> ( <i>ftsZ<sub>1-383</sub></i> ) in pWM176	32
pWM1201	Tet <sup>r</sup> ; pWM176- <i>ftsZ\Delta C2</i> ( <i>ftsZ<sub>1-316</sub></i> )	33
pDSW209	<i>P<sub>trc</sub>-gfp</i> (no stop codon) pBR322 derivative (Amp <sup>r</sup> )	49
pWM2244	<i>pflag-MAC</i> cloning vector (Amp <sup>r</sup> )	Sigma-Aldrich
pWM2270	<i>pflag-ftsE</i>	This study
pWM2529	<i>pflag-ftsE ftsX</i>	This study
<i>pflag-Spe-Sse1D8N</i>	<i>pflag-MAC</i> with SpeI site expressing <i>Saccharomyces cerevisiae</i> Sse1 D8N mutant with N-terminal <i>flag</i>	44
<i>pflag-BAP</i>	<i>pflag-MAC</i> expressing <i>E. coli phoA</i> with N-terminal <i>flag</i>	Sigma-Aldrich
pWM2800	pDSW209 with <i>P<sub>trc</sub>-ftsE, (gfp-ftsE, ftsX)</i>	This study
pWM2853	pDSW209 with <i>P<sub>trc</sub>-ftsE (gfp-ftsE)</i>	This study
pWM2654	pWM176 with <i>P<sub>tac</sub>-ftsZ<sub>1-268</sub></i>	This study
pWM2655	pWM176 with <i>P<sub>tac</sub>-ftsZ<sub>1-218</sub></i>	This study

NaCl or other electrolytes, including NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl, and CaCl<sub>2</sub> (11, 16). Recently, Reddy demonstrated that high-osmolarity conditions alone suppress the loss of FtsE and further showed that increased expression of *ftsQAZ* or *ftsN*, either of which can suppress the loss of FtsK (15, 20), can also compensate for the loss of FtsE (42). Consistent with the idea that FtsE and FtsX are cell division proteins, disruption of FtsE/FtsX functions in other bacteria is also associated with cell division defects (3, 29, 36). Because of the position of FtsE and FtsX relatively early in the recruitment pathway, we became interested in understanding how these proteins might function in cytokinesis and influence the activity of the Z ring. In this study, we provide evidence for how FtsE is targeted to the Z ring.

#### MATERIALS AND METHODS

**Growth conditions.** LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl) was used for routine growth experiments. High-salt and no-salt LB media contained 1% or 0% NaCl, respectively, instead of 0.5%. Kanamycin (50  $\mu$ g/ml), ampicillin (100  $\mu$ g/ml), or tetracycline (10  $\mu$ g/ml) was used as necessary. IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) at 400 mM was kept as a stock solution in *N,N*-dimethyl formamide.

**Strain construction.** Strains and plasmids are listed in Table 1. To delete *ftsX* from the chromosome, we constructed PCR primers CTCACCTGAGCGAT

GGTCACTTGCATGGAGGCGTGGGCCGCCACGTTGTGTCTC and ACGGAACCCATTGCAGGGAAGAGTATAACACGCTTTTAGAAAACTCA TCGAGC to amplify the kanamycin resistance cassette from plasmid pUC4K. The PCR product consisted of the 813-bp Kan<sup>r</sup> cassette with an additional 40 bp upstream and 36 bp downstream, corresponding to the flanking regions of *ftsX* (underlined in the primer sequence). The linear DNA product was cut with DpnI and transformed into DY330 competent cells as described previously (50), and transformants were selected on high-salt LB containing kanamycin. A phage P1 lysate was made from this strain (WM2650) and used to transduce W3110 to Kan<sup>r</sup> on high-salt LB to generate strain WM2712. We confirmed that this strain had *ftsX* deleted, as we were unable to amplify *ftsX* from the chromosome with PCR primers internal to the gene and could obtain a PCR product corresponding to the size of the kanamycin cassette following amplification of *ftsX* flanking regions. In addition, cell division was largely rescued in high salt; most cells were highly filamentous in salt-free medium, whereas 50% of the cells were slightly filamentous in high salt.

**Plasmid construction.** Plasmid pWM2270 (*pflag-ftsE*) was constructed by amplifying *ftsE* from the chromosome of TX3772 using primers FtsE fp EcoR1b (CGGGAATTCATGATTCGCTTTGAA; restriction site is underlined) and FtsE rv *Kpn* (CGGGGTACCTTCATGCCCCAGCC). The product was then cloned as a EcoRI-KpnI fragment into pWM2244. Plasmid pWM2529 was constructed by amplifying *ftsE* and *ftsX* from the chromosome using primers FtsE fp EcoR1b (CGGGAATTCATGATTCGCTTTGAA) and FtsX rp *Kpn* (CGGGTACCTTCAGGCGTAAAGTG). This 1,728-bp DNA product was then cloned as an EcoRI-KpnI fragment into pWM2244.

To make C-terminal deletions of FtsZ, we used primer FtsZfwd (ACGAAGCTTAGGCGACAGGCACAAATCGGAGAG) and reverse primer ftsZdelta804 (AAAACCTGCAGTTAGAAGCCCCGCGTGATGTTAA) or ftsZdelta654 (AAA

ACTGCAGTTAAGACATTACGGTGCCTACGT). FtsZ PCR products were cloned as HindIII-PstI fragments into pMK4. To make an N-terminal FtsZ deletion, we amplified FtsZ using primer Zdelta93 (ACGAAGCTTAGCGACAGGCACAAATCGGAGAGAACTATGGAGCGCATTGAAGGT) and primer Zrprtruncations (CGCTCTAGAAGTACTGGATCCCCGGGCTGCAGAA) and cloned the PCR product as a HindIII-PstI fragment into pMK4.

Plasmid pDSW209 (49), containing a weakened *trc* promoter, was used to express IPTG-inducible *gfp* fusions to *ftsE*. The *ftsE* gene was amplified from TX3772 chromosomal DNA by PCR using primers GFP-ftsEX-F-EcoRI (CCG GAA TTC ATG ATT CGC TTT GAA CAT) and GFP-ftsE-R-HindIII (CGG AAG CTT TTA TTC ATG GCC CAC GCC T). The PCR product was cloned as an EcoRI-HindIII fragment into pDSW209, resulting in pWM2853 (expressing *gfp-ftsE*). Plasmid pDSW209-*ftsE-ftsX* was constructed using the same strategy, except that the *ftsEX* genes from the chromosome were amplified in tandem by PCR using the antisense primer GFP-ftsEX-R-HindIII (CGG AAG CTT TTA TTC AGG CGT AAA GTG) and the sense primer GFP-ftsEX-F-EcoRI as described above. The resulting plasmid, pWM2800, coexpressed *gfp-ftsE* and *ftsX*. Top10 or XLI-blue was used as a recipient strain for the initial cloning and characterization of these plasmids.

**Immunoprecipitation.** Unless otherwise indicated, bacterial cultures (5 ml) were grown in LB plus antibiotic to an optical density at 600 nm (OD<sub>600</sub>) of 0.1, and then 0.5 mM IPTG was added to induce expression of *pflag-ftsE* for 2 to 4 h. Equal numbers of cells, as measured by OD<sub>600</sub>, were pelleted, resuspended in 1 ml RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% beta-mercaptoethanol, 50 μl protease inhibitor cocktail (1 tablet Roche complete, mini, EDTA-free, dissolved in 1.5 ml RIPA buffer), and 100 μg/ml lysozyme, and incubated on ice for 45 min. The cells were then sonicated for four 10-s cycles (20% duty cycle; output, 4) using a Branson sonifier 250 microtip apparatus. The suspension was then incubated at 4°C for 4 h with vigorous shaking, followed by centrifugation for 20 min to remove insolubilized material. The supernatant fraction was then collected and mixed with a 20-μl bed volume of protein A-Sepharose (Sigma-Aldrich) for 1 h to remove nonspecific binding proteins. An aliquot (50 μl) of precleared supernatant fraction was used as a control for total protein levels prior to immunoprecipitation. The remainder of this mixture was collected and incubated with a 50-μl bed volume of protein A-Sepharose and 2 μl of anti-FLAG M2 monoclonal antibody (Sigma) for 2 h or overnight. Beads were washed three times with RIPA buffer, resuspended in loading buffer, and boiled for 15 min. Samples were subsequently analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting.

FtsZ depletion strains used to perform immunoprecipitation reactions were grown at 42°C for 5 h (at which time 0.5 mM IPTG was added to induce expression of the pWM176-*ftsZ* derivative) and allowed to grow for an additional 2 h before being harvested. To conduct immunoprecipitation reactions from the *ftsA12*(Ts) strain WM1115, cells were grown at the permissive temperature of 30°C to early log phase and then shifted to the nonpermissive temperature of 42°C for 1 h before being harvested. For immunoprecipitation from the ZipA depletion strain WM1282, the strain was grown at 42°C for 4 h, followed by IPTG induction (0.5 mM) of *flag-ftsE* for 1 h before the cells were harvested. For immunoprecipitation from the *ftsX* deletion strain WM2712, 10-ml cultures were grown in LB plus 1% NaCl medium at 30°C for 4 h. Then, the culture was split, with 5 ml pelleted and resuspended in LB without added NaCl. IPTG (0.5 mM) was added to both high-salt and no-salt cultures, which were grown for one additional hour at 30°C before being harvested.

**Protein purification and immunoblotting.** FtsZ was purified from WM971 as described previously (4). For purification of FLAG-FtsE to test for FtsZ copurification, W3110 cells harboring pWM2270 (WM2933) were grown to an OD<sub>600</sub> of 0.6 at 37°C and then induced for four more hours with 0.5 mM IPTG. The cell pellet was then incubated with buffer and 0.4 mg/ml lysozyme at 0°C for 1 h, sonicated, and centrifuged at 19,800 × g for 25 min. The membrane fraction was resuspended in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl)–1% NP-40–0.1 mM CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} for 1 h at 0°C, sonicated, kept at 0°C for 1 h, and centrifuged at 264,000 × g for 30 min. The clear supernatant fraction was passed twice through an anti-FLAG M2 (Sigma-Aldrich; A2220) affinity column and washed with 20 column volumes of TBS, and FLAG-FtsE was eluted with 0.1 M glycine (pH 3.5) and neutralized by combining aliquots with 1/10 volume of 1 M Tris-HCl, pH 8.0; the column was also neutralized with the same buffer and stored in TBS-50% glycerol-0.02% NaN<sub>3</sub>.

Proteins from this experiment and from immunoprecipitations were separated by SDS-polyacrylamide gel electrophoresis and either stained with Coomassie blue or transferred to nitrocellulose membranes for immunoblotting. To detect

FtsZ or green fluorescent protein (GFP), blots were probed with affinity-purified polyclonal antibodies against either FtsZ or GFP and goat anti-rabbit antibody conjugated to horseradish peroxidase. To detect FLAG, blots were probed with monoclonal anti-FLAG and goat anti-mouse secondary antibody conjugated to horseradish peroxidase. The membranes were then developed using standard enhanced-chemiluminescence reagents (Amersham or Pierce).

**Localization of GFP fusion proteins in live cells.** GFP-FtsE produced from pWM2800 (coproduced with FtsX) was visualized in wild-type cells either with or without IPTG to induce higher levels of expression. In general, uninduced levels of GFP fusion protein were sufficient for visualization; the promoter on this plasmid is sufficiently weak that induction with IPTG only modestly increased the fluorescence intensities of the bands. To examine GFP-FtsE localization in the *ftsA12*(Ts) strain WM1115 or the *ftsZ84*(Ts) strain WM1125, cells with the pWM2800 plasmid were grown at 30°C to early exponential phase and then shifted to the nonpermissive temperature of 42°C for 90 to 120 min to inactivate FtsA or FtsZ. To examine GFP-FtsE localization in the ZipA depletion strain WM1282 or the FtsA depletion strain WM1281, cells with the pWM2800 plasmid were grown to early exponential phase and shifted to 42°C for 2.5 to 4 h to dilute out the thermosensitive plasmid carrying *zipA* or *ftsA* and thus deplete the cells of the ZipA or FtsA protein. To visualize GFP-FtsE in other strains, such as WM1657, WM1659, or the *ftsE* null mutant WM2497, cells with pWM2800 or the plasmid producing GFP-FtsE without FtsX (pWM2853) were grown in LB at 30°C to mid-exponential phase. In all cases, the cells were immobilized in LB containing low-melting-point agarose and visualized by fluorescence microscopy with an Olympus BX60 epifluorescence microscope containing a 100× oil immersion objective. Images were captured with QED software and a Photometrics Coolsnap fx charge-coupled device camera.

**Localization of FLAG-FtsE by immunofluorescence microscopy.** To observe the localization of FLAG-FtsE, cells were grown to mid-log phase and fixed with methanol as described previously (51). Each sample was processed using anti-FLAG M2 monoclonal antibody (1:200) and goat anti-mouse secondary antibody conjugated to rhodamine red (1:500; Molecular Probes) with an incubation period of 1 h.

## RESULTS

**A *flag-ftsEX* fusion construct is functional.** To elucidate the function of FtsE and to facilitate purification, the entire *ftsE* gene, coding for a 24-kDa protein, was cloned downstream of the *tac* promoter in a *pflag*-MAC cloning vector. This newly created vector, referred to as pWM2270, produced FtsE with an amino-terminal FLAG tag. Most cells producing the FLAG-FtsE fusion protein had a phenotype similar to that of wild-type cells, although a small percentage of cells were 1.5 to 3 times longer than normal. Immunofluorescence microscopy experiments with antibody directed against the FLAG epitope revealed that the majority of cells examined contained intense fluorescent staining at midcell (Fig. 1A), suggesting that the FLAG-FtsE fusion protein targeted the Z ring correctly. However, there was also a high background of extraneous fluorescence that made it difficult to evaluate the dependency of FLAG-FtsE localization on other cell division proteins in filamentous cells; this was addressed with a GFP fusion (see below).

Although FtsE is required for bacterial cell division, the requirement can be largely suppressed when cells are grown in high salt (1% NaCl). In salt-free medium (0% NaCl), however, cell division was blocked as growth continued, resulting in cell filamentation and loss of viability, as observed previously (43). To further characterize the functionality of the FLAG-FtsE fusion construct, we investigated whether this fusion was sufficient to complement an *ftsE* allele disrupted by insertion of a kanamycin cassette (WM2497; *ftsE::kan*) under no-salt growth conditions. Analysis of cell growth on salt-free plates incubated overnight revealed that the *ftsE::kan* strain expressing FLAG-FtsE was capable of forming colonies (Fig. 1E, spot 3), whereas

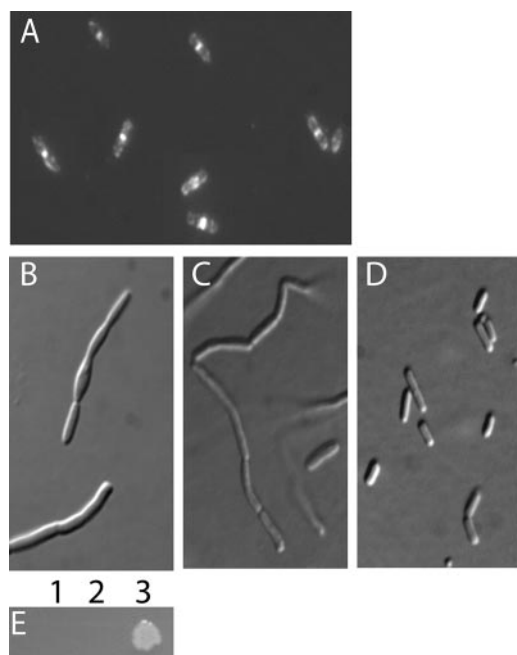


FIG. 1. A *flag-ftsE* fusion is functional when coexpressed with *ftsX*. (A) W3110 cells expressing *flag-ftsE* from pWM2270 that were fixed and immunostained with anti-FLAG antibody. (B to D) Micrographs (differential interference contrast) of the *ftsE::kan* strain (WM2497) grown in LB no-salt growth medium with no plasmid (B), *pflag-ftsE* plasmid pWM2270 (C), or plasmid pWM2529 expressing both *flag-ftsE* and *ftsX* (D). IPTG (100  $\mu$ M) was added to cells in panels A to C to induce expression of *flag-ftsE* from pWM2270, but basal levels of expression were sufficient to allow complementation by pWM2529 in panel D. (E) Colony growth overnight on LB no-salt agar of the *ftsE::kan* strain (WM2497) containing either no plasmid (lane 1), *pflag* vector pWM2244 (lane 2), or *pflag-ftsE* plasmid pWM2270 (lane 3).

cells containing no plasmid, or empty vector alone, failed to grow (Fig. 1E, spots 1 and 2). Although expression of FLAG-FtsE permitted colony growth, the cells in the colonies were filamentous (data not shown), similar morphologically to the *ftsE::kan* strain (Fig. 1B) or *ftsE::kan*/FLAG-FtsE cells grown in no-salt LB with expression of FLAG-FtsE induced with 100  $\mu$ M IPTG (Fig. 1C). This suggested that our FLAG-FtsE fusion, even when expressed at high levels, was not completely able to complement the *ftsE::kan* strain.

We reasoned that this might be caused by polarity of the Tn5 insertion within *ftsE* on the downstream *ftsX* gene and therefore cloned *ftsX* downstream of the *flag-ftsE* construct in the plasmid. This plasmid carrying *flag-ftsE-ftsX* (pWM2529) was sufficient to complement the *ftsE::kan* strain completely, allowing growth of colonies and mostly normal division of cells without IPTG induction (Fig. 1D). Therefore, we can conclude that our FLAG-FtsE fusion protein is fully functional when co-synthesized with FtsZ.

**FtsE interacts with FtsZ.** To decipher the role of FtsE in bacterial cell division, and to rectify the specific gap in knowledge about which proteins interact with FtsE, we used co-immunoprecipitation to investigate possible interaction partners. Using antibodies directed against the FLAG epitope, we were able to immunoprecipitate FLAG-FtsE (Fig. 2A, lane 4, top). Because FtsZ is abundant in the cell and we have a good

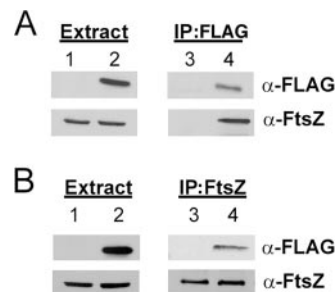


FIG. 2. Identification of an interaction between FtsE and FtsZ. (A) Immunoblots from a typical immunoprecipitation experiment performed on RIPA-solubilized total cell protein with anti-FLAG. (B) Immunoblots from a typical immunoprecipitation experiment performed on RIPA-solubilized total cell protein with anti-FtsZ. Antibodies used to probe the blots are outlined on the right. In all panels, lanes 1 and 3 contain cell extracts from Top10 cells expressing a *pflag* vector (pWM2244) control, whereas lanes 2 and 4 contain cell extracts from Top10 cells expressing *pflag-ftsE* (pWM2270).

polyclonal antibody against it, we wanted to see if FtsE could pull down a complex that contained FtsZ. Probing with anti-FtsZ, we found that FLAG-FtsE efficiently pulled down FtsZ (Fig. 2A, lane 4, bottom). In the converse experiment, we could immunoprecipitate FtsZ with anti-FtsZ (Fig. 2B, lanes 3 and 4, bottom) and were able to detect FLAG-FtsE in the precipitated mixture (Fig. 2B, lane 4, top).

FtsZ was immunoprecipitated equally efficiently in cells expressing either the *pflag* vector control (Fig. 2B, lane 3, bottom) or *pflag-ftsE* (Fig. 2B, lane 4, bottom), but a band corresponding to FLAG-FtsE was detected only in the reactions that included FLAG-FtsE (Fig. 2B, compare lanes 3 and 4, top). At the time the immunoprecipitation reactions were performed, the concentrations of FtsZ protein were similar in all extracts tested (Fig. 2A and B, lanes 1 and 2, bottom). The anti-FLAG antibody was specific in detecting the FLAG-FtsE protein (Fig. 2A and B, lanes 1 and 2, top).

To confirm that FtsZ interacted with the FtsE portion of FLAG-FtsE and not with the FLAG tag, we performed parallel immunoprecipitations with FLAG-FtsE and other FLAG-tagged proteins. These included FLAG-SSE1, a yeast chaperone protein, and FLAG-AP (*E. coli* alkaline phosphatase). Both were efficiently expressed in *E. coli* cells from a derivative of the same *pflag*-MAC vector (Fig. 3B, right). Blots of the immunoprecipitated proteins from the corresponding cell extracts revealed that FtsZ was pulled down only by FLAG-FtsE, indicating that FtsZ interacts with FLAG-FtsE but not FLAG-SSE1 or FLAG-AP (Fig. 3A, left). FtsZ levels were similar in all the extracts used (Fig. 3A, right).

Coomassie blue staining of the corresponding SDS gels of immunoprecipitated extracts showed that the predominant bands were the FLAG-tagged proteins and the immunoglobulin G (IgG) heavy and light chains (Fig. 3B, left). Interestingly, several faint bands between 40 and 50 kDa were detectable in the FLAG-FtsE lane but absent in the others, suggesting that they were specifically pulled down by FtsE. One band at  $\sim$ 40 kDa (Fig. 3B) was suggestive of FtsZ. Probing with anti-ZipA antibodies revealed a faint signal just below the IgG heavy chain in the FLAG-FtsE lane that might be ZipA (data not shown). Two other faint bands larger than 80 kDa

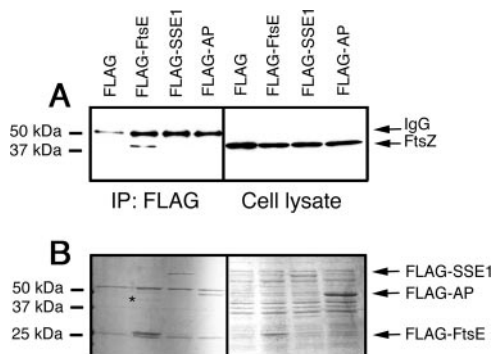


FIG. 3. FtsZ is pulled down by FLAG-FtsE but not by other FLAG-tagged proteins. (A) Immunoblots of RIPA-solubilized total cell protein (right) or after immunoprecipitation with anti-FLAG (left) probed with anti-FtsZ antibodies. The positions of protein size markers, FtsZ, and the IgG heavy chain are shown. The sources of extracts were strains overproducing either FLAG alone, FLAG-FtsE, FLAG-SSE1, or FLAG-AP (see Materials and Methods). (B) Coomassie-stained SDS-polyacrylamide gels corresponding to the blots in panel A; size markers and the positions of the overproduced FLAG fusion protein bands are shown. A band in the FLAG-FtsE lane presumed to be FtsZ is indicated by the asterisk.

were also visible only in the FLAG-FtsE pull-down, but their identities are unknown.

**FtsZ-FtsE interaction is independent of FtsA or ZipA.** According to the linear-dependency order of recruitment, the only two proteins localizing to the septum following FtsZ, but prior to FtsE, are ZipA and FtsA (43). Thus, we investigated if we could delete ZipA or inactivate FtsA and still retain an FtsE-FtsZ complex. To inactivate FtsA, we utilized a strain containing an *ftsA12*(Ts) allele and performed immunoprecipitation reactions on FLAG-FtsE and FtsZ from cells grown at both permissive and nonpermissive temperatures. To remove ZipA, we utilized a strain harboring a gain-of-function mutation in FtsA, R286W (also known as FtsA\*), which allowed the viability of a  $\Delta zipA$  null allele (14).

When ZipA was absent (Fig. 4, lane 3 top and bottom) or FtsA was thermoinactivated (Fig. 4, lane 4, bottom), immunoprecipitation of FLAG-FtsE with anti-FLAG still resulted in the efficient detection of FtsZ. The intensities of the FtsZ bands on the immunoblot were roughly equivalent to those from the permissive temperature for *ftsA12*(Ts) (Fig. 4, lane 4, top) or from wild-type cells (lane 2, top and bottom). As expected, no FtsZ was detected when only the *pflag* vector was used in the immunoprecipitation reaction (lane 1), indicating that the FtsZ pull-down was FtsE-specific. These results suggest that FtsE and FtsZ can interact in the absence of functional FtsA or in the absence of ZipA when compensated for by the FtsA R286W bypass mutant.

To further confirm that the FtsZ-FtsE interaction was independent of ZipA, we tested for the ability of FLAG-FtsE to pull down FtsZ from extracts of a ZipA depletion strain lacking the *ftsA*\* suppressor. After ZipA depletion in this strain at 42°C, cells cease dividing and form long filaments, similar to those carrying the *ftsA12*(Ts) allele. As with the  $\Delta zipA$  *ftsA*\* strain, FtsZ was pulled down by FLAG-FtsE (Fig. 4A, lane 5) after significant depletion of ZipA (Fig. 4B). Therefore, inactivation of the two known intermediary proteins between FtsE

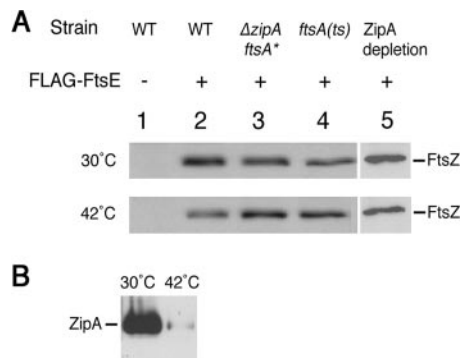


FIG. 4. The FtsE-FtsZ interaction is independent of FtsA or ZipA. (A) Immunoblots of immunoprecipitation experiments performed on RIPA-solubilized total cell protein by using anti-FLAG and subsequently probed with anti-FtsZ antibodies. Cells were grown at permissive (top) or nonpermissive (bottom) temperatures. Solubilized total proteins were from cells expressing either the *pflag* vector pWM2244 (lane 1) or *pflag-ftsE* plasmid pWM2270 (lanes 2 to 5). Cell lysates were from the TX3772 wild-type (WT) parent strain (lanes 1 and 2), a strain (WM1657) that lacks *zipA* but contains the *ftsA*\* (R286W) suppressor mutation and therefore lacks ZipA at both 30°C and 42°C (lane 3), cells containing an *ftsA12*(Ts) allele (WM1115) that is functional at 30°C but thermoinactivated at 42°C (lane 4), or a ZipA depletion strain that contains ZipA at 30°C but is depleted of ZipA after 4 h at 42°C (lane 5). Lanes 1 to 4 are all from one blot, and lane 5 is from a separate blot. (B) Extent of ZipA depletion for the experiment in lane 5 of panel A; extracts from equivalent densities of cells grown at 30°C or 42°C for 4 h were probed with anti-ZipA antibodies, and the resulting ZipA protein band, which migrates at ~50 kDa, is highlighted.

and FtsZ in the linear protein recruitment pathway failed to disrupt formation of an FtsE-FtsZ complex by coimmunoprecipitation, further supporting the idea that FtsZ and FtsE interact.

**FtsZ copurifies with FLAG-FtsE.** To provide further evidence for an interaction between FtsE and FtsZ, we tested whether FtsZ could copurify with FLAG-FtsE. FLAG-FtsE was overproduced, and the soluble and membrane fractions were then subjected to affinity purification with M2-agarose beads containing anti-FLAG. Whereas the soluble fraction became purer, we were more successful in obtaining largely pure FLAG-FtsE from the membrane fraction (Fig. 5, left), although two bands of larger molecular mass remained visible in the affinity-purified preparation. It is possible that one of these, running at ~55 kDa, was GroEL, an abundant protein that was shown previously to specifically interact with FtsE (7).

This partially purified FLAG-FtsE preparation was then used to probe for FtsZ on immunoblots. As shown in Fig. 5 (right), FtsZ was present at roughly equivalent levels in the crude membrane fraction of overproduced FLAG-FtsE, where it represented a very small fraction of the total protein, and the purified FLAG-FtsE preparation. Other purified FLAG-FtsE preparations also contained FtsZ (data not shown). These results strongly suggest that FtsZ copurifies with overproduced FLAG-FtsE and support the idea that FtsZ and FtsE interact.

**The conserved C-terminal tail of FtsZ is not required for the interaction with FtsE.** FtsZ interacts with FtsA and ZipA via 12 amino acids within its conserved C-terminal tail (23, 26, 30, 33). Because this C-terminal tail of FtsZ is essential in medi-

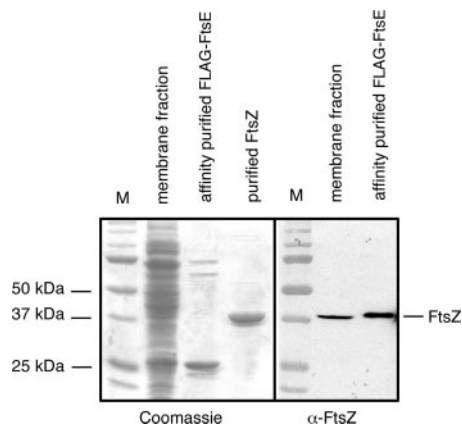


FIG. 5. FtsZ copurifies with FLAG-FtsE. FLAG-FtsE was overproduced from WM2933 and purified as described in Materials and Methods. The Coomassie-stained SDS-polyacrylamide gel of the crude membrane preparation and affinity-purified FLAG-FtsE is shown in the left panel (middle lanes), along with molecular mass markers (M) and purified FtsZ (outer lanes). An immunoblot made from the same preparations and probed with anti-FtsZ is shown in the right panel, along with the same mass markers, showing that FtsZ copurifies with FLAG-FtsE.

ating its interaction between ZipA and FtsA, we hypothesized that the domain might be important for FtsE binding.

To test this idea, we performed immunoprecipitation reactions with anti-FLAG, using FLAG-FtsE and several C-terminal truncations of FtsZ. Because FtsZ is essential for viability, we utilized an FtsZ depletion strain that contains a null allele of the chromosomal *ftsZ*, a thermosensitive plasmid expressing *ftsZ* for depletion of wild-type FtsZ upon temperature shift, and an additional IncP-based low-copy-number plasmid that expresses various deletions of *ftsZ* from the *tac* promoter (33). After growth at 42°C and induction with IPTG, the plasmid with the wild-type *ftsZ* was removed, depleting the wild-type protein and leaving the truncated FtsZ derivatives as the sole FtsZ proteins in the cells. Extracts from these cells were then used in coimmunoprecipitations with FLAG-FtsE, and the abilities of various FtsZ truncation derivatives to be pulled down with FLAG-FtsE were examined.

Immunoblot analysis of FtsZ protein levels within the cell confirmed that at the time the immunoprecipitation reactions were performed, the only detectable form of FtsZ within the cell was the full-length FtsZ (Fig. 6A, lane 3) or the C-terminal FtsZ truncation (Fig. 6A, lane 4). These levels of FtsZ were comparable to those of native FtsZ in wild-type cells (data not shown). Cell extracts of control strains containing an empty IncP vector with (Fig. 6A, lane 2) or without (lane 1) expression of *flag-ftsE* from the *pflag* vector did not contain detectable levels of FtsZ at the time they were used for the immunoprecipitation experiments.

Surprisingly, the truncated FtsZ derivative (FtsZ<sub>1-316</sub>) missing its entire C-terminal 67 amino acids was reproducibly pulled down by FLAG-FtsE (Fig. 6B, lane 4), although the amount coimmunoprecipitated was reproducibly less than that for full-length FtsZ, based on band intensities (Fig. 6B, compare lanes 3 and 4). Because deletion of just 12 residues from the C terminus of FtsZ is normally sufficient to abolish its interaction with ZipA and FtsA, it was striking that we could

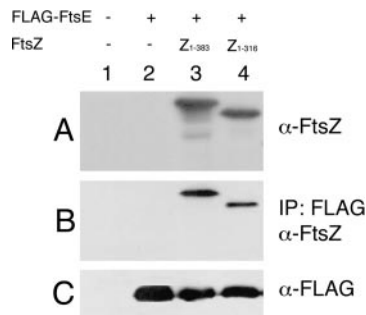


FIG. 6. The FtsZ C-terminal tail is not required for the FtsE-FtsZ interaction. Immunoprecipitation experiments were performed with anti-FLAG antibody on RIPA-solubilized total protein from cells depleted of native FtsZ but expressing various forms of FtsZ from a plasmid. Immunoblots from these experiments are shown. (A) Extracts of cell lysates prior to immunoprecipitation probed with anti-FtsZ antibody. (B) Immunoprecipitation reactions probed with anti-FtsZ antibody. (C) Whole-cell lysates immunoprecipitated and probed with anti-FLAG antibody. Lane 1 contains extracts from cells that do not express FtsZ or the pFLAG empty vector. Extracts in lane 2 are from cells producing FLAG-FtsE but not FtsZ. Lanes 3 and 4 contain extracts from cells synthesizing wild-type FtsZ or FtsZ<sub>1-316</sub>, respectively. WM1099 was the strain background used for all of the FtsZ depletion extracts.

remove this domain, as well as much of the flexible linker domain (residues 317 to 371), and still detect a functional interaction between FtsE and FtsZ.

This result prompted us to investigate whether more extensive C-terminal truncations of FtsZ were also able to interact with FtsE. FtsZ<sub>1-268</sub> and FtsZ<sub>1-218</sub> lack not only the C-terminal tail and flexible linker regions, but also large portions of the conserved C-terminal globular domain (37). These FtsZ derivatives were synthesized in the depletion strain but were probably insoluble, as they were not detectable in the lysate just prior to immunoprecipitation (data not shown) and consequently were not pulled down by FLAG-FtsE (data not shown). Therefore, the only firm conclusion we can draw is that the C-terminal tail and flexible linker regions of FtsZ are not required for its interaction with FtsE.

**The FtsE-FtsZ interaction is independent of FtsX.** FtsX is the membrane component of the hypothetical FtsEX ABC transporter. Subcellular fractionation studies have shown that FtsE is incorporated into the membrane upon overexpression of FtsX. In addition, FtsE has been shown to pull down FtsX upon overexpression and copurify with a histidine-tagged FtsX (11). Because FtsE interacts with FtsX and is incorporated into the membrane upon overexpression of FtsX, we wanted to investigate whether FtsX was necessary for the FtsE-FtsZ interaction.

To address this question, we first constructed a  $\Delta$ *ftsX::kan* strain, which could survive when grown on high salt. We then used extracts from  $\Delta$ *ftsX::kan* cells with or without *pflag-ftsE* for immunoprecipitation reactions to determine if FLAG-FtsE could pull down FtsZ in a  $\Delta$ *ftsX::kan* background. Cells were either grown in 1% NaCl to suppress the division defect of the  $\Delta$ *ftsX::kan* allele or switched from 1% to 0% for 1 h to create nonpermissive conditions for cell division. Immunoblots probed for FtsZ revealed that FLAG-FtsE could pull down FtsZ in extracts lacking FtsX (Fig. 7, top, lanes 3 and 6) at

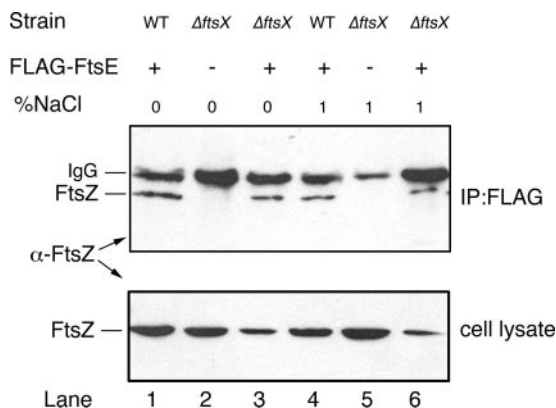


FIG. 7. The FtsE-FtsZ interaction is not dependent on FtsX. Shown are immunoblots, probed with anti-FtsZ, of immunoprecipitations (IP) with anti-FLAG antibody (top) or RIPA-solubilized total cell protein (bottom). Extracts were prepared from cells containing either the *pflag* empty vector (lanes 2 and 5) or *pflag-ftsE* (lanes 1, 3, 4, and 6) grown in LB with either 0% or 1% NaCl added, as described in Materials and Methods. The extracts used in lanes 2 and 3 and lanes 5 and 6 are from cells with a  $\Delta ftsX::kan$  insertion (WM2712), whereas cell extracts used in lanes 1 and 4 are from the wild-type (WT) parent strain W3110. The positions of FtsZ (~40 kDa) or the IgG heavy chain (~50 kDa) are shown. Lower levels of FtsZ in lanes 3 and 6 may have resulted from toxicity of FLAG-FtsE overproduction in the absence of chromosomal *ftsX*.

levels similar to the wild-type background (top, lanes 1 and 4). The ability of FtsZ to be pulled down by FLAG-FtsE in all cases was independent of the presence or absence of NaCl in the growth medium. Control immunoprecipitations from extracts lacking FLAG-FtsE did not pull down FtsZ (Fig. 7, top, lanes 2 and 5). FtsZ levels were similar in *ftsX*<sup>+</sup> strains expressing FLAG-FtsE and *ftsX* mutant strains not expressing FLAG-FtsE (Fig. 7A, lanes 1, 2, 4, and 5). The band intensities of FtsZ were lower in extracts from *ftsX* mutant strains expressing FLAG-FtsE, possibly because of toxicity; nevertheless, FtsZ was still pulled down by FLAG-FtsE in these extracts (Fig. 7A, lanes 3 and 6). These results suggested that the FtsE-FtsZ interaction does not require FtsX, is not affected significantly by FtsX, and is independent of added NaCl.

**Targeting of GFP-FtsE to Z rings and its dependence on ZipA and FtsA.** To provide *in vivo* evidence for FtsE-FtsZ interaction, we asked whether FtsE could localize to Z rings independently of other cell division proteins. Other division proteins that interact directly with FtsZ, such as FtsA, ZipA, and ZapA of *E. coli*, do not depend on any known divisome proteins other than FtsZ for their localization to Z rings (21, 23, 31). As mentioned above, detection of FLAG-FtsE by immunofluorescence was complicated by the high background fluorescence often observed, particularly in filamentous cells. Therefore, we constructed a GFP-FtsE fusion to detect FtsE localization in live cells. However, in wild-type cells, GFP-FtsE fluorescence was usually uniformly distributed throughout the cell, and midcell bands were difficult to detect. This poor localization of GFP-FtsE to Z rings was also noted in a previous study (43).

Because FtsE and FtsX interact and the *ftsEX* gene cluster was more effective at complementing the *ftsE* null allele than *ftsE* alone, as shown above, we reasoned that including the *ftsE*

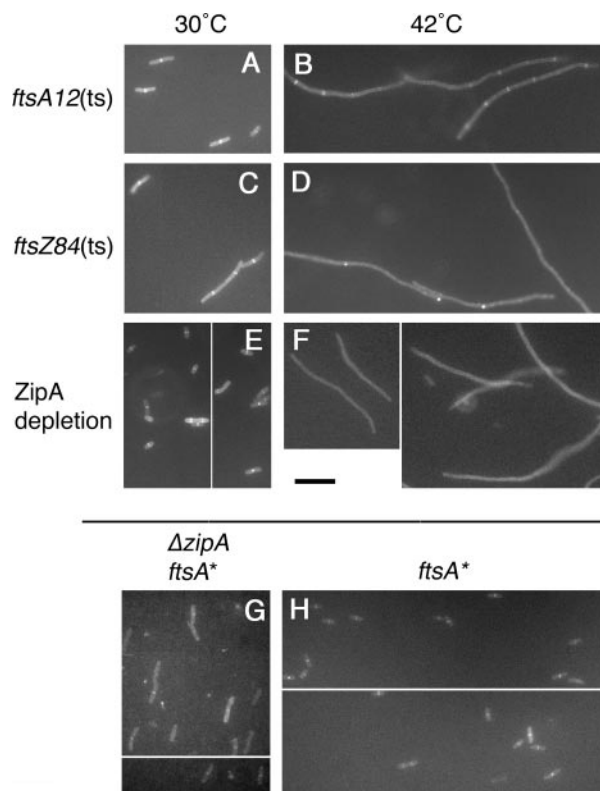


FIG. 8. Requirements for localization of GFP-FtsE to Z rings. Shown are fluorescence micrographs of representative fields of cells producing GFP-FtsE and FtsX from plasmid pWM2800 in the *ftsA12(Ts)* strain WM1115 at 30°C (A) or after 90 min at 42°C (B), in the *ftsZ84(Ts)* strain WM1125 at 30°C (C) or after 90 min at 42°C (D), in the ZipA depletion strain WM1282 at 30°C (E) or after 5 h at 42°C (F), and in an *ftsA*<sup>\*</sup> strain either lacking (G; WM1657) or containing (H; WM1659) the *zipA* gene, both at 30°C. Panels E, F, G, and H are subdivided to show two representative fields of cells. Scale bar, 10 μm.

gene downstream of the *gfp-ftsE* construct might provide the proper ratio of FtsE to FtsX to potentially stabilize the GFP-FtsE fusion. This indeed seemed to be the case, as expression of *gfp-ftsE-ftsX* from pDSW209-*ftsE-ftsX* gave rise to fluorescent bands at midcell in most cells in the population in a variety of strains that were proficient for cell division (Fig. 8A, C, E, and H). Moreover, the GFP-FtsE fusion protein made from pDSW209-*ftsE-ftsX* was functional, because it permitted colony formation of the *ftsE::kan* strain WM2497 on LB plates lacking added NaCl and produced fluorescent bands at division sites in these cells (data not shown). The failure of GFP-FtsE alone to localize was not simply because of protein degradation, as the low levels of the fusion protein were similar to those made in the presence of coproduced FtsX, as determined by immunoblotting with anti-GFP (data not shown). GFP-FtsE also did not cause destabilization of Z rings, because wild-type cells producing GFP-FtsE alone without extra FtsX divided mostly normally (data not shown).

We then tested whether localization of GFP-FtsE (again, with *ftsX* expressed *in cis*) to potential division sites required FtsZ, ZipA, or FtsA, with the idea that GFP-FtsE should be able to localize to Z rings in the absence of ZipA or FtsA if FtsE interacted directly with FtsZ. As expected, GFP-FtsE

exhibited diffuse localization in *ftsZ84*(Ts) cells at 42°C, a temperature at which Z rings are absent in this mutant, indicating that FtsE localization to division sites requires the presence of Z rings (Fig. 8D). GFP-FtsE also failed to localize to Z rings in filaments depleted of ZipA (Fig. 8F) but localized proficiently to potential division sites in *ftsA12*(Ts) filaments at 42°C (Fig. 8B) or in filaments depleted of FtsA in strain WM1281 (data not shown). These results indicate that localization of FtsE was dependent on ZipA but independent of FtsA.

The dependence of FtsE localization on ZipA would seem at first to contradict the idea that FtsE and FtsZ interact independently of ZipA. However, our previous isolation of the *ftsA*\* (R286W) mutant that could bypass the requirement for ZipA for cell division prompted a reassessment of how proteins are recruited to the divisome: proteins dependent on ZipA for recruitment, such as FtsK, FtsQ, or FtsN, are not actually recruited directly by ZipA but instead most likely depend on an indirect effect of ZipA on the Z ring that can be bypassed by an altered FtsA. As a result, we explored the possibility that the requirement for ZipA in FtsE recruitment was also indirect. GFP-FtsE (with coproduced FtsX) was localized in WM1657, which contains *ftsA*\* and a deletion of *zipA*. As predicted, GFP-FtsE could localize to midcell in the slightly elongated cells of this strain (Fig. 8G), although the midcell bands were sometimes more difficult to visualize than those in the *zipA*<sup>+</sup> parent strain, WM1659 (Fig. 8H). These results support the idea that targeting of GFP-FtsE to the Z ring does not require ZipA itself, but rather a state of the Z ring enhanced by ZipA.

**Minicells induced by expression of *flag-ftsE-ftsX*.** Overproduction of FtsE and FtsX together, as well as FtsX alone, causes cell filamentation and death (11). Cell division is also inhibited when some other cell division proteins, such as FtsZ or ZipA, are overproduced to high levels, suggesting that stoichiometries of different components of the divisome are crucial for proper function (10, 12, 22). However, overproduction of FtsZ and FtsA severalfold over native levels resulted in the formation of minicells, which result from cytokinesis at cell poles, as well as midcell, indicating that cell division was being stimulated (2, 47). We were interested to explore whether production of lower levels of FtsE and/or FtsX might give rise to more subtle and perhaps more interesting cell division phenotypes in cells containing chromosomal *ftsEX* genes. Top10 cells were transformed with pWM2529, containing an IPTG-dependent *flag-ftsE* fusion as well as *ftsX* that was able to complement the *ftsE::kan* strain (Fig. 1D). Growth of the transformants in Top10, which also contains a chromosomal copy of *ftsEX*, required addition of 1% glucose to inhibit expression of the plasmid-borne *flag-ftsE-ftsX*. Colonies were then inoculated into LB broth with or without glucose, and the cell morphology was examined after a few hours of growth.

Cells grown in 1% glucose had a normal morphology, with only 0.3% of the cell population displaying a minicell phenotype (Fig. 9A). However, cells grown without glucose, which presumably allowed higher expression levels of *flag-ftsE-ftsX*, failed to grow on agar plates, although they were able to grow in broth, where they exhibited a high proportion (~20%) of minicells (Fig. 9B). It is not clear why cells without glucose were unable to form colonies on plates yet formed minicells without significant filamentation in broth, but this phenotype is

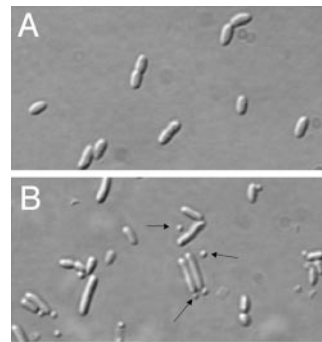


FIG. 9. Minicells in a strain expressing FLAG-FtsE-FtsX. Shown are composite differential interference contrast micrographs of Top10 cells containing the *pflag-ftsE ftsX* plasmid pWM2529 with (A) or without (B) glucose to control expression. The arrows highlight minicells.

reminiscent of moderate overproduction of FtsZ and FtsA and suggests that higher levels of FtsE and FtsX may make FtsZ more resistant to the MinC division inhibitor (38). A smaller proportion of minicells was also observed when *flag-ftsE-ftsX* from pWM2529 was expressed without IPTG in the *ftsE::kan* strain (data not shown). However, this effect was not observed in several other strain backgrounds, including TX3772, suggesting that a rather narrow window of FtsE-FtsX protein overproduction and/or strain background may be required to cause minicell formation.

## DISCUSSION

Most of the proteins of the *E. coli* divisome have likely been identified, and several previous studies have identified or inferred a number of interactions among cell division proteins. However, the interactions between FtsE, which was recently demonstrated to be a component of the divisome, and other proteins of the divisome were not investigated in those studies.

In the present work, we identified an interaction between FtsE and FtsZ by coimmunoprecipitation of FLAG-FtsE and FtsZ from *E. coli* cell extracts. We also showed that affinity-purified FLAG-FtsE is enriched for FtsZ and that the interaction is not mediated via the FLAG epitope. These results suggest that FtsZ-FtsE interaction may be direct. This idea is supported by the ability of FLAG-FtsE to pull down FtsZ in the absence of functional FtsA, ZipA (with or without FtsA R286W), or FtsX (with or without suppressing levels of salt), which are the nearest neighbors of FtsE in the Z-ring recruitment pathway. The ability of FtsE to localize to Z rings in the absence of FtsA is entirely consistent with a direct interaction with FtsZ, as ZipA and ZapA, which interact directly with FtsZ, also localize to Z rings independently of FtsA. All other known *E. coli* divisome proteins require FtsA for their recruitment to the Z ring.

Nevertheless, if FtsE interacts directly with FtsZ, why would GFP-FtsE fail to localize to Z rings in ZipA-depleted filaments? ZipA is not well conserved among bacteria, and FtsA R286W can bypass the requirement for ZipA (14). However, FtsA R286W can also suppress other divisome defects, and the molecular mechanism of suppression by R286W is not known. Therefore, it is possible that R286W does not specifically re-



place ZipA but instead generally stabilizes divisome components, compensating for the weakening of the divisome when a component, such as ZipA, is inactivated. We propose that ZipA probably does not directly recruit cell division components that depend on ZipA for proper targeting to the Z ring, including FtsEX, but instead indirectly enhances the Z ring's ability to recruit these proteins. The mechanism of this enhancement is unknown but may be more efficient anchoring of the Z ring to the membrane (39) and/or increased bundling of FtsZ protofilaments (25, 41).

There are other potential explanations for the failure to detect GFP-FtsE rings in ZipA-depleted filaments. One is that FtsZ did not localize to rings in these filaments, which would prevent GFP-FtsE localization to division sites. However, while the frequency of rings was lower in ZipA-depleted strains producing GFP-FtsEX, many rings remained, ruling out this possibility (data not shown). Another possibility is that the GFP-FtsE fusion was unstable under these conditions. Immunoblotting indicated that the fusion was expressed at low but detectable levels after the several hours at 42°C needed to deplete ZipA compared to the time at 30°C, and there was no detectable increase in degradation of the fusion protein at 42°C (data not shown). It also should be noted that GFP-FtsE localized proficiently at 42°C in the *ftsA*(Ts) and FtsA depletion strains, so it is likely that protein degradation is not a major issue. We cannot, however, rule out possible subtle effects of the GFP tag on the conformation of FtsE, which would lead to the apparent dependence on ZipA.

The presence of FtsX, which interacts with FtsE, potentially complicates any interpretations of localization requirements. For example, FtsX may also interact directly with FtsA and FtsQ (28). In support of this, previous results showed that localization of GFP-FtsX to Z rings required both ZipA and FtsA (43), in contrast to the clearly FtsA-independent localization of FtsE we observed here. Because our GFP-FtsE fusion without coproduced FtsX did not localize well and because of high nonspecific fluorescence with the FLAG-tagged FtsE, we were unable to determine conclusively whether localization of FtsE depends on FtsX. The ability of FLAG-FtsE to coimmunoprecipitate FtsZ in the absence of FtsX, however, suggests that FtsE probably does not need FtsX for its targeting to the Z ring. This idea is consistent with the ability of GFP-FtsE to localize to Z rings independently of FtsA, in contrast to GFP-FtsX. However, it is also possible that FtsX helps target FtsE to Z rings via its association with the membrane. Such membrane targeting is important for the proper midcell localization of other FtsZ-interacting proteins, such as FtsA or the C-terminal domain of MinC (27, 39). The requirement for coproduced FtsX for localization of GFP-FtsE to Z rings suggests that the ratio between FtsE and FtsX is critical for their proper function. Another possibility, which cannot be ruled out at present, is that the GFP tag causes FtsE to fail to localize under conditions (such as lack of ZipA or coproduced FtsX) that are normally permissive for its localization.

The C-terminal tail of FtsZ is required for its interaction with FtsA and ZipA and anchoring to the cytoplasmic membrane (25, 33, 45). However, this domain of FtsZ is not required for interaction with FLAG-FtsE by coimmunoprecipitation. Although the SOS-inducible division inhibitor SulA binds to the conserved C-terminal globular domain of FtsZ (9),

this is the first evidence of a domain of FtsZ apart from its C-terminal tail interacting with an essential divisome protein. Clearly, considerably more work will be needed to define precisely which determinants of FtsZ are necessary for its interaction with FtsE.

The role of FtsEX in *E. coli* cell division is unknown. The fact that FtsEX function is not essential for cell division at high osmotic strength suggests that other factors can compensate under some conditions. Indeed, it was recently found that expression of extra *ftsQAZ* or *sufI* can bypass the requirement for FtsEX (42). This is consistent with other reports of multiple overlapping functions of some other essential cell division proteins of *E. coli* (14, 15, 40). Whereas the localization of a number of cell division proteins is dependent on FtsEX (43), this dependence is probably a reflection of its effect on the Z ring and not direct recruitment by FtsEX, similar to the ZipA effect discussed above, because FtsEX is dispensable under certain conditions. Therefore, FtsEX, like ZipA, probably functions to maintain the integrity of the Z ring during its assembly and constriction. This idea is supported by the appearance of filamentous  $\Delta$ *ftsE::kan* cells grown in no salt, which have clear indentations that may be aborted attempts at septation. The efficient production of minicells when FtsEX is overproduced under certain conditions suggests that FtsEX has a stimulatory effect on Z-ring activity. It remains to be determined precisely how FtsEX influences the Z ring, what other proteins are contacted, and how the putative transporter function of FtsEX acts in this process.

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