Interaction between Cell Division Proteins FtsE and FtsZ

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Received 11 October 2006/Accepted 2 February 2007

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 cytoplasmic 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 an ftsA‡ 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, 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 provided to 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%
In this study, we provide evidence for how FtsE is targeted to function in cytokinesis and influence the activity of the Z ring. We became interested in understanding how these proteins might be recruited to the Division Site. We identified FtsE and FtsX relatively early in the recruitment pathway, we begin to understand how these proteins might function in cytokinesis and influence the activity of 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 μg/ml), ampicillin (100 μg/ml), or tetracycline (10 μg/ml) was used as necessary. IPTG (isopropyl-β-D-thiogalactopyranoside) at 400 mM was kept as a stock solution in N,N-dimethyl formamide.

**Plasmids.** For construction of Kanr or Crer strains, plasmids pPM2244 and pPM2270 were used. For construction of Cre recombinase strains, plasmid pPM176 was used.

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

\[
\text{GTCACCTGCACTGAGGCGGCGCGCCACGTTGTCTC} \quad \text{and ACGGAACCTTAGGAGGAAAGAAGTATAACGCTTGAAGAAACTCA} \quad \text{TCGACG} \\
\text{TAC} \quad \text{GAGTTCACTCATGATTGCAGTGGG} \quad \text{TGCCTAGATTGAAGAATTCA} \quad \text{TAC} \\
\text{TCGACG} \quad \text{amp} \quad \text{kan} \quad \text{resistance} \quad \text{cassette} \quad \text{from} \quad \text{plasmid} \quad \text{pUC4K}.
\]

The PCR product consisted of the 813-bp Kan 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 D330 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’. The resulting strain was then used 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 filamentous in high salt, whereas 50% of the cells were filamentous in salt-free medium, whereas 50% of the cells were filamentous in high salt.

**Plasmid construction.** Plasmid pPM2270 (pFlag-ftsX) was constructed by amplifying ftsX from the chromosome of T. thermophilus. The PCR product consisted of the 813-bp Kan 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 D330 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’ 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.

**Strain or plasmid** | **Description** | **Source or reference**
--- | --- | ---
**E. coli** strain | | |
TX3772 | MG1655 ΔlacU169 | Laboratory collection
Top10 | F- mcrA (mcr-hsdRMS-mcrBC) Δ80lacZΔM15 lacY74 recA1 ara139 (ara-lev)7697 galU galK rpsL endA1 mupG | Invitrogen
XL1-Blue | lac (F'proAB lacP2ΔZΔM15 Tn5) | Stratagene
W3110 | Wild-type strain | Laboratory collection
DY330 | W3110 ΔlacU169 galU900 lacI857 Δ(cro-bioA) | 50
WM746 | WX7/pCMX1, leu::Tn10 ftsZ’ (repA4(Ts) ftsZ’ Δ) | 46
WM971 | pET11α-ftsZ in BL21(DE3); FtsZ overproducer | H. Erickson
WM1099 | WM746 with leu::Tn10 replaced by leu::Tn5 | 33
WM1109 | TX3772 leu::Tn10 | 52
WM1115 | TX3772 leu::Tn10 ftsA12(Ts) | 14
WM1125 | TX3772 ftsZ84(Ts) | 52
WM1281 | CH2 (recA::Tn10, ftsA4) pDB280 (repA4(Ts) ftsA4) | 23
WM1282 | CH5 (recA::Tn10 ΔzipA::kan) pCH32 (repA4(Ts) zipA Δ ftsZ’ Δ) | 22
WM1657 | TX3772 ΔzipA::kan, ftsA’(R268W) | 14
WM1659 | TX3772 ftsA’(R268W) | 14
WM2497 | MG1655 ftsE::kan (FB2123, Tn5-Sce-I at position 284) | 52
WM2650 | DY330 ΔftsX::kan | This study
WM2712 | W3110 ΔftsX::kan | This study
WM2933 | W3110 + pPM2270 | This study

**Plasmids** | | |
pWM176 | Tet’, IncP plasmid containing the tac promoter | 35
pMK4 | Tet’, E. coli wild-type ftsZ (ftsZ1-268) in pWM176 | 32
pWM1201 | Tet’, ftsZ1-268 in pPM2264 | 33
pDSW209 | Prc-gfp (no stop codon) pBR322 derivative (Amp‘) | 49
pPM2244 | pflag-MAC cloning vector (Amp‘) | Sigma-Aldrich
pPM2270 | pflag-ftsZ | This study
pPM2529 | pflag-ftsZ ftsX | This study
pflag-Spe-SseID8N | pflag-MAC with SpeI site expressing Saccharomyces cerevisiae Sse1 D8N mutant with N-terminal flag | 44
pflag-BAP | pflag-MAC expressing E. coli phoA with N-terminal flag | Sigma-Aldrich
pPM2800 | pDSW209 with Prc-ftsE, gfp-ftsE, ftsX | This study
pPM2853 | pDSW209 with Prc-ftsE, gfp-ftsE | This study
pPM2654 | pPM176 with Phac-ftsZ1-268 | This study
pPM2655 | pPM176 with Phac-ftsZ1-218 | This study

NaCl or other electrolytes, including NaH₂PO₄, Na₂SO₄, NH₄Cl, and CaCl₂ (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.
FtsZ. PCR products were cloned as HindIII-PstI fragments into pMK4. For purification of FLAG-FtsE to test for FtsZ copurification from the ftsX gene, except that the ftsX gene was cloned as an EcoRI-HindIII fragment into pDSW209, resulting in pWMS283 (expressing gfp-fze). Plasmid pDWS209 (49), containing a weakened trc promoter, was used to express IPTG-inducible fze fusions to fze. The fze gene was amplified from TX3772 chromosomal DNA by PCR using primers GFP-ftsEX-EcoRII (CCG GAA TCC TTC ATT GTC CCT TGG GAT CAT) and GFP-EcoRII-HindIII (CCG AAG CTT TTA TTC ATG GCC CAC GAC T). The PCR product was cloned as an EcoRI-HindIII fragment into pDSW209, resulting in pWMS285 (expressing gfp-fze). Plasmid pDWS209-gfp-fze was constructed using the same strategy, except that the fzeEX genes from the chromosome were amplified in tandem by PCR using the antisense primer GFP-ftsEX-EcoRII (CCG GAA CTT TTA TTC ATG GCC CAC GAC T) and the sense primer GFP-ftsEX-EcoRII as described above. The resulting plasmid, pWMS280, coexpressed gfp-fze and fzeX. 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 (OD600) of 0.1, and then 0.5 mM IPTG was added to induce expression of pWMS280 for 2 to 4 h. Equal numbers of cells, as measured by OD600 nm, 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 µg protein inhibitor cocktail (1 tablet Roche complete, mini, EDTA-free, dissolved in 1.5 mM RIPA buffer), 100 µg/ml lysozyme, and incubated on ice for 45 min. The cells were then sonicated for 10-s cycles (20% duty cycle; output, 4) using a Branson sonifier 250 micropip apparatus. The suspension was then incubated at 4°C for 4 h with vigorous shaking, followed by centrifugation for 20 min to remove unsolubilized 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 non-specific 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-Aldrich) 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 pWMS270-fze derivative) and allowed to grow for an additional 2 h before being harvested. To conduct immunoprecipitation reactions from the ftsX84(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 30 min before being harvested. For immunoprecipitation from the ZipA deletion strain WM1282, the strain was grown at 42°C for 4 h, followed by IPTG induction (0.5 mM) of ftsX84 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 hour at 30°C to mid-exponential phase and then shifted to the nonpermissive temperature of 42°C for 90 to 120 min to inactivate FtsA or FtsX. To examine GFP-FtsE localization in the ZipA deletion strain WM1282 or the ftsX deletion strain WM1281, cells with the pWMS280 plasmid were grown to early exponential phase and then shifted to 42°C for 2.5 to 4 h to dilute out the thermosensitive plasmid carrying zipA or ftsX and thus deplete the cells of the ZipA or FtsX protein. To visualize GFP-FtsE in other strains, such as WM1657, WM1659, or the ftsX null mutant WM2497, cells with pWMS280 or the plasmid producing GFP-FtsE without FtsX (pWMS283) 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 100x 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-fzeEX fusion construct is functional. To elucidate the function of FtsE and to facilitate purification, the entire fze gene, coding for a 24-kDa protein, was cloned downstream of the trc promoter in a pFLAG-MAC cloning vector. This newly created vector, referred to as pWMS270, 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 fse allele disrupted by insertion of a kanamycin cassette (WM2497; fse::kan) under no-salt growth conditions. Analysis of cell growth on salt-free plates incubated overnight revealed that the fse::kan strain expressing FLAG-FtsE was capable of forming colonies (Fig. 1E, spot 3), whereas
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 ftsX strain. Two other faint bands larger than 80 kDa were the FLAG-tagged proteins and the immunoglobulin G heavy chain in the FLAG-FtsE lane that might be ZipA (data not shown). The anti-FLAG antibody was specific in detecting the FLAG-FtsE band at 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.

**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 coimmunoprecipitation 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 FtsE is abundant in the cell and we have a good 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 FtsE-FtsZ 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 ~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.
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 ΔzipA 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 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.

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 FLAG-FtsE and support 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-
with (Fig. 6A, lane 2) or without (lane 1) expression of Cell extracts of control strains containing an empty IncP vector ble to those of native FtsZ in wild-type cells (data not shown). truncation (Fig. 6A, lane 4). These levels of FtsZ were compara-
ments. Because FtsZ is essential for viability, we utilized an FtsZ depletion strain that contains a null allele 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). FtsZ in extracts lacking FtsX (Fig. 7, top, lanes 3 and 6) at

To test this idea, we performed immunoprecipitation reac-
ments were synthesized in the depletion strain but were prob-
that the domain might be important for FtsE binding.

To address this question, we first constructed a

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 de-

FIG. 5. FtsZ copurifies with FLAG-FtsE. FLAG-FtsE was over-

FIG. 4. FtsE and FtsZ interact in vivo. Immunoprecipitation reactions performed with anti-FLAG, using FLAG-FtsE and several C-termi-

FIG. 3. FtsE and FtsZ interact in vitro. Immunoblot made from 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

FIG. 2. FtsE coimmunoprecipitates with FtsZ and FtsA. Immunoblot made from the left lane (middle lanes), along with molecular mass markers (M) and purified FtsZ (outer lanes). A panel, along with the same mass markers, showing that FtsZ copurifies with FLAG-FtsE.

FIG. 1. FtsZ migrates as a single protein in SDS-polyacrylamide gels. 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.

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FIG. 1. FtsZ migrates as a single protein in SDS-polyacrylamide gels. 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.

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 exten-
sive C-terminal truncations of FtsZ were also able to interact with FtsE. FtsZ1-268 and FtsZ1-218 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 deriv-
atives were synthesized in the depletion strain but were prob-
ably insoluble, as they were not detectable in the lysate just prior to immunoprecipitation (data not shown) and conse-
quitently 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 in-
teraction.

To address this question, we first constructed a ∆ftsX::kan strain, which could survive when grown on high salt. We then used extracts from ∆ftsX::kan cells with or without pflag-ftsE for immunoprecipitation reactions to determine if FLAG-FtsE could pull down FtsZ in a ∆ftsX::kan background. Cells were either grown in 1% NaCl to suppress the division defect of the ∆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

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teraction.
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" 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 FtsE-FtsZ interaction 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 ftsX 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 F). 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 and FtsX from plasmid pWM2800 in the *ftsZ84 (Ts)* strain WM1125 at 30°C (D), or after 90 min at 42°C (E), in the ZipA depletion strain WM1282 at 30°C (F) or after 5 h at 42°C (G), and in an ftsA" 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.
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+ 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 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 communoprecipitation 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-FtsE, 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 conformations 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 sufl 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 ΔftsE::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.

ACKNOWLEDGMENTS

We thank Eric Cascales, Kevin Morano, and members of his laboratory for helpful advice. We are grateful to Fred Blattner for providing the ftsE::kan strain, Harold Erickson for the FtsZ overproducer (referred as WM971), and Patrick Gibney of the Morano laboratory for the SEE1 and BAP control plasmids.

This work was supported by a grant from the National Institutes of Health (R01-GM61074) to W.M. and a UT-TORCH Training Grant (5 T32 DE015355-04) to Y.W.

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