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A Predicted ABC Transporter, FtsEX, Is Needed for Cell Division in *Escherichia coli*

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FtsE and FtsX have homology to the ABC transporter superfamily of proteins and appear to be widely conserved among bacteria. Early work implicated FtsEX in cell division in *Escherichia coli*, but this was subsequently challenged, in part because the division defects in *ftsEX* mutants are often salt remedial. Strain RG60 has an *ftsE*::*kan* null mutation that is polar onto *ftsX*. RG60 is mildly filamentous when grown in standard Luria-Bertani medium (LB), which contains 1% NaCl, but upon shift to LB with no NaCl growth and division stop. We found that FtsN localizes to potential division sites, albeit poorly, in RG60 grown in LB with 1% NaCl. We also found that in wild-type *E. coli* both FtsE and FtsX localize to the division site. Localization of FtsX was studied in detail and appeared to require FtsZ, FtsA, and ZipA, but not the downstream division proteins FtsK, FtsQ, FtsL, and FtsI. Consistent with this, in media lacking salt, FtsA and ZipA localized independently of FtsEX, but the downstream proteins did not. Finally, in the absence of salt, cells depleted of FtsEX stopped dividing before any change in growth rate (mass increase) was apparent. We conclude that FtsEX participates directly in the process of cell division and is important for assembly or stability of the septal ring, especially in salt-free media.

In *Escherichia coli*, the division septum forms via the coordinated inward growth of all three layers of the cell envelope—the cytoplasmic membrane, the peptidoglycan wall, and the outer membrane. To date, about a dozen *E. coli* genes are known to be specifically required for septation (3, 11). These genes share two important properties: (i) loss of function mutations result in the formation of long, aseptate filaments with regularly spaced nucleoids (the *fts*, or filamentation temperature-sensitive phenotype), and (ii) the proteins encoded by these genes localize to the division site. Because cell division genes are generally essential and because lesions in many housekeeping genes can affect cell division indirectly, there have not been any exhaustive screens for division mutants. Thus, it seems likely that more division genes remain to be described.

A number of mutant hunts, starting with the pioneering work of Hirota and coworkers in the 1960s, suggested that there is an important cell division gene located at about 76 min on the *E. coli* chromosome (30). This locus was originally designated *ftsE*. One interesting property of *ftsE* mutants is that many are salt remedial, meaning that viability is restored by inclusion of NaCl in the growth medium. The amount of salt required for rescue is strain dependent, but generally in the range of 0.5%. Studies by Salmond and colleagues in the 1980s revealed that "*ftsE*" comprised two genes, which were then designated *ftsE* and *ftsX* (13). Moreover, the sequence of these genes revealed clear homology to ABC transporters; FtsE is the ATP-binding cassette (ABC) component, while FtsX is the membrane component. ABC transporters use energy from ATP to transport a wide variety of substrates either into or out of cells (or subcellular compartments). These observations led to the proposal that FtsEX transports an ion needed for division but not for growth per se (13).

Subsequently, Woldringh and colleagues questioned whether *ftsE* is really a division gene, after studying one allele and finding that this mutant produced filaments in broth but not minimal medium (34). Their thinking was influenced by having just completed a study of *ftsB*, which only filamented at high growth rates and turned out to be an allele of *nrd*, a gene needed for synthesis of DNA precursors (35). The view that *ftsEX* affects cell division indirectly seems to have gained ascendancy, as most of the review articles on bacterial cell division published in the last 10 years make no mention of *ftsE* or *ftsX* (e.g., references 11, 22, and 31), and recent work in *E. coli* has explored potential connections to membrane protein insertion (10, 37). During this same time period, however, mutants of *ftsE* and/or *ftsX* have been reported in *Flavobacterium*, *Neisseria*, and *Aeromonas* (2, 20, 24). Interestingly, these mutants are viable but have morphological defects suggestive of impaired division.

Here we report on experiments intended to determine whether FtsE and FtsX participate directly in cell division in *E. coli*. Our findings establish that FtsE and FtsX are bona fide division proteins.

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MATERIALS AND METHODS

Strains. Strains used in this study are listed in Table 1. RG60 has been described previously (10). Phage P1 grown on RG60 was used to transduce MG1655/pDSW610 to Kan^r to create EC1335. EC1335 was transduced to Amp^r

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant features	Source or reference
Strains		
MG1655	Wild type	Lab collection
DHB4	Wild type with F′ *lacI*^{q}	5
EC549	*ftsI*::Tn*phoA* (Kan^r)/pBAD33-*ftsI*	38
JOE170	*ftsQ*::Tn*phoA* (Kan^r)/pBAD33-*ftsQ*	8
JOE563	*ftsK*::*cat*/pBAD42-*ftsK*	7
JMG265	*ftsL*::Tn*phoA* (Kan^r)/pBAD33-*ftsL*	12
PS223	W3110 *zipA1*(Ts)	28
RG60	MG1655 *ftsE*::*kan*	10
EC1063	MG1655 Δ(*attL-lom*)::(*kan lacI*^{q} P_{204}-*ftsX-gfp*)	This study
EC1065	MG1655 Δ(*attL-lom*)::(*kan lacI*^{q} P_{206}-*gfp-ftsX*)	This study
EC1116	MG1655 *attϕ80*::pDSW533 (P_{206}-*gfp-ftsX*)	This study
EC1152	EC1116 *ftsA12*(Ts) leu::Tn*10*	This study
EC1158	EC1116 *ftsZ84*(Ts)	This study
EC1159	EC1116 *ftsW*::*kan*/pBAD33-*ftsW*	This study
EC1179	JOE170 *attϕ80*::pDSW533 (P_{206}-*gfp-ftsX*)	This study
EC1180	JMG265 *attϕ80*::pDSW533 (P_{206}-*gfp-ftsX*)	This study
EC1181	EC549 *attϕ80*::pDSW533 (P_{206}-*gfp-ftsX*)	This study
EC1295	JOE563 Δ(*attL-lom*)::(*kan* P_{204}-*ftsX-gfp*)	This study
EC1335	MG1655 *ftsE*::*kan*/pBAD33-*ftsEX*	This study
EC1340	PS223 *attϕ80*::pDSW533 (P_{206}-*gfp-ftsX*)	This study
EC1363	EC1335 Δ(*attL-lom*)::(*bla lacI*^{q} P_{206}-*ftsA-gfp*)	This study
EC1366	EC1335 Δ(*attL-lom*)::(*bla lacI*^{q} P_{204}-*gfp-ftsI*)	This study
EC1386	EC1335/pCH205 [P_{lac}-*ftsK(1–266)-gfp*]	This study and ref. 18
EC1391	EC1335 Δ(*attL-lom*)::(*bla lacI*^{q} P_{206}-*zipA-gfp*)	This study
EC1392	EC1335 Δ(*attL-lom*)::(*bla lacI*^{q} P_{206}-*gfp-ftsQ*)	This study
Plasmids		
pBAD33	Arabinose regulation (P_{BAD}), p15A *ori*, Cam^r	15
pDSW206	P_{206} promoter, ColE1 *ori*, Amp^r	38
pDSW209	*gfp*-fusion vector, P_{206} promoter, ColE1 *ori*, Amp^r	38
pDSW210	*gfp*-fusion vector, P_{206} promoter, ColE1 *ori*, Amp^r	38
pDSW533	pJC69-P_{206}-*gfp-ftsX* (*lacI*^{q}, *oriR*$_{R6Ky}$, *attP*$_{ϕ80}$, Spc^r)	This study and ref. 7
pDSW609	pTH18kr-*ftsE*-3xHA (P_{lac}, Kan^r, pSC101 *ori*)	This study and ref. 19
pDSW610	pBAD33-*ftsEX*	This study
pDSW621	pBAD33-*ftsE*	This study
pDSW622	pBAD33-*ftsX*	This study
pDSW636	pDSW209-*ftsX*	This study
pDSW637	pDSW210-*ftsX*	This study
pDSW638	pDSW206-*ftsE*-3xHA	This study

with P1 phage grown on EC447, EC436, EC535, and EC442 (8, 23, 38) to create EC1363, EC1366, EC1391, and EC1392, respectively. EC1335 was transformed with pCH205 (18) to create EC1386. EC1063 and EC1065 are derivatives of MG1655 and were constructed from pDSW513 and pDSW512 using λInCh1 pSX102(Amp^r) (5). EC1116 was constructed by integrating pDSW533 into *att*$_{ϕ80}$ of MG1655 (16). EC1116 was transduced to Tet^r with P1 grown on MM61 [*ftsA12*(Ts) *leu*::Tn*10*] or DRC14 [*ftsZ84*(Ts) *leu*::Tn*10*] to create EC1152 and EC1158, respectively. EC1159 was constructed by transducing EC1116/pDSW406 to Kan^r with P1 grown on EC912 (23). EC1179, EC1180, and EC1181 were constructed by integrating pDSW533 into *att*$_{ϕ80}$ of JOE170, JMG265, and EC549 (8, 12, 38). EC1295 was constructed by transducing JOE563 (7) to Kan^r with P1 grown on EC1063. EC1340 was constructed by transducing PS223 (28) to Kan^r with P1 grown on EC1065. EC1384 was constructed by transforming DHB4 (5) with pDSW609.

Plasmids. Plasmid pDSW512 (P_{206}-*gfp-ftsX*) was constructed by amplifying *ftsX* from the chromosome of MG1655 using primers P481 (CGAGAATTCAA CAACAACGTCACTTGCATGGAGGCGTGG) and P482 (TGCTCTAGATA TTCAGGCGTAAAGTGGCG). The 1,111-bp product was cut with *Eco*RI and *Xba*I (sites underlined) and ligated into the same sites of pDSW286, a Kan^r derivative of pDSW209 (38). pDSW513 (P_{204}-*ftsX-gfp*) was constructed similarly using primers P483 (CAGGAATTCGTCACTTGCATGGAGGCGTGG) and P484 (CTGCTGCAGGTTGTTGTTTTCAGGCGTAAAGTGGCGTAA), with the product being cloned into the *Eco*RI-*Pst*I sites of pDSW256, a Kan^r derivative of pDSW208 (38). To construct pDSW533 (P_{206}-*gfp-ftsX*, Spc^r, *attP*$_{ϕ80}$), pDSW512 was digested with *Mun*I and *Xba*I, and the 1,252-bp fragment carrying *ftsX* was isolated and ligated into the same sites of pJC118 (7). Plasmid pDSW609 was constructed in several steps. First, the triple hemagglutinin (HA) tag was PCR amplified from pMYP-3xHA (32) using primers P578 (catggaggcg tgggccatgaaaacaacaacTCTAGATACCCATACGATGTTCCTGAC) and P577 (CTGAAGCTTACTaAGCAGCGTAATCTGGAACGTC). The upstream primer has 20 bases homologous to the 3′ end of *ftsE* (lowercase letters), omitting the stop codon. The resulting 144-bp product was isolated and used as a primer in a second PCR together with P579 (CACGAATTCATAACACTTTT TGCCCGAGAGGATTAAC), which anneals to the 5′ end of *ftsE*. This reaction produced a 742-bp *ftsE*-3xHA fusion product that was digested with *Eco*RI and *Hin*dIII (sites underlined in P579 and P577) and ligated into the same sites of pTH18kr (19). Plasmid pDSW610 (pBAD33-*ftsEX*) was constructed in two steps. First, *ftsEX* was amplified from the chromosome of MG1655 with primers P477 (CAGCCATGGTTCGCTTTGAACATGTCAGC) and P488 (GTCAAGCTTA TTCAGGCGTAAAGTGGCGT). The 1,734-bp product was cut with *Nco*I and *Hin*dIII (sites underlined) and ligated into the same sites of pBAD24 (15) to create pDSW519. Then, the 1,799-bp *Bam*HI-*Hin*dIII fragment carrying *ftsEX* from pDSW519 was moved into the same sites of pBAD33 (15) to create pDSW610. The *ftsX* gene was deleted from pDSW610 by digestion with *Psh*AI and *Hin*dIII, followed by treatment with T4 DNA polymerase, and then ligation to create pDSW620 (pBAD33-*ftsE*). Similarly, *ftsE* was deleted by digesting pDSW610 with *Pci*I and *Sph*I, treatment with T4 DNA polymerase, and ligation to create pDSW621 (pBAD33-*ftsX*). Plasmid pDSW636 (P_{206}-*gfp-ftsX*) was constructed by ligating the 1,098-bp *Eco*RI-*Xba*I fragment carrying *ftsX* from pDSW512 into pDSW209 (38). The 1,095-bp *Eco*RI-*Pst*I fragment carrying *ftsX* from pDSW513 was ligated into the same sites of pDSW210 (38) to create pDSW637 (P_{206}-*ftsX-gfp*). Plasmid pDSW638 (P_{206}-*ftsE*-3xHA) was constructed

by cloning the 790-bp *Eco*RI-*Hin*dIII fragment carrying *ftsE* with three tandem repeats of the HA epitope tag into the same sites of pDSW206.

Media, chemicals, and molecular biological procedures. Luria-Bertani medium (LB) consisted of 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl (1% NaCl) and, for plates, 15 g of agar per liter. LB always contained 1% NaCl except where stated that it was omitted. Antibiotics were used at the following concentrations: 40 μg of kanamycin/ml, 30 μg of chloramphenicol/ml, 50 μg of spectinomycin/ml. L-Arabinose and D-glucose were used at 0.2%, unless otherwise indicated, to modulate expression from the *araBAD* promoter P_{BAD}. Isopropyl-β-D-galactopyranoside (IPTG) was added at the following concentrations: 5 μM (*gfp-ftsI*), 1 mM (*zipA-gfp*), 100 μM (*ftsA-gfp*), 50 μM (*ftsX-gfp*), 100 μM (*gfp-ftsX*), 40 μM (*ftsK (1-266)-gfp*), or 10 μM (*gfp-ftsQ*). Enzymes used to manipulate DNA were from New England Biolabs (Beverly, Mass.). Oligonucleotides were from Integrated DNA Technologies (Coralville, Iowa). DNA sequencing was performed by the DNA Core Facility of the University of Iowa. All constructs made by PCR were sequenced to verify their integrity.

Depletion of FtsEX. A culture of EC1335 (*ftsE*::*kan*/pBAD33-*ftsEX*) was grown overnight at 30°C in 5 ml of LB containing kanamycin, chloramphenicol, and arabinose. The next morning, this culture was diluted 1:20 into LB with no NaCl but containing kanamycin, chloramphenicol, 0.02% arabinose, and 0.2% glucose. This culture was grown at 30°C for 2 h to an optical density at 600 nm (OD_{600}) of ~0.5. Cells were washed to remove sugars by pelleting 1 ml of culture in a microcentrifuge and resuspending in 1 ml of LB with no NaCl. The washed cells were diluted 1:150 into a flask of LB with no NaCl but containing chloramphenicol and either arabinose or glucose. Growth and cell morphology were monitored periodically by the OD_{600} and with microscopy, respectively.

Localization of GFP fusions to FtsX. Strains EC1063 (P_{204}-*ftsX-gfp*) and EC1065 (P_{206}-*gfp-ftsX*) are MG1655 derivatives that harbor fusions of *gfp* to *ftsX* integrated into the chromosome in single copy at the λ attachment site. These strains were grown overnight at 30°C in LB-kanamycin. The next morning cultures were diluted 1:1,000 into LB without antibiotic but containing IPTG to induce expression of the *gfp* fusion, and cultures were grown to an OD_{600} of ~0.3 and then fixed in the growth medium with cross-linking agents and processed for fluorescence microscopy as described previously (38). Dependence of green fluorescent protein (GFP)-FtsX localization on other division proteins was determined in strains that harbored conditional alleles of these proteins. Growth of such strains under permissive and nonpermissive conditions has been described elsewhere (38). Briefly, cultures were grown in LB with IPTG to induce the *gfp* fusion and antibiotics to maintain any plasmids until early exponential phase, at which time cultures were shifted to the nonpermissive condition. Cells were fixed when they appeared filamentous. Typically, this was 1 h after shift to 42°C for Ts mutants and 4 h after dilution into glucose-containing medium for arabinose-dependent depletion strains.

Localization of GFP-Fts fusions in an FtsEX depletion background. Fusions of *gfp* to various division genes were introduced into the FtsEX depletion strain EC1335 by transduction or transformation. Depletion of FtsEX was done in LB with no NaCl as described above, except that the medium contained IPTG to induce the respective *gfp* fusion. Cells were fixed when the glucose-grown culture became filamentous and were examined by fluorescence microscopy.

Localization of FtsE-3xHA. A culture of DHB4/pDSW609 (P_{lac}-*ftsE*-3xHA) was grown overnight at 30°C in LB with kanamycin. This culture was diluted 1:200 into LB with 100 μM IPTG and grown at 30°C to an OD_{600} of ~0.5. Then, cells were fixed and processed for immunofluorescence microscopy as described previously (33). FtsE-3xHA was detected with anti-HA monoclonal antibody (HA.11; BabCo, Berkeley, Calif.) diluted 1:200 and incubated overnight, followed by goat anti-mouse antibody conjugated to Alexa488 (Molecular Probes, Eugene, Oreg.) diluted 1:200 and incubated for 2 h.

Localization of FtsN by immunofluorescence microscopy. Wild-type MG1655 or *ftsE*::*kan* mutant RG60 cells grown at 30°C in LB with 1% NaCl were fixed with cross-linking agents and processed for immunofluorescence as described previously (29). Purified anti-FtsN serum (39) was used at a dilution of 1:500 overnight at 4°C. The secondary antibody was goat anti-rabbit conjugated to Texas Red (Molecular Probes) at 1:400 for 2 h.

RESULTS

Localization of FtsN in RG60. Strain RG60 has been described elsewhere (10). It is an MG1655 derivative with an *ftsE*::*kan* insertion, now designated *ftsE400*::*kan*, that is predicted to be polar onto *ftsX*. RG60 was reported to be filamentous and required at least 0.5% NaCl for viability. Other electrolytes found to rescue RG60 on plates included NaH_2PO_4, Na_2SO_4, KCl, NH_4Cl, and $CaCl_2$, but not K_2SO_4, $MgSO_4$, or $MnCl_2$. Osmolytes such as sucrose, glycine betaine, and proline also failed to rescue RG60.

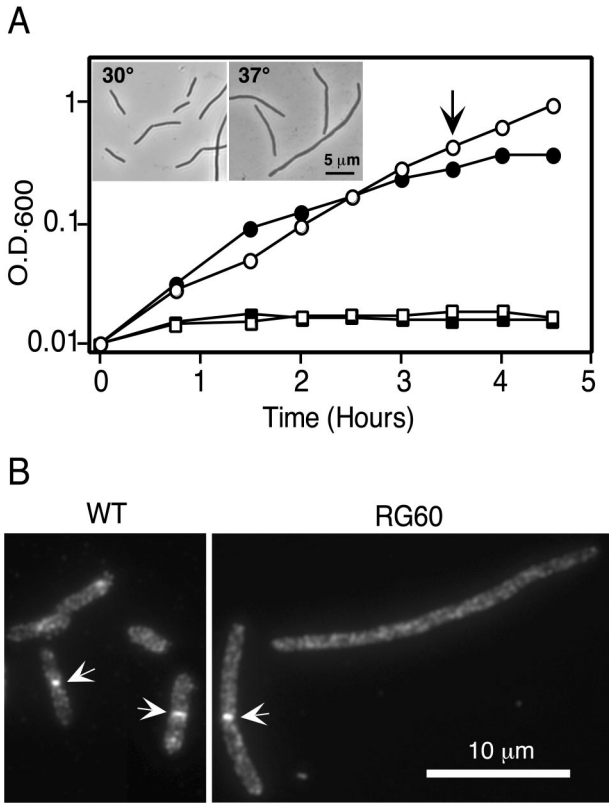

FIG. 1. (A) Effect of salt and temperature on growth of RG60. RG60 (*ftsE400*::*kan*) growing in LB with 1% NaCl at 30°C was subcultured into LB with 1% (circles) or no (squares) NaCl at 30°C (open symbols) or 37°C (closed symbols). The inset shows a phase-contrast micrograph of cells harvested at the time indicated by the arrow from the cultures growing with salt. (B) Localization of FtsN. Cells of wild type (MG1655) or RG60 in exponential growth in LB with 1% NaCl at 30°C were fixed, and FtsN was visualized by immunofluorescence microscopy. Arrows point to septal localization of FtsN.

We found that RG60 grew somewhat slowly and was mildly filamentous in standard LB broth, which contains 1% NaCl (Fig. 1A). These defects were more pronounced at elevated temperatures. Upon shift to LB broth that lacked NaCl, growth and division essentially stopped.

We attempted to transduce various *gfp-fts* fusion genes into RG60 so that we could determine whether the division block in this strain occurs before, during, or after assembly of the septal ring. These efforts were generally unsuccessful, although we could move the fusions into the parental strain, MG1655 (data not shown). Other approaches, such as transducing the *ftsE400*::*kan* allele into the *gfp* fusion strains or transforming RG60 with plasmids that express the fusions, also failed. In some cases we simply never recovered transductants or transformants, while in others we could recover them and confirm by PCR that they were correct, but they had a small colony phenotype and lysed when grown in broth, even with high salt. These findings suggest that the *gfp-fts* fusion genes are toxic

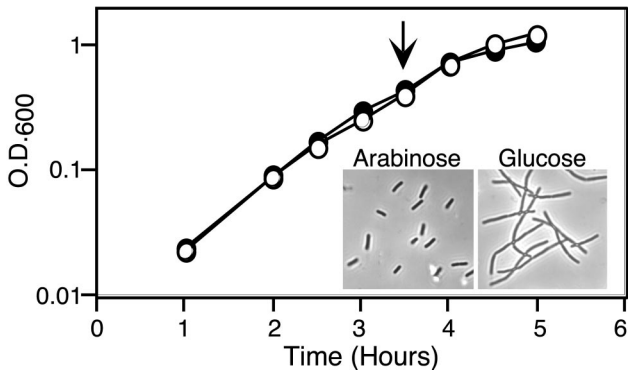

FIG. 2. Effect of FtsEX depletion on growth and division. EC1335 (*ftsE*::*kan*/pBAD33-*ftsEX*) was grown in LB with no NaCl but containing either arabinose (closed symbols) or glucose (open symbols) to modulate expression of the plasmid-borne *ftsEX* genes. Samples were removed periodically to monitor growth by OD_{600} or cell morphology. The inset shows a phase-contrast micrograph of cells harvested at the time indicated by the arrow.

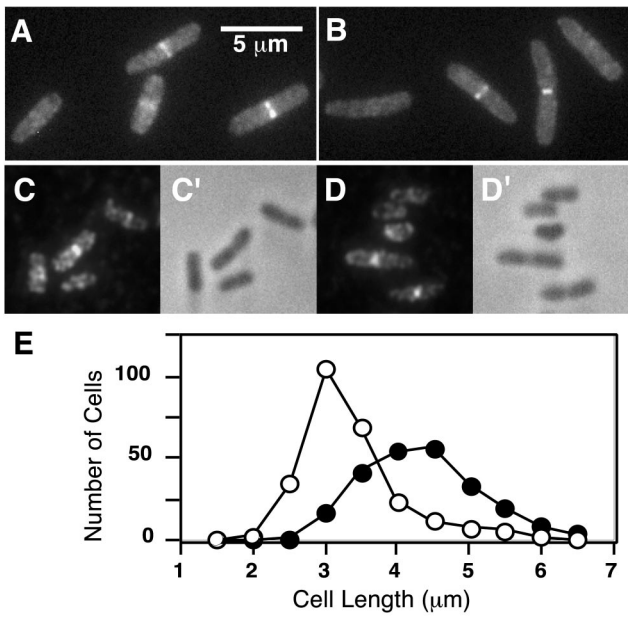

FIG. 3. Localization of FtsE and FtsX to the division site. (A to D) Cells in exponential growth in LB with NaCl were fixed and examined by fluorescence microscopy directly (A and B), by indirect immunofluorescence microscopy (C and D), or by phase-contrast microscopy (C′ and D′). Strains shown are EC1063 (P_{204}-*ftsX-gfp*) (A); EC1065 (P_{206}-*gfp-ftsX*) (B); DHB4/pDSW609 (P_{lac}-*ftsE*-3xHA) (C and D). (E) Relationship between cell length (age) and septal localization of FtsX. 509 cells of EC1063 were measured and scored for the presence (closed symbols) or absence (open symbols) of a fluorescent band at the midcell.

when combined with the *ftsE*::*kan* mutation, which is somewhat surprising because they are not very toxic in a wild-type background and some of them, like *gfp-ftsI*, complement (38).

Ultimately, we were able to use immunofluorescence microscopy to show that FtsN, a late recruit to the division site (1), can localize in RG60 grown under permissive conditions (1% NaCl, 30°C). About 60% of the RG60 cells exhibited FtsN localization, compared to about 35% of the cells of a wild-type control strain, MG1655 (Fig. 1B). However, the RG60 cells are long enough that close to 100% should exhibit FtsN localization if the *ftsE*::*kan* mutation has no effect on assembly or stability of the septal ring. Thus, FtsE improves, but is not required for, assembly or stability of the septal ring. This finding is consistent with RG60 having a leaky division defect.

Depleting cells of FtsEX blocks cell division. It is not obvious why an ABC transporter would be needed specifically for cell division, and strain RG60 grows poorly even in the presence of salt. These considerations suggested that the division defect in RG60 might be a secondary consequence of a metabolic defect that renders the cells generally unhealthy. We therefore constructed a strain, EC1335, in which FtsEX expression is under control of an arabinose-dependent promoter, P_{BAD} (15). In the presence of glucose, which prevents *ftsEX* expression, EC1335 formed colonies on LB plates that contained 1% NaCl, but not on LB plates that lacked NaCl. Colony formation in the absence of NaCl was rescued by arabinose. To investigate the relationship between cell division and overall health, EC1335 was grown in LB broth with no salt but containing 0.2% arabinose or 0.2% glucose. Both cultures grew at the same rate as judged by OD_{600}, but the glucose-grown cells stopped dividing after about 2 h and were clearly filamentous after about 3.5 h (Fig. 2). We conclude that the division defect is a primary defect rather than a secondary consequence of the cells becoming unhealthy.

FtsE and FtsX localize to the division site. To determine whether FtsE and FtsX localize to the division site, we fused *ftsE* and *ftsX* to *gfpmut2* (9), which encodes a bright variant of GFP. The fusion genes were integrated into the chromosome at the λ attachment site by selecting for a tightly linked kanamycin resistance marker (5). Expression of the *gfp* fusions was under control of an IPTG-inducible promoter. Strains were grown in LB containing IPTG to an OD_{600} of ~0.3, fixed with cross-linking agents, and examined by fluorescence microscopy. With FtsX-GFP and GFP-FtsX, about half of the cells had a bright band of fluorescence at the midcell (Fig. 3A and B). More precisely, the fraction of cells exhibiting septal localization was about 40% in an MG1655 background and about 60% in an MC4100 background. No other sites of localization were apparent. Convincing localization of GFP-FtsE and FtsE-GFP was not observed, perhaps because GFP interferes with the proper function of this protein. We therefore constructed a low-copy plasmid that expressed *ftsE* with three tandem copies of an HA epitope tag at the C terminus (32). Immunofluorescence microscopy revealed septal localization of the FtsE-3xHA fusion in about 50% of the cells (Fig. 3C and D). Similar fluorescent bands were not observed unless production of FtsE-3xHA was induced with IPTG, verifying the specificity of the antibodies used to detect the tagged protein. Cells with FtsE and FtsX at the midcell were on average longer than those without (Fig. 3E), indicating that these proteins are recruited to the division site during the later stages of cell growth and remain there until division is complete.

Complementation tests were used to determine whether the fusion genes used in the localization studies encoded functional division proteins. This was done by cloning the fusion genes into plasmids that confer Amp^r and express the fusions under control of a weak IPTG-inducible promoter. Plasmids

TABLE 2. Complementation of the *ftsE400*::*kan* allele in RG60[a]

Plasmid(s)	Arabinose	Glucose
pBAD33	−	−
pBAD33-*ftsE*	−	−
pBAD33-*ftsX*	−	−
pBAD33-*ftsEX*	+	−
pBAD33-*ftsE* + pDSW209 (vector)	−	−
pBAD33-*ftsE* + pDSW636 (*gfp-ftsX*)	+	−
pBAD33-*ftsE* + pDSW210 (vector)	−	−
pBAD33-*ftsE* + pDSW637 (*ftsX-gfp*)	+	−
pBAD33-*ftsX* + pDSW206 (vector)	−	−
pBAD33-*ftsE* + pDSW638 (*ftsE*-3xHA)	+	−

[a] Complementation was determined by colony formation when streaked onto LB plates with no NaCl but containing arabinose or glucose to modulate expression of genes under control of P_{BAD}. IPTG was not necessary for complementation.

that express *gfp-ftsX* or *ftsX-gfp* rescued growth of RG60 on LB plates lacking NaCl when cotransformed with pBAD33-*ftsE* (Table 2). Likewise, a plasmid that expressed *ftsE*-3xHA rescued RG60 when cotransformed with pBAD33-*ftsX*. We conclude that our fusions to *ftsE* and *ftsX* function in cell division. Moreover, these results confirmed the expected polarity of *ftsE400*::*kan* onto *ftsX*, since expression of *ftsE* alone was not sufficient to rescue RG60.

Position of FtsX in the recruitment pathway. Numerous studies of Fts protein localization in *E. coli* have revealed a set of dependencies that are generally interpreted to reflect the order of assembly of these proteins into a multiprotein complex, sometimes called the septal ring (reviewed in references 11, 22, and 31). The first protein to localize is FtsZ, which polymerizes into a ring on the inner surface of the cytoplasmic membrane. FtsA, ZipA, and ZapA (14) bind directly to FtsZ and localize next. Then come FtsK, FtsQ, FtsL/YgbQ (probably as a complex), FtsW, FtsI, FtsN, and AmiC. To determine where FtsX fits into this hierarchy, we examined the effects of inactivation of various *fts* genes on septal localization of FtsX fused to GFP. We also did a complementary set of experiments that investigated which Fts proteins do and do not localize properly upon depletion of FtsEX.

We introduced our fusions into strains that had conditional alleles of *ftsZ*, *ftsA*, *zipA*, *ftsK*, *ftsQ*, *ftsL*, or *ftsI*. Because several of these conditional mutants have kanamycin resistance elements inserted into the gene of interest, we subcloned our original *gfp-ftsX* fusion, which was linked to a kanamycin marker, into a plasmid that confers spectinomycin resistance (7). This plasmid, pDSW533, was then integrated into the chromosome of MG1655 at the attachment site for phage ϕ80 as described elsewhere (16) to create strain EC1116. About half the cells of EC1116 displayed localization of GFP-FtsX to the septal ring (Table 3), as was observed for the same fusion integrated at the phage λ attachment site in EC1065 (Fig. 3B).

Localization of GFP-FtsX was then assayed in filaments that formed upon inactivation or depletion of the indicated essential division proteins. These results are summarized in Fig. 4 and Table 3. GFP-FtsX did not localize in filaments that developed when FtsZ ring formation was inhibited by shift of an *ftsZ*(Ts) mutant to the nonpermissive temperature. Similar results were obtained when FtsZ ring assembly was blocked by induction of the FtsZ-binding protein *sulA* (25, 36) from a pBAD plasmid (38) in cells maintained at 30°C (data not shown). Inactivation of the *zipA1*(Ts) allele, or depletion of ZipA (17) (data not shown), also greatly reduced localization

TABLE 3. Localization of GFP-FtsX or FtsX-GFP in *fts* mutant backgrounds[a]

Mutant	Growth condition	No. of cells scored	Avg cell length (μm)	Total no. of FtsX rings	% Cells with a ring(s)	Spacing of rings[b]
Wild type	30°C	254	4.5	103	44	10
	42°C	225	7.2	91	40	18
Temperature-sensitive mutants						
ftsZ(Ts)	30°C	135	13	36	27	15
	42°C	101	34	0	0	>3,400
ftsA(Ts)	30°C	218	6.3	121	56	11
	42°C	112	36	95[c]	60	42
zipA(Ts)	30°C	174	4.0	53	30	13
	42°C	105	33	4	3.8	880
Depletion strains						
FtsK	Arabinose	208	3.0	112	54	5.7
	Glucose	145	18	261	98	10
FtsQ	Arabinose	187	6.9	123	66	11
	Glucose	132	28	231	91	18
FtsL	Arabinose	220	8.1	157	71	11
	Glucose	72	38	152	83	18
FtsW	Arabinose	265	4.5	113	43	11
	Glucose	61	29	104	89	17
FtsI	Arabinose	212	4.5	111	52	8.6
	Glucose	111	22	230	92	10

[a] Strains used were EC1116, EC1158, EC1152, EC1340, EC1295, EC1179, EC1180, EC1159, and EC1181.
[b] Units are micrometer per ring. The spacing is a measure of the frequency of rings per unit cell mass and was calculated by dividing the total number of rings (column 5) into the total length of cells or filaments scored (column 3 multiplied by column 4).
[c] These bands were faint, and so the numbers given understate the degree of dependence on FtsA.

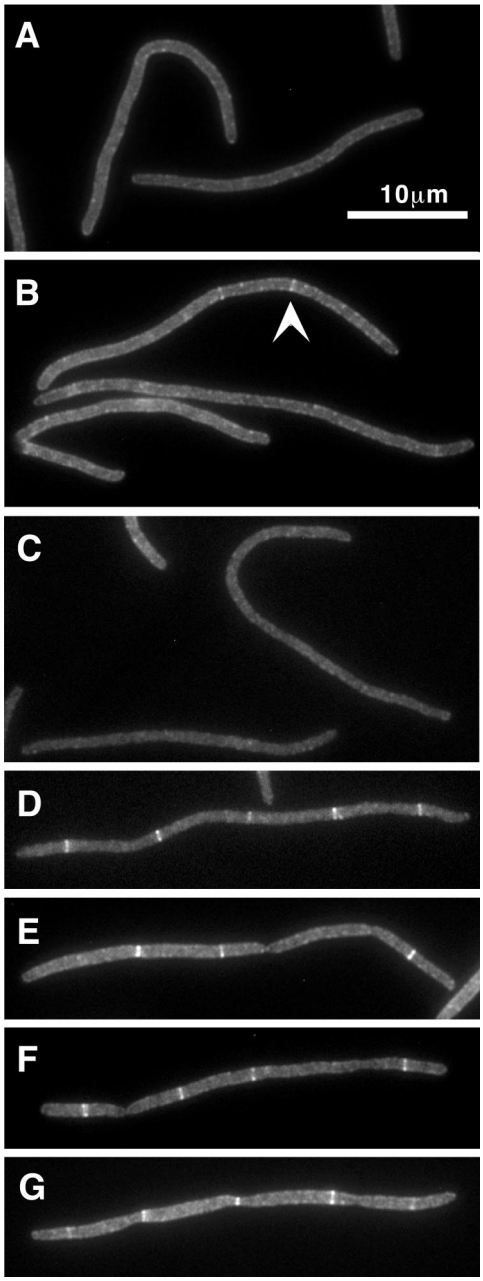

FIG. 4. Effect of *fts* mutations on localization of FtsX to potential division sites in filamentous *E. coli* cells. Strains induced to express GFP-FtsX or FtsX-GFP were grown under nonpermissive conditions until they became filamentous, at which time they were fixed and examined by fluorescence microscopy to visualize GFP. Relevant division mutations are as follows: *ftsZ84*(Ts) in EC1158 (A), *ftsA12*(Ts) in EC1152 (B), and *zipA1*(Ts) in EC1340 (C); and FtsK depletion in EC1295 (D), FtsQ depletion in EC1179 (E), FtsW depletion in EC1159 (F), and FtsI depletion in EC1181 (G). The arrowhead in panel B points to a faint band sometimes observed in *ftsA*(Ts) filaments. 4′,6′-Diamidino-2-phenylindole staining was done to verify proper nucleoid segregation (data not shown).

of GFP-FtsX. Similarly, GFP-FtsX localized poorly in an *ftsA12*(Ts) background at the nonpermissive temperature. Although some GFP-FtsX localization was seen in the *ftsA12*(Ts) filaments, these bands were invariably faint (e.g., Fig. 4B, top

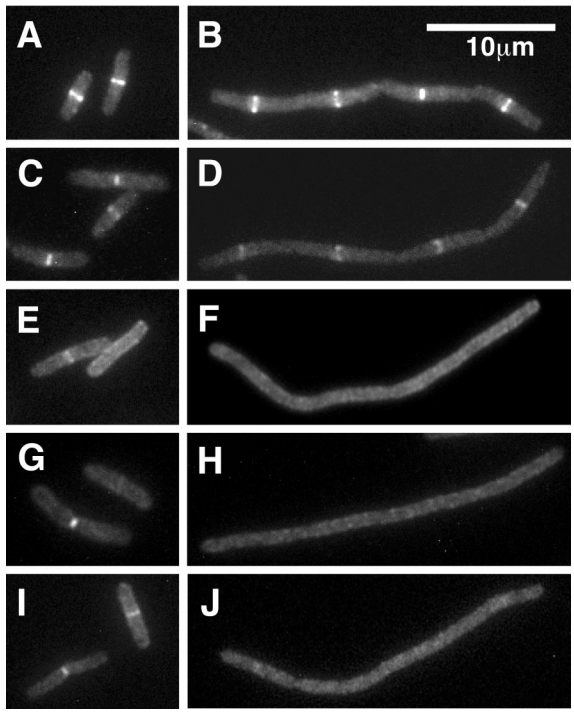

FIG. 5. Localization of various division proteins after depletion of FtsEX. The strains used express *ftsEX* under control of an arabinose-dependent promoter and harbor *gfp* fusions to different division genes. These strains were grown in parallel in media containing arabinose (short cells) or glucose (filaments) and then fixed and examined by fluorescence microscopy to visualize GFP. (A and B) FtsA-GFP in EC1363; (C and D) ZipA-GFP in EC1391; (E and F) FtsK (1-266)-GFP in EC1386; (G and H) GFP-FtsQ in EC1392; (I and J) GFP-FtsI in EC1366. 4′,6′-Diamidino-2-phenylindole staining was done to verify nucleoid segregation (data not shown).

filament). Whether this residual localization reflects leakiness of the Ts phenotype or a small degree of FtsA independence is not known. In contrast, most filaments formed by depleting cells of FtsK contained multiple fluorescent bands that were as bright as those seen in dividing cells. Likewise, GFP-FtsX localized well in filaments depleted of FtsQ, FtsL, or FtsI. Taken together, these results imply that FtsX localizes after FtsZ, FtsA, and ZipA, but before FtsK and subsequent proteins.

To determine which Fts proteins are dependent upon FtsEX for septal localization, we constructed a set of FtsEX depletion strains that harbored *gfp* fusions to various division genes. The results are summarized in Fig. 5 and Table 4. Depletion of FtsEX had only a minimal effect on localization of FtsA and ZipA. In contrast, localization of FtsK, FtsQ, and FtsI was markedly reduced in FtsEX depletion filaments, and the occasional fluorescent bands observed were quite faint. Sporadic localization of small amounts of FtsK, FtsQ, and FtsI might reflect partial assembly of the septal ring in the absence of FtsEX. Alternatively, there might be some residual FtsEX in the filaments. In either case, these findings imply that FtsEX localizes after FtsZ, FtsA, and ZipA, but before FtsK and other downstream proteins, and are consistent with the behavior of the GFP-FtsX protein in *fts* mutant backgrounds (see above).

TABLE 4. Localization of Fts proteins in FtsEX depletion background[a]

GFP fusion	Sugar	No. of cells scored	Avg cell length (μm)	% of cells with the indicated no. of rings						Spacing of rings[b]
				0	1	2	3	4	5	
FtsA-GFP	Arabinose	164	4.8	7	85	8	0	0	0	3.6
	Glucose	93	35	0	7	23	13	23	34	7.3
ZipA-GFP	Arabinose	224	4.1	20	79	1	0	0	0	5.0
	Glucose	112	28	4	10	35	19	19	13	9.5
FtsK(1–266)-GFP	Arabinose	243	6.3	49	51	0	0	0	0	12
	Glucose	101	21	67	29[c]	4[c]	0	0	0	55
GFP-FtsQ	Arabinose	217	7.2	35	65	0	0	0	0	11
	Glucose	131	24	69	27[c]	4[c]	0	0	0	67
GFP-FtsI	Arabinose	108	5.0	63	37	0	0	0	0	14
	Glucose	66	23	68	32[c]	0	0	0	0	72

[a] Strains used were EC1363, EC1391, EC1386, EC1392, and EC1366.
[b] See Table 3, footnote b.
[c] These bands were faint, and so the numbers given understate the degree of dependence on FtsEX.

The above experiments concerning localization of various Fts proteins in cells depleted of FtsEX were done in media without salt. Depletion of FtsEX in LB with 1% NaCl caused the cells to form short filaments (~10 μm), about 40% of which exhibited localization of GFP-FtsI (data not shown). Because we lack antibodies against FtsEX, we do not know whether the better localization of GFP-FtsI was due to salt rescue of localization (as expected) or less effective depletion of FtsEX.

DISCUSSION

We have shown that FtsE and FtsX localize to the septal ring, which implies that FtsEX participates directly in the division process. In a culture growing with a doubling time of about 30 min, about half of the cells exhibited septal localization of FtsE and FtsX. These cells were the longer (older) ones in the population and included cells with deep constrictions. Apparently, FtsEX is not present at the division site in newborn cells but gets recruited to that site during the later stages of cell growth and remains there until division is complete. This pattern of timed localization to the septal ring is similar to what has been reported for many other Fts proteins (e.g., references 8, 12, and 38). An additional line of evidence for direct participation in cell division is that during depletion of FtsEX, cells stop dividing before any change in growth rate (mass increase) is apparent. Thus, filamentation does not appear to be a secondary consequence of some metabolic defect or ion imbalance only tangentially related to septum assembly. This is not a trivial observation, as there are numerous examples of mutations that exert indirect effects on cell division, such as mutations in *nrdB*, *dnaK*, *secA*, and *ffh* (6, 26, 27, 35).

Previous studies from several laboratories have produced a model for the order of assembly of proteins into the septal ring in *E. coli* (for a recent review, see reference 11). A version of this model, revised to incorporate our new findings, is presented in Fig. 6. We infer that FtsEX localizes after FtsZ, FtsA, and ZipA and is important for recruitment of FtsK and all subsequent division proteins. Although we only demonstrated this directly for FtsK, FtsQ, and FtsI, previous work has established that septal localization of FtsL, YgbQ, FtsW, FtsN, and AmiC (3) requires prior localization of upstream proteins.

The ability of salt to rescue division in an FtsEX null mutant is enigmatic if FtsEX is needed for proper assembly or stability of the septal ring. Presumably, the downstream essential division proteins, FtsK through FtsN, all of which are required for division even on media containing salt, have some ability to localize in the absence of FtsEX. Consistent with this, RG60 is sensitive to β-lactams that target FtsI (D. Weiss, unpublished data) and we observed localization of FtsN, which is a late recruit to the division site (1), when RG60 was grown under permissive conditions. It is tempting to speculate that the ability of downstream proteins to localize independently of FtsEX is why we observed some residual localization of FtsK, FtsQ, and FtsI in filaments depleted of FtsEX in LB with no salt, but we cannot exclude the less interesting possibility that there is residual FtsEX in these filaments. Our depletion strains express *ftsEX* from a multicopy plasmid, so we sought to reduce the potential for leaky expression by placing a single copy of P_{BAD}-*ftsEX* on the chromosome. Unfortunately, this configuration resulted in very poor complementation, presumably owing to too little *ftsEX* expression in the presence of arabinose.

To explain the salt-remedial nature of *ftsEX* null mutants, we propose that ionic conditions affect the folding, assembly, and/or function of one or more of the downstream division proteins, FtsK through FtsN. We further suggest that there is a synergistic effect when combined with loss of FtsEX such that the septal ring fails to assemble or function properly if both salt and FtsEX are lacking, but salt can rescue the ring in the

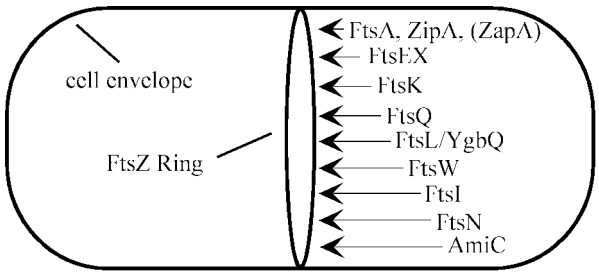

FIG. 6. Model for recruitment of proteins to the septal ring. The first event is polymerization of FtsZ into the Z-ring. FtsA, ZipA, and ZapA bind directly to FtsZ and localize next or concomitantly with Z-ring assembly. Once either FtsA or ZipA has joined the septal ring, the remaining proteins localize in the order indicated. Whether any *E. coli* proteins are dependent upon ZapA is not yet known.

absence of FtsEX, albeit poorly (recall that RG60 is filamentous even in the presence of salt).

An important question is whether FtsEX has any role in cell division beyond serving as an assembly or stability factor. In particular, one wonders whether FtsEX really is an ABC transporter and, if so, what it transports. Sequence comparisons indicate that FtsEX groups with importers rather than exporters (4). If FtsEX were an importer, it would be expected to function in conjunction with a periplasmic binding protein, although none has been associated with FtsEX as of yet. It has been speculated that FtsEX imports an ion (13), in part because of the salt-remedial nature of the defect in *ftsEX* mutants, but this notion is difficult to reconcile with the lack of specificity with respect to which salts rescue an *ftsEX* mutant (10). Moreover, FtsX does not appear to have any charged amino acids in its transmembrane domains, so it is difficult to envision how FtsX would accommodate an ion (R. Arends and D. Weiss, unpublished data). Finally, preliminary transcriptional profiling of an *ftsEX* null mutant has revealed a number of genes whose expression is altered, and none of these appears to be related to ion transport, ion homeostasis, or osmotic regulation (R. Arends and D. Weiss, unpublished data).

Not all ABC systems that group with importers actually import, or even transport, a substrate. Two interesting examples are the MacAB system and the LolCDE system, both of which are phylogenetically close to FtsEX (4). MacAB is an exporter that confers resistance to macrolides (21), while the LolCDE system is not a transporter at all—it is involved in release of lipoproteins from the cytoplasmic membrane (40). These observations make it worth considering functions for FtsEX that are unrelated to import. It has been suggested that FtsEX might be needed for insertion of a division protein into the cytoplasmic membrane (4, 36). An altogether different possibility is that FtsX serves as a membrane anchor, while FtsE uses ATP hydrolysis to promote constriction of the septal ring.

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