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How FtsEX localizes to the Z ring and interacts with FtsA to regulate cell division

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Abstract

In *Escherichia coli* FtsEX, a member of the ABC transporter superfamily, is involved in regulating the assembly and activation of the divisome to couple cell wall synthesis to cell wall hydrolysis at the septum. Genetic studies indicate FtsEX acts on FtsA to begin the recruitment of the downstream division proteins but blocks septal PG synthesis until a signal is received that divisome assembly is complete. However, the details of how FtsEX localizes to the Z ring and how it interacts with FtsA are not clear. Our results show that recruitment of FtsE and FtsX are codependent and suggests that the FtsEX complex is recruited through FtsE interacting with the conserved tail of FtsZ (CCTP), thus adding FtsEX to a growing list of proteins that interact with the CCTP of FtsZ. Furthermore, we find that the N-terminus of FtsX is not required for FtsEX localization to the Z ring but is required for its functions in cell division indicating it interacts with FtsA. Taken together, these results suggest that FtsEX first interacts with FtsZ to localize to the Z ring and then interacts with FtsA to promote divisome assembly and regulate septal PG synthesis.

Abbreviated Summary

FtsEX regulates assembly and activation of the divisome as well as daughter cell separation in *E. coli* by interacting with FtsZ and FtsA. Here we show that FtsEX localizes to the Z ring through an interaction between FtsE and FtsZ. Also, our results suggest FtsE binds to the conserved C-terminal peptide of FtsZ and FtsX binds to FtsA through its N-terminal cytoplasmic domain. Thus, FtsEX is recruited to the Z ring as it forms through the FtsE-FtsZ interaction and then regulates division through the FtsX-FtsA interaction.

Keywords

FtsEX; FtsZ; FtsA; divisome assembly; divisome activation; peptidoglycan

Introduction

In *E. coli* cell division is carried out by the divisome which is assembled in two temporarily distinct steps (Aarsman *et al.*, 2005, Du & Lutkenhaus, 2017). In the first step, FtsZ

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filaments tethered to the membrane by FtsA and ZipA coalesce at midcell to form the Z ring (Pichoff & Lutkenhaus, 2002). This step is aided by FtsZ filament cross-linking proteins that also serve to link the Z ring to the terminus of replication (Huang *et al.*, 2013, Buss *et al.*, 2013). FtsEX joins the Z ring soon after it is formed and, after a delay, the remaining essential proteins are recruited (FtsK, FtsQLB, FtsWI, FtsN) (Schmidt *et al.*, 2004) (Fig. 1). FtsN is the last essential protein to arrive (Goehring *et al.*, 2005, Addinall *et al.*, 1997) and it activates the divisome to synthesize septal peptidoglycan (PG) (Gerding *et al.*, 2009).

FtsEX is a member of the Type VII family of the ABC-superfamily (Greene *et al.*, 2018). Members of this family are not typical transporters but couple ATP hydrolysis in the cytoplasm with transmembrane conformational changes to perform mechanical work in the periplasm. FtsX is the inner membrane component and FtsE is the ATPase (Gill & Salmond, 1987). In *E. coli* FtsEX has two essential roles in cell division when cells are grown at low to moderate osmolarity. First, following recruitment to the Z ring FtsEX acts on FtsA to promote the recruitment of the downstream proteins in a step that does not require ATP (Arends *et al.*, 2009, Du *et al.*, 2016) (Fig. 1). It is thought that FtsEX promotes the formation of less oligomeric FtsA which is active in recruiting downstream proteins (Du *et al.*, 2016, Pichoff *et al.*, 2012). Second, once the divisome is fully assembled FtsEX must hydrolyze ATP for constriction to start (Du *et al.*, 2016, Arends *et al.*, 2009).

In the current model for divisome activation, FtsN acts on FtsA in the cytoplasm and possibly on FtsQLB in the periplasm to activate septal PG synthesis (Liu *et al.*, 2015, Tsang & Bernhardt, 2015) (Fig. 1). An FtsEX ATPase mutant blocks division by preventing the activation by FtsN in the cytoplasm (Du *et al.*, 2016, Liu *et al.*, 2015, Tsang & Bernhardt, 2015). This block is eliminated by *ftsA* mutations that reduce the FtsA-FtsX interaction suggesting that the FtsEX ATPase mutant acts on FtsA. This result also indicates that under normal conditions FtsEX must be stimulated to undergo ATP hydrolysis to allow the activation of septal PG synthesis.

The FtsEX ATPase cycle is also required to regulate EnvC in the periplasm to control amidase activity (Yang *et al.*, 2011) (Fig. 1). The large periplasmic loop in FtsX interacts with EnvC to regulate amidases AmiA and AmiB (Yang *et al.*, 2012). ATP hydrolysis by FtsEX likely induces conformational changes in EnvC causing it to overcome AmiB's auto-inhibition. Thus, the ATPase activity of FtsEX couples PG synthesis to PG hydrolysis at the septum (Yang *et al.*, 2011, Mavrici *et al.*, 2014, Sham *et al.*, 2011). However, *in vitro* EnvC is able to activate AmiB in the absence of FtsEX, suggesting that its role in regulating PG hydrolysis is not yet fully understood (Peters *et al.*, 2013, Uehara *et al.*, 2010).

The roles of FtsEX in PG synthesis and hydrolysis can be readily separated. A small deletion within the periplasmic loop of FtsX eliminates the interaction with EnvC but does not interfere with the essential roles of FtsEX (Du *et al.*, 2016, Yang *et al.*, 2011). Thus, an intact periplasmic loop is required for PG hydrolysis but not for FtsEX's role in divisome recruitment and PG synthesis. Also, increased osmolarity suppresses the requirement of FtsEX for recruitment and PG synthesis but not for PG hydrolysis. As a result, a *ΔftsEX* strain grows at high osmolarity but displays a mild chaining phenotype due to a deficit in cell separation (Yang *et al.*, 2011).

FtsEX has been extensively studied in various bacteria because of its role in PG hydrolysis, often, but not always, at the septum (Sham *et al.*, 2011, Meisner *et al.*, 2013, Mavrici *et al.*, 2014, Yang *et al.*, 2011). Nonetheless, how FtsEX is recruited to the Z ring is not entirely clear, although it is known that it does not require ATP binding or hydrolysis (Arends *et al.*, 2009, Yang *et al.*, 2011). In an initial study in *E. coli* it was determined that localization of FtsX to the Z ring required FtsZ, ZipA and FtsA (Schmidt *et al.*, 2004). It was suggested that FtsX was the main determinant for localization since FtsX localized in the absence of FtsE whereas FtsE did not localize in the absence of FtsX (Arends *et al.*, 2009, Schmidt *et al.*, 2004). co-PI experiments indicated that FtsE interacted directly with FtsZ independent of FtsX or the conserved C-terminal peptide (CCTP) of FtsZ. Surprisingly, however, FtsE required FtsX to localize to the Z ring *in vivo*.

The above studies demonstrated that *ftsE* localizes to the Z ring when co-expressed with *ftsX*. Furthermore, localization is efficient and likely occurs as the Z ring is formed (Yang *et al.*, 2011, Corbin *et al.*, 2007, Du *et al.*, 2016). However, it is not clear why FtsE interacts with FtsZ *in vitro* in the absence of FtsX but FtsE localization *in vivo* depends upon FtsX. To better understand the localization of FtsEX and its effect on the divisome we further explored the requirements for localization and how FtsEX interacts with FtsA.

Results

FtsE and FtsX are co-dependent for localization to the Z ring

To examine how FtsEX localizes to the Z ring we constructed GFP fusions to FtsE and FtsX and tested their ability to localize and correct the chaining phenotype of a *ΔftsEX* strain. A fusion of GFP to the N-terminus of FtsE was unable to correct the chaining phenotype of a *ΔftsEX* strain and it did not localize to the division site (Fig. 2). However, when coexpressed with its partner (*GFP-ftsEX*) it corrected the chaining phenotype and localized to the division site. A GFP fusion to the C-terminus of FtsX did not correct the chaining phenotype of a *ΔftsEX* strain or localize to the division site. However, when it was coexpressed with *ftsE* (*ftsEX-GFP*) it also complemented the morphological defect and localized to the division site (Fig. 2). FtsEX-GFP appeared to localize more efficiently to the division site than GFP-FtsEX as the fluorescence was more focused in rings at the septum and less was on the membrane away from midcell. The main conclusion from these experiments is that the function and localization of FtsE and FtsX to the Z ring are codependent.

Dependencies for localization of FtsEX-GFP

Since the requirements for the localization of FtsEX to the division site were not entirely clear, we decided to re-investigate its localization dependency using FtsEX-GFP which localized more efficiently than GFP-FtsEX. When *ftsEX-gfp* was expressed in a WT strain it localized to the division site and this localization was not affected by a temperature shift from 30°C to 42°C (Fig. 3). When *ftsEX-gfp* was expressed in the *ftsZ84* (Ts) strain fluorescence was observed in crossbands at midcell at 30°C but some fluorescence was also observed at the poles (Fig. 3). At 42°C crossbands were not observed but fluorescence was observed in sporadic spots indicating possible aggregation. In the *ftsA12* (Ts) strain the fluorescence was observed in crossbands at both 30°C and 42°C indicating that FtsEX-GFP

does not require FtsA to localize to the Z ring. In the *zipA1* (Ts) strain, crossbands were observed at 30°C but not at 42°C suggesting that localization of FtsEX-GFP depends upon ZipA as previously reported (Corbin *et al.*, 2007).

Using strains in which FtsA or ZipA could be depleted we confirmed that ZipA, but not FtsA, was required for localization of FtsEX-GFP (Fig. S1). At this temperature we did not see aggregation (spots of fluorescence) which is sometimes seen at higher temperature. We also confirmed (Corbin *et al.*, 2007) that FtsEX localized when *zipA* was deleted in a strain containing the *ftsA** (*ftsA*^{R286W}) mutation (which allows the bypass of ZipA (Corbin *et al.*, 2007) indicating the ZipA requirment is indirect (Fig. S2). To confirm that the localization of FtsEX under these conditions was independent of the FtsX-FtsA interaction, we added the *ftsA*^{G366D} mutation which eliminates the interaction between FtsX and FtsA (Du *et al.*, 2016). The FtsA^{*,G366D} mutant is able to complement an FtsA depletion strain because the *ftsA** mutation suppresses the recruitment defect of *ftsA*^{G366D} allowing deletion of *zipA*. FtsEX-GFP still formed crossbands in a *ΔzipA ftsA*$^{*/G366D}$ strain confirming that localization in the absence of ZipA did not depend upon the FtsX-FtsA interaction (Fig. S2).

FtsEX disrupts Z rings formed with only FtsA

The above results showed that FtsEX-GFP requires ZipA for localization when wild type FtsA is present, but not if FtsA* is present, which is consistent with previous observations (Corbin et al. 2007). Why there is this difference between strains carrying *ftsA* versus *ftsA** was not clear. However, the previous report (Corbin *et al.*, 2007) had not checked whether Z rings were still present when ZipA was inactivated in cells expressing a GFP fusion to FtsEX. Its possible that Z rings anchored by only FtsA are more sensitive to expression of FtsEX-GFP than Z rings anchored by only FtsA* since FtsA* interacts stronger with FtsZ (Pichoff *et al.*, 2012). To test this, we examined FtsEX-GFP localization along with the localization of ZapA-mCherry (as a proxy for the Z ring) in various temperature sensitive mutants.

At 30°C we observed colocalization of FtsEX-GFP and ZapA-mCherry in all strains tested consistent with FtsEX localizing to Z rings as they form (Fig. 4 & S3). Quantification in WT cells revealed that the frequency of FtsEX-GFP localization to midcell slightly exceeded Zap-mcherry (93.3% to 92%, Table S1). This slight difference is due to Zap-mcherry disappearing from deeply constricting cells before FtsEX-GFP. This result confirms that FtsEX localizes to Z rings as they form.

Following a shift to 42°C for 30 minutes, FtsEX-GFP and ZapA-mCherry still localized in crossbands in wild type and *ftsA12* (Ts) cells, however, no crossbands were observed in *ftsZ84* (Ts) or *zipA1* (Ts) cells (Fig. 4). In FtsZ84 (Ts) cells, ZapA-mCherry was present in faint cytoplasmic foci along the length of the filaments, consistent with it forming FtsZ-independent structures with ZapB between nucleoids (Buss *et al.*, 2017). In *zipA1* (Ts) cells FtsEX-GFP localized at the membrane at 42°C and formed sporadic spots on the membrane, whereas ZapA-mCherry appeared to localize in spots in the cytoplasm. This ZapA-mCherry localization pattern is very similar to FtsZ localization in the absence of FtsA and ZipA reported previously (Pichoff & Lutkenhaus, 2002). Indeed, when a *ftsA12 zipA1* (Ts) double mutant was shifted to 42°C for 30 minutes a similar localization pattern of ZapA-mCherry

was observed (Fig. S4). These results demonstrate that expression of *ftsEX-GFP* in the absence of ZipA results in the disruption of Z rings indicating that ZipA is required to "stabilize" Z rings when FtsEX-GFP is expressed as previously suggested (Corbin *et al.*, 2007). Together, these results indicate that FtsEX depends only upon FtsZ to localize and the apparent ZipA dependency arises from Z rings anchored by only FtsA being sensitive to ectopic expression of FtsEX-GFP. In contrast, Z rings anchored by ZipA or FtsA* are more resistant. Furthermore, this sensitivity of Z rings anchored by only FtsA to FtsEX-GFP raised the possibility that FtsEX competes with FtsA at the Z ring and we set out to test this.

FtsEX competes with FtsA at the Z ring

In the above studies we noticed that expression of *ftsEX-gfp* was toxic with temperature sensitive mutants being more sensitive at permissive temperature than the WT strain and this was confirmed in spot tests (Fig. 5A). Phase microscopy revealed that induction of *ftsEX-gfp* with 60 μM IPTG at 30°C resulted in filamentation of a WT strain as well as the temperature sensitive mutants (Fig. 5B). Fluorescence microscopy revealed that the WT, *ftsA12* (Ts) and *zipA1* (Ts) strains contained Z rings and FtsEX-GFP was associated with each ring demonstrating the block to division occurred at a step after Z ring assembly (Fig. 5B). To determine why these Z rings did not result in division we used immunostaining to determine if FtsA was absent (Fig. 6). The staining revealed that ZipA and FtsZ were present in crossbands in the filaments but FtsA rings were much less frequent (Table S2). Furthermore, FtsA rings were virtually absent in the *ftsA12* (Ts) strain where the filamentation was more extensive (dramatic increase in μM/ring, Table S2). These results suggest that FtsEX competes with FtsA at the Z ring, consistent with Z rings anchored by just FtsA being sensitive to the expression of FtsEX-GFP as shown above.

The displacement of FtsA from Z rings by mild overexpression of FtsEX-GFP is similar to what we observed previously with $MinC^{C}$/MinD which binds the CCTP of FtsZ but cannot disrupt FtsZ filaments because it lacks $MinC^{N}$ (Shen & Lutkenhaus, 2010, Park *et al.*, 2018). Mild overexpression of $MinC^{C}$/MinD displaces FtsA, but not ZipA, from Z rings indicating that ZipA has a higher affinity for the CCTP of FtsZ than FtsA (Shen & Lutkenhaus, 2009). However, at higher levels of expression of $MinC^{C}$/MinD Z rings disappear suggesting $MinC^{C}$/MinD also competes with ZipA for the CCTP of FtsZ (Shen & Lutkenhaus, 2010). To see if FtsEX could also disrupt Z rings we overexpressed *ftsEX* at a higher level than in Fig. 6. As controls we overexpressed *ftsX*, *ftsE* and *ftsA,* which is known to be toxic due to disruption of Z rings (Pichoff & Lutkenhaus, 2005). Overexpression of *ftsX*, *ftsEX* and *ftsA* were toxic whereas *ftsE* was not (Fig. 7A). To see if Z rings were disrupted cells overexpressing *ftsEX* and *ftsX* were immunostained for FtsZ. Although overexpression of FtsX caused cell chaining and filamentation, Z rings were present just as they were in a control in which filamentation was induced by cephalexin (Fig. 7B). In contrast, the overexpression of *ftsEX* caused a loss of Z rings. Taken together, these results indicate that mild overexpression of *ftsEX* displaces FtsA from the Z ring but that higher levels of overexpression disrupt the Z ring. These results suggest that FtsEX competes with FtsA and ZipA for the CCTP of FtsZ just like MinC/MinD.

Isolation of *ftsE* mutations affecting localization and function

A previous study concluded that FtsE interacted directly with FtsZ, but independent of the CCTP of FtsZ. However, as shown above our genetic tests suggest FtsEX competes with FtsA for the CCTP of FtsZ. We hypothesized that FtsE within the FtsEX complex interacts with the CCTP of FtsZ and set out to identify residues in FtsE responsible for localization of FtsEX to the Z ring. To do this we used the GFP-FtsEX fusion even though it localizes less well than FtsEX-GFP, since fluorescence at the membrane but not at the septum would indicate that GFP-FtsE is properly folded and interacts with FtsX but is deficient in localization to the Z ring. In contrast, fluorescence at the division site would indicate the mutations have no effect.

First a structural model of FtsEX was generated (as described in Experimental Procedures) and tested by mutation. Since members of the ABC transporter superfamily have a helical segment (coupling helix) in the membrane component that fits into a groove of the ATPase subunit (Wilkens, 2015) we made mutations that affect this putative helix in FtsX (I255E) (Fig. 8A, dark cyan, and Fig. S5A) or the putative groove in FtsE (L90A and L91A). These mutations markedly reduced the ability of *ftsEX* to complement a Δ*ftsEX* mutant (Fig. S5B) and reduced the ability of GFP-FtsE to localize to the membrane when co-expressed in *cis* with FtsX. They also prevented FtsX-GFP from localizing to midcell when co-expressed with FtsE (Fig. S6). Together, the results are consistent with these mutations disrupting the FtsE-FtsX interaction and support the homology model which allowed us to limit our mutagenesis to surface exposed residues that are away from the dimer interface and the surface interacting with FtsX.

A number of exposed residues scattered on the surface of FtsE were substituted with alanine or other amino acids and their effect on localization and the ability to complement a *ΔftsEX* strain examined (Fig. 8B, S7-S10, Table S3). Although many of these substitutions had no effect, some affected complementation. Among these, those in the region around 61 to 77 significantly reduced the ability of GFP-FtsEX to localize to the division site (Fig. 8B and S9). Single amino acid changes, such as F61A, H64D, I66A, L69A, E73A, F76A and L77A resulted in a markedly reduced frequency of crossbands. Similar results were obtained when E73 was changed to lysine and F76 was changed to glutamate. Combining two or three of these substitutions (E73K/F76E, H64D/E73K/F76E) resulted in a further reduction in the frequency of crossbands without affecting membrane localization (Fig. 8B). When these mutations were introduced into *ftsEX*-gfp, localization to midcell was also significantly reduced (Fig. S10). Western blot analysis showed that these GFP-FtsE mutants were expressed at similar level as wild type GFP-FtsE (Fig. S11). Moreover, expression of *gfp-ftsEX* in the *ΔftsEX* strain resulted in WT morphology whereas expression of the *gfp-ftsEX* mutants did not (Fig. 8B). The longer cell and chaining morphology of strains lacking a functional FtsEX is due to a deficiency in amidase activity at the septum (Yang *et al.*, 2011).

To confirm the inability of the FtsE mutants to complement a *ΔftsEX* deletion strain the E73K and F76E mutations were introduced into *ftsE* on a plasmid expressing *ftsEX* without the GFP tag. Expression of WT *ftsEX* allowed the strain to grow on LB without sucrose, whereas the double mutant did not (Fig. 9A). As shown previously (Du *et al.*, 2016), expression of a mutant of FtsE predicted to be defective in ATP hydrolysis ($ftsE^{D162N}X$) is

toxic in a WT strain. Since FtsE^{D162N}X must localize to the Z ring to block division, reducing its ability to localize should decrease its toxicity (Arends *et al.*, 2009, Du *et al.*, 2016). Indeed, this is what was observed as the presence of two of these mutations (E73K, F76E) into *ftsE^{D162N}X* dramatically reduced its toxicity (Fig. 9B). Unlike *ftsE^{D162N}X, ftsEX* is not toxic at relatively low levels of expression, however, it disrupts Z rings when expressed at a higher level as shown above (Fig. 7). Since the disruption of Z rings is likely due to interaction of FtsEX with the CCTP of FtsZ, then the mutations in *ftsE* should also reduce this toxicity. Indeed, overexpression of *ftsEX* in a *ΔftsEX* strain containing the *ftsA** mutation led to filamentation whereas overexpression of *ftsE$^{H64D/F73K/F76E}$X* did not (Fig. S12). The same result was obtained if the strain carried the *ftsA^{*,G366D}*, confirming that filamentation was not due to the interaction of FtsX with FtsA.

To assess whether the mutations in *ftsE* that reduce FtsEX localization to the Z ring affected the interaction of FtsEX with FtsZ we used the bacterial two-hybrid (BACTH) system (Karimova *et al.*, 1998). As shown in Fig. 9C, FtsE did not interact with FtsZ unless it was co-expressed with FtsX, which is consistent with GFP-FtsE having to be co-expressed with FtsX to localize to the Z ring. The presence of two (E73K, F76E) or three mutations (E73K, F76E, H64D) in *ftsE* reduced the color intensity (Fig. 9C). This reduction coincided with a reduction in the β-galatosidase activity (Fig. 9D). Thus, mutations in *ftsE* that reduce FtsEX localization to the Z ring (without affecting the interaction with FtsX) reduce the interaction with FtsZ.

The N-terminal FtsX^{cyto} domain is not required for localization but is required for FtsX function in division

Having determined the region of FtsE that is required for the localization of FtsEX to the Z ring, we wanted to determine the region of FtsX that interacts with FtsA. We previously reported that FtsEX regulates division through FtsX acting on FtsA and the interaction is abrogated by the *ftsA^{G366D}* mutation (Du *et al.*, 2016). In cells in which FtsA and FtsZ are overproduced FtsA filaments are about 8 nm from the membrane, which is halfway between FtsZ filaments and the membrane (Szwedziak *et al.*, 2014). Most of the FtsX molecule that is present on the cytoplasmic side of the membrane is predicted to be masked by FtsE, however, FtsX has an extended N-terminal cytoplasmic domain (FtsX^{Ncyto}) that is of sufficient length to reach FtsA (Fig. 8A).

To see if FtsX^{Ncyto} was required for FtsX function, a plasmid expressing *ftsEX$^{Δ4-69}$* (a mutant missing FtsX^{Ncyto}) was tested to see if it could complement a *ΔftsEX* strain on LB plates or in LB liquid medium. It failed to complement (Fig. 10A and Fig. S13). To determine if this lack of complementation was due to a localization defect the deletion was introduced into an *ftsEX-gfp* fusion on a plasmid. Expression of *ftsEX$^{Δ4-69}$-gfp* in a *ΔftsEX* strain revealed fluorescent crossbands within the filamentous cells indicating that FtsX$^{Δ4-69}$ was not affected for membrane insertion or interaction with FtsE (Fig. 10B). Thus, the FtsX^{Ncyto} domain of FtsX is required for FtsEX to correct the cell morphology but is not required for localization of FtsEX to Z rings.

In our model (Du *et al.*, 2016) FtsEX acts on FtsA to allow it to start recruitment of downstream proteins (Fig. 1). To see if this process was affected by the lack of FtsX^{Ncyto},

ftsEX$^{\Delta 4-69}$ was expressed from an IPTG-inducible promoter in a Δ*ftsEX* strain with wild type *ftsEX* expressed under the control of an arabinose promoter. This strain also contained chromosomally encoded *zapA-mCherry* as a marker for Z rings and a plasmid expressing *gfp-ftsB*, as a representative downstream gene. Following removal of arabinose *ftsEX* or *ftsEX*$^{\Delta 4-69}$ was induced with IPTG and cells examined by fluorescence microscopy. Cells expressing *ftsEX*$^{\Delta 4-69}$ or the vector were filamentous and had ZapA-mCherry rings but failed to display localization of GFP-FtsB (Fig. 10C). In contrast, control cells expressing *ftsEX* were normal-sized and displayed localization of both ZapA-mCherry and GFP-FtsB at midcell (Fig. S14). These results indicate that the FtsX^{Ncyto} domain is required for FtsX to act on FtsA to start the recruitment of downstream proteins.

Since *ftsE*^{D162N}*X* (ATPase mutant) blocks division by acting on *ftsA* we deleted FtsX^{Ncyto} from *ftsE*^{D162N}*X*. Whereas expression of *ftsE*^{D162N}*X* causes filamentation and was toxic as shown previously (Du *et al.*, 2016), *ftsE*^{D162N}*X* $^{\Delta 4-69}$ was not toxic and did not cause filamentation suggesting it did not interact with FtsA (Fig. 11 A & B). In summary, the FtsX^{Ncyto} domain is not required for localization of FtsEX to the Z ring but is required for FtsEX to promote recruitment of downstream division proteins and for FtsEX to inhibit division in the absence of its ATPase activity. Together, these results indicate FtsX^{Ncyto} is required for FtsEX to act on FtsA.

Discussion

In *E. coli* the FtsEX complex is recruited to the Z ring as it forms and recruits EnvC to regulate amidases to promote cell separation (Yang *et al.*, 2011). It then acts on FtsA to promote recruitment of downstream proteins including FtsWI for septal PG synthesis, but also blocks septal PG synthesis if it is unable to hydrolyze ATP (Du *et al.*, 2016, Schmidt *et al.*, 2004, Arends *et al.*, 2009). Upon ATP hydrolysis FtsEX releases the block to septal PG synthesis and promotes amidase activity coupling these two processes. In this study we find that FtsEX localizes to the Z ring through FtsE interacting with FtsZ and we identified a region of FtsE required for its localization. The observation that FtsEX can displace FtsA from the Z ring along with geometric constraints (FtsE is close to the membrane, while FtsZ filaments are ~16 nm from the membrane) indicates that this region of FtsE interacts with the CCTP of FtsZ (Fig. 12). We also found that the FtsX^{Ncyto} domain is dispensable for FtsEX to localize to the Z ring and prevent septal PG synthesis when ATP hydrolysis is blocked. However, FtsX^{Ncyto} is required for FtsEX to correct the chaining phenotype of a *ΔftsEX* mutant and to promote recruitment of downstream proteins suggesting that it is required for FtsX to interact with FtsA. Thus, FtsEX is part of the divisome recruitment pathway and functions as a checkpoint to couple PG synthesis with amidase activity at the septum.

How FtsEX localized to the Z ring was not clear since FtsE was reported to interact with FtsZ (independent of the CCTP of FtsZ and FtsX) (Corbin *et al.*, 2007) and FtsX has been reported to interact with FtsA and FtsQ (Karimova *et al.*, 2005, Du *et al.*, 2016). Also, FtsX was reported to localize in the absence of FtsE but FtsE did not localize in the absence of FtsX (Corbin *et al.*, 2007, Arends *et al.*, 2009). However, in our previous work we found that FtsEX localized even when the interaction between FtsX and FtsA was blocked by mutation

(Du *et al.*, 2016). Here we find that localization of FtsE and FtsX to the septum is codependent. Neither GFP-FtsE nor FtsX-GFP localized to the Z ring in the absence of their partner but both localized when expressed with their partner. The previous report (Arends *et al.*, 2009) that FtsX-GFP localized to the Z ring weakly in the absence of FtsE had a slightly different linker connecting the two proteins. Perhaps that FtsX-GFP fusion can localize to the Z ring weakly by interacting with FtsA or FtsQ. Regardless of the reason for the difference, FtsE contains the main localization determinant for the FtsEX complex to localize to the Z ring.

Previously, it was determined that ZipA, but not FtsA, was required for the localization of FtsEX but this was considered indirect since the *zipA* requirement could be bypassed by the *ftsA** mutation (Corbin *et al.*, 2007). Here we show that the apparent ZipA requirement can be explained by the sensitivity of Z rings supported only by FtsA to mild overexpression of wild type *ftsEX-gfp*. In contrast, rings formed by only ZipA or FtsA* are more resistant. This is consistent with ZipA having higher affinity for the CCTP of FtsZ than FtsA as inferred previously (Shen and Lutkenhaus, 2009). Also, the resistance provided by FtsA* is consistent with its ability to stabilize the Z ring due to stronger interactions between FtsA* and the CCTP of FtsZ as well as between FtsN and FtsA* (Du *et al.*, 2016, Pichoff *et al.*, 2018). What is not clear though is why FtsEX or MinC^{C}/MinD, which binds the membrane and the CCTP of FtsZ, cannot function as a membrane anchor for the Z ring when two quite disparate proteins, ZipA and FtsA, can. With the addition of FtsEX to the list of proteins in *E. coli* that interact with the CCTP of FtsZ, the number is at least 6 (FtsA, ZipA, MinC/D, SlmA, ZapD).

Using a homology model and limited site-directed mutagenesis of *ftsE* we identified critical residues required for FtsEX to localize to the Z ring. Mutating these residues resulted in loss of FtsEX localization to the Z ring and the ability of *ftsEX* to complement an *ΔftsEX* mutant. Furthermore, the toxicity of the ATPase mutant (*ftsE*^{D162N}*X*) which blocks septal PG synthesis was markedly reduced. The region of FtsE critical for FtsEX localization to the Z ring is at the opposite ends of the FtsE dimer close to the membrane (Fig. 12). Since FtsZ filaments have been shown by electron cryotomography to be ~16 nm from the cytoplasmic membrane, only the CCTP at the end of the intrinsically disordered region of FtsZ is of sufficient length to reach FtsE (Szwedziak *et al.*, 2014). Consistent with FtsE interacting with the CCTP of FtsZ, mild overexpression of FtsEX displaces FtsA from the Z ring and higher overexpression results in dissolution of the Z ring suggesting it also displaces ZipA, similar to what was observed previously with MinC^{C}/MinD (Shen and Lutkenhaus, 2009). A previous report concluded that the interaction of FtsE with FtsZ was independent of FtsX and the CCTP of FtsZ, however in that study FtsE was overexpressed (without FtsX) and we suspect the *in vitro* results may be affected by the tendency of FtsE to aggregate (Corbin et al. 2007; Yang *et al.*, 2011). Although our attempts to reconstitute the interaction *in vitro* were unsuccessful so far, our observations that GFP-FtsE has to be co-expressed with FtsX to localize to the Z ring and FtsE only interacts with FtsZ in the presence of FtsX in BACTH suggest that FtsE is unlikely to interact with FtsZ when expressed alone.

FtsX has been shown to interact with FtsA (Karimova *et al.*, 2005) and we previously found that this interaction is important for FtsEX to promote the recruitment of downstream

proteins and inhibit cell constriction when its ATPase was inactivated (Karimova *et al.*, 2005, Du *et al.*, 2016). Although we identified the interaction site for FtsX on FtsA in that study, the part of FtsX that interacts with FtsA was not determined. Cryo-tomography of FtsA filaments *in vivo* indicate they are 8 nm away from the membrane (Szwedziak *et al.*, 2014). FtsX has three regions in the cytoplasm, the N-terminal cytoplasmic domain ($FtsX^{1-69}$), the coupling helix that binds FtsE (confirmed in this work) and the C-terminal cytoplasmic end (Arends *et al.*, 2009). Only the N-terminal cytoplasmic domain is of sufficient length to reach FtsA. In agreement with this, we found that a truncation mutant of FtsX lacking residues 4–69 was unable to complement the *ΔftsEX* strain, although it localized to the Z ring. More importantly, this inability to complement was due to a deficiency in recruitment of downstream proteins (GFP-FtsB as a proxy), indicating that it is deficient in interaction with FtsA (Fig. 12). Deletion of this region also abolished the toxicity of the ATPase mutant ($FtsE^{D162N}X$) which has been shown to depend on the interaction between FtsX and FtsA. Together, the evidence indicates that FtsX interacts with FtsA through its $FtsX^{Ncyto}$ domain.

If FtsEX plays such an important role in divisome assembly and in coupling PG synthesis with hydrolysis at the septum, why can it be bypassed by high osmolarity (Reddy, 2007)? In part the high osmolarity promotes the interaction of FtsN with FtsA leading to the back recruitment of the other division proteins (Pichoff *et al.* 2018). Although divisome assembly is restored, the coupling is lost and how this might affect division is not clear. FtsEX was probably added to the division machinery during evolution to promote the robustness and efficiency of division. Cells can survive without it under certain conditions but have many defects, including increased membrane permeability, cell chaining, induction of the SOS response and sensitivity to oxidative damage (O'Reilly & Kreuzer, 2004, Samaluru *et al.*, 2007). We can envision two roles for the coupling of PG synthesis and hydrolysis at the septum in a Gram-negative bacterium like *E. coli*. The first is to ensure the outer membrane invaginates as the cell wall is split. This coordination of invagination of the layers of the envelope ensures integrity of the outer membrane at the newly forming poles and maintains the barrier to hydrophobic compounds (Heidrich *et al.*, 2002). The second concerns the thin layer of PG surrounding Gram-negative bacteria. Under conditions of low osmolarity it may be more important to couple PG synthesis and hydrolysis to prevent minor perturbations in septal PG synthesis causing cell lysis, something which may be suppressed at high osmolarity. This coupling may be necessary for *E. coli* and related organisms that have evolved to occupy niches with wide ranges in osmolarity (Egan, 2018).

Experimental procedures

Media, bacterial strains, plasmids and growth conditions

Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.05 g/L thymine) or LB with 0.2 M sucrose at indicated temperatures. When needed, antibiotics were used at the following concentrations: ampicillin = 100 μg/ml; spectinomycin = 25 μg/ml; kanamycin = 25 μg/ml; tetracycline = 25 μg/ml; and chloramphenicol = 20 μg/ml. When cells carried the *bla* or *cat* marker integrated in the chromosome, ampicillin or chloramphenicol was used at 25 μg/ml and 10 μg/ml, respectively. *ftsE* mutations, strains, plasmids and

primers used in this study are listed in Table S4-S6, respectively. Construction of strains and plasmids is described in detail in Supplemental Information.

BACTH (bacterial two-hybrid) and β-Galatosidase assay

To detect the interaction between FtsZ and FtsEX or its mutants by BACTH, appropriate plasmid pairs were co-transformed into BTH101. The next day, single colonies were resuspended in 1 ml of LB medium and 3 μL of each aliquot was spotted on LB plates containing ampicillin, kanamycin, 40 μg/ml X-gal and 100 μM IPTG. Plates were incubated at 30 °C overnight before imaging.

The BACTH results were quantitated using a β-galatosidase assay following the protocol as previously described (Park *et al.*, 2017) with minor modifications. 3 colonies were picked from each of the transformation plates from the BACTH assay (BTH101 containing the appropriate plasmid pairs) and cultured overnight in LB containing 0.2% glucose, ampicillin and kanamycin at 30°C. The next day, the cultures were diluted 1/100 into fresh LB containing ampicillin, kanamycin and 100 μM IPTG and cultured for 3.5 hours. The cultures were then put on ice for 20 min to stop bacterial growth and OD_{600} of each culture was measured. 0.5 ml of each culture was mixed with 0.5 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO4, pH 7.5, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol) and cells were permeabilized with 0.0015% SDS (wt/vol) and 2.5% chloroform (vol/vol) and vigorous vortexing. The mixtures were then kept at 30°C for 5 minutes and 0.25 ml of ONPG (*o*-nitrophenyl β-d-galactopyranoside, 4 mg/mL) (Sigma) was added to the mixtures. The reactions were incubated at 30°C for 10 minutes and stopped with 400 mM Na_2CO_3. OD_{420} and OD_{550} values were recorded and converted into Miller units as described (Miller, 1992).

Immunofluorescence microscopy

Immunostaining of FtsZ, FtsA and ZipA in cells overexpressing FtsEX-GFP.—

Overnight cultures of S3 (*leu*::Tn*10*) and PS236 (*leu::Tn10 ftsA12)* containing pSD242 (pDSW210, P_{206}::*ftsEX-gfp*) were diluted 1:100 in fresh LB medium with ampicillin and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium and IPTG was added to a final concentration of 60 μM. After growth at 30 °C for 2 hours, samples were taken, the cells were fixed with 2% paraformaldehyde and 0.4% glutaraldehyde, and prepared for immunostaining and photography as previously described (Addinall *et al.*, 1996). Antisera were used as the following concentration: FtsZ (1/5000), FtsA (1/1000) and ZipA (1/1000).

Immunostaining of FtsZ in W3110 cells overexpressing ftsEX or ftsX.—

Overnight cultures of W3110 carrying plasmid pSEB426 (P_{204}::*ftsE*), pSEB427 (P_{204}::*ftsX*) or pSEB428 (P_{204}::*ftsEX*) were diluted 1:100 in fresh LB medium with ampicillin and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium and IPTG was added to a final concentration of 1 mM. After induction for 2 hours, cells were fixed and prepared for immunostaining as above.

Localization of GFP-fusion proteins

The localization of FtsEX-GFP in the TS mutants.—Overnight culture of the TS strains (*ftsZ84*^{ts}*, ftsA12* ^{ts} *and zipA1* ^{ts}) carrying plasmid pSD242 were diluted 100X in fresh LB medium with antibiotics and grown at 30 °C for 2 h. The cultures were then diluted 1:10 in fresh LB medium and IPTG was added to a final concentration of 15 μM. Two hours post induction, the cultures were split in half. One half was kept at 30 °C, while the other half was shifted to 42 °C. Thirty min later, cells growing at 30 °C were photographed, while cells growing at 42°C were fixed with paraformaldehyde and glutaraldehyde before photographing.

Localization of ZapA-mcherry in the TS Strains expressing FtsEX-GFP.—Experiments were carried out as described above.

Determination of the localization of FtsEX-GFP upon depletion of FtsA.—An overnight culture of strain CH2/pDB280 (*ftsA*^{0} *recA56*-Tn*10*/pSC101^{ts}, P_{ftsA}::*ftsA*) harboring the plasmid pSD242 was diluted 1:100 in fresh LB medium with appropriate antibiotics and grown at 30 °C for about 3 hours. The culture was then diluted 1:10 in LB medium with 15 μM IPTG and shifted to 37 °C (a control culture induced at 30 °C was also prepared). To keep the cells in exponential phase, the cultures were diluted once after they were shifted to 37°C for 1.5 hours. Samples were taken for photography 3 hours after the cultures were shifted to 37°C.

Determination of the localization of FtsEX-GFP upon depletion of ZipA.—An overnight culture of strain PS195 (W3110, P_{ara}::*zipA-kan*) carrying plasmid pSD242 was diluted 1:100 in fresh LB medium with 1% arabinose and grown at 37 °C for about 2 hours. Cells were collected and washed 3 times with LB medium and diluted in LB medium with or without arabinose. IPTG was added to 15 μM to induce expression of FtsEX-GFP. The cultures were then grown at 37 °C for 3 hours and cells were immobilized on an agarose pad for photography.

Localization of FtsEX-GFP in ΔzipA cells.—An overnight culture of PS1323 (W3110, *leu*::Tn10, *ftsA**, *zipA*::*kan*) or SD480 (W3110, *leu*::Tn10, *ftsA**^{G366D,} *zipA*::*kan*) harboring plasmid pSD242 was diluted 1:100 in LB medium with antibiotics and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium and IPTG was added to a final concentration of 15 μM. After growth at 30°C for 2 hours, cells were immobilized on an agarose pad for photography.

Localization of GFP-FtsE, GFP-FtsEX, FtsX-GFP, FtsEX-GFP or their variants in ΔftsEX cells.—Overnight cultures of SD220 (S3, *ftsEX::cat*) harboring a plasmid expressing the fusions were diluted 1:100 in LB medium containing 0.2 M sucrose and ampicillin and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium with 0.2 M sucrose and IPTG was added to a final concentration of 15 μM. After growth at 30°C for 2 hours, cells were immobilized on an agarose pad for photography.

Localization of GFP-FtsB.—Overnight cultures of SD446 (W3110, *zapA-mcherry cat<>frt*, *ftsEX<>frt* P_{ara}::*ftsEX-cat*) harboring plasmids pSD332 (pSC101, P_{syn}::*gfp-ftsB*) and pEXT22, or pSD221 (pEXT22, P_{tac}::*ftsEX*) or pSD318 (pEXT22, P_{tac}::$ftsEX^{\Delta 4-69}$) were diluted 1:100 in LB medium with appropriate antibiotics and 0.2% arabinose. The cultures were grown at 30 °C for about 3 hours and then cells were collected by centrifugation and washed 3 times with LB medium. The washed cells were then resuspended in the same volume of LB medium and diluted 1:10 in LB medium with or without arabinose (IPTG was not added because the basal level of expression of *ftsEX* from pSD221 is sufficient for complementation). The diluted cultures were grown at 30 °C for 4 hours and cells were immobilized on an agarose pad for photography.

Western blot

To measure the level of GFP-FtsE mutants, strains were grown as described for fluorescence microscopy. Overnight cultures of SD220 (S3, *ftsEX::cat*) harboring the respective plasmid were diluted 1:100 in LB medium with 0.2 M sucrose and ampicillin and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium with 0.2 M sucrose and IPTG was added to a final concentration of 15 μM. After growth at 30°C for 2 hours, OD_{600} of each culture was measured and samples were taken for western blot. Cells were collected, resuspended in SDS-PAGE sample buffer and boiled for 10 min before they were loaded on the SDS-PAGE gel for analysis. Western blotting and detection of GFP-FtsE were performed as previously described for other division proteins (Addinall & Lutkenhaus, 1996). Anti-GFP antibody was used at a dilution of 1/1000.

Modeling of the FtsEX dimer complex

To do this, a homology model of an FtsE dimer was generated based upon the structure of the nucleotide component of an ABC transporter from *Aquifex aeolicus* (2PCL). It was then superimposed on an ABC transporter complex from *Staphylococcus aureus* (PDB:2ONJ). Also, a model of FtsX was produced using I-TASSER (Zhang, 2009, Roy *et al.*, 2012, Yang & Zhang, 2015) and both it and the FtsE model were superimposed upon the ATP bound form of MacB (5LJ7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Aarsman ME, Piette A, Fraipont C, Vinkenvleugel TM, Nguyen-Disteche M & den Blaauwen T, (2005) Maturation of the Escherichia coli divisome occurs in two steps. Molecular microbiology 55: 1631–1645. [PubMed: 15752189]

Addinall SG, Bi E & Lutkenhaus J, (1996) FtsZ ring formation in fts mutants. Journal of bacteriology 178: 3877–3884. [PubMed: 8682793]

Addinall SG, Cao C & Lutkenhaus J, (1997) FtsN, a late recruit to the septum in Escherichia coli. Molecular microbiology 25: 303–309. [PubMed: 9282742]

Addinall SG & Lutkenhaus J, (1996) FtsA is localized to the septum in an FtsZ-dependent manner. Journal of bacteriology 178: 7167–7172. [PubMed: 8955398]

Arends SJ, Kustusch RJ & Weiss DS, (2009) ATP-binding site lesions in FtsE impair cell division. Journal of bacteriology 191: 3772–3784. [PubMed: 19376877]

Buss J, Coltharp C, Huang T, Pohlmeyer C, Wang SC, Hatem C & Xiao J, (2013) In vivo organization of the FtsZ-ring by ZapA and ZapB revealed by quantitative super-resolution microscopy. Molecular microbiology 89: 1099–1120. [PubMed: 23859153]

Buss JA, Peters NT, Xiao J & Bernhardt TG, (2017) ZapA and ZapB form an FtsZ-independent structure at midcell. Mol Microbiol 104: 652–663. [PubMed: 28249098]

Corbin BD, Wang Y, Beuria TK & Margolin W, (2007) Interaction between cell division proteins FtsE and FtsZ. Journal of bacteriology 189: 3026–3035. [PubMed: 17307852]

Crow A, Greene NP, Kaplan E & Koronakis V, (2017) Structure and mechanotransmission mechanism of the MacB ABC transporter superfamily. Proc Natl Acad Sci U S A 114: 12572–12577. [PubMed: 29109272]

Du S & Lutkenhaus J, (2017) Assembly and activation of the Escherichia coli divisome. Molecular microbiology 105: 177–187. [PubMed: 28419603]

Du S, Pichoff S & Lutkenhaus J, (2016) FtsEX acts on FtsA to regulate divisome assembly and activity. Proceedings of the National Academy of Sciences of the United States of America 113: E5052–5061. [PubMed: 27503875]

Egan AJF, (2018) Bacterial outer membrane constriction. Mol Microbiol 107: 676–687. [PubMed: 29315884]

Geissler B, Shiomi D & Margolin W, (2007) The ftsA* gain-of-function allele of Escherichia coli and its effects on the stability and dynamics of the Z ring. Microbiology 153: 814–825. [PubMed: 17322202]

Gerding MA, Liu B, Bendezu FO, Hale CA, Bernhardt TG & de Boer PA, (2009) Self-enhanced accumulation of FtsN at Division Sites and Roles for Other Proteins with a SPOR domain (DamX, DedD, and RlpA) in Escherichia coli cell constriction. Journal of bacteriology 191: 7383–7401. [PubMed: 19684127]

Gill DR & Salmond GP, (1987) The Escherichia coli cell division proteins FtsY, FtsE and FtsX are inner membrane-associated. Mol Gen Genet 210: 504–508. [PubMed: 3323846]

Goehring NW, Gueiros-Filho F & Beckwith J, (2005) Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in Escherichia coli. Genes & development 19: 127–137. [PubMed: 15630023]

Greene NP, Kaplan E, Crow A & Koronakis V, (2018) Antibiotic Resistance Mediated by the MacB ABC Transporter Family: A Structural and Functional Perspective. Front Microbiol 9: 950. [PubMed: 29892271]

Heidrich C, Ursinus A, Berger J, Schwarz H & Holtje JV, (2002) Effects of multiple deletions of murein hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in Escherichia coli. J Bacteriol 184: 6093–6099. [PubMed: 12399477]

Huang KH, Durand-Heredia J & Janakiraman A, (2013) FtsZ ring stability: of bundles, tubules, crosslinks, and curves. Journal of bacteriology 195: 1859–1868. [PubMed: 23457247]

Karimova G, Dautin N & Ladant D, (2005) Interaction network among Escherichia coli membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. Journal of bacteriology 187: 2233–2243. [PubMed: 15774864]

Karimova G, Pidoux J, Ullmann A & Ladant D, (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proceedings of the National Academy of Sciences of the United States of America 95: 5752–5756. [PubMed: 9576956]

Liu B, Persons L, Lee L & de Boer PA, (2015) Roles for both FtsA and the FtsBLQ subcomplex in FtsN-stimulated cell constriction in Escherichia coli. Molecular microbiology 95: 945–970. [PubMed: 25496160]

Mavrici D, Marakalala MJ, Holton JM, Prigozhin DM, Gee CL, Zhang YJ, Rubin EJ & Alber T, (2014) Mycobacterium tuberculosis FtsX extracellular domain activates the peptidoglycan

hydrolase, RipC. Proceedings of the National Academy of Sciences of the United States of America 111: 8037–8042. [PubMed: 24843173]

Meisner J, Llopis P.Montero, Sham LT, Garner E, Bernhardt TG & Rudner DZ, (2013) FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in Bacillus subtilis. Molecular microbiology 89: 1069–1083. [PubMed: 23855774]

Miller JH, (1992) A short course in bacterial genetics : a laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

O'Reilly EK & Kreuzer KN, (2004) Isolation of SOS constitutive mutants of Escherichia coli. J Bacteriol 186: 7149–7160. [PubMed: 15489426]

Park KT, Dajkovic A, Wissel M, Du S & Lutkenhaus J, (2018) MinC and FtsZ mutant analysis provides insight into MinC/MinD-mediated Z ring disassembly. J Biol Chem 293: 5834–5846. [PubMed: 29414773]

Park KT, Villar MT, Artigues A & Lutkenhaus J, (2017) MinE conformational dynamics regulate membrane binding, MinD interaction, and Min oscillation. Proceedings of the National Academy of Sciences of the United States of America 114: 7497–7504. [PubMed: 28652337]

Peters NT, Morlot C, Yang DC, Uehara T, Vernet T & Bernhardt TG, (2013) Structure-function analysis of the LytM domain of EnvC, an activator of cell wall remodelling at the Escherichia coli division site. Mol Microbiol 89: 690–701. [PubMed: 23796240]

Pichoff S, Du S & Lutkenhaus J, (2018) Disruption of divisome assembly rescued by FtsN-FtsA interaction in Escherichia coli. Proc Natl Acad Sci U S A.

Pichoff S & Lutkenhaus J, (2002) Unique and overlapping roles for ZipA and FtsA in septal ring assembly in Escherichia coli. The EMBO journal 21: 685–693. [PubMed: 11847116]

Pichoff S & Lutkenhaus J, (2007) Identification of a region of FtsA required for interaction with FtsZ. Mol Microbiol 64: 1129–38. [PubMed: 17501933]

Pichoff S, Shen B, Sullivan B & Lutkenhaus J, (2012) FtsA mutants impaired for self-interaction bypass ZipA suggesting a model in which FtsA's self-interaction competes with its ability to recruit downstream division proteins. Molecular microbiology 83: 151–167. [PubMed: 22111832]

Roy A, Yang J & Zhang Y, (2012) COFACTOR: an accurate comparative algorithm for structure-based protein function annotation. Nucleic Acids Res 40: W471–477. [PubMed: 22570420]

Samaluru H, SaiSree L & Reddy M, (2007) Role of SufI (FtsP) in cell division of Escherichia coli: evidence for its involvement in stabilizing the assembly of the divisome. Journal of bacteriology 189: 8044–8052. [PubMed: 17766410]

Schmidt KL, Peterson ND, Kustusch RJ, Wissel MC, Graham B, Phillips GJ & Weiss DS, (2004) A predicted ABC transporter, FtsEX, is needed for cell division in Escherichia coli. Journal of bacteriology 186: 785–793. [PubMed: 14729705]

Sham LT, Barendt SM, Kopecky KE & Winkler ME, (2011) Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsXSpn cell division protein in Streptococcus pneumoniae D39. Proceedings of the National Academy of Sciences of the United States of America 108: E1061–1069. [PubMed: 22006325]

Shen B & Lutkenhaus J, (2009) The conserved C-terminal tail of FtsZ is required for the septal localization and division inhibitory activity of MinC(C)/MinD. Molecular microbiology 72: 410–424. [PubMed: 19415799]

Shen B & Lutkenhaus J, (2010) Examination of the interaction between FtsZ and MinCN in E. coli suggests how MinC disrupts Z rings. Molecular microbiology 75: 1285–1298. [PubMed: 20132438]

Szwedziak P, Wang Q, Bharat TA, Tsim M & Lowe J, (2014) Architecture of the ring formed by the tubulin homologue FtsZ in bacterial cell division. Elife 3: e04601. [PubMed: 25490152]

Tsang MJ & Bernhardt TG, (2015) A role for the FtsQLB complex in cytokinetic ring activation revealed by an ftsL allele that accelerates division. Molecular microbiology 95: 925–944. [PubMed: 25496050]

Uehara T, Parzych KR, Dinh T & Bernhardt TG, (2010) Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. The EMBO journal 29: 1412–1422. [PubMed: 20300061]

Wilkens S, (2015) Structure and mechanism of ABC transporters. F1000Prime Rep 7: 14. [PubMed: 25750732]

Yang DC, Peters NT, Parzych KR, Uehara T, Markovski M & Bernhardt TG, (2011) An ATP-binding cassette transporter-like complex governs cell-wall hydrolysis at the bacterial cytokinetic ring. Proceedings of the National Academy of Sciences of the United States of America 108: E1052–1060. [PubMed: 22006326]

Yang DC, Tan K, Joachimiak A & Bernhardt TG, (2012) A conformational switch controls cell wall-remodelling enzymes required for bacterial cell division. Molecular microbiology 85: 768–781. [PubMed: 22715947]

Yang J & Zhang Y, (2015) I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res 43: W174–181. [PubMed: 25883148]

Zhang Y, (2009) I-TASSER: fully automated protein structure prediction in CASP8. Proteins 77 Suppl 9: 100–113. [PubMed: 19768687]

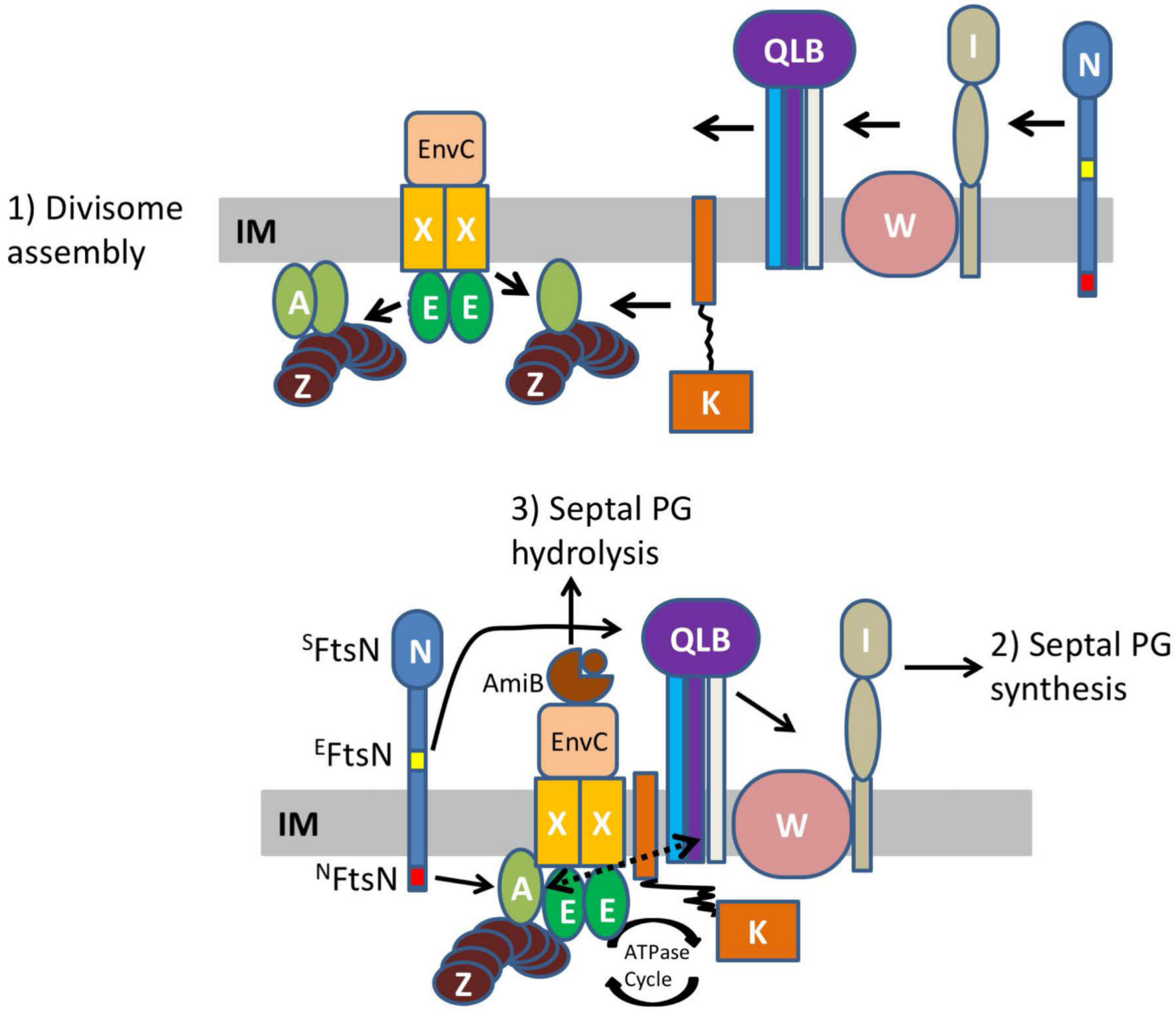

Fig. 1.

Model for the role of FtsEX in divisome assembly and activation. FtsEX plays three roles in divisome function in *E. coli*: 1) FtsEX localizes to the Z ring, recruits EnvC and acts on FtsA to promote the monomeric form which is active in recruitment of downstream proteins; 2) FtsEX is involved in transmitting the signal from FtsN to activate FtsWI to synthesize septal PG; and 3) FtsEX regulates amidases (AmiB/A) to hydrolyze peptidoglycan. The latter two roles require the ATPase activity of FtsEX. In the absence of the ATPase activity septal PG synthesis is not initiated and AmiB is not localized.

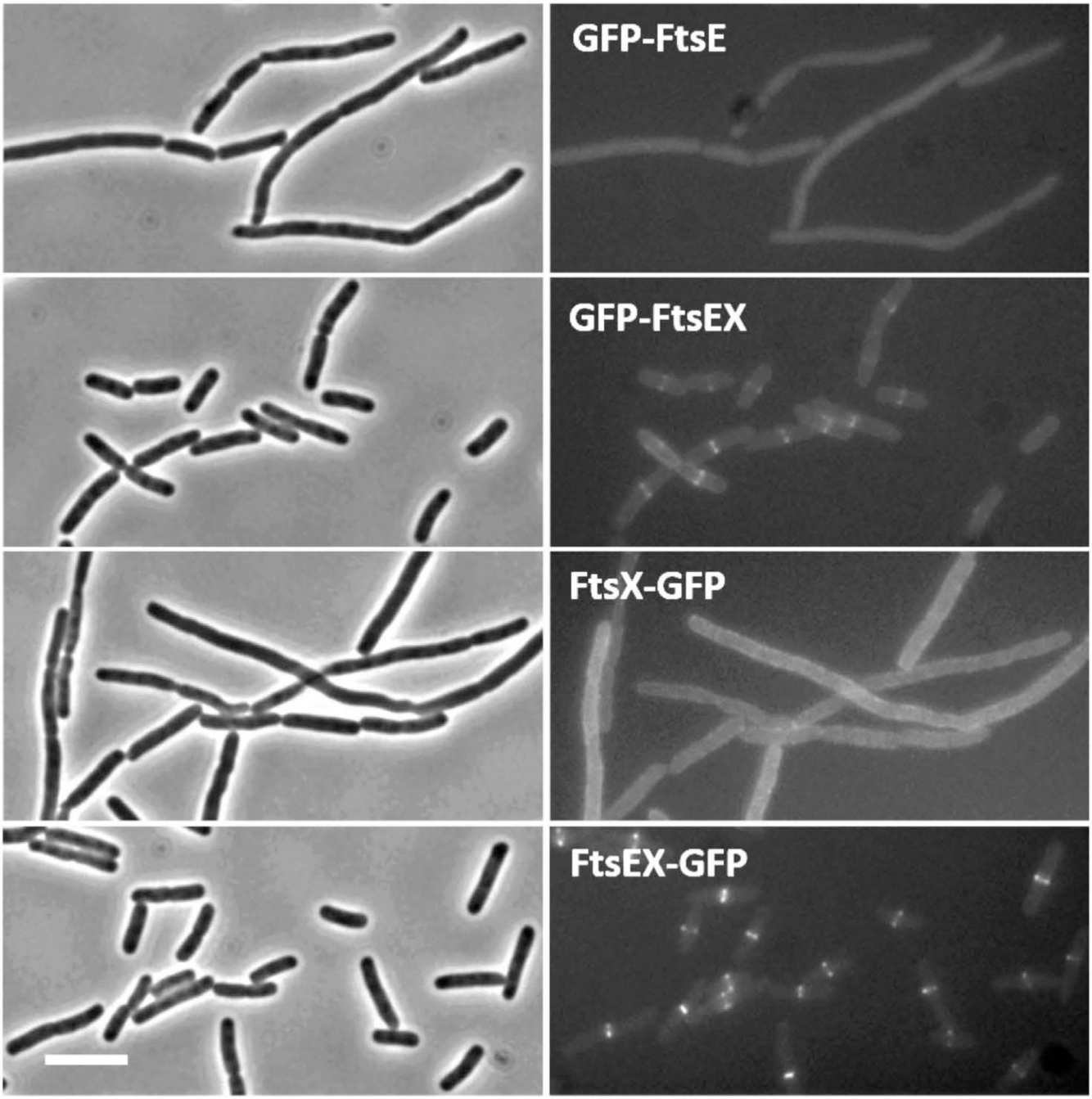

Fig. 2.

The localization of FtsE and FtsX to the Z ring is codependent. Overnight cultures of SD220 (*ΔftsEX*) carrying plasmids expressing *gfp-ftsE* (pSD229), *gfp-ftsEX* (pSD241), *ftsX-gfp* (pSD226) or *ftsEX-gfp* (pSD242) were diluted 1:200 in LB with 0.2 M sucrose and ampicillin. After growth at 30 °C for 1 hour, IPTG was added to a final concentration of 15 μM. Two hours post induction, 2 μl of cells were immobilized on an agarose pad containing LB with 0.2 M sucrose to examine the cell morphology and determine the localization of the fusion proteins. Scale bar, 3 μm.

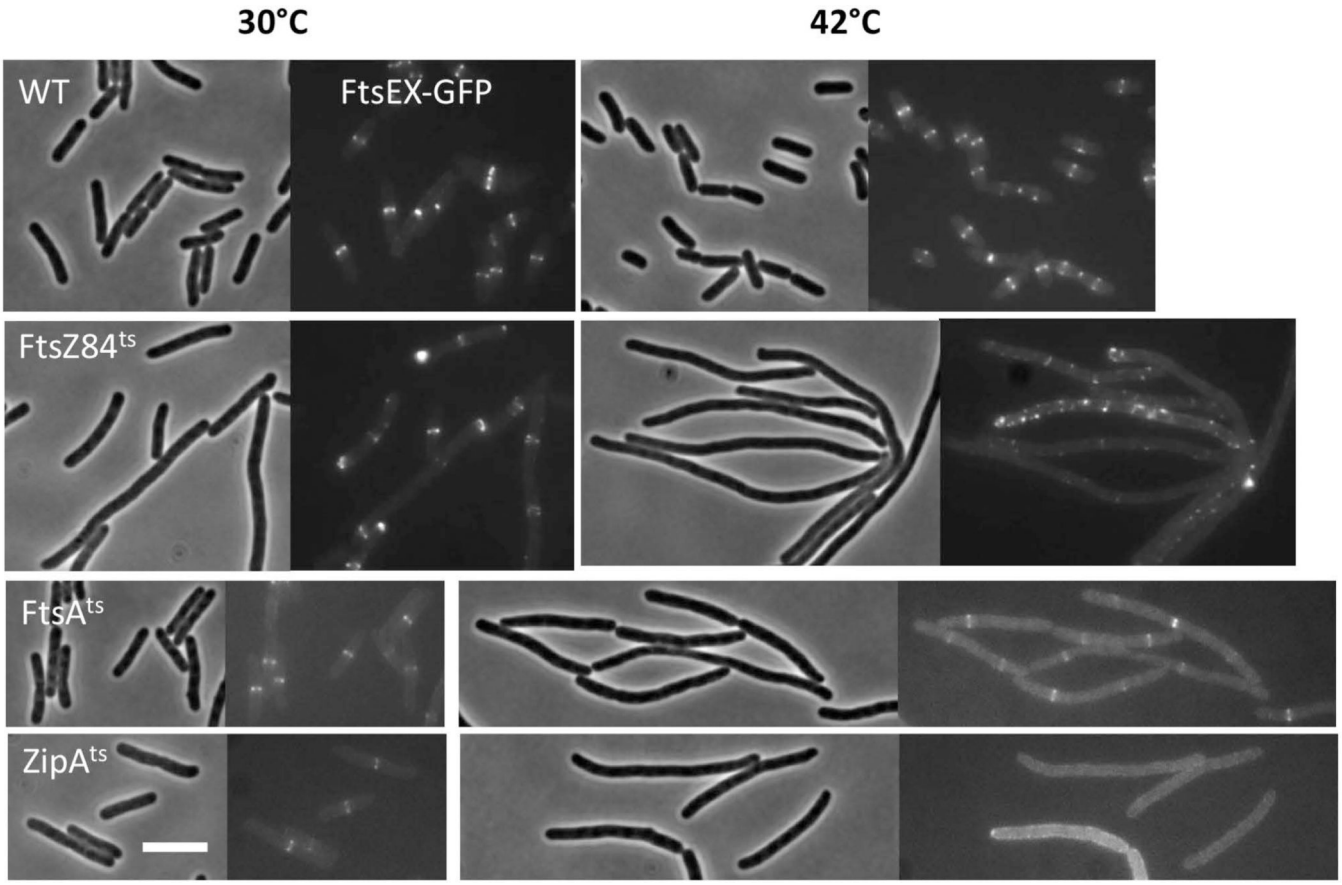

Fig. 3.

FtsEX-GFP depends on FtsZ and ZipA for localization. Overnight cultures of W3110, PS106 (*ftsZ84*^{ts}), PS223 (*zipA1*^{ts}) and PS236 (*ftsA12*^{ts}) carrying plasmid pSD242 (pDSW210, P_{206}::*ftsEX-gfp*) were diluted 100X in fresh LB medium with antibiotics and grown at 30°C for 2 h. The cultures were then diluted 1:10 in fresh LB medium with antibiotics and IPTG was added to a final concentration of 15 μM. Two hours post induction, the cultures were split in half. One half was kept at 30 °C, while the other half was shifted to 42°C. After 30 minutes, cells growing at 30 °C were taken for photography while cells growing at 42 °C were fixed with paraformaldehyde and glutaraldehyde before photographing. Scale bar, 3 μm.

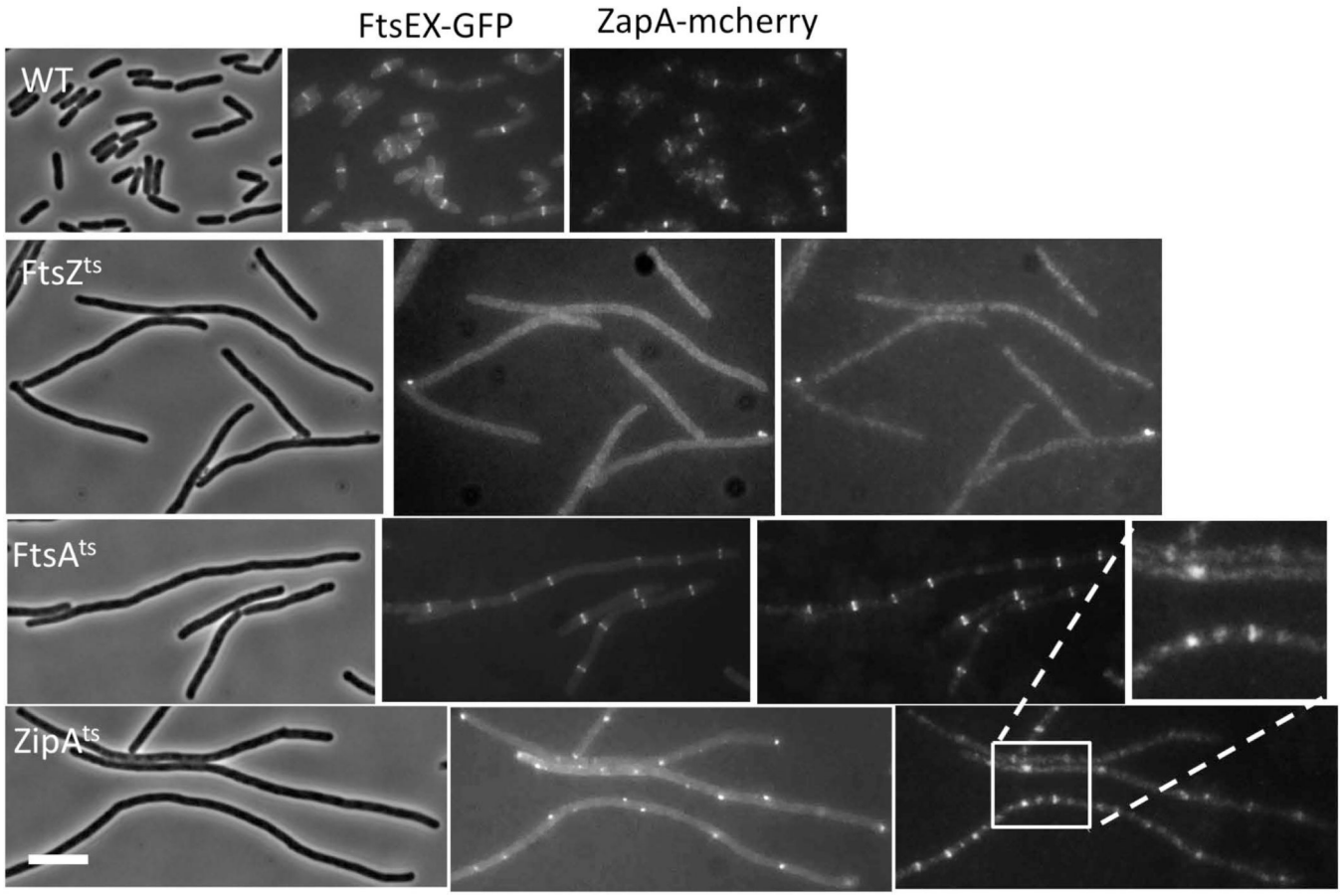

Fig. 4.

FtsEX-GFP disrupts Z rings assembled with only FtsA (ZipA^{ts} strain). Overnight cultures of SD335 (*zapA-mCherry*), SD336 (*ftsZ84* [Ts], *zapA-mCherry*), SD337 (*ftsA12* [Ts], *zapA-mCherry*) and SD338 (*zipA* [Ts], *zapA-mCherry*) carrying plasmid pSD242 (pDSW210, P_{206}::*ftsEX-gfp*) were diluted 1:100 in fresh LB medium with antibiotics and grown at 30°C for 2 hours. The cultures were then diluted 1:10 in fresh LB medium with antibiotics and IPTG was added to a final concentration of 15 μM. The cultures were split in half 2 hours later and one half was kept at 30°C, while the other half was shifted to 42°C. Thirty minutes later cells growing at 30°C were taken for photography while cells growing at 42°C were fixed with paraformaldehyde and glutaraldehyde before photographing. Samples from the 30°C cultures are shown in Fig. S3. Scale bar, 3 μm.

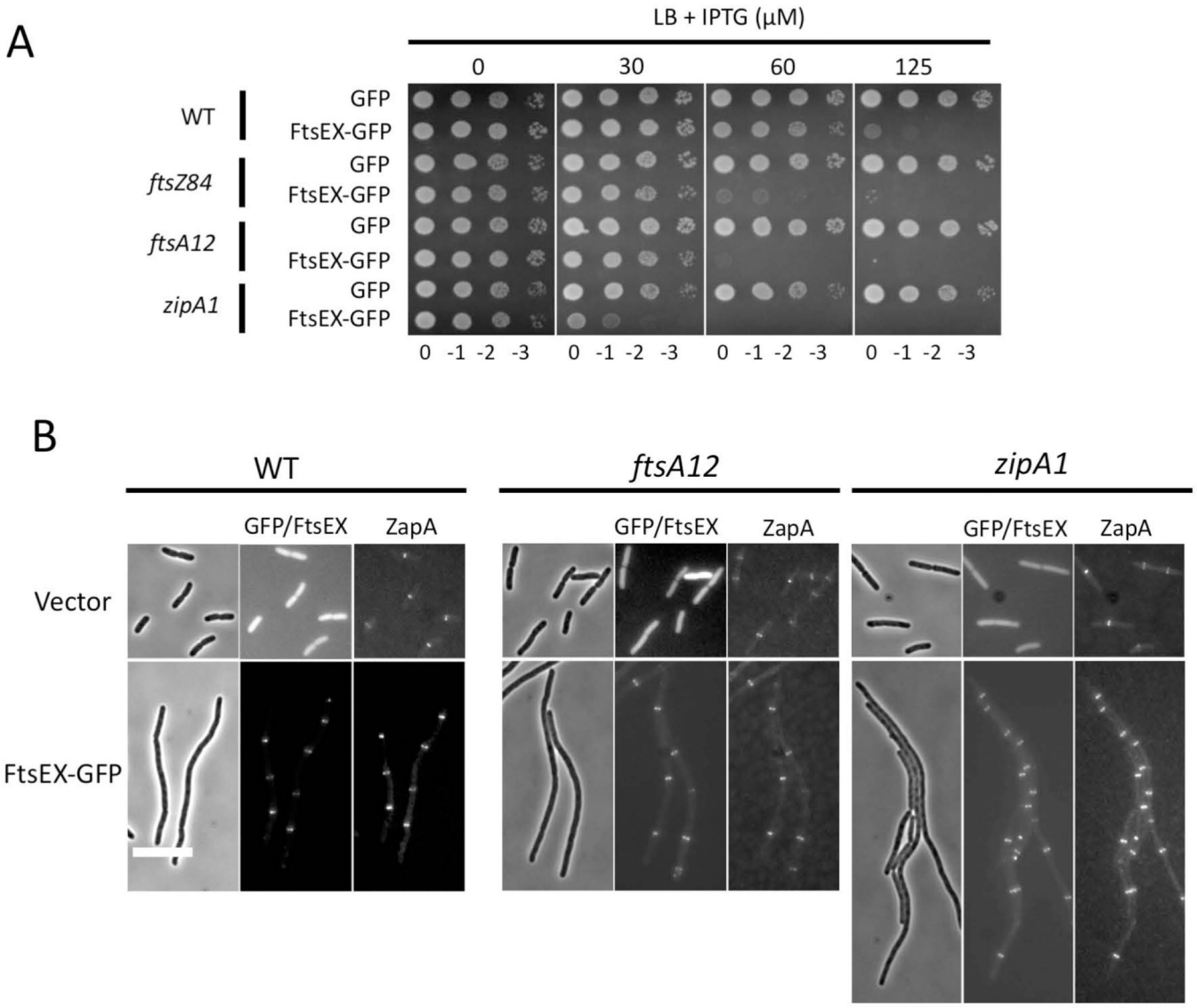

Fig. 5.

Moderate overexpression of *ftsEX-gfp* blocks cell division without disrupting Z rings. (A) The sensitivity of strains to expression of *ftsEX-GFP* was assessed at permissive temperature with a spot assay. In this and all subsequent spot assays cultures were serially diluted 10-fold in LB and spotted on LB plates with antibiotics with and without IPTG. The strains tested were SD335 (*zapA-mcherry*), PS106 (*ftsZ84* [Ts], *zapA-mcherry*), SD337 (*ftsA12* [Ts], *zapA-mcherry*) and SD338 (*zipA1* [Ts], *zapA-mcherry*) carrying plasmid pDSW210 (P_{206}::*gfp*) or pSD242 (P_{206}::*ftsEX-gfp*). Plates were incubated at 30°C overnight before photographing. (B) The effect of *ftsEX-gfp* expression on Z rings. The strains in (A) were grown to exponential phase in LB with ampicillin and 60 μM IPTG was added. Two hours later samples were taken and cells examined by phase and fluorescence microscopy. Results with strain SD335, SD337 and SD338 are shown. Scale bar, 5 μm

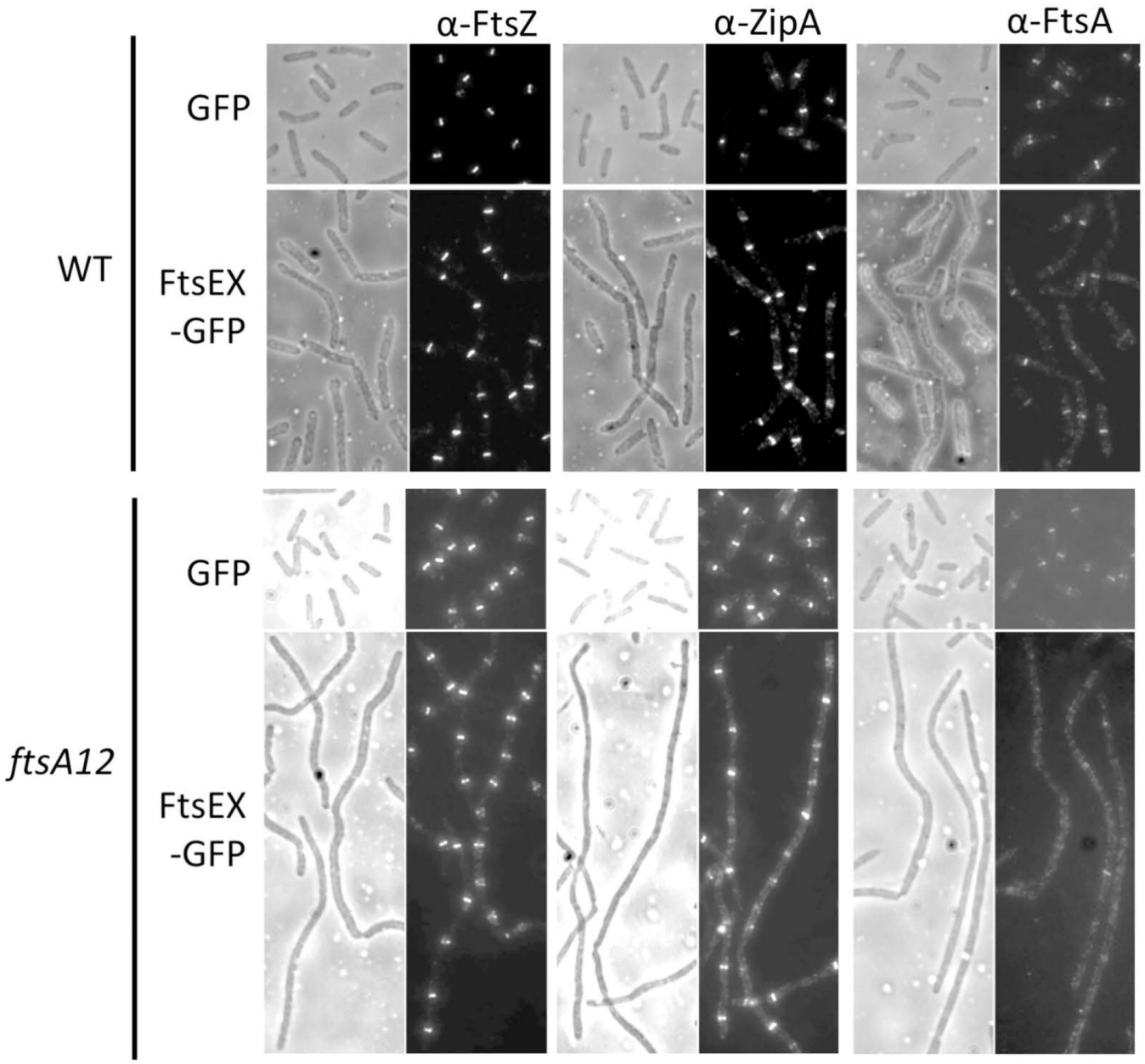

Fig. 6.

Moderate overexpression of *ftsEX-gfp* competes with FtsA at the Z ring. Strains S3 (WT) and PS236 (*ftsA12* [Ts]) containing a plasmid expressing *ftsEX-gfp* (pSD242) and growing exponentially at 30 °C in LB with ampicillin were induced with 60 μM IPTG for 2 hours. Cells were fixed with glutaraldehyde and paraformaldehyde and immunostained for FtsZ, ZipA and FtsA and examined by phase and fluorescence microscopy.

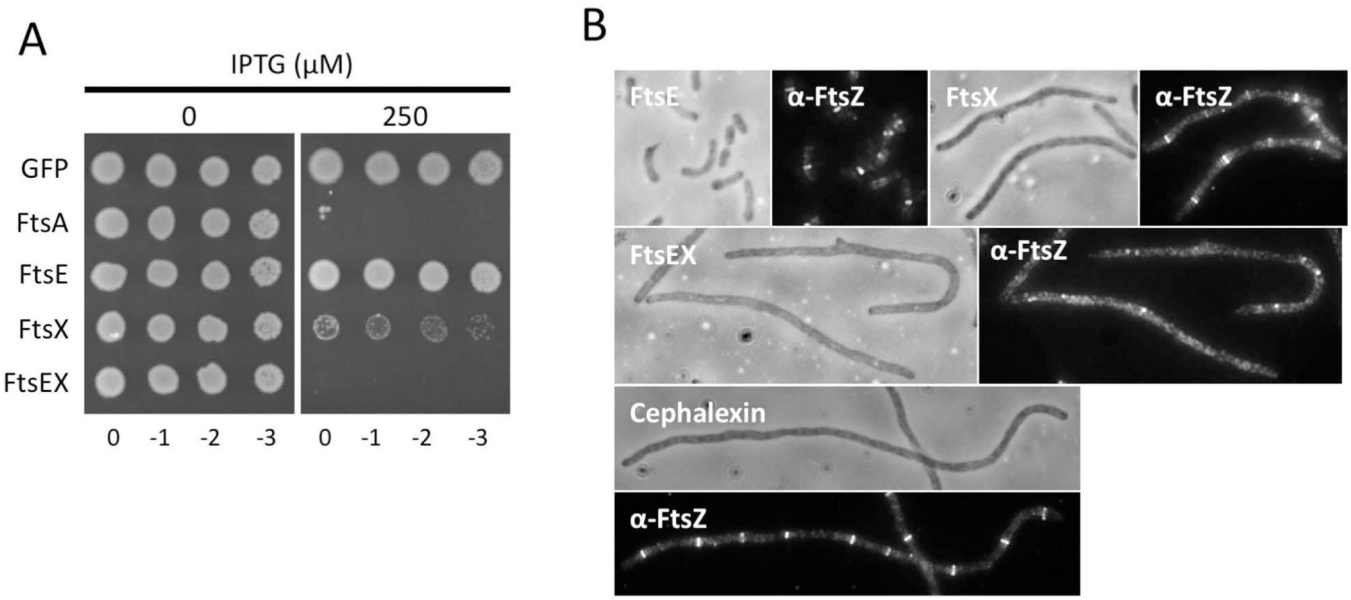

Fig. 7.

Higher overexpression of *ftsEX* blocks cell division by preventing Z ring formation. (A) Spot test to determine the toxicity of *ftsE*, *ftsX* and *ftsEX* overexpression. Cultures of strain W3110 containing plasmids expressing *ftsE* (pSEB426[P_{204}::*ftsE*]), *ftsX* (pSEB427[P_{204}::*ftsX*]), *ftsA* (pSEB306^{+}[P_{204}::*ftsA*]), *ftsEX* (pSEB428 [pDSW208, P_{204}::*ftsEX*]) or *gfp* (pDSW208, P_{204}::*gfp*) growing in LB with ampicillin were induced with IPTG. Plasmids expressing *gfp* or *ftsA* were used as controls. (B) Samples from liquid cultures of some of the strains in (A) were taken two hours after the addition of IPTG (1000 μM), immunostained for FtsZ and analyzed by phase and fluorescence microscopy. A control sample of W3110 treated with 20 μg/ml of cephalexin for 2 hours was also analyzed as a control.

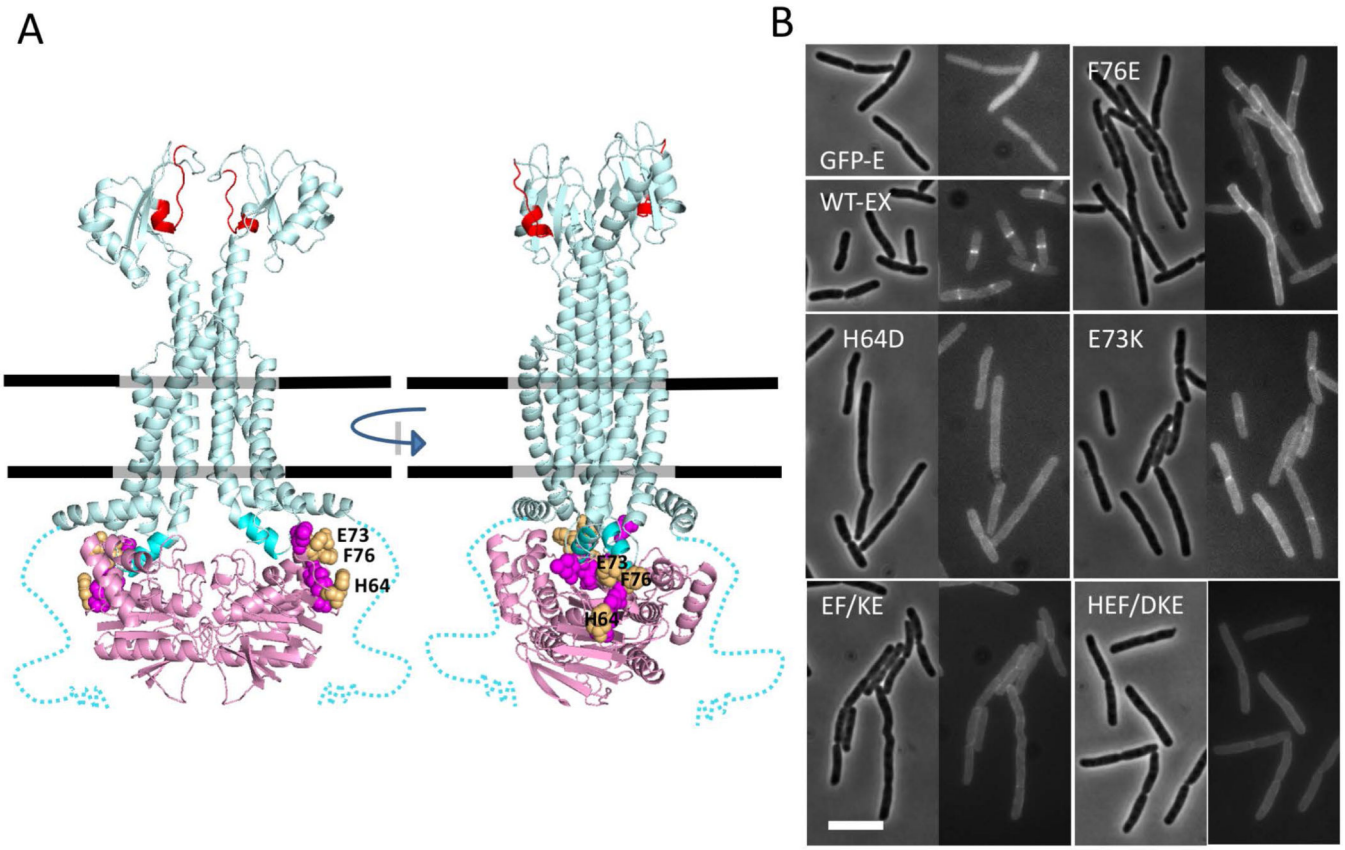

Fig. 8.

Model of FtsEX and identification of a region in FtsE that is important for localization of GFP-FtsEX to the Z ring. (A) Model of FtsEX. A model of FtsE was generated by using the nucleotide component of an ABC transporter from *Aquifex aeolicus* (2PCL) and then superimposing it on an ABC transporter complex from *Staphylococcus aureus* (PDB:2ONJ). A model of the FtsX monomer was generated using I-TASSER and it along with the FtsE dimer model were superimposed on the structure of MacB (Crow *et al.*, 2017). FtsE is in pink and FtsX is colored cyan. The first 46 residues of FtsX are not in the model and are indicated by a dotted line. The residues important for localization of FtsEX to the Z ring are shown in space filled (magenta and light orange). The region in FtsX colored red is required for the interaction between FtsX and EnvC (residues 152–161)(Yang *et al.*, 2011). (B) Effect of *ftsE* mutations on the localization of GFP-FtsEX. SD220 (W3110 *leu*::Tn*10*, *ftsEX::cat*) containing pSD241 or derivatives with *ftsE* mutations were grown in LB with ampicillin and 0.2 M sucrose. Localization was determined following low level of expression (15 μM IPTG) from pSD241 (P_{206}::*gfp-ftsEX*) and its derivatives. The altered residues are indicated in light orange in panel A. Scale bar, 3 μm.

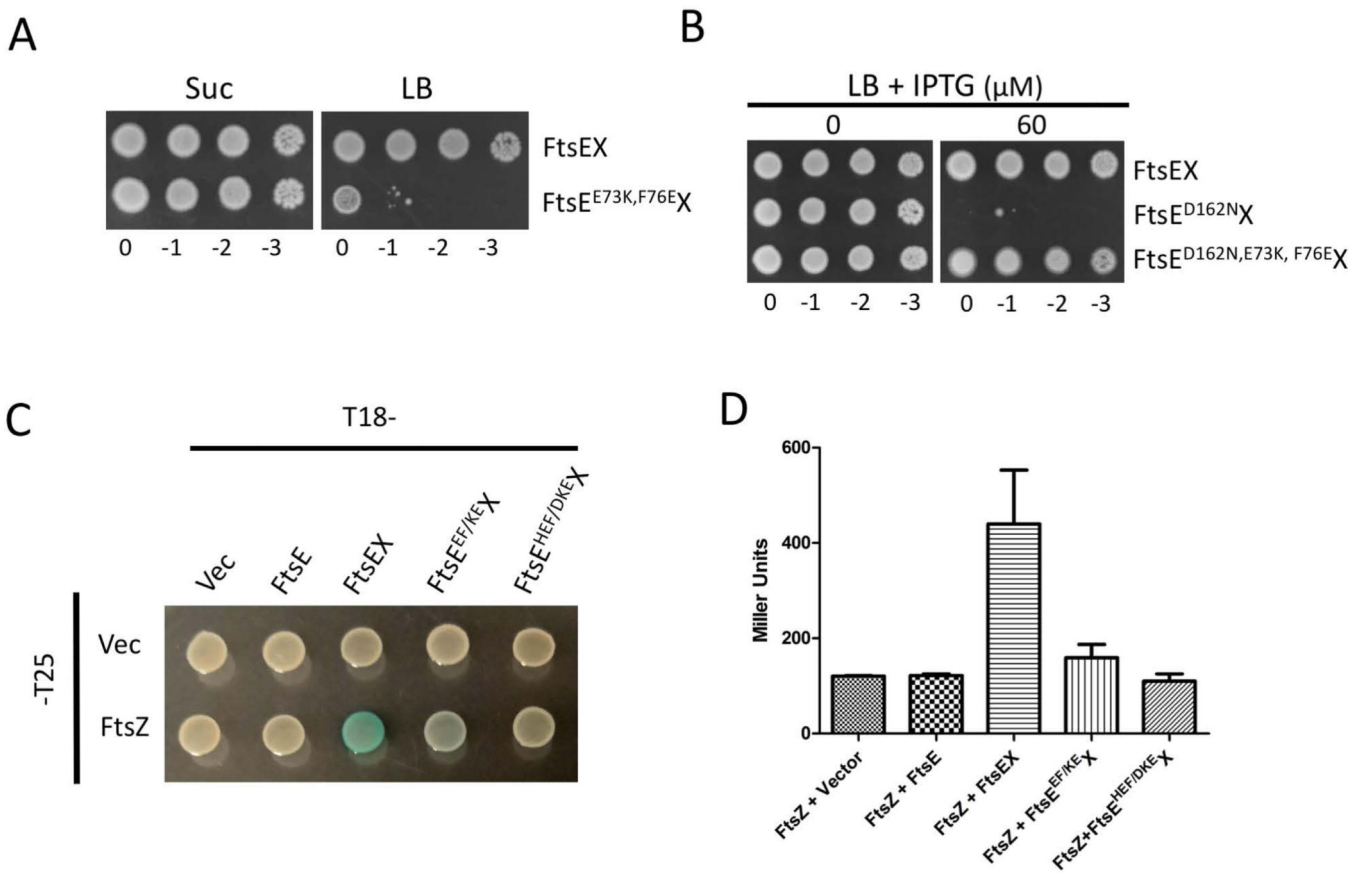

Fig. 9.

The effect of *ftsE* mutations on complementation, toxicity of FtsE^{D162N}X and the interaction of FtsE with FtsZ. (A) Effect of *ftsE* mutations on complementation. The ability to complement *ΔftsEX* was assessed in strain SD220 (W3110 *leu*::Tn*10*, *ftsEX::cat)* using plasmids pSD221 (pEXT22, P_{tac}::*ftsEX*), expressing *ftsEX* or a derivative pSD221-EF/KE expressing *ftsE*^{E73K,F76E}*X*. Single colonies were picked into LB, diluted in 10 fold increments and spotted on LB plates containing ampicillin with and without sucrose. Plates were incubated at 37 °C overnight before photographing. (B) The effect of *ftsE* mutations on the toxicity of the ATPase mutant of FtsEX. Plasmid pSD221 or derivatives expressing various alleles of *ftsEX* were transformed into strain S3 and the toxicity assessed with a spot assay. The plates were incubated at 37 °C overnight before photographing. (C) The effect of various *ftsE* mutations on the interaction between FtsE (interaction is only observed if *ftsE* is expressed with *ftsX*) and FtsZ was assessed with the BACTH. Plasmid pairs were co-transformed into BTH101. The next day, single colonies of each strain were resuspended in 1 ml LB medium and 3 μl of each aliquot was spotted on LB plates containing ampicillin, kanamycin, 40 μg/ml X-gal and 100 μM IPTG. Plates were incubated at 30°C overnight before imaging. (D) Quantification of the interactions in panel C was determined by a β-galatosidase assay as described in Experimental Procedures.

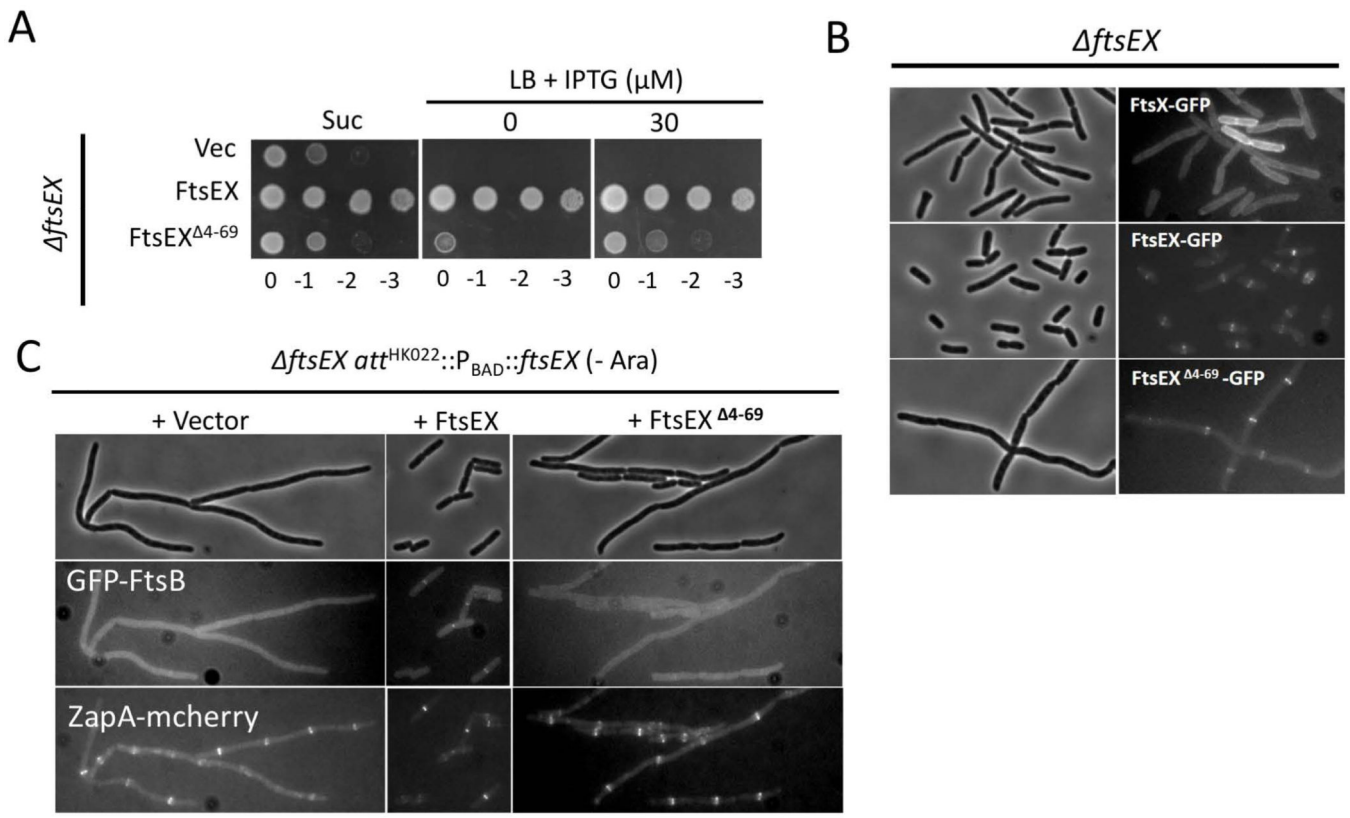

Fig. 10.

The N-terminal cytoplasmic domain of FtsX ($FtsX^{Ncyto}$) is dispensable for FtsEX localization but is required for FtsEX function. (A) Complementation test of $ftsEX^{\Delta 4-69}$. Plasmids expressing *ftsEX* (pSD221[P_{tac}::*ftsEX*]) or $ftsEX^{\Delta 4-69}$ (pSD318[P_{tac}::$ftsEX^{\Delta 4-69}$]) were transformed into strain SD220 (W3110 *leu*::Tn*10*, *ftsEX::cat)*. Single transformants were analyzed by a spot assay on LB plates containing kanamycin with or without sucrose. (B) Localization of $FtsEX^{\Delta 4-69}$-GFP in *ΔftsEX* cells. Overnight cultures of SD220 carrying plasmids pSD226 (P_{206}::*ftsX-gfp*), pSD242 (P_{206}::*ftsEX-gfp*) or pSD321(P_{206}::$ftsEX^{\Delta 4-69}$-*gfp*) were diluted 1:200 in LB with 0.2 M sucrose and ampicillin. After growth at 30 °C for 1 h, 15 μM IPTG was added to the culture and two hours later cells were analyzed by phase and fluorescence microscopy. (C) $FtsX^{Ncyto}$ is required for recruitment of downstream division proteins. Overnight cultures of SD446 (W3110, *zapA-mcherry cat<>frt*, *ftsEX<>frt* P_{ara}::*ftsEX-cat*) carrying plasmids pSD332 (pSC101, P_{syn}:*gfp-ftsB*) and pEXT22 or derivatives expressing alleles of *ftsEX* (pSD221 or pSD318) were diluted 1:100 in LB medium with antibiotics and 0.2% arabinose and grown at 30°C for 2 hours. Cells were centrifuged, washed 3 times in LB and resuspended in LB with antibiotics and with or without arabinose. After growth at 30°C for 3 hours, samples were taken and analyzed by phase and fluorescence microscopy. Shown are the samples without arabinose, the control with arabinose is shown in Fig. S14.

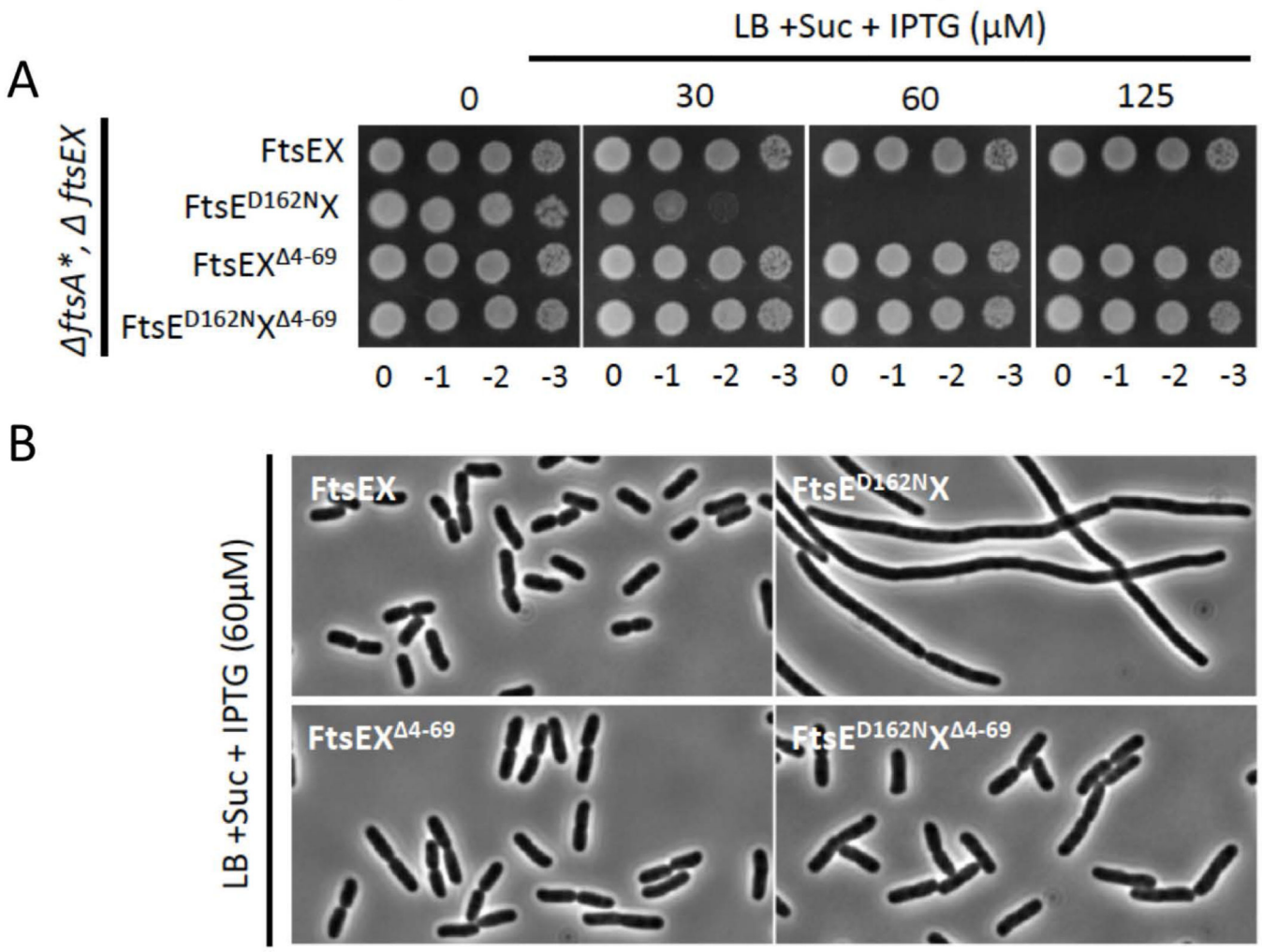

Fig. 11.

The toxicity of the ATPase mutant requires FtsX^{Ncyto}. (A) The toxicity of different *ftsEX* alleles. Derivates of pEXT22 expressing various alleles of *ftsX* and *ftsE* were transformed into strain SD221 (W3110, *leu*::Tn*10 ftsA*, ftsEX::cat*) and transformants tested by a spot assay. Single colonies of each strain growing on LB with 0.2 M sucrose and kanamycin were resuspended in LB, serially diluted and spotted on plates containing sucrose, kanamycin and increasing concentrations of IPTG. (B) Overnight cultures of strains from (A) were diluted 1:200 in LB with 0.2 M sucrose and kanamycin. After growth at 30 °C for 1 hour, IPTG was added to final concentration of 60 μM. Two hours after the addition of IPTG, samples were analyzed by phase microscopy.

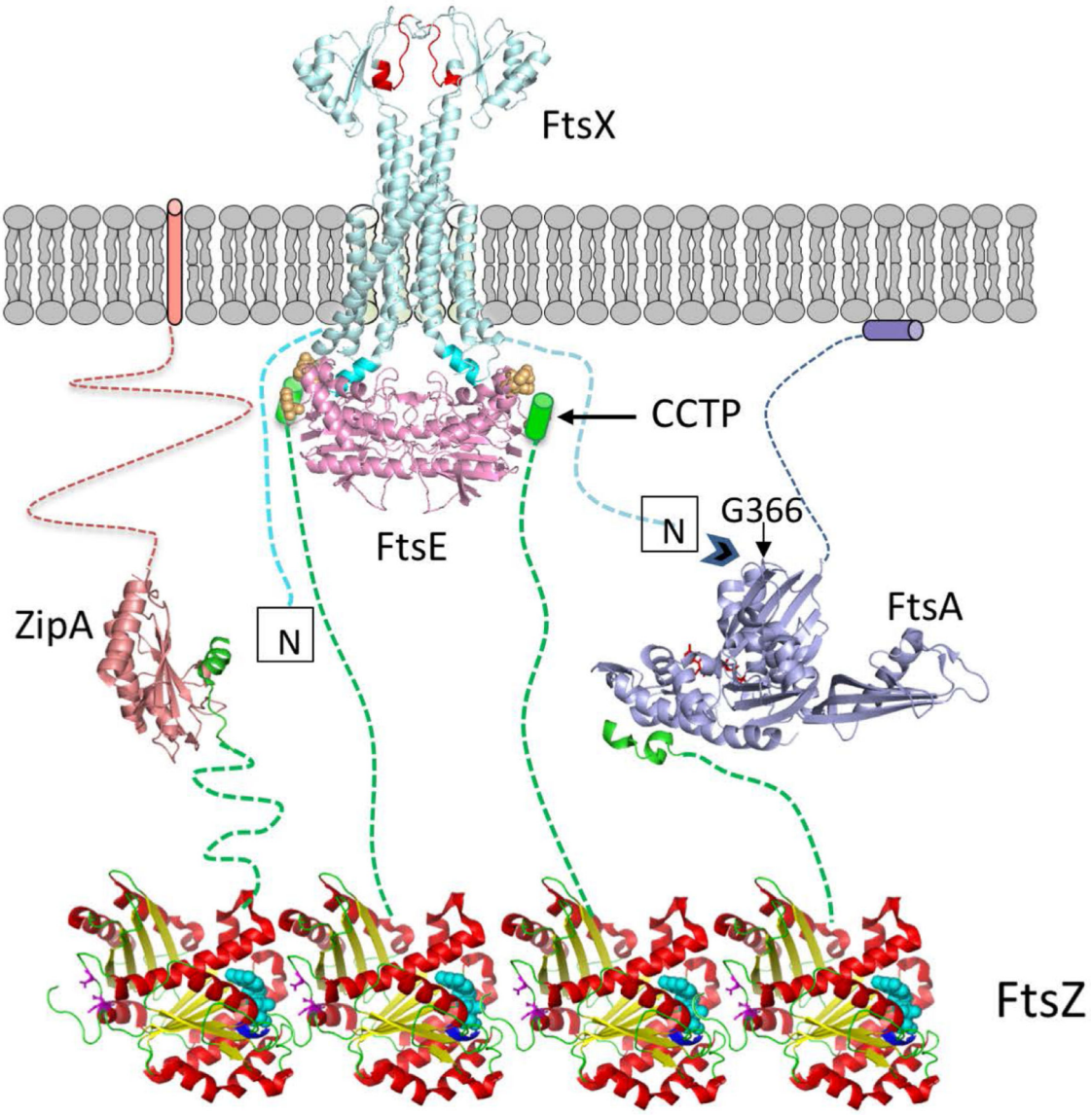

Fig. 12.

Model for FtsEX interactions with other division proteins in *E. coli*. An FtsZ filament is tethered to the membrane by the CCTP of FtsZ interacting with FtsA and ZipA. The CCTP is connected to the tubulin domain of FtsZ by a 50 amino acid intrinsically disordered linker (green dotted line). 2) FtsEX is recruited to the filament through FtsE bound to FtsX interacting with the CCTP. 3) The N-terminal region of FtsX (indicated by the boxed N) interacts with FtsA (region around residue G366) making FtsA less oligomeric. 4) Less oligomeric FtsA recruits the remaining divisome proteins (FtsK, FtsQLB, FtsWI and FtsN).