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Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA

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Summary

The cytokinetic Z ring is required for bacterial cell division. It consists of polymers of FtsZ, the bacterial ancestor of eukaryotic tubulin, linked to the cytoplasmic membrane. Formation of a Z ring in *Escherichia coli* occurs as long as one of two proteins, ZipA or FtsA, is present. Both of these proteins bind FtsZ suggesting that they might function to tether FtsZ filaments to the membrane. Although ZipA has a transmembrane domain and therefore can function as a membrane anchor, interaction of FtsA with the membrane has not been explored. In this study we demonstrate that FtsA, which is structurally related to eukaryotic actin, has a conserved C-terminal amphipathic helix that is essential for FtsA function. It is required to target FtsA to the membrane and subsequently to the Z ring. As FtsA is much more widely conserved in bacteria than ZipA, it is likely that FtsA serves as the principal membrane anchor for the Z ring.

Introduction

Bacterial cell division requires the cytokinetic Z ring consisting of polymers of FtsZ tethered to the cytoplasmic membrane at the division site (Bi and Lutkenhaus, 1991; Lutkenhaus and Addinall, 1997; Errington *et al.*, 2003; Romberg and Levin, 2003). The ring is positioned at the division site through the activity of two negative regulatory systems, Min and Noc, which prevent its formation elsewhere in the cell (de Boer *et al.*, 1989; Yu and Margolin, 1999; Wu and Errington, 2004). It is a highly dynamic structure with FtsZ in the ring turning over with a half-life on the order of 10 s (Stricker *et al.*, 2002). The Z ring functions to recruit additional cell division proteins, of which more than a dozen have been identified in *Escherichia coli*. These proteins are recruited in an ordered fashion resulting in a complex (septal ring) capable of carrying out cytokinesis (Buddelmeijer and Beckwith, 2002). Two of these proteins, FtsA and ZipA, interact directly with FtsZ and appear to localize simultaneously with FtsZ at the Z ring (Addinall and Lutkenhaus, 1996; Wang *et al.*, 1997; Hale and de Boer, 1999). A third protein ZapA also interacts directly with FtsZ but is not essential for cell division (Gueiros-Filho and Losick, 2002; Johnson *et al.*, 2004). Only after FtsA and ZipA are present at the Z ring, are additional downstream cell division proteins recruited resulting in the formation of a septal ring capable of carrying out cytokinesis (Weiss *et al.*, 1999; Bernhardt and de Boer, 2003; Schmidt *et al.*, 2004).

In addition to functioning as a scaffold for the assembly of the septal ring, the Z ring may have a more direct role in providing the force for constriction. Models have been proposed in which energy from depolymerization of FtsZ filaments is used to power the invagination of the septum (Erickson *et al.*, 1996; Bramhill, 1997). However, there has been no direct evidence to date for this model.

One of the main questions in bacterial cell division is how the Z ring is linked to the cytoplasmic membrane. In *E. coli* either one of two proteins, FtsA or ZipA, is capable of supporting formation of a Z ring (Addinall and Lutkenhaus, 1996; Hale and de Boer, 1999; Pichoff and Lutkenhaus, 2002). Both proteins bind to a short conserved sequence located at the extreme carboxy end of FtsZ (Ma and Margolin, 1999; Mosyak *et al.*, 2000; Haney *et al.*, 2001; Pichoff and Lutkenhaus, 2002). As long as one of these two proteins is present a Z ring forms, however, a complete septal ring is not formed as additional downstream proteins are not recruited (Pichoff and Lutkenhaus, 2002). Whether additional proteins are also required at this early stage of assembly is not clear. However, when both FtsA and ZipA are inactivated Z rings do not form (Pichoff and Lutkenhaus, 2002). Also, FtsZ mutants, unable to bind FtsA or ZipA because of substitutions or deletions of its conserved C-terminal motif, are unable to assemble into Z rings in the absence of wild-type FtsZ (Ma and Margolin, 1999; Pichoff and Lutkenhaus, 2002).

It is clear that ZipA could link FtsZ polymers to the membrane. The FtsZ interacting domain of ZipA is located in a C-terminal domain that is linked to an amino terminal transmembrane domain by an extended linker region (Hale and de Boer, 1997). This transmembrane domain of ZipA is required for ZipA to be functional so it likely serves

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a membrane-anchoring function. However, ZipA is unlikely to serve as the primary link to the membrane because it is only found in some Gram-negative bacteria (Hale and de Boer, 1997).

FtsA is structurally homologous to actin and is more conserved than ZipA (van den Ent and Lowe, 2000). It is present in most bacteria suggesting it may have a more important role in Z-ring formation than ZipA (Rothfield *et al*., 1999). The *ftsA* and *ftsZ* genes form one of the more highly conserved pair of tandemly linked genes in bacteria. Significantly, a gain of function mutation in the *ftsA* gene of *E. coli* bypasses the requirement for ZipA (Geissler *et al*., 2003). This *ftsA* allele allows all downstream division proteins to be recruited to the Z ring in the absence of ZipA, suggesting that FtsA plays a more direct and important role in Z ring assembly and in the recruitment of downstream proteins.

Since FtsA is a key protein interacting with FtsZ, how does it link FtsZ polymers to the membrane? One possibility is that FtsA interacts with some as yet unidentified membrane protein. Another is that FtsA interacts directly with the membrane even though no sequence interacting with the membrane has been identified. Cell fractionation studies revealed about 30% of FtsA is in the membrane fraction (Pla *et al*., 1990). Also, FtsA–GFP fusions show some tendency to be at the membrane and deletion analysis suggests that an internal domain is responsible (Ma *et al*., 1996). These results raise the possibility that FtsA is a peripheral membrane protein but do not differentiate whether FtsA binds directly to the membrane or to a protein in the membrane.

In this study, we explored the possibility that FtsA binds directly to the membrane. Previous deletion analysis of the C-terminal region of FtsA revealed that four residues could be deleted without affecting FtsA function; however, further deletion resulted in inactivation and failure of the truncated FtsA to localize to the septum (Yim *et al*., 2000). Our results indicate that a conserved C-terminal motif in FtsA functions as a membrane targeting sequence (MTS) by forming an amphipathic helix that anchors FtsA, and therefore, the Z ring to the membrane.

Results

Analysis of FtsA sequences reveals a conserved C-terminal motif with the potential to form an amphipathic helix

Aligning FtsA sequences from Gram-negative and Gram-positive bacteria reveals a highly conserved segment of approximately 375 residues. This segment is preceded by a short variable N-terminal region and a much larger and more variable C-terminal domain. Despite the variability in the length and sequence of the C-terminal domain, the extreme C-terminus contains a region of approximately 15 residues that is conserved and present in all FtsA sequences (Fig. 1A). This conserved motif was previously observed; however, its function has not been identified (Lowe and van den Ent, 2001). In the crystal structure of the *Thermatoga maritima* FtsA the carboxy terminal 30 amino acids are not present indicating that they are disordered (van den Ent and Lowe, 2000).

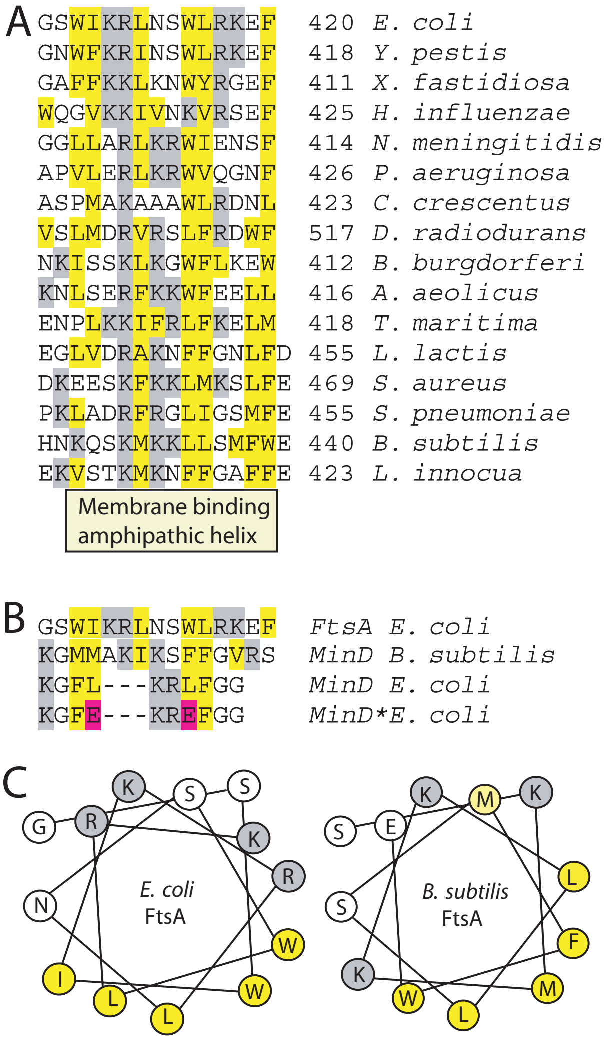

Fig. 1. The C-terminal region of FtsA contains a conserved motif that has the capacity to form an amphiphatic helix.
A. Comparison of the C-termini of FtsAs from various bacteria reveals a conserved motif with the capacity to form an amphipathic helix.
B. Comparison of the conserved motif of FtsA to the MTS of MinD from *B. subtilis* and *E. coli*.
C. Helical wheel diagram of the conserved carboxy motif from FtsA of *E. coli* and *B. subtilis*. The diagram of the *E. coli* FtsA motif does not include the last two residues as they are not essential for FtsA function. The diagram for the *B. subtilis* FtsA motif does not include the first four residues as the hydrophobic character of the putative helix is shifted towards the carboxy end of the motif in Gram-positive bacteria (the last five sequences in part A). Large hydrophobic residues are highlighted in yellow and basic residues are highlighted in grey. The glutamate substitutions in the MTS of the *E. coli* MinD (MinDEc*) are indicated in red.

Examination of these 15 moderately conserved residues reveals a pattern with the potential to form an amphipathic helix (Fig. 1C). The hydrophobic character of the helix lies towards the amino end of the segment in FtsA from Gram-negative bacteria, whereas it is shifted towards the carboxy end of the segment in FtsA from Gram-positive bacteria. This potential helix displays similarity to the C-terminal MTS of MinD which forms an amphipathic helix that mediates MinD binding to the membrane (Szeto *et al*., 2002; 2003; Hu and Lutkenhaus, 2003; Zhou and Lutkenhaus, 2003). The C-terminal motif in FtsA is more similar to the MinD MTS of Gram-positive bacteria, which is longer than the MTS present in MinD of Gram-negative bacteria (Fig. 1B). The longer sequence confers stronger membrane binding (Szeto *et al*., 2003). We set out to test if the conserved C-terminal motif of FtsA could promote FtsA binding to the membrane and therefore constitute an MTS by forming an amphipathic helix.

The C-terminal motif is required for localization to the membrane and the Z ring

As the localization of FtsA to the membrane has been reported but not well characterized, we set out to examine it more closely (Sanchez *et al*., 1994; Ma *et al*., 1996). GFP fusions to the N-terminus of FtsA and a mutant deleted for the carboxy terminal 15 residues were constructed. The behaviour of these GFP fusions was then examined in various genetic backgrounds including wild type, *ftsA12* (Ts) and *ftsZ84* (Ts). The fusions were induced at 30°C for 2.5 h and then shifted to 42°C to inactivate the temperature-sensitive proteins.

Expression of the GFP–FtsA fusion in the wild-type strain W3110 revealed patterns observed previously (Ma *et al*., 1996) (Fig. 2A). In some cells the fluorescence was located in bands at midcell (indicative of localization to Z rings, arrowheads) whereas in other longer cells the fluorescence was in spirals located at expected division sites (arrow). These spirals are thought to arise from excess expression of the GFP–FtsA fusion causing the Z ring to form spirals (Ma *et al*., 1996). In some cells the fluorescence not present at Z rings appears at the membrane (double arrowheads). At this time after induction, cells are longer than uninduced cells indicating the fusion is interfering with division (data not shown). This is perhaps not too surprising as increasing the level of FtsA results in a block to division (Dai and Lutkenhaus, 1992).

The GFP–FtsA fusion was not fully functional as it could not complement the *ftsA12* (Ts) mutation (data not shown). In fact, the presence of the *ftsA12* (Ts) mutation in W3110 made the strain sensitive to the GFP–FtsA fusion as it already started to filament during the induction at the permissive temperature. Nonetheless, at 42°C the fluorescence was distributed in evenly spaced bands

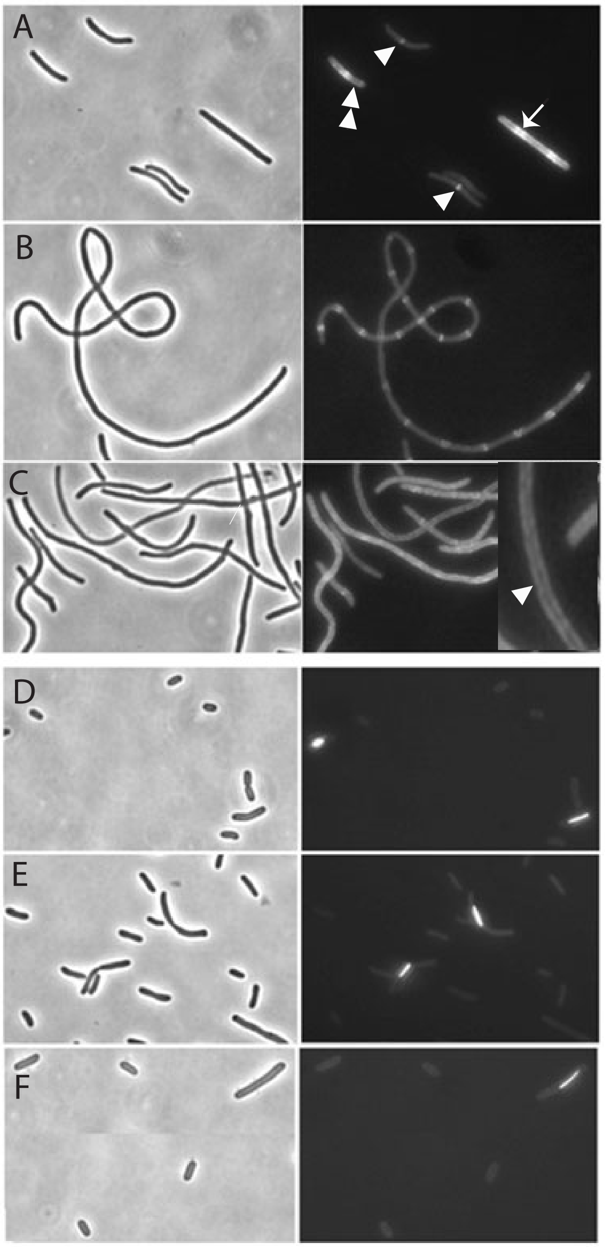

Fig. 2. The conserved carboxyl motif of FtsA is required for localization to the membrane and the Z ring. Strains carrying pSEB293 (*gfp-ftsA*) (A–C) or pSEB294 (*gfp-ftsAΔ15*) (D–F) were grown exponentially at 30°C for 2.5 h in presence of 0.001% arabinose at which time the fluorescence was visible. The cultures were shifted to 42°C for 30 min. Samples were taken, fixed and observed as described in *Experimental procedures*. The strains were as follows: W3110 (A and D); PS236 [W3110 *ftsA12* (Ts)] (B and E); PS106 [W3110 *ftsZ84* (Ts)] (C and F). The exposure times for D–F were less that A–C to avoid overexposure of the fluorescent rods. A single arrowhead indicates localization to the Z ring; double arrowhead indicates membrane localization and an arrow indicates spirals centred at expected division sites. The inset in (C) shows an expanded view to better illustrate the membrane localization (arrowhead).

along the filament indicating the GFP–FtsA fusion was localizing to Z rings (Fig. 2B). Previous results have shown that Z rings are formed at the non-permissive temperature in *ftsA* (Ts) cells because of the presence of ZipA (Addinal *et al*., 1996; Pichoff and Lutkenhaus, 2002). Of course, the GFP–FtsA could also be supporting Z-ring formation in these cells even though it is unable to complement *ftsA12* (Ts).

In both of the above cases the GFP–FtsA was primarily localized to Z rings or spirals and was therefore at the membrane. However, in some cells the fusion was expressed somewhat higher and the fluorescence appeared distributed throughout the membrane. To examine the location of GFP–FtsA in the absence of FtsZ polymers, we used the temperature-sensitive *ftsZ84* mutation. The presence of this mutation results in rapid disassembly of Z rings following a shift to the non-permissive temperature (Addinall *et al*., 1997). As with the *ftsA12* (Ts) mutation the presence of the *ftsZ84* (Ts) mutation made cells more sensitive to the GFP–FtsA fusion and cells started to filament at permissive temperature following induction of the fusion. At the non-permissive temperature the GFP–FtsA was not localized to well spaced bands indicative of localization to division sites but was at the cell membrane throughout the cell indicating that FtsA localizes to the membrane independent of FtsZ structures (Fig. 2C). This result also argues that under normal conditions the membrane-bound FtsA coalesces to the Z ring.

In our study, GFP–FtsA seems to be more obviously localized to the membrane than FtsA–GFP used previously (Ma *et al*., 1996). The juxtaposition of GFP to the conserved carboxy motif in that study may have affected its ability to mediate membrane association.

To analyse the role of the conserved C-terminal motif identified from the sequence analysis, we examined the localization of the truncated GFP–FtsAΔ15 fusion and the phenotype resulting from its expression. There were several notable differences between the expression of this fusion and the full-length fusion. First, the fluorescence did not localize to Z rings in either the wild-type background or the temperature-sensitive mutants, but was instead, present in the cytoplasm with some cells containing bright rods that also appeared to be in the cytoplasm (Fig. 2D–F). The appearance of the bright rods was associated with cell curvature in some of the cells. Also, cell division was not readily inhibited by this truncated derivative as filamentation did not occur during induction at the permissive temperature. Thus, removing the last 15 amino acids from FtsA reduced its ability to localize to the membrane and to Z rings and reduced its ability to inhibit division. The truncated FtsA fusion was present in the cytoplasm and formed rods. It has been reported previously that expression of a C-terminally truncated FtsA (5–28 residues removed) results in curved cells and the appearance of cytoplasmic filaments (Gayda *et al*., 1992; Yim *et al*., 2000). Our results demonstrate that these cytoplasmic filaments are formed by the truncated FtsA.

FtsA's C-terminal motif can substitute for MinD's MTS

MinD binds to the membrane through its MTS and recruits the division inhibitor MinC resulting in high affinity for a septal component (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999; Johnson *et al*., 2002; Zhou and Lutkenhaus, 2003). Deletion of this MTS or substitution of the hydrophobic amino acids with charged residues within the MTS dramatically diminishes MinD's ability to localize to the membrane and activate MinC (Szeto *et al*., 2002; Hu and Lutkenhaus, 2003; Zhou and Lutkenhaus, 2004). To determine if the C-terminal motif of FtsA could function to recruit MinD to the membrane we replaced MinD's MTS (the C-terminal 10 amino acids) with the C-terminal motif from FtsA (the C-terminal 15 residues). Our results below demonstrate that this motif from FtsA is a membrane targeting sequence so we refer to it as MTS.

To quantitatively assess the membrane localization we determined the ability of MinD–MTS^{FtsA} to activate MinC. We used the plasmid pCS104CD which is a low copy plasmid containing the *minCD* genes downstream of the arabinose promoter. The presence of this plasmid in JS964 (Δ*min*) leads to arabinose sensitivity resulting from expression of *minCD* blocking cell division (Fig. 3). With as little as 0.006% arabinose growth is reduced and at higher concentrations colony formation is inhibited. This

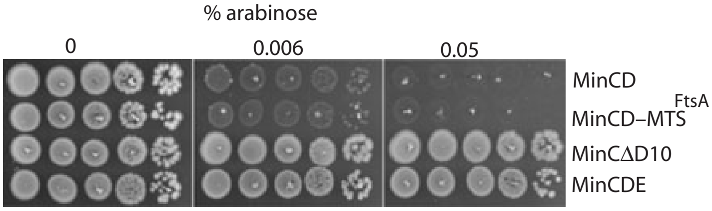

Fig. 3. The MTS of FtsA can substitute for MinD's MTS to activate MinC-mediated inhibition of cell division. Plasmids pCS104CDE, pCS104CDΔ10, pCS104CD and pSEB311 which express MinCDE, MinCDΔ10, MinCD and MinCD–MTS^{FtsA}, respectively, under P_{BAD} promoter control were introduced in JS964 (Δ*min*). One colony of each strain was resuspended in 300 μl of LB medium and serially diluted by 10. Aliquots were spotted on plates containing Spc with or without arabinose (as indicated) and incubated overnight at 37°C.

growth inhibition is accompanied by extensive cell filamentation (data not shown). Removing MinD's MTS (by using *minD*Δ10, which lacks the carboxy 10 amino acids; Hu and Lutkenhaus, 2003) blocks the arabinose sensitivity. Also, placing *minE* downstream of *minCD* restores the *min* operon and suppresses the arabinose sensitivity. Importantly, substituting the C-terminal motif from FtsA for MinD's MTS completely restored the arabinose sensitivity. By examining a range of arabinose concentrations we observed that MinD–MTS^{FtsA} was as efficient as wild-type MinD in blocking cell division and inhibiting colony formation. This result indicates that the MinD–MTS^{FtsA} fusion is just as effective as MinD in recruiting MinC to the membrane and argues that the C-terminal motif of FtsA binds at least as strongly to the membrane as MinD's MTS.

Direct evidence that the C-terminal motif is an MTS was obtained by examining cells expressing a GFP–MinD–MTS^{FtsA} fusion. Such cells displayed a halo appearance in fluorescence microscopy indicating that the fusion was present on the membrane (Fig. 4A). Also, addition of the FtsA MTS to GFP resulted in the appearance of cells with a halo appearance in fluorescence microscopy indicating that the MTS from FtsA could target GFP to the membrane (Fig. 4B).

MTS from MinD allows FtsA to go to the Z ring

As our results indicated that the FtsA C-terminal motif functions as an MTS, we did the reverse swap in which MinD's MTS was added to FtsAΔ15 to determine if it could substitute for FtsA's MTS. If successful, it would argue that it is the membrane targeting function that is important and not the sequence per se. To test this, the MTS from MinD of *E. coli* or *Bacillus subtilis* was added to the truncated GFP–FtsAΔ15 fusion. Expression of either of these fusions in a wild-type strain led to the appearance of the fluorescence at Z rings (Fig. 4C and D). This result indicates that the MTS of either of the MinDs is sufficient to target FtsA to the membrane which allows it to interact with FtsZ and avoids the formation of the cytoplasmic rods. Furthermore, overexpression of these fusions led to the formation of spirals and inhibition of cell division, the same pattern as observed with overexpression of the full-length GFP–FtsA fusion (data not shown). These results indicate that MinD's MTS can substitute for FtsA's MTS allowing FtsA to go to the membrane and localize to the Z ring.

Support for the above interpretation was provided by examining GFP–FtsA–MTS^{MinDEc*} in which two of the large hydrophobic residues within the MTS were substituted with charged residues. These same substitutions in the *E. coli* MinD prevent it from localizing to the membrane and efficiently activating MinC (Zhou and Lutkenhaus, 2003). This GFP–FtsA–MTS^{MinDEc*} fusion failed to localize to the Z ring and instead formed bright fluorescent rods similar to that observed with expression of the truncated GFP–FtsA fusion (Fig. 4E). This result indicates that the inactivation of a functional MTS in FtsA results in its failure to localize to the Z ring which leads to a cytoplasmic location and assembly into rods. At higher levels of expression this fusion did not cause dramatic filamentation, instead it caused twisted cell morphology and lysis similar to the truncated GFP–FtsAΔ15. The phenotypes because of overexpression are analysed further below in the absence of the GFP tag.

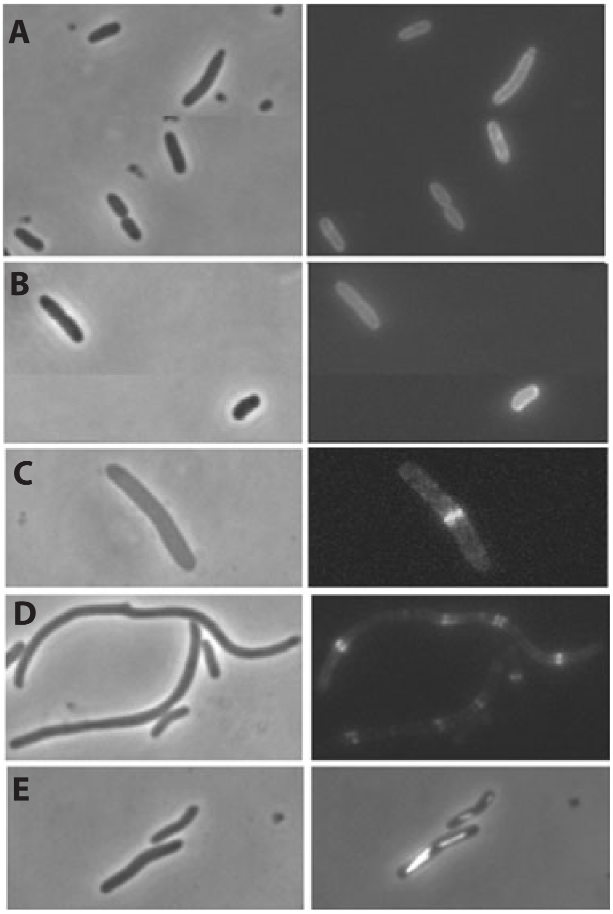

Fig. 4. FtsA's conserved C-terminal motif is a transplantable MTS and can be replaced by the MTS from MinD. The addition of MTS of FtsA to GFP–MinDΔ10 (A) or GFP (B) promotes their binding to the membrane. Effect of the MinD MTS from *B. subtilis* (C), *E coli* (D) or a mutated *E coli* (E) on the localization of FtsA. (A) JS964/pSEB296 (GFP–MinD–MTS^{FtsA}), (B) JS238/pSEB295 (GFP–MTS^{FtsA}), (C) pSEB297 (FtsA–MTS^{MinDBs}), (D) pSEB327 (FtsA–MTS^{MinDEc}) or (E) pSEB331(FtsA–MTS^{MinDEc*}). Strains containing plasmids were subcloned on plates containing ampicillin and 0.0001% arabinose and incubated for 5 h at 37°C before microscopic observation.

MinD MTS can functionally substitute for FtsA MTS

The above experiments indicated that the MTS from either the *B. subtilis* or *E. coli* MinD can substitute for the FtsA

MTS to allow FtsA to go to the membrane and localize to the Z ring. The results raised the possibility that the MinD MTS could fully substitute for the FtsA MTS. To test this, the MinD MTSs were substituted for the FtsA MTS in FtsA without GFP so that complementation tests could be carried out. The various *ftsA* constructs were cloned into pDSW210 and pDSW208, plasmids with promoters of different strengths (Ghigo *et al.*, 1999), and introduced into strain JFL110 [*ftsA12* (Ts), *recA*] and tested for their ability to complement the temperature-sensitive *ftsA12* mutation (Fig. 5, only results with pDSW210 derivatives are shown). The full-length FtsA complemented at low and intermediate levels of IPTG, whereas the truncated FtsA failed to complement at all IPTG concentrations. Interestingly, both FtsA–MTS^{MinDEc} and FtsA–MTS^{MinDBs} complemented indicating that the MTS from either MinD could functionally replace the FtsA MTS (Fig. 5). The MinD MTSs do not appear quite as efficient as the FtsA MTS as a slightly higher IPTG concentration was required for complementation.

Similar to the wild-type FtsA, both the FtsA–MTS^{MinDEc} and the FtsA–MTS^{MinDBs} fusions inhibited colony formation at high IPTG concentrations (observed with pDSW208 derivatives that produce higher levels of expression) and this was associated with filamentation just as observed with FtsA [Fig. 6B(f) and data not shown]. In contrast, the FtsA–MTS^{MinDEc*} fusion containing the charged substitutions did not complement the *ftsA12* (Ts) mutation (Fig. 5) and when overexpressed caused a twisted phenotype and cell lysis as observed with the truncated FtsA [Fig. 6B(e) and data not shown].

Hydrophobic residues within FtsA's MTS are important for FtsA function

The results so far demonstrate that the FtsA carboxy conserved motif can function as an MTS, presumably by forming an amphipathic helix that binds directly to the phospholipid bilayer. Peptides corresponding to the MinD MTSs have been shown to adopt a helical conformation in the presence of a lipid bilayer (Szeto *et al.*, 2003). Changing any of the large hydrophobic residues within the MinD MTS to tryptophan did not affect membrane binding and revealed that the tryptophan residues actually embed into the hydrophobic interior of the bilayer (Zhou and Lutkenhaus, 2003). In contrast, changing any of these large hydrophobic residues to a negatively charged amino acid resulted in failure of MinD to bind to the membrane. The FtsA MTS contains six large hydrophobic amino acids. If they are important in binding to the membrane as part of an amphipathic helix, then changing them to a negatively charged amino acid would be expected to disrupt membrane binding.

To test this, the large hydrophobic amino acids were changed individually to glutamate and the resulting mutants checked for localization and complementation. Previous analysis of deletion mutants revealed that the last four residues, including F420 are not essential. Therefore, the F420 may lie beyond the helix and may not be required for membrane binding (Fig. 1). We also constructed an R411E mutation because a basic residue is observed at this position in all FtsA sequences (Fig. 1A). The results were similar for five of the hydrophobic substitutions. W408E, I409E, W415E, L416E and L412E all failed to complement *ftsA12* (Ts) (Fig. 6A). When expressed at a higher level these mutants caused cells to become twisted and lyse [Fig. 6B(c–e), three of five are shown] similar to the phenotype caused by overexpression of the truncated FtsA and in contrast to the dramatic filamentation caused by any of the FtsA derivatives that are able to go to the membrane and localize to the Z ring. Furthermore, when these mutations were introduced into the GFP–FtsA fusion they prevented localization to the Z ring. Instead the fusions were present in the cytoplasm

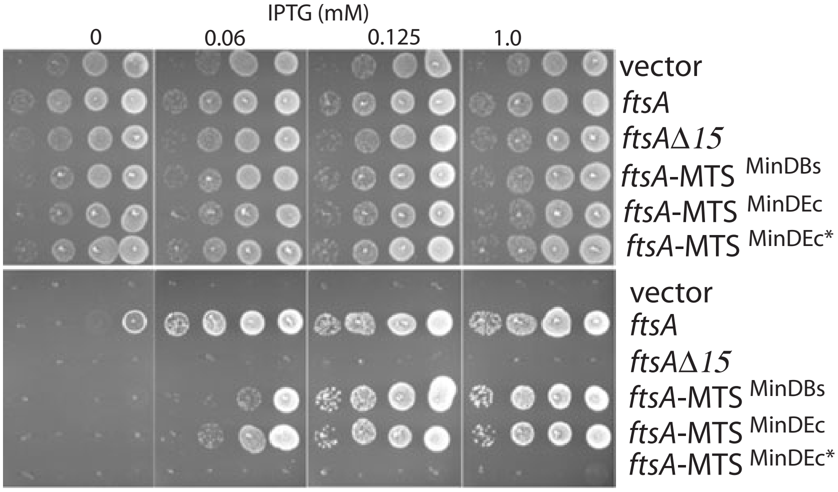

Fig. 5. MTS from MinD can substitute for FtsA's MTS. Plasmids pDSW210 (vector) pSEB306 (*ftsA*), pSEB307 (*ftsA*Δ*15*), pSEB308 (*ftsA*-MTS^{MinDBs}), pSEB330 (*ftsA*-MTS^{MinDEc}) and pSEB332 (*ftsA*-MTS^{MinDEc*}) were introduced into JFL110 [*ftsA12* (Ts)]. In accordance with the protocol described in the legend to Fig. 3, samples were spotted on plates containing ampicillin with increasing concentrations of IPTG and incubated overnight at 30°C (top) or 42°C (bottom).

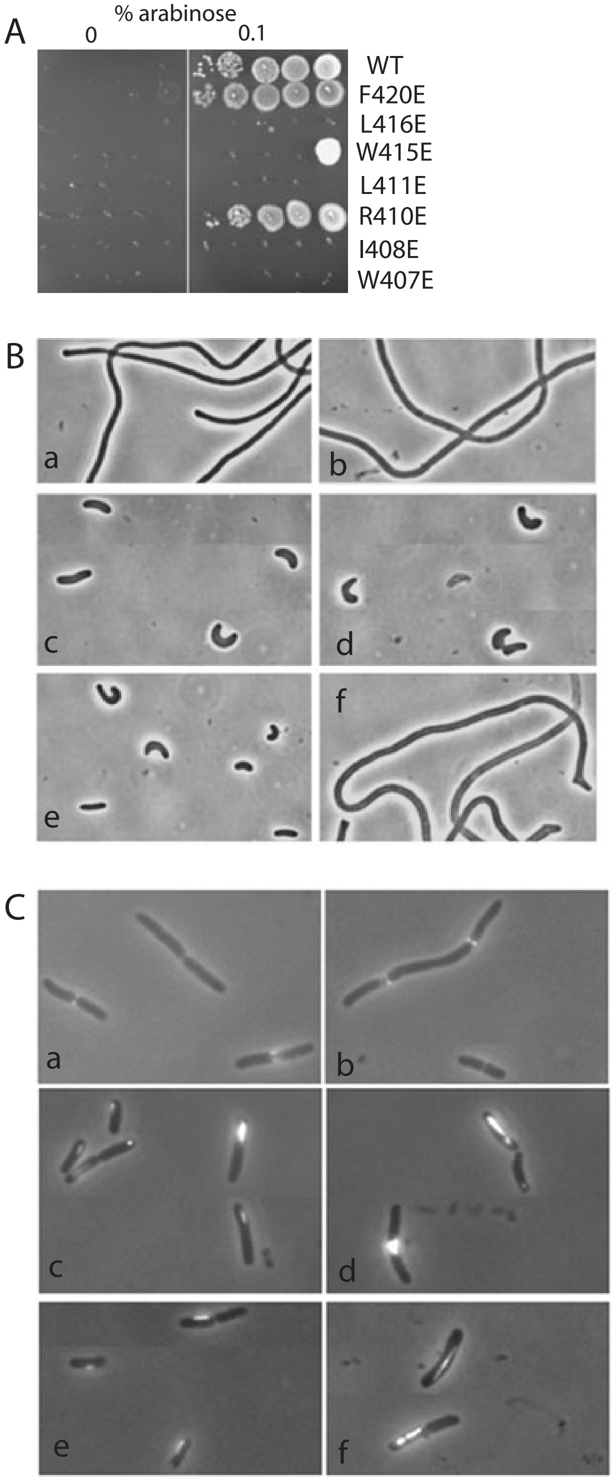

Fig. 6. The hydrophobic residues within FtsA's amphiphatic helix (MTS) are required for the localization and function of FtsA.
A. Complementation of *ftsA12* (Ts) at 42°C. Plasmids pSEB324 (*ftsA*) and derivatives carrying the following *ftsA* mutations: F420E, L416E, W415E, L412E, R411E, I409E and W408E were introduced into JFL110. In accordance with the protocol described in the legend to Fig. 3, samples were spotted on plates with ampicillin with or without arabinose and incubated overnight at 30°C or 42°C (only plates incubated at 42°C are shown).
B. Morphology of JFL110 cells overexpressing FtsA mutants from derivatives of pSEB324: (a) F420E, (b) R411E, (c) L416E, (d) W415, (e) FtsAΔ15 and (f) FtsA. Shown are phase contrast micrographs of cells prepared from spots obtained at 30°C on plates (not shown) containing ampicillin and 0.1% arabinose.
C. Effect of amino acid substitutions on the localization of GFP–FtsA mutant proteins. Combination phase plus fluorescence microscopy of the JS238 cells expressing different GFP–FtsA mutants. The mutants were expressed from derivatives of pSEB323. The cells were prepared according as described in the legend to Fig. 4. The mutants were as follows: (a) F420E, (b) R411E, (c) I409E, (d) L412E, (e) W415E and (f) L416E.

and formed rods, very similar to that observed for the truncated FtsA [Fig. 6C(c–f), four of the five are shown]. In contrast to these results, F420E and R411E complemented *ftsA12* (Ts) (Fig. 6A) and when overexpressed, caused cells to filament [Fig. 6B(a,b)]. When fused to GFP these two mutants localized to Z rings at low levels of expression [Fig. 6C(a,b)] and were clearly on the membrane at higher levels of expression (data not shown). Thus, the F420E and R411E substitutions did not affect FtsA localization or function.

Discussion

The first event readily detected in bacterial cytokinesis is the formation of a Z ring at the division site just beneath the cytoplasmic membrane. In *E. coli*, FtsZ interacts with FtsA and ZipA and either of these proteins is sufficient to promote Z-ring formation suggesting both are able to tether the Z ring to the membrane. For ZipA, which has a transmembrane domain, the mechanism appears straight forward; however, for FtsA, which did not appear to contain a specific sequence suggesting a membrane interaction it was less clear. In this report, we present clear and convincing evidence that FtsA is a membrane binding protein and that this interaction occurs through a conserved C-terminal amphiphatic helix that serves as an MTS. We also report that the interaction of FtsA with the membrane is essential for its function in cell division and that it regulates the interaction of FtsA with FtsZ. As the FtsZ–FtsA pair is well conserved in evolution, we suggest that FtsA tethers Z rings to the membrane in most bacteria.

FtsA is targeted to the membrane through a conserved C-terminal amphipathic helix

The conserved motif at the extreme C-terminus of FtsA is not present in the crystal structure indicating that it is not organized although computer predictions indicate that this motif is a potential amphiphatic helix (van den Ent and Lowe, 2000; Lowe and van den Ent, 2001). When GFP–FtsA was overexpressed in cells, the fluorescence was clearly located at the membrane and this was in sharp contrast to the cytoplasmic localization of the truncated version, GFP–FtsAΔ15, which lacks the conserved C-terminal motif (MTS). Importantly, this MTS was sufficient to target GFP to the membrane and when added to MinDΔ10 was as effective as the MinD MTS as assessed by MinC activation and membrane localization.

Similar to results with MinD (Zhou and Lutkenhaus, 2003), we found that substitution of the large internal hydrophobic residues within FtsA's putative amphipathic helix with a negatively charged amino acid led to loss of function. GFP fusions of the substituted proteins were unable to localize at the membrane and had a cytoplasmic localization just like the C-terminally truncated FtsA. Taken together these results demonstrate that the FtsA conserved C-terminal motif forms an amphipathic helix that serves as an MTS responsible for tethering FtsA to the membrane. Consistent with this motif functioning as a membrane targeting sequence, it could be functionally replaced by the well characterized membrane targeting helices of MinD.

Binding of FtsA to the membrane is essential for its function and regulates its interaction with FtsZ

Deletion of the MTS from FtsA or mutagenesis of the large hydrophobic residues to charged residues abolished the ability of FtsA to localize to the membrane and complement the *ftsA12* (Ts) mutation. In contrast, FtsA-hybrid proteins containing amphiphatic helices from the MinD proteins of *E. coli* or *B. subtilis* complemented *ftsA12* (Ts) as long as these replacement helices were a functional MTS. These results strongly suggest that targeting of FtsA to the membrane by an amphiphatic helix is essential for its function but that the actual sequence or length of the helix, as long as it is sufficient to promote membrane binding, is not important.

All FtsA constructs with ability to bind to the membrane localize to Z rings and cause filamentation when overexpressed indicating that they disturbed the normal cell division process. In contrast, overexpression of GFP–FtsAΔ15, which lacks the MTS, or FtsA mutants in which hydrophobic residues within the MTS were replaced by charged residues did not localize efficiently to Z rings or cause filamentation but produced twisted cells which eventually lysed. Even when GFP–FtsAΔ15 is expressed at a high enough level to form fluorescent rods in the cytoplasm, the cells remained about wild type in length. As the presence of these rods did not significantly affect cell division, FtsZ and FtsA must not be present in these rods. Immunofluorescence experiments confirmed that FtsZ was not present in these fluorescent rods but was still present in rings at the division site (data not shown). If we assume that the truncated FtsA protein retains the FtsZ interaction domain (and we assume that the MTS does not have a direct role in the interaction with FtsZ because it can be replaced with a MinD MTS) then the lack of targeting to the membrane results in a low affinity for FtsZ. In other words, these results strongly argue that FtsA must first to be targeted to the membrane before it interacts with FtsZ.

Interestingly, FtsA truncated for the MTS forms an organized structure in the cytoplasm which appear as fluorescent rods when the protein is fused to GFP. Other bacterial actin homologues, MreB and ParM are known to polymerize (Jensen and Gerdes, 1999; van den Ent *et al*., 2001; Jones *et al*., 2001); however, FtsA is unusual because one subdomain has an altered position and topology. Although FtsA has been shown to self-interact by yeast two hybrid (Yim *et al*., 2000) and purify as a dimer (Feucht *et al*., 2001), polymerization *in vitro* has not been demonstrated. One possibility is that interaction of FtsA with the membrane regulates the self-interaction and that removal of the membrane targeting motif allows the protein to undergo unregulated polymerization in the cytoplasm.

Yim *et al*. (2000) observed that removing 5–27 residues from the carboxy end of FtsA led to loss of complementation and a failure to localize to the Z ring. They also observed, as Gayda *et al*. (1992) had, that overexpression of C-terminally truncated FtsA results in a curved cell phenotype. Using the yeast two hybrid system they showed that the truncated mutants self-interacted but did not interact with the full-length protein. From this they proposed that the conserved C-terminal motif regulated the FtsA self-interaction. By integrating all of these results we suggest a model in which the interaction of FtsA with the membrane promotes self-interaction and interaction with FtsZ resulting in cooperative linkage of FtsZ polymers to form the Z ring.

FtsA in evolution and tethering the Z ring to the membrane

The *ftsA* gene is highly conserved among cell division genes and is almost always found in tandem with *ftsZ*. This relationship could reflect the need to maintain a set ratio between these two proteins for cell division to take place (Dai and Lutkenhaus, 1992) and possibly reflects coevolution of these two proteins because they work together.

The direct interaction of FtsA with FtsZ has been well characterized from the FtsZ perspective but it is not clear yet what domain of FtsA is involved in this interaction (Haney *et al*., 2001). Also, the interaction is well conserved in evolution because it is observed between proteins from as distantly related bacterial species as *E. coli* and *B. subtilis* (Wang *et al*., 1997). Interestingly, subdomain IC of FtsA, the subdomain that occupies a unique position among actin-like proteins, is essential for cell division but not required for FtsA to localize to the Z ring (Rico *et al*., 2004).

Our findings indicate that FtsA interacts directly with the membrane through a well conserved C-terminal amphiphatic helix that acts as an MTS. This result is significant because it suggests, along with the widespread presence of FtsA in bacteria, that FtsA might be the predominant mechanism for tethering the Z ring to the membrane in bacteria. However, some bacteria have a homologue of FtsZ, but no obvious FtsA, such as mycobacteria, mycoplasma and cyanobacteria. In these bacteria another protein must perform this function. In such bacteria or eukaryotic organelles (chloroplasts) with no FtsA, other proteins able to interact with both FtsZ and the membrane have been identified. One such protein, ZipN in cyanobacteria and its homologue Arc6 in chloroplasts, appears to be essential for the cell division process of these organisms (Vitha *et al*., 2003; Mazouni *et al*., 2004). In addition, FtsZ from *Mycobacterium tuberculosis* interacts with FtsW, an integral membrane protein, through sequences in these proteins that appear to be unique to this genus (Datta *et al*., 2002). These examples might represent the evolution of the anchoring of the Z ring to the membrane to other proteins.

Possible role of FtsA in Z-ring formation

Since FtsA is targeted to the membrane by an amphipathic helix, it raises questions as to whether the binding is reversible and regulated by ATP hydrolysis similar to MinD from *E. coli*? FtsA binds and hydrolyses ATP (Feucht *et al*., 2001) and can undergo phosphorylation (Sanchez *et al*., 1994). Fractionation studies indicate that FtsA bound to the membrane is unphosphorylated and cannot bind ATP while the cytoplasmic form is phosphorylated and binds ATP (Sanchez *et al*., 1994). This would suggest that the interaction of FtsA with the membrane is somehow regulated; however, mutants deficient in phosphorylation and ATP binding appeared functional.

Our studies demonstrate that FtsA's MTS can tether GFP to the membrane and is more similar in sequence to the MinD MTS from *B. subtilis* than *E. coli*, suggesting that oligomerization is not required for membrane binding. On the other hand, the MTS from the *E. coli* MinD, which requires oligomerization for membrane binding (Szeto *et al*., 2003), can functionally substitute for FtsA MTS indicating that oligomerization at the membrane is occurring.

Our results indicate that binding of FtsA to the membrane increases the affinity of FtsA for FtsZ and possibly itself suggesting a new model for Z ring formation. A major difference between this and an earlier model (Lutkenhaus, 1993) is that Z ring formation is not controlled at the level of a nucleation site but instead depends upon a reduction in the influence of negative factors that limit FtsZ polymers from associating to form the Z ring. In the new model FtsZ is mostly associated with FtsA and ZipA at the membrane anywhere in the cell. Importantly, this membrane tethered FtsZ constantly undergoes assembly throughout the cell to form dynamic membrane-tethered polymers. Evidence for such highly dynamic FtsZ polymers occurring throughout the cell has been provided recently (Thanedar and Margolin, 2004). The preferential assembly of the Z ring at midcell is driven by the Min and Noc systems (Wu and Errington, 2004), which act to limit polymer length at the membrane away from midcell (Fig. 7). At midcell, the combined influence of these negative effectors is reduced as cells elongate. Polymers emerging from midcell but growing parallel to the long axis will encounter the negative effect of Min/Noc thus limiting their growth. However, polymers growing perpendicular to the long axis will continue to grow. The increased polymer length allows FtsZ polymers to associate laterally resulting in formation of a Z ring. The lateral association could be driven by ZipA, which can bundle FtsZ filaments *in vitro* (RayChaudhuri, 1999; Hale *et al*., 2000) and possibly by FtsA through interaction between FtsA molecules bound to different FtsZ polymers. As we have shown previously (Pichoff and Lutkenhaus, 2002), either is sufficient to support Z ring formation.

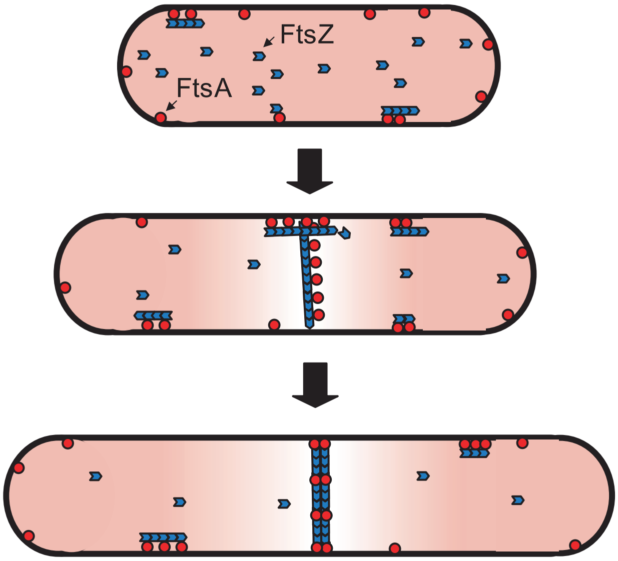

Fig. 7. Model for assembly of the Z ring. In this model FtsZ polymers are linked to the membrane through FtsA or ZipA (only FtsA is depicted because either can support Z-ring assembly). These membrane-linked polymers are formed throughout the cell; however, their length is limited by the combined negative action of the Min and Noc systems (indicated by the shading). As the cell division cycle progresses the influence of Min and Noc at midcell is reduced allowing longer FtsZ polymers to form. Polymer growth parallel to the long axis of the cell is limited by Min/Noc whereas growth perpendicular to the long axis is not. Formation of the Z ring may be favoured by interaction between the longer FtsZ protofilaments as well as ZipA-induced bundling and interaction between FtsA molecules bound to different FtsZ polymers.

Experimental procedures

Bacterial strains and media

All bacterial strains are derivatives of *E. coli* K-12. JS238 is a *recA malP::lacI*^{q} derivative of MC1061 (Pichoff *et al*., 1995). JS964 is a Δ*min::kan malP::lacI*^{q} derivative of MC1061 (Pichoff *et al*., 1995). Derivatives of W3110 carrying temperature-sensitive mutations were made by P1 transduction: PS106 is W3110 *ftsZ84 leu::Tn10* with MCZ84 (Addinall *et al*., 1996) as donor and PS236 is W3100 *ftsA12 leu::Tn10* with JFL110 (Addinall *et al*., 1996) as donor. Bacteria were grown in LB medium at 30°C, 37°C or 42°C as indicated. Antibiotics were added at the following concentrations: ampicillin (Amp) 100 µg ml^{-1} and spectinomycin (Spc) 50 µg ml^{-1}. Glucose was added at 0.2% and IPTG or arabinose were added as specified in the text.

Plasmid constructions

Plasmids are listed in Table 1. Since all the mutations or swap constructs involve the 3′ end of the *ftsA* or *minD* coding sequences we used polymerase chain reaction (PCR) involving PAGE-purified oligonucleotides (sequence available upon request) that carry the desired sequences (the codons for the last 15 or 10 amino acids). All PCRs were carried out using *pfx* polymerase (Invitrogen) using W3110 chromosomal DNA as template unless otherwise specified. All plasmids were sequenced to check the accuracy of the constructs.

A series of plasmids were made that express derivatives of *ftsA, gfp* or *minD* under arabinose promoter control. pSEB293, pSEB294, pSEB297, pSEB327 and pSEB331 express GFP fused to the N-terminus of FtsA, FtsAΔ15, FtsA-MTS$^{\text{MinDBs}}$, FtsA-MTS$^{\text{MinDEc}}$ and FtsA-MTS$^{\text{MinDEc*}}$ in pBAD18 (Guzman *et al*., 1995). In order to construct these plasmids different *ftsA* fragments were obtained by PCR using the oligos 5′*Xba*I-L-FtsA and 3′*Sph*I-FtsA, 3′ FtsAΔ-*Sph*I, 3′*Sph*I-A-MinDBs, 3′-*Sph*I-A-MinD2 or 3′-*Sph*I-A-MinD2mut. These PCR fragments were then cut by *Xba*I and *Sph*I and cloned into pJC106 (a pBAD18 derivative containing *GFPmut2* with an *Xba*I site at the 3′ end of the ORF (Hu and Lutkenhaus, 1999) using the same restriction sites. pSEB295 contains GFP with the last 15 amino acids of FtsA fused to its carboxy end. It was obtained by PCR using the oligos 5′*Xba*I-L-Cter FtsA and 3′-*Sph*I-FtsA and the fragment was cloned in pJC106 as described above. pSEB296 expresses *gfp-minD*-MTS$^{\text{FtsA}}$ in pBAD18 and was obtained by PCR using the oligos 5′GFPD and 3′*Hin*dIII D-FtsAend using pZH106 as template (Hu and Lutkenhaus, 1999). The PCR fragment was then cloned into the *Xba*I and *Hin*dIII sites of pJC106.

pSEB311 was constructed by replacing the *Bst*XI-*Hin*dIII fragment of pCS104CD by the equivalent one from pSEB296 in order to express the construct MinD–MTS$^{\text{FtsA}}$ along with MinC under control of the P$_{\text{BAD}}$ promoter on a pGB2 derivative.

pSEB306, pSEB307, pSEB308, pSEB330 and pSEB332 contain PCR fragments encoding FtsA, FtsAΔ15, FtsA–MTS$^{\text{MinDBs}}$, FtsA–MTS$^{\text{MinDEc}}$ and FtsA–MTS$^{\text{MinDEc*}}$ cloned into pDSW210 (low expression) or pDSW208 (moderate expression, noted pSEB306+, etc.) at the *Nco*I–*Sca*I sites. The inserts for these plasmids were obtained by *Nco*I and *Sca*I digestion of PCR products obtained with the using the oligos 5′*Nco*I-FtsA and 3′Amp with pSEB293, pSEB294, pSEB297, pSEB327 and pSEB331 as templates.

Plasmids encoding *ftsA* or the following alleles F420E, L416E, W415E, L412E, R411E, I409E and W408E were constructed by cloning PCR fragments obtained with oligos 5′-*Nhe*I-FtsA and 3′*Sph*I-FtsAWT, 3′*Sph*I-FtsAF420E, 3′*Sph*I-FtsAW415E, 3′*Sph*I-FtsAL412E, 3′*Sph*I-FtsAR411E, 3′*Sph*I-FtsAI409E or 3′*Sph*I-FtsAW408E, cut with *Nhe*I-*Sph*I and

Table 1. Plasmids used in this study.

Plasmids	Description	Reference
pZH106	*gfp-minD* under P$_{\text{BAD}}$ control on pBAD18, AmpR	Hu and Lutkenhaus (1999)
pJC106	pBAD18 derivative containing *GFPmut2*, AmpR	Hu and Lutkenhaus (1999)
pSEB293	*gfp-ftsA* in pBAD18, AmpR	This study
pSEB294	*gfp-FtsA*Δ*15* in pBAD18, AmpR	This study
pSEB295	*gfp*-MTS$^{\text{FtsA}}$ in pBAD18, AmpR	This study
pSEB296	*gfp-minD*$^{\text{FtsA}}$ in pBAD18, AmpR	This study
pSEB297	*gfp-ftsA*-MTS$^{\text{MinDBs}}$ in pBAD18, AmpR	This study
pGB2	Vector, low copy, SpcR	Churchward *et al.* (1984)
pCS104CDE	*minCDE* in pGB2 under P$_{\text{BAD}}$ control, SpecR	Zhou *et al.* (2005)
pCS104CD	*minCD* in pGB2 under P$_{\text{BAD}}$ control, SpecR	Zhou *et al.* (2005)
pCS104CDΔ10	*minCD*Δ*10* in pGB2 under P$_{\text{BAD}}$ control, SpecR	Zhou *et al.* (2005)
pSEB311	*minCD*-MTS$^{\text{FtsA}}$ (derivative of pCS104CD), SpecR	This study
pBAD18	pBR322 derivative containing P$_{\text{BAD}}$ promoter	Guzman *et al.* (1995)
pSEB327	*gfp-ftsA*-MTS$^{\text{MinDEc}}$ in pBAD18, AmpR	This study
pSEB331	*gfp-ftsA*-MTS$^{\text{MinDEc*}}$ in pBAD18, AmpR	This study
pSEB306	*ftsA* in pDSW210	This study
pSEB307	*FtsA*Δ*15* in pDSW210	This study
pSEB308	*ftsA*-MTS$^{\text{MinDBs}}$ in pDSW210	This study
pSEB330	*ftsA*-MTS$^{\text{MinDEc}}$ in pDSW210	This study
pSEB332	*ftsA*-MTS$^{\text{MinDEc*}}$ in pDSW210	This study
pSEB323series	*ftsA*, *F420E*, *L416E*, *W415E*, *L412E*, *R411E*, *I409E* and *W408E* in pBAD18	This study
pSEB324series	*gfp* fusions to *ftsA*, *F420E*, *L416E*, *W415E*, *L412E*, *R411E*, *I409E* and *W408E* in pBAD18	This study
pDSW208	Vector with moderate IPTG-inducible expression	Chen *et al.* (1999)
pDSW210	Similar to pDSW208 but with lower expression	Chen *et al.* (1999)

cloned into the *Nhe*I–*Sph*I sites of pBAD18. The resultant plasmids were designated the pSEB323 series. GFP fusions to the N-terminus of the above alleles were obtained by cloning PCR fragments obtained with 5′*Xba*I-L-FtsA and the 3′ oligos indicated above and cut with *Xba*I–*Sph*I and cloned into pJC106 at the *Xba*I–*Sph*I sites to give the pSEB324 series.

Microscopy protocol

Cells were fixed by adding 1% formaldehyde (final concentration) to a sample of a culture growing exponentially in LB. Samples were observed and photographed with a Nikon Eclipse E600 fluorescence microscope equipped with a 100× E Plan oil immersion lens (Nikon Instruments, Melville, NY) and a MagnaFire CCD camera S99802 from OPTRONICS (Goleta, CA). Images were imported to Adobe Photoshop software and assembled.

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