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An altered FtsA can compensate for the loss of essential cell division protein FtsN in *Escherichia coli*

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Summary

FtsN is the last known essential protein component to be recruited to the *Escherichia coli* divisome, and has several special properties. Here we report the isolation of suppressor mutants of *ftsA* that allow viability in the absence of *ftsN*. Cells producing the FtsA suppressors exhibited a mild cell division deficiency in the absence of FtsN, and no obvious phenotype in its presence. Remarkably, these altered FtsA proteins also could partially suppress a deletion of *ftsK* or *zipA*, were less toxic than wild-type FtsA when in excess, and conferred resistance to excess MinC, indicating that they share some properties with the previously isolated FtsA* suppressor mutant, and bypass the need for *ftsN* by increasing the integrity of the Z ring. TolA, which normally requires FtsN for its recruitment to the divisome, localized proficiently in the suppressed *ftsN* null strain, strongly suggesting that FtsN does not recruit the Tol–Pal complex directly. Therefore, despite its classification as a core divisome component, FtsN has no unique essential function but instead promotes overall Z ring integrity. The results strongly suggest that FtsA is conformationally flexible, and this flexibility is a key modulator of divisome function at all stages.

Introduction

Many complex cellular tasks are performed by protein machines. Cell division in *Escherichia coli* is no exception, with at least 10 proteins known to be required for cytokinesis that colocalize to the site of division at midcell. One of these, FtsZ, is a tubulin homologue that assembles into a structure, called the Z ring, which encircles the inner side of the cytoplasmic membrane. Once the Z ring forms, it recruits other proteins to the divisome complex (Weiss, 2004). FtsA and ZipA associate first, and are required for tethering the ring to the membrane (Hale and de Boer, 1997; Pichoff and Lutkenhaus, 2005). The rest of the complex, consisting of mostly integral membrane proteins, assembles in a second stage (Aarsman *et al*., 2005). Although some information is available about the protein–protein interactions among these membrane components, only FtsI, a transpeptidase, has a well-defined biochemical function in cell division.

One of the hallmarks of this protein machine is an apparent linear dependency order of recruitment to the Z ring (Goehring and Beckwith, 2005). FtsN, for example, cannot localize

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to the Z ring until all of the other essential components are present (Addinall *et al*., 1997; Chen and Beckwith, 2001), while FtsZ is first because it can localize in the absence of any one of the later proteins. The dependency order is FtsZ-ZipA/FtsA-FtsEX-FtsK-FtsQ-(FtsB-FtsL)-FtsW-FtsI-FtsN, with the pairs of proteins separated by a slash exhibiting mutually independent localization, while those in parentheses show co-dependence. However, recent evidence suggests that this order is misleading. First, FtsZ can localize in the absence of either FtsA or ZipA, but cannot form Z rings in the absence of both (Pichoff and Lutkenhaus, 2002). Second, many Z rings fail to localize to potential division sites when FtsN is depleted from cells (Addinall *et al*., 1997; Chen and Beck-with, 2001). Third, multiple interactions among these proteins exist that are outside the normal dependency order (Di Lallo *et al*., 2003; Corbin *et al*., 2004; Karimova *et al*., 2005). Fourth, the linear dependency order can be bypassed under certain conditions (Geissler *et al*., 2003; Geissler and Margolin, 2005; Goehring *et al*., 2005; 2007a). Fifth, overproduction of some cell division proteins can efficiently suppress the requirement for others, indicating functional redundancy, at least under laboratory conditions (Dai *et al*., 1993; Geissler and Margolin, 2005). These results strongly suggest that the divisome assembles not solely by sequential binary interactions but rather via a web of protein–protein contacts that may also include sequential interactions (Goehring and Beckwith, 2005; Vicente *et al*., 2006).

FtsN was originally discovered as a multicopy suppressor of an *ftsA*(ts) mutant, and when overproduced can partially suppress other *fts* mutants, including *ftsQ*, *ftsI* (Dai *et al*., 1993) and *ftsK* (Draper *et al*., 1998; Geissler and Margolin, 2005; Goehring *et al*., 2007b). This property of FtsN suggested that it had a potentially unique regulatory role. Its late localization was difficult to reconcile with its ability to affect the function of early proteins, such as FtsA. Nevertheless, because it is essential for viability, FtsN has been thought to be part of the core divisome machinery despite the lack of conservation of FtsN outside the enteric bacteria. In support of the importance of FtsN in late divisome functions, the recruitment of non-essential components of the divisome such as AmiC and the Tol–Pal complex to division sites requires FtsN (Bernhardt and de Boer, 2003; Gerding *et al*., 2007).

Despite its classification as a late divisome protein, recent molecular evidence suggests that FtsN may interact directly with early divisome components such as FtsA. When FtsA or subdomain 1c of FtsA was ectopically localized to cell poles, GFP-FtsN was re-localized from midcell to the poles (Corbin *et al*., 2004). GFP-FtsI also was recruited to poles, but other divisome proteins were not, suggesting that FtsI was back-recruited by FtsN. Bacterial two-hybrid assays also revealed an interaction between FtsA and FtsI/FtsN (Di Lallo *et al*., 2003; Karimova *et al*., 2005), although, as with the polar recruitment assays, a direct protein–protein interaction could not be proven. More recently, premature targeting of FtsQ to midcell in the absence of functional FtsA resulted in the recruitment of all downstream proteins to the septum, except for FtsN (Goehring *et al*., 2005). Although FtsN is a bitopic protein that has only a small segment in the cytoplasm (Dai *et al*., 1996; Yang *et al*., 2004), these results suggest that septal targeting of FtsN has a special requirement, potentially for FtsA.

These intriguing results prompted us to take the next logical step: remove FtsN entirely and ask whether the cells can compensate for its loss. This would provide important insights into

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whether FtsN has a unique or redundant function, and the nature of the compensatory changes would suggest how FtsN normally functions. Such an approach has been valuable in identifying redundant functions of ZipA and FtsK that are compensated by alterations in FtsA, as well as in understanding the function of FtsA itself. Here, we show that FtsN can indeed be removed, provided there are once again compensatory changes in FtsA. These compensatory changes are distinct from the FtsA mutation that is able to bypass ZipA, but share some overlapping functions.

Results

Isolation of *ftsN* bypass suppressors that map to a cloned *ftsA*

Previous studies implicated an interaction between FtsA and FtsN, and an altered FtsA (R286W) could compensate for the loss of ZipA or FtsK. Therefore, we hypothesized that an altered FtsA might suppress the loss of FtsN. As a first step, we constructed an FtsN depletion strain (WM2355), which contains a *cat* cassette in place of a complete deletion of chromosomal *ftsN*, and an Ap^R plasmid with a thermosensitive replication origin that expresses *ftsN*. At the permissive temperature of 30°C, WM2355 cells (carrying a control plasmid pBAD18kan, see below) were not as short as cells of the parent strain W3110 (Fig. 1A), probably because the levels of *ftsN* expressed from the plasmid are suboptimal. However, when shifted to 42°C, which causes dilution-mediated depletion of FtsN, the cells became highly filamentous (Fig. 1B) and lost viability (see below), consistent with the requirement of FtsN for cell division. Whereas cells of this strain had Z rings at most potential division sites at 30°C, many potential division sites in the filamentous cells at 42°C lacked rings (data not shown). This absence of Z rings at many division sites after FtsN depletion has been reported previously (Addinall *et al*., 1997; Chen and Beckwith, 2001). These results support the idea that although FtsN is a late recruit to the septum, it also has a role in enhancing the integrity or activity of the Z ring.

If an altered FtsA could allow survival in the absence of FtsN, then we reasoned that depletion of FtsN in the presence of mutagenized FtsA might allow rare colonies to survive. The *ftsA* gene was mutagenized using error-prone PCR, then cloned into pET28a, a Kan^R plasmid which expresses a histidine-tagged *ftsA* at basal levels sufficient to allow growth of an *ftsA12*(ts) mutant at the non-permissive temperature (Geissler *et al*., 2003). This plasmid was then introduced into the FtsN depletion strain, and the strain was then grown at 42°C for many generations to remove the *ftsN* expression plasmid and to select for variants that could survive in the absence of the plasmid. Several colonies were obtained, and the absence of the *ftsN* plasmid was confirmed by loss of Ap^R and by PCR analysis (data not shown).

The *ftsA* genes in these survivor strains were confirmed to carry the suppressor locus, as they could confer survival at 42°C after re-cloning into other plasmids and introducing them into the parent WM2355 FtsN depletion strain. Moreover, this indicated that the plasmid-borne suppressor was sufficient to bypass the requirement for FtsN, and that no secondary suppressors were involved. Sequencing of several of the cloned *ftsA* genes from suppressor strains yielded identical mutations (see below), so one isolate was chosen for further analysis. The complete absence of FtsN protein in suppressor strains containing *ftsN::cat* was confirmed by PCR (data not shown) and immunoblotting (Fig. 1K). The altered *ftsA*,

which we will call *ftsA* ^{sup}, is a true gain of function allele, because it complemented an FtsA depletion strain (Fig. 1J) or an *ftsA12*(ts) mutant (data not shown).

Suppressed cells lacking FtsN have a mild cell division defect

Having confirmed that FtsN was dispensable for colony formation in the presence of a suppressor, we then tested the effectiveness of the suppressor in restoring cell division in the absence of FtsN. In contrast to cells of the FtsN depletion strain containing the pBAD18kan plasmid vector (Fig. 1A and B), cells containing pBAD18kan with the cloned *ftsA* suppressor were relatively short both at 30°C and at 42°C, although the average length of suppressed cells lacking *ftsN* was higher than those containing *ftsN* (Fig. 1E and F). As with pET28a, basal levels of expression of the histidine-tagged *ftsA* ^{sup} gene cloned into pBAD18kan were sufficient for the *ftsN* bypass. Production of wild-type (WT) FtsA from pBAD18kan did not significantly suppress the filamentation caused by FtsN depletion (Fig. 1C and D), confirming that FtsA^{sup} was required for the bypass.

The effectiveness of FtsA^{sup} in bypassing *ftsN* was also assessed independently by introducing the *ftsN::cat* allele by transduction into W3110 recipient strains containing either pBAD18kan, pBAD18kan-*ftsA* or pBAD18kan-*ftsA* ^{sup}. Consistent with the above data, only the strain expressing the cloned *ftsA* ^{sup} allele allowed efficient transduction of the *ftsN::cat* allele (~200 transductants, Fig. 1I, see also below). This was consistently well above the typical background of 0–10 colonies per plate for the negative controls (Fig. 1G and H); it is possible that these background colonies represent some type of rearrangement, as a few that were tested were Cm^{R} but positive for *ftsN (*data not shown). We cannot rule out the possibility that some of these result from acquisition of suppressors.

To characterize the suppression more thoroughly, we compared cell lengths and FtsZ-GFP rings in an FtsN^{+} strain (EC448) versus the isogenic strain lacking FtsN and containing *ftsA* ^{sup} (WM2600). Cells of the control EC448 strain averaged 3.0 ± 0.7 μm in length, with 88% having a single Z ring at midcell. The calculated cell length per Z ring was 3.4 μm (Fig. 2A). In contrast, cells of WM2600 were about three times longer, averaging 9.7 ± 4.8 μm, and Z rings were sparser, with 5.6 μm per ring (Fig. 2B). DAPI staining indicated that the longer cells had four or more nucleoids (data not shown), suggesting that cell division is delayed under these conditions.

We then asked how the viability of suppressed *ftsN* cells compared with *ftsN*$^{+}$ cells with or without expression of *ftsA* ^{sup}. Mid-exponential cultures of WM2600 or EC448 carrying pBAD18kan derivatives at equivalent densities were spotted onto LB plates to measure colony growth. Consistent with its moderate increase in cell length, strain WM2600 formed colonies with a slightly lower (approximately twofold) efficiency compared with the equivalent *ftsN*$^{+}$ strains (Fig. 2C, compare rows 1 and 2).

One possibility was that if *ftsA* ^{sup} encoded an activated form of FtsA, perhaps it would be deleterious in the presence of FtsN. However, the colony viabilities of EC448/pBAD18kan-FtsA^{sup} and EC448/pBAD18kan were essentially identical (Fig. 2C, compare rows 2 and 4). Moreover, other plasmid derivatives expressing *ftsA* ^{sup} in W3110 showed no differences from W3110 alone either in cell morphology or in colony viability (data not shown). Finally,

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an equivalent *ftsA* suppressor mutant in the absence of WT *ftsA* also had no detectable phenotype on its own (see below). These results indicate that despite their dramatic suppression of *ftsN::cat*, the *ftsA* suppressor alleles themselves have no obvious phenotype on their own.

FtsA^{sup} is a triple missense mutant

Sequence analysis of several of the plasmids conferring the *ftsN* bypass phenotype revealed that they all expressed *ftsA* genes encoding three altered amino acids: K48R, K117R and E124G. Interestingly, the latter two of these mutations map to subdomain 1c, the region of FtsA sufficient to interact with GFP-FtsN in a polar recruitment assay (Corbin *et al*., 2004). Figure 3 (bottom)

An alignment of divergent FtsA sequences show that aspartate or glutamate residues are quite conserved at position 124 among diverse species of bacteria (Fig. 3, top). Nevertheless, the ability of the suppressor mutant to function normally in the absence of WT *ftsA* indicates that an acidic residue at this position must not be critical for normal function. Moreover, as can be seen in the alignment, the next most common residue at this position is glycine, which is present in several alpha-proteobacteria but also is present in *Haemophilus influenzae*, a close relative of *E. coli* that contains an *ftsN* homologue (Dai *et al*., 1996). Lysines (or arginines) at position 48 are also quite well conserved, although basic residues at position 117 are less well conserved (Fig. 3, top).

The E124G mutation is sufficient for the *ftsN* bypass but less efficient than the triple mutant

To identify which of the three mutations were crucial for bypassing the requirement for FtsN, they were separated into single and double combinations, and then tested them for their ability to confer survival of WM2355, the FtsN depletion strain. We cloned these *ftsA* derivatives into plasmid pCSB1, containing the weakened Trc99 promoter, so their expression levels could be regulated by isopropyl-β-D-thiogalactopyranoside (IPTG).

As shown in Fig. 4A, neither pCSB1 plasmid vector alone (row 1) nor WT FtsA (row 2) could permit significant growth after FtsN depletion at 42°C. However, induction of expression of the triple mutant with different levels of IPTG after FtsN depletion resulted in efficient colony formation (row 10, columns 2–4). On the other hand, no suppression occurred in the absence of IPTG (row 10, column 1). IPTG induction from this plasmid system also complemented *ftsA* mutants (data not shown). The levels of WT or mutant FtsA produced at 0, 0.1 and 1 mM IPTG at 30°C were measured by immunoblotting with anti-FtsA antibodies (Fig. 4B). Although all *ftsA* derivatives in pCSB1 are under identical transcriptional and translational controls, FtsA protein levels were somewhat variable from plasmid to plasmid; nonetheless, levels consistently increased with IPTG as expected.

Importantly, the single E124G mutation was sufficient to permit significant growth in the absence of FtsN, although the efficiency was not as high as with the triple mutant (Fig. 4A, compare rows 6 and 10). When this was tested independently by transducing the *ftsN::cat* allele into W3110 containing IPTG-induced pCSB1 derivatives containing the triple mutant

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and the E124G mutant, similar results were observed: the transductant colonies were more numerous and larger for the triple mutant than for E124G (data not shown).

The effects of the other two mutations were more subtle but reproducible. The K117R or K48R single mutants were unable to bypass the need for FtsN, and neither could the K48R K117R double mutant (Fig. 4A, rows 4, 5, 7). However, K48R enhanced the suppression ability of E124G, resulting in colony-forming efficiency similar to that of the triple mutant (row 8). K117R, on the other hand, had no significant effect on E124G (row 9). Therefore, we can conclude that E124G is sufficient to bypass partially the requirement for FtsN, and K48R enhances this activity to the point that the efficiency of colony formation at 42°C is similar to that at 30°C.

E124A is an efficient chromosomal bypass suppressor of *ftsN* and equivalent to *ftsA* ^{sup}

We also undertook an unbiased selection for spontaneous *ftsN* bypass suppressors by selecting for rare surviving colonies of a WM2355 derivative after long-term FtsN depletion. We have characterized one suppressor in detail. Genetic mapping and sequencing revealed that the suppressor mutation mapped to *ftsA*, and is a single missense mutation that results in an E124A change (strain WM2417). When *ftsA(E124A)* was cloned into pCSB1 and tested for the efficiency of transduction of *ftsN::cat*, E124A and FtsA^{sup} were indistinguishable in their ability to bypass *ftsN*, and both were significantly more efficient than E124G (Fig. 4, compare lane 6 with lanes 10 and 11; Fig. 5A; and data not shown). E124A was similar to FtsA^{sup} by other criteria, including requirement of IPTG for efficient *ftsN* bypass activity when in pCSB1, lack of overproduction toxicity upon IPTG induction from the same plasmid (Fig. 4, row 11), and ability to suppress other cell division mutations (see below). We conclude that a single amino acid change in FtsA can bypass the requirement for FtsN. Interestingly, whereas a glycine was often present at this position in some bacteria, alanine was not (Fig. 3).

Because E124A in WM2417 replaced the WT version of *ftsA* in the chromosome, we asked whether this allele of *ftsA* was as efficient a suppressor as when expressed in a merodiploid. Like cells of *ftsN::cat* strains expressing E124A (or *ftsA* ^{sup}) from a plasmid, such as WM2600 (see above), cells of strain WM2417 were longer on average than the corresponding *ftsN*$^+$ strain WM2886 (7.5 ± 2.6 μm versus 4.1 ± 1.0 μm, at 37°C). Length per Z ring (6.9 μm) was also longer than that for the *ftsN*$^+$ parent strain (4.4 μm). Furthermore, as shown in Fig. 2D, colony growth of WM2417 was similar to that of its *ftsN*$^+$ counterparts, indicating that the chromosomal E124A allele is an efficient suppressor of *ftsN::cat*.

We also asked whether W3110 containing E124A in place of WT *ftsA* displayed any noticeable phenotype compared with W3110 itself. The average lengths of WM2886 (W3110 *ftsA E124A*) and WM2887 (W3110) were the same (4.1 ± 1.0 μm versus 4.0 ± 0.8 μm, at 37°C). FtsZ-GFP localization (in WM2891 and WM2892) and colony growth phenotypes of the two strains were identical (Fig. 2D and data not shown). Growth rates of strains WM2886, WM2887 and WM2417 were essentially the same (data not shown). These three strains were also grown to stationary phase (18 h) and plating revealed that viability was equivalent among all three strains, indicating that the suppression was effective in stationary phase (data not shown). Therefore, we conclude that despite its dramatic

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suppression of cell division defects in cells lacking *ftsN*, the E124A mutant of FtsA has no obvious effect on growth and division of *E. coli* when in an otherwise WT context.

Gain of function *ftsA* mutants are less toxic than WT *ftsA* when expression is increased

Massive overproduction of FtsA, $FtsA^{sup}$ or FtsA* from the strong *araBAD* promoter after arabinose induction of the pBAD18kan plasmids blocked cell division and sharply reduced viability (data not shown), consistent with the known negative effects of excess FtsA on cell division (Dai and Lutkenhaus, 1992; Dewar *et al*., 1992; Geissler *et al*., 2007). However, during induction of the weak Trc99 promoter on pCSB1 derivatives, we noticed that increased expression of WT *ftsA* abolished viability as IPTG was increased (Fig. 4A, row 2 at 30°C), but similar increased expression of R286W (*ftsA**) (row 3), *ftsA* ^{sup} (row 10) or E124A (row 11) caused no detectable reduction in viability. This lack of toxicity of the mutant proteins was observed despite somewhat higher levels of R286W, $FtsA^{sup}$ or E124A protein relative to WT FtsA protein at a given IPTG concentration, as measured by immunoblotting (Fig. 4B). Interestingly, similar induction of K117R or K48R expression from pCSB1 derivatives caused toxicity similar to that of WT *ftsA* (Fig. 4A, rows 4 and 5).

It is notable that the degree of toxicity correlated with the inability to bypass FtsN. However, the data indicate that the failure of some of the derivatives to bypass FtsN is not solely because of toxicity. For example, cells overproducing FtsA* (Fig. 4B) had normal viability at 1 mM IPTG, but were only able to suppress the loss of FtsN partially at 0.1 mM IPTG and not at 0.5 or 1 mM IPTG (Fig. 4A, row 3). Likewise, cells overproducing K48R were viable at 0.1 mM IPTG (Fig. 4A, row 4, and Fig. 4B), but were not able to suppress the loss of FtsN at this or any other IPTG concentrations (Fig. 4A, row 4).

As R286W or $FtsA^{sup}$ can localize to the Z ring as GFP fusions (Geissler *et al*., 2003 and data not shown) and hence must interact with FtsZ, it is not surprising that very high levels of these FtsA derivatives still cause toxicity. Nevertheless, the lower toxicity of the gain of function mutants compared with WT FtsA suggests that the mutant FtsA proteins may resist perturbations in the crucial FtsA:FtsZ ratio by increasing the overall integrity of the Z ring.

$FtsA^{sup}$ or E124A can also partially compensate for the inactivation of ZipA or FtsK

FtsA* (R286W) can suppress multiple cell division gene mutants to varying degrees. We explored whether $FtsA^{sup}$ or E124A had similar gain of function activities. To allow introduction of *ftsK::kan* or *zipA::kan* alleles, the *ftsA**, *ftsA* ^{sup} and *ftsA(E124A)* mutant genes were cloned into the Ap^R plasmid pRR48, which allows for their IPTG-dependent expression. Remarkably, W3110 expressing these three *ftsA* alleles allowed efficient transduction of either *zipA::kan* or *ftsK::kan* null alleles compared with the vector control (Fig. 5A). In addition, an *ftsK44*(ts) strain was complemented efficiently at the non-permissive temperature by pBAD18kan-*ftsA** or pBAD18kan-*ftsA* ^{sup} (data not shown).

Nonetheless, *ftsA* ^{sup} or E124A expressed from pRR48 were not able to compensate for the loss of ZipA as efficiently as the *ftsA** allele R286W, with a 10- to 100-fold loss of viability (Fig. 5B). The same was true for either R286W or E124A alleles replacing WT *ftsA* in the chromosome (Fig. 5C), further supporting the idea that the E124A suppressor can

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compensate for the loss of ZipA, but not as effectively as R286W. The *ftsK::kan* allele was suppressed equally well by R286W or E124A, with *ftsA* ^{sup} showing a modest decrease (Fig. 5E); the significance of this is unclear. When the R286W and E124A alleles replaced chromosomal *ftsA*, *ftsK::kan* transductants were always smaller with R286W, which may reflect the prevalence of long cell chains seen with R286W compared with short filaments with E124A (data not shown).

Ability of FtsA R286W or increased levels of early Z ring components to compensate for the loss of FtsN

The functional overlap between FtsA^{sup} and FtsA R286W prompted us to investigate whether the latter could compensate for the loss of FtsN. Although *ftsN::cat* transductants could be isolated in W3110 strains with R286W, the colonies were small compared with those expressing *ftsA* ^{sup} or E124A (Fig. 5A, right) and had low viability compared with *ftsN::cat* strains containing FtsA^{sup} or E124A (Fig. 4). This was true whether the R286W or E124A alleles were expressed from a plasmid or replaced the native *ftsA* at the chromosomal locus (Fig. 5D). Therefore, despite their overlapping functions, FtsA R286W and FtsA^{sup} have somewhat different suppression profiles, suggesting that the lack of FtsN imposes different requirements for compensatory changes in FtsA.

Because the loss of *zipA* or *ftsK* was suppressed quite efficiently by merely increasing expression of *ftsZ*, *ftsA* and *ftsQ* from the pZAQ plasmid (Geissler *et al*., 2003), we investigated whether pZAQ might help to suppress the cell division defects in the absence of FtsN. However, when W3110 cells containing pZAQ were transduced with the *ftsN::cat* allele, no transductants were obtained, while several hundred transductants were isolated with *zipA::kan* in W3110/pZAQ or with *ftsN::cat* in W3110 derivatives expressing *ftsA* ^{sup} or E124A (data not shown). This suggests that the presence of extra early Z ring components cannot compensate for the lack of FtsN, and supports the idea that the *ftsN* bypass suppression may act via a distinct mechanism.

The E124A mutation in *ftsA* increases resistance to the MinC division inhibitor

One way to test whether E124A or FtsA^{sup} might increase the integrity of the Z ring was to determine if these mutant FtsA proteins, like R286W, could resist the inhibitory effects of MinC, a key inhibitor of FtsZ assembly. As we have shown that E124A and FtsA^{sup} are essentially equivalent, we used WM2886, which contains the E124A allele in place of the WT *ftsA* gene on the chromosome. A plasmid expressing an IPTG-inducible functional *flag–minC* fusion (pWM2801) was introduced into this strain and WM2887, the isogenic strain containing WT *ftsA*.

Without IPTG, cells of both strains containing the *flag–minC* fusions were short and divided normally, although WM2887 cells were slightly longer (Fig. 6A and B). As expected, WM2887 cells filamented after several hours of FLAG–MinC overproduction upon IPTG induction (Fig. 6D). WM2886 cells, on the other hand, remained fairly short, with only occasional filamentation (Fig. 6C). FLAG–MinC levels in both strains were equivalent at a given IPTG concentration (Fig. 6E), indicating that the E124A allele in WM2886 conferred resistance to Z ring disassembly caused by overproduction of MinC. Viability tests

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confirmed a ~10-fold decrease in colony-forming units in IPTG-induced WM2887 versus WM2886 at the time the micrographs were taken (data not shown). Therefore, despite having no visible phenotype in otherwise WT strains, including no observed minicells, the mutant FtsA subtly increases in the integrity of the Z ring.

GFP-FtsN localizes normally in cells with the E124A mutant of FtsA

In WT cells, FtsN is the last essential cell division protein to localize, and its septal localization depends on all other known essential cell division proteins. However, the ability of FtsN to be bypassed by an altered FtsA prompted us to test whether FtsN could still be targeted to the Z ring in the presence of the altered FtsA. One possible scenario was that the FtsA suppressor promotes a bypass of the normal divisome protein assembly pathway. As FtsN depends on prior localization of other divisome components, this model predicts that FtsN would fail to localize to the divisome in an E124A mutant (WM2886).

To test this idea, we introduced a plasmid expressing *gfp-ftsN* into WM2886, WM2887 and WM2417, which contains both *ftsN::cat* and *ftsA* (*E124A*). GFP-FtsN localized to the septum in approximately half of the cells of WM2887 and WM2886, and no other differences between the two strains were detected (Fig. 7A and B). GFP-FtsN also localized to division septa in WM2417 cells (Fig. 7C). Therefore, we conclude that FtsN, at least in the form of GFP-FtsN, retains the ability to localize to division septa in the presence of E124A. This suggests that the normal recruitment properties of the divisome, at least involving FtsN, are unchanged.

The requirement of FtsN for localization of the Tol–Pal complex to the cell division site is indirect

Recently, Gerding *et al*. (2007) showed that the Tol–Pal complex localizes to division sites and helps to co-ordinate invagination of the outer membrane with division septum formation. Using GFP fusions to several components of the complex, including TolA, they demonstrated these fusions failed to localize after FtsN depletion, indicating that FtsN is required for the localization of this complex to the divisome. This suggests two possible models. The first is that FtsN directly recruits the Tol–Pal complex, either via direct protein–protein interactions or via an intermediate protein(s). If this model were correct, FtsN should be indispensable for recruitment of Tol–Pal. The second is that FtsN does not directly recruit Tol–Pal components, but instead FtsN activity is indirectly required for Tol–Pal to localize to the divisome. If this model were correct, FtsN activity could theoretically be replaced by another compensatory activity.

The FtsN bypass suppressors allowed us to test these models directly. We constructed a GFP–TolA fusion and introduced it into WM2886 (E124A), WM2887 (WT) and WM2417 (E124A *ftsN::cat*). Importantly, GFP–TolA localized to the divisome in all three strains (Fig. 8). This rules out the first model and supports the second model, in which FtsN does not directly recruit Tol–Pal but instead increases the integrity of the Z ring, which then becomes competent to recruit Tol–Pal. To provide additional support for this idea, we grew WM2417 in LB with no added salt, conditions that normally cause chaining of cells in *tol* mutants (Gerding *et al*., 2007). As expected, WM2417 was unchanged from its normal

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phenotype observed in regular LB medium, with no cell chains detected (data not shown). This result suggests that as long as the appropriate *ftsA* suppressor is present to compensate, the lack of FtsN does not inhibit the function of the Tol–Pal complex in cell–cell separation.

Discussion

We have shown that the essential cell division gene *ftsN* can be bypassed by mutations in another cell division gene, *ftsA*. The immediate conclusion is that FtsN, while normally essential, cannot be a core component of the divisome. This is a significant finding, as a number of papers have been published about FtsN, but no unique function has been ascribed to it. FtsN is also poorly conserved outside the enteric bacteria, consistent with its revised role as an accessory factor for the divisome.

Despite the robust viability of cells with *ftsA* ^{sup} or *ftsA*(*E124A*) but lacking *ftsN*, there is a modest cost. For example, suppressed cells lacking FtsN are longer than WT cells with more space between Z rings, suggesting that Z rings are less stable or less active, resulting in less efficient septation. On the other hand, without the FtsA suppressor, depletion of FtsN results in formation of long filaments and death, probably because the remaining Z rings are too inactive to divide cells sufficiently for viability. Therefore, the gain-of-function mutations in FtsA reported here probably increase ring integrity or activity to mostly counteract the loss of FtsN. The resistance of the E124A mutant to the toxic effects of MinC overproduction directly supports this idea. Further work will be needed to determine whether additional factors can reduce the cost of not having FtsN. One prediction would be that removal of other Z ring destabilizing factors or activation of ring stabilizing factors might further compensate for its absence. However, it is also possible that FtsN has a unique function that, while not essential for cell division in the presence of a suppressor, increases its efficiency.

The stronger FtsN bypass activity of the E124A mutant FtsA versus the E124G mutant protein suggests that any conformational changes in FtsA resulting from E124A are more dramatic than those resulting from E124G. Because the triple mutation in FtsA^{sup} has FtsN bypass activity equivalent to E124A alone, and K48R combined with E124G are sufficient for this activity, it is likely that K48R induces additional subtle conformational changes in the E124G mutant protein that allow it to function more efficiently to bypass the FtsN requirement. The fact that the gain-of-function mutants of FtsA have no detectable phenotype by themselves and seem to recruit FtsN normally, at least in the form of GFP-FtsN, suggests that the divisome has tolerance for conformational flexibility of the FtsA molecule.

Our results provide additional support for the idea that FtsN enhances the activity of the divisome. The mutations in FtsA that bypass the requirement for FtsN also partially suppress the inactivation of other divisome proteins, including ZipA or FtsK, suggesting that FtsA^{sup} and E124A are not FtsN-specific, but instead have a general role in divisome integrity similar to R286W. Importantly, however, suppression efficiencies suggest that FtsA^{sup} and E124A are biased towards suppressing FtsN, while R286W fails to suppress the loss of FtsN significantly. Consistent with the bias of R286W away from suppressing FtsN, increased levels of FtsQ, FtsA and FtsZ from pZAQ phenocopy R286W and suppress the loss of ZipA

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and FtsK to some degree, but do not confer viability on a strain lacking FtsN. Therefore, we speculate that different subtle alterations in FtsA function result in overlapping but distinct suppression profiles because different divisome subcomplexes, as outlined in a recent review (Vicente *et al*., 2006), are influenced differentially by FtsA binding. The recent isolation of an *ftsA* mutant that suppresses a defective *ftsQ* allele but that also suppresses defects in some other cell division genes (Goehring *et al*., 2007a) is consistent with this idea.

This model is supported by additional evidence. First, despite the ability of E124A or $FtsA^{sup}$ to suppress the loss of either ZipA or FtsN, neither allele permitted both ZipA and FtsN to be inactivated simultaneously (T. Hammerstrom and W. Margolin, unpubl. results). This suggests that the effects of ZipA and FtsN on the divisome are synergistic and distinct and cannot be replaced by a single form of FtsA, and is consistent with the partial specificity of suppression that we have observed. We also attempted to delete both *zipA* and *ftsN* in a strain expressing *ftsA** from the chromosome and $ftsA^{sup}$ from a plasmid, but could only make the single deletions (W. Margolin, unpubl. results). There are many potential explanations for this, but once again it is consistent with a synergistic effects of ZipA and FtsN and a lack of simple additive effects of the suppressors.

Second, recent evidence suggests that FtsA interacts, probably indirectly, with the late divisome proteins FtsN and/or FtsI. In one of these approaches, premature targeting of FtsQ to the Z ring can recruit all known later divisome proteins in the absence of FtsA or FtsK except for FtsN. Therefore, FtsN likely has a direct requirement for FtsA to be stably recruited to the divisome. Third, overproduced FtsN can partially suppress a number of *fts* mutants much like $FtsA^{sup}$ and E124A, suggesting that the FtsA suppressor mutants allow FtsA to function more efficiently to bypass the FtsN requirement. Finally, as shown here, $FtsA^{sup}$, E124A and R286W seem to be less toxic than WT FtsA at equivalent levels of IPTG induction. These alleles may alter the requirement for a strict stoichiometric ratio between FtsZ and FtsA, although the mechanistic explanations for this are not clear. The challenge for the future is to understand how the various mediators of divisome integrity and activity function at the molecular level.

An alternative model for FtsN function is that it senses the completion of divisome assembly, triggering Z ring contraction (Corbin *et al*., 2004). Although it is not required for FtsN function, FtsN binds peptidoglycan (Ursinus *et al*., 2004), suggesting that part of the putative signal may originate from FtsI-mediated septal peptidoglycan biosynthesis. The known roles of FtsN in stimulating septation under conditions normally unfavourable for septation are consistent with this idea, as is its (probably indirect) interaction with FtsA, which might undergo a conformational change in response to FtsN and transduce the signal to FtsZ. In the absence of FtsN, the sensing pathway would be inactive, but could be compensated by a mutation that would mimic the FtsN-induced conformational change in FtsA. While this model is intriguing, it predicts that the *ftsA* mutants on their own would display a significant phenotype, which they do not, either when produced from a plasmid or as the sole copy of *ftsA* in the cell. The absence of *ftsN* homologues in most species also argues against a special signalling role for FtsN.

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Another potential role for FtsN is the recruitment of proteins involved in cell–cell separation, including AmiC and the Tol–Pal complex. These proteins are not recruited to the septum after FtsN depletion, indicating that FtsN is required for their recruitment (Bernhardt and de Boer, 2003; Gerding *et al*., 2007). However, we found that FtsN is not required for recruitment of a key Tol–Pal component, TolA, in the presence of the E124A suppressor mutant of FtsA. This result is important because it eliminates the most obvious model, which proposes that FtsN directly recruits these proteins to the septum via specific protein–protein interactions or a specific enzymatic activity. Instead, it strongly suggests that FtsN helps to recruit these later proteins by its indirect effects on divisome integrity, which can be replaced by an altered FtsA.

Despite previous evidence strongly suggesting that FtsN is a special component of the divisome (Corbin *et al*., 2004; Vicente *et al*., 2006; Goehring *et al*., 2007a), the present study demonstrates conclusively that FtsN is not a unique divisome component. Instead, FtsN is one of several factors that potentially regulate conformational changes within divisome components such as FtsA, which in turn regulate the activity of the Z ring. The fact that point mutations of FtsA can bypass the requirement for an early acting, FtsZ-binding protein (ZipA), a middle-stage protein (FtsK) or a late-stage protein (FtsN) strongly supports the model that FtsA is conformationally flexible, and can exert subtle effects on the divisome at different stages. In contrast to FtsN, this points to a key and core role for FtsA in regulating divisome function.

Experimental procedures

Growth conditions, microscopy and protein detection

Unless stated otherwise, cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl). SOC medium (2% tryptone, 0.5% yeast extract. 8.6 mM NaCl, 2.5 mM KCl, 20 mM glucose, 20 mM $MgSO_4$) was used for outgrowth of transformed cells. Antibiotics were added to LB at 50 μg ml^{-1} for ampicillin or kanamycin (kan), and 10 μg ml^{-1} for tetracycline (tet) or chloramphenicol (cam). Top10 was used as a recipient in transformations of ligations. TOE44, which is *ftsK44*(ts) and *thyA*, was supplemented with 50 μg ml^{-1} thymine. Immunoblotting was performed as described previously (Geissler *et al*., 2003) with rabbit polyclonal antibodies against purified FtsN or FtsA. Loading of gel lanes was normalized to cell density. FtsN protein was purified for antibody production by overproduction from a pET28-FtsN construct. Microscopy to detect GFP fusions in live cells or in fixed cells with immuofluorescence was performed as described previously (Sun and Margolin, 2001). Cell lengths were measured with Object Image 2.19 freeware (Norbert Vischer).

Plate viability spot assays were performed by spotting 10-fold dilutions of exponentially growing cells that were at equivalent densities, starting with a 10^{-1} dilution. For assays of plasmid derivatives in the FtsN depletion strain WM2355, cultures were grown overnight in LB kan at 30°C, diluted 1:100 and grown at 30°C for 2 h, then split into 30°C and 42°C cultures and grown for an additional 2 h each prior to spotting on plates with different IPTG concentrations at 30°C or 42°C.

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Constructing the FtsN depletion strain

All strains are listed in Table 1. To make a strain with the *ftsN::cat* allele, the GFP-FtsN plasmid pWM1152 (Corbin *et al*., 2004) was first introduced into strain DY329 (Yu *et al*., 2000). The *ftsN::cat* allele was assembled by PCR amplification of the cat cassette from the chromosomal *ftsK1::cat* allele (Diez *et al*., 1997) and, flanking the *cat* cassette, 30 bp regions flanking the chromosomal *ftsN* gene. This PCR product was then used to replace the native *ftsN* gene in DY329/pWM1152, using lambda-mediated recombineering (Yu *et al*., 2000), to make WM1932. A linked *argE*::Tn*10* marker was introduced from CAG12185 (Singer *et al*., 1989) to permit selection for Tc^R as well as Cm^R. To construct the FtsN depletion strain, the *ftsN* gene in pBAD30-FtsN (Geissler and Margolin, 2005) was cloned as a SacI–HindIII fragment into the thermosensitive Ap^R plasmid pTSA29 (Phillips, 1999). A correct plasmid clone was then selected in Top10 cells, and plasmid DNA was transformed into WT W3110 to make WM2245. This strain in turn was transduced with a P1 lysate from the strain with *ftsN::cat* linked to *argE*::Tn*10*, selecting Ap^R Tc^R Cm^R transductants. Several transductants were picked, and did not form colonies when streaked on LB tet cam plates and incubated at 42°C, suggesting that they were FtsN depletion strains. One strain was chosen and called WM2355. Depletion of FtsN in WM2355 was confirmed by the formation of long filamentous cells after shifting growth from 30°C to 42°C and loss of viability.

Mutagenesis of cloned *ftsA*

Plasmid pET28-FtsA, containing the *ftsA* gene with an N-terminal hexahistidine tag and a T7 tag from pET28 (Geissler *et al*., 2003), was the template used for PCR mutagenesis and selection. As shown previously, these tags do not affect the ability of FtsA to complement an *ftsA* mutant. The *ftsA* gene on pET28-FtsA was subjected to mutagenic PCR, using primers 727 and 728, which anneal to vector sequences just upstream and downstream of the *ftsA* insert. Amplification was performed with 1 μl (2.6 U) of Fisher Taq DNA polymerase and buffer, 200 μM dNTPs, 5 mM $MgSO_4$ and 1 μM of each primer for 40 cycles. In each cycle, annealing was at 52°C for 30 s, followed by elongation at 72°C for 3 min.

The PCR product was digested with XbaI and EcoRI and ligated to XbaI–EcoRI-cleaved pET28a. Nine of these ligation reactions were transformed into Top10 cells and plated on LB kan at 37°C, yielding an average of 220 colonies per plate for a total of ~2000 colonies. These colonies were pooled and used to make a mutagenized plasmid bank. Two per cent of the bank was then transformed into WM2355 electrocompetent cells after 1 h of outgrowth at 30°C in SOC. Cells were plated on LB kan tet either at 30°C or at 42°C overnight and yielded 1800 and 15 colonies respectively. To determine whether the ability to survive at 42°C was conferred by the mutagenized plasmid, plasmid DNA was isolated from the 15 temperature-resistant strains and re-transformed into WM2355 at 30°C and 42°C, as were pET28 vector alone and pET28-FtsA plasmids as controls.

Plasmid pWM2425 was the initial isolate of the triple $ftsA^{sup}$ mutant. To rule out potential effects of vector sequences, the NdeI–EcoRI fragment containing *ftsA* from this plasmid was re-cloned into pET28a and a confirmed isolate was saved as pWM2491. Plasmid pWM2491 was then introduced into WM2355. To test for suppression of the loss of FtsN, these cells

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were incubated for 75 min at 30°C in 1 ml of SOC, and then half the culture was grown at 30°C for 1 h and plated on LB kan tet at 30°C, while the other half was grown at 42°C for 1 h and plated at 42°C. Unlike control cells containing pET28 vector alone, cells with pWM2491 gave rise to colonies at 42°C at the same efficiency as 30°C, indicating that the loss of FtsN was bypassed.

Plasmid constructions

Plasmids are listed in Table 2. Construction of *ftsA* mutant combinations was performed in pET28a prior to moving the mutant *ftsA* genes to pBAD18kan or pCSB1. To construct the FtsA K117R single mutant in pET28a (pWM2488), PCR mutagenesis was performed with primer pairs 826 + 863 and 862 + 528 in the first amplification, and primers 826 + 528 in the second amplification (see Table 3 for oligonucleotide sequences). The product was then cloned as a PstI–XhoI fragment into pET28a. To construct the FtsA E124G single mutant (pWM2489), PCR mutagenesis was performed with primer pairs 826 + 861 and 860 + 528 in the first amplification, and primers 826 + 528 in the second amplification. The product was cloned as a SacI–XhoI fragment into pET28a. To construct the FtsA K48R single mutant (pWM2504), PCR mutagenesis was performed with primer pairs 106 + 837 and 836 + 726-1 in the first amplification, and primers 106 + 726-1 in the second amplification. The product was cloned as a NdeI–BamHI fragment into pET28a. To construct the FtsA K117R E124G double mutant (pWM2490), the original pET28-FtsA plasmid was cleaved with BglII, which cuts both the vector portion and between the K48 and K117 residues encoded by *ftsA*. This fragment, which includes the N-terminal portion of WT *ftsA*, was then ligated into BglII-cleaved pWM2425 containing the triple mutant *ftsA*, resulting in restoration of the WT *ftsA* encoding the K48 residue but leaving the mutant K117R and E124G-encoding residues intact. To construct the K48R K117R double mutant, the BglII fragment of WM2488 (K117R single mutation) was replaced with the BglII fragment from pWM2491 containing the triple mutation. The K48R E124G double mutant (pWM2507) was constructed by PCR mutagenesis of pWM2489 (E124G), using primers 106 + 897 and 896 + 726-1 in the first amplification and 106 + 726-1 in the second amplification, followed by cloning the product as a NdeI–BamHI fragment into pET28a.

All cloned *ftsA* derivatives in pET28a were transferred to pBAD18kan by amplification of the *ftsA* gene, its ribosome binding site, and sequences encoding the N-terminal histidine tag with primers 902 + 903, then cloning the PCR product as a SacI–XbaI fragment into pBAD18kan. All cloned *ftsA* derivatives in pET28a were transferred to pCSB1 by amplification of the *ftsA* gene with primers 826 + 903, and cloning the PCR product as a SacI–XbaI fragment into pCSB1. These constructs fuse *ftsA* sequences to a ribosome binding site already present in the vector sequences, and as a result only the *ftsA* open reading frame was transferred. Therefore, unlike the pET28a and pBAD18kan derivatives, none of the pCSB1 derivatives synthesize an N-terminal histidine tag fused to FtsA. No functional differences between FtsA with or without a histidine tag were apparent throughout this study.

The E124A allele was isolated as a spontaneous suppressor in WM2391 after FtsN depletion at 42°C, and called WM2417. The absence of FtsN in this strain was initially implicated by

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the Ap^s phenotype of the strain, indicating that the pTSA29-*ftsN* thermosensitive plasmid was indeed lost. The absence of FtsN was subsequently confirmed by immunoblotting. DNA sequencing of a PCR product from the chromosomal *ftsA* gene confirmed that the E124A allele resulted from an A to C change at position 371 in the *ftsA* nucleotide sequence (the E124G mutation of *ftsA*^{sup} resulted from an A to G change at the same position). This A to C change serendipitously generated a unique FspI site in *ftsA* that was useful for screening. The E124A allele was introduced into W3110 by co-transduction with the *leu*::Tn*5* marker (~50% linkage) present in WM2391 to make WM2886; WM2887 was another isolate from the same transduction that contained the WT *ftsA* instead. To enable selection of *zipA::kan* or *ftsK::kan* transductants, the Tn*5* marker was replaced by a Tet^R Tn*10*. This was done by transducing WM2886 and WM2887 with a *leu*::Tn*10* marker from the *ftsA** strain WM2177, selecting Tet^R colonies, and screening for the presence of the E124A allele of *ftsA* by FspI digestion of a PCR product amplified from chromosomal *ftsA* (WM2934). Isolates containing the *ftsA** allele instead of E124A (WM2935) were confirmed by NcoI digestion of the *ftsA* PCR product (Geissler *et al*., 2003).

Plasmid pWM2060 was made by removing the gene encoding GFP from pDSW210 (Weiss, 2004); the EcoRI–ScaI fragment of pDSW210 was replaced by the EcoRI–ScaI fragment of pDSW209. Plasmid pCSB1 was then constructed by cloning the kanamycin cassette (*aph* gene) from pET28a into the ScaI site of pWM2060, replacing the *bla* gene with *aph*.

The pRR48 derivatives were made by inserting a PCR-amplified *flag*-tagged *ftsA* construct into NdeI–XhoI-cut pRR48, an Amp^R plasmid which contains the *tac* promoter, *lac* operator and a *lacI*^{q} gene (kindly supplied by Sandy Parkinson). The N-terminal FLAG tag had no detectable effect on function of any of the *ftsA* derivatives. Full details of the cloning will be described in a forthcoming paper.

The GFP–TolA fusion was constructed by PCR amplification of chromosomal *tolA* using the same primers described previously (Gerding *et al*., 2007) and cloning the product into pDSW209 with BamHI and SalI.

Other strain constructions

WM2600 was made by transforming the FtsZ-GFP expression strain EC448 (Weiss, 2004) with pBAD18kan-*ftsA*^{sup} (pWM2552), then introducing the *ftsN::cat* allele by P1 transduction. To test complementation of *ftsA* mutants, pBAD18kan plasmids containing *ftsA* or *ftsA*^{sup} were transformed into the *ftsA12*(ts) strain WM1115 or the FtsA depletion strain WM1281 (Geissler and Margolin, 2005), grown at the permissive temperature of 30°C, then plated at 30°C or 42°C to inactivate or deplete FtsA.

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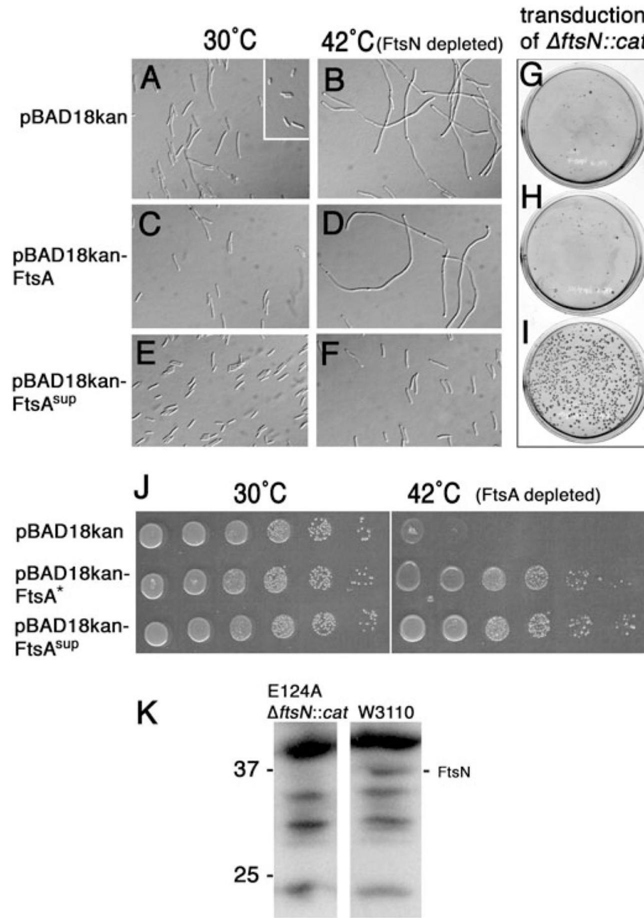

Fig. 1. The cloned *ftsA* suppressor mutant (*ftsA* ^{sup}) permits cell division and colony formation in the absence of FtsN or FtsA

A–F. DIC micrographs of FtsN depletion (WM2355) derivatives containing pBAD18kan (A and B), pBAD18kan-FtsA (C and D) or pBAD18kan-FtsA^{sup} (E and F) grown at either 30°C (A, C and E) or 42°C to deplete FtsN (B, D and F) for 4 h after initial growth for 2 h at 30°C (no arabinose added). Inset in (A) shows cells of the parent W3110 strain.

G–I. P1 transduction of *ftsN::cat* into W3110 containing pBAD18kan (G), pBAD18kan-FtsA (H) or pBAD18kan-FtsA^{sup} (I), selecting for Cm^{R} and Km^{R} at 37°C (no arabinose added).

J. Complementation of the FtsA depletion strain WM1281 by FtsA* or FtsA^{sup}. WM1281 cells containing the indicated plasmids were either grown at 30°C or induced at 42°C for 2 h prior to spotting on plates, which were then incubated overnight at the indicated temperatures.

K. Anti-FtsN immunoblot of lysates from strains WM2417, containing the chromosomal E124A suppressor and *ftsN::cat*, or parent strain W3110. Markers to the left are in kDa. The position of the FtsN protein band is shown to the right.

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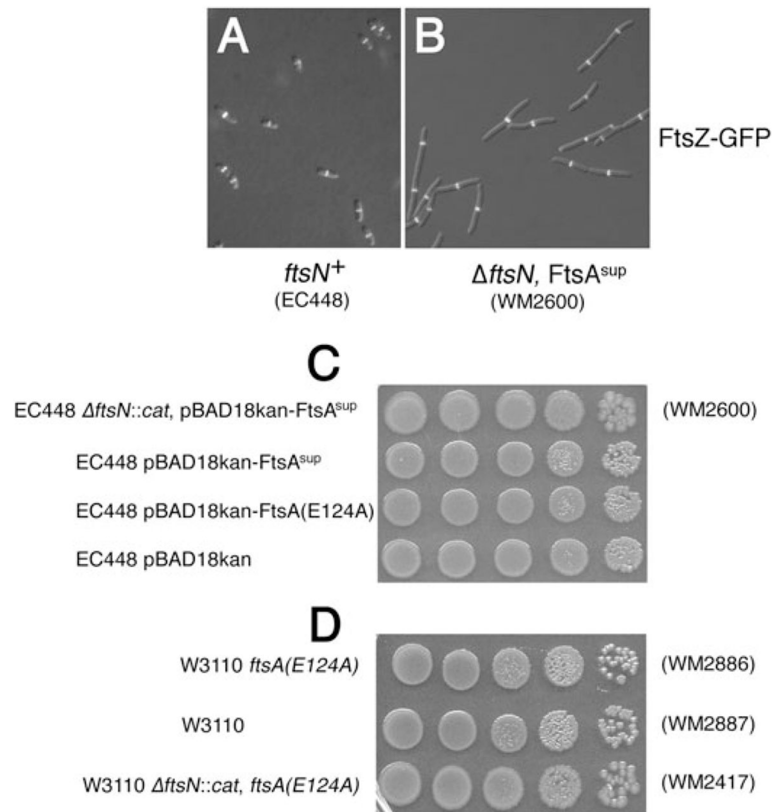

Fig. 2. Z rings and relative viability of *ftsN*$^{+}$ cells with the suppressor and suppressed cells lacking *ftsN*

A and B. Representative fluorescence/DIC overlays of logarithmically growing cells of EC448 (FtsZ-GFP) (A) or of WM2600, which is EC448 plus pBAD18kan-FtsA^{sup} and *ftsN::cat* (B).

C and D. Colony viabilities of EC448 carrying derivatives of pBAD18kan (C) or W3110 derivatives (also containing *leu*::Tn*5*) with or without the chromosomal E124A allele (D), in the presence or absence of *ftsN*.

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      48             117      124
       |               |        |
ANNCIRHGCVHN--//--AEMRG..ASFP.DKEVLDVTDPLFYVDG P. gingivalis
PSEGMKRGSVIN--//--ENARA..VPLDPSLEILHTIPQEYVVDG D. radiodurans
PSRGLRAGMVTN--//--ETAKA..INIPPDQKILDAVVQDYIIDT N. meningitidis
ESKGLKKGVVVN--//--ETAKA..INIPTDQQVLHILTQEFIIDG B. mallei
PSRGLKKGVVVN--//--DSARA..VAIPADQKILHILPQEFVIDR M. capsulatus
PSRGLKKGVVVN--//--DAAQA..VAIPADQRVLHTLPQDYVIDN P. putida
ESRGLKRGVVVD--//--DAAKA..VAIPADQRILHAIPREYVLDD X. campestris
PNTGIRQGVVVN--//--EAAKA..VAVPTDRSVLHVLPREFKVDG B. bacteriovorus
PSSGLKKGVVYN--//--RAAQT..VKISQNQNIIHVLPQEYMVDD D. psychrophila
ISRGVRKGVLIN--//--EAAKA..IVIPMDREILHVIPQEFIVDG B. burgdorferi
PAKGIDKGNITD--//--HTASS..IKMPEGLVVLHRLPQEYAVDQ P. multocida
PSKGIDRGSITD--//--HTASS..IKLPEGLSLLHVIPQEYAVDR H. influenzae
TSRGMDKGGVND--//--HAAQS..VPIAKERRILHVLPQEYVIDS I. ioihiensis
PSRGMDKGGVND--//--HTAKS..VRVRDEHRVLHVIPQEYAIDY E. coli
KSDGLKNGIIVD--//--QNALM..RGIVPEREIIAVETKEFTVDG L. lactis
KSDGLNRGIVVD--//--SAALV..RSTPPERQIVAILPQDFTVDG E. faecalis
PSEGLKKGSIVD--//--AAAQV..VSVPHEQLIVDVIPKQFIVDG B. subtilis
PCTGIKKGVVID--//--NASRI..ITIPSNKEIIGVIPEEYIVDG C. tetani
KTNGVKKGAITN--//--STAKHT.ANLPSGYEIIHVLPYNFKVND C. jejunii
KADGIKKGSITN--//--QTAIYN.AVIPEGHTVIHALPYEFRLDD H. hepaticus
QSKGIKQGSVIN--//--KTAKA..VPMSADTEMLHVLQRDYIVDG F. tularensis
PTKGMRHGKIVD--//--VTAID..TALKDGREAIAFLPNKFLIDG L. johnsonii
HSKGIKSGVILD--//--QKALV..EFKFKNQEIIHYFPLEFTLD. R. typhi
PSRGIVKGVIND--//--ERCLM..KAKEEGYEIVYTAPRKYILDD A. aeolicus
QSRGVRGGAIVN--//--ASALA..QVRIPGRKPIHLLPIAWSVDG C. crescentus
ESLGVRRGYLFD--//--ATGRD..AIDPDGHMVLHAQPALYTLDG Z. mobilis
YTSGIKNGLIDD--//--EGIRE..KNDVQETEVINVFPIRFIVDK S. aureus
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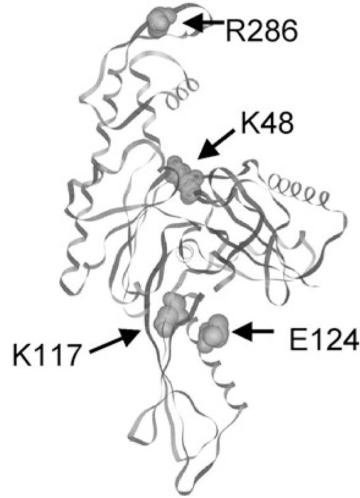

Fig. 3. Conservation of *ftsA* suppressor mutations in FtsA

Top. An alignment of segments comprising the K48, K117 and E124 residues involved in suppression. Conserved residues at these three positions, denoted by numbers at the top, are shown in bold. Species containing a likely homologue of FtsN, defined as an *E*-value < 0.001 after a BLAST search with *E. coli* FtsN, are underlined.

Bottom. Positions of the three FtsA^{sup} mutations and R286W (FtsA*) in the corresponding crystal structure of *Thermotoga maritima* FtsA. shows the positions of the three mutations mapped onto the crystal structure of FtsA from *Thermotoga maritima*.

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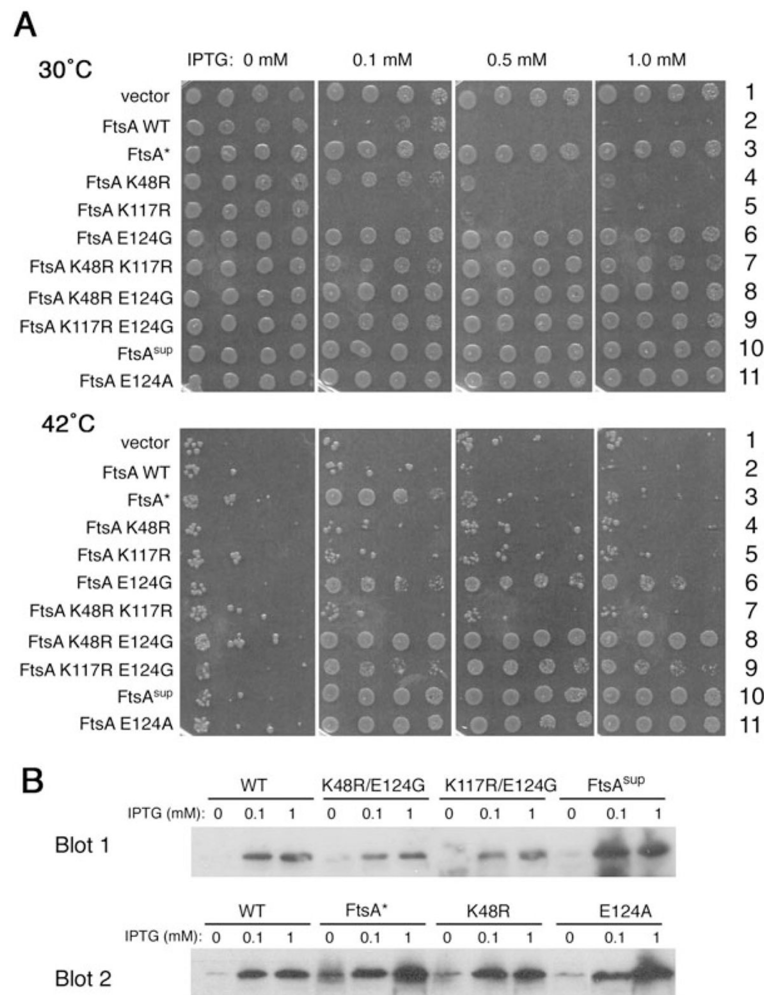

Fig. 4. Effects of various *ftsA* mutants on bypassing the requirement for FtsN and inhibiting colony formation

A. FtsN depletion strains (WM2355) containing pCSB1 plasmid derivatives expressing IPTG-inducible alleles of *ftsA* shown at left were tested for colony viability on plates at 30°C (permissive) or 42°C (to deplete FtsN). IPTG was present in the plates at 0, 0.1, 0.5 or 1 mM, as shown at the top.

B. Immunoblots of representative cultures used for the 30°C spots were probed with polyclonal anti-FtsA antibodies. Both blots show cultures expressing WT FtsA as controls.

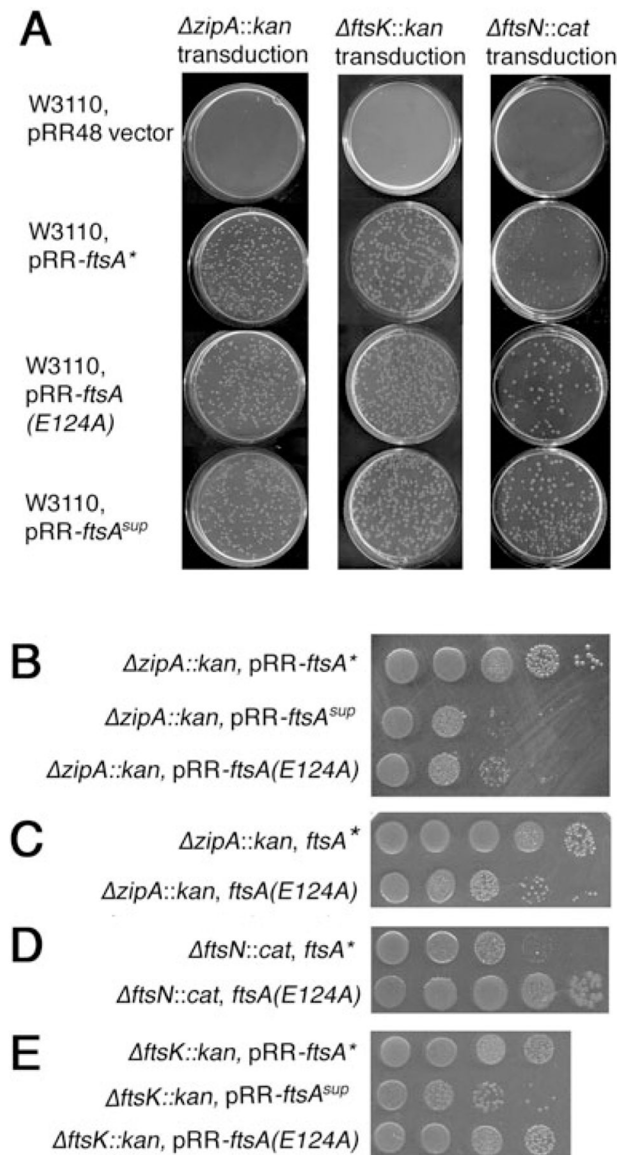

Fig. 5. Suppressor mutants of *ftsA* can suppress the loss of *ftsK* or *zipA* in addition to the loss of *ftsN*, with varying efficiencies

A. The *zipA::kan*, *ftsK::kan* or *ftsN::cat* alleles were introduced by P1 transduction into W3110 carrying the plasmids indicated, and plated on selective plates containing 0.1 mM IPTG to show the frequency and size of the initial transductants.

B–E. Colony viabilities of various combinations of *ftsA* suppressor mutants and cell division gene mutants are shown in each row. The *ftsA* suppressor mutants were expressed either from the native chromosomal locus in the absence of WT *ftsA* (C and D), or from plasmid pRR48 derivatives (supplemented with 0.1 mM IPTG) in addition to the WT *ftsA* in the chromosome (B and E).

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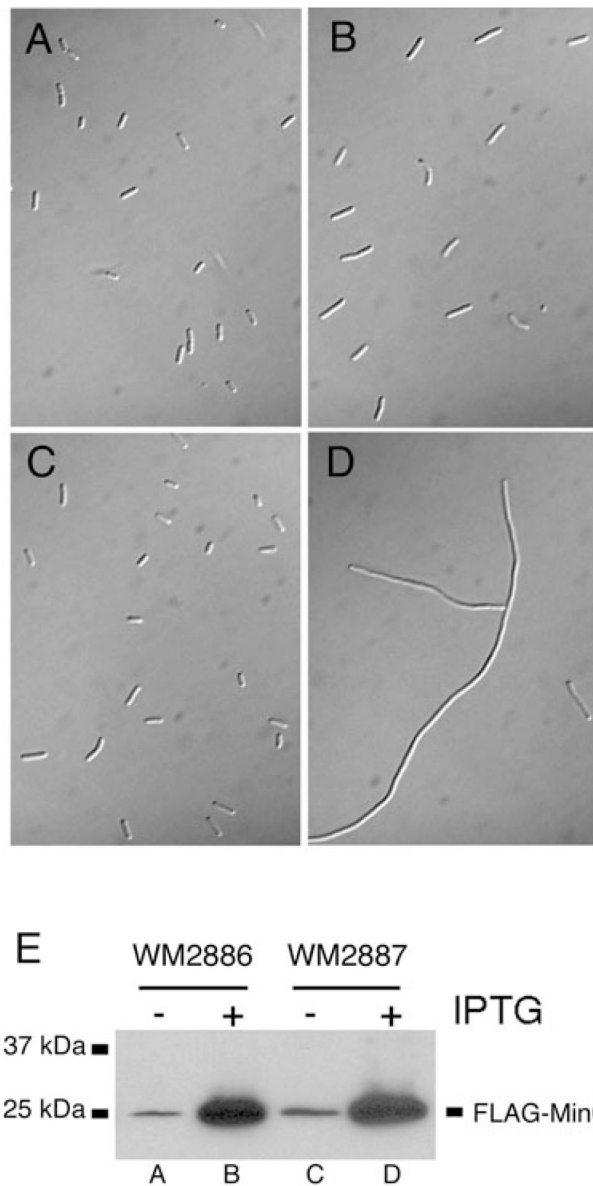

Fig. 6. The E124A allele of FtsA is resistant to inhibition caused by overproduction of MinC. WM2886 (*ftsA E124A*) or WM2887 (WT *ftsA*) containing plasmid pWM2801, which expresses *flag–minC* under control of IPTG, were grown at 37°C with either 0 mM or 1 mM IPTG for 5 h. Cells were then harvested for microscopy and for immunoblotting

A–D. WM2886/*flag–minC* without (A) or with IPTG (B); WM2887/*flag–minC* without (C) or with IPTG (D).

E. Immunoblot of cell extracts from (A)–(D), probed with anti-FLAG antibody, showing the overproduction of FLAG–MinC after IPTG addition. Equal cell density equivalents were loaded in each lane.

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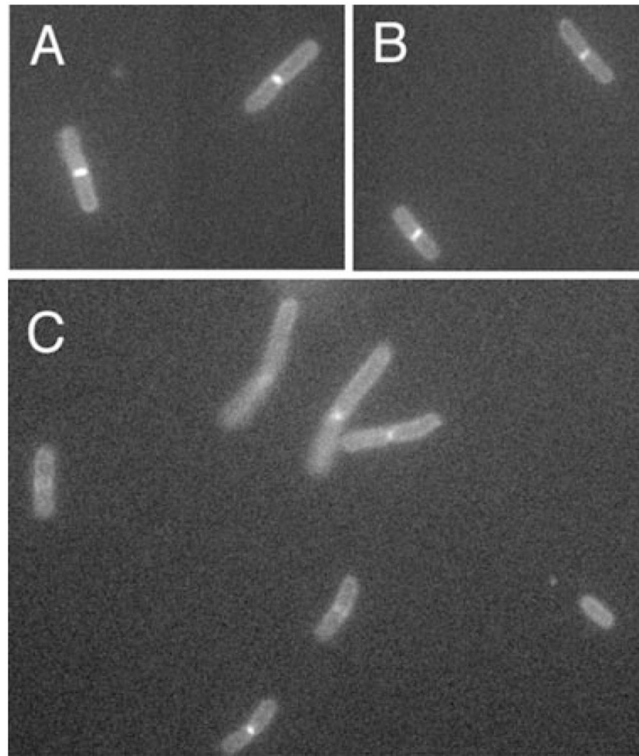

Fig. 7.
GFP-FtsN localizes normally in cells with the E124A suppressor. Shown are representative fluorescence micrographs of (A) WM2886 (*ftsA E124A*), (B) WM2887 (WT *ftsA*) or (C) WM2417 (*ftsA E124A*, *ftsN::cat*) producing GFP-FtsN from pWM1152. Cells were grown in LB without IPTG for 4 h at 37°C to mid-exponential phase.

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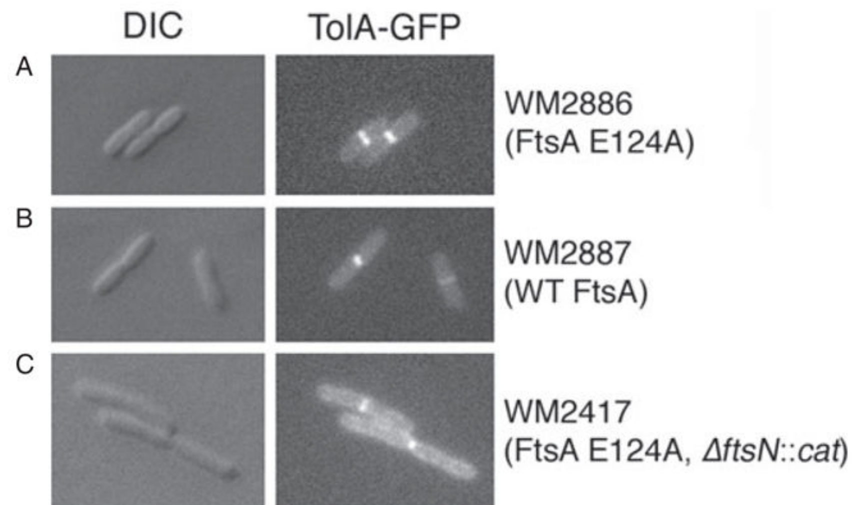

Fig. 8.

GFP–TolA localizes to division septa of cells lacking FtsN when the E124A suppressor is present. Shown are representative DIC (left) and fluorescence (right) micrographs of GFP–TolA producing cells of (A) WM2886 (*ftsA E124A*), (B) WM2887 (WT *ftsA*) or (C) WM2417 (*ftsA E124A*, *ftsN::cat*). Cells were grown in LB without IPTG for 5 h at 37°C to mid-exponential phase.

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Table 1

Strains used in this study.

Strains	Description	Source
CAG12185	*argE*::Tn*10* linked to *ftsN*	*E. coli* stock centre
WM640	TOE44 (*ftsK44)*	Begg *et al*. (1995)
TX3772	MG1655 *lacU169*	Laboratory collection
W3110	Wild-type strain	Laboratory collection
DY329	W3110 *lacU169 nadA*::Tn*10 gal490* λ*cI857* *(cro-bioA)*	Yu *et al*. (2000)
EC448	MC4100 (λ *attL-lom*)::*bla lacI* ^{q} P_{trc}-*ftsZ-gfp*	Weiss *et al*. (1999)
WM1115	TX3772 *leu*::Tn*10 ftsA12*(Ts)	Geissler *et al*. (2003)
WM1281	CH2 (*recA*::Tn*10*, *ftsA0*)/pDB280 *(repA* ^{ts}*ftsA+)*	Hale and de Boer (1999)
WM1657	TX3772 *ftsA* (R286W)* *zipA::kan*	Geissler *et al*. (2003)
WM1932	*ftsN::cat* in DY329/pWM1152	This study
WM2109	DY329+pWM1747 (GFP-$FtsK_{1-859}$) *ftsK:kan*	This study
WM2177	W3110 *leu*::Tn*10 ftsA* (R286W)*	This study
WM2227	W3110 + pZAQ	This study
WM2245	W3110 + pWM2245	This study
WM2354	WM2177 *zipA::kan*	This study
WM2294	W3110 + pWM1747 (*ftsK*) *ftsK::kan*	Geissler and Margolin (2005)
WM2355	W3110 + pWM2245, *argE*::Tn*10*, *ftsN::cat*	This study
WM2391	WM2355 *leu*::Tn*5*	This study
WM2417	WM2391 *ftsA*(*E124A*)	This study
WM2600	EC448/pBAD18kan-*ftsA* ^{sup}, *ftsN::cat*	This study
WM2701	W3110/pRR48-*ftsA**	This study
WM2702	W3110/pRR48-*ftsA* (*E124A*)	This study
WM2706	W3110/pRR48-*ftsA* ^{sup}	This study
WM2886	W3110 *leu*::Tn*5 ftsA*(*E124A*)	This study
WM2887	W3110 *leu*::Tn*5*	This study
WM2888	WM2701 *ftsK::kan* (P1 from WM2294)	This study
WM2889	WM2706 *ftsK::kan* (P1 from WM2294)	This study
WM2890	WM2702 *ftsK::kan* (P1 from WM2294)	This study
WM2893	WM2701 *zipA::kan* (P1 from WM1657)	This study
WM2891	P_{trc}-*ftsZ-gfp* in WM2886	This study
WM2892	P_{trc}-*ftsZ-gfp* in WM2887	This study
WM2894	WM2706 *zipA::kan* (P1 from WM1657)	This study
WM2895	WM2702 *zipA::kan* (P1 from WM1657)	This study
WM2921	pWM2537 in WM2355	This study
WM2922	pWM2538 in WM2355	This study
WM2923	pWM2539 in WM2355	This study
WM2924	pWM2540 in WM2355	This study
WM2925	pWM2541 in WM2355	This study
WM2926	pWM2542 in WM2355	This study

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Strains	Description	Source
WM2927	pWM2543 in WM2355	This study
WM2928	pWM2544 in WM2355	This study
WM2929	pWM2545 in WM2355	This study
WM2930	pWM2931 in WM2355	This study
WM2934	W3110 *leu*::Tn*10 ftsA* (R286W)*	This study
WM2935	W3110 *leu*::Tn*10 ftsA (E124A)*	This study
WM2936	WM2934 *zipA::kan* (P1 from WM1657)	This study
WM2937	WM2935 *zipA::kan* (P1 from WM1657)	This study

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Table 2

Plasmids used in this study.

Plasmids	Description	Source
pZAQ	*ftsQAZ* in pBR322, Tc^R	Bi and Lutkenhaus (1990)
pDSW207	P_{trc}-*gfp* pBR322 derivative with strong promoter, Ap^R	Weiss *et al*. (1999)
pDSW209	P_{trc}-*gfp* pBR322 derivative with weak promoter, Ap^R	Weiss *et al*. (1999)
pDSW210	P_{trc}-*gfp* pBR322 derivative with weak promoter, Ap^R	Weiss *et al*. (1999)
pRR48	Derivative of pJC30, P_{tac} expression, Ap^R	Ames *et al*. (2002)
pBAD18kan	Expression vector with *araBAD* promoter, Kan^R	Guzman *et al*. (1995)
pTSA29	Thermosensitive replication plasmid, Ap^R	Phillips (1999)
pWM2060	pSDW210 deleted for *gfp*, Ap^R	Geissler and Margolin (2005)
pCSB1	pWM2060 with *aph* (Kan^R) replacing *bla*	This study
pET28a	Expression vector, Kan^R	Novagen
pWM1152	pDSW207 expressing *gfp-ftsN*	Corbin *et al*. (2004)
pWM2022	pBAD30-*ftsN*	Geissler and Margolin (2005)
pWM2245	pTSA29-*ftsN*	This study
pWM2425	pET28-*ftsA* ^{sup}*(K48R K117R E124G)*	This study
pWM2488	pET28-*ftsA (K117R)*	This study
pWM2489	pET28-*ftsA (E124G)*	This study
pWM2490	pET28-*ftsA (K117R E124G)*	This study
pWM2491	pET28-*ftsA* ^{sup}*(K48R K117R E124G)* re-cloned	This study
pWM2504	pET28-*ftsA (K48R)*	This study
pWM2506	pET28-*ftsA (K48R K117R)*	This study
pWM2507	pET28-*ftsA (K48R E124G)*	This study
pWM2537	pCSB1-*ftsA from pET28-ftsA*	This study
pWM2538	pCSB1-*ftsA* (R286W)*	This study
pWM2539	pCSB1-*ftsA (K48R)*	This study
pWM2540	pCSB1-ftsA (K117R)	This study
pWM2541	pCSB1-*ftsA (E124G)*	This study
pWM2542	pCSB1-*ftsA (K48R K117R)*	This study
pWM2543	pCSB1-*ftsA (K48R E124G)*	This study
pWM2544	pCSB1-*ftsA (K117R E124G)*	This study
pWM2545	pCSB1-*ftsA* ^{sup}*(K48R K117R E124G)*	This study
pWM2546	pBAD18kan-*ftsA*	This study
pWM2547	pBAD18kan-*ftsA* (R286W)*	This study
pWM2548	pBAD18kan-*ftsA (K48R)*	This study
pWM2549	pBAD18kan-*ftsA (K48R K117R)*	This study
pWM2550	pBAD18kan-*ftsA (K48R E124G)*	This study
pWM2551	pBAD18kan-*ftsA (K117R E124G)*	This study
pWM2552	pBAD18kan-*ftsA* ^{sup}*(K48R K117R E124G)*	This study
pWM2658	pCSB1-*ftsA (E124A)*	This study

Plasmids	Description	Source
pWM2701	pRR48-*ftsA* (R286W)*	This study
pWM2702	pRR48-*ftsA (E124A)*	This study
pWM2706	pRR48-*ftsA* ^{sup}*(K48R K117R E124G)*	This study
pWM2931	pBAD18kan-*ftsA (E124A)*	This study
pWM2801	pDSW210 expressing *flag–minC*	Shiomi and Margolin (2007)
pWM2984	pDSW209 expressing *gfp–tolA*	This study

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Table 3

Oligonucleotides used in this study.

Oligonucleotide No.	Sequence
727	TAAGATCTCGATCCCGCGAAATT
728	TAGCTTTGTTAGCAGCCGGATCT
826	TATATAGAGCTCATGATCAAGGCGACGGACAGA
836	GGGGAAGTTCTGCCCNNNGGTATGGTCAATATC
837	GATATTGACCATACCNNNGGGCAGAACTTCCCC
860	GCGTGTGCGCGATGGCCATCGTGTGCTGCA
861	TGCAGCACACGATGGCCATCGCGCACACGC
862	CGTCCATACCGCGCGATCGGTGCGTGTGCGC
863	GCGCACACGCACCGATCGCGCGGTATGGACG
902	TATATAGAGCTCAATAATTTTGTTTAACTTTAAGAA
903	ATATATCTAGATTAAAACTCTTTTCGCAGCCA
528	TTTCTCGAGTTAAAACTCTTTTCGC
106	AACATATGATCAAGGCGACGGAC
726-1	TATATAGGATCCTTAAAACTCTTTTCGCAG