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Identification of a region of FtsA required for interaction with FtsZ

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Summary

The assembly of the Z ring is the earliest step in bacterial cell division. In *Escherichia coli* this assembly requires either FtsA or ZipA which bind to a conserved, C-terminal 17 amino acid motif in FtsZ and to the membrane. The FtsZ–ZipA interaction is well characterized; however, nothing is known about the region of FtsA involved in the interaction with FtsZ even though the FtsA–FtsZ interaction is nearly ubiquitous in Eubacteria. FtsA is proposed to bind to the membrane through its conserved C-terminal amphiphatic helix before efficiently interacting with FtsZ. Based upon this model we designed a genetic screen to identify mutants specifically impaired for the FtsA–FtsZ interaction. The mutants obtained retain the ability to be targeted to the membrane but fail to be recruited to the Z ring or interact with FtsZ in the yeast two-hybrid system. These mutants do not complement an *ftsA*-depletion strain. Through this approach we have identified a region of FtsA containing some invariant residues which is required for binding to FtsZ. The results support our model that FtsA is targeted to the membrane before it interacts with FtsZ and demonstrates that this interaction plays an essential role in *E. coli* cell division.

Introduction

Division of *E. coli* is carried out by a cytokinetic septal ring that consists of at least 10 essential proteins (Weiss, 2004). Together these proteins must invaginate the cytoplasmic membrane and bring about the synthesis of septal peptidoglycan. Murein hydrolytic enzymes are also required to split the newly formed septal cell wall but no single one is essential as this activity appears redundant (Heidrich *et al*., 2001).

The structural basis of the cytokinetic septal ring is the cytoskeletal Z ring, consisting of protofilaments of the bacterial tubulin FtsZ. These protofilaments are anchored to the membrane through ZipA and FtsA. Either of these proteins is sufficient to permit a Z ring to form; however, this ring is non-functional unless both are present as additional downstream division proteins are not recruited (Pichoff and Lutkenhaus, 2002). Although both of these proteins can tether FtsZ to the membrane, FtsA is thought to be the more important because mutations in *ftsA* can completely bypass the requirement for ZipA (Geissler *et al*., 2003). Also, several lines of evidence suggest that FtsA interacts directly with several downstream division proteins including FtsN and FtsI (Di Lallo *et al*., 2003; Corbin *et al*., 2004; Karimova *et al*., 2005).

The cytokinetic ring is assembled in two distinct temporal stages (Aarsman *et al*., 2005). In the first stage FtsZ localizes to the division site along with FtsA and ZipA to form the Z ring (Pichoff and Lutkenhaus, 2002). Recent studies indicate that FtsZ forms dynamic helices that move along the membrane outside of the Z ring (Thanedar and Margolin, 2004). It is likely that these helices are dynamic polymers of FtsZ that are attached to the membrane through FtsA and ZipA. Consistent with this interpretation, FtsZ and FtsA can be cross-linked in cells of *Bacillus subtilis* that lack the Z ring (Jensen *et al*., 2005). The Z ring ultimately forms, possibly by coalescence of these helices, at midcell where the activity of negative regulators of division, Min and Noc, is the lowest (Pichoff and Lutkenhaus, 2002). Once the Z ring is formed there is a considerable lag before the remaining division proteins are recruited. This lag is equivalent to about half of the cell cycle in rapidly growing cells (Aarsman *et al*., 2005). Once these remaining proteins are localized septation commences.

ZipA is anchored to the membrane through an N-terminal transmembrane domain that is linked to a C-terminal FtsZ binding domain by a long linker that could extend up to 60 nm (Hale and de Boer, 1997). FtsA is related to actin; however, it is unusual in that one of the four subdomains found in the actin family is uniquely located in the FtsA structure (van den Ent and Lowe, 2000). This subdomain (designated IC) is implicated in the interaction of FtsA with FtsI and FtsN (Corbin *et al*., 2004). FtsA is a peripheral membrane protein that binds to the membrane through a C-terminal amphipathic helix, designated the membrane targeting sequence (MTS) (Pichoff and Lutkenhaus, 2005). Removal of this MTS reduces the

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ability of FtsA to localize to the membrane and to the Z ring. Instead, FtsA deleted of its MTS assembles into rod-like structures in the cytoplasm. These rod-like structures indicate that FtsA is capable of polymerization but that it is regulated by the MTS. More recently FtsA from *Streptococcus pneumoniae* has been shown to assemble into highly stable polymers *in vitro* (Lara *et al*., 2005).

Both FtsA and ZipA interact with FtsZ through a short conserved sequence of ~17 residues present at the C-terminus of FtsZ (Liu *et al*., 1999; Ma and Margolin, 1999; Haney *et al*., 2001). This sequence motif is not involved in FtsZ polymerization but is essential for FtsZ function (Wang *et al*., 1997). Mutations that alter conserved residues within this peptide motif prevent interaction with FtsA and ZipA in the yeast two-hybrid system. Such mutations also prevent the mutant FtsZ from assembling into a ring in the absence of the wild-type FtsZ (Pichoff and Lutkenhaus, 2002). The domain of ZipA involved in binding to FtsZ has been crystallized in a complex with the C-terminal peptide (Mosyak *et al*., 2000). The peptide sits in a crevice in ZipA and makes mostly hydrophobic contacts. A comparable region is not apparent in the FtsA structure. In this article we set out to identify the region of FtsA responsible for interaction with FtsZ in order to further explore the function of FtsA in Z ring formation.

Results

Strategy for screening for ftsA *mutations deficient in interaction with FtsZ*

Although a GFP–FtsA fusion is not fully functional, it localizes to the Z ring. Increased expression of the fusion is toxic as it leads to the disintegration of the Z ring which appears to spiral away from the division site resulting in a failure in division (Ma *et al*., 1996; Pichoff and Lutkenhaus, 2005). This toxicity is also observed with FtsA itself as the ratio of FtsA to FtsZ has to be within a narrow window for a Z ring to form (Dai and Lutkenhaus, 1992). In contrast, expression of a GFP–FtsA fusion lacking the MTS is considerably less toxic as it targets very poorly to the Z ring and assembles into relatively non-toxic, rod-like structures in the cytoplasm (Pichoff and Lutkenhaus, 2005). These structures do not interfere with division as they do not recruit other division proteins to the rods, including intact FtsA. At quite elevated levels GFP–FtsAΔMTS (like FtsAΔMTS) causes the cells to become curved (Gayda *et al*., 1992; Yim *et al*., 2000; Pichoff and Lutkenhaus, 2005). This behaviour of the GFP fusions has led to a model in which FtsA has to bind to the membrane to efficiently interact with FtsZ and then itself (Pichoff and Lutkenhaus, 2005). Removal of the MTS is thought to bypass the requirement for FtsA to bind to the membrane and FtsZ before interacting with itself. Thus, screening for mutants that still localize to the membrane, but not the Z ring, should yield FtsA mutants specifically deficient in interaction with FtsZ. Furthermore, such mutants should not interfere with division as they would not be expected to disrupt the Z ring.

One possible problem with such a screen is that inactive or folding mutants could be targeted to the membrane by the MTS and therefore be the predominant isolates. As a preliminary experiment to better understand the requirements for membrane binding by FtsA we introduced mutations that should affect the interaction with ATP. If these mutants localize to the membrane it would argue that the mere presence of the MTS is sufficient for membrane binding. On the other hand, a failure of such mutants to localize to the membrane would suggest that FtsA has to be somewhat functional to bind to the membrane. Two residues were targeted. Glycine 336 of *E. coli* FtsA is totally conserved in all the members of the actin family and is thought to play an essential role in forming a loop structure for binding adenosine. Furthermore, FtsA102 (G336D) is non-functional and is unable to bind ATP *in vitro* (Sanchez *et al*., 1994). The second residue is aspartic acid at position 210 which is equivalent to the aspartic acid at position 170 in ParM of plasmid R1 and 165 in MreB from *E. coli* (both bacterial actin homologues). This residue is involved in binding of Mg^{2+} and mutants in which it was changed to alanine or glutamic acid have been described for both ParM and MreB. Such changes result in a loss of function and abolish the ATPase activity of these proteins (Moller-Jensen *et al*., 2002; Kruse *et al*., 2003).

GFP–FtsA fusions that carried mutations D210A or G336D were non-toxic. They did not localize to the membrane, but instead were present in the cytoplasm and formed amorphous aggregates distinctly different from the rods formed by GFP–FtsAΔMTS (Fig. 1n). These results indicate that a completely inactive FtsA is unlikely to bind to the membrane and therefore plague our screen.

A plasmid library containing a *gfp–ftsA* fusion in which *ftsA* had been subjected to polymerase chain reaction (PCR) mutagenesis as outlined in the *Experimental procedures* was introduced into XL1-Blue in the presence of glucose. 500 colonies were streaked on plates containing 0.001% arabinose to induce the fusion and were subsequently examined by fluorescence microscopy after 6 h of growth. At this time point cells carrying the wild-type fusion were very filamentous with the fluorescence distributed on the membrane and on the few Z rings still present whereas cells containing the fusion lacking the MTS were non-filamentous and the fluorescence was cytoplasmic, either diffuse or in bright rods. One consequence of using the arabinose system to regulate expression is that there is some variation in expression between cells in the popu-

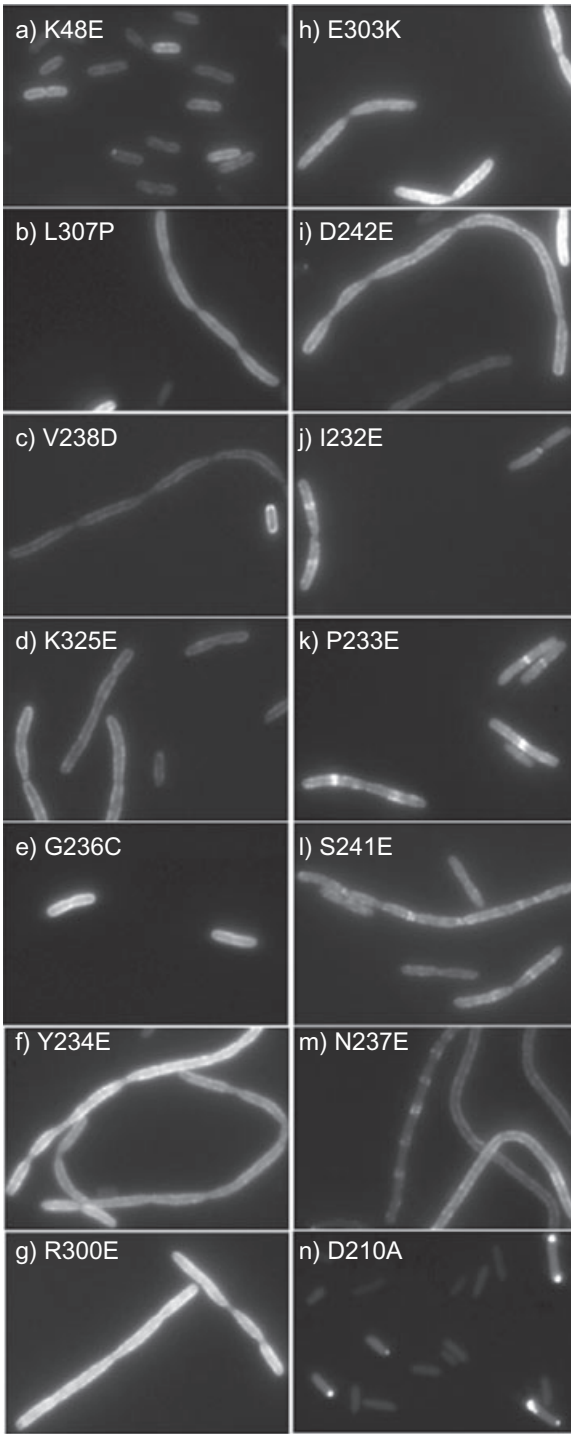

Fig. 1. Cellular localization of the GFP–FtsA mutants. The *ftsA* gene was mutagenized by random (panels A–E) or site-directed mutagenesis (panels F-N), cloned downstream of GFP under the control of the P_{BAD} promoter (pSEB293 series) and introduced into XL1-Blue. Colonies were streaked on 0.0001% arabinose plates and examined by fluorescence microscopy after 6 h at 37°C. The number in parenthesis after each mutation obtained by random mutagenesis indicates the number of independent clones obtained for each mutant: (a) K48E (4), (b) L307P (3), (c) V238D (1), (d) K325E (2), (e) G236C (1), (f) Y234E, (g) R300E, (h) E303K, (i) D242E, (j) I232E, (k) P233E, (l) S241E, (m) N237E, (n) D210A.

lation (Siegele and Hu, 1997).This proved useful in our screen because it allowed us to observe cells in the same microscopic field with a range of expression of the GFP fusion and to better compare their phenotypes with the one caused by GFP–FtsA. Of the 500 colonies screened, most displayed the phenotype observed with the wild-type GFP–FtsA fusion; however, 15 displayed cytoplasmic fluorescence with rod formation. Sequence analysis of these 15 mutants revealed mutations affecting the MTS, further confirming that loss of a functional MTS leads to rod formation. Another 32 mutants displayed cytoplasmic fluorescence without rod formation and were assumed to contain stop or frameshift mutations in *ftsA*. Interestingly, 11 mutants displayed fluorescence at the membrane but not at Z rings. They also appeared less toxic although several caused a mild filamentation with a 'pinched' septum. Sequencing these 11 revealed five distinct mutations and representative images are shown in Fig. 1a–e.

Mapping these mutations on a model of the *Thermotoga maritima* FtsA structure (van den Ent and Lowe, 2000) revealed that they mostly altered surface residues that are clustered on subdomain 2B (Fig. 2). An exception is G236 which is just beneath the surface. One possibility is that this region is responsible for binding FtsZ. Interestingly, this region contains a number of invariant charged amino acids (D242, R300 and E303; Fig. 2) that form a hydrogen bond network in the *T. maritima* FtsA structure. As a further examination of the function of this region, these conserved charged residues, as well as several surrounding residues were altered by mutation and the effect on the localization of GFP–FtsA examined (Fig. 1f–m). Many of these mutations yielded a phenotype similar to the original mutations; slightly elongated cells with extended septa and the fluorescence present on the membrane but not localized to Z rings. Those mutations yielding this phenotype included D242E, R300E, E303K and Y234E (Fig. 1f–i). In contrast, N237E (Fig. 1m) behaved like the wild type (extreme filamentation with fluorescence at the membrane and residual Z rings) whereas several displayed an intermediate phenotype (I232E, P233E and S241, Fig. 1j–l). These latter mutants localized to Z rings to varying degrees but caused much less filamentation than N237E or the wild type. Thus, mutations in this region, including those affecting the highly conserved residues, do not affect the ability of GFP–FtsA to bind to the membrane but do impair its ability to localize to the Z ring.

Effect of ftsA *mutations on self-interaction and interaction with FtsZ*

The above mutational analysis identified FtsA mutants that localized to the membrane but were not recruited to the Z ring. As shown previously (Pichoff and Lutkenhaus,

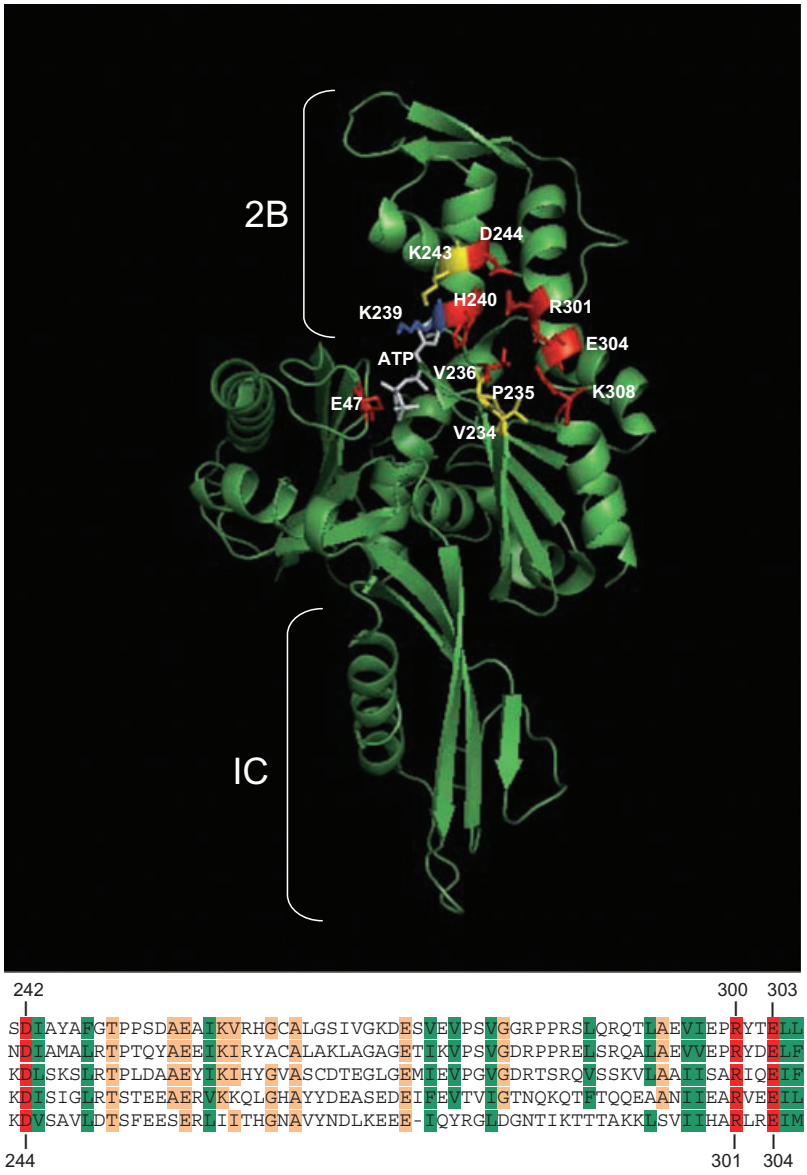

Fig. 2. Location of residues in FtsA required for interaction with FtsZ. The residues altered in the *E. coli ftsA* mutants are represented on the *Thermotoga maritima* FtsA (PDB-1E4G) structure. Mutation of residues that lead to loss of localization to the Z ring are coloured red [(*E. coli* number followed by *T. maritima* number) K48 – E47, Y234 – V236, V238 – H240, D242 – D244, R300 – R301, E303 – E304, L307 – K308]; those that reduce localization are coloured yellow (I232 – V234, P233 – P235, S241 – K243) and one that had no effect on localization is coloured blue (N237 – K239). Not indicated are K325 and G236 because the corresponding residues are not present in the structure. The alignment below the figure is from the region of FtsA containing the invariant charged residues. The residue numbers are for the *T. maritima* FtsA below the alignment and for *E. coli* FtsA above the alignment. The FtsA sequences are from the following bacteria: Ec, *E. coli*; Pa, *Pseudomonas aeruginosa*; Ng, *Neisseria gonorrhoeae*; Bs, *Bacillus subtilis*; Tm, *Thermotoga maritima*.

2005) and above, removing the MTS leads to FtsAΔMTS that assembles into rods in the cytoplasm. We have interpreted this rod-forming phenotype as indicative of self-interaction resulting in polymerization of FtsAΔMTS (Pichoff and Lutkenhaus, 2005). To determine if the mutations that prevent FtsA from localizing to the Z ring affect self-interaction we deleted the MTS from each of the alleles and examined rod formation. We observed that most mutations did not affect rod formation. Figure 3 shows some examples. Depicted are R300E, K325E, E303K and L307P. D242E, K48E and Y234E behaved similarly (data not shown). Thus, these mutations do not affect the ability of FtsA to self-interact under these conditions indicating that they do not disrupt the structure of FtsA. Exceptions are V238D which formed spots but not rods (Fig. 3B) and G236C which was diffuse in the cytoplasm (not shown).

FtsA self-interaction has also been demonstrated in the yeast two-hybrid system (Yim *et al.*, 2000). We observed that full length FtsA interacted extremely weakly in this system. In contrast, deleting the MTS from FtsA markedly enhanced the interaction. Its possible that removing the MTS reduces retention of the protein at the plasma membrane and increases the amount going to the nucleus as observed for another peripheral membrane-binding protein MinD (Taghbalout *et al.*, 2006). Also, FtsAΔMTS did not interact with the full length FtsA (data not shown). These results are similar to Yim *et al.* although we observed a much weaker interaction between the full length FtsAs. These results are

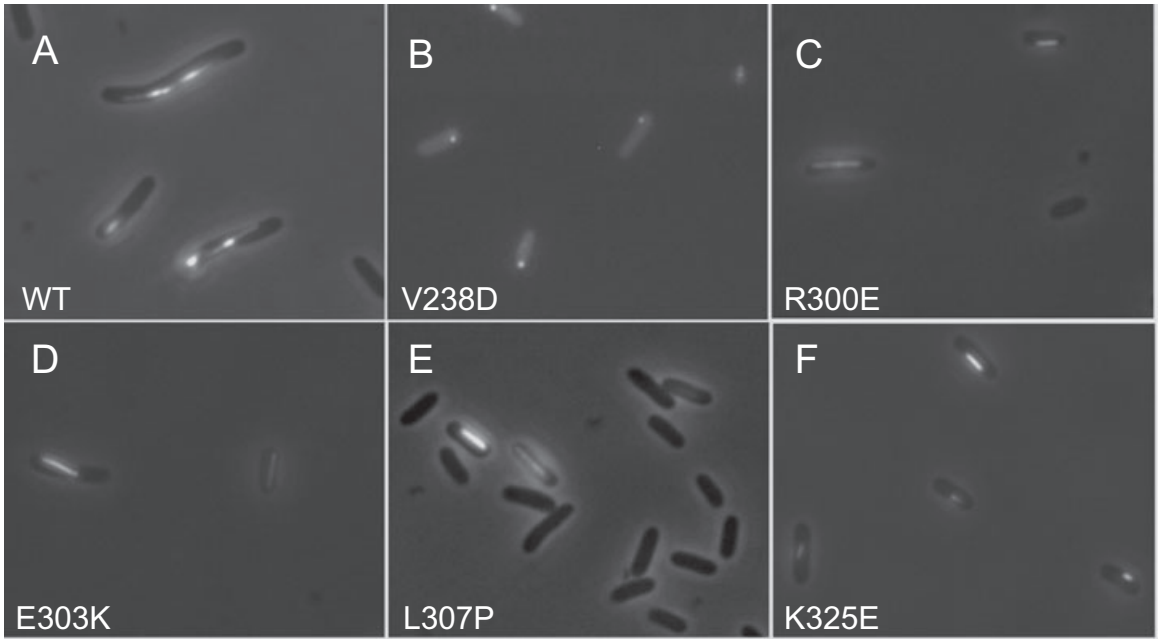

Fig. 3. Localization of GFP–FtsA mutants truncated for their MTS. *gfp–ftsA* fusions with their MTS removed (pSEB294 series) were analysed as described in Fig. 1. (A) wild-type FtsA, (B) V238D, (C) R300E, (D) E303K, (E) L307P, (F) K325E.

consistent with the failure of full length FtsA to be recruited to the cytoplasmic rods and the failure of GFP–FtsAΔMTS to be recruited to the Z ring, e.g. by interaction with full length FtsA. Because of the much stronger self-interaction observed with FtsAΔMTS the mutations were tested in this version (summarized in Table 1). As a preliminary test we examined the effect of two of the mutations that affect interaction with ATP. Both of these mutations, D210A and G336D, eliminated the self-interaction. Note that these mutations also eliminated the ability to form cytoplasmic rods.

Table 1. Summary of the yeast two-hybrid results.

Mutants tested	Vs FtsA	Vs FtsZ
WT	+++	+++
K48E	+++	–
L307P	+	–
V238D	+++	–
K325E	+++	–
G236C	–	–
D242E	+++	–
R300E	+++	–
E303K	+++	–
Y234D	+++	–
I232E	+++	–
P233E	+++	+
S241E	+++	–
N237E	+++	+
D210A	–	–
G336D	–	–

FtsA mutants lacking the MTS were cloned in frame with the DNA-binding domain of GAL4 and tested against $FtsA_{\Delta MTS}$ or full length FtsZ cloned in frame with the activating domain of GAL4. For each protein interaction tested, the β-galactosidase assay was done multiple times, on colonies obtained from several independent transformations using the filter lift assay as described in Clontech manual and gave reproducible results. +++, indicates a blue colour developing in less than 1 h; +, blue colour after 4 h; –, indicates no blue colour after overnight incubation.

The effect of the mutations that prevented FtsA from localizing to the Z ring was examined next. We observed that most of the mutations had no effect on the self-interaction. This is consistent with these mutations having no effect on cytoplasmic rod formation. Only L307P reduced the self-interaction even though it still produced rods. On the other hand, G236C, failed to produce the cytoplasmic rods and failed to self-interact in the yeast two-hybrid test. The G236 residue maps in the same area on the FtsA structure as the other mutants but it is not on the surface. It is located just beneath the conserved residues R300 and E303, at the hinge between FtsA's domains 2A and 2B. This glycine residue may be important for maintaining the structure of domain 2B of FtsA. Its change to a cysteine may disrupt the structure of domain 2B explaining both the lack of interaction with FtsZ and the loss of self-interaction.

The yeast two-hybrid system can also be used to detect the FtsA–FtsZ interaction (Wang *et al*., 1997). Interestingly, we observed that the interaction is much stronger between FtsZ and FtsA deleted for its MTS. This result was somewhat surprising because they do not appear to interact well in *E. coli*: FtsZ is not recruited to the cytoplasmic rods and GFP–FtsAΔMTS is not efficiently recruited to the Z ring (Pichoff and Lutkenhaus, 2005). It is

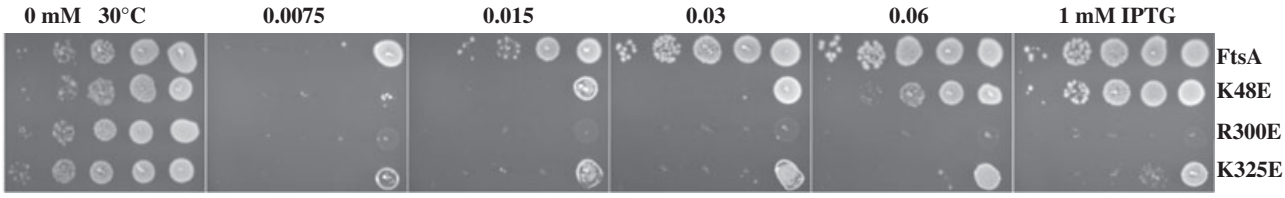

Fig. 4. Complementation of an FtsA-depletion strain. Plasmids expressing FtsA or FtsA mutants under the control of IPTG (pSEB306 series) were transformed into an *ftsA* depletion strain [CH2 *recA ftsA*^{0}/pDB280 (*rep*Ts *ftsA*)] at 30°C. Colonies of each strain were resuspended in LB, serially diluted, spotted onto plates containing increasing IPTG concentrations and incubated at 42°C.

not clear why this interaction is so strong in yeast; however, we used this test to examine the effect of the mutations on the FtsZ–FtsA interaction.

We observed that the mutations that affect interaction with ATP abrogated this interaction (Table 1). Importantly, each of the mutations that eliminated localization of GFP–FtsA to the Z ring also eliminated the interaction with FtsZ in this test (Table 1). The three mutations that reduced localization to the Z ring also reduced the interaction with FtsZ. N237E, which behaved like the wild-type protein, still interacted with FtsZ, although the interaction was weaker that wild type. These results provide further support that the mutations isolated here are deficient in the FtsZ–FtsA interaction.

Complementation

The mutations identified above that prevent FtsA from localizing to the Z ring would be expected to no longer support cell division. To test this, each of the alleles was examined for its ability to complement an *ftsA* depletion strain in which the chromosomal *ftsA* gene was inactivated and *ftsA* provided on a replicon that is temperature sensitive for replication. The various alleles were cloned in an expression vector that contained a weakened *lac* promoter. With this vector the wild-type *ftsA* complements the depletion strain well on plates containing 30 μM IPTG. R300E, K325E and most of the other mutations that prevented FtsA from localizing to the Z ring were unable to complement the temperature sensitive depletion even at high (1 mM) IPTG concentrations (Fig. 4 and data not shown). The only exception was K48E. This mutant complemented but only at higher IPTG concentrations than required for the wild type. It complemented well at 1 mM IPTG indicating that it was relatively inefficient and a higher level of the protein was required. Immunoblot analysis confirmed that K48E was stable (data not shown). Similar results were observed when our mutants were tested for the complementation of an FtsA depletion strain in which *ftsA* expression is repressed at 30°C and expressed at 37°C by using a thermosensitive repressor λC_I. This latter result indicates that the lack of complementation observed with the mutants in the earlier test was not due to a potential thermosensitivity of these mutant proteins (data not shown).

The complementation by K48E was unexpected but can perhaps be explained if it has a weak affinity for FtsZ and the overexpression compensates for this deficiency.

To further explore the basis of the complementation we determined if K48E was localized to the division site. The depletion strain carrying wild-type *ftsA* or K48E was cured of the temperature sensitive plasmid carrying wild-type ftsA by growth on media containing IPTG (0.03 mM for the wild type and 1 mM for K48E). The localization of FtsA and FtsZ was determined in liquid cultures at these IPTG concentrations. Examination of the cell morphology indicated that K48E complemented about as well as the wild-type FtsA under these conditions. Immunofluorescence of the strain carrying the wild-type *ftsA* revealed that Z rings were present in > 80% of the cells (Fig. 5A). FtsA was localized to midcell in a similar fraction of the cells as expected (Fig. 5B). The strain expressing K48E revealed a similar high percentage of cells with a Z ring (Fig. 5C); however, K48E was localized to the division site in a much lower percentage of cells, mostly those with a constriction (Fig. 5D). These results suggest that Z rings are forming in these cells with the aide of ZipA and that K48E is only recruited at a later stage about the time constriction commences. This late recruitment may reflect a residual weak interaction with FtsZ.

The ftsA *mutants are still toxic when overexpressed*

The basis of our screen was the inability of the mutant GFP–FtsA fusions to localize to the Z ring even though they could localize at the membrane. To determine if the various mutants interfered with cell division the effects of their overexpression (of the unfused proteins) in wild-type cells was compared with that of wild-type *ftsA*. Overexpression of wild-type *ftsA* disrupts Z ring formation leading to smooth filamentation as shown in Fig. 6C and previously (Dai and Lutkenhaus, 1992). Most of the mutants that failed to localize to Z rings still interfered with cell division; however, they blocked at a later stage than the wild type as evidenced by the 'pinched' phenotype of the filaments obtained (Fig. 6D, R300E is depicted as an

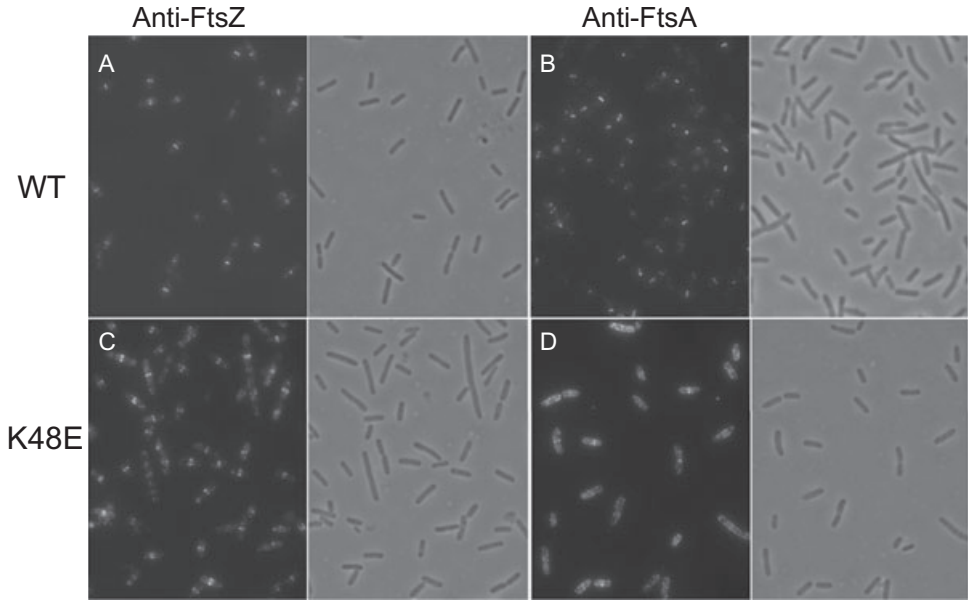

Fig. 5. Immunolocalizazion of FtsZ and FtsA under complementation conditions.CH2/pSEB306 (A and B) and CH2/pSEB306-K48E (C and D) were cured of pDB280 (*ftsA*$^+$) and are only expressing FtsA or FtsA K48E under the control of IPTG-inducible promoter. Both strains were grown at 37°C in LB and ampicillin with IPTG at 0.03 (A and B) or 1 mM (C and D) to an OD540 of 0.4, fixed and prepared for immunofluorescence using an antibody against FtsZ (A and C) or FtsA (B and D) as previously described. Note that the fluorescence from the K48E is stronger than for wild-type FtsA (compare B and D). This is due to the higher expression level of K48E and the distribution of the excess protein at the membrane.

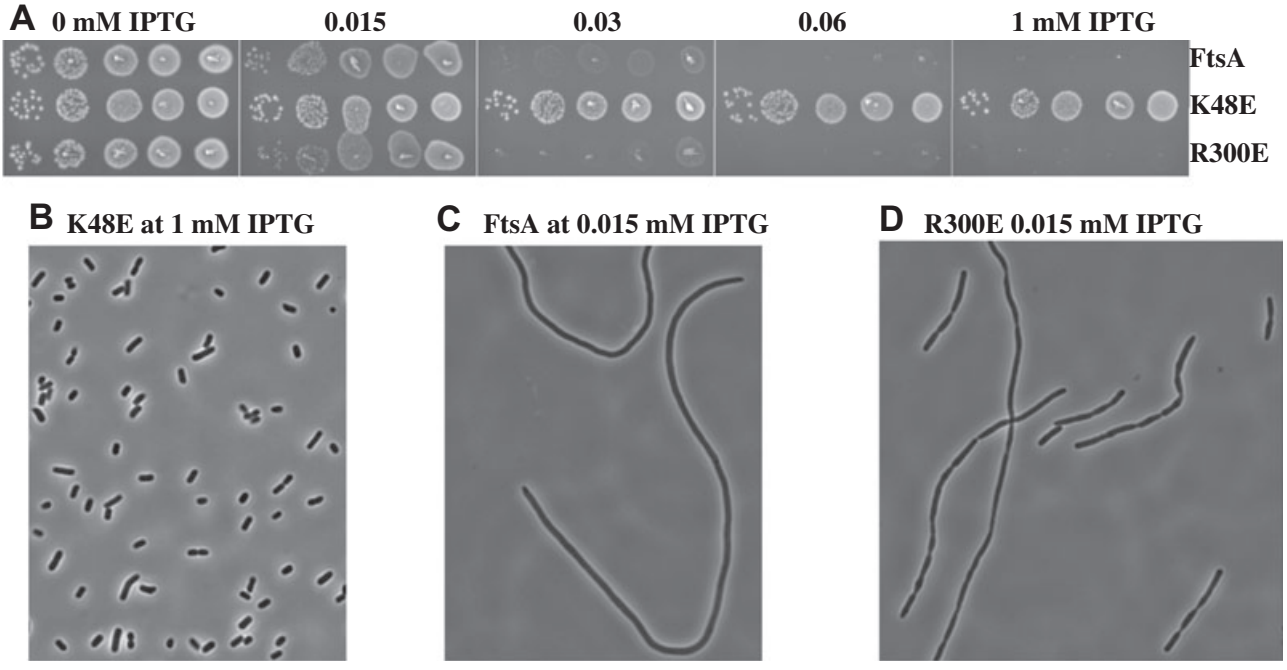

Fig. 6. Effect of overexpression of FtsA mutants.
A. Same experiment as in Fig. 4 but the plasmids (pSEB306+ series) were transformed into XL1-Blue and plates incubated at 37°C.
B–D. Phase contrast microscopy of cells taken from the spots at the indicated IPTG concentrations.

example). This phenotype indicates that Z rings are still forming but that the process of septation is slowed. Perhaps the FtsA mutants, which are distributed throughout the membrane but do not localize to the division site, are interfering with cell division by competing for later division proteins. Interestingly, K48E did not inhibit cell division (Fig. 6A–B).

Discussion

In order to isolate *ftsA* mutations deficient in interaction with FtsZ we designed a visual screen based on a model for FtsA function. In this model, which was deduced from our previous study on FtsA membrane targeting (Pichoff and Lutkenhaus, 2005), we proposed that FtsA's interaction with FtsZ, and also with itself, were dependent on it first interacting with the membrane. Based upon this model FtsA mutants that are unable to interact with FtsZ should localize to the membrane but not to the Z ring. Our results are consistent with this model.

In the initial PCR mutagenesis of *ftsA* we obtained mutants that produced rods in the cytoplasm. All of these were found to have mutations causing a truncation or a disruption of FtsA's conserved C-terminal amphipathic helix (15 out of 500 screened). This result demonstrated that our mutagenesis protocol was working and confirmed: (i) the amphipathic helix is required for FtsA to be targeted to the membrane and (ii) FtsA mutants unable to bind the membrane assemble into rods in the cytoplasm.

Our visual screen yielded mutations that resulted in a failure of GFP–FtsA to localize to the Z ring. The yeast two-hybrid system confirmed that this recruitment failure was due to a failure to interact with FtsZ. This random mutagenesis led us to residues in subdomain 2B of FtsA and subsequent analysis of neighbouring residues indicated they were also required for interaction with FtsZ leading us to propose that this region of the protein is essential for the interaction with FtsZ. Included in this region are charged residues conserved in all *ftsA*s sequenced to date (D242, R300 and E303; Fig. 2) which form a hydrogen bond network. Interestingly, the relatively subtle mutation D242E, which would disrupt this hydrogen bond network, is sufficient to disrupt the interaction with FtsZ. Our results suggest these charged residues are conserved because they are part of the FtsA–FtsZ interaction site, either binding the FtsZ tail directly or required to maintain the conformation of the binding site. The finding that conserved residues are part of the region required for binding FtsZ is not very surprising because both genes are conserved as a pair among most bacteria and we think that FtsA is the main membrane anchor for FtsZ polymers. In support of this, our complementation results also indicate that the FtsA–FtsZ interaction is essential for FtsA's role in cell division.

The MTS-deleted form of the mutant proteins self-interact strongly in the yeast two-hybrid system and form rods *in vivo*, indicating they self-interact. However, the full length fusions to GFP are not targeted to the Z ring even though cells are also expressing wild-type FtsA. These results strongly suggest that the self-interaction of FtsA at the membrane requires that FtsA first interact with FtsZ which is in accordance with our model (Pichoff and Lutkenhaus, 2005). Furthermore, it suggests that the interaction of FtsA with the membrane is modulating FtsA self-interaction. In the model, a molecule of FtsA bound to the membrane and to an FtsZ polymer becomes competent to interact with another FtsA bound to another FtsZ polymer. This interaction between occupied FtsAs could provide a mechanism for FtsA to assemble different FtsZ polymers into a ring and suggests a role for FtsA as an organizer of FtsZ polymers.

Most of the mutants lacking the ability to interact with FtsZ produce pinched filaments when they are overexpressed. This pinched phenotype indicates that Z rings are forming and initiating division. One possibility is that these mutants still interact with the late cell division proteins and interfere with the cell division process by competing with endogenous FtsA for later cell division proteins, causing the process of septation to be slowed. The pinched septa phenotype was observed with decreased activity of FtsI (Taschner *et al*., 1988), but it may occur with impaired activity of other late division proteins as well. Note, this phenotype is different than observed with complete loss of late proteins which results in a smooth phenotype. If so, this observation suggests that interaction of FtsA with late cell division proteins can occur independent of the interaction with FtsZ or itself, at least when FtsA is overexpressed. This would be consistent with previous reports showing that domain 1C of FtsA is involved in the interaction with FtsN and FtsI (Corbin *et al*., 2004; Rico *et al*., 2004).

One of the mutants (K48E) we identified as deficient in interaction with FtsZ has several differences from the other mutants. First, K48E maps at a different place on the FtsA structure. This mutation alters a residue near the γ-phosphate of the bound ATP and may cause a reduction in ATP binding or ATPase activity that could diminish K48E's ability to bind FtsZ. Second, it does not disrupt the cell division process when it is overexpressed. This suggests its interaction with the late cell division proteins may also be compromised. Third, K48E can complement the FtsA-depleted strain when it is overexpressed. These phenotypes are consistent with a diminished ability of K48E to function in cell division which can be overcome by increased expression. The impaired interaction with FtsZ could be too weak to be detected in the two-hybrid assay and could prevent GFP-K48E from localizing to the Z ring as it is unable to compete with the endogenous

FtsA. Perhaps K48E is only retained at the septum by weak cooperative interactions with FtsZ and the later cell division proteins which function to stabilize its association with the Z ring and allow it to function in septation.

Biochemical experiments using purified FtsA, FtsZ and lipid vesicles will be required to assess the dependence of the different FtsA interactions on each other and to confirm the model of FtsA role as an organizer of the Z polymers as we suggested. The FtsA mutant isolated here should be useful for such experiments.

Experimental procedures

Strains and plasmids

Plasmids pSEB293 and pSEB294 are pBAD18 derivatives that express *gfp–ftsA* or a MTS-deleted version (deletion of FtsA's last 15 amino acids) respectively. pSEB306 and pSEB306+ are derivatives of pDSW210 (lower expression) and pDSW208 (moderate expression), respectively, that express *ftsA* under an IPTG inducible promoter. These four plasmids were described previously (Pichoff and Lutkenhaus, 2005). The yeast two-hybrid plasmid pSEB135 (pGAD424-FtsZ) was described earlier (Pichoff and Lutkenhaus, 2002). The wild-type and MTS-deleted *ftsAs* (same deletion as in pSEB294) were amplified by PCR from the pSEB293 series using the oligonucleotides 3′FtsAdelPstI and EcforFtsA5′BamHI (sequences available upon request). The PCR fragments were digested with BamHI and PstI and cloned into pGT9 or into pGAD424 (both from Clonetech, Palo Alto, CA) digested with the same enzymes creating the collection of pSEB347 plasmids or pSEB304 (done only with wild-type *ftsA*) respectively. In pSEB347, the Gal4bd domain is fused to MTS-deleted *ftsA* or *ftsA* mutants. These plasmids allowed us to test interactions of these proteins with *ftsZ* (from pSEB135) or MTS-deleted *ftsA* (from pSEB304) in the yeast two-hybrid system as described previously (Pichoff and Lutkenhaus, 2002). None of the plasmids used in these Two Hybrid System assays was self-activating for the β-galactosidase assay (data not shown). Clonetech's XL1-Blue strain was used for screening of mutants, other GFP–FtsA localization studies and the overexpression assay. CH2/pDB280 [*ftsA*^{0} *recA*$^-$ strain containing a *repA*(Ts) *ftsA*$^+$ plasmid was provided by Piet de Boer (Hale and de Boer, 1999) and was used in complementation assays. All the bacterial cultures and microscopy techniques were done as described earlier (Pichoff and Lutkenhaus, 2005).

Random PCR mutagenesis

Random mutagenesis was done on pSEB293 using the Stratagene's Gene Morph II kit. The 1.3 kb PCR fragment was obtained using 350 ng of pSEB293 (6 kb) as template, 125 nM of each of the oligonucleotides 3′SphI-FtsAWT and 5′XbaI-L-FtsA (Pichoff and Lutkenhaus, 2005) and 1 μl of mutazyme II in a reaction total of 50 μl. The DNA was amplified for 25 cycles. Each cycle was 95°C/1 min, 65°C/1 min and 72°C/90 s. These conditions were designed according to Stratagene's protocol to cause 1–4 mutations per Kb of DNA amplified. The results obtained from the sequencing of the mutants we selected and studied were consistent with this rate of mutation. PCR fragments were cut with SphI and XbaI and cloned into pSEB293 cut with the same restriction enzymes. The plasmids carrying the *ftsA* mutants were named pSEB293 followed by the residue change such as pSEB293-K48E.

Site-directed mutagenesis

Site-directed mutagenesis of *ftsA* was done using Stratagene's QuickChange kit according to manufacturer recommendations. The sequences of the oligonucleotides used to introduce the mutations are available upon request. After mutagenesis, the presence of the mutation was confirmed by sequencing. The plasmids carrying the different mutated *ftsAs* were called pSEB306 followed by the residue change such as pSEB306-R300E for example.

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