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Role of two essential domains of *Escherichia coli* FtsA in localization and progression of the division ring

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Summary

The FtsA protein is a member of the actin superfamily that localizes to the bacterial septal ring during cell division. Deletions of domain 1C or the S12 and S13 β-strands in domain 2B of the *Escherichia coli* FtsA, previously postulated to be involved in dimerization, result in partially active proteins that do not allow the normal progression of septation. The truncated FtsA protein lacking domain 1C (FtsAΔ1C) localizes in correctly placed division rings, together with FtsZ and ZipA, but does not interact with other FtsA molecules in the yeast two-hybrid assay, and fails to recruit FtsQ and FtsN into the division ring. The rings containing FtsAΔ1C are therefore incomplete and do not support division. The production of high levels of FtsAΔ1C causes filamentation, an effect that has been reported to result as well from the imbalance between FtsA$^+$ and FtsZ$^+$ molecules. These data indicate that the domain 1C of FtsA participates in the interaction of the protein with other FtsA molecules and with the other proteins that are incorporated at later stages of ring assembly, and is not involved in the interaction with FtsZ and the localization of FtsA to the septal ring. The deletion of the S12–S13 strands of domain 2B generates a protein (FtsAΔS12–13) that retains the ability to interact with FtsA$^+$. When the mutated protein is expressed at wild-type levels, it localizes into division rings and recruits FtsQ and FtsN, but it fails to sustain septation at normal levels resulting in filamentation. A fivefold overexpression of FtsAΔS12–13 produces short cells that have normal division rings, but also cells with polar localization of the mutated protein, and cells with rings at abnormal positions that result in the production of a fraction (15%) of small nucleoid-free cells. The S12–S13 strands of domain 2B are not essential for septation, but affect the localization of the division ring.

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Introduction

We have investigated the effects of deletions of domain 1C and the β-strands S12 and S13 in domain 2B of the *Escherichia coli* FtsA protein (Fig. 1) on its activity in cell division and its ability to interact with FtsA and FtsZ. FtsA is required for cell division in many eubacteria. In *E. coli*, it assembles simultaneously with ZipA into a central ring shortly after the formation of the initial FtsZ ring (Pichoff and Lutkenhaus, 2002; Rueda *et al.*, 2003). The assembly of these three proteins in the middle of the cell is required for the subsequent incorporation of the remainder of the protein components into the final division ring (Addinall and Lutkenhaus, 1996a; Ma *et al.*, 1996; Hale and de Boer, 1999; Liu *et al.*, 1999; Pichoff and Lutkenhaus, 2002).

Structurally FtsA belongs to the superfamily of ATP-binding proteins represented by actin, Hsp70 and hexokinase (Bork *et al.*, 1992). The structure of FtsA from *Thermotoga maritima* shows that it strikingly differs from actin and other members of the family (including the bacterial actin homologue MreB) in the orientation of the whole 1C domain (van den Ent and Löwe, 2000). The *E. coli* FtsA protein is able to bind ATP (Sánchez *et al.*, 1994) and to interact with other division proteins (FtsN, FtsQ and FtsZ) and with itself (Hale and de Boer, 1997; Wang *et al.*, 1997; Yim *et al.*, 2000). FtsA obtained from *Bacillus subtilis* membranes has been reported to have a strong ATPase activity (Feucht *et al.*, 2001), although this has not been found in FtsA from *E. coli* or *T. maritima* (J. Mingorance and M. Vicente, unpubl. results).

Phe420, the very last carboxy-terminal residue, is dispensable both for the role of FtsA in septation and for the correct interaction of the protein with itself (Yim *et al.*, 2000). However, deletions that eliminate the last five carboxy-terminal residues of FtsA cause the loss of both the septation activity and the interaction, but they have no effect on the correct localization of the truncated protein in the septal ring. Extending the deletion into W415, the sixth residue from the carboxy end, results in a protein that is unable to localize into the septal ring. Therefore, the carboxy-terminal end is involved in the interaction between the *E. coli* FtsA molecules, and moreover, the

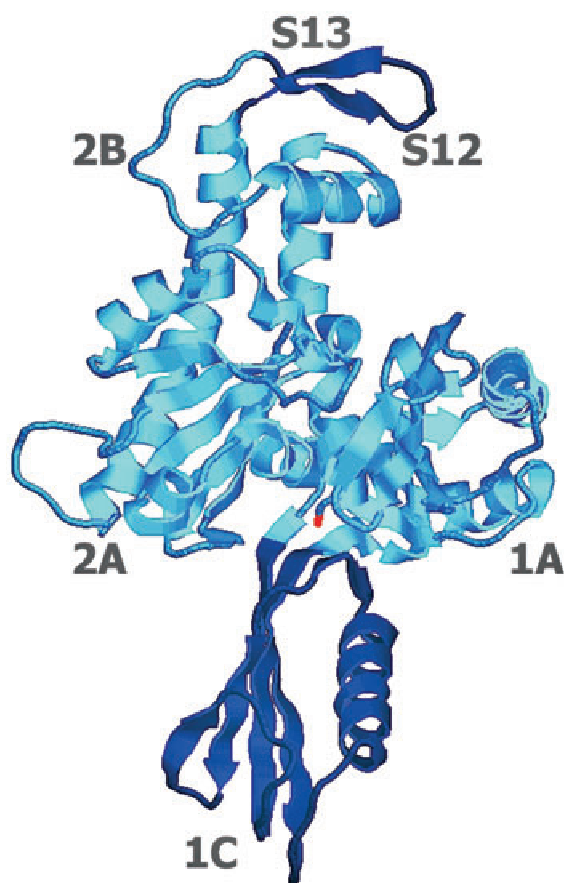

Fig. 1. Three-dimensional model of the *E. coli* FtsA (residues 7–385) obtained by homology modelling from the resolved crystal of *Thermotoga maritima* FtsA (Carettoni *et al*., 2003). Positions of the deleted fragments, domain 1C and the β-strands S12–S13 in domain 2B, are indicated in deeper blue and the carboxy-terminal residue of the modelled structure is coloured in red.

ability to establish these interactions correctly is required for septation.

A predictive model to describe the dimerization of *E. coli* FtsA (Carettoni *et al*., 2003) suggests that domains 1C and 2B include some regions that are crucial to establish the interaction between the *E. coli* FtsA molecules. A non-lethal gain-of-function point mutation, R286W, in the FtsA S13 β-strand (domain 2B) has already been isolated and found to bypass the requirement for a functional ZipA protein to complete septation (Geissler *et al*., 2003).

We have constructed variants of the *E. coli* FtsA protein in which either the 1C domain or the S12 and S13 β-strands in domain 2B have been deleted. Although none of the resultant proteins is fully functional in septation, both retain some partial activity. The absence of domain 1C abolishes the ability of the truncated protein to interact with other FtsA molecules, but does not prevent the localization of the otherwise inert truncated protein at unproductive potential septation sites correctly distributed along the length of the cell. On the contrary, the deletion of the S12–S13 β-strands generates a protein that still retains some ability to interact with $FtsA^+$ and provokes the misplacement of active septation sites in the cell, yielding DNA-less and other aberrant cells.

Results

FtsA lacking domain 1C fails to sustain division in E. coli

Comparison of the structure of ATP-binding proteins of the actin family reveals that in the structure of *T. maritima* FtsA domain 1C occupies a remarkably different position relative to that occupied by the equivalent domain 1B in other members of the family (van den Ent and Löwe, 2000). A predictive model for the structure of the *E. coli* FtsA protein, based on the crystal structure of FtsA from *T. maritima*, has been published (Carettoni *et al*., 2003). The model suggested that domain 1C may play a role in the interaction between FtsA molecules and this might be important for the activity of the protein in septation. To test these possibilities, we constructed a deletion mutant of FtsA lacking domain 1C. This domain is continuous and protrudes from the core of the molecule, suggesting that it might be removed without altering the structure of the remaining protein fragment. The deletion was designed using as a blueprint the three-dimensional (3D) model of the *E. coli* FtsA derived from the crystal structure of *T. maritima* FtsA (van den Ent and Löwe, 2000), and giving particular attention to two factors: the proximity of the residues located at the beginning and the end of the deletion and the localization of the initial residue in a flexible loop that after the deletion might allow the connection to the residue to prevent alterations in the 3D structure of the remainder of the molecule. According to these criteria a deletion was constructed spanning residues Gly84 to Ile161 (Fig. 1).

The deletion was introduced into pMSV20 (Sánchez *et al*., 1994), and into pLYV30, where it is under the control of the inducible P*tac* promoter (Yim *et al*., 2000), yielding the plasmids pARV1 and pARV11 respectively. The levels of the wild-type and mutated proteins produced by these plasmids in the *E. coli* strain OV16 were checked by Western blotting using an affinity-purified antibody raised against *E. coli* FtsA. OV16 contains an *ftsA16* amber allele in a temperature-sensitive suppressor background, and is therefore a thermonull mutant (Donachie *et al*., 1979; Lutkenhaus and Donachie, 1979). At permissive temperature, 30°C, the amount of FtsA in OV16 is ≈ 10% that of a wild-type strain grown under similar conditions. Transformation of OV16 with the plasmid pMSV20 increased the amount of FtsA to nearly wild-type levels. In the absence of IPTG, the cells transformed with pLYV30 had five times more FtsA than pMSV20 transformants, while they had about

50 times more after 2 h of induction with 50 μM of IPTG. The expression of the deletion gene from pARV1 and pARV11 produced truncated proteins with a molecular weight of 34 kDa, as predicted from the sequence. The levels of these proteins were similar to those of the wild-type protein (43 kDa) produced from pMSV20 or pLYV30 either at 30°C or at 42°C, showing that the deletion does not alter the stability of the remaining protein fragment.

To test the functionality of the mutant FtsAΔ1C protein OV16 was transformed with pARV1 and pMSV20 plasmids. The ability of the transformants to grow at 30°C or 42°C was tested on NBT plates. The *ftsAΔ1C* allele at 42°C did not complement *ftsA16* in OV16, while the control *ftsA*$^+$ allele did. The same results were obtained when we tested transformants yielding higher expression levels, resulting from the leakiness of the non-induced P*tac* promoter in pJF119 derivatives: pARV11 (*ftsAΔ1C*) and pLYV30 (*ftsA*$^+$).

In agreement with the lack of complementation of *ftsA16* by *ftsAΔ1C*, OV16/pARV1 (*ftsAΔ1C*) cells formed long filaments at 42°C (Fig. 2), while the length of the control strain OV16/pMSV20 (*ftsA*$^+$) was similar to the wild type (OV2; Yim *et al.*, 2000) and slightly shorter than the length of OV16 at 30°C. We conclude that FtsAΔ1C is stable but non-functional *in vivo*, suggesting that domain 1C is needed for FtsA to be active in septation.

Lack of interaction between FtsAΔ1C and FtsA$^+$

We have analysed the interacting properties of FtsAΔ1C and its ability to localize into the septal ring because both features have been already related to the activity of FtsA in cell division (Yim *et al.*, 2000; Carettoni *et al.*, 2003). Using the yeast two-hybrid system, we analysed the ability of FtsAΔ1C to interact with FtsA$^+$. The *ftsA*$^+$ and *ftsAΔ1C* genes were each fused to the GAL4-binding and to the GAL4-activation domain sequences, and the resulting plasmids were transformed into the yeast reporter strain Y187. The expression of these four fusions in yeast was checked by Western blotting, confirming that all of them produced similar amounts of fusion proteins. None of the single transformants producing either FtsA$^+$ or the mutated protein activated the expression of the reporter gene, while in the transformants containing the two *ftsA*$^+$ fusions the expression of the reporter was induced as previously reported (Yim *et al.*, 2000). The combination of the FtsAΔ1C, either at the GAL4 binding or activation domain, with the complete FtsA$^+$ did not activate the expression of the reporter, indicating that the absence of domain 1C in any of the two FtsA fusions impaired the interaction between two FtsA molecules. Accordingly, the combination of the two fusions with FtsAΔ1C did not activate the expression of the reporter gene either.

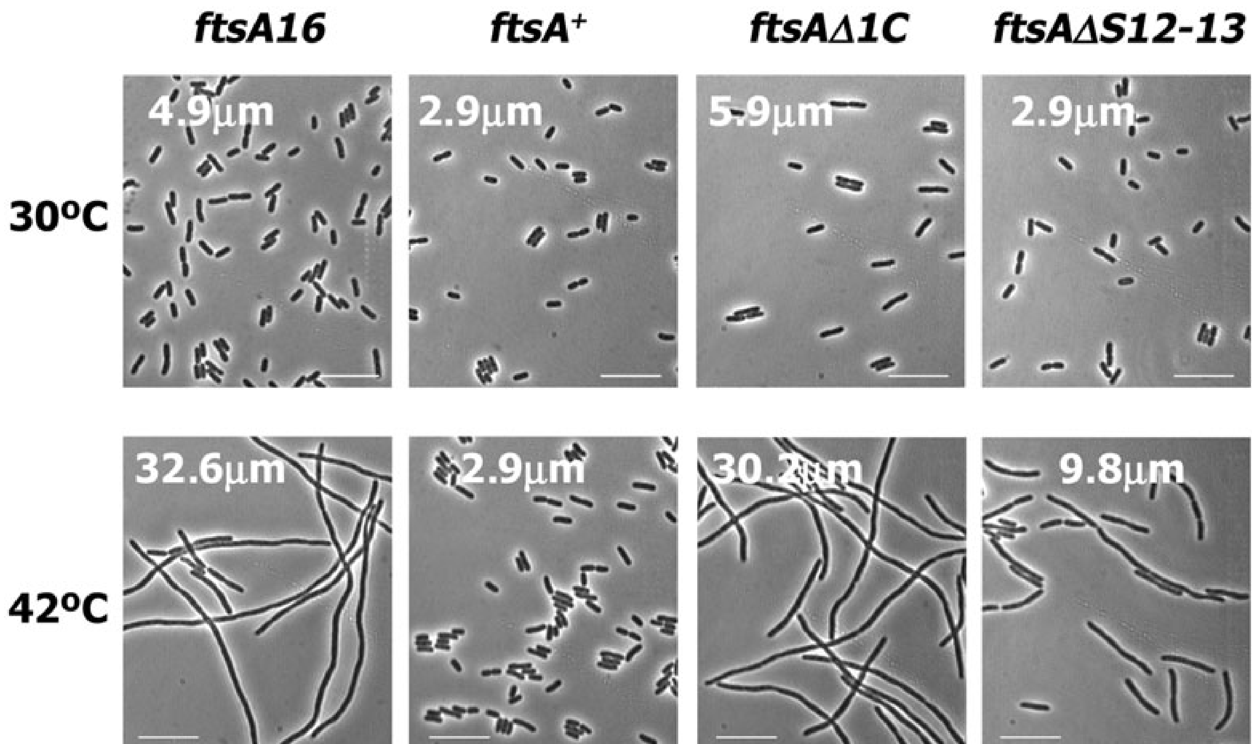

Fig. 2. Cell length and morphology of the OV16 cells (*ftsA16*) and its transformants harbouring pMSV20 (*ftsA*$^+$), pARV1 (*ftsAΔ1C*) or pARV31 (*ftsAΔS12–13*) at permissive (30°C) and restrictive (42°C) temperatures. The average length of at least 100 cells is indicated for each sample in μm. All the frames were corrected to minimize background differences using the Photoshop Levels adjustment. The reference bar marks 10 μm.

Localization of FtsAΔ1C into septal rings

The carboxy-terminal end of FtsA, which is required for the interaction between FtsA molecules, also plays a role in the localization of the protein into the septal ring (Yim *et al*., 2000). Although deletions that eliminate the last five carboxy-terminal residues of FtsA have no effect on the correct localization of the truncated protein, extending the deletion into W415, the sixth residue from the carboxy end, resulted in a protein that was unable to localize properly. Using immunofluorescence with specific anti-FtsA serum, we have examined the intracellular localization of FtsAΔ1C under conditions (OV16 background at 42°C) in which the amount of $FtsA^+$ is negligible (less than 1% of the wild-type contents; Fig. 3A).

Under these conditions, rings of the FtsAΔ1C protein that did not progress into active septa were detected at the potential division sites in OV16/pARV1 (*ftsAΔ1C*) grown at 42°C (Fig. 3F), similarly $FtsA^+$ localized correctly in OV16/pMSV20 (Fig. 3P), but in this case the rings proceeded normally into dividing septa. We can conclude then that deletion of domain 1C does not impair the ability of FtsA to be recruited into the Z-ring. Moreover, domain 1C properties, being required for septation and interaction but not for localization into the ring, are similar to those reported for the region comprising the last five carboxy-end residues of the protein (Yim *et al*., 2000). Taken together, these observations indicate that in the FtsA molecule the ability to interact with other FtsA molecules can be dissociated from the ability to localize in the division ring, and that both are required for the protein to be fully functional in septation.

Some cell division proteins fail to be recruited into the division ring in the presence of FtsAΔ1C

The imbalance between FtsZ and FtsA levels and their ability to interact with each other have been invoked to explain cell filamentation caused by high levels of $FtsA^+$ (Dai and Lutkenhaus, 1992; Dewar *et al*., 1992). The expression of both *ftsAΔ1C* and *ftsA*$^+$ at high levels (2 h of induction with 50 μM IPTG in OV16/pARV11 and OV16/pLYV30 respectively) caused filamentation. In both

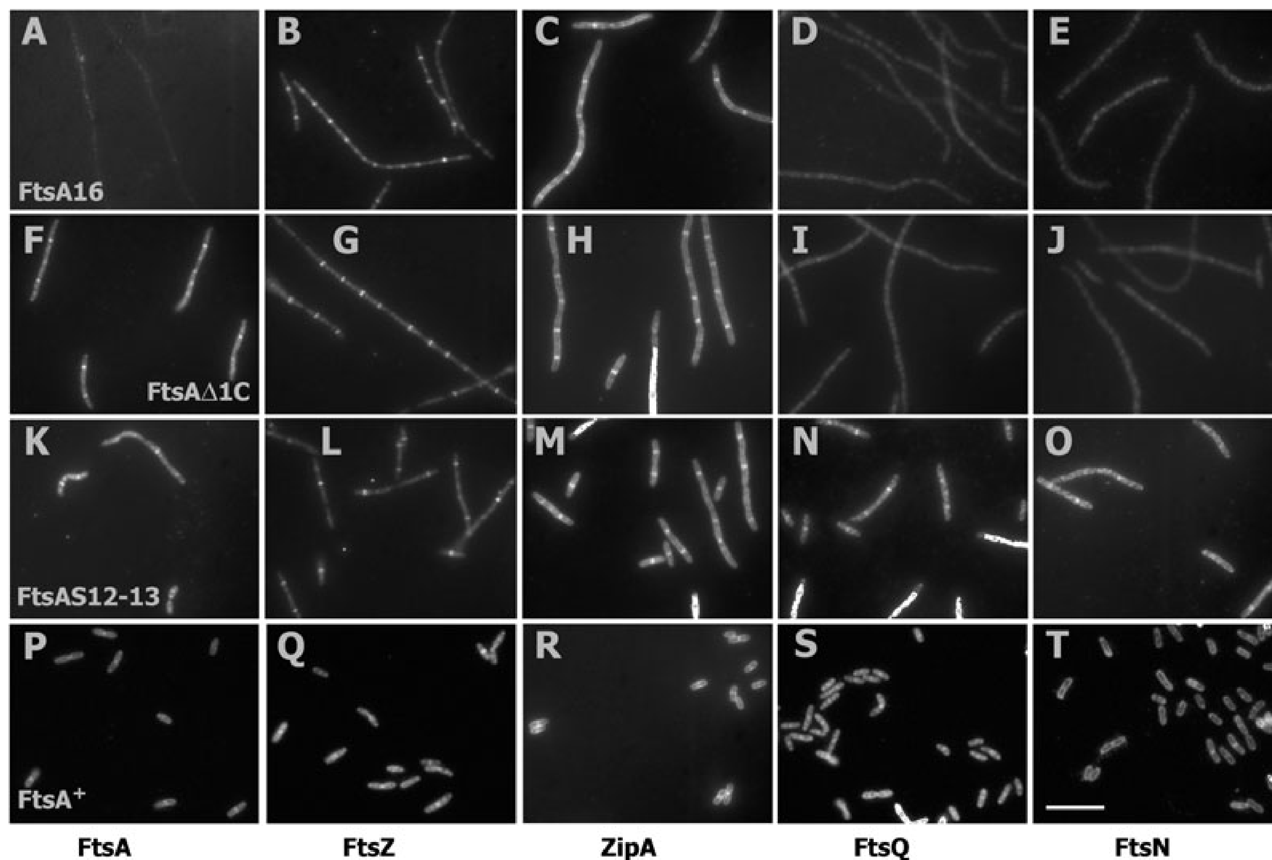

Fig. 3. Localization of the essential division proteins FtsA, FtsZ, ZipA, FtsQ and FtsN in *E. coli* cells without $FtsA^+$ but expressing the truncated forms of FtsA. OV16 strain (A–E) and its derivatives containing plasmids pARV1 (*ftsAΔ1C*; F–J), pARV31 (*ftsAΔS12–13*; K–O) and pMSV20 (*ftsA*$^+$ P–T) were grown at 42°C during 2 h before taking the samples for immunostaining. Purified polyclonal anti-sera against the *E. coli* proteins FtsA (A, F, K and P), FtsZ (B, G, L and Q), ZipA (C, H, M and R), FtsQ (D, I, N and S) and FtsN (E, J, O and T) were used. All the frames were corrected to minimize background differences using the Photoshop Levels adjustment. The reference bar in frame T marks 10 μm.

strains, the filaments were smooth and straight and lacked FtsZ or FtsA rings. The same morphology and absence of rings were observed when the experiment was performed in strain MC1061 (data not shown). On the contrary, when FtsAΔ1C was expressed at wild-type levels (OV16/pARV1), the FtsZ protein localized normally into Z-rings at potential division sites, as it did in the absence of FtsA (Fig. 3B and G). These results suggest that FtsAΔ1C retains the ability to interact with FtsZ, and that the localization of FtsAΔ1C in the division ring (Fig. 3F) might be directed by its interaction with FtsZ (Addinall and Lutkenhaus, 1996a; Ma *et al.*, 1996).

In the absence of FtsA, only FtsZ (Fig. 3B) and ZipA (Fig. 3C) were recruited into rings. This has been interpreted as being a consequence of the disruption in the normal sequence of incorporation of the division proteins into the ring (Addinall and Lutkenhaus, 1996a; Hale and de Boer, 1999; Chen and Beckwith, 2001). As expected, ZipA was correctly localized into rings at potential septation sites in OV16 transformants expressing *ftsAΔ1C* at 42°C (Fig. 3H). Immunolocalization of other ring components showed that both FtsQ and FtsN fail to localize into rings in cells containing the truncated FtsAΔ1C protein (Fig. 3I and J respectively) as in those altogether devoid of FtsA (Fig. 3D and E). FtsQ has been reported to be incorporated into the ring after FtsA, ZipA and FtsK (Chen and Beckwith, 2001), and FtsN is a multicopy suppressor of a temperature-sensitive *ftsA* mutation that is the last protein being recruited into the ring (Dai *et al.*, 1993; Addinall *et al.*, 1997). We conclude that FtsAΔ1C, although able to be recruited itself into the division ring, fails to promote the recruitment of further components of the ring (except FtsZ and ZipA that do not depend on FtsA for recruitment), and therefore aborts the progression of the ring into the formation of a functional division septum. Consequently, as expected from the complementation results already described, FtsAΔ1C is not able to sustain cell division.

A mutant lacking the S12 and S13 β-strands of domain 2B of FtsA is weakly active in septation

The FtsA dimerization model proposed by Carettoni *et al.* (2003) suggests that the S12 and S13 β-strands of domain 2B might be involved in the interaction between two FtsA molecules, but contrary to domain 1C, this domain is not continuous, and probably it cannot be deleted without altering the structure of the remaining protein fragment. Therefore, to test whether these β-strands are actually involved in the biological role of FtsA, we have constructed deletion mutants that express proteins in which both of them are absent but maintain most part of domain 2B. The constructions were engineered to minimize any other alteration in the structure of the resultant protein (Fig. 1). The two chosen residues located at the beginning and the end of the deletion, Ser274 and Gln289, respectively, are close in the *E. coli* 3D model. The serine 274 residue is in a loop, allowing therefore the connection of the two ends of the deletion without further alterations in the structure of the remainder of the molecule. Because of the generation of an additional unsought mutation, another deletion was also obtained spanning from Val275 to Arg290 (see *Experimental procedures*). All the experiments were performed with the two constructions, and in all cases their behaviour was identical, therefore we will simplify their nomenclature in this report referring to them collectively as FtsAΔS12–13.

Complementation analyses of *ftsA16* were conducted for *ftsAΔS12–13* in a way similar to that described above for *ftsAΔ1C*. The mutations were introduced into pMSV20 (Sánchez *et al.*, 1994): pARV31 (FtsAΔ275–290) and pARV32 (FtsAE273D/Δ274–289). OV16 was transformed with these two plasmids and the production of the mutated proteins was checked by Western blot analysis. In all of them, a protein of the expected molecular weight, 41.4 kDa, was produced at levels similar to those of the wild-type protein in the control strains. For complementation analysis, the cells were plated on NBT plates and incubated at 30°C or 42°C. None of them could grow at 42°C at low expression levels. It is concluded therefore that FtsAΔS12–13 cannot substitute for the wild-type FtsA protein at these levels.

The effect of expression of FtsAΔS12–13 at wild-type levels on cell morphology was analysed in the presence or absence of the wild-type FtsA (OV16 cells at 30°C and 42°C respectively). In agreement with the lack of complementation of *ftsA16* by *ftsAΔS12–13*, the cells containing FtsAΔS12–13 but devoid of $FtsA^+$ formed filaments (Fig. 2). However, the length of these filaments was three- to fourfold shorter than the OV16 filaments at 42°C, indicating that FtsAΔS12–13 might retain a weak activity, enough to support infrequent divisions, but not to maintain cell viability.

Even at permissive temperature, the amount of FtsA in OV16 is about 10% that of the wild type, probably because of the low efficiency of the temperature-sensitive *tyrT* suppressor (*supFA81*(Ts); Celis *et al.*, 1973). As a result, OV16 cells at 30°C are nearly 25% longer than the wild-type MC1061 (Fig. 2). Expression of the *ftsAΔS12–13* allele at the temperature permissive for the suppressor had the effect of shortening the length of the OV16 transformants containing pARV31 or pARV32 (*ftsAΔS12–13*) to values close to those found for the $ftsA^+$ OV16 transformants (Fig. 2). These results are consistent with the idea that the FtsAΔS12–13 proteins retain some activity at division.

A point mutation R286W in the FtsA S13 β-strand has been recently reported to be able to bypass the require-

ment for the ZipA protein in cell division (Geissler *et al*., 2003). The possibility that the FtsAΔS12–13 proteins could equally rescue CH5/pCH32, a *zipA* thermonull mutant strain (Hale and de Boer, 1999), was tested. The CH5/pCH32 strain was transformed with pMSV20 or, pARV31 and pARV32, and also pLYV30 (*ftsA*+ expressed from a P*tac* promoter) and its derivatives pARV33 (*ftsA*Δ*275–290*) and pARV34 (*ftsAE273D/*Δ*274–289*). The ability of the transformants to grow at the restrictive temperature, when ZipA is not synthetized, was checked. Neither FtsA+ nor the FtsAΔS12–13 deletions, even if overproduced from P*tac*, could rescue CH5/pCH32.

The FtsAΔS12–13 proteins interact with FtsA+, localize at division rings and recruit other components into them

If, as stated above, the FtsAΔS12–13 proteins still have some cell division activity, they should retain those activities that have been proven essential for the role of FtsA in septation, among them they might be able to interact with the FtsA+ protein. The ability of the FtsAΔS12–13 proteins to interact with FtsA+ was assayed using the yeast two-hybrid system. We have found that FtsA–GAL4 fusions are often difficult to clone in *E. coli*, and once cloned they inhibit cell division and growth poorly, although they can be propagated in yeast. The FtsAΔS12–13 deletion could be fused to the GAL4-DNA-binding domain, but after several attempts the fusion with the GAL4 activation domain could not be obtained neither with the FtsAΔ274-289 nor with the FtsAΔ275–290 deletions, therefore the interaction could only be tested in one sense. The two deletions fused to the GAL4-DNA-binding domain were probed against the wild-type FtsA fused to the GAL4-activation domain. As with the FtsAΔ1C mutants, the expression of the fusion in yeast was confirmed by Western blotting. The two deletion mutants produced blue colonies in colony-lift filter assays that were indistinguishable from the assay with the wild-type gene fused to both GAL4 domains, indicating that the FtsAΔS12–13 protein interacts with wild-type FtsA. The self-interaction of the mutant could not be tested.

Immunofluorescence labelling of filaments formed at 42°C by OV16/pARV31 and OV16/pARV32 showed localization into septal rings of both FtsZ (Fig. 3L) and the mutant FtsAΔS12–13 proteins (Fig. 3K). Contrary to the case of OV16 filaments containing FtsAΔ1C, if the rings formed with the truncated FtsAΔS12–13 proteins retained some cell division activity, they should be proficient at localizing the rest of the cell division proteins. Immunofluorescence microscopy using sera against ZipA, FtsQ or FtsN revealed that these proteins did also localize at rings within the filaments (Fig. 3M–O). As both FtsQ and FtsN are dependent for their recruitment into the division ring on the previous localization of FtsA in the septal rings, we conclude that the truncated FtsAΔS12–13 proteins retain the activity of FtsA+ that is necessary for the recruitment of other division proteins into the septum.

Septation sites containing the FtsAΔS12–13 proteins are frequently misplaced

When OV16 transformants that harbour the deleted *ftsA*Δ*S12–13* genes under the control of P*tac* in pARV33 and pARV34 were grown at 42°C in the absence of inducer, they expressed the mutant gene at about five times the wild-type levels. Under these conditions the cells did not filament. After 2 h of exponential growth at 42°C, the average length of the cells was 3.9 μm for OV16/pARV33 and 4.1 μm for OV16/pARV34, while the control strain transformed with pLYV30 (FtsA+) had an average of 4.3 μm, and the strain with the vector alone had 31.6 μm. In spite of this, the mutated proteins gave a partial complementation of OV16. Immunofluorescence analysis showed that some cells had rings in anomalous, non-central, positions and others had polar foci (Fig. 4). Non-central division septa were observed either when FtsA+ was present at normal (MC1061), low (OV16 at 30°C; Fig. 5) or very low (OV16 at 42°C; Fig. 5) levels. Even in the absence of inducer, these cultures contained a small but noticeable proportion (15% at 30°C) of short, round or almost round nucleoid-free cells. These small cells measured from 1 to 1.5 μm, which is longer than the minicells observed in *min* mutants (for comparison see de Boer *et al*., 1988; Addinall and Lutkenhaus, 1996b; Yu and Margolin, 1999), suggesting that they were not formed by polar divisions, but more likely by misplaced septa. The misplaced rings were localized in DNA-free regions, conforming to the nucleoid occlusion model for the placement of the division ring (Woldringh *et al*., 1990). Immunofluorescence performed with anti-FtsZ serum revealed that in OV16/pARV33 cells, grown at 42°C for 2 h, the FtsZ protein was also localized at abnormal sites (not shown).

Overproduction of the FtsAΔS12–13 truncated proteins in these strains (50 μM IPTG during 2 h) led to the loss of cell viability and resulted in the production of aberrant cell morphologies (C-shaped cells in Fig. 6) similar to those reported for the overproduction of some FtsA proteins truncated in the carboxy-terminus (Gayda *et al*., 1992; Yim *et al*., 2000) and in cell lysis.

Discussion

Our results allow us to propose that two distinct regions of the cell division protein FtsA, domain 1C and the S12 and S13 β-strands of domain 2B, are associated to two functions that are essential for its role in septum formation. Domain 1C is necessary for the interaction with other

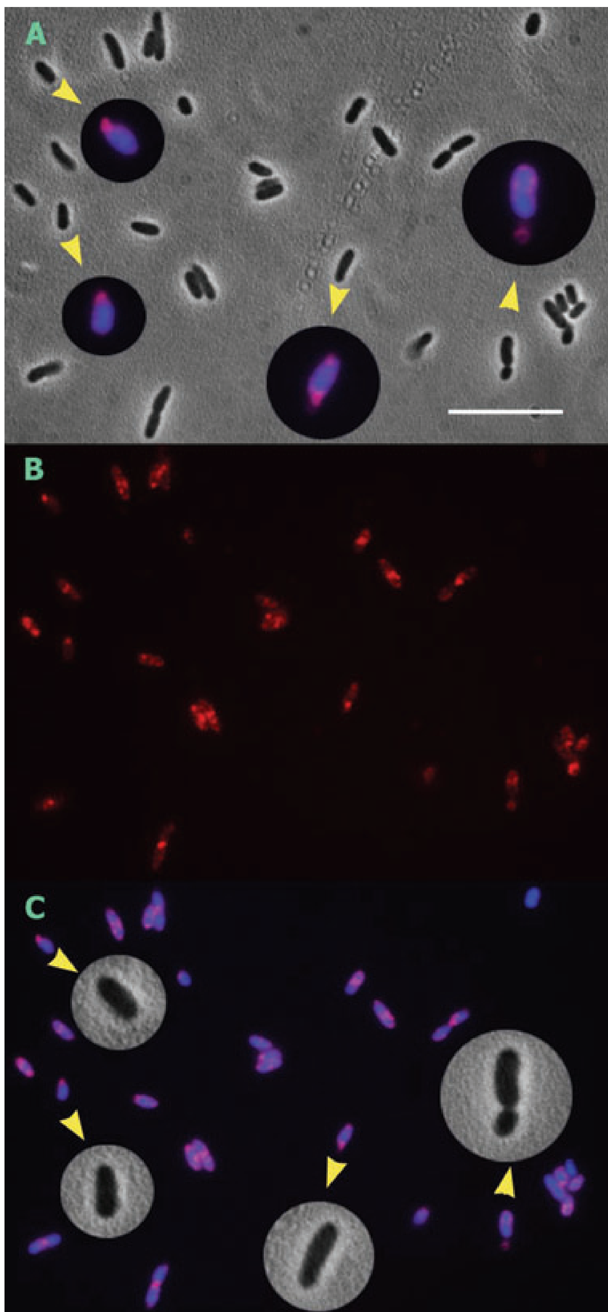

Fig. 4. Effect of the truncated FtsAΔS12–13 protein produced by the P*tac* promoter leakage. OV16 strains expressing the FtsAΔS12–13 from the plasmids pARV33 were grown at 42°C during 2 h before taking the samples for microscopy. Cells were stained for DNA (blue) with DAPI and FtsAΔS12–13 (red) with anti-FtsA by immunolabelling. (A) Phase-contrast image, (B) immunolocalization of FtsA and (C) overlay of the FtsA (red) and nucleoid (blue) images. The position of DNA (blue) and the FtsAΔS12–13 proteins (red) of some of the aberrant cells in detail are shown in (A), and the corresponding phase contrast images are shown in (C). The reference bar in (A) marks 10 μm.

FtsA molecules and the S12 and S13 strands of domain 2B affect the selection of the cell centre to form a division ring.

The role of domain 1C in the interaction between FtsA molecules and their activity at the division ring.

Carettoni *et al.* (2003) have proposed that the residues 126–133 in the domain 1C are involved in the homodimerization of FtsA and in its biological function. Our results using the yeast two-hybrid system support the involvement of domain 1C in the interaction between FtsA molecules, and suggests, as deduced from the absence of complementation, that the lack of self-interaction is associated to the loss of the FtsA activity in septation, failing to sustain the sequential incorporation into the division ring of proteins that are recruited at later stages.

FtsA interacts with the carboxy-end of FtsZ and this interaction is essential for the proper functioning of the division ring (Din *et al.*, 1998; Ma and Margolin, 1999; Yan *et al.*, 2000). Increasing the amount of FtsA or FtsZ induces filamentation, an effect that is thought to result from the imbalance between them (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992). We have found that FtsAΔ1C localizes correctly at the potential division sites, and that the production of high levels of FtsAΔ1C provokes filamentation and loss of Z-rings, suggesting that the absence of domain 1C in FtsAΔ1C does not prevent the interaction with FtsZ and indicating that domain 1C would not be directly involved in the FtsA–FtsZ interaction.

The FtsAΔ5 protein, which lacks the last five carboxy-end residues of *E. coli* FtsA, did not interact with $FtsA^+$ in the yeast two-hybrid system, had no activity in septation and was able to localize at division rings, although larger deletions did not localize (Yim *et al.*, 2000). Together with our results, this supports the idea that the FtsA self-interaction and the correct cellular localization of the protein can be dissociated, and that both are needed for the role of this protein in cell division.

The S12 and S13 β-strands in domain 2B of FtsA are dispensable for the interaction with $FtsA^+$ and for the completion of septa.

The analysis of strains producing the FtsAΔS12–13 proteins showed that the S12 and S13 β-strands in domain 2B are dispensable for the interaction with $FtsA^+$. The model of Carettoni *et al.* (2003) postulated an interaction between FtsA molecules involving the domains 1C and 2B, and within domain 2B the S12 and S13 β-strands were proposed to interact with the residues 126–133 of domain 1C. According to our data, this last interaction would not be essential for dimerization. A possible explanation might be that even in the absence of the S12 and

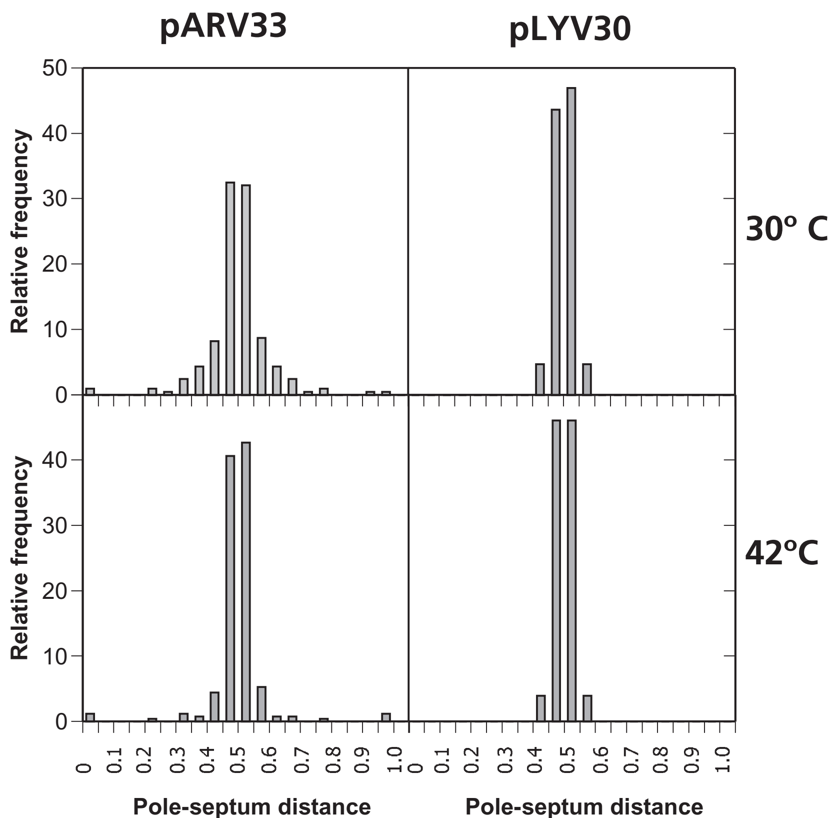

Fig. 5. Effect of the expression of *ftsAΔS12–13* on the position of division septa. OV16 transformants containing the plasmids pLYV30 (FtsA^{+}) and pARV33 (FtsAΔS12–13) were grown to exponential phase and then for 2 h at the permissive and restrictive temperatures. Phase-contrast microscopy images were taken and the distance from the septum to each pole was measured. Cells without a constriction were not included in the analysis. The histograms show the distribution of division septa as a function of the relative cell length. Cell numbers were $n = 75$ for pLYV30 at 30°C, $n = 76$ for pLYV30 at 42°C, $n = 103$ for pARV33 at 30°C and $n = 123$ for pARV33 at 42°C.

S13 β-strands there is still a large contact surface between domains 1C and 2B that might be enough to maintain an interaction, although the complex formed would not be fully functional. The FtsAΔS12–13 proteins colocalize with FtsZ at septation sites where they can recruit other components of the division ring, among them we have specifically detected FtsQ and FtsN (Fig. 3N and O). At 42°C, OV16 transformants that produce FtsAΔS12–13 proteins at wild-type levels (pARV31) formed filaments shorter than those of the parental strain (Fig. 2). Moreover, increasing five times the amount of mutated protein decreased the length of the cells to the wild-type length. These results indicate that the rings containing the FtsAΔS12–13 proteins as their only form of FtsA are weakly functional in septation. Further increasing the amount of mutated protein produces severe morphological alterations similar to those produced by the overexpression of FtsA carboxy-terminal deletion mutants (Gayda *et al*., 1992; Yim *et al*., 2000).

The distribution of division rings becomes altered in the presence of the FtsAΔS12–13 proteins

Our results indicated that the production of these proteins was accompanied by the presence of an unusually high proportion (15%) of short cells deprived of DNA in the population and of misplaced division rings (Fig. 4). The precise localization of a septum along the cell length seems to be determined by several factors, the absence of the MinCD inhibitor of FtsZ polymerization and the simultaneous local absence of a nucleoid being the best documented ones (Bi and Lutkenhaus, 1993; Sun *et al*., 1998; Yu and Margolin, 1999; Harry, 2001). Our results do not allow us to unequivocally define a causative sequence for the observed abnormal morphologies. A role for FtsA and ZipA on the stabilization of the *E. coli* FtsZ ring has been suggested (Hale and de Boer, 1999; RayChaudhuri, 1999; Pichoff and Lutkenhaus, 2002; Geissler *et al*., 2003). A point mutation in the S13 β-strand of FtsA has been reported to bypass the need for ZipA in division and to counteract the inhibition of Z-ring formation by MinC (Geissler *et al*., 2003). A plausible interpretation of our results would be that the putative FtsZ stabilizing activity of the FtsAΔS12–13 proteins is increased relative to that of the wild type, in this case nucleation of Z-rings could occur at abnormal locations, including places that are normally inhibited by MinC. The FtsA and FtsZ polar foci found in a fraction of the cells might be remnants of previous division rings that remain after cell division because of an increase in the stability of ring structures (Addinall and Lutkenhaus, 1996b).

These and previous results (Yim *et al*., 2000; Carettoni *et al*., 2003; Geissler *et al*., 2003) suggest that domain 2B

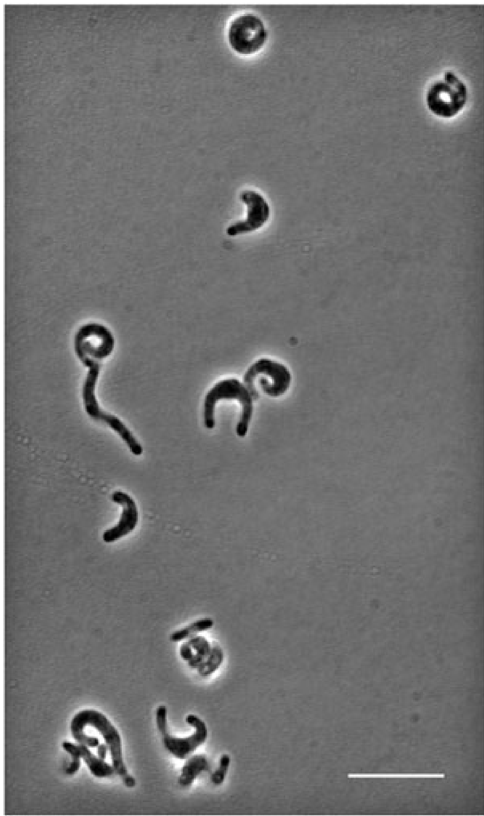

Fig. 6. Morphology of OV16 cells producing FtsAΔS12–13 protein by the induction with IPTG (50 μM) during 2 h at the restrictive temperature. The reference bar marks 10 μm.

of FtsA might affect Z-ring localization while domain 1C might be involved both in the FtsA–FtsA self-interaction and in the recruitment of downstream division proteins. The role of the carboxy-end in septation is less clear, because a deletion of five residues affects the self-interaction of FtsA, but not the localization at the septal ring, while longer deletions affect both. The position of the carboxy-terminal end within the 3D structure of the FtsA molecule is not known, because being flexible, it was not rendered in the crystal structure of *T. maritima* FtsA, and the model shown in Fig. 1 ends therefore 35 residues before the end of the protein sequence. The involvement of both, domain 1C and the carboxy-terminus, in the FtsA self-interaction supports the model of Carettoni *et al.* (2003), while the fact that the cap of domain 2B is not essential for the FtsA self-interaction in the yeast two-hybrid assay does not discard a role for the whole domain. The model of Carettoni *et al.* (2003) proposed a head-to-tail interaction between FtsA molecules to form a dimer (or even higher oligomeric or polymeric structures). Therefore, although domains 2B and 1C are involved in the FtsA self-interaction, there would be a free domain 2B, and a free domain 1C in each extreme that might be available for interaction with other division proteins.

Experimental procedures

Escherichia coli *and yeast strains and growth conditions*

The strains used in this study and their relevant characteristics are described in Table 1.

Luria–Bertani (LB) broth (Sambrook *et al.*, 1989) and LB agar, supplemented with antibiotics when required (ampicillin, 100 $\mu g\ ml^{-1}$, kanamycin, 50 $\mu g\ ml^{-1}$ and/or chloramphenicol, 50 $\mu g\ ml^{-1}$) were used for routine cultures of *E. coli* at 37°C. Thermosensitive strain OV16 used for complementation assays was grown in nutrient broth medium (Oxoid no. 2 nutrient broth) supplemented with thymine (50 $\mu g\ ml^{-1}$) (NBT) and antibiotics when required and incubated at the permissive (30°C) or restrictive (42°C) temperatures. Complementation tests were performed as described by Sánchez *et al.* (1994).

Yeast strains were grown in YEPD or SD medium, supplemented with required amino acids and glucose as described in the Matchmaker GAL4 Two-Hybrid System 3 manual (Clontech Laboratories, Inc.).

Cell parameter measurements, photography and immunofluorescence microscopy

Cultures were grown at the permissive temperature in liquid media in a shaking water bath so that balanced growth was maintained for several doublings (not less than four) before the beginning of the experiment. For observing the cell morphology at different conditions, as 42°C or/and overexpression, the samples were taken after 2 h in the desired conditions. The optical density at 600 nm (OD_{600}) was measured using a Shimadzu UV-1203 spectrophotometer or a CO8000 Cell Density Meter from WPA biowave; the optical density was always kept below 0.40–0.50 by appropriate dilution with prewarmed medium. Particles were fixed in 0.75% formaldehyde. Cells were spread on thin agar layers and photographed under phase-contrast optics using a Sensys charge-coupled device camera (Photometrics) coupled to a Zeiss Axiolab HBO 50 microscope with a 100× immersion oil lens. The software used for image capture was IPLab Spectrum, and Adobe Photoshop 7.0 software was used for processing. Cell lengths were measured with Object-Image version 1.62 (N. Vischer, University of Amsterdam). One hundred cells were analysed for each sample.

For immunofluorescence microscopy, exponentially growing cells were prepared as described by Addinall and Lutkenhaus (1996a). The cells were immobilized in poly L-lysine-pretreated slides. The final lysozyme concentration used was 4 $\mu g\ ml^{-1}$, and the permeabilization time was 1 min. The samples were incubated overnight at 4°C with primary antibodies. Polyclonal anti-sera used for the FtsA, FtsZ, ZipA and FtsQ immunolocalizations were MVM1 (*Sánchez et al.*, 1994), MVJ9 (Pla *et al.*, 1991), MVC1 (Rueda *et al.*, 2003) and MVJ11 (Dopazo *et al.*, 1992) respectively. The primary antibody used for FtsN immunolocalization was MVG1, a polyclonal anti-serum obtained from rabbits (Charles River Laboratories) after their immunization with purified His-FtsN. The specificity of this anti-serum was checked by Western blot with cellular extracts of JOE565, a strain containing the chromosomic *ftsN* gene interruped and a plasmid containing

Table 1. Strains and plasmids.

Strains	Genotype and relevant characteristics	Source/reference
E. coli		
BL21(DE3)	F⁻ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
pLysS	Expression of His-FtsN	
CH5	*dadR-*, *trpE-*, *trpA-*, *tna-*, *zipA::aph recA::Tn10* ZipA-thermonull strain	Hale and de Boer (1997)
DH5α	F⁻ *endA1 hsdR17 supE44 thi1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169* (Φ80λαχZΔM15) Host for cloning	Hanahan (1983)
JOE565	MC4100 araD⁺ *ftsN::kan*/pJC83 Conditional FtsN null strain	Chen and Beckwith (2001)
MC1061	F⁻ *araD139*, Δ*(ara-leu)76997*, Δ*(lac)X74*, *galU*, *galK*, *strA* Wild-type *E. coli* strain	Busby *et al.* (1982)
OV16	F⁻ *ilv leu thyA* (*deo*), *his*, *ara*(Am), *lac125*(Am) *galKu42*(Am) *galE trp*(Am) *tsx*(Am) *tyrT*(*supFA81* Ts) *ftsA16*(Am) *ftsA* amber allele in a thermosensitive suppressor background	Donachie *et al.* (1979)
Yeast		
Y187	MATα, *ura3–52*, *his3–200*, *ade2–101*, *trp1–901*, *leu2–3*, *112*, *gal4Δ*, *met⁻*, *gal80Δ*, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-*lacZ* Yeast two-hybrid assays	Clontech
Plasmids		
pARV1	pMSV20 derivative containing *ftsAΔ1C* by PCR-mediated mutagenesis, Amp	This study
pARV5	GAL4 AD::*ftsA*⁺ cloned in pGADT7, Amp, LEU	This study
pARV6	DNA BD::*ftsA*⁺ cloned in pGBKT7, Kan, TRP	This study
pARV11	*ftsAΔ1C* cloned under P_{tac} and *lacI*^{q} in pJF119EH, Amp	This study
pARV13	GAL4 AD::*ftsAΔ1C* cloned in pGADT7, Amp, LEU	This study
pARV14	DNA BD::*ftsAΔ1C* cloned in pGBKT7, Kan, TRP	This study
pARV29	DNA BD::*ftsAΔ275-290* cloned in pGBKT7, Kan, TRP	This study
pARV30	DNA BD::*ftsAΔ274-289* cloned in pGBKT7, Kan, TRP	This study
pARV31	pMSV20 derivative containing *ftsAΔ275-290* by PCR-mediated mutagenesis, Amp	This study
pARV32	pMSV20 derivative containing *ftsAΔ274-289* by PCR-mediated mutagenesis, Amp	This study
pARV33	*ftsAΔ275-290* cloned under P_{tac} and *lacI*^{q} in pJF119EH, Amp	This study
pARV34	*ftsAΔ274-289* cloned under P_{tac} and *lacI*^{q} in pJF119EH, Amp	This study
pBR322	Amp, Tet	Bolívar *et al.* (1977)
pCH32	*aadA*+ *repA*(Ts) ftsZ+ *zipA*+	Hale and de Boer (1997)
pET28a	Cloning and expression vector for His-tag fusion proteins	Novagen
pGADT7	Cloning vector for yeast two-hybrid assays. Fusions to GAL4 activation domain. Amp, LEU	Clontech
pGBKT7	Cloning vector for yeast two-hybrid assays. Fusions to DNA binding domain. Kan, TRP	Clontech
pJC83	pBAD33-*ftsN*	Chen and Beckwith (2001)
pJF119EH	Cloning vector containing a P_{tac} promoter and *lacI*^{q}, Amp	Fürste *et al.* (1986)
pJF119HE	as pJF119EH but with the multicloning site in the opposite orientation	Fürste *et al.* (1986)
pLYV30	*ftsA*⁺ cloned under P_{tac} and *lacI*^{q} in pJF119HE, Amp	Yim *et al.* (2000)
pMGV1	*ftsN* cloned into pET28a(+)	This study
pMSV20	*ftsA*⁺ maintaining its natural promoters cloned into pBR322, Amp	Sánchez *et al.* (1994)
pZAQ	*ftsQAZ*, Tet	Ward and Lutkenhaus (1985)

the *ftsN* gene under the control of the P_{BAD} promoter (Chen and Beckwith, 2001). All of them were previously purified by membrane affinity. Alexa594-conjugated anti-rabbit serum (Molecular Probes Inc.) was used at a 1 : 100 dilution as the secondary antibody. Cells were observed by fluorescence microscopy using the Zeiss Axiolab HBO 50 microscope fitted with a HQ: Cy3 filter (excitation, 545/30 nm; emission, 610/75 nm; beamsplitter, 565LP) and 100× immersion oil lens.

When indicated, 4′,6-diamidino-2-phenylindole (DAPI) was added to observe nucleoid segregation.

Plasmid constructions and DNA manipulation

Plasmid DNA isolation, cloning techniques and transformation procedures were performed as described by Sambrook *et al.* (1989). Restriction endonucleases and other enzymes were purchased from and used as recommended by Roche molecular Biochemicals. Two-hybrid system cloning vectors (pGADT7 and pGBKT7) were obtained from Clontech.

Plasmids encoding the deleted FtsA proteins for the complementation assays were obtained by polymerase chain reaction (PCR)-based method of site-directed mutagenesis as described by Weiner and Costa (1994), but using the Expand High Fidelity (EHF) and the Pwo polymerases (both supplied by Boehringer Mannheim). Plasmid pMSV20 is a pBR322 derivative containing the *ftsA* wild-type gene with its upstream region that was used as target for PCR-directed mutagenesis (Sánchez *et al.*, 1994). This sequence context, working together with any potential readthrough transcription from the plasmid promoters, at the copy number of the vector produces levels of each protein that fall within the level range found in strains expressing the wild-type *ftsA*⁺ from the chromosome. Primers AR1 (5′-CGACAGCGCCAGATATACCG AAG-3′) and AR2 (5′-ACATGTCACAACGATATGGCG-3′), phosphorylated by T4 polynucleotide kinase from Promega,

were used to amplify the sequence of pMSV20, except the sequence encoding the domain 1C of FtsA from Gly84 to Ile161. A clone containing the desired deletion of the entire domain 1C was obtained, although by sequencing we checked that it also contains a change in the residue after the deletion site, 162Thr to Ala. The plasmid was named pARV1, which produces a truncated FtsA lacking the entire domain 1C, named FtsAΔ1C, at levels close to the one obtained from the chromosomal *ftsA*$^+$ copy. In the same way, the phosphorylated primers AR5 (5′-CTCATCTTTTCCAAC GATGG-3′) and AR6 (5′-CGTCAGACACTGGCAGAGGTG-3′) were used to amplify the sequence of pMSV20, except the sequence encoding the FtsA region comprising the β-strands S12–S13 and the connecting loop between them. Two clones, pARV31 and pARV32, encoding two nearly identical FtsA proteins lacking the two β-chains S12 and S13, have been obtained. Both contain an additional point mutation next to the deletion site checked by sequencing. As a result, we have two different forms of this deletion, one in which residues 275–290 have been deleted and another in which the deletion spans residues 274–289 and contains an additional point mutation in the residue before the deletion site, 273Glu to Asp.

These truncated *ftsA* genes were cloned under an IPTG-inducible promoter in pJF119EH (Fürste *et al.*, 1986) for over-expressing the deleted proteins. The truncated genes were obtained by PCR amplification from the plasmids pARV1, pARV31 and pARV32. We used the upstream primer SR1 (5′-GGGCGAATTCATGATCAAGGCGACGGAC-3′), which introduces an *Eco*RI restriction site immediately upstream of the start codon, and the downstream primer LY20 (5′-CAG GTCGACCGTAATCATCGTCGGCCTC-3′), which introduces a *Sal*I restriction site immediately downstream of the stop codon of the truncated *ftsA* gene. The resulting plasmids were named pARV11, pARV33 and pARV34 respectively. The control plasmid pLYV30 (Yim *et al.*, 2000) contains the wild-type *ftsA* gene. The presence of all the mutations was confirmed by DNA sequencing.

Plasmids for the GAL4 two-hybrid assays were constructed as follows. The *ftsA*$^+$ coding sequence was obtained from pZAQ by PCR amplification with the upstream and downstream primers MF24 (5′-AATCGCATATGATCAAGGC GACGGACA-3′) and SR2 (5′-CCCGGAATTCAAACTCTTTT CGCAGCCAA-3′), which incorporate the restriction sites *Nde*I and *Eco*RI respectively. The amplified fragment was cloned into pGADT7, yielding plasmid pARV5. Plasmid pARV6 was obtained by subcloning with *Eco*RI and *Pst*I the *ftsA*$^+$ coding sequence from pLYV44 (Yim *et al.*, 2000) into the same sites in pGBKT7. The coding sequence for the domain 1C of FtsA in isolation was obtained by PCR amplification with the upstream and downstream primers AR10 (5′-GGAATTCGGTAAGCACATCAGCTGCC-3′) and AR11 (5′-CGGGATCCCGGATCAGGTGCACTTTTGCC-3′), which incorporate the restriction sites *Eco*RI and *Bam*HI respectively. The amplified fragment was cloned into pGADT7 and pGBKT7, yielding plasmids pARV8 and pARV9 respectively. The *ftsAΔ1C* coding sequence was obtained from pARV1 by PCR amplification with the upstream primer SR1 incorporating the restriction site *Eco*RI, and the downstream primers AR14 (5′-CCATCGATGTTAAAACTCTTT TCGCAGCC-3′) or LY20, which incorporate the restriction sites *Cla*I and *Sal*I respectively. The amplified fragment flanked by the restriction sites *Eco*RI and *Cla*I was cloned into pGADT7, yielding plasmid pARV13, and the one flanked by the restriction sites *Eco*RI and *Sal*I was cloned into pGBKT7, yielding plasmid pARV14. The *ftsAΔ275–290* and *ftsAΔ274–289* coding sequences were obtained from pARV31 and pARV32, respectively, by using PCR amplification with the upstream and the downstream primers SR1 and LY20. The amplified fragments were cloned into the restriction sites *Eco*RI and *Sal*I of vector pGBKT7, yielding plasmids pARV29 and pARV30 respectively. All these constructions were also checked by sequencing.

To construct the His-FtsN fusion protein for the obtention of anti-FtsN antibodies, the coding sequence of *ftsN* was amplified from pJC83 (Chen and Beckwith, 2001) by PCR with the upstream and downstream primers MG1 (5′-CGGAATTCATGGTCGCTATTGCTGCC-3′) and MG2 (5′-CCCAAGCTTTCAACCCCCGGCGGCGAG-3′), which incorporate the restriction sites *Eco*RI and *Hin*DIII respectively. The amplified fragment was cloned into pET28a(+) (Novagen) yielding the plasmid pMGV1, which was checked by sequencing.

Structural modelling of E. coli *FtsA*

The structural model for *E. coli* FtsA (Carettoni *et al.*, 2003) was made using homology modelling procedures based on the multiple alignment of the members of the FtsA family of proteins including the known 3D structure of the *T. maritima* FtsA (Apo form structure: Protein Data Bank accession number 1E4F; ATP bound form: 1E4G; van den Ent and Löwe, 2000) The model for *E. coli* FtsA residues 7–385 was built using the program Swiss-Pdb Viewer and the SWISS-MODEL server facilities (http://http://www.expasy.ch/swissmod/SWISS-MODEL.html; Guex and Peitsch, 1997). The quality of the model was checked using the WHAT-CHECK routines from the WHAT IF program (Vriend, 1990) and the PROCHECK validation program from the SWISS-MODEL server facilities; briefly, the quality values of the model are within the expected region for protein structural models.

Expression and purification of His-FtsN

Cultures of BL21(DE3)pLysS containing pMGV1 were grown at 37°C in LB medium supplemented with antibiotics (kanamycin and chloramphenicol) to an OD_{600} of 0.4. Overexpression of the proteins was induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Two hours after addition of the inducer, cells were harvested by centrifugation and resuspended in Binding Buffer [5 mM Imidazole; 0.5 M NaCl; 20 mM Tris-HCl (pH 7.9)] supplemented with 1% Triton X100. The bacteria were lysed by sonic disruption and centrifuged for 15 min at 11 000 *g* at 4°C. Pellets containing the fusion protein in the pellet were resuspended in Binding Buffer supplemented with 1% Triton X-100 and 6 M Urea. The denatured His-tagged proteins recovered in the supernatant were purified by metal affinity chromatography on a nickel column (His·Bind Resin; Novagen) eluting with a step gradient of imidazole ranging from 20 to 200 mM in a 0.5 M NaCl; 1% Triton X100; 6 M Urea and 20 mM Tris-HCl (pH 7.9) buffer.

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Integrity and purity of proteins were checked by SDS–PAGE and quantified by the Bradford method with a commercial assay (Bio-Rad).

Yeast two-hybrid assays

Yeast strains were transformed using the Li acetate method (Gietz *et al*., 1992) and selected in SD medium supplemented with the required amino acids and glucose at 30°C. Qualitative β-galactosidase assays were performed using Whatman no. 5 filters as described in the Matchmaker GAL4 Two-Hybrid System 3 manual (Clontech Laboratories, Inc.). For colour development, filters were incubated up to 16 h at room temperature (although no changes were usually observed after 3 h). Cell lysis was achieved by two freeze (liquid N2) and thaw (37°C) cycles. The expression of the fusion proteins was checked by Western blotting using the affinity-purified anti-FtsA anti-serum and yeast protein extracts obtained following the protocol described in the Matchmaker manual.

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