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# Premature targeting of cell division proteins to midcell reveals hierarchies of protein interactions involved in divisome assembly

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## Summary

**In order to divide, the bacterium *Escherichia coli* must assemble a set of at least 10 essential proteins at the nascent division site. These proteins localize to midcell according to a linear hierarchy, suggesting that cell division proteins are added to the nascent divisome in strict sequence. We previously described a method, 'premature targeting', which allows us to target a protein directly to the division site independently of other cell division proteins normally required for its localization at midcell. By systematically applying this method to probe the recruitment of and associations among late cell division proteins, we show that this linear assembly model is likely incorrect. Rather, we find that the assembly of most of the late proteins can occur independently of 'upstream' proteins. Further, most late proteins, when prematurely targeted to midcell, can back-recruit upstream proteins in the reverse of the predicted pathway. Together these observations indicate that the late proteins, with the notable exception of the last protein in the pathway, FtsN, are associated in a hierarchical set of protein complexes. Based on these observations we present a revised model for assembly of the *E. coli* division apparatus.**

## Introduction

Following the replication and segregation of its DNA to the two halves of the elongating cell, the bacterium *Escherichia coli* begins a complex cell division programme that produces two viable and genetically identical daughter cells. This programme carries out the co-ordinated remodelling of all three layers of the Gram-negative cell envelope, including the inner and outer membrane and the peptidoglycan cell wall. At least 14 proteins are involved in this process, all of which assemble into a ring-like structure at midcell known as the divisome (Goehring and Beckwith, 2005; Vicente *et al*., 2006). Among these proteins, 10 are essential under standard laboratory conditions and localize to midcell according to a largely linear hierarchy (FtsZ > FtsA/ZipA > FtsK > FtsQ > FtsL/B > FtsW > FtsI > FtsN). Within this hierarchy, a given protein requires all upstream proteins (to the left) to localize and is, in turn, required for the localization of proteins further downstream (to the right). Several additional non-essential proteins are assembled as part of this pathway. FtsEX require FtsZ/ZipA/FtsA to localize and in turn help facilitate the localization of FtsK, especially under conditions of low salt (Schmidt *et al*., 2004). The amidase, AmiC, requires FtsN to localize and is implicated in cell separation (Bernhardt and de Boer, 2003). Finally, the FtsZ-binding protein ZapA binds FtsZ directly and is not required for the localization of any other cell division proteins (Gueiros-Filho and Losick, 2002; Johnson *et al*., 2004; Goehring *et al*., 2005).

Although these dependency relationships have been known for some time, we are only beginning to develop a molecular picture of the mechanisms that drive assembly of this ring structure and the arrangement of these proteins within it. The initial steps involving FtsZ and FtsZ-binding proteins are best understood. FtsZ, a tubulin homologue, polymerizes into a highly dynamic ring-like structure at midcell (Mukherjee *et al*., 1993; Lowe and Amos, 1998; Stricker *et al*., 2002; Romberg and Levin, 2003; Mingorance *et al*., 2005). The ring is then stabilized and tethered to the membrane via two membrane-associated proteins, FtsA and ZipA, which bind directly to the C-terminus of FtsZ (Hale and de Boer, 1997; Wang *et al*., 1997; Ma and Margolin, 1999; Mosyak *et al*., 2000; Moy *et al*., 2000; Yan *et al*., 2000; Pichoff and Lutkenhaus, 2002; 2005). This Z-ring and its associated proteins in turn form a scaffold for the assembly of the 'late' or downstream proteins.

The mechanism(s) governing the localization of these late proteins, and how they give rise to the apparent linear assembly pathway remain poorly understood. In fact, several findings indicate that the pathway may not be as linear

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as first expected. Bacterial two-hybrid analysis suggests a highly interconnected network of proteins (Di Lallo *et al*., 2003; Karimova *et al*., 2005) and at least some cell division proteins assemble independently of their normal association with the divisome (Buddelmeijer and Beckwith, 2004; Goehring *et al*., 2005). Moreover, several of the division components, including ZipA and FtsK, can be completely bypassed via suppressor mutations or overexpression of other divisome components (Geissler *et al*., 2003; Geissler and Margolin, 2005). Together these results hint at a model in which multiple cooperative interactions are involved in the recruitment of late proteins.

One problem in addressing assembly of the divisome has been the lack of an efficient assay with which to generate a physical map of the proteins within it. Biochemical approaches have been used with some success, revealing that at least four of the cell division proteins are associated with higher-order complexes. Three divisome components, FtsQ, FtsL and FtsB, can be co-immune precipitated and they and their homologues have been the subject of several *in vitro* studies (Sievers and Errington, 2000; Robson *et al*., 2002; Buddelmeijer and Beckwith, 2004; Noirclerc-Savoye *et al*., 2005). In addition, the division-specific transpeptidase FtsI (PBP3) is likely associated with multiple proteins as it can be cross-linked to a set of proteins involved in peptidoglycan synthesis. These proteins include several other penicillin-binding proteins and murein hydrolases (Holtje, 1996; Alaedini and Day, 1999; Vollmer *et al*., 1999).

We recently described an *in vivo* method, premature targeting, which permits a direct assessment of a protein's ability to recruit other cell division components in the absence of upstream components normally required for its own localization (Goehring *et al*., 2005). In this approach, we localize a protein directly to the Z-ring via fusion to the FtsZ-binding protein ZapA. Upstream components normally required for the protein's recruitment can then be selectively depleted without disrupting the protein's localization at midcell. We can then assess how the localization of other divisome components is affected as these upstream components are removed.

This method was originally applied to FtsQ in order to understand how recruitment of cell division proteins by FtsQ was affected by the depletion of upstream proteins. This analysis showed that forward recruitment of FtsL, FtsB, FtsW and FtsI is independent of FtsA and FtsK, while recruitment of the final protein, FtsN, requires the presence of FtsA at midcell. We also provided evidence that FtsK and FtsQ are likely physically associated within the cell by showing that FtsQ can back-recruit FtsK to the septum in conditions normally non-permissive for FtsK's localization. This association presumably reflects the reverse of the normal pathway in which FtsK recruits FtsQ.

In this work we extend this analysis to four additional late cell division proteins that are broadly conserved across bacterial species. By fusing each in turn to the FtsZ-binding protein, ZapA, we have been able to successfully target these proteins to midcell in the absence of two upstream components, the Z-ring associated protein, FtsA, and the late protein, FtsQ. Analysis of recruitment by these prematurely targeted proteins has revealed several physical associations that drive recruitment and likely underlie assembly of late-division proteins. This new information allows for a more accurate model for divisome formation (see Fig. 5).

## Results

### *Premature targeting of FtsL and FtsB in* ftsA12*(Ts) cells*

Our earlier report on the premature targeting technique involved a ZapA fusion to one cell division protein, FtsQ. In this work, we first extend this approach to the next two proteins in the assembly pathway, FtsL and FtsB. The ability to co-immune precipitate FtsQ, FtsL and FtsB from membrane extracts provided the initial indication that these three proteins are associated in a stable complex (Buddelmeijer and Beckwith, 2004). This conclusion is supported by the observation that a ZapA–FtsQ fusion protein can readily recruit FtsL and FtsB to midcell in the absence of both FtsA and FtsK (Goehring *et al*., 2005). If FtsL, FtsB and FtsQ are in fact part of a stable complex, it is reasonable to expect that a ZapA–FtsL fusion would recruit FtsB and FtsQ under such conditions, while a ZapA–FtsB fusion would recruit FtsL and FtsQ.

We constructed a plasmid expressing a ZapA–FtsL fusion protein (pNG156), in which ZapA is fused in frame to the amino-terminal cytoplasmic tail of FtsL using three asparagines (3×Asn) as a linker. This construction was identical to that used for the original ZapA–FtsQ fusion, except that in this case, the fusion is expressed from a low-copy plasmid rather than in single copy on the chromosome (Goehring *et al*., 2005). When ZapA–FtsQ is expressed from this plasmid, the results are similar to those found with the published chromosomal expression construct (see *Experimental procedures*).

Expression of the ZapA–FtsL fusion in cells functionally depleted for FtsA efficiently restored localization of both FtsQ– and FtsB–GFP fusions (Fig. 1). The appearance of regularly spaced rings of FtsQ and FtsB can be seen in the representative fluorescence images and in the quantification of recruitment as represented by the average ring spacing (total length of cells/total number rings – see *Experimental procedures* and Fig. 1). Positive recruitment is reflected by a ring spacing of approximately 10 μm per ring. This number is derived from the observation that FtsZ rings form with an average spacing of 7–12 μm per

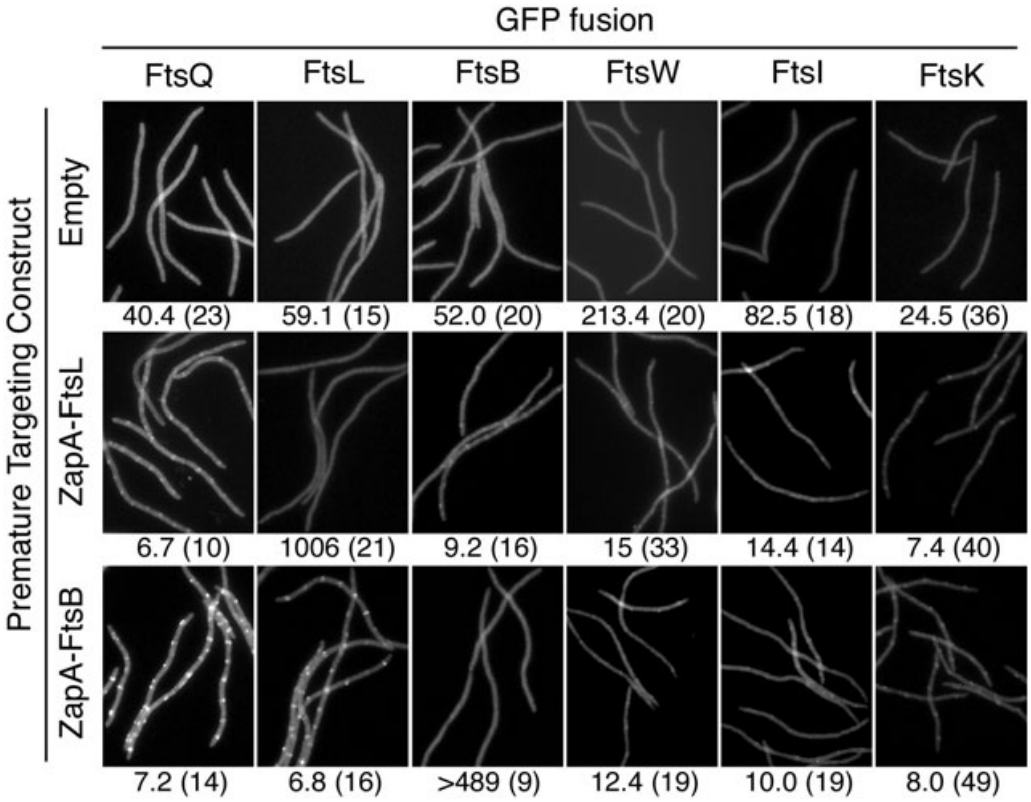


**Fig. 1.** Premature recruitment by ZapA–FtsL and ZapA–FtsB in an *ftsA12*(Ts) strain. Cells were grown at the restrictive temperature of 42°C to inactivate FtsA. For simplicity, we refer to this condition as 'FtsA depletion'. For this and all subsequent experiments, a representative fluorescence micrograph for each condition is shown along with the quantification of ring spacing in μm per ring for that experimental replicate indicated directly below the corresponding image. Ring spacing values from 5 to 15 μm per ring are consistent with a positive localization signal based on previous published values. Values > 20 are considered negative. Values from 15 to 20 are intermediate. The total number of cells analysed for the experimental replicate shown is indicated in parenthesis. Strains and plasmids used: EC455, EC461, JOE95, NWG501, NWG531, NWG567, pNG156, pNG162, pNG170.

ring in cells depleted for any late cell division protein and thus represents optimal recruitment to potential division sites (Weiss *et al*., 1999; Chen and Beckwith, 2001).

We initially constructed a ZapA–FtsB fusion construct (pNG166) in which we simply replaced the FtsL coding sequence with that of FtsB. Analysis of recruitment of FtsL and FtsQ, however, yielded only a faint recruitment signal (data not shown). We considered that this weak signal might be due to the extremely short cytoplasmic tail of the FtsB protein (three amino acids versus >30 in the case of FtsL), which would not allow sufficient distance or flexibility between the transmembrane segment of FtsB and ZapA. Therefore, we created an alternative protein fusion with a larger distance between ZapA and FtsB. This was done by inserting the first cytoplasmic domain of the membrane protein MalF between the sequences of ZapA and FtsB flanked by 3×Asn linkers on either side. Expression of this version of the ZapA–FtsB fusion protein (pNG170) efficiently recruited both FtsL and FtsQ in FtsA-depleted cells (Fig. 1). Thus, both ZapA–FtsL and ZapA–FtsB are targeted to midcell and are able to recruit the other members of the Q–L–B complex as expected. Together with our prior results using the ZapA–FtsQ protein, these results also indicate that as long as one member of the Q–L–B complex is targeted to potential division sites via fusion to ZapA, the remaining two members will be efficiently targeted as well.

We have previously shown that when the Q–L–B complex is targeted using a ZapA–FtsQ fusion in FtsA-depleted cells, not only are two downstream proteins FtsW and FtsI recruited, but FtsK is back-recruited as well. We wished to determine whether the ability of the Q–L–B complex to recruit FtsK, FtsW and FtsI was affected by how the complex was targeted to the division site. Specifically, would ZapA fusions to FtsL and FtsB also result in the localization of this same set of proteins to division sites in FtsA-depleted cells? As expected, all three proteins are efficiently recruited in cells expressing either ZapA–FtsL or ZapA–FtsB (Fig. 1). Thus, localization of any one member of the Q–L–B complex is sufficient to restore the localization not only of the Q–L–B complex but of the majority of late proteins as well.

 

*Detection of an FtsQ-independent interaction between FtsL and FtsB*

We next asked how the ZapA fusions to FtsL and FtsB would behave in cells deficient for FtsQ. Co-immune precipitation suggested that the interaction between FtsL and FtsB is highly dependent on FtsQ, an observation that is not surprising given that the three proteins are present in a stable complex (Buddelmeijer and Beckwith, 2004). Our ZapA fusions to FtsL and FtsB provided an opportunity to assess this interaction directly within cells. Specifically, we could analyse *in vivo* the ability of prematurely targeted FtsL and FtsB to recruit GFP-tagged FtsB and FtsL, respectively, in cells depleted for FtsQ.

Plasmids expressing ZapA fusions to either FtsL (pNG156) or FtsB (pNG170) were introduced into strains expressing GFP– fusions to FtsL or FtsB and which also carried the *ftsQ1*(Ts) allele. Previous work indicated that the level of FtsQ in such a strain is reduced to undetectable levels upon shift to 42°C (Buddelmeijer *et al*., 1998) and that neither FtsL or FtsB is localized under these conditions (Ghigo *et al*., 1999 and Fig. 2A). In these FtsQ-deficient cells, expression of ZapA–FtsL resulted in the localization of FtsB, and expression of ZapA–FtsB resulted in the localization of FtsL (Fig. 2A). We also repeated these experiments in an FtsQ-depletion strain (*ftsQE14::kan*), in which the only copy of wild-type *ftsQ* is placed under the control of a $P_{BAD}$ promoter. In these experiments, transfer of cells into media lacking arabinose results in the repression of the *ftsQ* gene and the eventual depletion of FtsQ protein to undetectable levels (data not shown). Upon sufficient depletion, cells lose the ability to divide and begin growing as filaments. Again, under these depletion conditions, FtsL and FtsB are not localized (Buddelmeijer *et al*., 2002 and Fig. 2B). In this background, expression of ZapA–FtsL restores localization of FtsB, while expression of ZapA–FtsB restores localization of FtsL. Thus, FtsL and FtsB are able to recruit each other, and hence interact, in the absence of FtsQ. These results appear to conflict with our previous report that FtsL and FtsB could not be co-immune precipitated in the absence of FtsQ. However, we have recently discovered that previous failures to co-immune precipitate FtsL and FtsB (Buddelmeijer and Beckwith, 2004) were due to the use of a C-terminal Flag tag on FtsB for detection. This tag

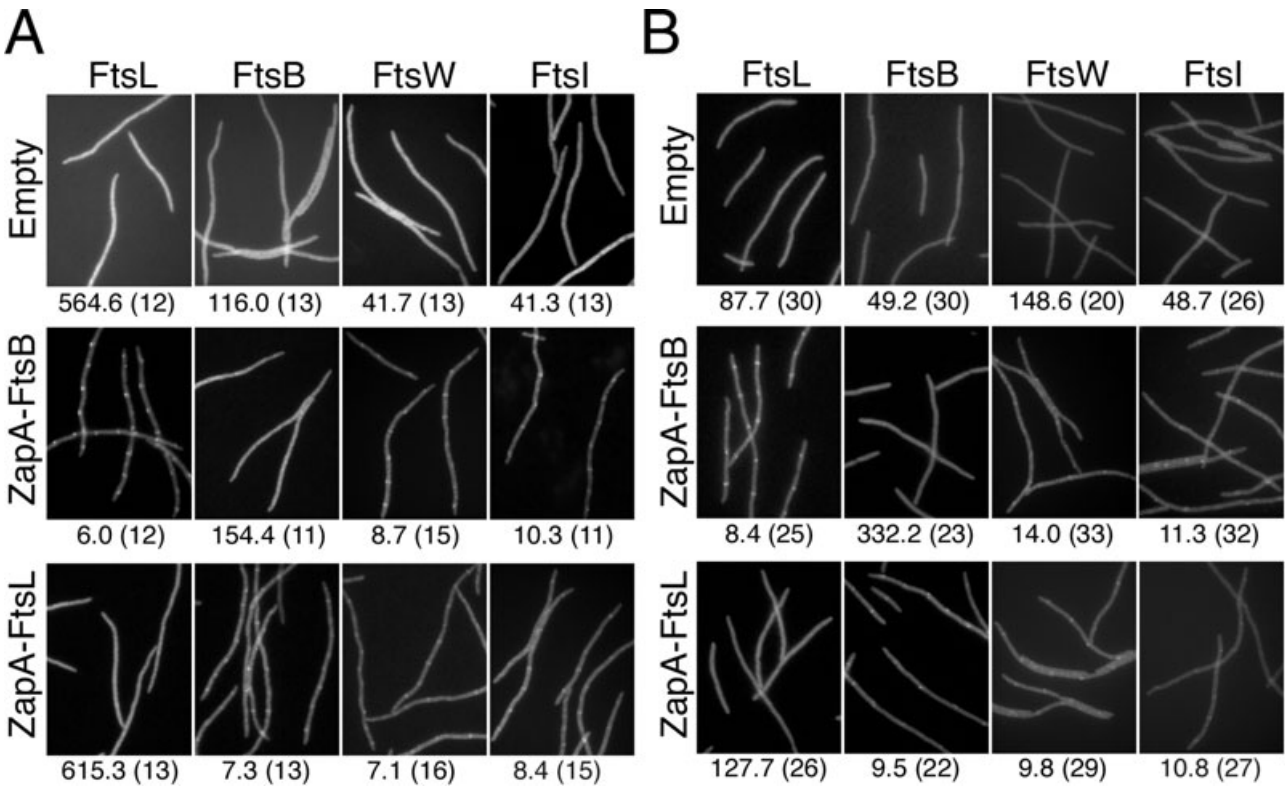


**Fig. 2.** FtsL and FtsB can recruit each other and FtsW and FtsI in the absence of FtsQ.
A. Results for cells carrying the *ftsQ1*(Ts) allele grown at the restrictive temperature of 42°C.
B. Results for cells in which the native *ftsQ* gene is disrupted and in which *ftsQ* expression is provided by an arabinose-inducible construct. Expression has been repressed by growth in glucose-containing media lacking arabinose resulting in depletion of FtsQ protein. Strains and plasmids used: JOE150, JOE153, NWG536, NWG568, NWG577, NWG644, NWG645, pNG156, pNG162, pNG170.

is cleaved under conditions of FtsQ depletion (M. Gonzalez and J. Beckwith, in preparation).

These results, then, raise the question: is formation of the entire Q–L–B complex required for the subsequent recruitment of downstream proteins such as FtsW and FtsI? Or is FtsQ simply required to recruit the FtsL–FtsB subcomplex to the division site, the targeting of which is itself sufficient to recruit downstream proteins? We repeated the above experiment in strains expressing GFP fusions to either FtsW or FtsI. Expression of ZapA–FtsL or ZapA–FtsB proteins resulted in the recruitment of both FtsW and FtsI in both sets of FtsQ-deficient cells (Fig. 2A and B). It must be noted that the ability of ZapA–FtsB to recruit FtsW is somewhat different in the temperature-sensitive and depletion strain backgrounds as quantified by ring spacing and estimated from signal intensity. However, recruitment of FtsI occurs largely to a similar extent. Because FtsI requires FtsW for its recruitment, FtsW is likely being recruited, although perhaps with somewhat different efficiencies in the two depletion conditions. Thus, we conclude that the L–B complex is sufficient to recruit FtsW and FtsI, provided the L–B complex is targeted in some manner to midcell.

### *Premature targeting of FtsW and FtsI*

We next applied this method to address potential recruitment by prematurely targeted FtsW and FtsI. We first determined whether the localization of FtsW via premature targeting would be sufficient for FtsI localization, even in the absence of a variety of upstream proteins. As shown in Fig. 3, expression of ZapA–FtsW results in the efficient localization of GFP–FtsI in cells depleted for either FtsA or FtsQ. We then performed the reciprocal experiment in which we asked whether prematurely targeted FtsI was able to 'back-recruit' FtsW in these same backgrounds. Back-recruitment, too, turned out to be quite efficient (Fig. 3). Hence, the localization of either FtsW or FtsI is sufficient to allow the localization of the other.

As prematurely targeted FtsQ, FtsL and FtsB can recruit FtsW and FtsI, we next asked whether FtsW and FtsI could back-recruit members of the Q–L–B complex. Results of such an analysis should give indication of the ability of these two potential protein complexes to interact. In an *ftsA12*(Ts) background, we detected back-recruitment of FtsQ, FtsL and FtsB by both ZapA–W and ZapA–I at the restrictive temperature, with back-recruitment of FtsQ and FtsL being somewhat more efficient (Fig. 4). In contrast, when cells are depleted for FtsQ, back-recruitment is weaker, with ZapA–FtsW showing some back-recruitment of FtsL and FtsB, and ZapA–FtsI showing no detectable back-recruitment.

We conclude that the Q–L–B and W–I complexes have affinity for one another as targeting of the Q–L–B complex

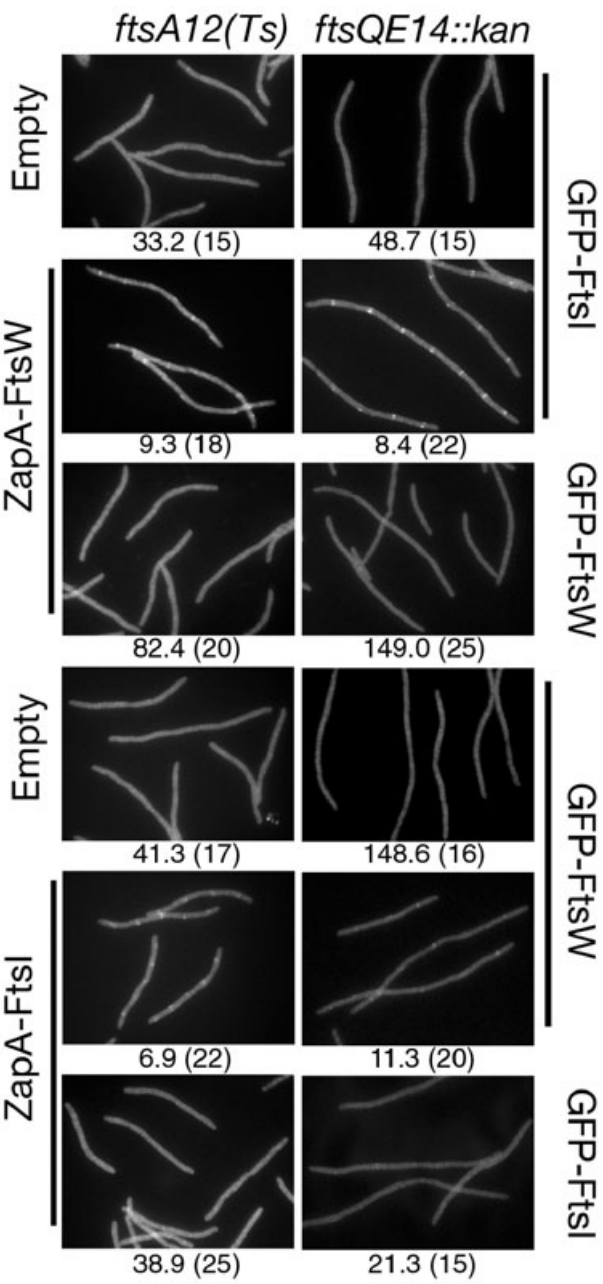


**Fig. 3.** FtsW and FtsI can recruit one another to midcell. The left column shows results for cells carrying the *ftsA12*(Ts) allele grown at 42°C. The right column shows results for cells depleted for FtsQ as in Fig. 2B. Strains and plasmids used: EC455, NWG567, NWG577, NWG646, pNG162, pNG177, pNG178.

results in targeting of the W–I complex and targeting of W–I complex restores localization of the Q–L–B complex in cells depleted for FtsA. It is unclear why forward recruitment of FtsW and FtsI by ZapA–FtsL and ZapA–FtsB is more efficient in the absence of FtsQ than back-recruitment of FtsL and FtsB by ZapA—W and ZapA–I (compare Figs 2 and 4). If these results reflected the simple interaction between two complexes, one would imagine that

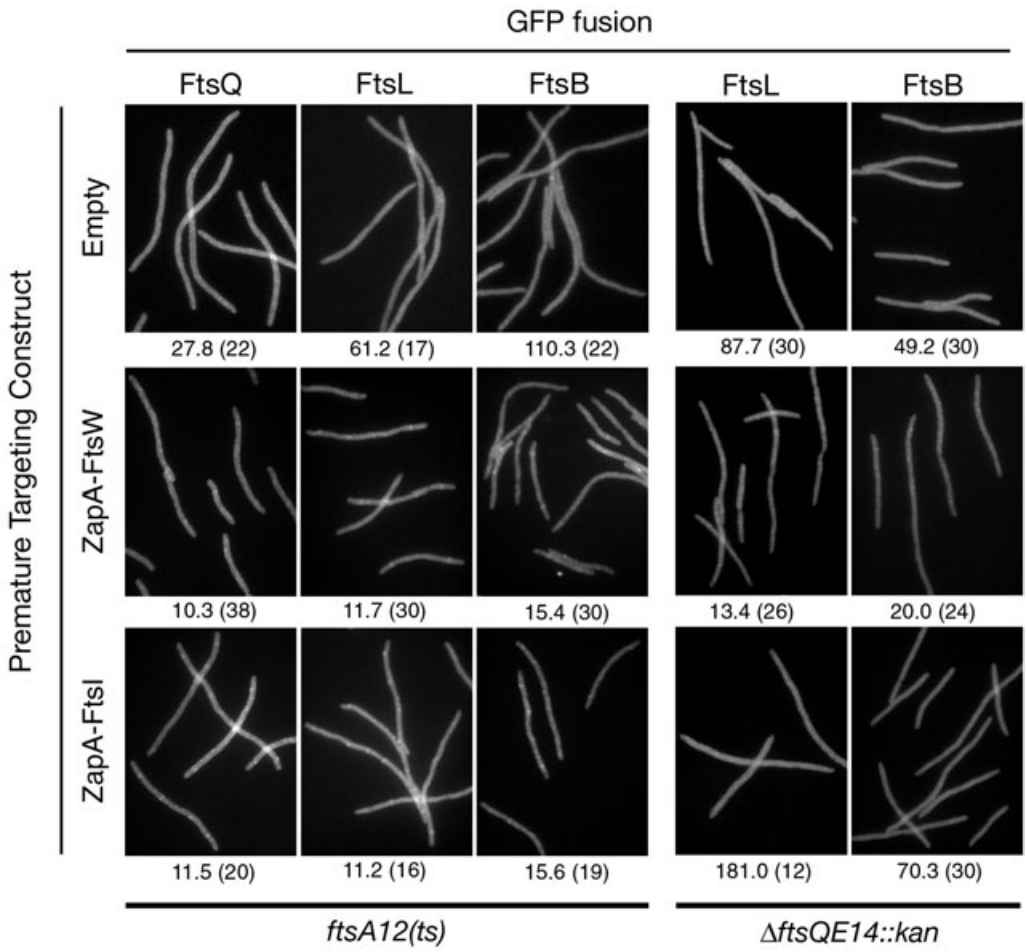


**Fig. 4.** Back-recruitment by ZapA–FtsW and ZapA–FtsI. Results for both *ftsA12*(Ts) cells and cells depleted for FtsQ protein (as in Fig. 2B) are shown. Strains and plasmids used: JOE95, EC461, NWG501, NWG644, NWG645, pNG162, pNG177, pNG178.

the direction of recruitment would be irrelevant. Nonetheless, that back-recruitment between the putative L–B and W–I complexes is inefficient under the same conditions in which efficient reciprocal recruitment is seen within the two protein pairs, suggests at a minimum that the L–B and W–I subcomplexes form independently of one another and are likely discrete entities.

### *FtsN is not recruited by prematurely targeted proteins*

We previously reported that FtsQ, when prematurely targeted in cells deficient for FtsA or both FtsA and FtsK, recruited FtsI but not the next protein in the pathway, FtsN (Goehring *et al.*, 2005). We have extended this analysis to the premature targeting constructs with FtsL, FtsB, FtsW and FtsI in strains depleted for either FtsA or FtsQ. In no case were any of the ZapA– fusions able to recruit FtsN (Table 1 and Fig. S1). Thus, FtsN does not simply follow FtsI to midcell, but requires the presence, at minimum, of FtsA, FtsQ and FtsI at midcell, and possibly many additional proteins.

## Discussion

In building a model for divisome assembly, we must address several questions regarding the localization of cell division proteins to midcell. Is divisome assembly mediated by protein interactions? If so (as most data suggest that it is), among the set of division proteins, which proteins are associated? How do these associations depend on the presence of other proteins? Finally, are proteins pre-assembled into discrete subcomplexes or is the divisome built up one protein at a time as suggested by the linear assembly pathway?

We report here the use of the premature targeting approach to systematically analyse the mechanisms by which the late cell division proteins associate and recruit one another to the division site. By allowing one not only to establish whether two proteins are associated within the cell, but also to determine whether that association depends on the presence, or prior assembly, of other divisome components, this assay overcomes many limitations inherent to previously used methods. Bacterial two-

**Table 1.** Summary of premature targeting results.

| ZapA-Fusion | Depletion | Recruitment[a] of FtsK | FtsQ | FtsL | FtsB | FtsW | FtsI | FtsN |
|---|---|---|---|---|---|---|---|---|
| FtsQ | FtsA[b] | **yes** | no | **yes** | **yes** | **yes** | **yes** | no |
| | FtsQ | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| FtsL | FtsA | **7.4** | **6.7** | 1006 | **9.2** | ***15*** | **14.4** | 70.1 |
| | FtsQ[c] | n/a | n/a | 615/128 | **7.3/9.5** | **7.1/9.8** | **8.4/10.8** | 131 |
| FtsB | FtsA | **8.0** | **7.2** | **6.8** | > 489 | **12.4** | **10.0** | 134 |
| | FtsQ | n/a | n/a | **6.0/8.4** | 154/332 | **8.7/14.0** | **10.3/11.3** | 57.4 |
| FtsW | FtsA | n/d | **10.3** | **11.7** | ***15.4*** | 82.4 | **9.3** | 118 |
| | FtsQ | n/a | n/a | **13.4** | ***20.0*** | 149 | **8.4** | 208 |
| FtsI | FtsA | n/d | **11.5** | **11.2** | ***15.6*** | **6.9** | 38.9 | 88.9 |
| | FtsQ | n/a | n/a | 181 | 70.3 | **11.3** | 21.3 | 49.6 |

**a.** Experiments yielding a positive recruitment signal are indicated in **large bold** and indicate a ring spacing of ~10 μm/ring and enhancement of localization compared negative controls. Experiments yielding no recruitment (ring spacing > 20 μm/ring) are in normal font. Intermediate signals which show significant enhancement compared to negative controls, but exhibit a ring spacing > 15 μm/ring, are in ***bold italic***.
**b.** Results are taken from (Goehring et al., 2005) and data not shown and are presented for purposes of comparison.
**c.** Data from both depletion conditions is shown where appropriate (*ftsQ1*/Δ*ftsQE14::*kan).
n/a, not applicable (these conditions cannot be assayed using this method); n/d, not done.

hybrid experiments can detect potential physical associations within the cell, yet they cannot distinguish between direct and indirect interactions. Moreover, the two-hybrid assays currently in use cannot determine whether these pair-wise interactions depend on other known proteins. Biochemical approaches such as co-immune precipitations and *in vitro* interaction assays using purified proteins have the potential to answer these questions. However, the low abundance of these proteins, that nearly all are membrane-embedded or membrane-associated and that many of the interactions may be highly sensitive to the cellular environment in which the proteins are found, all place limits on our ability to detect these complexes.

We have fused five broadly conserved late-division proteins to the Z-binding protein ZapA and analysed the ability of these proteins, when prematurely targeted to midcell, to recruit other late-division proteins. The results of this analysis, summarized in Table 1, indicate that not only are the late proteins largely physically associated with each other in the cell, but they engage in a hierarchical set of protein interactions.

We confirm the ability of FtsQ, FtsL and FtsB to interact in the absence of other known cell division proteins. This conclusion is based on both the ability to target the Q–L–B complex to midcell in the absence of FtsA and FtsK via premature targeting of any of the component proteins (this work and Goehring *et al.*, 2005) and the ability to co-immune precipitate these proteins in the absence of FtsK, FtsW and FtsI (Buddelmeijer and Beckwith, 2004). The recent reconstitution of the homologous Q–L–B complex from *Streptococcus pneumoniae* indicates that this complex is a broadly conserved unit of the division machinery (Noirclerc-Savoye *et al.*, 2005).

Despite forming a stable complex with FtsQ, we find that FtsL and FtsB interact independently of FtsQ and likely independently of other known cell division components. This observation resolves several recent issues regarding the interactions between FtsL and FtsB. Previous work in *E. coli* suggested that the association of these proteins was dependent on FtsQ. These experiments, however, were unable to provide an explanation for the fact that the stability of FtsL, postulated to require an interaction with FtsB, was maintained in the absence of FtsQ, a condition in which these proteins were observed not to interact (Buddelmeijer and Beckwith, 2004). In *Bacillus subtilis*, because FtsQ is not essential at low temperatures, FtsL and the FtsB homologue DivIC must interact to some degree in the absence of FtsQ *in vivo* (Harry *et al.*, 1993). However, there are contradictory reports in the literature as to the ability of the FtsL and DivIC to interact *in vitro* (Sievers and Errington, 2000; Robson *et al.*, 2002). Here, we assessed recruitment by FtsL and FtsB in the absence of proteins (including FtsQ) that are normally required for their localization to midcell. This allowed us to observe directly the *in vivo* association between FtsL and FtsB in the absence of FtsQ. That the FtsQ-independent interaction between FtsL and FtsB is conserved in both *E. coli* and *B. subtilis* suggests that assembly in these two distantly related organisms follows similar rules, despite the fact that the localization of cell division proteins in *B. subtilis* does not follow a linear recruitment hierarchy as in *E. coli* (Errington *et al.*, 2003).

The finding that FtsW and FtsI can reciprocally recruit each other to the division site is also informative with regard to the assembly pathway. It has been speculated that FtsI-like proteins may be recruited via localized substrate availability, perhaps via substrate export by FtsW-like proteins that are themselves localized at midcell.

 

There is evidence to support this mechanism in *Staphylococcus aureus* (Pinho and Errington, 2005). While the reciprocal nature of recruitment between these proteins does not rule out the contribution of substrate recognition to the assembly or stability of the divisome, it indicates that protein–protein interactions are sufficient to drive localization of FtsI in *E. coli*. This interpretation is consistent with observations that the transmembrane segment of FtsI is sufficient for localization (Piette *et al*., 2004; Wissel *et al*., 2005) and that FtsI can localize even if its active site is blocked by β-lactam antibiotics (Weiss *et al*., 1999). FtsI is likely associated with a variety of other murein-remodelling proteins, including lytic hydrolases and PBP1a,b as part of a cell division peptidoglycan machine (Romeis and Holtje, 1994; Holtje, 1996; Alaedini and Day, 1999; Vollmer *et al*., 1999). Thus, FtsW may well play a molecular scaffolding role, linking this FtsI-containing, peptidoglycan synthesis complex to the upstream Q–L–B complex. Further analysis of recently isolated mutations in FtsW and FtsI using this system may help elucidate the nature of this association and help resolve the potential of substrate binding to contribute to this process (Pastoret *et al*., 2004; Wissel and Weiss, 2004; Wissel *et al*., 2005).

Thus, there appear to be at minimum three distinct subcomplexes that make up the divisome. First, there is the Z-ring-associated proteins composed of FtsZ and the Z-binding proteins FtsA, ZipA and ZapA. Next, there is the Q–L–B complex, which itself contains an independently forming subcomplex containing (and likely consisting of) FtsL and FtsB. Finally, there is a complex containing FtsW and FtsI, which, based on cross-linking experiments and *in vitro* biochemical analysis, likely contains a variety of other peptidoglycan synthesis enzymes. The Q–L–B subcomplex and the W–I complex in turn can associate with each other into a larger divisomal complex of late proteins (for summary, see Fig. 5). Based on the observation of reciprocal recruitment between FtsK and members of the Q–L–B complex, it is reasonable to expect that FtsK is part of this larger complex as well. However, our assay cannot determine whether this association can occur away from the Z-ring.

That most of these cell division components appear fully capable of associating into a relatively large complex in the absence of a Z-ring component essential for their localization to midcell also suggests that this complex of late proteins can be pre-assembled within the cell and then recruited once the Z-ring is established. This model is consistent with the recent observation of a time lag between Z-ring formation and the localization of late proteins (Aarsman *et al*., 2005; Vicente *et al*., 2006). Such a model then raises the critical question of what this complex is doing when it is not present at midcell, a particular concern in cells growing at a slow growth rate where the Z-ring is only present for a fraction of the cell cycle. Specifically, the cell must ensure that this complex of cell division proteins remains inactive until it is targeted to the fully assembled Z-ring at midcell. Failure to do so could lead to unregulated peptidoglycan modification and lysis. In this regard, it is striking that FtsN appears unable to associate with this prematurely targeted complex of late proteins and is unique among late proteins in this aspect. Based on its peculiar localization requirements and its ability to act as a multicopy suppressor of various division defects, we have speculated previously on a possible role for FtsN in regulating constriction initiation (Goehring and Beckwith, 2005; Goehring *et al*., 2005).

Notably, we do not detect dimerization of any late cell division protein under our assay conditions. Two recent bacterial two-hybrid assays detected dimerization of FtsQ

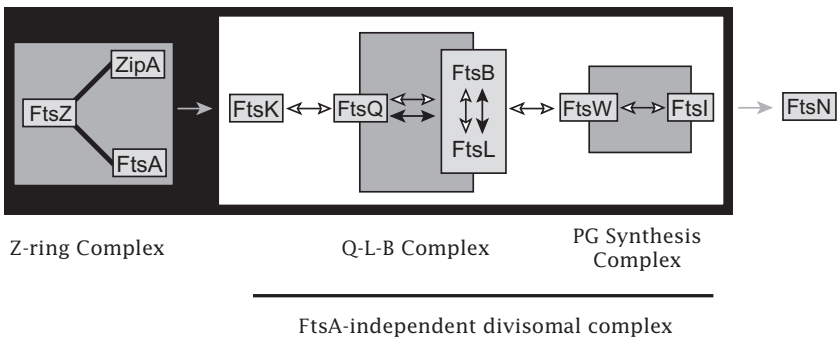


**Fig. 5.** A model for divisome organization. Physical associations underlie the majority of assembly steps and are indicated by double-sided arrows. Solid lines connect FtsZ with FtsA and ZipA, reflecting a verified direct protein–protein interaction. Black closed double arrows indicate proteins that can be co-immune precipitated. Open double arrows represent physical association as measured by reciprocal recruitment in the premature targeting assay. Two recruitment steps, those of FtsK and FtsN, remain uncharacterized and are shown as grey arrows. Given the observed patterns of protein association, divisome assembly involves a hierarchy of protein complex formation. Light grey boxes represent individual division proteins or FtsL/B, which for these purposes can be considered to act as a single unit in the assembly pathway. These units assemble into a set of discrete subcomplexes that are indicated by dark grey boxes. The subcomplexes of late proteins can, in turn, assemble into an FtsA-independent higher-order complex indicated here by the white box. Once the Z-ring is established, this complex can be recruited to midcell. Only after these proteins are all assembled at midcell (represented by the outer black box), can FtsN localize.

 

and one detected dimerization of FtsI (Di Lallo *et al*., 2003; Karimova *et al*., 2005). Neither work detected dimerization of FtsL, FtsB or FtsW. Given the caveats we discuss in our previous work (Goehring *et al*., 2005), our data argue that none of these proteins form homodimers, at least under the conditions in which this assay is performed. Consistent with these observations, we are unable to co-immune precipitate homodimeric FtsQ under conditions that permit co-immune precipitation of the Q–L–B complex (N.W. Goehring, unpubl. results).

This re-examination of recruitment among late proteins yields a picture of divisome assembly (summarized in Fig. 5) that is quite different from what was initially inferred from the linear localization hierarchy, in which assembly order was deduced by determining the set of proteins that localize in each *fts* mutant. Underlying these experiments was the assumption that the set of localizing proteins in a given *fts* mutant comprised a defined assembly intermediate on the path to building the divisome. As such, it appeared that cell division proteins were added to the septum in sequential fashion. However, by focusing only on localization to midcell, some assembly steps that occur among proteins that have not yet been added to the ring structure were missed. In contrast, the premature targeting analysis we present here along with the aforementioned biochemical analyses provides a more complete description of the associations and hierarchies of interactions among late-division proteins that we believe likely drive divisome assembly.

This map of protein associations underlying the assembly process casts doubt on the sequential addition model for divisome assembly, at least for the late division proteins. However, we readily concede that these experiments do not reveal the actual temporal order in which these associations take place within a normal cell. Ribosome assembly was recently revisited with novel tools that allowed the association of all components to be monitored simultaneously (Talkington *et al*., 2005). This analysis provided a previously unattainable kinetic map of the assembly process. Similarly, a complete description of the divisome will require the development of techniques that provide temporal insight into the protein associations, structural changes and peptidoglycan modifications that occur during the assembly process.

## Experimental procedures

### *Bacterial strains, plasmids and media*

Bacterial strains and plasmids used in this work are listed in Table 2. All experiments were performed in NZY medium (Guzman *et al*., 1992). Antibiotics were added when appropriate at the indicated concentrations. D-glucose and L-arabinose were added at 0.2% to repress or induce the expression of genes under control of the $P_{BAD}$ promoter. Isopropyl-β-D-thiogalactoside (IPTG) was added at the appropriate concentrations as indicated.

Standard laboratory techniques were used for cloning and analysis of DNA, PCR, electroporation, transformation and P1 transduction (Miller, 1992). Chromosomal constructs were integrated using λ InCh (Boyd *et al*., 2000). Individual bacterial strains and plasmids were constructed as described or as indicated in Table 2. λ*att* integrated constructs were transduced by P1 using resistance to ampicillin (25 μg $ml^{-1}$). The temperature-sensitive alleles *ftsA12*(Ts) and *ftsQ1*(Ts) were transduced by linkage to *leu*::Tn*10* ($tet^R$) and resulting transductants screened for temperature sensitivity.

The plasmid pNG164 was created by ligation of the EcoRI–HindIII fragment containing the *ftsW* cloning sequence from pDSW311 into pDSW207. The ligation was transformed into *pcnB80* strain DHB250 and plated on media containing 0.2% glucose. pNG164 was integrated using λ InCh except that recombinant λ InCh containing *gfp–ftsW* was recovered directly from DHB250 containing pNG164.

For ease of use, we created a low-copy plasmid to express our ZapA fusions. Briefly, we excised an NsiI–HindIII fragment containing $lacI_Q$ $P_{204}$-*zapA–QQQ* from plasmid pNG132, which is identical to the published plasmid pNG101(Goehring *et al*., 2005) except the $P_{99a}$ promoter was replaced with that from pDSW204 to reduce the expression level. This fragment was ligated into the same sites of pBAD42 replacing an *araC*–$P_{BAD}$ fragment to create pNG153, a low-copy, spectinomycin-resistant plasmid expressing ZapA–QQQ. Expression of ZapA–QQQ from this plasmid resulted observations identical to those produced from previously characterized chromosomally expressed constructs (data not shown). QQQ is described elsewhere (Guzman *et al*., 1997; Chen *et al*., 1999). Briefly, it is an allele of FtsQ containing two additional restriction sites and was used to facilitate cloning steps (Goehring *et al*., 2005).

To create the additional *zapA–* fusion constructs, *ftsL*, *ftsB*, *ftsW* and *ftsI* were excised as EcoRI–HindIII fragments from the respective *gfp–* gene fusions in pDSW207 (Chen *et al*., 1999; Ghigo *et al*., 1999; Weiss *et al*., 1999; this study). Ligation of these fragments into the same sites of pNG93 (Goehring *et al*., 2005) resulted in the formation of *yfp–zapA–* fusion constructs repressed by a $P_{BAD}$ promoter to minimize toxicity (plasmids pNG114, pNG115, pNG135 and pNG150). The *zapA–ftsL* and *zapA–ftsB* regions of these constructs were then subcloned into pNG52 (Goehring *et al*., 2005) as PstI–HindIII fragments to create pNG116 and pNG117. The $P_{99a}$-*zapA–ftsL* region from pNG116 was then introduced into pNG153 using MluI, HindIII sites to create pNG156. The entire $lacI_Q$-$P_{99a}$-*zapA–ftsB* region was subcloned into the NsiI–HindIII sites of pBAD42 to create pNG160. The first cytoplasmic domain of MalF (MDVIKKKHWWQSDAG) was used as an extension between *zapA* and *ftsB*, *ftsW* and *ftsI* to create the final ZapA– fusions. A set of overlapping oligonucleotides encoding 3×Asn in frame with $malF_{cyto}$ was synthesized (F_Eco_F: 5′-aatttaacaacaacatggatgtcattaaaaagaa acattggtggcaaagcgacgccggcc; F_Eco_Rv: 5′-atttggccggcgtc gctttgccaccaatgtttctttttaatgacatccatgttgttgtta) and inserted into the EcoRI site of pNG115, pNG135 and pNG150. The resulting *zapA–*$malF_{cyto}$*–* fusions were introduced into pNG166 replacing the *zapA–ftsB* fusion construct to create pNG170, pNG177 and pNG178. The empty vector control

 

**Table 2.** Strains and plasmids.

| Strain or plasmid | Relevant genetic marker(s) and/or features[a] | Construction, source or reference[b] |
|---|---|---|
| Previously described and/or general strains | | |
| MC4100 | F⁻*araD139* Δ*lacU169 relA1 rpsL150 thi mot flb5301 deoC7 ptsF25 rbsR* | Laboratory collection |
| MM61 | F⁻*araD139* Δ*lacU169* str$^R$*leu*::Tn*10 ftsA12*(Ts) | Laboratory collection |
| EC433 | MG1655 *leu*::Tn*10 ftsQ1*(Ts) | Chen *et al*. (1999) |
| JOE309 | MC4100 *ara*$^+$ | Chen and Beckwith (2001) |
| JOE417 | JOE309 *ftsQE14::kan*/pBAD33-*ftsQ* | Chen *et al*. (2002) |
| EC436 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsI* | Weiss *et al*. (1999) |
| EC438 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsL* | Ghigo *et al*. (1999) |
| JOE86 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsN* | J. Chen (unpublished) |
| NB805 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsB* | Buddelmeijer *et al*. (2002) |
| FX209 | W1485 *thy leu thi deoB/C supE strepR ftsK–yfp (cam$^R$)* | F.X. Barre |
| NWG351 | JOE309 *ftsK–yfp (cam$^R$)* | P1(FX209) × JOE309 |
| NWG563 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsW* | This study |
| FtsA depletion strains | | |
| EC455 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsI leu*::Tn*10 ftsA12*(Ts) | Weiss *et al*. (1999) |
| JOE95 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsQ leu*::Tn*10 ftsA12*(Ts) | Chen *et al*. (1999) |
| EC461 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsL leu*::Tn*10 ftsA12*(Ts) | Ghigo *et al*. (1999) |
| JOE105 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsN leu*::Tn*10 ftsA12*(Ts) | J. Chen (unpublished) |
| NWG501 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsB leu*::Tn*10 ftsA12*(Ts) | P1(MM61) × NB805 |
| NWG531 | JOE309 *ftsK–yfp (cam$^R$) leu*::Tn*10 ftsA12*(Ts) | P1(MM61) × NWG351 |
| NWG567 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsW leu*::Tn*10 ftsA12*(Ts) | P1(MM61) × NWG563 |
| FtsQ depletion strains | | |
| JOE150 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsI leu*::Tn*10 ftsQ1*(Ts) | J. Chen (unpublished) |
| JOE153 | MC4100 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsL leu*::Tn*10 ftsQ1*(Ts) | J. Chen (unpublished) |
| NWG536 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsB leu*::Tn*10 ftsQ1*(Ts) | P1(EC433) × NB805 |
| NWG568 | JOE309 Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsW leu*::Tn*10 ftsQ1*(Ts) | P1(EC433) × NWG563 |
| NWG577 | JOE309 *ftsQE14:kan* Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsI*/pBAD33-*ftsQ* | P1(EC436) × JOE417 |
| NWG578 | JOE309 *ftsQE14:kan* Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsN*/pBAD33-*ftsQ* | P1(JOE86) × JOE417 |
| NWG644 | JOE309 *ftsQE14:kan* Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsL*/pBAD33-*ftsQ* | P1(EC438) × JOE417 |
| NWG645 | JOE309 *ftsQE14:kan* Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsB*/pBAD33-*ftsQ* | P1(NB805) × JOE417 |
| NWG646 | JOE309 *ftsQE14:kan* Δ(λ*attL-lom*)::*bla lacI*$^q$ $P_{207}$-*gfp–ftsW*/pBAD33-*ftsQ* | P1(NWG563) × JOE417 |
| Plasmids | | |
| pDSW204 | IPTG-regulated $P_{Trc}$ promoter, –35 down mutation, ampicillin$^R$ | Weiss *et al*. (1999) |
| pDSW207 | pDSW204-*gfp*–MCS (fusion vector) | Weiss *et al*. (1999) |
| pBAD18 | $P_{BAD}$ ampicillin$^R$ (high copy) | Guzman *et al*. (1995) |
| pBAD42 | $P_{BAD}$ spectinomycin$^R$ (low copy, pSC) | L.M. Guzman |
| pNG52 | pTrc99a-*zapA–myc* | Goehring *et al*. (2005) |
| pNG93 | pBAD18-*gfp–zapA–QQQ* | Goehring *et al*. (2005) |
| pNG101 | pTrc99a-*zapA–QQQ* | Goehring *et al*. (2005) |
| pDSW311 | pDSW209-*ftsW* | Mercer and Weiss (2002) |
| pNG114 | pBAD18-*gfp–zapA–ftsL* | This study |
| pNG115 | pBAD18-*gfp–zapA–ftsB* | This study |
| pNG116 | pTrc99a-*zapA–ftsL* | This study |
| pNG117 | pTrc99a-*zapA–ftsB* | This study |
| pNG132 | pDSW204-*zapA–QQQ* | This study |
| pNG135 | pBAD18-*gfp–zapA–ftsI* | This study |
| pNG150 | pBAD18-*gfp–zapA–ftsW* | This study |
| pNG153 | pSC (Spc$_R$) lacI$_Q$ $P_{204}$-*zapA–QQQ* | This study |
| pNG156 | pSC (Spc$_R$) lacI$_Q$ $P_{99a}$-*zapA–ftsL* | This study |
| pNG162 | pSC (Spc$_R$) lacI$_Q$ $P_{204}$ (empty) | This study |
| pNG164 | pDSW207-*ftsW* | This study |
| pNG166 | pSC (Spc$_R$) lacI$_Q$ $P_{99a}$-*zapA–ftsB* | This study |
| pNG170 | pSC (Spc$_R$) lacI$_Q$ $P_{99a}$-*zapA–$F_{cyto}$–ftsB* | This study |
| pNG177 | pSC (Spc$_R$) lacI$_Q$ $P_{99a}$-*zapA–$F_{cyto}$–ftsI* | This study |
| pNG178 | pSC (Spc$_R$) lacI$_Q$ $P_{99a}$-*zapA–$F_{cyto}$–ftsW* | This study |

**a.** pSC denotes a low-copy pSC101 origin derived from pAM238.
**b.** P1 indicates P1 transduction. For example, to create NWG531, a P1 lysate grown on FX209 was used to infect JOE309.

pNG162 was made by ligating an NsiI–HindIII fragment containing lacI$_Q$ and the promoter region from pDSW204 into pBAD42.

### *Premature targeting assay conditions*

For depletion experiments utilizing temperature-sensitive alleles, single colonies were resuspended into NZY media containing 0.2% glucose plus 50 μg ml$^{-1}$ spectinomycin and grown in a shaking water bath at 30°C either overnight (*ftsA12*) or until $OD_{600}$ ~ 0.5 (*ftsQ1*). Cultures were adjusted to approximately equal density and back-diluted 1:100 into fresh media for 3 h at which time cultures were diluted 1:5 into media pre-warmed to 42°C and grown in a 42°C shaking

 

water bath for 60–70 min at which point extensive filamentation was evident. IPTG was added to a concentration of 20 μM for the final 30 min of growth to induce both *gfp*– and *zapA*– fusions.

For depletion experiments using conditional expression from $P_{BAD}$ promoters, single colonies were resuspended into NZY media containing 10 μg ml$^{-1}$ chloramphenicol and 50 μg ml$^{-1}$ spectinomycin supplemented with 0.2% arabinose and grown at 30°C in a shaking water bath. Once cultures reached an $OD_{600}$ of ~0.5, cultures were adjusted to approximately equal density and back-diluted 1:100 into fresh arabinose-supplemented media for 3 h. Cultures were then diluted 1:200 into pre-warmed (30°C) media supplemented with 0.2% glucose and returned to 30°C. Depletion was monitored by microscopy until filamentation was clearly visible, usually ~4 h, at which point IPTG was added to a final concentration of 20 μM and the cells were grown 30–35 min. Depending on the fusion construct, expression varied from slightly less than wild type to several-fold higher (data not shown).

In order to control for day-to-day variation in the extent of depletion, ZapA fusion-expressing strains were always paired with control strains carrying empty vectors. If possible, all strains for a given depletion condition were grown together.

### *Microscopy*

Cells were harvested, fixed (Chen *et al*., 1999) and mounted on agarose cushions for microscopy as described (van Helvoort and Woldringh, 1994). In some cases, cells were stained with 0.2 μg ml$^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) in PBS and washed twice in PBS before mounting. Cells were examined for fluorescence using an Axioskop 2 microscope (Zeiss) equipped with a 100× plan-Apochromat oil immersion objective and a 100 W mercury lamp. Filter sets to visualize EGFP (HQ:FITC/Bodipy/Fluo3), EYFP and DAPI (UV) were from Chroma Technology. Images were captured using an Orca-100 CCD camera (Hamamatsu Photonics) and Openlab (Improvision) and subsequently processed and analysed in Openlab. All measurements were limited to intact cells exhibiting normal segregation of nucleoids as evidenced by DAPI staining. Ring spacing is calculated as the total length of all cells measured divided by the total number of rings observed. In all cases, experiments were verified multiple times and data from representative experimental replicates are shown. Final processing of images for presentation was performed in Adobe Photoshop.

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** FtsN is not recruited when late proteins are prematurely targeted to midcell.

This material is available as part of the online article from http://www.blackwell-synergy.com

 