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# Polymer Stability Plays an Important Role in the Positional Regulation of FtsZ

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**We conducted a series of experiments examining the effect of polymer stability on FtsZ localization dynamics in *Bacillus subtilis*. A loss-of-function mutation in *ezrA*, a putative polymer-destabilizing factor, suppresses the defects in FtsZ polymer stability associated with *minCD* overexpression. In addition, a mutation that is predicted to stabilize the FtsZ polymer leads to the formation of polar FtsZ rings. These data support the hypothesis that carefully balanced polymer stability is important for the assembly and localization of FtsZ during the bacterial cell cycle.**

Cell division is tightly regulated to ensure accurate and efficient segregation of chromosomal material and to maintain cell size and shape. Throughout the bacterial and archaeal kingdoms, the position of the division septum appears to be controlled largely via the localization of the tubulin-like GTPase FtsZ (17). In response to one or more cell cycle signals, FtsZ forms a ring at the nascent division site. This ring then serves as a framework for assembly of the division apparatus (17, 30). Although FtsZ has been shown to be required for cell division in several organisms, the presence of an FtsZ ring alone is not sufficient for cytokinesis (14, 17).

Two factors are known to play important roles in modulating the position of the division septum in the gram-positive bacterium *Bacillus subtilis*: MinC and MinD, which function as a complex (18), and the 65-kDa protein EzrA (13). MinCD is concentrated at the cell poles, where it inhibits aberrant FtsZ ring formation (24). Null mutations in *minC* or *minD* lead to the formation of polar FtsZ rings and polar septa (12, 15, 16, 27). *Escherichia coli* MinC inhibits FtsZ polymerization in vitro (9), and it is likely that MinCD inhibition of polar FtsZ ring formation in *B. subtilis* also takes place through direct interactions between FtsZ and MinC. The polar localization of MinCD in *B. subtilis* is dependent on the 164-residue DivIVA, whereas in *E. coli* the MinCD complex is kept away from midcell by MinE, an 88-residue protein that shares no apparent homology with DivIVA (7, 24).

Null mutations in *ezrA*, like those in *minCD*, lead to the formation of polar FtsZ rings (13). However, in contrast to MinCD, EzrA is distributed throughout the plasma membrane and is concentrated at the nascent septal site in an FtsZ-dependent manner (13). A *minCD ezrA* double mutant displays a more severe division defect than either single mutant, suggesting that the two factors act independently to inhibit inappropriate FtsZ ring formation. The loss of EzrA apparently results in hyperstabilization of the FtsZ polymer. First, a null mutation in *ezrA* suppresses the temperature-sensitive phenotype of a conditional allele of *ftsZ* (13). Also, the loss of *ezrA* significantly lowers the concentration of FtsZ required to initiate ring formation in vivo (13). These data support a model in which EzrA acts throughout the plasma membrane to destabilize the FtsZ polymer and inhibit inappropriate FtsZ assembly. At the same time, however, a positively acting factor (perhaps a component of the putative FtsZ nucleation site) overcomes EzrA inhibition at midcell, permitting the formation of a medial FtsZ ring even in the presence of EzrA. Although it does not prevent FtsZ assembly at midcell, EzrA presumably contributes to the dynamic nature of the medial FtsZ ring.

Our observation that a loss-of-function mutation in *ezrA*, a putative destabilizer of the FtsZ polymer, leads to the formation of polar FtsZ rings led us to consider the idea that polymer stability plays an important role in the spatial regulation of FtsZ. In support of this model, increasing the intracellular concentration of FtsZ leads to polar divisions and the formation of anucleate minicells in *E. coli* (29) and the formation of polar FtsZ rings in *B. subtilis* (P. A. Levin, unpublished data). A twofold increase in expression of the FtsZ-stabilizing protein ZipA in *E. coli* also results in the formation of polar FtsZ rings (19). In this report, we examine the effect of factors predicted to stabilize the FtsZ polymer on FtsZ assembly and localization dynamics.

**Overexpression of *minCD* leads to filamentation in *B. subtilis*.** Overexpression of *minCD* in *E. coli* leads to extensive filamentation and cell death (3, 11). Similarly, Marston and Errington have shown that overexpression of either *GFP-minD* alone or *GFP-minCD* is sufficient to inhibit cell division in otherwise wild-type *B. subtilis* cells (18). To establish that overexpression of wild-type *minCD* is sufficient to inhibit cell division in *B. subtilis*, we constructed a strain in which a second copy of *minCD* was placed under the control of a modified version of the LacI-repressible, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter $P_{spac}$ (J. D. Quisel, W. Burkholder, and A. D. Grossman, submitted for publication) at the amylase locus (PL1138). This promoter ($P_{spac\text{-}hy}$) is 10- to 20-fold stronger than $P_{spac}$ (Quisel et al., submitted).

Induction of $P_{spac\text{-}hy}$-*minCD* strongly inhibited cell division, resulting in severe filamentation and eventually cell death.

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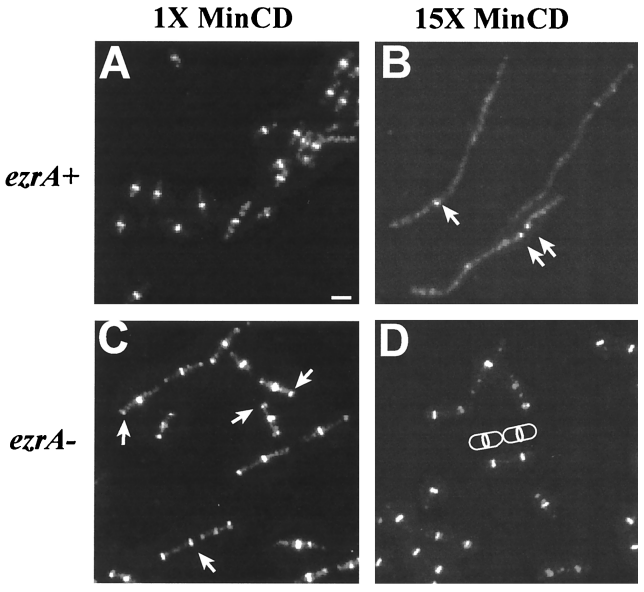


FIG. 1. Stabilization of the FtsZ polymer in the absence of *ezrA* suppresses the division defect associated with *minCD* overexpression. The figure shows the immunolocalization of FtsZ in cells in which *minCD* expression is under the control of a strong IPTG-inducible promoter. Cells were grown to an $OD_{600}$ of approximately 0.5 in Luria-Bertani medium, after which they were resuspended at a 1:10 dilution in fresh medium in the absence (A and C) or presence (B and D) of 1 mM IPTG. Samples were taken 90 min after resuspension, fixed in methanol, and stained essentially as described elsewhere (13). (A and B) PL1138 (*amyE*::$P_{spac\text{-}hy}$-*minCD ezrA*$^+$); (C and D) PL1152 (*amyE*::$P_{spac\text{-}hy}$-*minCD ezrA*::*spc*). Arrows point to rings of FtsZ in panel B and examples of polar rings of FtsZ in panel C. A cartoon illustrates a pair of cells with medial rings in panel D. Bar = 2 μm.

Viability assays indicated that the plating efficiency of this strain (PL1138) is 50- to 100-fold lower in the presence of 1 mM IPTG than in the absence of inducer. Cells were sampled from a mid-exponential-phase culture (optical density at 600 nm [$OD_{600}$] of 0.5) and serially diluted onto solid medium in the presence or absence of IPTG. The CFU per milliliter were $1.7 \times 10^5$ in the absence of IPTG and $4.0 \times 10^3$ in the presence of IPTG for the $P_{spac\text{-}hy}$-*minCD ezrA*::spc strain. In the absence of IPTG, MinD levels in these cells were at most twofold above wild type, as measured by quantitative immunoblotting using antibodies against MinD (data not shown). However, in the presence of IPTG, the intracellular concentration of MinD was increased approximately 15-fold within 90 min of induction (data not shown).

**Overexpression of *minCD* inhibits FtsZ ring formation.** To examine the effect of *minCD* overexpression on FtsZ ring formation, we used antibodies against FtsZ and immunofluorescence microscopy to localize FtsZ in cells encoding $P_{spac\text{-}hy}$-*minCD* grown in the absence and presence of IPTG (Fig. 1A and B). We found that overexpression of *minCD* severely inhibited FtsZ ring formation (Fig. 1B). Filaments examined 90 min after induction of *minCD* were 10 to 15 times longer than wild-type cells. Before induction, the frequency of FtsZ ring formation in this strain background was approximately one ring per 1.3 cell lengths. After induction of *minCD*, the frequency of FtsZ ring formation fell to less than one ring per 6 cell lengths. (Average cell length was determined by measuring wild-type cells grown under conditions identical to those used to induce $P_{spac\text{-}hy}$-*minCD*). These data suggest that an increase in the intracellular concentration of MinCD leads to a mislocalization of the complex and prevents FtsZ from forming stable rings at the nascent division site.

The target of MinCD division inhibition in *E. coli* is somewhat controversial. Biochemical data indicate that *E. coli* MinC is sufficient to inhibit FtsZ assembly in vitro (9). However, genetic and cell biological data suggest that the primary target of the MinCD division inhibitor in *E. coli* may not be FtsZ directly but rather the interaction between FtsZ and the putative polymer-stabilizing protein FtsA (11). In contrast to *E. coli*, FtsA is not essential for viability in *B. subtilis* (1), and although we cannot rule out the possibility that FtsA is also involved, we believe our data favor the hypothesis that FtsZ is the primary target of MinCD in *B. subtilis*.

**A null mutation in *ezrA* suppresses the growth defect associated with *minCD* overexpression.** A null mutation in *ezrA* leads to the formation of polar FtsZ rings in *B. subtilis* (13). Although other interpretations are possible (e.g., EzrA binds to and inactivates a membrane-bound FtsZ localization determinant at cell poles), these data suggest that an EzrA deficiency may stabilize FtsZ polymers such that they are resistant to MinCD activity at the cell poles, leading to the formation of polar FtsZ rings. We determined whether the increase in polymer stability observed in the absence of EzrA was sufficient to overcome the inhibition of FtsZ polymerization at midcell resulting from overexpression of *minCD*.

As expected, in the absence of IPTG, the *ezrA* null mutant (PL1151 *amyE*::$P_{spac\text{-}hy}$-*minCD ezrA*::*spc*) exhibits a typical *ezrA* phenotype with regard to the frequency and position of FtsZ ring formation (Fig. 1C). Cells often had an FtsZ ring at one or both poles in addition to the medial ring, and the frequency of FtsZ ring formation was approximately one ring per 0.8 cell lengths, consistent with the high frequency of FtsZ ring formation.

In contrast to the congenic *ezrA*$^+$ strain (PL1138), the plating efficiency of the *ezrA* null mutant is identical in the presence and the absence of IPTG, indicating that the loss of *ezrA* completely suppresses the growth defect associated with overexpression of *minCD*. The CFU per milliliter were $4.2 \times 10^8$ in the absence of IPTG and $3.9 \times 10^8$ in the presence of IPTG for the $P_{spac\text{-}hy}$-*minCD ezrA*::*spc* strain. Consistent with this observation, immunofluorescence microscopy indicates that the loss-of-function mutation in *ezrA* restores normal FtsZ ring formation to cells in the presence of excess MinCD (Fig. 1D). The frequency of FtsZ ring formation in the *ezrA* null mutant background in the presence of 1 mM IPTG was one ring per cell length, approximately sixfold higher than the *ezrA*$^+$ background. Polar FtsZ rings were not visible in the *ezrA* null cells following induction of *minCD,* indicating that the loss of one division inhibitor (EzrA) can be compensated for by increasing the level of a second division inhibitor (MinCD). Quantitative immunoblotting demonstrates that there is no difference in MinCD concentration in the presence and the absence of *ezrA* (data not shown). These results suggest that the loss of EzrA stabilizes the FtsZ polymer to an extent sufficient to overcome the inhibition of ring formation associated with *minCD* overexpression.

**A mutation in the GTP binding site of *B. subtilis* FtsZ suppresses the *minCD* overexpression phenotype.** FtsZ polymerizes in vitro in a GTP-dependent manner (5, 17). Work with GTPase-defective alleles of *ftsZ* suggests that GTP hydrolysis is essential for turnover of FtsZ polymers in vitro and for FtsZ activity in vivo (6, 20, 26). Structural and biochemical data indicate that dimerization of FtsZ creates an active site for the hydrolysis of a single shared GTP molecule (8). GDP-bound multimers of FtsZ are unstable and quickly fall apart (21, 22, 25). Factors that inhibit GTPase activity but do not affect GTP binding are, therefore, expected to stabilize the FtsZ polymer.

Overexpression of *minCD* in *E. coli* can be suppressed by four- to fivefold overexpression of *ftsZ* (4) or by one of several *rsa* (also called *sulB*) mutations in *ftsZ* (10) originally isolated on the basis of their ability to suppress filamentation caused by expression of the division inhibitor SulA (2, 4). Although the resistance of these *ftsZ* alleles to *minCD* overexpression could be explained by the loss of residues important for FtsZ-MinCD interaction, we favor the hypothesis that the *rsa*/*sulB* mutations in *ftsZ* stabilize the FtsZ polymer, rendering it resistant to both MinCD and SulA. Consistent with this model, six of the *E. coli rsa*/*sulB* mutations have been shown to alter the interaction between FtsZ and GTP and several of them permit GTP binding but specifically inhibit hydrolysis (6, 26).

To determine if a similar mutation in the GTP binding site of *B. subtilis ftsZ* can also render cells insensitive to *minCD* overexpression, we constructed an allele of *B. subtilis ftsZ* (D213G) corresponding to *E. coli ftsZ2* (D212G) based on sequence alignment data (8; H. P. Erickson, personal communication). *E. coli* FtsZ2 binds GTP almost as well as wild-type FtsZ but is at least 200-fold reduced in GTPase activity (6, 26). Although we cannot be certain that the D213G mutation in *B. subtilis* FtsZ has a phenotype identical to that of *E. coli* FtsZ2 with regard to GTP binding and hydrolysis, Asp212 is conserved among all known FtsZs and has been implicated in coordinating a metal ion and the nucleotide at the interface of two FtsZ monomers (23). A similar mutation in *Caulobacter crescentus ftsZ* (DVR216AVA) functions as a dominant lethal mutation when expressed in conjunction with wild-type *ftsZ,* suggesting that it incorporates into the FtsZ ring at midcell and prevents division altogether (28). Like *E. coli ftsZ2*, *B. subtilis ftsZ2* is not able to support cell division in the absence of wild-type *ftsZ* (data not shown).

To test the ability of *B. subtilis ftsZ2* to suppress *minCD* overexpression, *ftsZ2* was placed under the control of a xylose-inducible promoter at the threonine locus. Using this construct in conjunction with $P_{spac\text{-}hy}$-*minCD* (strain PL1190), we found that, like its *E. coli* counterpart, *B. subtilis ftsZ2* is able to suppress the division defect and restore ring formation to cells in the presence of excess MinCD (Fig. 2A and B). In contrast, expression of a second copy of wild-type *ftsZ* from the same xylose promoter in an identical strain background (PL1192) was not sufficient to overcome the actions of the MinCD division inhibitor (Fig. 2C and D). FtsZ protein levels are identical in the $P_{xyl}$-*ftsZ* and $P_{xyl}$-*ftsZ2* strains in the presence of xylose (data not shown). Thus, suppression of the lethality associated with *minCD* overexpression by *ftsZ2* is not simply the result of an increase in FtsZ protein levels. Instead, these results support our model that an increase in FtsZ polymer stability in the presence of the GTPase-defective FtsZ protein overcomes the effect of *minCD* overexpression.

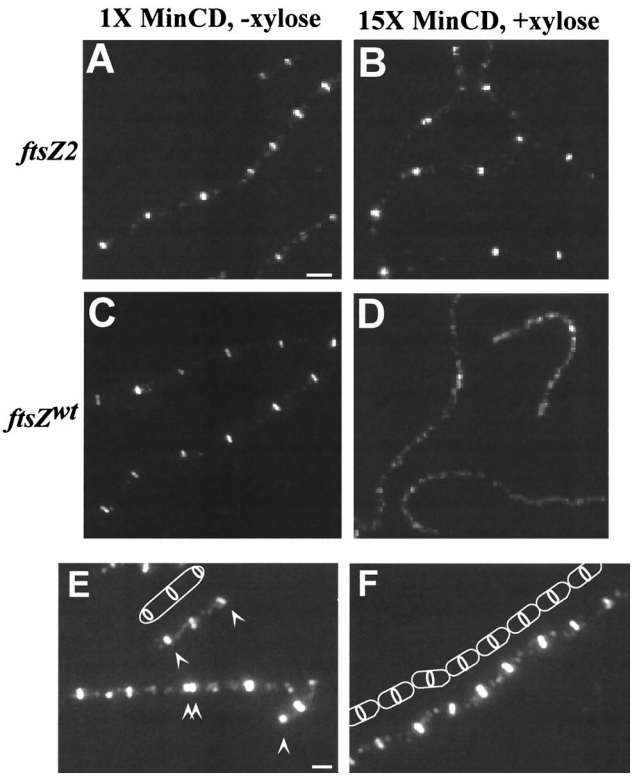


FIG. 2. The GTPase-defective allele of *ftsZ* (*ftsZ2 D213G*) suppresses the division defect associated with *minCD* overexpression and leads to the formation of aberrant polar FtsZ rings. (A to D) Immunolocalization of FtsZ in cells encoding both a copy of *minCD* under the control of a strong IPTG-inducible promoter at the *amyE* locus and a second copy of *ftsZ* under the control of a xylose-inducible promoter at the *thrC* locus. Cells were grown to an $OD_{600}$ of approximately 0.5 in Luria-Bertani medium and diluted 1:10 in fresh medium in the absence (A and C) or presence (B and D) of 1 mM IPTG and 0.5% xylose. Cells were fixed and stained for immunofluorescence microscopy 90 min after resuspension. (A and B) PL1190 (*amyE*::$P_{spac\text{-}hy}$-*minCD thrC*::$P_{xyl}$-*ftsZ2*). (C and D) PL1192 (*amyE*::$P_{spac\text{-}hy}$-*minCD thrC*::$P_{xyl}$-*ftsZ*). Bar = 2 μm. (E and F) Immunolocalization of FtsZ in cells expressing either a copy of the GTPase-defective *ftsZ2* allele, $P_{xyl}$-*ftsZ2* (E), or a second copy of wild-type *ftsZ*, $P_{xyl}$-*ftsZ2* (F), under the control of a xylose-inducible promoter at *thrC*. Cells were grown to an $OD_{600}$ of approximately 0.5 in Luria-Bertani medium, diluted 1:10 in fresh medium in the presence of 0.5% xylose, and fixed and stained for immunofluorescence microscopy 90 min after resuspension. Arrows point to polar rings of FtsZ in panel E. Cartoons illustrate a cell with medial and polar FtsZ rings in panel E and cell boundaries and FtsZ rings in the chain of cells in panel F. Bar = 1 μm.

**The GTPase-defective allele of *B. subtilis ftsZ* leads to the formation of extra FtsZ rings at polar positions in otherwise wild-type cells.** Based on data from the *ezrA* null mutant, we predicted that stabilization of the FtsZ polymer by *ftsZ2* (and not $ftsZ^{wt}$) would lead to the formation of extra FtsZ rings at polar positions in otherwise wild-type cells. Using a strain (PL1187) encoding the xylose-inducible allele of *ftsZ2* at the threonine locus (*thrC*::$P_{xyl}$-*ftsZ2*), we found that a 90-min induction was sufficient to cause the formation of extra FtsZ rings at cell poles, similar to those observed in *ezrA* null mutant cells (Fig. 2E). In contrast, a congenic strain (PL1118) encoding a wild-type allele of *ftsZ* under the same xylose promoter

(*thrC*::$P_{xyl}$-*ftsZ*) had only single medial FtsZ rings after 90 min of growth in the presence of xylose (Fig. 2F). Quantitative immunoblotting showed no difference in FtsZ protein levels between the *thrC*::$P_{xyl}$-*ftsZ* strain and the *thrC*::$P_{xyl}$-*ftsZ2* strain in the presence of xylose (data not shown).

**Polymer stability and the assembly and localization of FtsZ.** Our results suggest that the relative stability of the FtsZ polymer plays an important role in modulating the frequency and position of FtsZ ring formation. Both the loss of *ezrA*, an inhibitor of FtsZ ring formation, and a mutation in *ftsZ* that is predicted to stabilize the FtsZ polymer, *ftsZ2,* lead to the formation of aberrant polar FtsZ rings. These aberrant polar FtsZ rings are apparently the result of the hyperstable FtsZ polymer overcoming MinCD inhibition at cell poles. Consistent with this, both an *ezrA* null mutation and coexpression of *ftsZ2* with wild-type *ftsZ* are able to suppress the growth defect associated with *minCD* overexpression, restoring normal growth and ring formation in the presence of excess MinCD.

These data support a model in which the localization of FtsZ, and hence the spatial regulation of cell division, is governed by an assortment of factors that modulate FtsZ polymer dynamics throughout the cell and throughout the cell cycle. The activity of some factors, like MinCD, is normally restricted to one region of the cell, while others, like EzrA, function in a more global manner, to alter the concentration of FtsZ required for assembly at the plasma membrane. This model predicts the existence not only of negative-acting factors that inhibit FtsZ ring formation but also of positive-acting factors, such as components of the putative FtsZ nucleation site at midcell, that promote FtsZ ring formation, ensuring that it occurs at both the correct position and the appropriate stage of the cell cycle.

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