MinC and FtsZ mutant analysis provides insight into MinC/MinD-mediated Z ring disassembly

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The Min system negatively regulates the position of the Z ring, which serves as a scaffold for the divisome that mediates bacterial cytokinesis. In Escherichia coli, this system consists of MinC, which antagonizes assembly of the tubulin homologue FtsZ. MinC is recruited to the membrane by MinD and induced by MinE to oscillate between the cell poles. MinC is a dimer with each monomer consisting of functionally distinct MinCN and MinCC domains, both of which contact FtsZ. According to one model, MinCC/MinD binding to the FtsZ tail positions MinCN at the junction of two GDP-containing subunits in the filament, leading to filament breakage. Others posit that MinC sequesters FtsZ–GDP monomers or that MinCN caps the minus end of FtsZ polymers and that MinCC interferes with lateral interactions between FtsZ filaments. Here, we isolated minC mutations that impair MinCN function and analyzed FtsZ mutants resistant to MinC/MinD. Surprisingly, we found mutations in both minC and ftsZ that differentiate inhibition by MinC from inhibition by MinD/MinD. Analysis of these mutations suggests that inhibition of the Z ring by MinC alone is due to sequestration, whereas inhibition by MinC/MinD is not. In conclusion, our genetic and biochemical data support the model that MinC/MinD fragments FtsZ filaments.

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in the presence of MinC, they argued against a severing mechanism that should produce a random distribution.

To investigate the MinC mechanism under more physiological conditions, Arumugam et al. (17) devised reconstitution experiments in which FtsZ filaments were artificially directed to a planar membrane. A 1:1 mixture of FtsZ–YFP–MTS (the membrane targeting sequence from Escherichia coli MinD) and FtsZ, or alternatively FtsZ–MTS and FtsZ–F268C–Cy5, was added to a supported lipid bilayer. Once a stable but dynamic FtsZ bundle network emerged, enhanced green fluorescent protein–MinC was introduced to induce disassembly. Unlike FtsZ in solution, FtsZ–YFP–MTS has a low critical concentration (0.1 μM) for polymerization likely due to membrane interaction. A kinetic analysis of the concentration- and time-dependent disassembly indicated that MinC decreases the FtsZ attachment rate and increases the detachment rate. Because no interaction between MinC and FtsZ–F268C–Cy5 was observed in solution, they excluded a sequestration mechanism. Instead, they proposed that MinCC promotes subunit detachment by decreasing bundling and that MinCN binds to the minus end of FtsZ filaments to hinder the addition of new subunits (capping).

To gain further insight into the mechanism of FtsZ depolymerization mediated by MinCN, we isolated additional mutations in this domain and further characterized FtsZ mutants identified by Shen and Lutkenhaus (13, 14) that provide resistance to MinC/MinD. Our results indicate that depolymerization of FtsZ by MinC is distinct from that caused by MinC/MinD. Inhibition of FtsZ assembly by MinC is due to sequestration, whereas inhibition by MinC/MinD is not due to sequestration or capping the ends of FtsZ filaments. Our genetic and biochemical evidence, instead, points to the idea that MinC/MinD fragments FtsZ filaments.

Results

Isolation of minC mutations affecting MinCN activity

Only one point mutation affecting the MinCN domain (minCG10D) has been characterized so far (6, 18). To try and gain additional insight into the mechanistic basis of FtsZ inhibition by MinC, we isolated additional mutations in this domain and further characterized FtsZ mutants identified by Shen and Lutkenhaus (13, 14) that provide resistance to MinC/MinD. Our results indicate that depolymerization of FtsZ by MinC is distinct from that caused by MinC/MinD. Inhibition of FtsZ assembly by MinC is due to sequestration, whereas inhibition by MinC/MinD is not due to sequestration or capping the ends of FtsZ filaments. Our genetic and biochemical evidence, instead, points to the idea that MinC/MinD fragments FtsZ filaments.
there is no selection for such mutants it was necessary to find conditions to distinguish them from clones with no mutation as well as clones with mutations inactivating MinCC. The desired mutants should display reduced toxicity (loss of MinCC function) but localize to the Z ring in the presence of MinD (retain MinCC function and therefore some toxicity) (13, 14). Mutagenized minC was cloned into pMCW26 (P\textsubscript{lac}:gfp-minC\textsuperscript{G10D}/minD) to replace minC\textsuperscript{G10D} and the mutant library transformed into JS964 (\Delta min/pSB12) containing the min operon as well as derivatives with minC mutations. The white arrows indicate minicells. C, quantitation of the minicell production by the of cells shown in B was done by counting the number of polar septa. The frequency of polar septa is shown on the x axis (n > 600 cells from two separate cultures for each).

### Identification of an inhibitory region within MinCN that is critical for Z ring regulation

The MinCN domain from Salmonella typhimurium (PDB code 3GHF) and E. coli (PDB code 4L1C) crystallize as dimers due to domain swapping of the first β strand of each subunit (Fig. S2) (20). We crystallized MinC\textsuperscript{101} and also obtained a domain-swapped dimer (data not shown). We observed that MinC\textsuperscript{101} forms a dimer in the presence of high salt, but this was prevented by the presence of FtsZ. In the structure of full-length MinC from Thermotoga maritima (PDB code 1HF2) the MinCN domain does not form a swapped dimer (21). For these reasons, it is likely that the swapped MinCN dimer observed with the enteric MinCs is a crystallographic artifact.

The two residues, Lys-35 and Ala-39, identified above were located on the EcMinC structure assuming it is not a swapped dimer (Fig. S1). This revealed that they are close together at the distal end of α11 with their side chains exposed to solvent. More over, these residues are on the same surface as Gly-10 (Fig. S2). The residues corresponding to Gly-10, Lys-35, and Ala-39
are also located on the same surface on the TmCnN structure (Fig. 1B).

To further examine the region of MinCnN identified by the above mutations we used site-directed mutagenesis to alter the residues around this area. We introduced five point mutations, minCnK115, minCnK35E, minCnA39D, and minCnF42E into pMCW84 (Plac::gfp-minCn/minD) and examined their effect on the inhibitory activity of MinCn. The K115 substitution was made because a positive charge at this position is conserved in all MinCs (Fig. 1A). Three of these mutations (minCnK115, minCnK35E, and minCnA39D) did not affect MinCn function as Z rings were readily disrupted (only K35E and Q238A are shown). Furthermore, when these three mutations were introduced into the min operon on pSEB12 the Δmin phenotype of JS964 was complemented (Fig. 2, B and C, and Fig. S1). The other two mutants, however, MinCnK9A and MinCnF42E, behaved similarly to MinCnG10D, MinCnK35E, and MinCnA39D. At 2 h after induction of pMCW84 (Plac::gfp-minCn/minD) with 10 μM IPTG the derivatives with these mutations contained fluorescent cross-bands although some were distorted (Fig. 2A). Consistent with this, pSEB12 carrying minCnK9A, minCnA39D, or minCnF42E failed to complement the Δmin phenotype (Fig. 2, B and C, and Fig. S1). Because the point mutations affecting residues on the α1–β1 surface (Fig. 1B and Fig. S2) compromise MinCn activity without affecting protein stability (Fig. S3), they define the FtsZ inhibitory region of MinCn.

Because the MinCn mutants, MinCnK9A, MinCnK35E, MinCnA39D, and MinCnF42E, are impaired for function in vivo we tested their effect on FtsZ polymerization in vitro using a sedimentation assay. A MalE–MinCn fusion protein prevents FtsZ sedimentation in a dose-dependent manner and this activity is due to MinCn (6, 12). The four MinCn mutants, as well as MinCnG10D, had little effect on FtsZ sedimentation (Fig. 3A). Thus, the inability to inhibit FtsZ sedimentation correlates with the reduced ability of these mutants to disrupt Z rings and their inability to correct a minicell phenotype.

**MinCn overexpression inhibits cell division by sequestration**

MinCn inhibits cell division in the absence of MinD when it is expressed at 40-fold over the physiological level (22), whereas MinCnG10D does not, indicating the inhibitory activity is mediated by MinCn (6). Also, in the absence of MinD, MalE–MinCn is as active as MalE–MinCn, whereas MalE–MinCn has no activity (12). To test the four new MinCn mutants on the inhibitory activity of MinCn in the absence of MinD, we used a vector expressing just MinCn (pT7::minC115-6×his). This plasmid as well as derivatives with the mutations were transformed into BL21 (DE3). At 50 μM IPTG, expression of minC115 blocked colony formation (Fig. 4A). In contrast, expression of minC115(R9A), minC115(G10D), minC115(K35E), minC115(A39D), and minC115(F42E) did not (Fig. 4, A, B, and D). Expression of these alleles from another vector (pT7::6×his-minC115) in JS964 produced the same pattern (Fig. S4). Thus, mutations that reduce MinCn’s ability to prevent FtsZ sedimentation in vitro also reduce the ability of MinCn to inhibit colony formation.

Based on our previous findings (14) and this study, we assume that the inhibitory region of MinCn binds to FtsZ in a region that includes helix 10. To test how the minCn mutations affect MinCn binding to FtsZ, we used a biosensor assay (see “Experimental procedures”). Purified MinCnHis6 and the various mutants were loaded onto Ni-NTA biosensor pins and incubated with FtsZ. Most of the mutants (MinCnG10D, MinCnK35E, MinCnA39D, and MinCnF42E) were significantly impaired for FtsZ binding relative to MinCn (Fig. 5A). The binding of MinCnR9A was the least affected (only reduced 2-fold) suggesting that 50% loss of binding affinity is sufficient to prevent MinCnR9A from blocking FtsZ sedimentation in vitro and colony formation in vivo.

Because the binding affinity under our conditions is in the micromolar range we tested whether the interaction between MinCn and FtsZ could be detected by gel filtration. Because some FtsZ oligomers are present when FtsZ is purified, we used FtsZL178E, which exists as a monomer regardless of the presence or absence of GTP and Mg2+ (23). Each protein eluted as a single peak indicative of monomers (Fig. S5A), however, when the two proteins were mixed, a single peak was obtained that was shifted to a lower volume (Fig. S5B) at a position consistent with a 1:1 stoichiometric complex. The presence of the two proteins in the peak was confirmed by SDS-PAGE (Fig. S5C). In contrast, when MinCnG10D-His6 was mixed with FtsZL178E,
the proteins eluted in discrete peaks that were identical to their respective monomeric peaks (data not shown), indicating that it had markedly reduced affinity for FtsZL178E.

Because MinC115 and FtsZL178E form a complex that can be detected by gel filtration in the same buffer we used for the in vitro FtsZ sedimentation experiments, we reasoned that MinC blocks FtsZ polymerization by sequestering FtsZ monomers. This is consistent with the report by Hernandez-Rocamora et al. (16) that MinC shortens FtsZ filaments by forming a 1:1 complex. However, in our studies, the apparent $K_d$ values were in the range of $1 \times 10^{-6}$ M, whereas Hernandez-Rocamora et al. (16) determined a value of $\sim 10$ M. Examination of these studies revealed different buffer conditions were used in the polymerization assays so we employed the biosensor assay to assess the effects of buffer on the interaction. An increase in pH clearly weakened the interaction of MinC115–His6 with FtsZL178E (Fig. S6A). In agreement with Hernandez-Rocamora et al. (16) increasing the salt concentration also reduced the binding (Fig. S6B). In contrast, varying the salt concentration had no effect on FtsZ binding to His6-ZipA (Fig. S6C). At low ionic strength (50 mM KCl) and pH 6.5 the apparent dissociation constant for the MinC–FtsZ interaction was comparable with one that we previously reported ($1 \times 10^{-7}$ M) (Fig. S7, A and B), however, it shifted to $1 \times 10^{-4}$ M at pH 7.5 (Fig. S7, C and D). These results indicate a significant electrostatic contribution to the interaction between MinC and FtsZ. Consistent with the biosensor assay and Hernandez-Rocamora et al. (16), MinC did not block FtsZ sedimentation in a high salt buffer (500 mM KCl) (Fig. 3B). In contrast, MalE–SulA prevented FtsZ polymerization regardless of the ionic strength.

FtsZ is estimated to be in $\sim 10$-fold excess over MinC in vivo (24). Hence, a $\sim 40$-fold overexpression of MinC in the absence of MinD will likely result in substantial sequestration of FtsZ even if biochemical parameters such as pH and salt concentrations are taken into consideration. Also, the FtsZ level only has to be decreased by $\sim 30\%$ to inhibit division (25). Therefore, our results indicate that inhibition of FtsZ assembly by MinC in vitro correlates with inhibition of cell division by overexpression of MinC in the absence of MinD in vivo. In both cases inhibition of FtsZ assembly is caused by MinC binding to free FtsZ subunits, thereby reducing the FtsZ subunit pool available for polymerization.

Figure 4. The effect of minC mutations on the inhibitory activity of MinC115. Plasmid pET2aMinC115 (P$_f$=minC115-6xhis) and derivatives were introduced into BL21 (DE3). A colony of each strain was re-suspended in 300 μl of LB and serially diluted 10-fold. 3 μl of each dilution was spotted on plates containing ampicillin and IPTG, and incubated at 37 °C overnight. The panels represent different plates and the vector and WT MinC115 were included in each panel as controls.

Figure 5. The MinC inhibitory region mutants exhibit diminished affinity for FtsZ. A, the effect of minC mutations on the binding of MinC to FtsZ. MinC115–His$_6$ and various mutants were immobilized on Ni-NTA biosensor pins and incubated with FtsZ. The graphs show the kinetics of MinC–FtsZ attachment to the pins (30–180 s), followed by FtsZ binding to MinC (180–300 s), and FtsZ dissociation (300–420 s). B and C, the effect of the E7K mutation on MinC binding to FtsZ. The binding was done as in A.
Genetic evidence for an electrostatic contribution to the inhibitory activity of MinC

The above results suggest that MinC\textsuperscript{N} binding to FtsZ involves an electrostatic component. Consistent with this, mutations that reduced the activity of MinC\textsuperscript{N} either removed a positively charged residue (K9A or K35E) or introduced a negatively charged residue (G10D, A39D, and F42E). Mutations that did not reduce MinC activity either increased the positive charge (E7K), did not affect the charge (Q34A), or removed a negative charge (E7A). One possibility is that a positive electrostatic potential in the inhibitory region might be critical for MinC function, so we examined several mutations in more detail.

Interestingly, JS964 (min::kan) cells containing pSEB12-E7K (p\textsubscript{BAD}:min\textsuperscript{E7K}/minD/minE) were somewhat longer than those expressing the wildtype min operon (Fig. 2B and Fig. S1). Because no minicells were detected (Fig. 2C), min\textsuperscript{E7K} might represent a more active form of MinC due to an effect on the electrostatics. To test this possibility, we introduced min\textsuperscript{E7K} into pQE80L-E (p\textsubscript{lac}:6xhis-min\textsuperscript{C115}) and expressed it in JS964. Indeed, at 100 \textmu M IPTG MinC\textsuperscript{115(E7K)} suppressed cell division and colony formation more effectively than MinC\textsuperscript{115} (Fig. S4). Reexamination of inhibition by GFP-MinC\textsuperscript{E7K}/MinD revealed that it was also more active than the wildtype (data not shown).

Because we suspected that electrostatics played a role in the MinC mechanism, we wondered what effect the E7K mutation would have on those min\textsuperscript{C} mutations that introduce a negatively charged residue in the FtsZ inhibitory region. Interestingly, pSEB12 containing min\textsuperscript{CE7K/K35E}/minD/minE complemented the \Delta min phenotype of JS964 indicating E7K was an intragenic suppressor of K35E (Fig. 2, B and C, and Fig. S1). In addition, induction of GFP-MinC\textsuperscript{E7K/K35E}/MinD with 10 \textmu M IPTG led to a loss of cross-bands (JS964/pMCW84–7/35 (p\textsubscript{lac}:gfp-min\textsuperscript{CE7K/K35E}/minD), suggesting that the double mutant is at least as active as wildtype MinC (Fig. 2A). In addition, adding E7K to min\textsuperscript{C935E}, min\textsuperscript{CAS9D}, and min\textsuperscript{C42F}, as well as min\textsuperscript{C115D}, in the context of the min operon, restored MinC activity as the Min\textsuperscript{N} phenotype was complemented (Fig. 2, B and C, and Fig. S1). E7K also rescued the inhibitory activity of these mutations when expressed from pSEB104CD (p\textsubscript{ara}:min\textsuperscript{C}/minD) (Fig. 6). Thus, those mutants that have lost inhibitory activity due to introduction of a negative charge in MinC\textsuperscript{N} were rescued by the addition of the E7K mutation.

In light of the above findings, we speculated that the loss of activity due to the min\textsuperscript{G10D} mutations could be due to the introduction of the negatively charged aspartate residue. Therefore, lysine was substituted for glycine. JS964/pSEB12 containing min\textsuperscript{C10G} displayed a wildtype phenotype (Fig. 2, B and C, and Fig. S1). Consistent with min\textsuperscript{C10G} behaving like WT MinC, we did not detect any cross-bands when GFP-MinC\textsuperscript{G10K}/MinD was induced with 10 \textmu M IPTG in JS964/pMCW84–10 (p\textsubscript{lac}:gfp-min\textsuperscript{G10K}/minD) (Fig. 2A). These results suggest that it is not the size of the residue at this position but the charge that results in the loss of the activity. As controls, we altered charged residues just outside of the putative FtsZ inhibitory region of MinC\textsuperscript{N}. Mutations min\textsuperscript{C94A} and min\textsuperscript{C72E} (Fig. 1) did not affect MinC activity as pSEB12 containing these mutations complemented the Min\textsuperscript{N} phenotype (Fig. 2, B and C). In summary, the loss of activity of MinC due to addition of a negative charge in the inhibitory region can be recovered by increasing the positive charge nearby.

Mechanism of Z ring disassembly by MinC/MinD on the membrane is distinct from sequestration

Although our study of MinC\textsuperscript{N} indicated inhibition by sequestration, the level of MinC is about 10-fold lower than FtsZ (22, 24) suggesting a sequestration mechanism is unlikely for MinC/MinD. We therefore tested whether E7K, which rescues min\textsuperscript{C} mutations in the context of MinC/MinD, also rescues in the context of min\textsuperscript{D115}. To do this E7K was combined with each of the min\textsuperscript{C} mutations and expressed in BL21 (ADE3)/pET2a-MinC\textsuperscript{115} (p\textsubscript{lac}:min\textsuperscript{C115,6xhis}). In contrast to what was observed with MinC/MinD (Fig. 6) most mutations were not rescued (Fig. 4, A–C). Only min\textsuperscript{C115(K9A)} was rescued (Fig. 4B). Similar results were obtained when min\textsuperscript{C7K} was added to min\textsuperscript{C} mutations and then expressed in JS964/pQE80L-MinC\textsuperscript{115} (p\textsubscript{lac}:6xhis-min\textsuperscript{C115}) (Fig. S4). Because E7K failed to rescue the \textit{in vivo} activity of these mutants we suspected it would not rescue the binding to FtsZ. Consistent with the \textit{in vivo} results, E7K rescued K9A binding...
MinC/MinD mechanism

but not that of the other mutants (Fig. 5, B and C). In summary, we find that E7K rescues mutations that introduce a negative charge in the MinC\textsuperscript{N} domain when present in the MinC/MinD context but it does not rescue these mutations in the context of just MinC\textsuperscript{N}, either in vivo or when testing the interaction with FtsZ in vitro. With MinC\textsuperscript{N} we see very good correlation between the affinity for FtsZ in vitro and inhibition of Z ring formation in vivo indicating inhibition of FtsZ is by sequestration. However, our data strongly indicate that MinC/MinD antagonizes Z rings through a distinct mechanism.

A minC mutation that differentially affects inhibition by MinC\textsuperscript{N} versus MinC/MinD

To further explore the difference between inhibition by MinC/MinD and MinC\textsuperscript{N} we substituted the conserved lysine residue at position 9 with arginine (K9R). A lysine is present in most MinCs and therefore might be critical for MinC function (Fig. 1A). Importantly, this substitution replaces this highly conserved lysine with a bulky arginine residue while maintaining the positive charge. MinC\textsuperscript{115(K9R)} inhibited cell division, albeit a little less effectively than MinC\textsuperscript{115} (Fig. 4D). This was also observed in JS964/pQE80L-MinC\textsuperscript{115} (Fig. S4). Consistent with the K9R substitution not affecting inhibitory activity, the binding of MinC\textsuperscript{115(K9R)} to FtsZ was unaffected (Fig. S5A). Surprisingly, however, when MinC\textsuperscript{K9R} was expressed along with MinD (JS964/pSEB104C\textsuperscript{K9R}/D [P\textsuperscript{RAD}:minC\textsuperscript{K9R}/minD]) inhibition was not observed (Fig. 6B). It was not only defective in inhibiting colony formation but it also failed to cause cell filamentation (Fig. S8). Also, JS964/pSEB104C\textsuperscript{K9R}/D (P\textsuperscript{RAD}:minC\textsuperscript{K9R}/minD/minE) failed to complement a Δmin phenotype (data not shown). These results show that the K9R mutation eliminates MinC activity in the context of MinC/MinD but has little effect in the context of MinC\textsuperscript{N}. Thus, MinC\textsuperscript{K9R} in the cytoplasm functions as an FtsZ inhibitor, consistent with a sequestration mechanism, whereas MinC\textsuperscript{K9R}/MinD on the membrane is inactive even though it should localize to the Z ring and bind to the ends of FtsZ polymers. Thus, the K9R mutation differentially affects the activity of MinC depending on the context and indicates distinct mechanisms.

Characterization of FtsZ mutants resistant to MinC in vivo

The differential effect of the K9R mutation on inhibition by MinC\textsuperscript{N} (cytoplasmic MinC) versus MinC/MinD (on the membrane) indicates distinct mechanisms. To gain additional support for this we tested ftsZ mutations that we previously isolated as resistant to MinC/MinD for resistance to MinC\textsuperscript{115}. Consistent with our previous reports, S7/pBang112 (P\textsuperscript{con}:ftsZ) carrying ftsZ mutations that alter residues in helix 10 provided resistance to MinC/MinD (Fig. S9). However, resistance to MinC\textsuperscript{115} (P\textsuperscript{tac}:minC\textsuperscript{115}) was more varied (Fig. 7A). FtsZ\textsuperscript{E276D} and FtsZ\textsuperscript{I294T} exhibited resistance to MinC\textsuperscript{115}, whereas FtsZ\textsuperscript{K271G} and FtsZ\textsuperscript{D291E} exhibited much less resistance. Strikingly, FtsZ\textsuperscript{E276D}, although resistant to MinC/MinD, was completely susceptible to MinC\textsuperscript{115} (Fig. 7A).

To determine whether resistance to MinC\textsuperscript{115} correlated with FtsZ binding the interaction of MinC\textsuperscript{115}/His\textsubscript{6} with the various FtsZ mutants was examined using the biosensor assay. Two mutants were at the extreme. FtsZ\textsuperscript{K271G}, which showed the most resistance to MinC\textsuperscript{115}, displayed no binding (Fig. 7B). In contrast, FtsZ\textsuperscript{E276D}, which was as sensitive to MinC\textsuperscript{115} as WT FtsZ, bound to MinC\textsuperscript{115} as well as wildtype FtsZ. The two mutants that showed some resistance showed intermediate binding (FtsZ\textsuperscript{N280D} and FtsZ\textsuperscript{I294T}). The interaction of FtsZ\textsuperscript{E276D} (and the lack of interaction of FtsZ\textsuperscript{K271G}) with MinC\textsuperscript{115} was verified by gel filtration (data not shown). Because the proximal region of helix 10 (Leu-270 and Arg-271) seemed important for resistance to MinC/MinD and MinC\textsuperscript{115}, as well as binding to MinC\textsuperscript{115}, we made the ftsZ\textsuperscript{L272E} mutation. This mutation, like ftsZ\textsuperscript{K271G}, eliminated MinC\textsuperscript{115} binding (Fig. 7B). Resistance to MinC could not be tested, however, due to the inability of L272E to complement in vivo (data not shown). Nonetheless, this result supports the idea that binding of MinC\textsuperscript{115} to FtsZ involves helix 10 and the degree of resistance to MinC\textsuperscript{115} correlates with a reduction in binding.

Because FtsZ\textsuperscript{E276D} is resistant to MinC/MinD, expression of GFP–MinC/MinD in a strain carrying FtsZ\textsuperscript{E276D} should not cause filamentation and cells should contain fluorescent cross-bands. To confirm this, pBang85 (P\textsuperscript{tac}:gfp-minC/MinD) was introduced into S7/pBang112-E276D. For controls pBang85 was also introduced into S7/pBang112-WT, S7/pBang112-E276D/I374V, and S7/pBang112-I374V. When GFP–MinC/MinD was induced with 10 μM IPTG for 30 min in the strain with WT FtsZ, cross-bands were rarely observed and cells were filamentous (Fig. 8). In contrast, cells were not that filamentous with WT FtsZ, cross-bands were rarely observed and cells were filamentous (Fig. 8). In contrast, cells were not that filamentous with WT FtsZ, cross-bands were rarely observed and cells were filamentous (Fig. 8).
in the absence of MinD correlates with the ability of MinC to sequester FtsZ by binding MinD mutants from plasmid pBANG112. An overnight culture of each strain was diluted 1000-fold in LB containing spectinomycin and ampicillin and IPTG was added after the cultures reached exponential phase. Each strain was grown until Abs = 0.4 (2–3 h) and phase (left panel) and fluorescence (right panel) photographs were taken.

Figure 8. The effect of ftsZ and minC mutations on the localization of GFP-MinC/MinD. Plasmid pBang85 (PLex-gfp-minC/minD) was introduced into S7 (ftsZ ᵗn:nic:S7::kan) expressing FtsZ or FtsZ mutants from plasmid pBANG112. An overnight culture of each strain was diluted 1000-fold in LB containing spectinomycin and ampicillin and IPTG was added after the cultures reached exponential phase. Each strain was grown until Abs = 0.4 (2–3 h) and phase (left panel) and fluorescence (right panel) photographs were taken.

S7/pBang112-I374V (Fig. 8) and no cross-bands were observed (unable to localize to Z137⁴V rings). We also analyzed MinCK⁹R as it should localize to Z without disrupting them. We transformed S7/pBang112-WT with pBang85 carrying gfp-minC/kg9p/minD. Following induction, cross-bands were observed and cells were not filamentous consistent with the mutation inactivating MinCN in the MinC/MinD context (Fig. 8). Thus, GFP–MinCK⁹R/MinD behaves similarly to GFP–MinC⁴⁰D/MinD and the other MinC mutants we isolated (Fig. 2). Also, FtsZ⁺²⁷⁶D is resistant to MinC/MinD even though it is sensitive to MinC¹¹⁵ in vivo and binds MinC¹¹⁵ in vitro. These results support the notion that the mechanisms of inhibition by MinC in the cytoplasm and MinC/MinD on the membrane are different.

Characterization of FtsZ mutants resistant to MinC in vitro

As stated earlier, the prevention of FtsZ sedimentation by MinC in vitro correlates with the ability of overexpressed MinC¹¹⁵ to inhibit cell division and colony formation in vivo. This suggests that the polymerization of FtsZ⁺²⁷⁶D should be susceptible to MinC¹¹⁵. To test this FtsZ was incubated with MinC¹¹⁵–His₅ in the presence of GTP and filaments recovered by sedimentation. Although FtsZ⁺²⁷⁶D polymerization was a little less efficient than wildtype FtsZ, it was indeed susceptible to MinC¹¹⁵ (Fig. 9A). This is consistent with MinC¹¹⁵ inhibiting FtsZ⁺²⁷⁶D by sequestration. On the other hand, the sedimentation of FtsZ⁻²⁷¹G, which is resistant to MinC¹¹⁵ in vivo, was largely unaffected by MinC¹¹⁵. Also, MinC¹¹⁵(K⁹R) antagonized FtsZ polymerization as effectively as MinC¹¹⁵, whereas MinC¹¹⁵(G₁⁰D) did not (Fig. 9B). These results further demonstrate that the inhibition of division by overexpression of MinC in the absence of MinD correlates with the ability of MinC to bind FtsZ and prevent FtsZ sedimentation in vitro. However, as detailed above this ability is separable from the mechanism used by MinC/MinD to inhibit division.

Discussion

Our earlier studies (13, 14) led to a model for the mechanism of MinC/MinD in antagonizing Z ring assembly. In this model MinC²⁷⁶D/MinD captures the CCTP of FtsZ in a filament positioning the MinCN domain to interact with the H10 helix of FtsZ at the interface of two FtsZ subunits, and if GTP has been hydrolyzed, to cause filament breakage. This model highlights synergistic cooperation between MinC²⁷⁶D and MinCN, and also explains why FtsZ mutants defective in GTPase activity are resistant to MinC²⁷⁶D/MinD (6). However, due to a paucity of direct evidence that MinC/MinD severs FtsZ filaments, questions remain about the mechanism. In this study, we sought to advance our understanding of the mechanism employed by MinC by isolating and characterizing mutations in the MinCN domain defective in blocking Z ring assembly. Our study revealed that residues important for MinCN function are clustered on the MinC structure, which we designated the FtsZ inhibitory region, and verified that residues located at the proximal end of helix H10 of FtsZ are required for MinC activity. Also, our examination of MinC²⁷⁶D, MinC⁹R, and FtsZ⁻²⁷⁶D clearly distinguished inhibition by MinC (in the absence of MinD) from inhibition by MinC/MinD. The results reveal that MinC inhibits by sequestration, whereas MinC/MinD, the physiologically relevant mechanism, prevents FtsZ polymerization through a distinct mechanism not involving sequestration or capping suggesting the mechanism is severing.

Cell division inhibition by MinC/MinD has largely been considered to be similar to the inhibition observed by overexpression of MinC, which correlates with the prevention of FtsZ sedimentation in vitro (6). MinD activation of MinC was largely thought to be due to recruiting MinC to the membrane and increasing its affinity for the CCTP of FtsZ (13, 26). Many MinC mutants isolated here in the context of MinC/MinD (K⁹A, K³⁵E, A³⁹D, and F⁴²E) were defective in inhibiting cell division when overexpressed in the absence of MinD. In addition, they were compromised for binding to FtsZ and were unable to prevent FtsZ sedimentation in vitro. Consistent with this, size
exclusion chromatography revealed that MinC interacts with FtsZ through its MinCN with a 1:1 stoichiometry, whereas the MinC mutants failed to form a complex. Even though this interaction is quite strong at low pH and low ionic strength (Kd 1 M) it is weaker under physiological conditions. However, we see a good correlation between binding in vitro and inhibition of Z ring formation in vivo indicating that sequestration is the likely mechanism. However, inhibition of FtsZ by MinC/MinD is unlikely to be due to sequestration because FtsZ is in 10-fold excess over MinC. Although capping remained a possibility, the CCTPs internal to the filament would compete with the terminal subunit CCTP for MinC/MinD, making capping less likely.

**Mutations differentially affecting MinC versus MinC/MinD**

Three different mutations clearly differentiate inhibition by MinC from that of MinC/MinD. The K9R mutation in the minC115 background inhibited cell division and colony formation and as expected, the MinCK9R mutant bound to FtsZ in a biosensor assay and prevented FtsZ polymerization in vitro. Surprisingly, however, MinCK9R did not inhibit cell division nor induce cell filamentation in the presence of MinD. In addition, fluorescence microscopy revealed that Z rings were not disrupted by MinCK9R/MinD and instead were decorated with GFP fusions to MinCK9R/MinD as expected for loss of MinCN function. These results indicate that MinC/MinD inhibits cell division via a mechanism distinct from FtsZ sequestration. Because MinCK9R can still bind FtsZ, and therefore, should bind to helix H10 of the terminal subunit in an FtsZ filament, a capping mechanism can also be ruled out.

This difference between inhibition by MinC and MinC/MinD is also supported by the FtsZE276D mutant. A strain carrying the ftsZE276D allele was sensitive to MinC115 overexpression. Consistent with this, FtsZE276D bound MinC115 as well as WT FtsZ and was unable to polymerize in the presence of MinC115 in vitro. Nonetheless, FtsZE276D was resistant to MinC/MinD in vivo and ZE276D rings were decorated with GFP-MinC/MinD and not readily disrupted. Study of the E7K mutation also highlighted the difference between inhibition by MinC and MinC/MinD. The E7K mutation produced a more active MinC and suppressed minC mutations that decreased the positive electrostatic potential. Although this mutation suppressed the minC mutations in the context of MinC/MinD it had little effect on these mutations in the context of MinC115.

**Implications of the mutations on mechanism**

Besides further confirming differences between the mechanisms of the two forms of MinC the mutations lead to speculation about the mechanism of MinC/MinD. In SatFtsZ filaments produced with PC190723, the loop and proximal region of H10 constituting the FtsZ intersubunit interface is only partially

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**Figure 10.** The FtsZ residues important for MinC activity are located at the interface of the subunits. A, three subunits of a Staphylococcus aureus FtsZ filament assembled with PC197023 (PDB code 3VOB) (40). The residues important for MinC/MinD activity are indicated (colored red and green). The C-terminal tail of FtsZ emerges from the magenta colored residue and is indicated by a dotted line with the CCTP at the end. B, model for the interaction of MinC/MinD with an FtsZ filament. A MinD dimer is bound to the membrane and interacts with a MinC dimer through the MinC domain, which enhances its affinity for FtsZ's CCTP. This interaction puts the MinC domains in position to interact with the negatively charged FtsZ filament, which is tethered to the membrane by FtsA and ZipA. It is possible that the Lys-9 residue of MinC interacts with the Glu-276 residue of FtsZ to weaken the interaction between adjacent FtsZ subunits, which helps to sever the filament. It is also possible that another MinD dimer is bound to the other MinC domain in the MinC dimer (43).
exposed to solvent (Fig. 10A). Thus, we presume that the proximal (residues Leu-270 and Arg-271) and the central region of H10 (residues Glu-276 and Asn-280) become more exposed to solvent once GTP hydrolysis occurs. Loss of the T7 loop contacts may weaken the subunit–subunit interaction and expose the proximal end of helix 10 (41, 42). Although the mechanistic details are beyond the scope of this work, we would suggest that once MinC/MinD is bound to a CCTP in an FtsZ filament the MinCN domain interacts with the filament through an electrostatic interaction (Fig. 10B). Mutations that introduce a positive charge or reduce the positive charge in MinCN (K35E, A39D, G10D, F42E, and K9A) are unable to interact with the filament, but this deficiency can be restored by addition of the E7K mutation. In contrast, MinC^{K9R} retains the positive charge but is still inactive in the context of MinC/MinD suggesting that a positive charge is not sufficient and that a specific steric interaction is required. A specific steric interaction is also suggested by the failure of the FtsZ^{L270D} mutant to respond to MinC/MinD. This suggests that once the MinCN domain interacts with the FtsZ filament the Lys-9 residue is required in some way to break the filament, possibly by interacting with residue Glu-276 of FtsZ (Fig. 10B). However, the observation that E7K suppresses K9A would argue that a lysine at position 7 can also accomplish this task.

Comparison of MinC/MinD with other inhibitors

Both SlmA and MinC/MinD are positioned in the cell and antagonize Z ring formation where they take up residence; MinC/MinD on the membrane with the highest concentration at the poles, whereas SlmA is bound to DNA around the origin of replication (3, 4, 11). In both cases binding to their respective surface potentiates their activity. SlmA bound to its DNA-binding site is activated to disassemble FtsZ filaments with little activity in the absence of DNA (4, 27). The DNA binding results in a conformational change in SlmA that leads to increased affinity for the CCTP of FtsZ (15, 28). Upon binding the CCTP, SlmA severs FtsZ filaments provided GTP has been hydrolyzed. The precise mechanism is not clear, but it requires a positive charge at position 190 at the midpoint of the long helix connecting the two globular domains of FtsZ (15). How SlmA bound to the CCTP takes advantage of this positive charge to elicit filament breakage is not clear.

Although MinC prevents FtsZ assembly in vitro, this activity is pH- and salt-sensitive (16), and as shown here separable from the mechanism employed by MinC/MinD. In vivo the activity of MinC is activated 40-fold by MinD (26), but this enhancement has not been observed in vitro. We have been unable to see any enhancement of MinC’s activity by MinD in vitro despite some effort. Perhaps, MinD has to be bound to the membrane to effectively activate MinC. We know that the conformation of MinD is affected by membrane binding because it can only be induced to undergo ATP hydrolysis by MinE when membrane bound (29). Also, we have only observed interaction between MinC/MinD and the CCTP of FtsZ when MinD/MinC are on the membrane (13, 30). Arumugam et al. (17) observed some enhancement in their reconstituted system but not to the degree observed in vivo. This may be related to the FtsZ bundles observed in their system that may deviate from FtsZ organization in the Z ring.

Interestingly, many inhibitors of FtsZ interact with the region around the proximal end of H10. Although this does not include SlmA, it does include MinC, SulA (31) as well as MciZ from Bacillus subtilis (32). SulA inhibits by sequestration (33, 34), whereas MciZ inhibits by capping. One of the main differences between the two is that MciZ has a much higher affinity for FtsZ, about 0.15 μM, in contrast to about 1 μM for SulA. This is likely related to their physiological role. Although inhibition by SulA needs to be rapidly reversible (35), the MciZ inhibition occurs in the dying mother cell during sporulation and does not have to be reversible (36).

Experimental procedures

Strains and media

The E. coli K12 strain JS964 (MC1061 malP::lacI^{q} minC::kan) was primarily used for complementation of the Min^ phenotype, cell division inhibition by MinC/MinD, and MinC purification (37). JS238 (MC1061, araD (ara leu) galU galK hsdS rpsL rpsL \Delta( lac/OPZYA)X74 malP::lacI^{q} srlC::Tn10 recA1) was used for most cloning experiments and FtsZ purification. BL21 (ADE3) (F^{-} ompT gal dcm lon hsdS (r_{B} m_{B}) A(DE3) [lac lacI^{Q} lacIV5-T7 gene1 ind1 sam7 min5]) was used for MinC^{115-166}. His^{6} expression and purification (38). Other protein purifications were carried out using JS964 grown at 37 °C. Construction of S7 (ftsZ^{recA::Tn10 min::kan/pKD3C [ftsZ^{+}]}) was previously described and is a derivative of W3110 (13). Unless stated otherwise, Luria-Broth (LB) medium containing 0.5% NaCl was used and antibiotics (ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml), IPTG, glucose (0.2%), and arabinose (0.2%) were added accordingly.

Plasmids

The construction of pBang59 (P_{TAC}::minC/MinD), pBang85 (P_{TAC}::gfp::minC/MinD), pBang112 (P_{CON}::ftsZ), pHJZ108 (P_{LAC}::gfp-minC^{G10D}/minD), pHJZ109 (P_{LAC}::gfp::minC^{G10D}/minD), pSEB12 (P_{CON}::minC/minD/minE), pET21αC{^{N}} (P_{T7}::minC^{15}), pSEB104CD (P_{BAD}::minC/MinD), pSEB104EDE (P_{BAD}::minC/MinD/minE), and pZH101 (P_{BAD}::malE-mem) were previously described (6, 13, 14, 39). An XmaI site was introduced between gfp-minC^{G10D} and minD via QuikChange (Stratagene) on the template pHJZ108 (gfp-minC^{G10D}/minD) giving rise to the plasmid pMCW26. The minC^{G10D} allele in pMCW26 was reverted back to wildtype by site-directed mutagenesis to generate pMCW84. Derivatives of pZH101 and pMCW84 with various nonsense mutations in minC were generated using the QuickChange mutagenesis kit (Stratagene). To create pQE80LC (P_{T7-LOCA}::minC^{115}), E. coli minC ORF was PCR amplified and digested with EcoRI/HindIII prior to ligation into the pQE80L vector. Plasmid pR100–MinCN (P_{T7}::minC^{115}) was created by ligating a PCR fragment of minC^{115} cut with EcoRI/HindIII into the pSC101 vector. Plasmid pSUMO FtsZ (P_{T7}::sumo-ftsZ) was constructed by ligating XbaI/Bsal-digested E. coli/ftsZ ORF into pSUMO vector (LifeSensors, Inc.) linearized with Bsal. Site-directed mutagenesis was carried out to create pSUMO FtsZ230 (P_{T7}::sumo-ftsZ_{230}) and pSUMO FtsZ_{320}–L178E (P_{T7}::sumo-ftsZ_{320}–L178E).
**Microscopy**

To visualize the location of GFP–MinC in cells grown in liquid media, JS964 containing pMCW84 (R<sup>hes–gfpr–minC<sub>GIOD</sub>/minD</R>) and various derivatives with mutations were grown overnight at 37 °C to A<sub>600</sub> ~ 0.02 at which point they were induced with 100 μM IPTG. The cells were analyzed by fluorescence microscopy 0.5–2 h later. At the same time point, the cell morphology was assessed by phase-contrast microscopy. The toxicity of the MinC mutants was assessed by the degree of filamentation and lysis during growth. To determine whether minC mutations affected normal cell division, they were introduced into the mini-F plasmid pSEB12 containing the complete min operon. The resultant plasmids and the wildtype plasmid were transformed into JS964 (Δmin) and selected on LB medium plates containing chloramphenicol. The morphology of cells was examined during exponential growth. The frequency of cells with polar septa was determined with at least 200 cells scored per experiment. The localization of GFP–MinC/MinD and its variants expressed with wildtype or mutants FtsZ were recorded according to the procedure described previously.

**Site-directed and random mutagenesis**

Various minC mutations such as K9A and G10D were introduced into pHJZ108, pSEB12, pET21αC<sup>N</sup>, pSEB104CD, and pSEB104CDE using a QuickChange II site-directed mutagenesis kit according to the manufacturer’s instruction (Agilent Technologies). The ftsZ mutations such as E276D and N280D were introduced into ftsZ on pBang12 using the same method. Random mutagenesis of the minC gene was performed by PCR with GeneMorph II (Stratagene). The amplification reaction mixture had a volume of 100 μl and used 1 μg of pSEB104CD plasmid template (115 ng of minC target sequence). The amplified product was digested with XbaI/Xmal and ligated into the same site of pMCW26. The resulting plasmid pool was introduced into JS964 by electroporation and selection for spectinomycin resistance on LB plates. The colonies were then patched in a grid pattern onto spectinomycin plates that contain either 10 or 100 μg/ml IPTG. The colonies were screened as described in the main text.

**Protein expression and purification**

The purification of FtsZ, His<sub>6</sub>–MinC<sup>115</sup>, and MinC<sup>115</sup>–His<sub>6</sub> was previously described. MalE–MinC and MalE–MinC<sup>GIOD</sup> were purified from cultures of JS964 (Δmin) containing pZH101 and pZH102, respectively, as described previously (6). MalE fusions to various MinC mutants were purified in the same manner as for the wildtype fusion. For FtsZ<sub>320</sub>(L178E) purification, a 1-liter volume of BL21(DE3)/pSUMO FtsZ<sub>320</sub>(L178E) was cultured at 37 °C and IPTG (1 mM) was added at A<sub>600</sub> ~ 0.5. After 3 h of induction, cells were harvested and stored at −80 °C until processed. Thawed cell pellet was resuspended in lysis buffer (25 mM Tris–HCl, pH 7.9, 100 mM NaCl, 10 mM imidazole, 10% glycerol) and lysed with a French press. Cell lysate was centrifuged at 10,000 × g at 4 °C for 30 min and the supernatant was applied to a nickel-agarose column equilibrated with lysis buffer. The column was washed with washing buffer (25 mM Tris–HCl, pH 7.9, 500 mM NaCl, 20 mM imidazole, 0.1% Nonidet P-40) and His<sub>6</sub> SUMO–FtsZ<sub>320</sub>–L178E was eluted with elution buffer (25 mM Tris–HCl, pH 7.9, 500 mM NaCl, 250 mM imidazole). The eluted fractions were pooled and dialyzed overnight in dialysis buffer (25 mM HEPES–NaOH, pH 7.0, 50 mM KCl, 10% glycerol). The next day His<sub>6</sub> SUMO was cleaved from His<sub>6</sub> SUMO–FtsZ<sub>320</sub>–L178E using SUMO protease and untagged FtsZ<sub>320</sub>–L178E was eluted from nickel-agarose column. The eluted samples were pooled and concentrated over ~10 mg/ml.

**Biosensor assays**

The Blitz system (Fortebio) was used to examine the protein–protein interaction and was previously described in detail. MinC<sup>115</sup>–His<sub>6</sub> and various mutants in 1× Pol buffer (25 mM HEPES–NaOH, pH 6.8, 50 mM KCl, 10 mM MgCl<sub>2</sub>) were incubated with Ni-NTA biosensors pre-equilibrated with 1× Pol buffer. Once MinC<sup>115</sup> or its variants were stably immobilized on the biosensor, FtsZ or FtsZ mutants in the same buffer were incubated with the MinC-coated biosensors for 2 min. Kinetics of MinC<sup>115</sup> and FtsZ bindings were monitored. Data analysis was carried out using GraphPad Prism (GraphPad Software) as described (23).

**Gel filtration**

MinC<sup>115</sup>–His<sub>6</sub> was incubated with FtsZ and FtsZ mutants in Pol buffer (50 mM HEPES–NaOH, pH 6.8, 50 mM KCl, 10 mM MgCl<sub>2</sub>) for 5 min at 25 °C. A 500-μl volume at a final concentration of 3 μg/ml for FtsZ and 1 μg/ml for MinC<sup>115</sup> were thereafter applied to Superdex 200 coupled to AKA-fast protein liquid chromatography (GE Healthcare) with a flow rate of 0.4 ml/min. UV absorbance at 280 nm was measured.

**FtsZ sedimentation**

The sedimentation of FtsZ was measured as described previously (6). In addition to MalE–MinC, we also used His<sub>6</sub>–MinC and MinC<sup>115</sup>–His<sub>6</sub> to exclude the effects of the MalE tag. Their biochemical activity was identical to MalE–MinC. For sedimentation assays, FtsZ at the indicated concentrations was incubated in Pol buffer (50 mM Mes–NaOH, pH 6.5 (or 50 mM HEPES–NaOH at pH 6.8), 50 mM KCl, 10 mM MgCl<sub>2</sub>) prior to mixing with MinC or SulA. The reactions were incubated at room temperature for 10 min with addition of GDP or GTP (2.5 mM) and centrifuged at 175,000 × g for 15 min in TLA 100.2 rotor (Beckman). Pellets and supernatants were analyzed on 12.5% PAGE and stained with Coomassie Blue.

**Western blot analysis**

JS964 (Δmin) containing pSEB12 or its derivatives with various minC alleles were grown in LB medium with chloramphenicol to exponential phase. At A<sub>600</sub> 0.3–0.6, cells from a 1-ml volume of culture was centrifuged and resuspended in 100 μl of SDS-loading buffer, boiled for 5 min, and subjected to SDS-12.5% PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane, and MinC was detected by using a rabbit antiserum raised against MalE–MinC as the primary antibody and goat alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Bio-Rad) as the secondary antibody.
References


MinC/MinD mechanism