Targeting of $^{D}\text{MinC}/\text{MinD}$ and $^{D}\text{MinC}/\text{DicB}$ Complexes to Septal Rings in *Escherichia coli* Suggests a Multistep Mechanism for MinC-Mediated Destruction of Nascent FtsZ Rings

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The MinC protein is an important determinant of septal ring positioning in *Escherichia coli*. The N-terminal domain ($^{\beta}\text{MinC}$) suppresses septal ring formation by interfering with FtsZ polymerization, whereas the C-terminal domain ($^{\alpha}\text{MinC}$) is required for dimerization as well as for interaction with the MinD protein. MinD oscillates between the membrane of both cell halves in a MinE-dependent fashion. MinC oscillates along with MinD such that the time-integrated concentration of $^{\alpha}\text{MinC}$ at the membrane is minimal, and hence the stability of FtsZ polymers is maximal, at the cell center. MinC is cytoplasmic and fails to block FtsZ assembly in the absence of MinD, indicating that recruitment of MinC by MinD to the membrane enhances $^{\alpha}\text{MinC}$ function. Here, we present evidence that the binding of $^{\alpha}\text{MinC}$ to MinD endows the MinC/MinD complex with a more specific affinity for a septal ring-associated target in vivo. Thus, MinD does not merely attract MinC to the membrane but also aids MinC in specifically binding to, or in close proximity to, the substrate of its $^{\alpha}\text{MinC}$ domain. MinC-mediated division inhibition can also be activated in a MinD-independent fashion by the DicB protein of cryptic prophage Kim. DicB shows little homology to MinD, and how it stimulates MinC function has been unclear. Similar to the results obtained with MinD, we find that DicB interacts directly with $^{\alpha}\text{MinC}$, that the $^{\alpha}\text{MinC}/\text{DicB}$ complex has a high affinity for some septal ring target(s), and that MinC/DicB interferes with the assembly and/or integrity of FtsZ rings in vivo. The results suggest a multistep mechanism for the activation of MinC-mediated division inhibition by either MinD or DicB and further expand the number of properties that can be ascribed to the Min proteins.

Assembly of the FtsZ ring (5) is the first visible event in formation of the septal ring organelle, which mediates cell constriction during cytokinesis in prokaryotes and eukaryotic organelles of prokaryotic origin (17, 33, 37, 38, 44, 49). The plane of cell division is determined by the plane of the initial Z ring (2). Therefore, a proper definition of the site of initial FtsZ assembly is crucial for the proper distribution of mother cell components to her progeny.

The MinC protein is an important negative regulator of FtsZ assembly in *Escherichia coli* (4, 10, 11, 29, 30). The peptide consists of 231 residues which fold into two domains of roughly equal sizes. The N-terminal domain (Z domain, designated here $^{\beta}\text{MinC}$) is responsible for inhibition of FtsZ assembly, whereas the C-terminal domain (D domain, $^{\alpha}\text{MinC}$) is responsible for both homodimerization of MinC and binding MinD (8, 26, 53). The effect of MinC on cell division is determined by its cellular location, which, in turn, is determined by the activities of the MinD and MinE proteins (28, 46).

MinD is an ATPase that associates with the membrane in a peripheral manner (9, 27, 48, 50). The protein directly binds MinC and MinE (30) and recruits both to the membrane (28, 46, 47). In the absence of MinD, overexpression of MinC (over 25-fold) is sufficient to block cell division (13). However, MinD is required for MinC function when the division inhibitor is present at physiological levels in the cell (10, 11, 13).

The MinE protein imparts topological specificity to MinC/MinD such that FtsZ assembly is not blocked at the normal division site at midcell. In the absence of MinE, MinC/MinD is found over the entire membrane, Z-ring assembly is blocked at all membrane sites, and cells form long nonseptate filaments (11, 28, 46, 48, 50). In wild-type cells, however, MinC/MinD action at midcell is prevented by MinE in an interesting way. MinE causes MinD to undergo a pole-to-pole oscillatory localization cycle during which the protein alternately accumulates on the membrane of one cell half every other 25 s or so (15, 22, 27, 48, 50). MinC is not required for oscillation (48) but oscillates along with MinD (28, 46). As a result, the concentration of membrane-associated MinC/MinD over time is maximal at the cell poles and minimal at the cell center. We proposed that it is this time-integrated concentration differential of MinC which limits stable assemblies of FtsZ to the middle of the cell (22, 46). The feasibility of such a mechanism is strongly supported by computer simulations of MinCDE and FtsZ dynamics, which closely resemble the behavior of these proteins observed in vivo (25, 41).

In addition to its normal role in division site placement, MinC is also involved in the division block which occurs upon induction of *dicB*. This gene is part of the *dic* operon, which resides on cryptic prophage Kim (Qin) (6). Under normal conditions, expression of *dicB* is actively repressed (3). When expression is induced, however, cell division rapidly ceases (34), and this division block is dependent on MinC (10, 35). In
contrast to MinC/MinD-mediated division inhibition, MinC/DicB-mediated division inhibition does not require MinD and is resistant to suppression by MinE. Both MinC-dependent division blocks can be suppressed by overexpression of FtsZ, however (10, 35). These results indicate that, although MinD (30 kDa) and DicB (7 kDa) have little sequence homology, either protein can independently activate MinC-mediated division inhibition (10, 35). Because MinD recruits MinC to the membrane, we and others proposed that MinD might activate MinC simply by causing the division inhibitor to accumulate at the membrane to a local concentration that is sufficiently high for blocking FtsZ assembly (28, 46). How DicB activates MinC function has not yet been addressed.

In this paper we investigate the mechanism(s) whereby MinD and DicB stimulate MinC-dependent division inhibition. We find that, like MinD, DicB interacts directly with the C-terminal D domain of MinC and that MinC/DicB blocks cell division by preventing the formation of stable FtsZ rings. Using MinC derivatives which lack a functional Z domain, we further show that complexes between MinC and either DicB or MinD have a high affinity for one or more septal ring components in vivo. The results suggest that both MinD and DicB activate MinC function by directing its D domain to, or close to, the substrate of its Z domain. For MinD, the bulk recruitment of MinC to the membrane is likely to contribute to its MinC-activating properties, and this step may normally precede the more specific targeting of MinC/MinD to membrane-associated septal ring components. For DicB, we find no evidence for binding of MinC/DicB to the membrane per se, suggesting that DicB stimulates MinC-mediated division inhibition primarily by directly targeting the inhibitor to its substrate.

MATERIALS AND METHODS

E. coli strains, plasmids, and phages. Relevant E. coli strains, plasmids, and phages used in this study are listed in Table 1. Strain L11 was obtained by P1-mediated transduction of lon::Tn10 from RC7 to PB114.

Phage JXE39 was obtained after crossing λNT5 with plasmid pJE39 (see below), as described previously (11).

Plasmids pBAD33 (20), pDB182 (10), pDR120 (21), and pDR175 (46) were Plasmids pBAD33 (20), pDB182 (10), pDR120 (21), and pDR175 (46) were obtained from commercial sources. Unless specified otherwise, Plasmids pBAD33, pDB182, pDR120, and pDR175 were obtained from commercial sources. Unless specified otherwise, Plasmids pBAD33, pDB182, pDR120, and pDR175 as the template) pJE44 was constructed in two steps. First, plasmid pDB182 was digested with EcoRI and SalI, yielding a 252-bp fragment, which was ligated to its SalI-compatible overhangs. This fragment was used to replace the mutant with the wild-type version of minD simply by causing the division inhibitor to accumulate at

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ACACTCTGACCTATG-3' and 5'-TATGTTGTGACCTAAACGACCTGGCT G-3' to amplify sfA. The product was treated with NdeI and SalI (sites underlined), and the 565-bp fragment was inserted into pET21b, yielding pDB276. The 611-bp XbaI-HindIII fragment of the latter was then inserted into pBAD33, placing sfA downstream of PBAD.

The plasmids listed above are derivatives of pSC101 derivative plDR175 (46). Except for pJE46, which lacks gfp, all encode fusion proteins in which Gfpmut2 (GFP) is linked, via the T7.tag peptide (T; Novagen) to the N-terminus of various portions of MinC. As indicated in Table 1, expression of these fusions is under the control of PBAD and c577.

Creation of these plasmids involved the use of two other new constructs: pDB411, a pUC18/pET21b hybrid vector, and pJE13, which was used as template in PCR described below.

To obtain pDB411, pUC18 DNA was treated with SalI and XhoI, followed by incubation with deoxynucleoside triphosphates and Klenow enzyme to produce blunt ends. Recircularization of the large fragment resulted in a pUC18 derivative (pDB410) in which EcoRI and XhoI sites were regenerated but in which no other restriction sites were deleted. Ligation of the 769-bp AphAminBael fragment of pDB410 to the 2,144-bp AphAminEael fragment of pET21b yielded pDB411.

Plasmid pJE13 encodes a fusion protein [STII-T-MinC(5-231)] in which the StreptagII (STII; Genosys) and T7.tag epitopes replace the STII epitope with His tag peptide (h) coding sequences, when these sequences are present in the genetic construct, are indicated.

<table>
<thead>
<tr>
<th>Strain, plasmid</th>
<th>Relevant genotypea</th>
<th>Source or reference</th>
</tr>
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<tr>
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<td></td>
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</tr>
<tr>
<td>PB103</td>
<td>dadr trpE trpA trnA</td>
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</tr>
<tr>
<td>PB147</td>
<td>PB103, ΔminDE</td>
<td>22</td>
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<tr>
<td>PB114</td>
<td>PB103, ΔminCDE::aph</td>
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<tr>
<td>RC7</td>
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<td>Phages</td>
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<td>ΔADR155</td>
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</tr>
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<td>10</td>
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<td>JXE39</td>
<td>imm31 bla lacF1+ Padd::gfp-t::dicB</td>
<td>This work</td>
</tr>
</tbody>
</table>

a The positions of in-frame gfp (gfp), linker peptide (l), and His tag peptide (h) coding sequences, when these sequences are present in the genetic construct, are indicated.
which encodes T7.tag-MinC(14-231). NheI was replaced with the 696-bp XbaI-MluI-NcoI fragment from pLL13 with the 948-bp NcoI-MluI fragment of pDR175.

For plasmid pPC105 we performed a PCR with primers 5'-TAGGTCCTCGAGATTTA and 3'-NcoI fragment of pLL19, yielding pLL21. Last, to construct pLL14, a PCR fragment was generated with primers 5'-gfp-ftsZ and 3'-NcoI fragment of pPC103 to produce plasmid pPC105. Plasmid pJE78 was used to replace the 1,636-bp BamHI-SalI fragment of pDR120 (21). The same 696-bp fragment was next isolated from the resulting plasmid (pDB379) and inserted into the MCS of pGAD-C1 and pGBDU-C1, respectively.

**Yeast methods and two-hybrid β-galactosidase assays.** Plasmids encoding BD fusions (pGAD derivatives) were introduced into strain PJ69-4A, and those encoding AD fusions (pGPD derivatives) were introduced into SL304 by the lithium acetate transformation method (16). Appropriate pairs of transformants were mated, and diploids were selected by growth on plates containing synthetic complete medium (19) lacking leucine and uracil (C-Leu-Ura).

**Microscopy and other methods.** Live cells were examined for most experiments. Sometimes, however, cells were chemically fixed as described previously (1), except that cells were incubated in the presence of formaldehyde, glutaraldehyde, and sodium phosphate buffer (pH 7.5) at final concentrations of, respectively, 2.40%, 0.04%, and 30 mM for cells grown in Luria-Bertani medium or 2.40%, 0.04%, and 30% for cells grown in M9 minimal salts medium supplemented with 0.2% Casamino Acids, 0.2% maltose, and 100 μM IPTG and then fixed as described above. The procedure preserved a level of GFP-MinF(C14-21) signal sufficient for imaging directly. For fluorescence and differential interference contrast (DIC) imaging, cells were applied to a microscope slide and viewed with a Zeiss Axioplan-2 microscope outfitted with a Hamamatsu C4742-95 progressive scan cooled charge-coupled device camera and a plan-NEOFULAR (100×, numerical aperture = 1.3) oil immersion objective, by using Cy3-specific (580-nm dichroic mirror, 510- to 560-nm excitation filter, and 590-nm barrier filter) and/or Cy5-specific (495-nm dichroic mirror, 450- to 490-nm excitation filter, and 500- to 550-nm barrier filter) filter sets for fluorescent images and Nomarski optics for DIC images. Images were captured with QED software and were further manipulated with Adobe Photoshop.

Cell lengths and positions of sepal and fluorescent rings were measured by using Object Image, version 1.6, software (55). Length-to-ring (L/R) ratios were determined as described previously (21).

Western analyses were performed essentially as described previously (13) with the following modifications. Nitrocellulose blots were incubated overnight at 4°C with a commercially available rabbit anti-GFP antibody (Sigma) diluted 1:5,000. After three washes, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma) diluted 1:4,000. After four additional washes, blots were developed with the ECL Western blotting analysis system (Amersham Pharmacia Biotech). Digital images were analyzed as described previously (21).
of blots were collected with a Bio-Rad Fluor-S-Max MultiImager using the accompanying Quantity One software.

RESULTS

MinC/DicB interferes with the assembly of FtsZ rings. To study the effect of MinC/DicB expression on the integrity of FtsZ rings, we used strain PB147(ADR120) [ΔminDE (Plac::gfp-ftsZ)], harboring either plasmid pJE44 [PBAD::dicB] or vector pBAD33. Cells were grown in the presence of 37 μM IPTG (to induce expression of GFP-FtsZ) to an OD₆₀₀ of 0.2. Arabinose was then added (to 0.05%) to induce expression of dicB, and aliquots were prepared for microscopic examination at 60-min intervals. The results are summarized in Table 2 and Fig. 1.

As expected, cells containing pBAD33 continued to divide normally upon addition of arabinose (Fig. 1C'), whereas those harboring pJE44 developed a pronounced division defect (Fig. 1A' and B'). The average length of the latter cells was already 3.5-fold (12.0 μm) that of the former (3.4 μm) at the time of arabinose addition. This was likely due to a low level of dicB expression from the plasmid even before arabinose was added. After 3 h, the average dicB-expressing cell was about ninefold longer (23.3 μm) than the control cells (2.7 μm).

Throughout the experiment, about 80% of PB147(ADR120)/pBAD33 cells showed brightly fluorescent ring structures, and the L/R ratio of the population as a whole increased only slightly from 3.1 (0 min) to 3.4 μm (180 min). In comparison, the localization of GFP-FtsZ in PB147(ADR120)/pJE44 filaments differed significantly in that the level of fluorescence in the cytoplasm was noticeably elevated and far fewer ring structures were present. This is reflected in the L/R value for the population as a whole, which increased substantially from 6.1 μm at 0 min to 27.1 μm at 120 min (Table 2). By 180 min, this value had decreased slightly to 26.0 μm. At this time, the culture also contained a significant number of small, ring-containing cells (an example is shown in Fig. 1B), resulting in a decreased average cell length compared to that at 120 min. Since PB147 is Ara⁺, we presume this reemergence of small cells was due to consumption of the inducing sugar.

In addition to finding a high L/R value, we found that many of the ring-like accumulations still present in the PB147(ADR120)/pJE44 filaments were very dim (Fig. 1A), indicating that they contained relatively little FtsZ and suggesting that they represent poorly assembling and/or disintegrating Z rings. We conclude that MinC/DicB interferes with the assembly and/or integrity of FtsZ rings.

MinC and DicB interact in a yeast two-hybrid system. To test whether MinC and DicB can interact directly, we used a yeast two-hybrid assay (31). As controls, we also tested the interaction between MinC and MinD and DicB with MinD, as well as the interaction of MinC, MinD, and DicB with themselves (Table 3). Consistent with previous reports, we observed

### Table 2. Quantitation of cell length and FtsZ rings after MinC/DicB-mediated filamentation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Time (min)</th>
<th>R− cells</th>
<th></th>
<th>R+ cells</th>
<th></th>
<th>R− + R+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (n)</td>
<td>Avg length (range) (μm)</td>
<td>% (n)</td>
<td>Avg length (range) (μm)</td>
<td>L/R (μm)</td>
<td>Avg length (range) (μm)</td>
</tr>
<tr>
<td>pJE44 [PBAD::dicB]</td>
<td>10</td>
<td>20 (9)</td>
<td>6.7 (3.2–20.0)</td>
<td>80 (36)</td>
<td>13.4 (4.4–33.5)</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7 (2)</td>
<td>21.9 (8.0–35.8)</td>
<td>93 (25)</td>
<td>15.1 (4.2–49.8)</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>35 (9)</td>
<td>39.0 (4.2–66.3)</td>
<td>65 (17)</td>
<td>30.3 (2.6–70.8)</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>30 (17)</td>
<td>51.4 (3.5–98.4)</td>
<td>70 (39)</td>
<td>11.0 (2.5–97.6)</td>
<td>8.6</td>
</tr>
<tr>
<td>pBAD33 (vector)</td>
<td>0</td>
<td>16 (14)</td>
<td>2.5 (1.9–3.5)</td>
<td>84 (72)</td>
<td>3.5 (1.9–15.9)</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>22 (13)</td>
<td>2.5 (1.8–3.8)</td>
<td>78 (47)</td>
<td>2.8 (1.8–4.5)</td>
<td>2.7</td>
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</table>

* Cells of strain PB147(ADR120) [ΔminDE(Plac::gfp-ftsZ)] harboring the indicated plasmid or vector were treated and analyzed as described in the text. Parameters were calculated separately for cells without fluorescent ring structures (R− cells), cells with one or more rings (R+ cells), and all cells combined (R− + R+ cells). n, number of cells examined. Any visible accumulation of fluorescence resembling a ring was counted as one.

**FIG. 1.** Inhibition of Z-ring assembly by MinC/DicB. Fluorescence (A to C) and corresponding DIC (A’ to C’) micrographs showing the distribution of GFP-FtsZ in cells which either were (A and B) or were not (C) subjected to MinC/DicB-mediated division inhibition. Cells of strain PB147(ADR120) [ΔminDE(Plac::gfp-ftsZ)] carrying either pJE44 [PBAD::dicB] (A and B) or vector pBAD33 (C) were grown at 37°C in the presence of 37 μM IPTG to an OD₆₀₀ of 0.2. Arabinose was added to 0.05%, and growth was allowed to continue for 60 (A) or 180 (B and C) min. Arrowheads (A), positions of strongly fluorescent ring structures. Arrow, position of faintly fluorescent structures. Bar, 4 μm.
a moderately strong interaction between MinC and MinD (24, 30, 39) and a weaker interaction of MinC with itself (26). Interestingly, MinC interacted very strongly with DicB in both genetic configurations, demonstrating a direct interaction between the two proteins. DicB did not appear to interact with itself to a significant degree. In contrast, MinD showed a strong interaction with itself (Table 3), as was recently also observed by Szeto et al. for both the Neisseria gonorrhoeae and the E. coli proteins (52). This self-interaction is consistent with the proposed ability of MinD to cooperatively assemble on the membrane during its dynamic localization cycle in vivo (22, 27, 41, 48).

**Localization of MinC in DicB-induced filaments.** MinD recruits MinC to the cytoplasmic membrane (28, 46). To determine whether DicB might similarly affect the cellular distribution of MinC, we studied cells of strain PB114(DB182)/pLL18 [ΔminCDE(pIC::dicB)/cI857. PcI::gfp-minC], lysogenic for either λDB182 [pλ::dicB] (A to C) or ADR155 [pλ::minD] (D). Cells were grown at 37°C in the presence of either 0.1% glucose (A) or 100 μM IPTG (B to D) and were chemically fixed prior to examination. Bar, 4 μm.

**FIG. 2.** Different effects of MinD and DicB on the distribution of GFP-MinC. Micrographs show the distribution of GFP-MinC in the presence of MinD (D) or DicB (B and C) or in the absence of either activator (A). Shown are cells of strain PB114/pLL18 [ΔminCDE/cI857. PcI::gfp-minC], lysogenic for either λDB182 [pλ::dicB] (A to C) or ADR155 [pλ::minD] (D). Cells were grown at 37°C in the presence of either 0.1% glucose (A) or 100 μM IPTG (B to D) and were chemically fixed prior to examination. Bar, 4 μm.

**TABLE 3. Yeast two-hybrid interactions**

<table>
<thead>
<tr>
<th>Plasmids (BD/AD)</th>
<th>Protein fused to:</th>
<th>BD Activity (U)</th>
<th>AD Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJE30/pJE10</td>
<td>MinC</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>pJE9/pJE10</td>
<td>MinD</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>pJE55/pJE10</td>
<td>DicB</td>
<td>132</td>
<td>13</td>
</tr>
<tr>
<td>pJE30/pJE54</td>
<td>MinC</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>pJE9/pJE54</td>
<td>MinD</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pJE55/pJE54</td>
<td>DicB</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pJE9/pJE8</td>
<td>MinD</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>pJE30/pGAD-C2</td>
<td>MinC</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pJE9/pGAD-C2</td>
<td>MinD</td>
<td>3</td>
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</tr>
<tr>
<td>pJE55/pGAD-C2</td>
<td>DicB</td>
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</table>

* BD and AD indicate fusions to the yeast Gal4 binding domain and activating domain, respectively.
* a, unfused AD.

septal rings, resulting in a filamentous phenotype as well as a redistribution of MinC back into the cytoplasm. To test this scenario, we used a fusion (GFP-DMinC) in which the first 13 aa of MinC are replaced by GFP. Whereas this fusion still interacts with DicB and MinD, it no longer inhibits cell division and, thus, lacks a functional Z domain (Table 4 and our unpublished data).

When strain PB114(ΔDB182)/pLL13 [ΔminCDE(pIC::dicB)/cI857. PcI::gfp-t-minC(14-231)] was grown at 37°C in the absence of inducer, cells displayed a Min− phenotype and the fluorescence signal was distributed throughout the cytoplasm (Fig. 3A and A'). This random distribution was not due to a possible release of the GFP moiety by proteolytic processing, as only the full-length fusion was detectable in Western analyses using anti-GFP antibodies (Fig. 4, lane 2). When grown in the presence of IPTG, the cells were still Min−. Strikingly, however, the expression of DicB caused the GFP-DMinC fusion to accumulate in bright rings (Fig. 3B and C; Table 4). Such rings were present in ~80% of the population (189 of 230 cells), and their cellular locations corresponded to those expected for septal ring structures, including sites of active cell wall invagination. To confirm this location, cells were fixed and immunostained with anti-FtsZ antibodies. As illustrated in Fig. 5A, the GFP-DMinC rings indeed colocalized with FtsZ to septal rings in these cells. We next examined the localization of GFP-DMinC in cells expressing both DicB and division inhibitor SfiA (SucA), which interferes with the assembly and maintenance of septal rings by blocking FtsZ polymerization (42, 54) independently of MinC (10). To this end, strain PB114(ΔDB182)/pLL13 was transformed with either vector

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<thead>
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<th>Protein fused to:</th>
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<td>MinC</td>
<td>7</td>
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<td>MinD</td>
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<td>MinD</td>
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<tr>
<td>pJE55/pJE54</td>
<td>DicB</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pJE9/pJE8</td>
<td>MinD</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>pJE30/pGAD-C2</td>
<td>MinC</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pJE9/pGAD-C2</td>
<td>MinD</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>pJE55/pGAD-C2</td>
<td>DicB</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
TABLE 4. Functionality and localization of GFP-MinC fusions

<table>
<thead>
<tr>
<th>Protein (plasmid)</th>
<th>LLI (ΔDB182) [ΔminCDE(P&lt;sub&gt;lac&lt;/sub&gt;−minD)]</th>
<th>LLI (ΔDR155) [ΔminCDE(P&lt;sub&gt;lac&lt;/sub&gt;−minD)]</th>
<th>LLI (ΔDB175) [ΔminCDE(P&lt;sub&gt;lac&lt;/sub&gt;−minD)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP−T−MinC(5−231) (pLL18)</td>
<td>Sep&lt;sup&gt;−&lt;/sup&gt;</td>
<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sep&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>GFP−T−MinC(14−231) (pLL13)</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
<td>R</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>GFP−T−MinC(108−231) (pPC105) (pLL14)</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
<td>C</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>GFP−T−MinC(108−231)−H (pJE78)</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
<td>R</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>GFP−T−MinC(108−208)−H (pJE79)</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
<td>C</td>
<td>Min&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lysozymes containing the indicated plasmids were grown for ~5 h at 37°C in the presence of 100 μM IPTG to an OD<sub>600</sub> of 0.1. Live cells were observed through fluorescence optics to determine the localization pattern of the GFP−MinC fusion (Loc.) and through phase-contrast optics to determine the division phenotype (Phen.). C, cytoplasmic; M, along the periphery of the cell; O, oscillating; R, stably associated with rings; O/R, transiently associated with rings during oscillation cycle. When grown in the absence of IPTG, cells were Min<sup>−</sup> and fluorescence was cytoplasmic in all cases.

<sup>b</sup> Ring-like accumulations were detected at a low frequency in these filaments (see text).

plasmid pBAD33 or with a derivative (pJE80) that carries sfbA downstream of the P<sub>BAD</sub> promoter. Cells carrying the control plasmid continued to divide and showed the DicB-dependent accumulation of GFP−DMinC in rings, regardless of the presence of arabinose in the growth medium (Fig. 6C). In contrast, cells carrying pJE80 ceased division upon addition of arabinose, and the resulting filaments were completely devoid of fluorescent rings (Fig. 6D). These results showed that, in the presence of DicB, MinC is targeted directly to one or more septal ring components.

To evaluate what domain of the MinC peptide is required for DicB-dependent targeting, we used pLL18 derivatives encoding GFP fusions to various portions of MinC. As shown in Table 4, fusions containing residues 108 to 231 of MinC still accumulated in rings in a DicB-dependent fashion, whereas fusions containing aa 141 to 231 or aa 108 to 208 failed to do so. These results are consistent with genetic analyses of minC missense alleles (43) and indicate that the domain of MinC required for interaction with DicB overlaps that responsible for its interaction with MinD (see below) (26).

DIC-dependent integrity and localization of DicB. The DicB-mediated targeting of MinC to a septal ring ligand could occur in several ways. (i) Without itself being targeted to a specific cellular location, DicB might bind cytoplasmic MinC and modify it to a form with affinity for a septal ring component. (ii) DicB itself might have affinity for a septal ring factor and recruit MinC. (iii) The binding of DicB to MinC might create a site on the heteromeric complex with a high affinity for a septal ring-associated target.

To discriminate between these possibilities, we studied the location of a GFP−DicB fusion in the absence and presence of ΔminC. The ΔminCDE strain PB114(ΔJE39)pJE46 is lysogenic for phage λJE39 [P<sub>lac</sub>−gfp−dicB], which encodes GFP−DicB under the control of the lac promoter and which also harbors plasmid pJE46 [cI857, P<sub>lac</sub>:minC(14−231)], which expresses MinC (aa 14 to 231) in a temperature-dependent fash-

![FIG. 3. Mutually dependent accumulation of ΔMinC and DicB on rings.](image-url)

![FIG. 4. Detection of GFP−ΔMinC and GFP−DicB by immunoblotting.](image-url)
ion. Upon growth in the presence of IPTG (DicB-GFP<sup>+</sup>) at 30°C (3<sup>MinC</sup>'), cells showed fluorescence throughout the cell body (Fig. 3D). Upon growth at 37°C (3<sup>MinC</sup>'), cells still showed a significant signal throughout the cytoplasm. However, the vast majority of cells also showed clear accumulations in rings, which were easily discernible above the cytoplasmic signal (Fig. 3E and F).

To evaluate the integrity of the GFP-DicB fusion in these experiments, we performed Western analyses using anti-GFP antibodies. Interestingly, whereas a significant amount of intact GFP-DicB (35 kDa) was present in cells coexpressing 3<sup>MinC</sup> (Fig. 4, lane 5), the full-length species was almost undetectable in cells lacking 3<sup>MinC</sup> (lane 4). In both cases, cell extracts contained multiple smaller products, which migrated as 30- to 31-kDa species during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Such species might still contain an intact GFP moiety (27 kDa) and contribute to the significant fluorescence signal seen in the cytoplasm of these cells. We obtained very similar results with a DicB-GFP fusion in which the GFP tag is fused to the C terminus of DicB (data not shown). Given the size of the DicB fusion breakdown products and our experience that many other GFP fusions are much more stable (e.g., GFP-MinC; Fig. 4, lane 2), the likeliest explanation of these results is that the binding of 3<sup>MinC</sup> to the DicB moiety of the fusion protects the latter from rapid proteolytic attack.

The Lon protease is a major protein-processing factor in <i>E. coli</i>, and the SfiA division inhibition protein is among its substrates (18). To assess whether Lon might be responsible for processing the GFP-DicB fusion, we also examined the integrity of the latter in LL1, a <i>lon</i> derivative of strain PB114. As in PB114, however, the concentration of intact GFP-DicB in LL1 was strongly dependent on the coexpression of 3<sup>MinC</sup> (data not shown). Thus, although Lon may contribute to the degradation of GFP-DicB, it is clearly not the sole factor responsible for its instability.

Due to the instability of the DicB fusions in the absence of MinC, we are not yet able to conclusively dismiss the possibility that a stable form of DicB by itself may have some intrinsic affinity for the septal ring. Nevertheless, our results strongly favor the possibility that 3<sup>MinC</sup> and DicB colocalize to the septal ring as part of a heteromeric complex.

**Interdependent targeting of 3<sup>MinC</sup> and MinD to the septal ring in MinE<sup>+</sup> cells.** In MinE<sup>+</sup> filaments, the bulk of both MinD and MinC is distributed along the membrane (28, 46, 48, 50). In many such filaments, however, this distribution is not completely even. Quite often, some small but distinct accumulations at the membrane are evident, especially in the smaller filaments. Figure 2D shows an example of such an accumulation in a filament of strain PB114(ADR155)/pLL18 [ΔminCDE(P<sub>lac</sub>−:minD)/c1857, P<sub>lac</sub>−:gfp-minC(5-231)]. As for the MinC/DicB filaments described above, we suspected that these accumulations could correspond to remnants of septal rings which had not yet been fully cleared by the action of 3<sup>MinC</sup>.

To test this idea, we introduced pLL13 into strain PB114(ADR155) and studied the localization of GFP-3<sup>MinC</sup> in the absence and presence of MinD by growing cells in the absence and presence of IPTG, respectively. As shown in Fig. 7A to D, the fusion accumulated in bright ring structures in a MinD-dependent manner. Again, these rings corresponded to septal rings, as shown by colocalization with FtsZ (Fig. 5B) and their sensitivity to Sfa (Fig. 6B). Furthermore, the minimal domain of MinC required for MinD-mediated targeting of 3<sup>MinC</sup> to septal rings was the same as that for DicB-mediated targeting (aa 108 to 231; Table 4).

To evaluate whether, conversely, MinD would be attracted to septal rings in a 3<sup>MinC</sup>-dependent manner, we used strain PB114(ADR119)/pJE46 [ΔminCDE(P<sub>lac</sub>−:gfp-minD)/c1857,
GFP-MinD in the absence (E) and presence (F) of DMinC. (A to D) in the absence (A) and presence (B to D) of MinD and that of GFP-MinD in the absence (E) and presence (F) of DMinC. (A to D) Cells of strain PB114(\lambda DR119)/pJE46/pJE44 [\Delta minCDE(P_{\text{suc}}::gfp-minD)c1857, P_{\text{AR}}::gfp-minC(14-231)] which were grown at 37°C in the absence (A) or presence (B to D) of 100 \mu M IPTG. (E and F) Cells of strain PB114(\lambda DR119)/pJE46 [\Delta minCDE(P_{\text{suc}}::gfp-minD)c1857, P_{\text{AR}}::minC (14-231)] which were grown in the presence of 37 \mu M IPTG at 30°C (E) or 37°C (F). Bar, 2 \mu m.

FIG. 7. Mutually dependent accumulation of DMinC and MinD on rings. Micrographs show the distribution of GFP-DMinC (A to D) in the absence (A) and presence (B to D) of MinD and that of GFP-MinD in the absence (E) and presence (F) of DMinC. (A to D) Cells of strain PB114(\lambda DR115)/pLL13 [\Delta minCDE(P_{\text{suc}}::minD)c1857, P_{\text{AR}}::gfp-minC(14-231)] which were grown at 37°C in the absence (A) or presence (B to D) of 100 \mu M IPTG. (E and F) Cells of strain PB114(\lambda DR119)/pJE46 [\Delta minCDE(P_{\text{suc}}::gfp-minD)c1857, P_{\text{AR}}::minC (14-231)] which were grown in the presence of 37 \mu M IPTG at 30°C (E) or 37°C (F). Bar, 2 \mu m.

Diesel competes with MinD for binding MinC in vivo. Expression of dicB induces filamentation in both wild-type (WT) and minD cells, suggesting that MinC targeting by DicB is dominant over that by MinD (10, 35). One possibility is that DicB efficiently competes with MinD for binding MinC. Alternatively, DicB might be able to bind the MinC/MinD complex, after which the tripartite complex targets some septal ring component. To discriminate between these possibilities, we introduced plasmid pJE44 into strain PB114(\lambda DR119)/pJE46. In the resulting transformants, expression of GFP-MinD can be regulated with IPTG, that of DMinC can be regulated with temperature, and that of DicB can be regulated with arabinose. Cells that were grown at 37°C in the presence of IPTG but without arabinose showed bright rings as described above (Fig. 8A). In contrast, rings were almost completely absent when cells were grown in the presence of 0.05% arabinose for 4 h. Instead, the GFP-MinD fusion was distributed more or less evenly over the entire membrane (Fig. 8C). In the presence of less arabinose (0.02%), cells with intermediate patterns predominated (Fig. 8B). These results indicate that DicB competes with MinD for complex formation with MinC.

Transient association of oscillating DMinC with septal rings. The affinity of DMinC/MinD for septal rings described above was observed most clearly in cells lacking MinE (see above). In WT cells, the MinC/MinD complex oscillates from pole to pole in a MinE-dependent fashion and an accumulation of MinC/MinD in ring structures is not, or at best very rarely, observed (28, 46, 48, 50). A rationale for the latter is that MinE prevents an association of Minc/MinD with the septal ring in WT cells by sweeping the complex away from the cell center and that noncentral septal rings simply are not present, or are highly unstable, due to the action of the intact Z domain of MinC (15, 22, 41). It can be predicted, therefore, that, even in the presence of MinE, MinC/MinD should be capable of decorating noncentral septal rings provided they are sufficiently stable.

To test this prediction, we compared the localization of fully functional MinC with that of DMinC, in cells expressing both MinD and MinE. For this purpose, pLL18 [c1857, P_{\text{AR}}::gfp-
introduced into strain LL1(H9261) and cells were grown in the presence of IPTG at 37°. Cells expressing the GFP-DMinC fusion were MinC accumulating on ring structures (Table 4; Fig. 9A). As expected, cells expressing the GFP-MinC fusion showed a WT division phenotype (A). Bar, 2 μm.

FIG. 9. Transient association of oscillating ΔMinC with rings. Time-lapse images show the oscillation of GFP-MinC (A) and GFP-ΔMinC (B and C) in the presence of MinD and MinE. Shown are cells of strain LL1(ΔDB175) [ΔminCDE(Pbac::minDE)] harboring either pLL18 [cI857, P_{AR::gfp-minC}(5-231)] (A) or pPCI05 [cI857, P_{AR::gfp-minC}(108-231)] (B and C). Cells were grown at 37°C in the presence of 100 μM IPTG. Times in seconds are indicated. Arrows in the DIC panels (B and C) mark the positions of rings to which GFP-DMinC transiently associated as it moved from one end of the cell to the other. Such transient associations are not observed when the Z domain of MinC is functional (A). Bar, 2 μm.

Multistep Mechanism for FtsZ Ring Destruction by MinC

Time-lapse images of individual cells and measured cell length and the distances between transiently fluorescent rings and the proximal cell pole. For comparison, we also measured the positions of division septa in a parallel culture. As shown in Fig. 10, transient association of GFP, ΔMinC/MinD occurred almost exclusively at non-centrally positioned rings, providing further support for the proposed role of MinE in keeping MinC/MinD away from septal ring structures located at the cell center (15, 22, 41).

Decoration of septal rings by ΔMinC/DicB is unaffected by MinE. In contrast to a MinC/MinD-induced division block, MinC/DicB-induced filamentation is not suppressed by MinE (10), suggesting that MinE should have little effect on the targeting of MinC/DicB to septal ring structures. To test this prediction, plasmid pJE75 [P_{BAD::minE}] or vector pBAD33 was introduced into strains PB114(ΔDR155)/pLL13 [ΔminCDE(Pbac::minD)/cI857, P_{AR::gfp-minC}(14-231)] and PB114(ΔDB182)/pLL13 [ΔminCDE(Pbac::dicB)/cI857, P_{AR::gfp-minC}(14-231)]. Transformants were grown at 37°C in the presence of both IPTG (100 μM) and arabinose (0.1%), and cells were examined for the localization of GFP, ΔMinC.

Interestingly, PB114(ΔDB182)/pLL13 cells harboring pJE75 showed fluorescent rings (Fig. 11D) at about the same frequency (~80% of cells) as cells of either strain carrying the control plasmid (Fig. 11A and C). In contrast, the GFP, ΔMinC fusion appeared almost completely cytoplasmic in virtually all PB114(ΔDR155)/pLL13/pJE75 cells (Fig. 11B). We note that although MinC oscillates from pole to pole when MinE is present at physiological levels (see above), MinC becomes cytoplasmic at high levels of MinE (J. E. Johnson and P. A. J. de Boer, unpublished data), which may be related to the ability of MinE to interfere with the interaction between MinC and MinD (30).

We conclude that, in contrast to the localization of ΔMinC/MinD, the targeting of ΔMinC/DicB to septal rings is indeed unaffected by MinE.
FIG. 11. GFP-DMinC/DicB rings are resistant to MinE. Fluorescence (A to D) and DIC (A′ to D′) micrographs show the localization of GFP-DMinC in MinC+ DicB+ (A and B) and MinC− DicB+ (C and D) cells in the absence (A and C) or upon (over)expression (B and D) of MinE. Shown are cells of strains PB114(ADR155)/pLL13/pBAD33 [ΔminCDE(Plac::minD)cI857, P_Ac::gfp-minC (14-231)/vector] (A), PB114 (ADR155)/pLL13/pJE75 [ΔminCDE(Plac::minD)cI857, P_Ac::gfp-minC (14-231)/Plac::minE] (B), PB114(ADR182)/pLL13/pBAD33 [ΔminCDE (Plac::dicB)cI857, P_Ac::gfp-minC (14-231)/vector] (C), and PB114 (ADR182)/pLL13/JE75 [ΔminCDE(Plac::dicB)cI857, P_Ac::gfp-minC (14-231)/Plac::minE] (D). Cells were grown for 3.5 h to an OD600 of 0.3 at 37°C in the presence of 100 μM IPTG and 0.1% arabinose before examination. Bar, 2 μm.

DISCUSSION

This study further elucidates the mechanism by which MinD and DicB stimulate the division-inhibitory activity of MinC. Both MinD and DicB were found to interact directly with the C-terminal D domain of the MinC peptide, and both the DMinC/MinD complex and the DMinC/DicB complex were shown to have a high affinity for septal ring structures in vivo. These observations indicate that the Z and D domains of MinC participate in separate interactions with components of the division apparatus. The Z domain is thought to interact directly with FtsZ polymers, resulting in depolymerization (26, 29). In contrast, the interaction of the D domain with division components requires the binding of this domain to MinD or DicB, and it appears to be the complex of DMinC with either activator which interacts with some septal ring factor(s).

These results suggest a three-step mechanism for MinC-mediated division inhibition as outlined in Fig. 12E. In the first step, the D domain of MinC binds to either MinD or DicB. Under normal conditions, only the interaction with MinD is relevant because dicB transcription is actively repressed (3). When transcription is induced, however, the DicB protein effectively competes with MinD for binding MinC. In the next step, the complex of MinC with either MinD or DicB binds a target which is closely associated with FtsZ polymers, bringing the Z domain of MinC in close proximity to its substrate. Finally, the Z domain stimulates depolymerization of FtsZ, which simultaneously leads to dispersal or destruction of the target recognized by the D domain complex.

Although the purified Z domain of MinC was shown to stimulate FtsZ depolymerization in vitro (26, 29), one recent report suggested that MinC might block cell division in vivo primarily by interfering with the interaction between FtsZ and FtsA rather than by acting on FtsZ polymers directly (32). Subsequent localization studies, however, using either GFP-tagged FtsZ in live E. coli (45) (C. Hale and P. A. J. de Boer, unpublished data) or anti-FtsZ antibodies in Bacillus subtilis (36) quite clearly show that MinC/MinD-induced filamentation coincides with a failure to assemble Z rings. Here we showed that an intact Z domain of MinC is also required for MinC/DicB-induced filamentation and that Z rings fail to assemble in such filaments as well. Therefore, it is highly likely that both MinC-dependent division blocks, whether stimulated by MinD or DicB, result from a direct interference with FtsZ polymerization by the Z domain of MinC.

It remains to be determined what septal ring component(s) is recognized by DMinC/MinD and DMinC/DicB and whether both complexes bind the same or different targets. In WT cells, the MinC/MinD complex is rarely, if ever, seen to decorate a ring. Rather the proteins rapidly move from the membrane at one cell end to the other in an oscillatory membrane association-dissociation cycle, which requires the activities of both MinD and MinE. Due to the action of MinE, MinC/MinD complexes are kept away from the assembling or assembled septal ring at the cell center, while they prevent assembly of complete Z rings at noncentral sites due to the activity of ZMinC in the complex (15, 22, 26–29, 46, 48, 50). Blocking the assembly of functional Z rings near cell poles requires a minimum oscillation frequency of at least 0.25 full cycles per min (22, 48). From this observation it can be inferred that FtsZ must be capable of rapidly initiating polymerization on the noncentral portion of the membrane soon after MinC/MinD has departed. Therefore, we expect that the physiological targets of MinC/MinD during normal growth are small septal ring intermediates that assembled within the previous 20 to 25 s (one-half oscillation cycle) on that half of the cell membrane from which MinC was temporarily absent (Fig. 12A to D). We imagine that such incipient structures consist of FtsZ polymers and factors that rapidly associate with the polymers. Associated factors could include FtsA and ZipA, which bind FtsZ directly, as well as one or more other division factors which can join the assembly subsequent to ZipA and FtsA (7). In the simplest scenario, the DMinC/MinD complex is directly attracted to (polymers of) FtsZ. However, any other septal ring component which associates with FtsZ before the assembly of a stable Z ring is completed might provide, or contribute to the formation of, a specific binding surface for DMinC/MinD (Fig. 12E).

It will also be interesting to determine what determinants within the DMinC/MinD and DMinC/DicB complexes provide specificity for the septal ring target(s). Binding specificity may be provided by a (sub)domain on DMinC which is exposed only upon binding to either MinD or DicB. Alternatively, specificity may be provided by domains on MinD and DicB that become exposed upon binding to DMinC or by a combination of determinants present on both partners in the complexes. In the crystal structure of MinC from Thermotoga maritima, the D domain is folded in a triangular, right-handed β-helix, one side of which provides the dimer interface (8). How MinD and DicB bind such a structure and how their binding might affect this structure are intriguing questions.

Especially with regard to the possibility that MinD might provide binding specificity to a septal ring component(s), it is interesting to compare our results with those obtained with B. subtilis, where the binding of the MinC/MinD complex to the
FIG. 12. Models for MinD- and DicB-dependent activation of MinC-mediated division inhibition. Under conditions of normal growth (A to D), MinC and MinD co-oscillate from the membrane on one cell half to the other in a MinE-dependent fashion (MinC/MinD is indicated by a thick black line or squares; MinE is omitted for simplicity). FtsZ polymerization (chains of open circles) is allowed to initiate on any site of the membrane not occupied by MinC/MinD and not subject to nucleoid occlusion (38). As soon as MinC/MinD returns to occupy that half of the membrane, nascent ring structures that formed in the previous half cycle and that are positioned off-center are the substrate for MinC/MinD-mediated destruction (C and D), while structures that may have formed at the cell center are protected because the oscillating behavior of the division inhibitor causes its time-averaged concentration to be minimal at the cell’s middle (22, 25, 41). Upon induction of dicB expression in WT cells (B’ and C’), DicB efficiently competes with MinD for binding MinC to form MinC/DicB complexes (shaded triangles). The latter do not oscillate and destroy nascent septal ring structures at all sites in the cell, resulting in filamentous growth of the bacterium. At a molecular level (E), MinC-mediated division inhibition in vivo is likely to occur in several steps. In step 1, MinD or DicB (open triangle) binds the C-terminal D domain of MinC (DMinC; black rectangle portion of molecule). This binding event produces a complex with a high affinity for a target that is part of both nascent and mature septal ring structures. In the simplest scenario, the inhibitor complex directly recognizes polymers of FtsZ (chain of Z-shaped monomers). Alternatively, additional septal ring components (open and shaded circles) may first need to associate with FtsZ polymers to create target sites. In step 2, DMinC/MinD or DMinC/DicB binds the target. This event is independent of any interaction involving the N-terminal domain of MinC (2MinC; black oval portion of molecule). Rather, step 2 serves to bring 2MinC in close proximity to its substrate, allowing efficient disassembly of FtsZ polymers in step 3. Disassembly of FtsZ polymers by 3MinC simultaneously destroys the septal ring ligand(s) recognized by the 3MinC complex (step 4). As a result, MinC/DicB is instantly released into the cytoplasm, whereas MinC/MinD remains associated with the surrounding membrane until it is forced off by the action of the next MinE wave (15, 22, 27).
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