Identification of *Escherichia coli* ZapC (YcbW) as a Component of the Division Apparatus That Binds and Bundles FtsZ Polymers\(^\dagger\)†

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Assembly of the cell division apparatus in bacteria starts with formation of the Z ring on the cytoplasmic face of the membrane. This process involves the accumulation of FtsZ polymers at midcell and their interaction with several FtsZ-binding proteins that collectively organize the polymers into a membrane-associated ring-like configuration. Three such proteins, FtsA, ZipA, and ZapA, have previously been identified in *Escherichia coli*. FtsA and ZipA are essential membrane-associated division proteins that help connect FtsZ polymers with the inner membrane. ZapA is a cytoplasmic protein that is not required for the fission process per se but contributes to its efficiency, likely by promoting lateral interactions between FtsZ protofilaments. We report the identification of YcbW (ZapC) as a fourth FtsZ-binding component of the Z ring in *E. coli*. Binding of ZapC promotes lateral interactions between FtsZ polymers and suppresses FtsZ GTPase activity. This and additional evidence indicate that, like ZapA, ZapC is a nonessential Z-ring component that contributes to the efficiency of the division process by stabilizing the polymeric form of FtsZ.

Cytokinesis in *Escherichia coli* and most other bacteria is driven by a complex ring-shaped organelle variously referred to as the divisome, septosome, or septal ring (SR). The mature, constriction-competent SR in *E. coli* contains more than two dozen different protein components. Ten of these (FtsA, -B, -I, -K, -L, -N, -Q, -W, and -Z and ZipA) are essential to the cell constriction process and can be considered to form the core of the apparatus. Cells lacking any of the core proteins fail to constrict and display the classical lethal division phenotype, and assembly of the cell division apparatus in bacteria starts with formation of the Z ring on the cytoplasmic face of the membrane. This process involves the accumulation of FtsZ polymers at midcell and their interaction with several FtsZ-binding proteins that collectively organize the polymers into a membrane-associated ring-like configuration. Three such proteins, FtsA, ZipA, and ZapA, have previously been identified in *Escherichia coli*. FtsA and ZipA are essential membrane-associated division proteins that help connect FtsZ polymers with the inner membrane. ZapA is a cytoplasmic protein that is not required for the fission process per se but contributes to its efficiency, likely by promoting lateral interactions between FtsZ protofilaments. We report the identification of YcbW (ZapC) as a fourth FtsZ-binding component of the Z ring in *E. coli*. Binding of ZapC promotes lateral interactions between FtsZ polymers and suppresses FtsZ GTPase activity. This and additional evidence indicate that, like ZapA, ZapC is a nonessential Z-ring component that contributes to the efficiency of the division process by stabilizing the polymeric form of FtsZ.

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activity in vitro (ZapC) as a new component of the division machinery in instead (25). It is a small and abundant protein that 78). The recently discovered ZapB appears restricted to the gammaproteobacteria. In vivo, FtsA* stimulates cell fission at reduced cell mass and 1394. 

The ZapA and ZapB proteins also associate with early Z- ring assemblies. Unlike FtsA and ZipA, these proteins are not also has the remarkable property of supporting efficient cell division in the complete absence of ZipA (27, 28). The ZapA and ZapB proteins also associate with early Z-rings. Unlike FtsA and ZipA, these proteins are not essential, and E. coli single mutants show only modest phenotypes (23, 25, 33, 45, 57). ZapA is well conserved and, like ZipA, binds and bundles FtsZ polymers in vitro and promotes the assembly and stability of the Z ring in vivo (33, 51, 57, 58, 78). The recently discovered ZapC appears restricted to the gammaproteobacteria. It is a small and abundant protein that forms antiparallel coiled-coil dimers that readily polymerize into filaments in vitro (23). Initially suspected to interact with FtsZ directly, it associates primarily with the Z ring via ZapA instead (25).

In genetic and cytological screens, we identified YebW (ZapC) as a new component of the division machinery in E. coli that binds FtsZ directly. In vivo, ZapC localizes to Z rings independently of FtsA, ZipA, ZapA, or ZapB, and overexpression of the protein results in coassembly of ZapC and FtsZ in aberrant structures and lethal filamentation. In vitro, ZapC suppresses FtsZ GTPase activity and promotes lateral association of FtsZ polymers. Cells lacking only ZapC divide almost normally, but its absence significantly aggravates filamentation of cells already lacking ZapA or a functional Min system. The results indicate that, similar to ZapA, ZapC is a nonessential division protein that contributes to the process by promoting interactions between FtsZ filaments in the Z ring.

**MATERIALS AND METHODS.**

*E. coli plasmids.* Relevant plasmids are listed in Table 1. The plasmids pET21b (Novagen), pGAD-C1 and pBDU-C1 (42), pDR10 (36), pDB361 and pDR120 (37), pCH151 (10), pKNT25 (45), pCH325 (6), pCH363, pE21, pTB97, pTB98, pTB146, and pTB183 (7) and pMG20 (29) have been described before. Unless indicated otherwise, TB28 chromosomal DNA was used as a template in amplification reactions. Sites of interest (e.g., relevant restriction sites or those engineered for targeted recombination) are underlined in primer sequences.

To construct pH3 (attHK022 P<sub>zapC</sub>::gfp-le), the 1,339-bp Apal-HindIII fragment of pCH315 (see below) was used to replace the 1,849-bp Apal-HindIII fragment of pTB183 (attHK022 P<sub>zapC</sub>::gfp-zapA).

For pBL3 (attHK022 P<sub>zapC</sub>::gfp-le), the 2,303-bp Apal-HindIII fragment of pMG6 (see below) was used to replace the 1,849-bp Apal-HindIII fragment of pTB183 (attHK022 P<sub>zapC</sub>::gfp-zapA).

To obtain pCH299 (P<sub>gal4BD::zapC-le</sub>), the 1,017-bp XbaI–XhoI fragment of pMG20 [P<sub>gal4BD::TorA-hsp70-BAI</sub>·gal4BD-zapC] was replaced with the 852-bp XbaI–XhoI fragment of pCH315.

To obtain pCH299 (P<sub>gal4BD::zapC-le</sub>), the zapC gene of pMG6 was amplified with the primers 5'-GAGGCCATATGCGAATTAAACCAGACGATAACTG-3' and 5'-GTACAGCTCGAGATCCATG-3' and 5'-GTCAGCTCGAGATCCATG-3' and 5'-GTCAGCTCGAGATCCATG-3'. The product was digested with NdeI and XhoI, and the 542-bp fragment was used to replace the 582-bp XbaI–XhoI fragment of pCH299.

For pBL3 (P<sub>gal4AD::zapC-le</sub>), the 2,103-bp ApaI–HindIII fragment of pCH325 (see below) was used to replace the 1,849-bp Apal-HindIII fragment of pTB183 (attHK022 P<sub>gal4AD::zapC</sub>::gfp-zapA).

For pBL3 (P<sub>gal4AD::zapC-le</sub>), the 2,303-bp Apal-HindIII fragment of pMG6 (see below) was used to replace the 1,849-bp Apal-HindIII fragment of pTB183 (attHK022 P<sub>gal4AD::zapC</sub>::gfp-zapA).

For pBL3 (P<sub>gal4AD::zapC-le</sub>), the 2,303-bp Apal-HindIII fragment of pMG6 (see below) was used to replace the 1,849-bp Apal-HindIII fragment of pTB183 (attHK022 P<sub>gal4AD::zapC</sub>::gfp-zapA).
CCTGTTCAGGGCTGAAGC-3'. The product was digested with BamHI and SalI, and the 549-bp fragment was used to replace the 12-bp BamHI-SalI fragments of pCH321 and pGAD-C1, respectively. The plasmid pCH322 except that pWM3632 [P\text{zapC}::\text{aph}]+ was used to replace the 17-bp SacI-XbaI fragment of pDSW208, placing trc promoter to amplify the zapC-gfp fusion. Two loss-of-function mutants of zapC were obtained as described above for pCH321 and pCH330, except that pWM3632 was used as a template.

The plasmid pCH438 [P\text{att}C::\text{padh}1::\text{gfp}-\text{ftsZ}] was obtained in a manner similar to that of pWM2978, as described below.

The plasmid pCH458 [P\text{att}C::\text{zapA}::\text{ftsZ}] was recovered as a solid-blue colony after plating an EZTnKan-2 (\text{aph}::\text{Tn}) transconjugant of TX3772. Strain Slm260 (\text{lac}IZYA::\text{ap}, \text{gfp}-\text{ftsZ} (R286W))44 was obtained by transduction of strain Slm260 into TX3772. Several colonies grew, and plasmids from these colonies were transformed back into TX3772 under the same selection conditions. Those that gave rise to transformants at high efficiency were then subjected to sequencing of the putative zapC mutant. Two loss-of-function mutants of zapC were identified encoding a L22P mutant and a E27G/R164A double mutant. Both encoded stable proteins, and they had similar phenotypes. The L22P mutant (encoded on pWM3632) was chosen for further study.

**Strains.** Relevant E. coli strains are listed in Table 2. Strains BL5 and BL6 were obtained by P1-mediated transduction (hereafter referred to simply as "transduction") of zapC\textsuperscript{minCDE} (zapC::EZTnKan-2) from SLm260 to TB28 and TB43, respectively.

CH41 was obtained by transduction of zapC::\text{cat} from CH21 to TB28. (The symbol "\text{\textsuperscript{-}}" denotes DNA replacement by recombineering.)

CH57 was created by a Red-mediated recombineering (16, 89). The cat cassette of pKD3 was amplified using the primers 5'-GGTACCTAGGAAGGCCTTTTTTGCAATATGGAATTACGCCG-3' and 5'-ATGCTGAACTCGTCTGACTCATGTGCTTCTTTTGTAT-3'.

The plasmid pCH322 was used to replace the 17-bp SacI-XbaI fragment of pDSW208, yielding a 1,097-bp zapC\textsuperscript{minCDE} fragment (chromosomal sequences are underlined), which was recombined with the chromosome of TB10. In CH56, cat replaces 570 bp of the zapC gene (from bp -37 to +533), while flanking genes (\text{padh}1 and \text{ych}X) are intact.

**TABLE 2. E. coli strains used in this study**

<table>
<thead>
<tr>
<th>Strain\textsuperscript{a}</th>
<th>Relevant genotype\textsuperscript{b}</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL5</td>
<td>TB28, zapC\textsuperscript{minCDE}::\text{cat}</td>
<td>This work</td>
</tr>
<tr>
<td>BL6</td>
<td>TB28, minCDE::\text{frt} zapC\textsuperscript{minCDE}::\text{cat}</td>
<td>This work</td>
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<tr>
<td>CH2\textsuperscript{*}</td>
<td>PB103, ftsA\textsuperscript{recA}:\text{Tim}</td>
<td>37</td>
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<tr>
<td>CH5\textsuperscript{*}</td>
<td>PB103, zipA::zfap recA::\text{Tim}</td>
<td>36</td>
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<tr>
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<td>44</td>
</tr>
<tr>
<td>CH41</td>
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<td>This work</td>
</tr>
<tr>
<td>CH56</td>
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</tr>
<tr>
<td>CH57</td>
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<td>This work</td>
</tr>
<tr>
<td>CH59</td>
<td>TB28, zapC\textsuperscript{minCDE}::\text{frt}</td>
<td>This work</td>
</tr>
<tr>
<td>CH63</td>
<td>TB28, zapC\textsuperscript{minCDE}::\text{frt} zapC\textsuperscript{minCDE}::\text{cat}</td>
<td>This work</td>
</tr>
<tr>
<td>CH64</td>
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<td>MG1655</td>
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<td>MB103</td>
<td>dada rpe::\text{trpA}</td>
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</tr>
<tr>
<td>PB143\textsuperscript{*}</td>
<td>PB103, ftsZ\textsuperscript{recA}:\text{Tim}</td>
<td>37</td>
</tr>
<tr>
<td>Slm260\textsuperscript{f}</td>
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<td>This work</td>
</tr>
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<td>TB10</td>
<td>MG1655, nadA::\text{Tim} \chi\text{185777} (\text{cros-bio})</td>
<td>44</td>
</tr>
<tr>
<td>TB28</td>
<td>MG1655, lac\text{U}\text{Z}\text{A}::\text{cat}</td>
<td>10</td>
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<tr>
<td>TB43</td>
<td>MG1655, lac\text{U}\text{Z}\text{A}::\text{cat}</td>
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<tr>
<td>TX3772</td>
<td>MG1655, lac\text{U} \text{Z}</td>
<td>81</td>
</tr>
<tr>
<td>WM3004</td>
<td>TX3772, zapC\textsuperscript{minCDE}::\text{cat}</td>
<td>This work</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Note that strains marked with "\text{\textsuperscript{-}}" required an appropriate plasmid, phage, and/or inducer for survival.

\textsuperscript{b} The symbol "\text{-}" denotes DNA replacement by recombineering, and "\text{frt}" indicates a scar sequence remaining after evocation of an \text{aph} or \text{cat} cassette by Flp recombinase (16, 89).

\textsuperscript{f} The Slm260 allele \((\text{aph}::\text{EzTnCan-2})\) encodes the first 20 residues of ZapC, followed by the nonsense peptide TSVYTHLNPEACMPGRL.

\textsuperscript{f} Cells were routinely grown at 30°C in LB (0.5% NaCl) or M9 minimal medium supplemented with 0.2% maltose, 0.2% Casamino Acids, 50 \mu M thiamine (M9-mal). When appropriate, medium was supplemented with 36 \mu M kanamicin (M9-car) and 50 \mu M ampicillin (M9-car). Strain Slm260 (\text{slm} \text{H} \text{L}22 \text{P}) was isolated by transduction of strain Slm260 into TX3772.
Protein purification. Native (untagged) FtsZ was purified as described previously (39). Untagged ZapC and ZapC(L22P) were purified using a SUMO fusion system (7, 53, 59). Strain BL21(ADE3)plysS (Novagen), harboring either pCH322 (P gal-sumo-zapC) or pCH372 (P gal-sumo-zapC(L22P)) was grown overnight in LB-Amp-Camp with 0.1% glucose. The culture was diluted 1:100 into 0.5 liter of LB-Amp-Camp with 0.04% glucose and grown at 37°C to an optical density at 600 nm (OD600) of 0.5. IPTG was added to 840 µM, and growth was continued at 30°C for 4 h. Cells were harvested by centrifugation (2,000 x g, 20 min, 4°C), washed once in 20 ml ice-cold 0.9% NaCl, resuspended in 20 ml ice-cold cell lysis (CL) buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0), flash frozen in dry ice-acetone, and stored at −80°C. Cells were broken by stirring the tube in a 37°C water bath to quickly thaw the suspension, followed by two more quick-freeze-thaw cycles. After addition of 1 µl Benzonase (Novagen), the lysate was incubated on ice for 30 min and, following brief sonication, subjected to centrifugation (175,000 x g, 90 min, 4°C). The majority of H-SUMO-ZapC or H-SUMO-ZapC(L22P) was present in the supernatant, 2.5 ml portions of which were loaded on 0.5-ml columns of Ni-NTA-agarose (Qiagen) pre-equilibrated in CL buffer. Columns were washed with 4 x 1.0 ml of CL buffer containing 20 mM imidazole and 3 x 0.35 ml of CL buffer containing 50 mM imidazole, and bound protein was eluted with 4 x 0.25 ml of CL buffer containing 250 mM imidazole. Peak fractions were pooled and dialyzed against buffer A (50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol [DTT], 10% glycerol, pH 8.0). The column was washed to 0.2% NP-40, H-ULp protease was added to a final molar ratio of 1:300 (protease/substrate), and the mixture was incubated overnight on ice. To capture the His-tagged protease and freed H-SUMO tag, the mixture was brought to 10 mM imidazole and loaded on a 0.5-ml Ni-NTA-agarose column equilibrated in buffer B (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 8.0) and further fractionated by anion-exchange chromatography on a Mono-Q column (Pharmacia) with a linear 100 to 600 mM NaCl gradient in the same buffer. The majority of ZapC or ZapC(L22P) eluted between 300 and 350 or 450 and 540 mM NaCl respectively. Peak fractions were pooled, dialyzed against buffer C (20 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 10% glycerol, pH 8.0), and stored at −80°C.

Gel filtration. A calibrated Superose-12 column was equilibrated in buffer (20 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, pH 8.0), loaded with 25 µg of purified ZapC or ZapC(L22P) (100 µl of 11 mM solution), and run at 400 µl/min with equilibration buffer on an Akta purifier UPC-10 system at 4°C. The column was calibrated with blue dextran (2,000 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine α-chymotrypsin (200 kDa), horse heart cytochrome C (12,400 kDa), and phosphorylase b (92,500 kDa). The column was eluted with 0.55 µM of ZapC rather than to possible polar effects of the HC022 site), and the distribution of FtsZ in such filaments was highly aberrant.

Coedensation assay. Reaction mixtures (100 µl final volume) containing buffer (50 mM HEPES· KOH, 50 mM KCl, 4 mM MgCl2, pH 7.0), bovine serum albumin (BSA) (3 µM), and other proteins as appropriate were assembled on ice. GTP or GDP was added to 1 mM, and after 5 min at room temperature, the mixtures were subjected to high-speed centrifugation (278,353 x g) for 15 min at 25°C in a Beckman TL-100 ultracentrifuge. Pellets were resuspended in 100 µl of buffer, and equal amounts (11 µl/lane) of pellet and supernatant fractions were loaded on SDS-PAGE gels. After electrophoresis, proteins were stained with Coomassie brilliant blue, gels were digitally imaged with a Fluor-S multi-imager (Bio-Rad), and band intensities were measured using accompanying software.

Light scattering assay. Light scatter (90° angle) was monitored in a Jobin Yvon Horiba FluoroMax-3 fluorometer using a wavelength of 350 nm and slit widths set at 1.5 nm. Reactions (150 µl final volume) were kept at 30°C using a water-bath jacket.

GTPase assay. Reactions were started by addition of 2 mM [γ-32P]GTP (~37.5 Ci mmol−1, corresponding to 3 µCi per reaction). The conversion of [γ-32P]GTP to [α-32P]GDP at the indicated time was determined by quantitative thin-layer chromatography, essentially as described before (17, 49).

Electron microscopy. Reactions (50 µl final volume) containing 50 mM HEPES·OH (pH 7.0), 50 mM KCl, 4 mM MgCl2, and proteins as needed were assembled without nucleotide and placed at 30°C for 2 min. GTP or GDP was added to 1 mM, and 5 min later a 10 µl aliquot was applied to a carbon-coated copper grid (300-mesh) that had been pretreated with 10 µl of a Bactracin solution (7.5 µg/ml in water) for 0.5 min and wicked dry. After 35 s, the grid was wicked dry, treated with 10 µl uranyl acetate (2%) for 45 s, and wicked dry again. Grids were allowed to dry further in air and were then examined with a JEOL 1200 EX transmission electron microscope (TEM) at 80 kV.

Other methods. Fluorescence and differential interference contrast (DIC) microscopy (7), immunofluorescence staining of cells with affinity-purified anti-FtsZ antibodies (37, 43), measurements of cell parameters (6), Western analyses with anti-green fluorescent protein (GFP) (10) or anti-FLAG (74) antibodies, and yeast two-hybrid assays (43, 44) were performed as described previously.

RESULTS

Identification of ZapC (YcbW) as a new septal ring component. Two initial observations suggested an involvement of the zapC (ycbW) gene in the cell division process of E. coli. First, in a survey of the localization patterns of E. coli proteins that are fused to GFP (ASKA library [47]), we noticed accumulation of a YcbW-GFP fusion at sites of cell constriction (Fig. 1B). Second, in screens for transposon insertion mutants that are particularly detrimental in the absence of a functional Min system (slm screens) (8, 9), we recovered multiple mutants in which EZTn Kan-2 had inserted in the ycbW open reading frame (ORF). Because subsequent evidence presented below showed that ycbW encodes a nonessential FtsZ-associating protein, we renamed the gene zapC. The gene is expected to encode a cytoplasmic protein of 180 residues (~20.6 kDa), and homologues can be identified in 98 other gammaproteobacterial species belonging to the Enterobacteriales, Vibrionales, Al teromonadales, or Aeromonadales (Pfam 24.0, DUF1379 [24]).

ZapC promotes cell division. To assess the phenotype associated with loss of zapC, we selected one EZTn Kan-2 insertion allele (zapCsin260) for further study and also created ΔzapC strains from which the complete ORF was removed (Fig. 1A). Phenotypes associated with either allele were identical (see below; also data not shown). When cultured to late-logarithmic growth (OD600 = 1.2), cells of strain BL5 (zapCsin260) were slightly longer on average (~8%) than those of the wild-type (wt) parent, TB28. Consistent with the recovery of the zapC lesions in the slm screen, however, this effect was significantly more pronounced in cells lacking a functional Min system. Thus, cells of strain BL6 (∆minCDE zapCsin260) were on average 74% longer than those of TB43 (∆minCDE), and the length-to-septum ratio (L/S) increased from 17 µm in TB43 to 55 µm in BL6 (Table 3). The filamentous phenotype of BL6 could be suppressed in an IPTG-dependent manner by ectopic expression of zapC from the lac promoter on pBL3 (P lac::zapC) (plasmid pBL3, integrated at the chromosomal arlHK022 site), indicating that inefficient division was indeed due to a lack of ZapC rather than to possible polar effects of the zapC lesion on nearby genes (Fig. 2A).

Overexpression of ZapC interferes with proper FtsZ assembly. While expression of zapC at low levels of IPTG (up to 25 µM) suppressed filamentation of B6(iBL3) cells, the average cell length increased again at higher levels of inducer (Fig. 2A and data not shown), suggesting that an excess of ZapC also interfered with cell division. Indeed, expression of zapC from multicopy plasmids led to a lethal division block and the formation of long nonseptate filaments (Fig. 2B). Interestingly, the distribution of FtsZ in such filaments was highly aberrant. Rather than forming canonical Z rings, FtsZ assembled into spots, spirals, and more linear rod-like structures (Fig. 2B, arrows).

Localization of ZapC-GFP to rings requires FtsZ but not FtsA, ZipZ, ZapA, or ZapB. To establish requirements for recruitment of ZapC to the division site, we monitored the
distribution of a ZapC-GFP fusion in cells lacking various other septal ring components. Depletion of FtsZ led to a cyttoplasmic distribution of ZapC-GFP in the resulting filaments (Fig. 3B), indicating that FtsZ is required for the normal accumulation of the protein in rings (Fig. 1B and 3A). In contrast, ZapC-GFP did localize to ring-like structures upon depletion of FtsA (Fig. 2C). Moreover, a ZapC(L22P)-GFP fusion failed to accumulate at division sites (Fig. 1C), and this failure was not due to excessive processing of the fusion as judged by Western analyses (Fig. 1D).

We then tested native and mutant ZapC for self-interaction and interaction with FtsZ in a yeast two-hybrid assay. As shown in Table 4, the results indicated a robust interaction between ZapC and FtsZ and little to no interaction of ZapC with itself or with ZapC(L22P). In contrast, ZapC(L22P) failed to interact with FtsZ in this assay but instead showed an appreciable level of self-interaction. Increased self-interaction associated with the L22P mutation was also indicated by the hydrodynamic properties of purified ZapC, a new division protein that binds FtsZ directly

TABLE 3. Division phenotypes of ΔzapC cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>No. of cells</th>
<th>Length (μm)</th>
<th>No. of septa</th>
<th>% polar</th>
<th>L/S (μm)</th>
</tr>
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<tbody>
<tr>
<td>TR28</td>
<td>wt</td>
<td>500</td>
<td>3.6 (1.0)</td>
<td>184</td>
<td>0</td>
<td>9.7</td>
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<tr>
<td>BL5</td>
<td>zapC&lt;sup&gt;mls260&lt;/sup&gt;</td>
<td>526</td>
<td>3.9 (1.1)</td>
<td>133</td>
<td>0</td>
<td>15.6</td>
</tr>
<tr>
<td>TB43</td>
<td>ΔminCDE</td>
<td>505</td>
<td>6.5 (3.7)</td>
<td>99</td>
<td>19.8</td>
<td>17.2</td>
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<tr>
<td>BL6</td>
<td>ΔminCDE&lt;sup&gt;zapC&lt;sup&gt;mls260&lt;/sup&gt;&lt;/sup&gt;</td>
<td>463</td>
<td>11.3 (7.1)</td>
<td>95</td>
<td>17.9</td>
<td>55.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were grown in LB at 30°C to OD<sub>600</sub> of 1.3 and chemically fixed before imaging and measurements of cell parameters.

<sup>b</sup> Total number of cells measured.

<sup>c</sup> Lengths are averages, and standard deviations are given in parentheses. Minicells were not included in length measurements.

<sup>d</sup> Total number of septa, including polar ones.

<sup>e</sup> Ratio of total cell length and total number of septa.
ZapC and ZapC(L22P) were purified using a SUMO fusion system (7, 53, 59) and analyzed by gel filtration on a calibrated Superose-12 column (Fig. 4). The majority (~90%) of purified ZapC eluted in a single peak corresponding to a molecular mass of 31 kDa and the rest in a minor peak at 62 kDa. Given the predicted molecular mass of ZapC (20.6 kDa), this result indicated that purified ZapC was mostly monomeric. In contrast, approximately half (~55%) of ZapC(L22P) eluted as an apparent dimer (57 kDa) and the rest as apparent tetramers (117 kDa) or higher-order oligomers. This result parallels that of the yeast two-hybrid assays in indicating that the L22P mutation increases the affinity of ZapC for itself.

In vitro interactions between ZapC and FtsZ. To obtain direct evidence for an interaction between ZapC and FtsZ, we next used purified components in cosedimentation assays. FtsZ (6 μM) was mixed in polymerization buffer (50 mM HEPES, KOH, 50 mM KCl, 4 mM MgCl₂, pH 7.0) containing 3 μM BSA (control), 2 μM ZapC or ZapC(L22P), and 1 mM GTP or GDP. After a short incubation at 25°C, reaction mixtures were subjected to high-speed centrifugation and sedimentable and soluble material was analyzed by SDS-PAGE. When incubated alone in the presence of GTP, about one-fifth (22%) of FtsZ sedimented under these conditions (Fig. 5, lane 1), and very little (4%) of ZapC sedimented in the absence of FtsZ (lane 7). When both proteins were incubated with GTP, however, ~80% ZapC cosedimented with ~45% FtsZ (lane 3), suggesting the formation of large heteromultimeric protein complexes. In contrast, ZapC(L22P) failed to increase the fraction of sedimentable FtsZ (lane 4), and ~20% of ZapC(L22P) sedimented regardless of the presence (lane 4) or absence (lane 8) of FtsZ. These results support the in vivo evidence that ZapC associates with FtsZ and that the L22P mutation interferes with the ability of ZapC to do so.

Further support for this came from 90°-angle light scatter analyses of FtsZ polymerization reactions. Addition of GTP (Fig. 6, traces 2 and 3) to a solution of FtsZ (6 μM) in polymerization buffer led to a modest increase in scatter (ΔS = 30 arbitrary units; t > 60 s and < 200 s), reflecting the assembly of FtsZ polymers. However, subsequent addition of ZapC (to 2 μM; trace 2) led to a significantly larger additional increase in scatter (ΔS = 95; t > 200 s and < 500 s), indicating the growth at 30°C in LB with the indicated concentration of IPTG to an OD₆₀₀ of 1.1 to 1.3. Cells were chemically fixed before imaging, and between 247 and 263 cells were measured in each case. Minicells were ignored. (B and C) Shown are wt cells expressing GFP-FtsZ from a weak constitutive promoter and overexpressing either ZapC (B) or ZapC(L22P) (C). Panel B shows that excess ZapC causes assembly of FtsZ into spiral-like, rod-like, or other aberrant configurations (arrows). A fraction of the population that probably lost the ZapC plasmid was of normal size and showed regular Z-ring structures (arrowheads). Panel C illustrates that overexpression of ZapC(L22P) had little to no effect on Z-ring assembly or cell division. Strain TB28(iTB198) [wt(Plac::gfp-ftsZ)] harboring either plasmid pCH315 (P₆₅₋::zapC) (B) or pCH388 (P₆₅₋::zapC(L22P)) (C) was grown at 30°C in M9-maltose with 20 μM IPTG. At an OD₆₀₀ of 0.5 to 0.6, live cells were imaged with fluorescence (left or upper portions of panels) and differential interference contrast (DIC) optics. Bars in panels B and C equal 4 μm.
formation of larger protein complexes. In contrast, addition of ZapC(L22P) (trace 3) instead of the native protein resulted in a much smaller increase in scatter (ΔS = 13; t > 200 s and < 500 s). Little to no increase in scatter was observed when GTP was replaced with GDP (trace 1), and neither ZapC nor ZapC(L22P) contributed significantly to the scatter signal in the absence of FtsZ (traces 4 and 5).

**ZapC promotes associations between FtsZ filaments.** Transmission electron microscopy was used to visualize the effect of ZapC on FtsZ polymers. Incubation of FtsZ (6 μM) with GTP in polymerization buffer at neutral pH led to the formation of mostly short (<400-nm) and thin (5- to 10-nm) filamentous structures, likely corresponding to single and paired FtsZ protofilaments (Fig. 7A). Strikingly, inclusion of 2 μM ZapC caused the formation of extended networks of polymer bundles. The latter were of various diameters (~10 to 40 nm), indicating they comprised various numbers of FtsZ protofilaments (Fig. 7B). Filamentous structures were absent when GDP was substituted for GTP (Fig. 7D) or when FtsZ was omitted from the reaction (not shown), indicating that the formation of bundle networks required FtsZ polymerization. As expected, and in contrast to ZapC (Fig. 7B), ZapC(L22P) had no obvious effect on the appearance of FtsZ polymers (Fig. 7C).

**ZapC suppresses FtsZ GTPase activity.** The ability of ZapC to promote lateral associations between FtsZ protofilaments likely promotes their stability, and this was supported by a significant reduction in the GTPase activity of FtsZ in the presence of ZapC (Fig. 8). Thus, GTPase activity was already reduced by ~40% at a molecular ratio of 1 ZapC to 5 FtsZ and was reduced further (by ~60%) as the ratio approached unity. In comparison, the mutant ZapC(L22P) protein reduced the GTPase activity by less than 10% in parallel assays (Fig. 8).

<table>
<thead>
<tr>
<th>BD-fused protein</th>
<th>AD-fused protein</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZapC</td>
<td>—</td>
<td>7</td>
</tr>
<tr>
<td>ZapC</td>
<td>FtsZ</td>
<td>90</td>
</tr>
<tr>
<td>ZapC</td>
<td>ZapC</td>
<td>12</td>
</tr>
<tr>
<td>ZapC</td>
<td>ZapC(L22P)</td>
<td>5</td>
</tr>
<tr>
<td>ZapC(L22P)</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>ZapC(L22P)</td>
<td>FtsZ</td>
<td>11</td>
</tr>
<tr>
<td>ZapC(L22P)</td>
<td>ZapC(L22P)</td>
<td>49</td>
</tr>
<tr>
<td>ZapC(L22P)</td>
<td>ZapC</td>
<td>12</td>
</tr>
</tbody>
</table>

*BD, binding domain; AD, activation domain.

TABLE 4. ZapC interacts with FtsZ in yeast two-hybrid system

## FIG. 3. Localization of ZapC-GFP in cells lacking various division proteins. Strains harboring plasmid pMG6 (Plac::zapC-gfp) were grown at 30°C in LB with 25 μM (B to D and F) or 30 μM (A and E) IPTG, and live cells were imaged with fluorescence (left or upper portions of panels) and differential interference contrast (DIC) optics. Strains used were as follows: PB103 (wt) (A), PB143/pDB346 [ftsZ0/cI857(ts)] (B), CH2/pDB355 [ftsA4/cI857(ts) PftsZ::ftsA] (C), CH5/pDB361 [zipA0/cI857(ts) PftsZ::zipA] (D), JE32 [zipA0/ftsA*(R286W)] (E), and CH65 (zapA::zapC) (F). Note the cytoplasmic localization of ZapC-GFP in FtsZ-depleted filaments (B), the accumulation of ZapC-GFP into nonring blobs and rods upon depletion of ZipA (D, arrows), and restoration of the normal localization pattern of the fusion in cells in which the essential function of ZipA is bypassed by the FtsA(R286W) variant (E). Bar = 4 μm.

## FIG. 4. Gel filtration analyses of purified ZapC and ZapC(L22P). Overlaid elution profiles of purified ZapC (black) and ZapC(L22P) (gray) are shown. Calculated molecular masses (in kDa) are indicated at corresponding peaks. The results indicate that purified ZapC (calculated molecular mass = 20.6 kDa) is mostly monomeric, while ZapC(L22P) elutes as dimers and tetramers. A calibrated Superose-12 column was equilibrated in buffer (20 mM Tris · Cl, 100 mM KCl, 1 mM EDTA, pH 8.0), loaded with 25 μg of purified protein, and run at 400 μl/min with equilibration buffer.
The ZapC and ZapC(L22P) preparations by themselves lacked measurable GTPase activities (not shown).

**ZapC protects against MinC overexpression.** The results described above are consistent with a role for ZapC in stabilizing FtsZ assemblies in the cell. This notion was also supported by an increased sensitivity of \(H_9004\) zapC cells to overexpression of the MinC division inhibitor, which directly binds and destabilizes FtsZ assemblies (15, 73). When a FLAG-tagged version of MinC encoded on plasmid pWM2801 was overproduced to a similar level (Fig. 9C) in isogenic wt or \(H_9004\) zapC cells, the latter were significantly more filamentous than the former (Fig. 9B) and showed correspondingly poor survival in spot-titer assays (Fig. 9A).

**ZapA and ZapC promote cell division synergistically.** Several of the properties of ZapC described above are reminiscent of those of ZapA (see Discussion), suggesting they play similar roles in stabilizing the division process. This idea was supported by division phenotypes of strains in which lesions of \(\Delta\)zapA and \(\Delta\)zapC were combined. Though the average cell length of \(\Delta\)zapC cells was slightly higher than that of wt cells during late-logarithmic growth (Table 3), they were of normal length when sampled during mid-logarithmic growth (4.4 \(\mu\)m at an OD_{600} of 0.6 to 0.8) (Table 5). In comparison, isogenic \(\Delta\)zapA cells were measurably longer by 34% (5.9 \(\mu\)m) (Table 5), as was also observed by others (25, 57). However, \(\Delta\)zap/\(\Delta\)zapC cells were more than twice (216%) as long as wt cells (9.5 \(\mu\)m) (Table 5). Introduction of an additional \(\Delta\)zapB lesion to create strain CH65 (\(\Delta\)zapABC) did not increase the average length much further (9.7 \(\mu\)m) (Table 5). As expected, the average cell length of CH65 could be reduced again by production of ZapC from iBL3 (\(P_{\text{lac}}\)::zapC) in an IPTG-dependent manner. Production of ZapC-GFP was also effective in suppressing the filamentous phenotype of CH65, indicating that the fusion retained at least some ZapC functionality (Table 5).

**DISCUSSION**

In this study, we showed that ZapC (YcbW) is a component of the division machinery in *E. coli* and that it interacts directly with the key division protein FtsZ. In addition to FtsA, ZipA, and ZapA, therefore, ZapC represents the fourth Z-ring protein that engages FtsZ directly and independently of other division proteins. ZapB was suspected of interacting with FtsZ as well but associates with the Z ring via an interaction with ZapA instead (23, 25).

In addition to binding FtsZ, ZapC shares other properties with ZipA and ZapA, indicating overlapping functions among the Z-ring proteins. Like ZipA (39, 62, 69) (C. A. Hale and P. A. J. de Boer, unpublished data) and ZapA (33, 51, 57, 71, 78), ZapC promotes the polymeric form of FtsZ by stimulating...
association of FtsZ protofilaments and suppression of FtsZ GTPase activity (Fig. 5 to 8).

Moreover, like ZipA (36, 69) and ZapA (25, 33, 57, 58), the stabilizing effect of ZapC on FtsZ polymers is likely to contribute to the integrity and function of the Z ring. Though ΔzapC cells divide almost normally under standard growth conditions, they are more sensitive to overexpression of the FtsZ polymerization antagonist MinC (Fig. 9) and show pronounced division defects when they additionally lack ZapA or a functional Min system (Tables 3 and 5). In the latter case, it is likely that competition for a limiting pool of FtsZ subunits between the multiple Z rings that form in Min− cells renders each structure more dependent on polymer-stabilizing factors for successful development into a functional septal ring (8, 25, 90).

Like FtsA, ZipA is a membrane-associated protein that is normally essential for division (36), even though certain hypermorphic variants of FtsA can compensate for its absence (27). In contrast, both ZapA (25, 33, 44, 57) and ZapC (Tables 3 and 5) are nonessential cytoplasmic proteins that each contribute only modestly to the division process in E. coli. However, the fact that ΔzapA ΔzapC double mutants divide significantly less efficiently than either single mutant (Table 5) indicates that the mild phenotypes of ΔzapA and ΔzapC single mutants can be at least partially explained by the overlapping biochemical activities of ZapA and ZapC identified in this study. Moreover, since ZipA also stabilizes FtsZ polymers (39, 62, 69), this activity may be sufficient to support division, albeit inefficiently, even when ZapA and ZapC are both lacking.

In this regard, we note that a partially redundant role for FtsA in stabilizing FtsZ polymers also seems likely. Even...
though the hypermorphic variant FtsA* (R286W) was recently found to stimulate depolymerization of FtsZ protofilaments (12), this activity is more compatible with a role of FtsA in Z-ring constriction during active cell envelope invagination than with its role in Z-ring assembly (65) and with the ability of FtsA* to compensate for the absence of ZipA (27). The latter indicates that \textit{in vivo} FtsA also plays a role in stabilizing FtsZ polymers, and it has been proposed that it is the self-interaction state of FtsA that determines whether it promotes stability or instability of FtsZ polymers (75). Thus, all four Z-ring partners of FtsZ may contribute to stability of FtsZ polymers in the ring, especially during early stages of SR development.

Overexpression of ZapC caused FtsZ to assemble in a variety of remarkable noncanonical structures (Fig. 2), and similar aberrant FtsZ assemblies were formed when ZapC-GFP was expressed at a low level in filaments that had been depleted for ZipA (Fig. 3D; see also Fig. S1 in the supplemental material). Cells also form filaments upon overexpression of FtsA (84) and ZipA (36, 69), but overexpression of FtsA leads to broad and diffuse FtsZ zones (75), suggesting FtsZ polymers become unstable, while overexpression of ZipA results in filaments with multiple sharply defined Z rings that fail to support constriction (69) (Hale and de Boer, unpublished observations). We suspect that excess ZapC leads to the indiscriminate stabilization and growth of FtsZ-ZapC coassemblies that can be seeded anywhere in the cell by normally short-lived FtsZ polymers and that compete effectively for FtsZ monomers with proper Z rings, especially when ZipA is lacking.

As with ZipA and ZapB, the presence of ZapC appears to be restricted to the gammaproteobacteria. This further highlights the variety of Z-ring components in different bacterial phyla that are known (or suspected) to modulate the assembly dynamics of FtsZ but appear to be phylogenetically unrelated. Other examples are SepF in Gram positives and cyanobacteria (40, 41, 54, 56, 77), EzrA (14, 35, 76) and UgtP (87) in \textit{Bacillus subtilis} and related Gram positives, ZipN (Ftn2) (55) and ZipS.

![FIG. 9. Supersensitivity of ΔzapC cells to overexpression of FLAG-MinC.](image)

(A) Wild-type (TX3772) or ΔzapC (WM3004) cells harboring either a plasmid expressing IPTG-inducible FLAG-MinC (pWM2801; +) or a control vector (pWM2784; −) were grown in LB medium with Amp to logarithmic phase. Tenfold serial dilutions of the cells were then spotted onto LB Amp plates containing either 0, 0.5, or 1 mM IPTG and incubated at 37°C overnight. (B) The same cells were imaged with DIC optics to show the effects on cell division. (C) Total protein from equal quantities of each strain in panel B was separated by SDS-PAGE and immunoblotted with anti-FLAG antibody to detect FLAG-MinC. The 25-kDa marker is shown at the left.

**TABLE 5. ZapC contributes to cell division**

<table>
<thead>
<tr>
<th>Strain</th>
<th>IPTG conc. (μM)</th>
<th>Length</th>
<th>% cells with:</th>
<th>Total no. of septa</th>
<th>L/S (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg (μm)</td>
<td>SD (μm)</td>
<td>1 septum</td>
<td>&gt;1 septum</td>
<td></td>
</tr>
<tr>
<td>TB28</td>
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<td>1.1</td>
<td>26.5</td>
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</tr>
<tr>
<td>CH59  (ΔzapC)</td>
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<td>1.1</td>
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<tr>
<td>CH41  (ΔzapA)</td>
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<td>5.9</td>
<td>3.3</td>
<td>33.0</td>
<td>2.2</td>
</tr>
<tr>
<td>CH65  (ΔzapA ΔzapB ΔzapC)</td>
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<td>9.5</td>
<td>12.4</td>
<td>24.8</td>
<td>1.3</td>
</tr>
<tr>
<td>CH65(iBL3) [ΔzapABC (P_{lac}::zapC)]</td>
<td>10</td>
<td>5.7</td>
<td>5.1</td>
<td>23.5</td>
<td>1.7</td>
</tr>
<tr>
<td>CH65(iBL3) [ΔzapABC (P_{lac}::zapC)]</td>
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<td>4.8</td>
<td>3.0</td>
<td>26.5</td>
<td>2.3</td>
</tr>
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<td>7.8</td>
<td>8.1</td>
<td>26.1</td>
<td>2.2</td>
</tr>
<tr>
<td>CH65(iBL4) [ΔzapABC (P_{lac}::zapC-gfp)]</td>
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<td>5.0</td>
<td>3.5</td>
<td>25.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Cells were grown in LB at 30°C with IPTG as indicated to OD_{600} = 0.6 to 0.8 and chemically fixed before imaging and measurements of cell parameters. Between 600 and 605 cells from each culture were analyzed.

b Average cell lengths and standard deviations are given.


27. Lackner, L. D., M. M. Raskin, and P. A. de Boer. 2003. ATP-dependent...