Site-directed Fluorescence Labeling Reveals a Revised N-terminal Membrane Topology and Functional Periplasmic Residues in the Escherichia coli Cell Division Protein FtsK*

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**Background:** FtsK is an essential membrane protein that links chromosome segregation and cell division. The N-terminal domain of FtsK (FtsKN) is essential for division, and the C terminus (FtsKC) is a well characterized DNA translocase. Although the function of FtsKN is unknown, it is suggested that FtsK acts as a checkpoint to ensure DNA is properly segregated before septation. This may occur through modulation of protein interactions between FtsKN and other division proteins in both the periplasm and cytoplasm; thus, a clear understanding of how FtsKN is positioned in the membrane is required to characterize these interactions. The membrane topology of FtsKN was initially determined using site-directed reporter fusions; however, questions regarding this topology persist. Here, we report a revised membrane topology generated by site-directed fluorescence labeling. The revised topology confirms the presence of four transmembrane segments and reveals a newly identified periplasmic loop between the third and fourth transmembrane domains. Within this loop, four residues were identified that, when mutated, resulted in the appearance of cellular voids. High resolution transmission electron microscopy of these voids showed asymmetric division of the cytoplasm in the absence of outer membrane invagination or visible cell wall ingrowth. This uncoupling reveals a novel role for FtsK in linking cell envelope septation events and yields further evidence for FtsK as a critical checkpoint of cell division. The revised topology of FtsKN also provides an important platform for future studies on essential interactions required for this process.

Bacteria rely on complex gene expression and a large number of coordinated protein systems to drive cell division. Together, these systems facilitate cell growth and elongation, chromosome replication and segregation, septum formation, and physical division of the cell (1–3). Temporal and spatial coordination is essential for each step of this process to ensure that replicated chromosomes are partitioned into their respective daughter cells before the septum forms at mid-cell and division occurs (4–6).

In *Escherichia coli*, the essential and bifunctional division protein FtsK is thought to link chromosome segregation and cell division. FtsK is a large multispanning membrane protein composed of 1329 amino acids and is a homolog of the protein SpoIIIE from *Bacillus subtilis* (7, 8). Together, these proteins make up the large, well conserved FtsK/SpoIIIE protein family (9–11) and possess a variety of functions, the most notable being double-stranded DNA (dsDNA) translocation during division and sporulation in organisms such as *E. coli* and *B. subtilis*, respectively (11–13). FtsK can be divided into three separate functional units. The first ~200 N-terminal amino acids of FtsK (FtsKN) act as the hydrophobic integral membrane anchor (14, 15). FtsKN is the only portion of FtsK essential for division (14–16), although the precise role that FtsKN plays in septation is still unknown. Immediately downstream of FtsKN is a highly flexible linker domain rich in proline and glutamine (FtsKc), whose length is variable between bacterial species (7, 17). This linker region connects FtsKN to the remaining ~500 amino acids at the C terminus of FtsK (FtsKC). FtsKC functions as a DNA translocase capable of both dsDNA translocation and chromosome dimer resolution, and it is the most well-characterized domain with respect to both its structure and function (10, 18–23).

Several theories have been proposed regarding the mechanism of action for FtsK, all of which rely on alterations to the protein-protein interactions between FtsK and various components of the division machinery, collectively known as the division (10, 17, 23, 24). In 2010, Dubarry et al. (17) speculated on the functional link between FtsK in cell division and DNA segregation and suggested a mechanism of action that highlights its potential role as a checkpoint of bacterial cell division. This proposed mechanism emphasizes an inverse relationship between septum formation and DNA translocation that is mediated by allosteric changes in FtsK and modulation of protein-protein interactions within the N-terminal domain. The
bifunctional nature of FtsK makes it an intriguing cell division checkpoint candidate, as it is proposed to sense both proper DNA segregation and complete accumulation of divisome proteins within the same molecule (17, 24). Previous biochemical evidence suggests that FtsK_N interacts with both cytoplasmic and membrane-bound proteins within the divisome (17, 25, 26). These include the major cell division protein FtsZ, a homolog of the eukaryotic cytoskeletal protein tubulin that forms the dynamic ring-like structure at the division site known as the Z-ring (27–30), and proteins involved in peptidoglycan synthesis, such as FtsQ, FtsL, and FtsI (31–37). To better characterize how FtsK may function as a checkpoint within the divisome, a better understanding of both the regions required for these interactions and ultimately its overall organization within the cytoplasmic membrane is required.

In 2000, a study by Dorazi and Dewar (38) explored the N-terminal membrane topology of FtsK using site-directed reporter fusions. With the help of previously reported hydrophobicity analysis (7), computer-generated topology predictions, and reporter fusion data, a topology map of FtsK_N was generated. In this proposed model, FtsK_N contains four transmembrane α-helices connected by a moderately sized periplasmic and cytoplasmic loop, as well as a third periplasmic segment containing a single amino acid residue (38). Because this topology mapping technique relies on severe truncation of the target protein, we must assume these truncated and usually inactive constructs maintain the same native topology as the target protein, although the single amino acid linking the transmembrane segments is not impossible, the steric constraints associated with these areas, and the single amino acid residue (38).

In this study, site-directed fluorescence labeling was used to refine the N-terminal membrane topology of FtsK. This technique relies on the differential reactivity of engineered cysteine residues with a thiol-specific fluorescent probe. By using the full integral membrane portion of the protein, the native topology and function are more likely to be preserved. Our revised topology confirmed the presence of four transmembrane segments, yet revealed a much larger periplasmic loop between the third and fourth transmembrane segments than previously reported (38). In addition, a series of residues in the newly identified periplasmic loop were identified that, when mutated, uncoupled invagination of the inner and outer membranes and resulted in visible voids in the cellular material.

**TABLE 1**

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| E. coli strain | rph::IN (rmd::rrnE) | Coli Genetic Stock Center |
| Lemo21(DE3) | fhuA2 [lon] omplT gal (λ DE3) [dcm] ΔhsdS::pλemo(Cam^®) Δ DE3 = λ sBarHio ΔEcoRI-B int::lac-PlacIV5::T7 gene1) Δ21 Δmini pLemo = pACYC184-PrhaBAD-lysY | New England Biolabs |
| DH5α | F–Φ80lacZAM15Δ (lacZYA-argF) U169 recA1 endA1 hisdR17 (rk–, mk+) phoA supE44 | Invitrogen |
| LP11-1 | W3110 ftsK44 araA::Tn10 | 41 |

**Plasmids**

- pBAD24 protein expression vector under the control of P_ara::Amp^R |
- pAB006-2 pBAD24 derivative encoding amino acids 1–220 of FtsK (His_10-FtsK_N(220)) from E. coli |
- pAB006-13 pBAD24 derivative encoding Cys-less (ΔCys) His_10-FtsK_N(220) |

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—Bacterial strains and plasmids used in this study are listed in Table 1. E. coli Lemo21 cultures were grown at 37 °C in lysogeny broth (LB) (BD Biosciences) in a rotary shaker at 200 rpm. Media were supplemented with 30 μg/ml chloramphenicol and 150 μg/ml ampicillin, as well as 0.2% (w/v) l-arabinose when appropriate. E. coli LP11-1 cultures were grown in Complementation Media (10% (w/v) tryptone, 5% (w/v) yeast extract, and 10% (w/v) NaCl (Fisher)). Media were supplemented with 15 μg/ml tetracycline and additionally with 150 μg/ml ampicillin for plasmid-carrying strains. Cultures were grown as outlined in the temperature-sensitive complementation assays described below.

**Plasmid Construction**—The N-terminal 220 amino acids of FtsK (FtsK_N220) were amplified from E. coli K12 W3110 genomic DNA by PCR using iProof high fidelity DNA polymerase (Bio-Rad) with custom primers AMB001Fb (5'TTCCATCAAGATGGTCACACCAACCACCACACCCACCACCACACTCTCCATTGAAGGTCTGATTTTGGACGAGGATACTATTTGAA-3') and AMB003R (5'-TAAACCCTCAGTACCGATGCTCTCTTATCTACATCCTATTTCCTGAGATGTCGGAATCATTATAAGTTTGGACGAGGATACTATTTGAA-3'). The custom primers (Operon) were designed to introduce EcoRI and KpnI restriction sites and an N-terminal decahistidine tag. PCR products were purified by gel extraction using a QIAquick gel extraction kit (Qiagen). Purified PCR products and plasmid DNA (pBAD-24) were digested using FastDigest® EcoRI and KpnI restriction endonucleases as per the manufacturer’s instructions (Thermo Scientific) and ligated using T4 DNA ligase (New England Biolabs). The ligation reaction was transformed into E. coli DH5α by chemical heat shock, and the resulting gene construct was isolated using a MEGAquick-spin total fragment DNA purification kit (FrogaBio) and then verified by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph) to produce pAB006-2.

**Site-directed Mutagenesis**—To probe FtsK_N topology, plasmids encoding cysteine-less (ΔCys) and single cysteine variants of FtsK_N(220) were created by site-directed mutagenesis. To generate ΔCys, native cysteine residues (Cys-94, Cys-127, and Cys-169) were mutated to alanine residues using a QuikChange...
Lightning Multi site-directed mutagenesis kit (Stratagene). All primers used for mutagenesis were used in combination to remove native Cys residues, using a Stratagene QuikChange Multi site-directed mutagenesis kit. See under “Experimental Procedures” for details.

**TABLE 2**

| Mutation | Sequence of mutagenic oligonucleotide (5' to 3') | Plasmid |
|----------|-----------------------------------------------|---------|
| C94A*   | TCTATTATGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| C127A*  | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| C169A*  | TCTATTATGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| R20C    | TGCTTACTGGCGAAGGTGAGTTTTTAAACATTCTCACC | pAB006-21 |
| S50C    | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| E58C    | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| I107C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| A134C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| D136C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| I137C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| W138C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| Y139C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| S148C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| S160C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| W181C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| W193C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| T126C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |
| Y217C   | GCCCTCATCCCTGTTGCGGTTCTGTGTTTCTGCGGTTCT | pAB006-21 |

* Base changes are underlined and in boldface.

| Primers were used in combination to remove native Cys residues, using a Stratagene QuikChange Multi site-directed mutagenesis kit. See under “Experimental Procedures” for details.

**Temperature-sensitive Complementation Assays**—To assess whether FtsK\(_{N(220)}\) \(\Delta\)Cys, and single cysteine mutants to a final concentration of 0.2% (w/v). Cultures were then incubated at either 30 or 42 °C for 2 h. Following incubation at their respective temperatures, cell length and morphology were assessed by phase contrast microscopy using a Leica DM2000 LED light microscope equipped with a ProgRes CT3 camera (Jenoptik AG). For each sample, 75 random cells were measured for cell length using the Imagej program (version 1.46r, National Institutes of Health). Measurements are reported as mean cell length ± S.E. Statistical analysis was completed using a one-way analysis of variance with Tukey-Kramer multiple comparison post-tests by Prism 5 software (GraphPad Software, Inc.). Individual tests were performed for each strain, and the level of significance was set at \(\alpha = 0.05\) for all tests.

To quantify cellular voids seen in the nonfunctional FtsK\(_{N(220)}\) mutants, LP11-1 strains carrying FtsK\(_{N(220)}\) and single cysteine mutants D135C, D136C, I137C, and W138C were grown and induced at 42 °C for 6 h as described above. For each strain, cells were sampled every hour and imaged by phase contrast microscopy. Cell length and the number of voids per cell were determined for 150 random cells and are reported as mean length ± S.E. and mean number of voids per cell ± S.E., respectively. For both cell length and cell void data, statistical analysis was completed using one-way anal-
yses of variance with Tukey-Kramer multiple comparison post-tests as described above.

Site-directed Fluorescence Labeling—E. coli Lemo21 strains carrying ΔCys and single cysteine mutants of FtsK_{N(220)} were grown at 37 °C for 1 h as 50-ml cultures in LB media inoculated from an overnight culture (1% v/v). Cells were induced with the addition of l-arabinose to a final concentration of 0.2% (w/v) and incubated for another hour. Induced cells were harvested as three 15-ml aliquots (labeled aliquots 1–3) by repeated centrifugation (1.5 ml × 10 tubes per aliquot; 14,000 × g, 2 min) in microcentrifuge tubes, and each aliquot was briefly washed in 1 ml of Wash Buffer (20 mM Tris-HCl, pH 7.0, 100 mM NaCl). Aliquot 3 was stored at 4 °C in 1 ml of Wash Buffer until the cell lysis step described below.

Fluorescence labeling was performed as described by Culham et al. (42), with minor modifications. Fresh stock solutions of Oregon Green 488 maleimide (OGM)2 (Molecular Probes) and methanethiosulfonate ethyltrimethylammonium (MTSET) (Toronto Research Chemicals Inc.) were prepared in N,N-dimethylformamide (Sigma) immediately prior to use. The OGM stock solution was stored at −20 °C when not in use, and all OGM solutions and OGM-treated samples were protected from light where possible.

Each cell aliquot was treated with a different combination of OGM and MTSET reagents to label periplasmic (Aliquot 1), cytoplasmic (Aliquot 2), or both periplasmic and cytoplasmic (Aliquot 3) cysteine residues. To label cysteine residues accessible to the periplasm, Aliquot 1 was resuspended in 4 ml of Wash Buffer containing 40 μM OGM and incubated at room temperature for 15 min with rocking. The labeling reaction was quenched with 3 mM β-mercaptoethanol. Similarly, Aliquot 2 was resuspended in 4 ml of Wash Buffer supplemented with 2 mM MTSET and incubated at room temperature for 15 min with rocking to block cysteine residues accessible to the periplasm. Both aliquots were then washed three times with Wash Buffer. Aliquots 1–3 were pelleted by centrifugation and resuspended in 2 ml of Wash Buffer containing 40 μM OGM and incubated at room temperature for 15 min with rocking to block cysteine residues accessible to the periplasm. Both aliquots were then washed three times with Wash Buffer. Aliquots 1–3 were pelleted by centrifugation and resuspended in 2 ml of Wash Buffer containing 5 mM EDTA, 40 μg/ml DNase I, and 300 μg/ml lysozyme. Following 15 min of incubation at room temperature, each aliquot was added to 18 ml of ice-cold Milli-Q H2O and vortexed briefly to complete cell lysis. Membrane pellets were collected by centrifugation at 12,000 × g for 10 min at 4 °C (Beckman Coulter Avanti J-E centrifuge, JA-25.50 rotor). Membrane pellets from Aliquots 1 and 3 were resuspended in 4 ml of Wash Buffer containing 40 μM OGM and incubated at room temperature for 15 min with rocking. Membranes were permeabilized by three freeze/thaw cycles using liquid nitrogen to label previously inaccessible cytoplasmic cysteine residues (Aliquot 2) or both periplasmic and cytoplasmic cysteine residues (Aliquot 3). Labeling reactions were quenched with 3 mM β-mercaptoethanol, diluted in 16 ml of ice-cold Milli-Q H2O, and centrifuged as before to collect the membrane fraction. Membrane pellets were washed twice more in 1 ml of ice-cold Milli-Q H2O and collected in microcentrifuge tubes by centrifugation (14,000 × g, 2 min, 4 °C).

Labeled membrane fractions from all three aliquots were resuspended in 3 ml of Purification Buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol) containing 2% lauryldimethylamine-oxide (LDAO) and 50 μl of Proximity Ni2+-charged IMAC resin (Bio-Rad) and were incubated at 4 °C for 1 h with rocking. Solubilized membrane/resin mixtures were collected by centrifugation at 21,000 × g for 2 min at 4 °C in microcentrifuge tubes and washed three times with 1 ml of Purification Buffer containing 0.1% LDAO. Proteins were eluted in 80 μl of Elution Buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, 0.1% (v/v) bromphenol blue), and boiled for 10 min in a covered beaker. Samples were immediately analyzed by loading 25-μl aliquots onto 13% SDS-polyacrylamide gels. Fluorescence was detected upon exposure of the gel to UV light using a Bio-Rad Gel Doc equipped with a CCD camera. Gels were stained with silver following UV exposure to visualize protein bands (43). The experiment was conducted in duplicate and produced the same results. Residue location data were entered into the HMMTOP version 2.0 protein topology prediction server to determine optimal positions and lengths of transmembrane segments based on experimental constraints (44, 45).

For residues found to be within a transmembrane segment, as indicated by no fluorescent labeling using the above protocol, the presence of the engineered cysteine residue was confirmed by labeling purified protein in an SDS-containing solution. Briefly, ΔCys, Cys-94, and Cys-169 FtsK_{N(220)} mutants were purified from 15-ml induced cultures as described above with omission of all OGM and MTSET treatments. To 80 μl of purified protein, 1% (w/v) SDS and 40 μM OGM were added, and samples were incubated at room temperature for 15 min. Labeling reactions were then quenched upon the addition of 20 μl of 5× Loading Buffer and boiled for 10 min in a covered beaker. SDS-PAGE analysis of fluorescence and protein visualization was completed as described above.

Membrane Isolation—For total membrane isolation, 40-ml cultures of LP11-1 carrying FtsK_{N(220)} and nonfunctional single cysteine mutants were induced and grown at 42 °C as described in the temperature-sensitive complementation assays above. Following induction, the entire culture was harvested by centrifugation (5,000 × g, 10 min, 4 °C) and resuspended in 4 ml of Wash Buffer containing 5 mM EDTA, 40 μg/ml DNase I, and 300 μg/ml lysozyme. Samples were incubated at room temperature for 15 min with rocking, then added to 32 ml of ice-cold Milli-Q H2O, and sonicated for 1 min (10 s on and 10 s off) to complete cell lysis. To remove cellular debris, samples were centrifuged at 8,000 × g for 10 min at 4 °C. The resulting supernatant was collected and ultracentrifuged at 120,000 × g for 1 h at 4 °C (Beckman L8–55 M ultracentrifuge, Ti70 rotor) to collect the membrane fraction. Membranes were resuspended in 200 μl of Wash Buffer. Total protein concentration of each sample was determined by a bicinchoninic acid protein assay.

2The abbreviations used are: OGM, Oregon Green 488 maleimide; MTSET, methanethiosulfonate ethyltrimethylammonium; LDAO, lauryldimethylamine oxide; TEM, transmission electron microscopy; HADA, hydroxylcoumarin carbonyl amino-L-alanine; HALA, hydroxylation coumarin carbonyl amino-L-alanine.
FtsK Functional Loop Couples Division of Bacterial Membranes

(Thermo Scientific) using bovine serum albumin (BSA) as a standard.

Western Blotting—To verify the presence of FtsK<sub>N(220)</sub> and nonfunctional single cysteine variants in the membrane fractions collected above, 30 μg of protein from each membrane sample was prepared for SDS-PAGE and Western blotting. Samples were separated using a 13% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane using a Trans-Blot® Turbo transfer system (Bio-Rad) on the Turbo Transfer setting. The blot was developed using a SNAP i.d.® 2.0 Protein Detection System (EMD Millipore) as per the manufacturer’s instructions. Primary and secondary antibodies used were mouse anti-His<sub>6</sub> (Clontech) and alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Sigma), respectively. The blot was developed using a solution of 33 mg of nitro blue tetrazolium and 17 mg of 5-bromo-4-chloro-3-indolyl phosphate in 10 ml of alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>). The blot was scanned, and band density was measured using the ImageJ program (version 1.46r, National Institutes of Health).

Immunofluorescence Microscopy—Immunofluorescence was performed as described by Hiraga et al. (46), with minor modifications. LP11-1 carrying FtsK<sub>N(220)</sub> and nonfunctional single cysteine mutants were induced and grown at 42 °C as 5-ml cultures as described in the temperature-sensitive complementation assays. To fix the cells, 2 ml of culture was added to 10 ml of 80% methanol in a 15-ml conical tube, inverted to mix, and allowed to evaporate overnight before samples were embedded in 100% HPLC grade acetone and placed into a Leica AFS2 freeze substitution unit for substitution under controlled temperatures. Following substitution, cells were washed three times in 100% HPLC grade acetone and infiltrated with 10–15% (v/v) Epon 812 (prepared as in Ref. 47) in acetone for 3 h. Samples were then infiltrated with 25% (v/v) Epon 812 in acetone overnight, followed by 50% (v/v) Epon 812 in acetone for at least 1 h. The acetone was allowed to evaporate overnight before samples were embedded in 100% Epon 812 and polymerized at 60 °C for 48 h. Ultrathin sections were cut using a Reichert UltraCut E ultramicrotome and placed on 100-mesh platinum/copper grids for viewing.

Transmission Electron Microscopy—Grids containing ultrathin sections were negatively stained for 7 min with 2% uranyl acetate, washed with HPLC-grade water, and then stained with Reynold’s lead citrate for 3 min prior to viewing. Samples were viewed using an FEI Tecnai G2 F20 transmission electron microscope operating at 200 kV equipped with a bottom mount Gatan 4k CCD camera under standard operating conditions.

FM4-64 Membrane Staining—Membranes of LP11-1 cells overexpressing FtsK<sub>N(220)</sub> and nonfunctional single cysteine mutants were stained using the red fluorescent lipophilic styryl dye FM4-64 (Invitrogen). Cultures of LP11-1 carrying FtsK<sub>N(220)</sub> were induced and grown at 42 °C as described in the temperature-sensitive complementation assays. FM4-64 was added to 500 μl of culture at a final concentration of 5 μg/ml and incubated at room temperature for 10 min. Following incubation, 3 μl of stained culture was placed on a glass slide, and a glass coverslip was applied and sealed with nail polish prior to imaging. Oblique illumination, dark field, and FM4-64 fluorescent micrographs were obtained using a Riveal Contrast Microscope (Quorum). FM4-64 fluorescence was detected using an N2.1 filter (Leica).

Peptidoglycan Labeling—To visualize the peptidoglycan layer, cells were stained with the fluorescent amino acids hydroxyxylumarin carbonyl amino-d-alanine (HADA) or hydroxylcumarin carbonyl amino-l-alanine (HALA) as described by Kuru et al. (48). Briefly, 25-ml cultures of LP11-1 carrying FtsK<sub>N(220)</sub> and nonfunctional single cysteine mutants were induced and grown at 42 °C as described in the temperature-sensitive complementation assays. After 1 h, 1-ml aliquots of each strain were transferred to two sterile test tubes containing 10 μl of 100 mM
HADA or 100 mM HALA as a negative control. Cultures were then grown at 42 °C for an additional hour. The cells were fixed by the addition of 2.3 ml of ice-cold 100% ethanol and incubated on ice for 20 min. Cells were harvested by centrifugation and washed three times in 1 ml of PBS. The final cell pellets were resuspended in 400 µl of PBS containing SlowFade® gold anti-fade reagent and mounted on poly-l-lysine-coated glass slides. Coverslips were sealed with nail polish prior to imaging as described for the immunofluorescence microscopy.

RESULTS

FtsK<sub>N(220)</sub> Single Cysteine Mutants Successfully Complement LP11-1 (ftsK44)—To generate the single cysteine mutants used to probe the topology of FtsK<sub>N(220)</sub>, the three native cysteine residues were removed by site-directed mutagenesis. In a single step, cysteine residues at amino acid positions 94, 127, and 169 were replaced by alanine residues to create a cysteine-less version (ΔCys) of FtsK<sub>N(220)</sub>. During the creation of ΔCys, two variants that retained single native cysteine residues (Cys-94 and Cys-169) were also isolated. In addition to these two single cysteine variants, ΔCys was used as a template to generate 17 new mutants containing single cysteine residues at targeted positions. Each position was chosen to verify the presence of additional transmembrane domains toward the C terminus of the protein. In total, 19 single cysteine variants of FtsK<sub>N(220)</sub> were generated.

Functional characterization of wild-type (WT) FtsK<sub>N(220)</sub> ΔCys, and all single cysteine mutants was performed using a temperature-sensitive complementation assay. Given that the precise role the N-terminal domain of FtsK plays in cell division is currently unknown, we tested the functionality of our mutants by assessing their ability to successfully complement the ftsK44 temperature-sensitive E. coli strain LP11-1. This strain harbors the FtsK44 temperature-sensitive G80A substitution first characterized by Begg et al. (7). E. coli LP11-1 divides normally when grown at 30 °C; however, when cultured at the nonpermissive temperature (42 °C), this strain is unable to successfully divide; and a filamentous phenotype is observed (Fig. 1A). All mutants were assayed at both the permissive and nonpermissive temperatures to assess their ability to suppress this filamentation by measuring cell length. To ensure that cells used each FtsK<sub>N(220)</sub> Variant rather than endogenous FtsK during division, constructs were overexpressed by the addition of 0.2% (w/v) 1-arabinose. In total, WT FtsK<sub>N(220)</sub> ΔCys; and 15 of the 19 single cysteine mutants were able to successfully complement LP11-1 and suppress filamentation (Fig. 1). It was necessary to ensure all constructs were functional before using them to assess the cellular location of their cysteine residues, because any nonfunctional mutants may not be folded properly and would therefore not accurately depict the correct membrane topology of FtsK. As such, single cysteine mutants D135C, D136C, I137C; and W138C were not used for the site-directed fluorescence labeling experiments described below.

Revised Membrane Topology of FtsK<sub>N(220)</sub> Reveals Altered Periplasmic Loop—Site-directed fluorescence labeling was used to generate a membrane topology model of the N-terminal portion of FtsK (Fig. 2A). This technique has been successfully used previously to determine the number and orientation of transmembrane segments for several inner membrane proteins (49–51). The location of targeted cysteine residues was determined by their relative accessibility to the thiol-specific fluorescent probe OGM (Fig. 2B). Given the membrane-impermeable nature of this reagent, treatment of intact bacterial cells with OGM allows for the detection of periplasmic cysteine residues only (Fig. 2B, top panel). Cytoplasmic cysteine residues are only accessible upon cell lysis. However, OGM treatment of lysed cells will label both periplasmic and cytoplasmic cysteine residues. Therefore, to definitively identify cysteine residues located in the cytoplasm, whole cells were pretreated with the impermeable, nonfluorescent, thiol-specific blocking reagent MTSET. Pretreatment of intact cells with MTSET blocks any cysteine residues located in the periplasm from further treatment with OGM. Consequently, OGM will label only cytoplasmic cysteine residues in MTSET-pretreated cells following cell lysis (Fig. 2B, middle panel). Cysteine residues located within a transmembrane segment are not labeled in either intact or lysed cells (Fig. 2B, bottom panel), but they are labeled upon exposure of the transmembrane segments by an SDS-containing solution following purification (Fig. 2C). In general, it was found that the labeling efficiency of intact cells harboring periplasmic cysteine variants was lower than for cytoplasmic and transmembrane cysteine residues. This difference in labeling efficiency could not be improved by increasing the concentration of OGM used to label intact cells, and it is therefore likely a consequence of localized protein folding rather than insufficient probe. Variation in labeling efficiency of targeted cysteine residues by OGM has been previously reported (50).

Of the 15 single cysteine variants used to generate the revised membrane topology, five were found to harbor cysteine residues located at a different position than reported previously (Table 3). All five residues were initially proposed to be within the fourth transmembrane domain or immediately downstream in the cytoplasmic loop (38). As such, it was suggested that the third and fourth transmembrane domains were located immediately after one another, containing residues 116–134 and 136–156, respectively (38). In contrast, our data suggest the fourth transmembrane domain is located much closer to the C terminus of the protein (residues 163–179) (Fig. 2A). This positioning of the fourth transmembrane segment is in agreement with domain IV identified by all computer predictions reported by Dorazi and Dewar (38). The cytoplasmic location of all targeted cysteine residues after this domain (W181C to Y217C) confirms the absence of additional transmembrane segments toward the C terminus and supports the view that FtsK<sub>N</sub> is composed of four transmembrane α-helices and not five as suggested previously (7, 52).

As a consequence of the shift in location by the fourth transmembrane segment, the topology results also revealed an extensive periplasmic loop connecting the third and fourth transmembrane domains (Fig. 2A, residues 133–162). This is in contrast to the single amino acid connection proposed by Dorazi and Dewar (38). The newly identified loop contains a combination of both hydrophobic and polar amino acids and is predicted to have 83.3% helical identity based on Chou and
Fasman secondary structure prediction (53, 54). This loop also contains two negatively charged aspartate residues (Asp-135 and Asp-136). Given that these residues are the only charged amino acids within this periplasmic segment, their role as potential functional residues of FtsKN was investigated.

Nonfunctional FtsKN(220) Mutants Localize to Cellular Voids—During the temperature-sensitive complementation assay, overexpression of four single cysteine mutants (D135C, D136C, I137C, and W138C) was unable to suppress filamentation of the LP11-1 strain when grown at the nonpermissive temperature and caused cell elongation at 30 °C (Fig. 1B). This suggested that mutation of these residues, which are all located in the newly identified periplasmic loop described above, disrupts the ability of FtsKN to function during cell division. To determine whether the inability to suppress filamentation was a result of a failure of FtsKN to insert into the membrane, total membrane fractions of cells overexpressing WT FtsKN(220) and each of the nonfunctional single cysteine mutants were collected and analyzed by Western blotting. FtsKN was detected in the membrane fraction of all strains (Fig. 2), and no large differences in expression levels were observed between the WT and nonfunctional FtsKN(220) mutants. This suggests the inability of these mutants to suppress filamentation was not a result of insufficient membrane targeting.

Closer inspection of these nonfunctional mutants by phase contrast microscopy revealed the majority of the filamentous cells also contained multiple voids in their cellular material at various positions along the cell (Fig. 1A, inset). This suggested that mutation of these residues, which are all located in the newly identified periplasmic loop described above, disrupts the ability of FtsKN to function during cell division. To determine whether the inability to suppress filamentation was a result of a failure of FtsKN to insert into the membrane, total membrane fractions of cells overexpressing WT FtsKN(220) and each of the nonfunctional single cysteine mutants were collected and analyzed by Western blotting. FtsKN was detected in the membrane fraction of all strains (Fig. 2D), and no large differences in expression levels were observed between the WT and nonfunctional FtsKN(220) mutants. This suggests the inability of these mutants to suppress filamentation was not a result of insufficient membrane targeting.

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ied depending on which FtsKn(220) mutant was expressed. In cells overexpressing FtsKn(220) mutants D135C, D136C, I137C, and W138C that contained a cellular void, FtsZ was predominantly found at the top and bottom edges of the voids in 75, 53, and 70% of cells, respectively. The remainder of cells containing a cellular void exhibited a diffuse FtsZ localization pattern. In cells overexpressing the FtsKn(220) mutant D135C, FtsZ was found at the top and bottom edges of the voids in only 45% of cells containing a cellular void, making the diffuse localization pattern the more predominant phenotype. In this strain, FtsZ was excluded from the cellular voids and exhibited very little co-localization with FtsKn. For each of the nonfunctional FtsKn(220) mutant strains, the cellular voids were also vacant of DNA. Moreover, the appearance of multiple nucleoids in each cell indicates these cells have undergone multiple rounds of

| Variant | Original location | Revised location |
|---------|------------------|-----------------|
| R20C    | Cytoplasm        | Cytoplasm       |
| S50C    | Periplasm        | Periplasm       |
| E58C    | Periplasm        | Periplasm       |
| W70C    | Periplasm        | Periplasm       |
| C94     | Membrane         | Membrane        |
| I107C   | Cytoplasm        | Membrane        |
| A134C   | Membrane         | Membrane        |
| Y139C   | Membrane         | Membrane        |
| S148C   | Membrane         | Membrane        |
| S160C   | Cytoplasm        | Cytoplasm       |
| C169    | Cytoplasm        | Cytoplasm       |
| W181C   | Cytoplasm        | Cytoplasm       |
| W193C   | Cytoplasm        | Cytoplasm       |
| T205C   | Cytoplasm        | Cytoplasm       |
| Y217C   | Cytoplasm        | Cytoplasm       |

* Data are based on Dorazi and Dewar (38).

* Residue locations that differ from the original topology are highlighted in boldface.

FIGURE 2. Revised membrane topology of FtsKn. A, revised topology determined by site-directed fluorescence labeling. Residues are color-coded based on amino acid property. Shaded circles indicate residues mutated to produce FtsKn(220) single cysteine variants used to probe topology. B, representative SDS-polyacrylamide gels of single cysteine mutants harboring periplasmic, transmembrane, and cytoplasmic cysteine residues labeled with OGM. The top panel for each variant shows the SDS-polyacrylamide gel after exposure to UV light to detect OGM labeling, and the bottom panel shows the same gel after silver staining to detect total protein. Periplasmic (P), cytoplasmic (C), and both periplasmic and cytoplasmic (P+C) cysteines were labeled as described under “Experimental Procedures.” C, purified ΔCys, Cys-94, and Cys-169 variants labeled with OGM in an SDS-containing solution to expose previously inaccessible transmembrane cysteine residues. SDS-polyacrylamide gels are shown after exposure to UV light (top panel) and subsequent silver staining (bottom panel). D, Western blot of total membrane isolated from LP11-1 strains overexpressing WT and nonfunctional single cysteine mutants (D135C, D136C, I137C, and W138C). Samples were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and subsequently probed with mouse anti-His6 antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse IgG.
chromosome replication with failed cellular division. Together, this suggests mutation of these periplasmic residues may have an impact on division at a specific stage of cell growth, potentially through alteration of protein-protein interactions with cytoplasmic division components such as FtsZ.

Cellular Voids Are Linked to Cell Growth and Increased Cell Size—To test whether the appearance of the cellular voids corresponded to a particular duration of growth, cell morphology of LP11-1 strains overexpressing WT FtsK\(_{N(220)}\) and nonfunctional mutants D135C, D136C, I137C, and W138C was monitored hourly by phase contrast microscopy. All LP11-1 strains exhibited an increase in growth over the first 2 h of incubation; however, cells overexpressing nonfunctional single cysteine mutants consistently showed less growth with prolonged incubation compared with cells overexpressing WT FtsK\(_{N(220)}\) (Fig. 4A). In addition, all nonfunctional mutants displayed a significant increase in cell length over the first 2 h of growth (Fig. 4B), in contrast to the WT FtsK\(_{N(220)}\) strain, which showed no significant change in cell length over the entire incubation period.

On average, the number of voids per cell increased over the incubation period and directly corresponded with both cell growth and significant increases in cell length (Fig. 4, C and D). In LP11-1 cells overexpressing FtsK\(_{N(220)}\) mutant D135C, cellular voids were observed in ~64% of cells after 2 h of growth and between 73 and 82% of cells after 3–6 h. Similar proportions and accumulation of cellular voids were also seen with the other three mutants. In all four strains, a significant increase in the mean number of voids per cell was seen within the first 2 to 3 h of growth (Fig. 4D). The mean number of voids per cell did not significantly increase in mutants D136C and I137C after this time, and cells maintained an average of ~2 voids per cell despite continued incubation. FtsK\(_{N(220)}\) mutants D135C and W138C exhibited a slightly later increase in the mean number of cellular voids, which occurred between 2 and 4 h \((p < 0.05)\) and 2 and 5 h \((p < 0.05)\), respectively. Overall, the appearance of cellular voids occurred at regular intervals over the incubation period, suggesting these voids may be a result of numerous failed division events.

Cellular Voids Are Produced by Division of the Inner Membrane—To further investigate the ultrastructural characteristics of LP11-1 strains overexpressing WT and nonfunctional FtsK\(_{N(220)}\) mutants, cell samples were processed by high pressure freezing followed by freeze substitution, ultrathin sectioning, and imaging by transmission electron microscopy (TEM) to visualize the cellular voids at high resolution. Cells overexpressing WT FtsK\(_{N(220)}\) exhibited typical cell morphology and invagination of the cell envelope during division (Fig. 5A). Small aggregates were observed within the cytoplasm of some WT cells, indicating the possible presence of inclusion bodies formed by the overexpression of FtsK\(_{N(220)}\). However, because these cells retain normal growth and morphology despite elevated protein

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**FIGURE 3.** Overexpression of nonfunctional FtsK\(_{N(220)}\) mutants results in altered localization of FtsK\(_N\) and FtsZ. LP11-1 cells overexpressing WT or nonfunctional FtsK\(_{N(220)}\) single cysteine mutants (D135C, D136C, I137C, and W138C) were fixed with methanol and adhered to glass slides pretreated with 0.1% poly-L-lysine. Cells were stained with mouse anti-His\(_6\) and rabbit anti-FtsZ antibodies followed by anti-mouse-conjugated Alexa Fluor\(^®\) 594 and anti-rabbit-conjugated FITC secondary antibodies to visualize FtsK\(_{N(220)}\) and FtsZ, respectively. Cells were then counterstained with DAPI and imaged using an upright Leica DMS0008 fluorescent microscope. Localized FtsK\(_{N(220)}\) and FtsZ are indicated by the white arrows. The white arrowheads indicate cellular voids. Bar, 5 \(\mu\)m.
levels, the presence of these aggregates does not interfere with normal cell function.

Visualization of LP11-1 strains overexpressing nonfunctional single cysteine FtsK_{N(220)} mutants by TEM confirmed the presence of cellular voids in all mutant strains (Fig. 5, B–E). These voids are free of any discernable cellular material and are completely enclosed from the extracellular environment by the outer membrane and what appears to be the peptidoglycan layer at the cell surface (Fig. 5B, inset). The cytoplasm is asymmetrically partitioned at either end of the void and is entirely bound by the inner membrane. Finally, protrusions of the inner membrane and cytoplasmic material along the edge of these voids are consistently seen in all mutants; however, the number and size of these protrusions vary between cells. The presence of these protrusions suggested that the cytoplasm might not be completely divided, giving rise to cytoplasmic bridges within the cellular voids. To verify the absence of such bridges, LP11-1 strains expressing WT FtsK_{N(220)} and nonfunctional mutants were imaged by dark field microscopy, and the bacterial membranes were stained with the red fluorescent lipophilic styryl dye FM4-64. Dark field illumination revealed a distinct difference in density between the cytoplasm and cellular voids (Fig. 6), suggesting these spaces are in fact devoid of cytoplasmic material. In addition, FM4-64 staining of the bacterial membranes indicates the presence of membrane on either side of the voids. Together with the high resolution TEM images, this
implies that the cytoplasm is completely membrane-bound and that the inner membrane has been fully divided.

Based on our ultrastructural analysis of the cell wall architecture above, it is evident that the cellular voids produced by overexpression of nonfunctional FtsK_{N(220)} mutants do not contain the defined linear septum typical of WT E. coli cells. Although density that is likely the peptidoglycan layer can be seen closely associated with the outer membrane throughout these voids (Fig. 5B, inset), diffuse material within the voids is apparent. It is unclear from our TEM analysis whether this material is in fact disorganized peptidoglycan or simply alternative periplasmic components; therefore, to verify the absence of septal peptidoglycan within these cellular voids, the cell wall was labeled using the fluorescent D-amino acid HADA. D-Amino acids are selectively incorporated into the growing peptidoglycan cell wall as peptide cross-links between glycan strands (48). When coupled with a fluorescent moiety, the incorporation of these amino acids into the cell wall allows for a robust and highly specific tool for visualizing the peptidoglycan layer of bacterial cells. Visualization of HADA-labeled LP11-1 cells overexpressing WT FtsK_{N(220)} revealed clear peptidoglycan ingrowth at the site of division (Fig. 7, top row). In contrast, the peptidoglycan layer of LP11-1 cells overexpressing nonfunctional FtsK_{N(220)} mutants showed no variation in fluorescent intensity or ingrowth along the length of the cell, including the cellular voids. Based on the lack of additional fluorescent density or obvious ingrowth in these areas, this suggests the cellular voids either do not contain septal peptidoglycan or contain disorganized peptidoglycan at an amount not detectable over the background fluorescence seen throughout the remainder of the cell.

**DISCUSSION**

The role of FtsK as a potential checkpoint of cell division relies upon its ability to efficiently couple both chromosome segregation and septation in bacteria. Although the precise role that FtsK plays in cell division is unknown, evidence suggests that multiple regions along FtsK connect essential components of the divisome, including the Z-ring and peptidoglycan synthesis machinery, and that modulation of these interactions may delay septation to allow for proper DNA segregation (17, 25, 26). The majority of these proposed interactions has been broadly mapped to the N-terminal domain of FtsK (17, 25). However, to fully understand how these contacts are formed and how they may impact the function of FtsK as a cell division checkpoint, a better understanding of the membrane topology of FtsK_{N} is necessary.

Consistent with the original topology map reported by Dorazi and Dewar (38), our revised topology confirms that FtsK_{N} is anchored into the membrane by four transmembrane segments. However, the substantial difference found in the position of the fourth transmembrane segment significantly alters the overall structure of the N-terminal domain. Given that truncated fusion proteins were used to elucidate the original topology (38), and a minimum of ~200 amino acids are required for FtsK to function in septation (14–16), expression

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**FIGURE 5. Ultrastructure of WT FtsK_{N(220)} and nonfunctional FtsK_{N(220)} mutants.** Shown are representative transmission electron micrographs of negatively stained ultrathin sections of LP11-1 cells overexpressing WT (A) and nonfunctional FtsK_{N(220)} single cysteine mutants D135C, D136C, I137C, and W138C (B–E). Cells were processed by high pressure freezing followed by freeze substitution and embedded in Epon resin prior to sectioning. The black arrowheads indicate cytoplasm visibly bound by the inner membrane, and the black arrows show protrusions of the inner membrane. A typical WT division site (A, inset) and cytoplasmic protrusion (B, inset) are highlighted showing the inner membrane (IM), peptidoglycan (PG), and outer membrane (OM) cell envelope components. Asterisk indicates the corresponding area for each inset. Bar, 100 nm, including insets.
of these nonfunctional constructs may have led to protein misfolding or an inability to target to the membrane, which could account for the differences seen between the two topology maps. It is important to note that Dorazi and Dewar (38) identified residue Glu-58 of FtsK\textsubscript{N} as the essential glutamic acid within a putative metalloprotease HE\textsubscript{XX}H motif, based on the inability of an E58A mutation to complement an \textit{ftsK}\textsubscript{44} temperature-sensitive strain (TOE\textsubscript{44}). Mutation of Glu-58 to a cysteine residue in the site-directed labeling experiments described in this study was able to fully complement strain LP11-1 (\textit{ftsK}\textsubscript{44}), and this single cysteine variant exhibited normal cell growth and morphology. This suggests that mutation of Glu-58 does not affect the ability of the protein to function in septation, as proposed previously, and that conservation of the glutamic acid residue is not strictly essential.

The most striking feature of the revised topology map reported herein is the presence of a large periplasmic loop connecting the third and fourth transmembrane domains (Fig. 2A). Within this loop, individual mutation of four residues (Asp-135, Asp-136, Ile-137, and Trp-138) was found to impair the ability of FtsK to function in cell division. Following complete accumulation of the divisome, septation is typically triggered by constriction of the cytoplasmic membrane by the Z-ring, and transition from cell wall elongation to septal peptidoglycan synthesis, followed by invagination of the outer membrane in Gram-negative bacteria (2–5, 57). Fluorescence microscopy
and ultrastructural analysis revealed that mutation of these four residues permits division of the cytoplasmic membrane, yet inhibits invagination of the outer membrane and potentially cell wall ingrowth, resulting in discernible voids in the cellular material (Figs. 5–7). Although current knowledge regarding the role of FtsZ and the Z-ring in cytoplasmic membrane constriction has been driven by in vitro and computational modeling data (30, 58–61), it is possible that this uncoupling of the cytoplasmic and outer membranes during division in vivo is caused by altered activity of FtsZ. This altered activity may be due to mislocalization or modified protein-protein interactions between FtsZ and other components of the divisome, such as FtsK. Given the appearance of the cellular voids in this study was found to correlate with both cell growth and significant increases in cell length (Fig. 4), this potential modulation of FtsZ activity may result in regular failed division attempts as cells proceed through multiple cell cycles.

Accumulation of the nonfunctional FtsK\textsubscript{N(220)} mutants at either end of the cellular voids disrupted proper localization of FtsZ (Fig. 3). A previous study by Draper et al. (14) suggested that overexpression of FtsK results in the inhibition of FtsZ assembly into septal rings. Although LP11-1 cells overexpressing WT FtsK\textsubscript{N(220)} show a decreased proportion of cells with a visible Z-ring compared with WT \textit{E. coli} strains (62, 63), we clearly observed proper localization and formation of Z-rings despite overexpression; therefore, it is unlikely that the mislocalization of FtsZ in the nonfunctional mutant strains is solely a result of FtsK overexpression. Rather, co-localization of FtsZ with FtsK\textsubscript{N(220)} mutants D136C, I137C, and W138C, or diffuse localization of FtsZ with mutant D135C, may be a result of altered interaction with FtsZ. This suggests that mutations within this periplasmic loop of FtsK\textsubscript{N} alter the activity of FtsZ, directly or indirectly, in the cytoplasm.

It was also observed that division of the cytoplasmic membrane in cells overexpressing nonfunctional FtsK\textsubscript{N(220)} was consistently asymmetric (Fig. 5, B–E). Asymmetric division of the cytoplasm has been observed in \textit{E. coli} mutants with deletions in multiple peptidoglycan hydrolases, which are responsible for the cleavage of peptidoglycan during cell growth and division (64). In these strains, cells still exhibit visible septal peptidoglycan ingrowth, but no septal cleavage, resulting in cell chains (64). In contrast, the absence of a defined linear septum and the extent of physical separation observed between the cytoplasmic compartments in LP11-1 strains overexpressing nonfunctional FtsK\textsubscript{N(220)} mutants in our study are unique. If the cellular voids are indeed free of septal peptidoglycan, this would suggest the Z-ring can generate sufficient force to completely divide the cytoplasm in the absence of septal cell wall synthesis. However, although a visible septum cannot be seen by TEM (Fig. 5, B–E) or by fluorescent cell wall labeling (Fig. 7), it is also possible that the cell wall is organized in an aberrant manner not easily detected within the limits of either TEM or fluorescence microscopy. This may include diffusely organized peptidoglycan throughout the cellular void or perhaps a thin peptidoglycan layer associated with the inner membrane at either end. In either case, it raises the possibility that FtsK may play a role in coupling Z-ring constriction with the transition from cell growth to septation, with a particular emphasis on the regulation of septal peptidoglycan synthesis. Specifically, the mechanism responsible for the shift between cell wall elongation to septation is unknown, although it is speculated that it may involve regulated transduction of a signal between FtsZ and the septal peptidoglycan synthesis proteins, namely FtsI, by at least one transmembrane protein (4, 57, 58, 65–67). Based on its bifunctional nature, the idea that FtsK may function as a checkpoint of bacterial cell division has been well established (10, 17, 23, 24, 68), and evidence suggests that FtsK interacts with both FtsZ and proteins involved in peptidoglycan synthesis, including FtsI (17, 25, 26). Therefore, it is possible that mutation within the newly identified periplasmic loop could modify or disrupt essential contacts between FtsK and these divisome proteins or perhaps alter the oligomeric state of FtsK\textsubscript{N} (52), thereby uncoupling the switch necessary for the cell to complete septation. An insertion mutation upstream of the C terminus of FtsK was shown to affect cell-cell separation in \textit{E. coli} (69). This defect occurs much later during septation, which resulted in cell chains attached by a small envelope structure (69), as opposed to the early septation defect seen with our mutants. Therefore, it has been speculated that FtsK might be involved in peptidoglycan hydrolysis during cell-cell separation (69). This is consistent with the fact that the ftsK\textsubscript{44} temperature-sensitive phenotype can be suppressed by deletion of \textit{dacA}, which codes for penicillin-binding protein 5 (PBPs) that catalyzes the removal of the terminal \textit{d}-alanine from peptidoglycan side chains during cell wall synthesis (7, 14). This together with the evidence provided in this study regarding the inability of nonfunctional FtsK\textsubscript{N(220)} variants to complete cell envelope septation suggest the FtsK checkpoint function might be specific to peptidoglycan modification and transmission of the cell signal to shift from cell wall elongation to septation in \textit{E. coli}. The revised membrane topology of FtsK\textsubscript{N} and identification of functional periplasmic residues reported here will provide a platform for future studies on the potential protein-protein interactions and regions of FtsK required for this process.

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REFERENCES
1. Nordström, K., Bernander, R., and Dasgupta, S. (1991) The \textit{Escherichia coli} cell cycle: one cycle or multiple independent processes that are coordinated? \textit{Mol. Microbiol.} 5, 769–774
2. Errington, J., Daniel, R. A., and Scheffers, D.-J. (2003) Cytokinesis in \textit{bacteria}. \textit{Microbiol. Mol. Biol. Rev.} 67, 52–65
3. Tuppas, A., Banzhaf, M., Gross, C. A., and Vollmer, W. (2012) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. \textit{Nat. Rev. Microbiol.} 10, 123–136
4. Goehring, N. W., and Beckwith, J. (2005) Diverse paths to midcell: assem-
FtsK Functional Loop Couples Division of Bacterial Membranes

bly of the bacterial cell division machinery. Curr. Biol. 15, R514–R526
5. Lutkenhaus, J., Pichoff, S., and Du, S. (2012) Bacterial cytokinesis: From Z ring to division. Cytoskeleton 69, 778–790
6. Thanischler, M. (2010) Synchronization of chromosome dynamics and cell division in bacteria. Cold Spring Harb. Perspect. Biol. 2, a00331
7. Siggia, E. J., Dewar, S. J., and Donachie, W. D. (1995) A new Escherichia coli cell division gene, ftsK. J. Bacteriol. 177, 6211–6222
8. Liu, G., Draper, G. C., and Donachie, W. D. (1998) FtsK is a bifunctional protein involved in cell division and chromosome localization in Escherichia coli. Mol. Microbiol. 29, 893–903
9. Demarre, G., Galli, E., and Barre, F. X. (2013) in DNA Helicases and DNA Motor Proteins (Spies, M., ed) pp. 245–262, Springer, New York
10. Bigot, S., Sivanathan, V., Possoz, C., Barre, F-X., and Cornet, F. (2007) FtsK, a literate chromosome segregation machine. Mol. Microbiol. 64, 1434–1441
11. Barre, F.-X. (2007) FtsK and SpolII E: the tale of the conserved tails. Mol. Microbiol. 66, 1051–1055
12. Burton, B., and Dubnau, D. (2010) Membrane-associated DNA transport machines. Cold Spring Harb. Perspect. Biol. 2, a000406
13. Wul, L. J., and Errington, J. (1994) Bacillus subtilis SpolIIe protein required for DNA segregation during asymmetric cell division. Science 264, 572–575
14. Grainge, I. (2010) FtsK–a bacterial cell division checkpoint? FEBS Lett. 582, 1315–1327
15. Guzman, L.-M., Barondess, J. J., and Beckwith, J. (1992) FtsL, an essential cytoplasmic membrane protein involved in cell division in Escherichia coli. J. Bacteriol. 174, 7716–7728
16. Spratt, B. G. (1975) Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12. Proc. Natl. Acad. Sci. U.S.A. 72, 2999–3003
17. Bogdanov, M., Zhang, X., Xie, J., and Dowhan, W. (2005) Transmembrane protein topology mapping by the substituted cysteine accessibility method (SCAM): application to lipid-specific membrane protein topogenesis. Methods 36, 148–171
18. Geissler, B., and Margolin, W. (2005) Evidence for functional overlap among multiple bacterial cell division proteins: compensating for the loss of FtsK. Mol. Microbiol. 58, 596–612
19. Potluri, L.-P., Kannan, S., and Young, K. D. (2012) ZipA is required for FtsZ-dependent preseptal peptidoglycan synthesis prior to invagination during cell division. J. Bacteriol. 194, 5334–5342
20. Culham, D. E., Hillar, A., Henderson, J., and Morkovskaya, L. Y., Racher, K. L., Boggs, I. M., and Woll, J. M. (2003) Creation of a fully functional cell cycle-specific variant of osmosensor and proton-osmoprotectant symporter ProP from Escherichia coli and its application to the transmembrane transporter orientation. Biochemistry 42, 11815–11823
21. Shevchenko, A., Schäffer, A., Hiller, A., Kowalczykowski, S., and Mann, M. (1996) Mass spectroscopic studies of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858
22. Grynberg, G., and Simon, I. (1998) Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J. Mol. Biol. 283, 489–506
23. Tunády, G. E., and Simon, I. (1998) The HMMTOP transmembrane topology prediction server. Bioinformatics 14, 895–890
24. Hiraga, S., Ichinose, C., Niki, H., and Yamazoe, M. (1998) Cell cycle-dependent duplication and bidirectional migration of SeqA-associated DNA-protein complexes in E. coli. Mol. Cell 1, 381–387
25. Beijerinck, T. J., Moles, D., and Harris, B. (2007) in Methods for General and Molecular Microbiology (Reddy, C. A., Beveridge, T. J., Berzina, I. A., Mazzafar, C., Schmidt, T. M., and Snyder, L. R., eds) 3rd Ed., pp. 54–81, American Society for Microbiology, Washington, D. C.
26. Kuro, E., Hughes, H. V., Brown, P. J., Hall, E., Tzekov, S., Cava, F., de Pedro, M. A., Brun, Y. V., and VanNieuwenhze, M. S. (2012) In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent 6-amino acids. Angew. Chem. Int. Ed. Engl. 51, 12519–12523
27. Ye, L., Jia, Z., Tang, T., and Maloney, P. C. (2001) Topology of OxlT, the outer membrane protein of Oxalobacter formigenes, determined by site-directed fluorescence labeling. J. Bacteriol. 183, 2490–2496
28. Wood, J. M., Culham, D. E., Hillar, A., Vernikovska, Y. I., Liu, F., Boggs,
51. Larue, K., Ford, R. C., Willis, L. M., and Whitfield, C. (2011) Functional and structural characterization of polysaccharide co-polymerase proteins required for polymer export in ATP-binding cassette transporter-dependent capsule biosynthesis pathways. J. Biol. Chem. 286, 16658–16668

52. Bisicchia, P., Steel, B., Mariam Debela, M. H., Löwe, J., and Sherratt, D. (2013) The N-terminal membrane-spanning domain of the Escherichia coli DNA translocase FtsK hexamerizes at midcell. MBio 4, e00800–e00813

53. Chou, P. Y., and Fasman, G. D. (1974) Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. Biochemistry 13, 211–222

54. Chou, P. Y., and Fasman, G. D. (1974) Prediction of protein conformation. Biochemistry 13, 222–245

55. Wang, X., Possoz, C., and Sherratt, D. J. (2005) Dancing around the divisome: asymmetric chromosome segregation in Escherichia coli. Genes Dev. 19, 2367–2377

56. Chen, J. C., and Beckwith, J. (2001) FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during Escherichia coli cell division. Mol. Microbiol. 42, 395–413

57. Rothfield, L. (2003) New insights into the developmental history of the bacterial cell division site. J. Bacteriol. 185, 1125–1127

58. Strauss, M. P., Liew, A. T. F., Turnbull, L., Whitchurch, C. B., Monahan, L. G., and Harry, E. J. (2012) 3D-SIM super resolution microscopy reveals a bead-like arrangement for FtsZ and the division machinery: implications for triggering cytokinesis. PLoS Biol. 10, e1001389

59. Allard, J. F., and Cytrynbaum, E. N. (2009) Force generation by a dynamic Z-ring in Escherichia coli cell division. Proc. Natl. Acad. Sci. U.S.A. 106, 145–150

60. Lan, G., Daniels, B. R., Dobrowsky, T. M., Wirtz, D., and Sun, S. X. (2009) Condensation of FtsZ filaments can drive bacterial cell division. Proc. Natl. Acad. Sci. U.S.A. 106, 121–126

61. Osawa, M., Anderson, D. E., and Erickson, H. P. (2008) Reconstitution of contractile FtsZ rings in liposomes. Science 320, 792–794

62. Addinall, S. G., Bi, E., and Lutkenhaus, J. (1996) FtsZ ring formation in fts mutants. J. Bacteriol. 178, 3877–3884

63. Sun, Q., and Margolin, W. (1998) FtsZ dynamics during the division cycle of live Escherichia coli cells. J. Bacteriol. 180, 2050–2056

64. Heidrich, C., Ursinus, A., Berger, J., Schwarz, H., and Hölting, J.-V. (2002) Effects of multiple deletions of murine hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in Escherichia coli. J. Bacteriol. 184, 6093–6099

65. Wientjes, F. B., and Nanninga, N. (1989) Rate and topography of peptidoglycan synthesis during cell division in Escherichia coli: concept of a leading edge. J. Bacteriol. 171, 3412–3419

66. Pogliano, J., Pogliano, K., Weiss, D. S., Losick, R., and Beckwith, J. (1997) Inactivation of FtsI inhibits constriction of the FtsZ cytokinetic ring and delays the assembly of FtsZ rings at potential division sites. Proc. Natl. Acad. Sci. U.S.A. 94, 559–564

67. Nanninga, N. (1991) Cell division and peptidoglycan assembly in Escherichia coli. Mol. Microbiol. 5, 791–795

68. Lesterlin, C., Pages, C., Dubarry, N., Dasgupta, S., and Cornet, F. (2008) Asymmetry of chromosome replichores renders the DNA translocase activity of FtsK essential for cell division and cell shape maintenance in Escherichia coli. PLoS Genet. 4, e1000288

69. Diez, A. A., Farewell, A., Nannmark, U., and Nyström, T. (1997) A mutation in the ftsK gene of Escherichia coli affects cell–cell separation, stationary-phase survival, stress adaptation, and expression of the gene encoding the stress protein UspA. J. Bacteriol. 179, 5878–5883