Investigating the Role of Divisome Protein FtsK as an Essential Checkpoint During Bacterial Cell Division

by

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ABSTRACT

INVESTIGATING THE ROLE OF DIVISOME PROTEIN FtsK AS AN ESSENTIAL CHECKPOINT DURING BACTERIAL CELL DIVISION

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University of Guelph, 2018

Advisor: Dr. Cezar M. Khursigara

Bacterial cell division is an essential and fundamental process required to sustain life. In *Escherichia coli*, division requires the recruitment and assembly of approximately thirty soluble and membrane-bound proteins, ten of which are essential (FtsZ, FtsA, ZipA, FtsK, FtsQ/B/L, FtsW/I, FtsN). Complete assembly of this macromolecular complex relies on formation of a dynamic ring-like structure known as the Z-ring. Following initial formation and stabilization of the Z-ring, cells must complete segregation of the bacterial chromosome and remodel the cell envelope to allow septum formation.

One key modulator linking early (Z-ring) and late (cell envelope remodeling) division complexes is the essential protein FtsK. FtsK is a bifunctional transmembrane protein that coordinates chromosome segregation with its C-terminus (FtsKC) and cell division with its membrane-anchored N-terminal domain (FtsKN). Although the structure and function of FtsKN during division is unclear, it is suggested that FtsK acts as a checkpoint to ensure DNA is properly segregated before septation can begin. In this capacity, we hypothesize that FtsK must modulate septum formation during division through the formation of dynamic and essential protein interactions with both the Z-ring and late stage division machinery.

Drawing on advanced molecular techniques and imaging technologies, this thesis refines the membrane topology of FtsKN using site-directed fluorescence labeling, and elucidates several protein interaction partners that are critical for its role as an essential division checkpoint. Our
revised topology revealed a novel functional periplasmic loop of FtsK_N that, when mutated, produces cellular voids. We extensively characterized this novel cell division defect by fluorescence microscopy and high-resolution transmission electron microscopy, which exposed a novel role for FtsK_N in linking cell envelope septation events. In addition, UV cross-linking and a genomic suppressor screen each uncovered potential interaction partners of FtsK_N involved in both cell elongation and division. Two of these proteins, rare lipoprotein A (RlpA) and FtsA, were confirmed as direct FtsK_N protein interactors by in vitro pull-down assays. Together, these findings provide critical evidence on how FtsK_N may mediate the transition between cell elongation and septation in E. coli, and significantly advances our understanding of what is necessary for bacteria to replicate and survive.
Dedicated to Mom and Dad – my loudest and most unwavering cheerleaders.
Acknowledgments

The work presented in this thesis could not have been possible without the encouragement and support from a great number of people. Firstly, I would like to thank my advisor, Dr. Cezar Khursigara. The guidance and unwavering patience you afforded me as I navigated my challenging (and at times, outright rebellious) project, all while I dabbled in a growing list of other collaborations, is greatly appreciated. Your excitement for each and every experimental finding, no matter how small, keeps our whole lab going (especially during those brief times when we may have lost our spark for science). Most importantly, I thank you for instilling in me an unbridled passion for microscopy. Your persistent advice to ‘Look at your cells!’ not only shaped my graduate project, but will shape my career for years to come.

I am also grateful for the expert knowledge and support given to me by my advisory committee members, Dr. Janet Wood and Dr. Matthew Kimber. Your tips, tricks and advice along the way have been invaluable, and I thank you for being exceptional mentors in all aspects of my graduate experience. I also thank Dr. Jonathan Krieger of the SPARC BioCentre at the Hospital for Sick Children, and Dr. Dyanne Brewer of the Mass Spectrometry Facility here at the University of Guelph. Together, we have endured the countless trials and tribulations of my mass spectrometry endeavor. I am grateful for your boundless expertise and willingness to troubleshoot every conceivable angle of my experiment to ensure we obtained the best data possible. As the managers of my home away from the lab, I also acknowledge Mr. Bob Harris and Dr. Michaela Strüder-Kypke of the Molecular and Cellular Imaging Facility. Without you the vast majority of my thesis would not have been possible. I have enjoyed the countless hours we have spent together in the Imaging Facility (I still stand by my notion that Bob needs a little bell so you can hear him coming), and I thank you for patiently teaching me everything you know about microscopy.

I have had the pleasure of working with a whole host of brilliant colleagues over the years, and I acknowledge the contributions each member of the Khursigara Lab has made to my thesis. In particular, to the first and last undergraduate students I proudly mentored, Ms. Mara Goodyear and Ms. Sabrina Glavota: you are both wonderful scientists and much of the work presented here is a direct result of your hard work and dedication. I thank you for making the lab so enjoyable, and to Mara, I couldn’t have asked for a better side-kick and friend over the years – I can’t wait to reunite the Dream Team when we start our state-of-the-art microscopy consortium someday. To Dr. Elyse Roach and Dr. Amber Park: thank you for all of your technical and philosophical help as I troubleshooting almost every aspect of my project. Your work ethics and brilliant ideas inspire me every day.

Finally, there really aren’t enough words to adequately thank my friends and family for all of the support they have given me. Emily, Kate and Sean, you never cease to put a smile on my face, even when experimental disaster strikes. Mom, Dad and Michael, I couldn’t have asked for a better support system than the three of you. I am honoured to be your sister and daughter, and hope I continue to make you proud.
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<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>A&lt;sub&gt;600&lt;/sub&gt;</td>
<td>absorbance at 600nm</td>
</tr>
<tr>
<td>ABC</td>
<td>ammonium bicarbonate</td>
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<tr>
<td>AGC</td>
<td>automatic gain control</td>
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<tr>
<td>ATc</td>
<td>anhydrotetraycline</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
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<td>charge coupled device</td>
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<tr>
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<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAZ</td>
<td>&lt;i&gt;diff&lt;/i&gt; activity zone</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetraacetic acid</td>
</tr>
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<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FM4-64</td>
<td>(N-(3\text{trimethylammonium propyl})-4-(6-(4\text{diethyl amino phenyl}) hexatrienyl) pyridinium dibromide)</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>fts</td>
<td>filamentous temperature sensitive</td>
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<td>FtsK&lt;sub&gt;C&lt;/sub&gt;</td>
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<td>N-terminal amber stop codon variant of FtsK</td>
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<td>guanosine triphosphate</td>
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<td>hydroxylcoumarin carbonyl amino-D-alanine</td>
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<tr>
<td>HALA</td>
<td>hydroxylcoumarin carbonyl amino-L-alanine</td>
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<tr>
<td>HCD</td>
<td>higher-energy collisional dissociation</td>
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<td>HMMTOP</td>
<td>hidden Markov model for topology prediction</td>
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<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IM</td>
<td>inner membrane</td>
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<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
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<td>IPTG</td>
<td>isopropyl- [β-D-thiogalactoside</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>KOPS</td>
<td>FtsK orienting polar sequences</td>
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<td>LB</td>
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<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
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<td>LDAO</td>
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<td>MTS</td>
<td>membrane targeting sequence</td>
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<td>MTSET</td>
<td>methanethiosulfonate</td>
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<tr>
<td>OM</td>
<td>outer membrane</td>
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<tr>
<td>ori</td>
<td>origin of replication</td>
</tr>
<tr>
<td>OGM</td>
<td>Oregon Green 488 maleimide</td>
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<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
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<tr>
<td>PBpa</td>
<td>(p)-benzoyl-L-phenylalanine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RlpA</td>
<td>rare lipoprotein A</td>
</tr>
<tr>
<td>S.E.</td>
<td>standard error</td>
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<td>sodium dodecyl sulfate</td>
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<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>super optimal broth with catabolite repression</td>
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CHAPTER 1: Introduction

Cell division is an essential and fundamental process required to sustain life. To drive replication, bacterial cells rely on complex gene expression and a large number of coordinated protein systems. Together, these systems work to allow for cell growth and elongation, chromosome replication and segregation, septum formation and physical division of the cell into genetically identical daughter cells (Figure 1.1)(1). Temporal and spatial coordination is essential at each step of this complex process to ensure replicated chromosomes are fully partitioned into their respective daughter cells before the septum begins to form at mid-cell and division occurs. In the absence of proper coordination, cells lose the ability to effectively divide both their genetic and cellular material, resulting in the inability to produce viable progeny (2, 3). It is this dependence on coupling multiple essential processes that makes cell division an excellent target for the development of novel antimicrobials and is a prominent focus for current drug research. However, although division is indispensable for the survival and replication of all bacterial cells, our limited knowledge of the proteins involved and the interplay between growth and division severely hinders our progress in exploiting this process for drug development.

1.1. Growth and Division of the Gram-Negative Cell Envelope

Growth and division of a Gram-negative, rod-shaped bacterium such as Escherichia coli requires coordinated modification of three distinct layers of the cell envelope. Unlike the relatively simple cell envelope of Gram-positive species, which contains a single plasma membrane and thick cell wall, the Gram-negative cell envelope contains an inner phospholipid bilayer (inner membrane, IM), an asymmetric outer membrane (OM), and a thin peptidoglycan layer located within the periplasmic space between these two membranes (4). Together, these
Figure 1.1. Stages of bacterial cell division. Diagrammatic representation of bacterial cell division in a Gram-negative bacterium. Before constriction of the bacterial cell envelope occurs, cells undergo various coordinated processes, including DNA replication, cell elongation and chromosome segregation. Upon completed assembly of the division machinery, which includes a protein scaffold known as the Z-ring and various associated proteins (collectively known as the divisome), the Z-ring constricts and a septum is formed at mid-cell. Completion of septation and physical separation of the newly formed cell poles results in the generation of two genetically identical daughter cells.

 Components form an essential barrier between the bacterium and the external environment. During the cell cycle of rod-shaped bacteria, cells undergo two distinct processes: elongation and septation. Elongation results in longitudinal growth of the bacterium while maintaining a relatively fixed diameter (5). Septation involves invagination of all three layers of the cell envelope followed by cleavage of the newly formed septum and physical separation of the two daughter cells (6). These two processes must be highly coordinated to maintain integrity of the cell envelope at all times and prevent cell lysis.

 Two large macromolecular protein complexes are responsible for the spatial and temporal coordination of elongation and division in E. coli. Lateral insertion of peptidoglycan along the long axis of the cell is facilitated by the elongasome (7, 8), while invagination of the cell envelope during septation is caused by action of the divisome (9, 10). The majority of proteins
involved in these processes are essential and many parallels in both overall organization and function of the proteins involved exist between both complexes (8).

1.2. The Elongasome

Growth and maintenance of the three-dimensional rod shape of \textit{E. coli} is mediated by coordinated elongation of the peptidoglycan layer. In general, elongation follows a series of essential steps: lipid-linked peptidoglycan precursors are generated in the cytoplasm, which are then transferred through the IM to the periplasm by a flippase protein, where they are polymerized and cross-linked by peptidoglycan synthases to form a completed sacculus (11). This process relies on the concerted action of the macromolecular protein complex known as the elongasome (Figure 1.2)(7, 8, 12). Recruitment of the proteins involved in elongation occurs in a hierarchical manner beginning with initial formation of a cytoskeletal scaffold made of the bacterial actin homolog MreB (5, 12, 13). In the presence of ATP, MreB polymerizes as antiparallel protofilaments to form a helical or patch-like structure that wraps around the long axis of the cell, and is bound directly to the membrane \textit{via} a hydrophobic loop and N-terminal amphipathic helix (5, 13–16). Assembly and localization of MreB is driven by association with the essential bitopic transmembrane protein RodZ and the structural protein complex formed by MreC and MreD (17–22). The building blocks of peptidoglycan, synthesized in the cytoplasm, are transported to the periplasm as lipid-linked precursors by the flippase RodA (23, 24). Here, the peptidoglycan binding proteins (PBPs) specifically required for cylindrical growth, PBP1a and PBP2, polymerize the glycan backbone (transglycosylation) and cross-link peptide side-chains (transpeptidation)(25). PBP1a is a bifunctional class A PBP that is regulated by the OM lipoprotein LpoA (26, 27) and is capable of both transglycosylation and transpeptidation (28).
Figure 1.2. The elongasome general protein topologies. Proteins shown promote lateral growth of the cell wall in E. coli. General protein topologies are shown in relation to the Gram-negative cell envelope. MreB, a homolog of the eukaryotic protein actin, polymerizes in an ATP-dependent manner to form a scaffold onto which the rest of the elongasome is assembled. Recruitment of all downstream proteins, including the structural proteins MreC and MreD, the peptidoglycan synthases PBP1a (controlled by the OM lipoprotein LpoA) and PBP2, as well as the flippase RodA, is dependent on proper assembly and localization of MreB filaments (driven by RodZ). Adapted from Ref. 12.

Alternatively, PBP2 is a monofunctional transpeptidase and is the only peptidoglycan synthase specifically essential for lateral cell wall growth (25, 29).

Following sufficient longitudinal growth of the cell to support physical division, a major switch in activity during cell envelope remodeling must occur. While it is known that multiple components of the elongasome interact with the divisome (30, 31), how the switch from elongation to septation is facilitated is currently unknown.
1.3. The Divisome

Similar to the elongasome, at a molecular level, septation requires the recruitment and assembly of a macromolecular protein complex known as the divisome (9, 10). The divisome, which forms at mid-cell, is made up of approximately thirty soluble and membrane-bound proteins, ten of which have been found to be essential for division (Table 1.1)(12, 32–34). The majority of these proteins were initially discovered in mutants that, when grown at a nonpermissive temperature (typically 42°C), are unable to physically divide despite continued replication of their DNA, which leads to the development of a filamentous phenotype (2, 35). For this reason, most genes encoding proteins in bacterial cell division are given the designation *fts* for filamentous temperature sensitive (2, 35).

Table 1.1. Essential proteins involved in cell division in *E. coli*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Proposed Function</th>
</tr>
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<tbody>
<tr>
<td>FtsZ</td>
<td>Filamentous, self-activating GTPase that forms the Z-ring scaffold; First protein recruited to mid-cell; Recruits all downstream proteins to the divisome</td>
</tr>
<tr>
<td>FtsA</td>
<td>Main Z-ring membrane anchor; May also be involved in Z-ring stabilization</td>
</tr>
<tr>
<td>ZipA</td>
<td>Z-ring stabilizing protein; Shown to induce bundling of FtsZ filaments in the Z-ring; Modulates FtsA self-interaction</td>
</tr>
<tr>
<td>FtsK</td>
<td>Involved in DNA translocation and chromosome dimer resolution during chromosome segregation; Poorly understood role in septum formation</td>
</tr>
<tr>
<td>FtsQ</td>
<td>Forms trimeric complex with FtsB and FtsL; Proposed to regulate constriction initiation in an FtsN-dependent manner</td>
</tr>
<tr>
<td>FtsB</td>
<td>Forms trimeric complex with FtsQ and FtsL; Proposed to regulate constriction initiation in an FtsN-dependent manner</td>
</tr>
<tr>
<td>FtsL</td>
<td>Forms trimeric complex with FtsQ and FtsB; Proposed to regulate constriction initiation in an FtsN-dependent manner</td>
</tr>
<tr>
<td>FtsW</td>
<td>Transports lipid-linked peptidoglycan precursors across the cytoplasmic membrane; Recruits FtsI to the divisome</td>
</tr>
<tr>
<td>FtsIe</td>
<td>Transpeptidase involved in peptidoglycan synthesis</td>
</tr>
<tr>
<td>FtsN</td>
<td>Involved in peptidoglycan binding and divisome stability; Proposed role in triggering constriction of the cell envelope</td>
</tr>
</tbody>
</table>

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*a* All information was obtained from Refs. 33, 34, unless otherwise noted.

*b* Refs. 36, 37

*c* Refs. 38–41

*d* Refs. 43–45

*e* Penicillin-binding protein 3 (PBP3)

*f* Ref. 42
Overall, the recruitment of the various proteins that make up the divisome occurs in a sequential or hierarchal manner, with downstream elements requiring the proper association of all upstream elements before they can bind (Figure 1.3A)(9, 46). In general, the localization of divisome proteins occurs in two stages: the early stage and the late stage (47).

**Figure 1.3. Hierarchical assembly of the divisome and general protein topologies.** A, hierarchal recruitment of division proteins during formation of the divisome at mid-cell in *E. coli*. Arrows point from the protein responsible for recruitment towards the component(s) recruited. Before any particular protein can be recruited, all upstream elements must first be properly assembled within the divisome. B, general topologies of the essential proteins within the divisome complex. Proteins are displayed in the general order of recruitment from left to right. Adapted from Ref. 9.
1.3.1. Early Stage Cell Division

Complete assembly of the divisome relies on the initial formation of a dynamic ring-like structure at the site of division known as the Z-ring (48–50). This ring is composed of the first and most critical protein recruited to mid-cell, FtsZ (48).

FtsZ is a small (40 kDa), globular protein that is a homolog of the eukaryotic cytoskeletal protein tubulin and has the ability to form short intracellular filaments upon binding GTP (Figure 1.4A)(49, 51–54). It is this GTP-dependent polymerization that allows for the formation of the Z-ring, which acts as a scaffold structure onto which the rest of the divisome complex is assembled (Figure 1.3B). In the absence of the Z-ring, the remainder of the divisome components are not recruited to mid-cell and division does not occur (55, 56). In vitro, FtsZ is able to form many different filamentous structures (51–53, 57); however, the organization of the Z-ring in vivo is still not completely understood. Drawing on electron cryotomography and recent advances in super-resolution fluorescence microscopy, the architecture of the Z-ring in vivo is becoming clearer. High-resolution images of the Z-ring in multiple organisms suggest that FtsZ forms numerous short protofilaments that are approximately 100 nm in length and condense to form overlapping bundles (Figure 1.4, B–E)(58–66). Although these studies show that FtsZ protofilaments assemble in a discontinuous and heterogeneous manner, the extensive bundling and lateral contacts made between filaments allows the Z-ring to span the entire circumference of the cell (58–66).

The remainder of the essential proteins recruited in the early stage of divisome formation work together to both stabilize and tether the Z-ring to the membrane. The main membrane anchor between FtsZ and the IM is FtsA. FtsA belongs to the actin family of proteins, although insertion of an additional subdomain (1C) that is involved in protein-protein interactions with
Figure 1.4. In vivo Z-ring architecture.  

A, Structure of FtsZ from *P. aeruginosa*, as determined by X-ray crystallography (PDB 2VAW). Ribbon model is coloured from blue (N-terminus) to red (C-terminus), with the bound GDP molecule depicted as red sticks (PyMOL 2.0; Schrödinger).  

B, Model for the organization of FtsZ within the Z-ring. FtsZ forms numerous protofilaments that are approximately 100 nm in length. Lateral contacts between filaments creates overlapping bundles, and allows the Z-ring to span the entire circumference of the cell.  

C, 3D segmentation of a cryoelectron tomogram showing overlapping FtsZ protofilament organization at a *Caulobacter crescentus* division site. The OM, IM and FtsZ filaments are pseudo-coloured in yellow, blue and red, respectively. Reproduced from Ref. 58.  

D, Brightfield (i), total fluorescence (ii) and super-resolution photoactivated light microscopy (iii) images of an *E. coli* cell expressing FtsZ-mEos2. Image demonstrates that the Z-ring is a heterogeneous structure. *Bar*, 500 nm. Reproduced from Ref. 59.  

E, 3D structured illumination micrograph of FtsZ-GFP Z-rings in *Bacillus subtilis*. The bacterial membrane (red) is stained with the dye FM4-64. Constricting Z-rings are indicated by the white arrows. Reproduced from Ref. 60.

downstream cell division proteins is unique to FtsA (Figure 1.5A)(67–70). Association of FtsA with the Z-ring is mediated by subdomain 2B, and the combined association of FtsA and the Z-ring with the IM is achieved by a C-terminal amphipathic helix of FtsA that acts as a membrane
targeting sequence (MTS) (71–73). The ATP binding ability of FtsA has been shown to enhance its binding to FtsZ (36, 73–76) and is required for the self-assembly of FtsA into polymers (Figure 1.5B) (36). Importantly, residue changes outside the ATP binding pocket of FtsA, and even outside the known FtsA-FtsZ interface, can allosterically enhance interaction with FtsZ (75), highlighting plasticity within FtsA to adapt to changes in divisome assembly.

Figure 1.5. Domain architecture of the essential cell division protein FtsA. A, Structure of FtsA from *Thermotoga maritima*, as determined by X-ray crystallography (PDB 1E4G). Ribbon model is coloured by subdomain, with the bound ATP molecule depicted as black sticks (PyMOL 2.0; Schrödinger). The C-terminal membrane targeting sequence (MTS) is not shown. Domain 1C is unique to FtsA and is involved in protein interactions with late stage divisome proteins. Domain 2B is responsible for interaction with FtsZ. The surfaces involved in FtsA polymerization are indicated by the dotted lines. B, Approximate arrangement of FtsA monomers in an FtsA polymer. Domains 2A and 1C from one FtsA monomer interact with domains 2B and 1A of the following monomer to form an actin-like, straight protofilament. Adapted from Refs. 67, 71.
In addition to FtsA, transmembrane protein ZipA acts as a secondary flexible membrane anchor, which has also been shown to induce bundling of FtsZ filaments within the Z-ring (77–79). Given homologs of ZipA are only found in Gammaproteobacteria (80), the precise role of ZipA has remained unclear. It was initially speculated that ZipA simply played a role in Z-ring stabilization and recruitment of downstream cell division proteins in \textit{E. coli} (80–83). More recently, it has been postulated that ZipA is responsible for modulating the ability of FtsA to self-interact at the Z-ring, thereby enhancing its interaction with FtsZ and facilitating division protein recruitment (36, 37). In addition, it has been shown that ZipA is required for initial stages of peptidoglycan synthesis at the site of division (84), although the role of ZipA is still unknown.

\textbf{1.3.2. Late Stage Cell Division}

Following initial formation and stabilization of the Z-ring at mid-cell, bacterial cells must begin synthesis and rearrangement of the peptidoglycan layer to allow for septum formation. This process is carried out by the various essential protein complexes recruited in the late stage of cell division: FtsQ/B/L, FtsW/I, and FtsN (7). FtsQ, FtsB, and FtsL are all bitopic transmembrane proteins that form a stable trimeric complex prior to recruitment to the site of division (38, 39, 85, 86). The precise function of this complex is still poorly understood; however, it may be involved in activation of septal peptidoglycan synthesis by the FtsW/FtsI sub-complex (40, 41). FtsW is a large polytopic membrane protein that is co-transcribed with a class B PBP known as PBP3 (FtsI)(87, 88). FtsW is thought to transport lipid-linked peptidoglycan precursors across the cytoplasmic membrane, and it functions to recruit FtsI to the site of division and regulate its activity (43–45, 88). Here, FtsI acts as a transpeptidase to cross-link peptidoglycan chains and aids in septal peptidoglycan synthesis (25, 89–91).
The last essential protein recruited to the divisome is FtsN. FtsN is a bitopic IM protein that contains a large periplasmic domain (92, 93). This periplasmic domain includes both the only region of FtsN essential for division, as well as the only conserved region of the protein, its C-terminal SPOR domain (93–95). This SPOR domain binds peptidoglycan and acts as a septal targeting domain for FtsN (95–98). While complete stability of the divisome and visible cell envelope constriction is triggered by the arrival of FtsN at the site of division (94, 95, 99, 100), the precise role of FtsN remains elusive. Current theories regarding FtsN-stimulated cell constriction rely on transmission of a cell signal through allosteric interactions between FtsN and other essential cell division proteins (37, 40–42). The majority of evidence points to a link between FtsN and FtsA as the main components of this cell signaling complex, with additional input from another transmembrane protein involved in division (37, 40–42, 68).

Following complete assembly of the divisome, the Z-ring must constrict to accommodate formation of a septum between the two daughter cells. As the Z-ring constricts, the bacterial membrane furrows and eventually the cell will pinch into two, completing division. Currently, there is no consensus on the origin of the force that causes constriction \textit{in vivo}. Debate exists over whether invagination of the cell envelope is solely driven by pulling on the IM by Z-ring constriction, by pushing on the IM from ingrowth of peptidoglycan in the periplasm, or by the concerted action of both processes (101, 102). There is considerable \textit{in vitro} evidence that condensation and bending of FtsZ filaments within the Z-ring may produce the necessary force to achieve division (53, 103–107); however, this has not been verified \textit{in vivo}. Alternatively, recent evidence indicates that peptidoglycan synthesis occurs progressively inward from the cell surface at discrete sites that move around the division plane (108–110). This movement, which would push the IM inward, is caused by FtsZ polymer treadmilling that directs both the amount and spatial distribution of septal peptidoglycan synthesis (108–110). In this instance, the Z-ring
would simply act as a passive scaffold to position the peptidoglycan synthesis enzymes, rather than generate the constrictive force itself. To unify both pulling and pushing modes of force generation, it is possible that both of these mechanisms work in tandem, where deformation of the IM by FtsZ filaments is reinforced by inward peptidoglycan synthesis to build the septum.

1.4. Filamentous Temperature Sensitive Protein K (FtsK)

As mentioned previously, in order for cell division to proceed properly, DNA must be fully partitioned into what will eventually become two separate daughter cells. Aberrations in segregating replicated DNA to each pole can uncouple chromosome segregation and physical septation, and lead to premature septum formation which can trap DNA in the closing septum. Therefore, there must be a mechanism or checkpoint available to ensure that this event has taken place before septum formation is initiated. In fact, prior to the recruitment of the six late division proteins mentioned above, a seventh protein called FtsK is recruited to mid-cell. Based on our current understanding of the roles each essential divisome protein plays, FtsK is unlike any other component. Rather than performing a single function, FtsK is a bifunctional protein that is involved in both cell division, as well as chromosome segregation (111). It is this role in coupling both processes essential for proper and efficient division of bacterial cells (chromosome segregation and septation), that makes FtsK a candidate for the role of essential checkpoint of bacterial cell division, and an excellent target for the development of novel antibiotics.

1.4.1. Discovery of FtsK

Similar to most genes encoding essential proteins involved in bacterial cell division, FtsK was first discovered by means of a temperature-sensitive mutation of the \textit{ftsK} gene in \textit{E. coli} (112). Begg et al. discovered the mutant TOE44 in 1995 using temperature-oscillatory enrichment (TOE)(112). TOE was developed to search for division genes that encode proteins
that are both temperature-sensitive and are required at a discrete time interval within the cell cycle (112, 113). It was found that the TOE44 mutant harbours a G to C nucleotide transversion at base pair 239, which leads to a missense mutation that replaces a glycine with an alanine at position 80 in the mutant FtsK44 protein (112). This \textit{ftsK44} mutation allows cells to divide normally at 30°C, yet form long, indented filaments when grown at 42°C. These cells also continue to replicate and segregate their DNA normally, which is typical of \textit{fts} mutants (112).

Begg \textit{et al.} were able to further characterize the stage at which this previously unidentified cell division protein functions using two additional mutant strains: \textit{rodA(Ts)} and \textit{pbpA(Ts)} (112). Each of these strains grows as normal rod-shaped cells at 30°C; however, when grown at a nonpermissive temperature (42°C), they replicate as spheres. Therefore, when a temperature-sensitive mutation is introduced into these strains, such as \textit{ftsK44}, they either grow as large, unconstricted spheres or show incomplete constrictions depending on whether the temperature-sensitive mutation affects early or late stage cell division, respectively (112). When the double-mutant \textit{rodA ftsK44} strain was grown at 42°C, deep, incomplete constrictions of the cells were observed, indicating that FtsK acts at a late stage of division. However, there is still debate over whether FtsK is a true late stage cell division component. Both Wang and Lutkenhaus, and Chen and Beckwith, observed a smooth filament phenotype using FtsK depletion strains (114, 115). This suggests that the division block occurs at an earlier stage of division than seen with the mutant \textit{ftsK44} phenotype. The precise time at which FtsK acts during cell division is still unclear.

It should also be noted that the mutant \textit{ftsK44} phenotype, and in some instances complete deletion of \textit{ftsK}, can be suppressed by gene deletion, or overexpression or mutation of other cell division proteins. Specifically, the loss of functional FtsK can be compensated by the deletion of the gene \textit{dacA} (112, 116), overexpression of the proteins FtsN or FtsQ (116–118), or point
mutations in the proteins FtsA (117–119) or FtsL (40). The \( \text{dacA} \) gene codes for a PBP (PBP5) that functions as a carboxypeptidase and catalyzes the removal of the terminal \( \text{D-alanine} \) from peptidoglycan side chains during cell wall synthesis (7, 120). Inhibition of PBP5 is proposed to increase accumulation of complete peptidoglycan chains with pentapeptide side chains in the periplasm, and allow cells to directly use these chains in septum closure (112). However, further evidence using FtsK depletion strains with a \( \text{dacA} \) deletion show that full loss of FtsK cannot be suppressed by the absence of \( \text{dacA} \), which indicates that deletion of PBP5 may simply help restore the function of the mutated FtsK44 protein rather than completely bypass its function (114). With respect to the bypass ability of overexpression of FtsN or FtsQ, or through point mutations of FtsA and FtsL, one thought is that these changes result in artificial stabilization of the divisome, suggesting that FtsK may help to indirectly stabilize the Z-ring and the remainder of the divisome complex when present (117). Alternatively, given both early (FtsA) and late-stage (FtsQ, FtsN) proteins are able to suppress the loss of functional FtsK, it is suggested that FtsK modulates a putative signaling pathway between these stages of division (40, 121). The exact mechanism of this signal transduction is unclear. In any case, with all bypass mechanisms for FtsK, cells are still partially elongated or exhibit cell chains while downstream division proteins are properly recruited (116–118), implying that the loss of FtsK cannot be completely suppressed and that hierarchical recruitment by fully functional FtsK is more efficient. Despite considerable experimental evidence supporting the essentiality of FtsK during division, many questions regarding its precise structure and function remain.

1.4.2. Structure and Function

FtsK is a large, multi-spanning membrane protein that is composed of 1329 amino acids and has a molecular weight of approximately 147 kDa (111, 112). It belongs to a large, well-
conserved SpoIIIE family of proteins that possess a variety of functions over a diverse range of organisms, the most notable being double-stranded DNA (dsDNA) translocation during division and sporulation in organisms such as *E. coli* and *Bacillus subtilis*, respectively (122, 123). In general, FtsK is comprised of three separate units, each of which contain different structural features (Figure 1.6)(124).

![Figure 1.6. Schematic representation for the proposed topology of *E. coli* FtsK.](image)

The N-terminal domain (FtsK<sub>N</sub>) is made of approximately the first 200 amino acids and is the membrane embedded portion of FtsK thought to play a role in cell division. Following FtsK<sub>N</sub> is a flexible linker region (FtsK<sub>L</sub>) that has a variable length between bacterial species and no currently known function. The C-terminal domain (FtsK<sub>C</sub>) of approximately 500 amino acids forms a hexameric DNA translocase involved in bacterial chromosome segregation. FtsK<sub>C</sub> contains three subdomains that constitute the translocation motor (α, β) and a regulatory domain that binds DNA (γ). Adapted from Ref. 124.

### 1.4.2.1. FtsK N-terminal Domain (FtsK<sub>N</sub>)

The first 200 N-terminal amino acids comprise the first functional region of FtsK (114, 116). This region (denoted FtsK<sub>N</sub>) acts as the hydrophobic integral membrane anchor for FtsK, and was initially thought to contain between four and five α-helical transmembrane segments (112, 116). Through various truncation experiments, FtsK<sub>N</sub> was found to be the only portion of FtsK absolutely essential for division. Complementation of FtsK depletion strains with a
truncated polypeptide containing the N-terminal 15% of FtsK (~202 amino acids) allows for restoration of cell viability and normal cell division (114, 116). In contrast, constructs lacking the N-terminal portion (i.e., containing the C-terminal half only) do not complement depletion strains (114). FtsK\textsubscript{N} is also responsible for targeting FtsK to mid-cell during division and is considered the septum-targeting domain (116, 125). Despite abundant experimental evidence on the essentiality and localization of FtsK\textsubscript{N}, there is no direct biochemical evidence regarding its precise role in cell division.

Currently, very little is known about the organization and positioning of FtsK\textsubscript{N} in the IM. An initial study by Dorazi and Dewar in 2000 assessed the N-terminal membrane topology of FtsK using alkaline phosphatase and β-galactosidase fusions to ten C-terminal truncations of FtsK\textsubscript{N} (126). While this low-resolution model served to verify that this region is composed of four transmembrane α-helices, not five (126), questions regarding the sizes and locations of the transmembrane α-helices remain. In addition to the rudimentary topology of the N-terminal domain, some evidence also suggests that FtsK\textsubscript{N} forms hexamers both \textit{in vitro} and \textit{in vivo} at mid-cell (127). This hexamerization of the N-terminal domain occurs independently from the hexamer formation typically seen by the C-terminal domain (127). Overall, there have been no further attempts to refine these initial structural models. This limited information serves as a considerable drawback to studies on potential protein-protein interactions between FtsK and other divisome components in this region.

\textbf{1.4.2.2. Variable Linker Domain (FtsK\textsubscript{L})}

The remaining two functional domains of FtsK are cytoplasmic in nature and make up approximately 85% of its total size (112). Immediately downstream of FtsK\textsubscript{N} is a highly flexible linker domain rich in proline and glutamine (denoted FtsK\textsubscript{L}). The length of the linker ranges from
approximately 600 amino acids in \textit{E. coli} down to approximately 100 amino acids in \textit{Pseudomonas} species (121, 128). In \textit{E. coli}, FtsKL contains six consecutive repeats of a 10-amino acid ‘PQ repeat motif’; PQQPV[A/P]PQ[P/Q]Q separated by YQQ (112, 128). It is this long linker region that makes FtsK much larger than most other proteins in the conserved SpoIIIE family (112, 129). The precise function of FtsKL is currently unknown. However, a few studies in \textit{E. coli} using various truncations and deletion of the linker region have shown that FtsKL can restore normal cell morphology in FtsK depleted strains when FtsKL is fused to another membrane-bound protein (121, 130). Simply due to the fact that the linker region physically connects FtsKN and the C-terminal domain, it is speculated that FtsKL may act to facilitate the coordination of septum formation and chromosome segregation.

\textbf{1.4.2.3. FtsK C-terminal Domain (FtsKC)}

The remaining approximately 500 amino acids at the C-terminus of FtsK (denoted FtsKC) comprise the most well-characterized domain of FtsK with respect to both its structure and function. When Begg \textit{et al.} first discovered FtsK in 1995, a BLAST search revealed very high primary sequence identity and homology between FtsKC and the SpoIIIE family of DNA translocases, especially those of \textit{B. subtilis} (Figure 1.7)(112). As such, it was initially postulated that FtsKC was likely to possess similar properties as other members of the family and facilitate chromosome partitioning during division (112, 131).

To date, three main systems have been implicated in bacterial chromosome segregation, highlighting extensive functional redundancy associated with this process (132). In particular, the FtsK/SpoIIIE family of DNA translocases is highly conserved throughout bacteria (132). The two main members of this family, FtsK and SpoIIIE, are both dsDNA transporters that share upwards of 50% identity within each of the various domains of their C-terminal regions (Figure 1.7)(122).
Figure 1.7. Domain conservation between *E. coli* FtsK and *B. subtilis* SpoIIIE. Alignment of the individual domains of FtsK (*top*) and SpoIIIE (*bottom*), and the corresponding percent identity and percent similarity of their amino acid sequences. The grey dotted line in the SpoIIIE structure indicates a gap in the sequence alignment. Identities and similarities of the translocation motor (α, β) and the regulatory domain (γ) of the DNA translocase region are reported individually. The approximate first and last amino acids of the N- and C-terminal domains, as well as the length of the linker regions, are also indicated under the corresponding structure. Percent identity and percent similarity were determined using Clustal Omega (EMBL-EMI) and Ident and Sim (Sequence Manipulation Suite) programs. Adapted from Ref. 122.

A specialized form of cell division in *B. subtilis*, called sporulation or endospore differentiation, results in movement of the site of division to one pole, and generates asymmetric division of the cell to produce a large mother cell and a small prespore compartment (122, 129, 133). After the spore septum has been formed between the two compartments, the protein SpoIIIE is required to pump the prespore chromosome across the closed septum from the mother cell into the forespore (133). FtsKC possesses a similar function; however, in contrast to SpoIIIE, FtsK acts before the septum fully closes in dividing bacteria (134).

SpoIIIE and FtsKC are both RecA-type ATPases that form a multimeric ring around dsDNA and possess consensus nucleotide-binding pockets that allow the use of ATP to drive its αβ domain motor (135, 136). Specifically, FtsKC forms a homohexameric structure in the presence of dsDNA, which was experimentally determined by analytical ultracentrifugation,
electron microscopy and X-ray crystallography (Figure 1.8)(136). Through recognition of an 8-base pair motif (5′-GGGNAGGG-3′) known as FtsK orienting polar sequences (KOPS) by the γ domain of FtsKₐ (137–142), DNA that stretches across the septum is actively moved at a rate upwards of 6.7 kbp/s at room temperature or 17 kbp/s at 37°C towards a site at the replication terminus called the dif site (142–145). The precise orientation of KOPS, with respect to this second 28 base pair dif site, ensures FtsKₐ rapidly pumps the DNA in the correct direction to facilitate activation of chromosome dimer resolution (CDR) at dif, a second function of the FtsKₐ domain (137, 140, 142).

**Figure 1.8. Structure of hexameric FtsKₐ.** Ribbon model of hexameric FtsKₐ from *Pseudomonas aeruginosa*, as determined by X-ray crystallography (PDB 2IUU), viewed from the side (*left*) and top (*right*). Each monomer is coloured individually, and the bound ADP molecule associated with each monomer depicted as grey spheres (PyMOL 2.0; Schrödinger). Double-stranded DNA is threaded through the center of the hexamer (i.e., through the central pore seen in the top view). Position of the α and β domains within the DNA translocation motor are shown on the left. Adapted from Ref. 136.
During circular chromosome replication, dimers form through homologous recombination between sister chromosomes in upwards of 15% of the bacterial population under standard laboratory conditions (Figure 1.9)(122, 146, 147). Without proper resolution of these dimers, DNA can become trapped and broken at the septum, which leads to chromosome degradation and cell death (148). In *E. coli*, CDR occurs at *dif* and relies on the interaction between FtsK<sub>C</sub> and two site-specific tyrosine recombinases, XerC and XerD (135, 149–152). Given the importance of CDR to cell survival during replication, Xer recombination is highly regulated through its interaction with FtsK<sub>C</sub> (135). In fact, it has been found that dimer resolution does not take place

![Diagram](https://via.placeholder.com/150)

**Figure 1.9. Role of FtsK<sub>C</sub> in *E. coli* during chromosome segregation.** During chromosome replication in *E. coli*, homologous recombination between sister chromosomes can result in the formation of a chromosome dimer (*A*). FtsK<sub>C</sub> functions to pump the replicated chromosome towards the *dif* site (marked by the purple dot, *) until the two *dif* sites come within the *dif* activation zone (*B*), where Xer recombination by the XerC/XerD complex occurs (*C*). Xer recombination results in the separation of DNA into two genetically identical sister chromosomes (*D*). In all panels, the origin of replication (*ori*) is marked by the blue dot (*•*). Adapted from Ref. 122.
in the absence of FtsK C (128, 146). Once both dif sites from opposite chromosomes have been positioned within the dif activity zone (DAZ), FtsK C activates recombination by direct interaction with the XerCD/dif complex (135, 152–155). Therefore, FtsK C is involved in two steps of CDR: correct positioning of the dif sites and activation of the recombination machinery.

In E. coli, genes encoding specific proteins suspected of being involved in chromosome segregation have been successfully disrupted without preventing normal segregation, which points to redundant processes that contribute to the proper packaging of DNA into daughter cells (132). With respect to FtsK C, cells are able to divide and segregate their DNA normally in the absence of this domain. This may be the result of functional redundancy and FtsK C might simply act as a checkpoint ‘helper’ function should DNA not be cleared properly from the closing septum.

1.4.3. FtsK as an Essential Checkpoint of Bacterial Cell Division

The bifunctional nature of FtsK makes it an intriguing candidate for the role of ‘checkpoint’ of bacterial cell division, as the protein holds the potential to sense both proper DNA segregation and complete accumulation of division machinery within the same molecule. Disruption of FtsK within the divisome may uncouple chromosome partitioning and formation of the division septum, and lead to DNA becoming guillotined by the septum followed by cell death. However, lack of biochemical characterization with respect to its N-terminal domain and its functional role in cell division severely impedes progress towards utilizing this uncoupling potential in the development of novel antibiotics.

Researchers have proposed various theories regarding a mechanism of action for FtsK, all of which rely on alterations to the interactions between FtsK and various components of the divisome, namely FtsZ, FtsQ, FtsL, and FtsI (156). In 2010, Dubarry et al. speculated on the
functional link between the roles of FtsK in cell division and DNA segregation, and generated the most recent and complete model for the mechanism of action used by FtsK to date (Figure 1.10)(121). This model highlights a relationship between septum formation and DNA translocation that is mediated by changes in FtsK protein interactions. During division, while DNA is still present at the site of septum formation, FtsK₆ is able to recognize KOPS located throughout the chromosome and actively pump DNA towards the dif site. While translocation takes place, strain is placed on the linker region and FtsK is unable to maintain stable protein contacts with both the Z-ring and peptidoglycan synthesis machinery (Figure 1.10A). This destabilization inhibits peptidoglycan synthesis and rearrangement, effectively delaying septum constriction until the DNA has been cleared. Alternatively, once DNA is removed from the site of division, hexameric FtsK₆ disassembles and FtsK is free to re-form stable contacts with FtsZ, FtsQ, FtsL, and FtsI (Figure 1.10B). This conformation is referred to as the constriction permissive configuration, and allows the cells to proceed with septum formation (121). Despite the fact that the mechanism proposed by Dubarry et al. in 2010 is based on biochemical evidence regarding protein-protein interactions between FtsK and proposed interaction partners, it is purely speculative and has yet to be experimentally verified. Given its reliance on protein contacts made along all regions of FtsK, this mechanism would benefit significantly from an in-depth exploration of the precise protein interaction sites and complexes that FtsK forms during division.

1.5. Coordination of Division via Protein-Protein Interactions and Protein Complex Formation

Protein complexes play an essential role in almost every biological process. With the large number of proteins involved in cell division and the tight regulation necessary to ensure that proper and efficient division occurs, it seems logical that elucidating the protein-protein
Figure 1.10. Proposed model for the role of FtsK in cell division. A, the presence of DNA at the site of division causes assembly of hexameric FtsK$_C$ around the chromosome. While FtsK actively pumps DNA towards the $\textit{dif}$ site, the linker region is extended and strain is placed on FtsK$_N$, resulting in a slight conformational change. The loss of contact points between FtsK and the division machinery destabilizes the divisome and inhibits formation of the septum. B, once DNA has been cleared from the septum, hexameric FtsK$_C$ disassembles and FtsK is free to reform multiple contacts with division components, thereby stabilizing the divisome and allowing septum formation to proceed. This conformation is referred to as the ‘constriction permissive conformation’. Adapted from Ref. 121.
interactions that mediate complex formation in the divisome would be at the forefront of division research. However, although the basic components of bacterial cell division have been identified, critical interactions between the various proteins involved are still poorly understood.

The hierarchy of recruitment employed by bacteria during formation of the divisome has mainly been determined through the use of depletion strains and fluorescence microscopy (46, 56). It was initially thought that each protein was recruited in a strictly linear fashion, with one protein localizing after the other. However, various sub-complexes, such as FtsQ/B/L, have been shown to associate prior to incorporation into the divisome, and others have been shown to back-recruit proteins typically thought to associate earlier in the recruitment hierarchy (38, 46, 117). This evidence points to a more cooperative approach to divisome assembly that relies heavily on various interactions between essential divisome components.

The protein-protein interactions in *E. coli* that mediate divisome formation reveal a complex web of interaction partners (Figure 1.11A)(9). The large majority of these interactions have been determined by bacterial two-hybrid analysis, with few further characterized by co-immunoprecipitation experiments and cross-linking analysis (38, 39, 70, 121, 157–160). Given the compact space occupied by the divisome, the large number of proteins involved and their low-level native expression, it has been suggested that some of the interactions detected by two-hybrid analysis may have resulted from artificial interaction caused by over-expression of the fusion constructs, or by indirect interaction with an endogenous bridging protein (9, 158). For example, initial detection of a protein interaction between FtsZ and the FtsZ associated protein ZapB was later found to be bridged by a third intermediate partner, ZapA (161, 162). As such, further biochemical evidence must be generated to verify that these interactions are
Figure 1.11. Proposed interactions between essential divisome components. A, interaction map of essential divisome components. Lines indicate a positive signal between two proteins in a least one two-hybrid assay (9, 157, 158). Circular arrows indicate self-interaction. Proposed FtsK interaction partners are highlighted in orange. Re-drawn from Ref. 9. B, summary of the proposed regions of interaction between FtsK and divisome components FtsZ (Z), FtsQ (Q), FtsL (L) and FtsI (I). Lines represent the broad region of FtsK thought to interact with each component. The dotted line indicates continued interaction with FtsL, but not FtsQ in this region. Re-drawn from Ref. 156.
physiologically relevant. This is especially true with respect to interactions involving FtsK, all of which have been proposed from the results of single bacterial two-hybrid experiments (9, 157, 158).

As mentioned previously, in total, two-hybrid analysis has revealed interactions between FtsK and FtsZ, as well as the peptidoglycan synthesis machinery (FtsQ, FtsL, and FtsI) (157). Few studies have attempted to pinpoint precise regions of interaction between FtsK and other components of the divisome (Figure 1.11B) (121, 156, 160). Both Grenga et al. and Dubarry et al. used truncated versions of FtsK in bacterial two-hybrid assays to test their interaction with FtsZ, FtsQ, FtsL and FtsI (121, 160). While each study was able to detect interaction between the truncated FtsK peptides and the various cell division proteins tested, the results of these two studies were not in agreement. For example, Grenga et al. detected interaction between FtsK and FtsZ within a short region of FtsK_L (residues 608 – 724) (160), whereas Dubarry et al. detected interaction between FtsK and FtsZ within the entire FtsK N-terminal domain (residues 1 – 179), as well as a different region of FtsK_L (residues 331 – 641) (121). Similar discrepancies can be seen with regards to FtsK interaction with FtsQ, FtsL, and FtsI (Figure 1.11B). Therefore, it becomes immediately apparent that there is still a lack of consensus on what regions within the structure of FtsK are involved in protein interactions, as well as which proteins actually interact in these regions. It is understood within the literature that these interactions represent only the strongest contacts made with FtsK; however, this begs the question if there are other more transient contacts made that could modulate the function of FtsK, but are too weak to be detected by two-hybrid analysis. Alternatively, FtsK may interact with other proteins outside of the classical divisome, a possibility that has not been previously explored. In order to further our understanding of how FtsK acts during cell division, especially with respect to the under-
characterized FtsK\textsubscript{N} domain, an understanding of the precise protein contacts made by FtsK during the process of division is required.

1.6. Summary and Rationale

Cell division is a complex and intricate process that requires a large number of varied protein interactions to ensure bacterial cells divide into genetically viable daughter cells. Specifically, the concept that FtsK may play a role of checkpoint in bacterial cell division relies on the premise that FtsK is involved in the coordination of multiple protein interactions, many of which have not been fully confirmed or characterized. To function as a checkpoint, FtsK must have the ability to alter the activity of the proteins involved in septum formation, likely through physical interaction with the Z-ring (FtsZ) and peptidoglycan synthesis machinery. Variation in the protein contacts FtsK makes throughout the process of division may alter these components’ abilities to form the divisome or enzymatically carry out septum formation. As such, we hypothesize that FtsK modulates septum formation during bacterial cell division through the formation of dynamic and essential protein-protein interactions with both the Z-ring and peptidoglycan synthesis machinery. To further our understanding of how FtsK may carry out this function within the divisome complex, we must first refine the structure of FtsK and ultimately characterize how these essential complexes are formed. In this thesis, we report a revised membrane topology of the N-terminal domain of FtsK (FtsK\textsubscript{N}) using site-directed fluorescence labeling, and characterize a novel cell division defect in \textit{E. coli}, caused by mutation of a newly identified functional periplasmic loop of FtsK\textsubscript{N}. Using fluorescence microscopy and high-resolution transmission electron microscopy, we show that this defect results from complete division of the IM in the apparent absence of peptidoglycan ingrowth or OM invagination. In addition, using both a targeted \textit{in vivo} UV cross-linking approach and a genetic suppressor screen,
we have identified two novel interaction partners of FtsK_N (RlpA and FtsA), whose interaction with this domain are shown to be involved in FtsK_N function during division. Together, this evidence points to an essential function of FtsK_N as a checkpoint of bacterial cell division, and specifically as a mediator of the transition between elongation and septation in *E. coli*. This thesis expands our understanding of what is necessary for bacteria to replicate and survive, and significantly advances our knowledge of the role of FtsK_N in division, a field of research that has seen very little progress in the last decade.
CHAPTER 2: A Revised Membrane Topology of FtsK and Microscopical Characterization of a Novel Cell Division Defect

This chapter has been published in The Journal of Biological Chemistry with the title “Site-directed Fluorescence Labeling Reveals a Revised N-terminal Membrane Topology and Functional Periplasmic Residues in the *Escherichia coli* Cell Division Protein FtsK” (163).

Statement of Contributions

The experiments described below were designed by Alison M. Berezuk and Dr. Cezar M. Khursigara. All experiments were performed by Alison M. Berezuk, with assistance from Mara Goodyear during the cloning of a subset of FtsK\textsubscript{N} single cysteine variants. The manuscript and all accompanying figures were prepared by Alison M. Berezuk, with drafting and editorial assistance from Dr. Cezar M. Khursigara.
2.1 Abstract

In *Escherichia coli*, FtsK is a large integral membrane protein that coordinates chromosome segregation and cell division. The N-terminal domain of FtsK (FtsK<sub>N</sub>) is essential for division, and the C terminus (FtsK<sub>C</sub>) is a well-characterized DNA translocase. Although the function of FtsK<sub>N</sub> 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 FtsK<sub>N</sub> and other division proteins in both the periplasm and cytoplasm; thus, a clear understanding of how FtsK<sub>N</sub> is positioned in the membrane is required to characterize these interactions. The membrane topology of FtsK<sub>N</sub> 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 FtsK<sub>N</sub> also provides an important platform for future studies on essential interactions required for this process.
2.2 Introduction

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, 7, 32). 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 (3, 9, 164).

In *Escherichia coli*, the essential and bifunctional division protein FtsK is thought to link chromosome segregation and cell division. FtsK is a large multi-spanning membrane protein composed of 1329 amino acids and is a homolog of the protein SpoIIIE from *Bacillus subtilis* (111, 112). Together, these proteins make up the large, well-conserved FtsK/SpoIIIE protein family (122, 123, 165) 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 (122, 133, 166). 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 (116, 125). FtsKN is the only portion of FtsK essential for division (114, 116, 125), 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 (FtsKL), whose length is variable between bacterial species (112, 121). 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 (131, 134–136, 146, 165, 167).
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 divisome (121, 134, 156, 165). In 2010, Dubarry et al. (121) 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 (121, 156). Previous biochemical evidence suggests that FtsK interacts with both cytoplasmic and membrane-bound proteins within the divisome (121, 157, 160). 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 (48–50, 168), and proteins involved in peptidoglycan synthesis, such as FtsQ, FtsL, and FtsI (25, 38, 86, 89, 113, 169, 170). 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 (126) explored the N-terminal membrane topology of FtsK using site-directed reporter fusions. With the help of previously reported hydrophobicity analysis (112), computer-generated topology predictions, and reporter fusion data, a topology map of FtsK was generated. In this proposed model, FtsK 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 (126). 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 full integral membrane portion of the protein (171). Although the proposed locations and size of the transmembrane α-helices are reasonable based on the amino acid composition in these areas, and the single amino acid linking the transmembrane segments is not impossible, the steric constraints associated with a single amino acid turn calls into question the likelihood that FtsK_N retains this conformation.

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 (126). 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.

2.3 Experimental Procedures

2.3.1 Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1. 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
Table 2.1. Bacterial strains and plasmids

The abbreviations used are as follows: Amp, ampicillin; Cam, chloramphenicol.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>rph-1N (rrnD-rrnE)</td>
<td>Coli Genetic Stock Center</td>
</tr>
<tr>
<td>Lemo21 (DE3)</td>
<td>fhuA2 [lon] ompT gal (λDE3) [dem] ΔhsdS' pLemo(CamR)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td></td>
<td>λ. DE3 = λ s BamHlo ΔEcoRI-B int:(lacI::PlacUV5::T7 gene1) i21 Δnin5 pLemo = pACYC184-PrhaBAD-lysY</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F−Φ80lacZΔM15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ-thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>LP11-1</td>
<td>W3110 fisK44 aroA::Tn10</td>
<td>(84)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBAD24</td>
<td>Protein expression vector under the control of P_{ara}; AmpR</td>
<td>Addgene</td>
</tr>
<tr>
<td>pAB006-2</td>
<td>pBAD24 derivative encoding amino acids 1-220 of FtsK (His₁₀-FtsK(N_{220})) from E. coli</td>
<td>This study</td>
</tr>
<tr>
<td>pAB006-13</td>
<td>pBAD24 derivative encoding Cys-less (ΔCys) His₁₀-FtsK(N_{220})</td>
<td>This study</td>
</tr>
</tbody>
</table>

well as 0.2% (w/v) L-arabinose when appropriate. E. coli LP11-1 cultures were grown in Complementation Media (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [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.

2.3.2 Plasmid Construction

The N-terminal 220 amino acids of FtsK (FtsK(N_{220})) were amplified from E. coli K12 W3110 genomic DNA by PCR using iProof high fidelity DNA polymerase (Bio-Rad) with custom primers AMB001Fb (5’-TTCCATCAAGATTGATGCACCACCACCACCACCACCA-CCACCACCACCACCTCCTCCATTGAAGGTCGAGTTTGAGCCAGGAATACATTGAA-3’) and AMB003Ra (5’-TAATCTTCAGGTTACCCCGAATTAGTCGCTTCTCTCCATCTCATCTTTCATCGACCAGGTATC-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 (pBAD24) 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 (FroggaBio) and then verified by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph) to produce pAB006-2.

### 2.3.3 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) with primers C94A, C127A, and C169A (*Table 2.2*) and pAB006-2 as a template, resulting in construct pAB006-13. During generation of the ΔCys mutant, single cysteine variants Cys-94 (pAB006-16) and Cys-169 (pAB006-11) were also obtained. The ΔCys mutant was used as the template to generate all other single cysteine variants using a QuikChange Lightning site-directed mutagenesis kit (Stratagene). All primers used for mutagenesis and resulting plasmids are shown in *Table 2.2*. All mutants were confirmed by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph).

### 2.3.4 Temperature-sensitive Complementation Assays

To assess whether FtsK_N(220), ΔCys, and all single cysteine mutants generated were functionally active, a temperature-sensitive complementation assay was developed, similar to that used by Geissler and Margolin (117). Plasmids carrying FtsK_N(220), as well as ΔCys and all single
Table 2.2. Oligonucleotide pairs used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence of mutagenic oligonucleotide (5′ to 3′)(^a)</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C94A(^b)</td>
<td>TCATTATGTGCGCCGTGCAGTTCGTTTTGCGCTGCGGATC</td>
<td>pAB006-21</td>
</tr>
<tr>
<td></td>
<td>GACGCCAGCAAAAACCAAGACCCGCCGACAAATAGA</td>
<td></td>
</tr>
<tr>
<td>C127A(^b)</td>
<td>GCTCATCCTACCCTCGCTTGCTCTGGCCGCAATC</td>
<td>pAB006-171</td>
</tr>
<tr>
<td></td>
<td>GATTTGCGCCAGACCCGGAGGTAAAGTGGACG</td>
<td></td>
</tr>
<tr>
<td>C169A(^b)</td>
<td>CTATTGCCTGCTCGCAGTTGGTTGCGCCGACGG</td>
<td>pAB006-31</td>
</tr>
<tr>
<td></td>
<td>GCGCTGGCCAAACACCGGAGCGACGGCAGAATAAG</td>
<td></td>
</tr>
<tr>
<td>R20C</td>
<td>AGTTAAGTAGCGGGCTGCCGCTTCGGAAG</td>
<td>pAB006-41</td>
</tr>
<tr>
<td></td>
<td>CTTCCAGAAGCGCGGACGGCTACTTAACTT</td>
<td></td>
</tr>
<tr>
<td>S50C</td>
<td>CCCCTTGCCGACCCCATGCTGGTGCCGAAAC</td>
<td>pAB006-51</td>
</tr>
<tr>
<td></td>
<td>CTTTGCACGACAGGGGGCTCGGAAAG</td>
<td></td>
</tr>
<tr>
<td>E58C</td>
<td>GCTGTCGCAAGACGGCCTGCTGCGATCAATTATTTTGGTATATTA</td>
<td>pAB006-151</td>
</tr>
<tr>
<td></td>
<td>CACCTAATTATGGATAGCGCGCAGCCAGCCCTTTGGCAGAC</td>
<td></td>
</tr>
<tr>
<td>W70C</td>
<td>GCCCCGTGCGGCCTGGCAGATAG</td>
<td>pAB006-61</td>
</tr>
<tr>
<td></td>
<td>GTATCTGGCAAGACGACCACG</td>
<td></td>
</tr>
<tr>
<td>I107C</td>
<td>GTCACTACGGTCCACGAGAATACATGGATTTTTGCGGTTTCG</td>
<td>pAB006-162</td>
</tr>
<tr>
<td></td>
<td>CGAAACCGAACAAATAATCAGATTTCTCGGACTGATGAC</td>
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<tr>
<td>A134C</td>
<td>CTGTCTTGCGCCGCAATCAACTGGTGACGTATCTGTTATTTTATT</td>
<td>pAB006-181</td>
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<td></td>
<td>AAAATACGAGGATATGCTCAGATTGGTTATTTGCTCAAGGC</td>
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<td>D135C</td>
<td>TGGTCTGGCCGCAATCACTGGCGATATCTGTTGATTTTG</td>
<td>pAB006-191</td>
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<td></td>
<td>CAAATAACGAGATATAGGCGCTGAGGTGATTTCGCCAGAC</td>
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<td>D136C</td>
<td>GGCGCGCAATCAGCTGACGTATCTGTTATTTGCTCC</td>
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<td>GAGGGCGAATACAGAGCTGGAACGCTGGAATTCCGCGGG</td>
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<td>CACCGGAGGCAAAAATAGCGAGATATCTGCTACG</td>
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<td>Y139C</td>
<td>GCTGACGATATCTGGTGTGTTTTTGCCCTGGCGG</td>
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<td>GCCACCGAGCACAACACAGAGATATCTGCTACG</td>
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<td>S148C</td>
<td>CGGTGGGCCATTTGGCTGATTCAAGCAGCATAC</td>
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<td></td>
<td>GTACTGTCTTTAGATGACGAGCCTGAGTACG</td>
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<td>S160C</td>
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</tr>
<tr>
<td></td>
<td>TAGGTCCCGCCGCTACAGTGACTTGCGGGAACGTA</td>
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<tr>
<td>W181C</td>
<td>CGTTGTACACCCTGCTGCTATTTTGCCCTCCGGTGC</td>
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</tr>
<tr>
<td></td>
<td>CAAATTTGCTACAGTGACTTGCGGGAACGTA</td>
<td></td>
</tr>
<tr>
<td>Y217C</td>
<td>TACCTGGGTGCGATAGTTGAGTGATGTTGAAAGCACTA</td>
<td>pAB006-61</td>
</tr>
<tr>
<td></td>
<td>TAGCTGCTTTACACTCATATTCTCTCATCGGACCCAGGTA</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Base changes are underlined and in boldface.

\(^b\) 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.
cysteine mutants described above, were transformed into *E. coli* strain LP11-1 (*ftsK*44) (84) by chemical heat shock. A 5-mL culture of each strain was inoculated in Complementation Media, supplemented with appropriate antibiotics, and grown at 30°C in a rotary shaker at 200 rpm overnight. Each strain was then subcultured to an *A*<sub>600</sub> of 0.1 in two separate 5-mL aliquots of fresh Complementation media. L-Arabinose was added to both aliquots of each strain carrying FtsK<sub>N(220)</sub>, ΔCys, and single cysteine mutants to a final concentration of 0.2% (w/v). Cultures were then incubated at either 30 or 42°C for 2h. 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 α = 0.05 for all tests.

To quantify cellular voids seen in the nonfunctional FtsK<sub>N(220)</sub> mutants, LP11-1 strains carrying FtsK<sub>N(220)</sub> and single cysteine mutants D135C, D136C, I137C, and W138C were grown and induced at 42°C for 6h 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 assessed 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 analysis of variance with Tukey-Kramer multiple comparison post-tests as described above.
2.3.5 Site-directed Fluorescence Labeling

*E. coli* Lemo21 strains carrying ΔCys and single cysteine mutants of FtsKN(220) were grown at 37°C for 1h 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.* (172), with minor modifications. Fresh stock solutions of Oregon Green 488 maleimide (OGM) (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 5 mM EDTA, 40 µg/mL DNaseI, 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 2 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 Profinity 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 M imidazole, 10% [v/v] glycerol, 0.1% LDAO), mixed with 20 µL of 5× Loading Buffer (250 mM Tris-HCl, pH 6.8, 10% [w/v] SDS, 30% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.1% [w/v] bromophenol 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 (173). 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 (174, 175).

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.

2.3.6 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 DNsae, 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 H_2O, 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 (Thermo Scientific) using bovine serum albumin (BSA) as a standard.

2.3.7 Western Blotting

To verify the presence of FtsK_{N(220)} 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-His6 (Clontech) and alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Sigma), respectively. The blot was developed using a solution of 3.3 mg of nitro blue tetrazolium and 1.7 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 MgCl₂). The blot was scanned, and band density was measured using the ImageJ program (version 1.46r, National Institutes of Health).

2.3.8 Immunofluorescence Microscopy

Immunofluorescence was performed as described by Hiraga et al. (176), with minor modifications. LP11-1 carrying FtsK_{N(220)} 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 incubated at room temperature for 1 h. Following
fixation, cells were collected by centrifugation and resuspended in 1 mL of 80% methanol. Methanol-fixed cells (20 μL) were placed on slides previously coated with 0.1% poly-L-lysine solution (Sigma) and air-dried for 20 min. Slides were then incubated in 100 μL of buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 2 mg/mL lysozyme at room temperature for 10 min. The lysozyme solution was removed by washing the slides three times in PBS containing 0.05% (v/v) Tween 20 (PBST). Samples were submerged in 99% methanol for 1 min, allowed to dry, and then submerged in acetone for 1 min and air-dried. Cells were blocked with 100 μL of 10% normal goat serum (Invitrogen) for 30 min at room temperature to eliminate nonspecific antibody binding. The blocking solution was then replaced with a 100-μL mixture of primary antibodies (1:100 diluted mouse anti-His6 [Clontech] and 1:100 diluted rabbit anti-FtsZ [Cedarlane] in 1% [w/v] BSA/PBST). Slides were incubated for 1 h at room temperature before removing the primary antibodies by washing three times in PBST. In a dark room, a 100-μL mixture of secondary antibodies (1:200 diluted goat anti-mouse conjugated to Alexa Fluor® 594 [Molecular Probes] and 1:200 diluted goat anti-rabbit conjugated to FITC [Sigma] in 1% [w/v] BSA/PBST) was placed on the slides and incubated for 1 h at room temperature. Secondary antibodies were removed by washing three times in PBST, and then cells were counterstained with 100 μL of DAPI (10 μg/mL) for 1 min in the dark and washed with PBST. Coverslips were mounted on the slides with SlowFade® gold antifade reagent (Invitrogen) and sealed with nail polish. Slides were imaged using an upright Leica DM5000B fluorescent microscope equipped with a Hamamatsu ImagEM EM-CCD digital camera (Quorum Technologies). Images were analyzed using Volocity software (version 6.3, PerkinElmer Life Sciences). To verify specificity of the fluorescent signal, control samples were immunostained as above with omission of incubation in the primary antibody mixture. Quantification of the FtsK and FtsZ localization patterns was determined from a random sampling of 75 cells for each strain.
2.3.9 High Pressure Freezing and Freeze Substitution

Samples used for high-resolution electron microscopy analysis were induced and grown at 42°C as 20-mL cultures as described in the temperature-sensitive complementation assays. Cells were harvested from 9 mL of culture by centrifugation (500 × g, 2 min), resuspended in 1 mL of 250 mM sucrose, and pelleted again. The majority of the sucrose was removed, leaving a few drops for cell resuspension. Cells were then immediately frozen using a Leica EM HPM100 high pressure freezer. Cryofixed cells were transferred to vials containing 1 mL of Substitution Media (1% OsO₄, 2.5% glutaraldehyde, 1-2% uranyl acetate in 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. 177) 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.

2.3.10 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.
2.3.11 *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>, D135C, D136C, I137C, and W138C 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).

2.3.12 *Peptidoglycan Labeling*

To visualize the peptidoglycan layer, cells were stained with the fluorescent amino acids hydroxylcoumarin carbonyl amino-<i>α</i>-alanine (HADA) or hydroxylcoumarin carbonyl amino-<i>β</i>-alanine (HALA) as described by Kuru <i>et al.</i> (178). 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 antifade reagent and mounted on poly-<i>β</i>-lysine-coated glass slides. Coverslips were sealed with nail polish prior to imaging as described for the immunofluorescence microscopy.
2.4 Results

2.4.1 *FtsK*$_{N(220)}$ Single Cysteine Mutants Successfully Complement *LP11-1* (*ftsK*44)

To generate the single cysteine mutants used to probe the topology of *FtsK*$_{N(220)}$, 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*$_{N(220)}$. 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 proposed transmembrane segments and to confirm the absence of additional transmembrane domains toward the C terminus of the protein. In total, 19 single cysteine variants of *FtsK*$_{N(220)}$ were generated.

Functional characterization of wild-type (WT) *FtsK*$_{N(220)}$, 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 *ftsK*44 temperature-sensitive *E. coli* strain LP11-1. This strain harbours the FtsK44 temperature-sensitive G80A substitution first characterized by Begg *et al.* (112). *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 (Figure 2.1A). 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
Figure 2.1. Complementation of *E. coli* LP11-1 (*ftsK*44) with WT FtsK$_N$(220) and FtsK$_N$(220) mutants. *A*, representative phase contrast micrographs, and *B*, mean cell lengths of LP11-1 cells overexpressing WT and FtsK$_N$(220) variants grown at permissive (30°C) and nonpermissive (42°C) temperatures. An example of a typical cellular void is shown, as indicated by the *arrowhead* (D135C, *inset*). Bar, 5 µm, including *inset*. Cells were measured for cell length using the ImageJ program and are reported as mean cell length (µm) ± S.E. (*n* = 75 cells per strain per temperature). *, *p* < 0.001 versus WT FtsK$_N$(220) grown at the same temperature.

Each FtsK$_N$(220) variant rather than endogenous FtsK during division, constructs were overexpressed by the addition of 0.2% (w/v) L-arabinose. In total, WT FtsK$_N$(220), ΔCys, and 15 of the 19 single cysteine mutants were able to successfully complement LP11-1 and suppress filamentation (Figure 2.1). 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.

2.4.2 Revised Membrane Topology of FtsK_{N(220)} Reveals Altered Periplasmic Loop

Site-directed fluorescence labeling was used to generate a membrane topology model of
the N-terminal portion of FtsK (Figure 2.2A). This technique has been successfully used
previously to determine the number and orientation of transmembrane segments for several inner
membrane proteins (179–181). The location of targeted cysteine residues was determined by their
relative accessibility to the thiol-specific fluorescent probe OGM (Figure 2.2B). Given the
membrane-impermeable nature of this reagent, treatment of intact bacterial cells with OGM
allows for the detection of periplasmic cysteine residues only (Figure 2.2B, 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 (Figure 2.2B, bottom panel). Cysteine residues located within
a transmembrane segment are not labeled in either intact or lysed cells (Figure 2.2B, middle
panel), but they are labeled upon exposure of the transmembrane segments by an SDS-containing
solution following purification (Figure 2.2C). In general, it was found that the labeling efficiency
of intact cells harbouring periplasmic cysteine variants was lower than for cytoplasmic and
Figure 2.2. Revised membrane topology of FtsK

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 FtsK_{N(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-His_{6} antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse IgG.
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 (180).

Of the 15 single cysteine variants used to generate the revised membrane topology, five were found to harbour cysteine residues located at a different position than reported previously (Table 2.3). All five residues were initially proposed to be within the fourth transmembrane domain or immediately downstream in the cytoplasmic loop (126). 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 (126). In contrast, our data suggest the fourth transmembrane domain is located much closer to the C terminus of the protein (residues 163-179) (Figure 2.2A). This positioning of the fourth transmembrane segment is in agreement with domain IV identified by all computer predictions reported by Dorazi and Dewar (126). 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_N is composed of four transmembrane α-helices and not five as suggested previously (112, 127).

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 (Figure 2.2A, residues 133-162). This is in contrast to the single amino acid connection proposed by Dorazi and Dewar (126). 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 (182, 183). This loop also
Table 2.3. Location of targeted cysteine residues

<table>
<thead>
<tr>
<th>Variant</th>
<th>Original location</th>
<th>Revised location</th>
</tr>
</thead>
<tbody>
<tr>
<td>R20C</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>S50C</td>
<td>Periplasm</td>
<td>Periplasm</td>
</tr>
<tr>
<td>E58C</td>
<td>Periplasm</td>
<td>Periplasm</td>
</tr>
<tr>
<td>W70C</td>
<td>Periplasm</td>
<td>Periplasm</td>
</tr>
<tr>
<td>C94</td>
<td>Membrane</td>
<td>Membrane</td>
</tr>
<tr>
<td>I107C</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
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<td>Membrane</td>
<td><strong>Periplasm</strong></td>
</tr>
<tr>
<td>Y139C</td>
<td>Membrane</td>
<td><strong>Periplasm</strong></td>
</tr>
<tr>
<td>S148C</td>
<td>Membrane</td>
<td><strong>Periplasm</strong></td>
</tr>
<tr>
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<td>Cytoplasm</td>
<td><strong>Periplasm</strong></td>
</tr>
<tr>
<td>C169</td>
<td>Cytoplasm</td>
<td><strong>Membrane</strong></td>
</tr>
<tr>
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<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>W193C</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>T205C</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
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<tr>
<td>Y217C</td>
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</table>

\(^a\) Data are based on Dorazi and Dewar (126).
\(^b\) Residue locations that differ from the original topology are highlighted in boldface.

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 FtsK\(_N\) was investigated.

2.4.3 Nonfunctional FtsK\(_N\)(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 (Figure 2.1B). This suggested that mutation of these residues, which are all located in the newly identified periplasmic loop described above, disrupts the ability of FtsK\(_N\) to function during cell division. To determine whether the inability to suppress filamentation was a result of a failure of FtsK\(_N\) to insert into the membrane, total membrane fractions of cells overexpressing WT FtsK\(_N\)(220) and each of the nonfunctional single cysteine mutants were collected and analyzed.
by Western blotting. FtsK<sub>N</sub> was detected in the membrane fraction of all strains (Figure 2.2D), and no large differences in expression levels were observed between the WT and nonfunctional FtsK<sub>N(220)</sub> 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 (Figure 2.1A, inset). Given that FtsK<sub>N</sub> is known to localize to the mid-cell during cell division in <i>E. coli</i> (114, 115, 125, 184), we used immunofluorescence to determine whether filamentation and these cellular voids were a result of improper localization of FtsK<sub>N</sub> and, consequently, other essential divisome proteins such as FtsZ during division. Co-localization of both FtsK<sub>N(220)</sub> and FtsZ at mid-cell was observed in 52% of LP11-1 cells overexpressing WT FtsK<sub>N(220)</sub> (Figure 2.3, top row). These cells also exhibited proper chromosome segregation as detected by staining with the DNA reactive dye DAPI. In all cells overexpressing nonfunctional FtsK<sub>N(220)</sub> mutants that contained a cellular void, FtsK<sub>N</sub> localized to the top and bottom edges of the voids (Figure 2.3). In contrast, distribution of FtsZ varied depending on which FtsK<sub>N(220)</sub> mutant was expressed. In cells overexpressing FtsK<sub>N(220)</sub> mutants 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 FtsK<sub>N(220)</sub> 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 FtsK<sub>N</sub>. For each of the nonfunctional FtsK<sub>N(220)</sub> mutant
Figure 2.3. Overexpression of nonfunctional FtsK<sub>N(220)</sub> mutants results in altered localization of FtsK<sub>N</sub> and FtsZ. LP11-1 cells overexpressing WT or nonfunctional FtsK<sub>N(220)</sub> 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-His6 and rabbit anti-FtsZ antibodies followed by anti-mouse-conjugated Alexa Fluor® 594 and anti-rabbit-conjugated FITC secondary antibodies to visualize FtsK<sub>N(220)</sub> and FtsZ, respectively. Cells were then counterstained with DAPI and imaged using an upright Leica DM5000B fluorescent microscope. Localized FtsK<sub>N(220)</sub> and FtsZ are indicated by the white arrows. The white arrowheads indicate cellular voids. Bar, 5 µm.
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 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.

2.4.4 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\textsubscript{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\textsubscript{N(220)} (Figure 2.4A). In addition, all nonfunctional mutants displayed a significant increase in cell length over the first 2 h of growth (Figure 2.4B), in contrast to the WT FtsK\textsubscript{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 (Figure 2.4, C and D). In LP11-1 cells overexpressing FtsK\textsubscript{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 (Figure 2.4D). The mean number of voids per cell did not significantly increase in
Figure 2.4. Appearance of cellular voids in nonfunctional FtsK_{N(220)} mutants as a function of cell growth. A, growth curves, and B, mean cell length for LP11-1 strains overexpressing WT or nonfunctional FtsK_{N(220)} single cysteine mutants grown at 42°C. Cells were measured for cell length using the ImageJ program and are reported as mean cell length (µm) ± S.E. (n = 150 cells per time point). Mean cell length of LP11-1 cells overexpressing WT FtsK_{N(220)} did not significantly change over the time course and is represented by the dotted line. C, representative phase contrast micrographs, and D, quantitative assessment of cellular voids of LP11-1 strains overexpressing nonfunctional FtsK_{N(220)} mutants. Micrographs depict typical LP11-1 cells overexpressing FtsK_{N(220)} mutant D135C after 0, 2, 4, and 6 h of growth at 42°C (bar, 1 µm). The number of voids per cell was assessed every hour by phase contrast microscopy and is reported as mean number of voids per cell ± S.E. (n = 150 cells per time point). Pairwise comparisons of within-strain differences for both cell length and cell void data were made using a one-way analysis of variance with Tukey-Kramer multiple comparison post-tests (*, p < 0.05; ** p < 0.01; *** p < 0.001).
mutants D136C and I137C after this time, and cells maintained an average of ~2 voids per cell despite continued incubation. FtsK\textsubscript{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.

2.4.5 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\textsubscript{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\textsubscript{N(220)} exhibited typical cell morphology and invagination of the cell envelope during division (\textbf{Figure 2.5A}). Small aggregates were observed within the cytoplasm of some WT cells, indicating the possible presence of inclusion bodies formed by the overexpression of FtsK\textsubscript{N(220)}. However, because these cells retain normal growth and morphology despite elevated protein levels, the presence of these aggregates does not interfere with normal cell function.

Visualization of LP11-1 strains overexpressing nonfunctional single cysteine FtsK\textsubscript{N(220)} mutants by TEM confirmed the presence of cellular voids in all mutant strains (\textbf{Figure 2.5, 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 (\textbf{Figure 2.5B, 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
Figure 2.5. Ultrastructure of WT FtsK$_{(220)}$ and nonfunctional FtsK$_{(220)}$ mutants. Shown are representative transmission electron micrographs of negatively stained ultrathin sections of LP11-1 cells overexpressing WT (A) and nonfunctional FtsK$_{(220)}$ 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 (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.

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$_{(220)}$ 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 (Figure 2.6), 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 (Figure 2.5B, 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 (178). 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 (Figure 2.7, 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
Figure 2.6. Cellular voids result from complete division of the cytoplasm. Representative oblique illumination, dark field, and fluorescent micrographs of FM4-64 stained LP11-1 cells overexpressing WT or nonfunctional FtsK(N220) single cysteine mutants. Cells were stained with 5 µg/mL FM4-64 for 10 min prior to imaging using a Riveal Contrast Microscope. The white arrowheads indicate typical cellular voids. The white arrows indicate the border between the cellular voids and cytoplasmic compartments. Bar, 5 µm.
Figure 2.7. Cellular voids lack septal peptidoglycan. The peptidoglycan layer of LP11-1 cells overexpressing WT or nonfunctional FtsK_{N(220)} single cysteine mutants was stained with the fluorescent amino acid HADA for 1 h as described under “Experimental Procedures”. Ethanol-fixed cells were adhered to glass slides pretreated with 0.1% poly-L-lysine and imaged using an upright Leica DM5000B fluorescent microscope. The white arrows in the WT strain images indicate visible invagination of the peptidoglycan layer. The white arrowheads indicate cellular voids. Bar, 5 µm, including insets.
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.

2.5 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 (121, 157, 160). The majority of these proposed interactions have been
broadly mapped to the N-terminal domain of FtsK (121, 160). 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 (126), 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 (126), and a minimum of ~200 amino
acids are required for FtsK to function in septation (114, 116, 125), expression 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 (126) identified residue Glu-58 of FtsKN as the essential glutamic acid with a putative metalloprotease HEXXH motif, based on the inability of an E58A mutation to fully complement an \emph{ftsK44} temperature-sensitive strain (TOE44). 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 (\emph{ftsK44}), 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 (\textbf{Figure 2.2A}). 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 (7, 9, 32, 164, 185). 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 discernable voids in the cellular material (\textbf{Figures 2.5-2.7}). Although current knowledge regarding the role of FtsZ and the Z-ring in cytoplasmic membrane constriction has been driven by \emph{in vitro} and computational modeling data (60, 103–105, 168), it is possible that this uncoupling of the cytoplasmic and outer membranes during division \emph{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 (Figure 2.4), 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<sub>N(220)</sub> mutants at either end of the cellular voids disrupted proper localization of FtsZ (Figure 2.3). A previous study by Draper et al. (116) suggested that overexpression of FtsK results in the inhibition of FtsZ assembly into septal rings. Although LP11-1 cells overexpressing WT FtsK<sub>N(220)</sub> show a decreased proportion of cells with a visible Z-ring compared with WT E. coli strains (55, 186), 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<sub>N(220)</sub> 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<sub>N</sub> 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<sub>N(220)</sub> was consistently asymmetric (Figure 2.5, B–E). Asymmetric division of the cytoplasm has been observed in E. coli mutants with deletions in multiple peptidoglycan hydrolases, which are responsible for the cleavage of peptidoglycan during cell growth and division (187). In these strains, cells still exhibit visible septal peptidoglycan ingrowth, but no septal cleavage, resulting in cell chains (187). 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<sub>N(220)</sub> 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 (Figure 2.5, B–E) or by fluorescent cell wall labeling (Figure 2.7), 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 (9, 60, 90, 185, 188, 189). Based on its bifunctional nature, the idea that FtsK may function as a checkpoint of bacterial cell division has been well established (121, 134, 156, 165, 190), and evidence suggests that FtsK interacts with both FtsZ and proteins involved in peptidoglycan synthesis, including FtsI (121, 157, 160). Therefore, it is possible that mutation within the newly identified periplasmic loop could modify or disrupt essential contact between FtsK and these divisome proteins or perhaps alter the oligomeric state of FtsK\textsubscript{N} (127), 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} (191). This defect occurs much later during septation, which resulted in cell chains attached by a small envelope structure (191), 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 (191). This is consistent with the fact that the \textit{ftsK44} temperature-sensitive phenotype can be suppressed by deletion of \textit{dacA}, which codes for penicillin-binding protein 5 (PBP5) that catalyzes the removal of the terminal D-alanine from peptidoglycan side chains
during cell wall synthesis (112, 116). This, together with the evidence provided in this study regarding the inability of nonfunctional FtsK_{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 elongation to septation in *E. coli*. The revised membrane topology of FtsK_{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.
CHAPTER 3: Identification and Biochemical Characterization of Periplasmic FtsK Interaction Partners – Rare Lipoprotein A (RlpA)

Statement of Contributions

The experiments described below were designed by Alison M. Berezuk and Dr. Cezar M. Khursigara. All experiments were performed by Alison M. Berezuk, with the cloning, expression and purification of FLAG-rlpA performed by Sabrina K. Glavota and Dr. Elyse J. Roach. The FtsKN-RlpA pull-down assays were conducted collaboratively by Alison M. Berezuk and Dr. Elyse J. Roach. Additional assistance was provided by Mara Goodyear during the cloning of a subset of FtsKN amber codon variants. Liquid chromatography tandem-mass spectrometry was performed at the Hospital for Sick Children in Toronto, ON by Dr. Jonathan Krieger. Data analysis, preparation of the manuscript and all accompanying figures were completed by Alison M. Berezuk, with drafting and editorial assistance from Dr. Jonathan Krieger and Dr. Cezar M. Khursigara.
3.1 Abstract

Bacterial cell division relies on multiple macromolecular protein complexes to coordinate cell elongation and septation. In *Escherichia coli*, cell elongation is mediated by the elongasome, while division is mediated by the divisome. Proper transition between these two critical cell events is essential to ensure viable progeny are produced; however, the components of each complex responsible for transmission of the cell signal to shift from cell elongation to septation is unclear. Recently, a novel functional periplasmic loop within the N-terminal domain of the essential divisome protein FtsK (FtsKN) was identified that points to a key role for FtsK as a checkpoint of cell envelope remodeling events during division. Here, we used site-specific *in vivo* UV cross-linking to probe the periplasmic loops of FtsKN for protein interaction partners critical for FtsKN function. Mass spectrometry analysis of five unique FtsKN periplasmic cross-links revealed a network of potential FtsKN interaction partners, including cell division proteins FtsN and DamX, and elongasome protein PBP1a. The septal peptidoglycan binding protein rare lipoprotein A (RlpA) was further verified as a novel protein interaction partner of FtsKN by an *in vitro* pull-down assay. Deletion of *rlpA* from the temperature-sensitive *E. coli* strain LP11-1 (*ftsK44*) partially restored cell growth in the absence of functional FtsK and largely suppressed cellular filamentation compared to the wild-type strain. This restoration suggests that interaction with RlpA may be critical in suppressing septation until proper assembly of the divisome occurs, and yields further evidence for FtsKN as a potential regulator of cell envelope remodeling during division.
3.2 Introduction

In a Gram-negative, rod-shaped bacterium, the interplay between cell growth and division is highly coordinated. As with most Gram-negative bacteria, the cell envelope of *Escherichia coli* contains three layers; a thin peptidoglycan layer enclosed within a periplasmic space by two structurally distinct cell membranes (4). Together, these layers form an essential, highly impermeable barrier to the external environment. Based on the indispensable nature of the cell envelope, the precise, simultaneous modification and rearrangement of all three layers during growth and division is necessary to ensure strict maintenance of its barrier function at all stages.

Through the combined action of two large macromolecular protein complexes, cells undergo two broad morphological changes during division. In *E. coli*, lateral insertion of peptidoglycan along the long axis of the cell is facilitated by the elongasome (7, 8). Following sufficient elongation, invagination of the cell envelope is driven by the divisome complex (9, 10). Collectively, the elongasome and divisome are made up of over 30 essential and non-essential proteins (7, 11). Each complex is composed of a membrane-anchored protein scaffold and associated peptidoglycan synthesis enzymes. During elongation, positioning of the elongasome peptidoglycan synthesis machinery is driven by MreB, a homolog of the eukaryotic protein actin (5, 13, 192, 193). Similarly, the septal peptidoglycan synthesis machinery is anchored at mid-cell by the Z-ring, which is mainly composed of the eukaryotic tubulin homolog FtsZ (48, 49, 108, 193).

Although many parallels exist between the functions and general organization of each complex (7, 8), it is still unclear how the switch from dispersed lateral peptidoglycan synthesis to concentrated synthesis at the new cell poles and invagination of the cell envelope during septation occurs. As discussed in *Section 2.5*, the prevailing theory is that transduction of a cell
signal from the peptidoglycan synthesis machinery of the elongasome to the divisome in the periplasm is then transmitted by at least one transmembrane protein to the Z-ring in the cytoplasm to initiate constriction (9, 60, 90, 185, 188, 189). Co-localization and protein interaction analysis of several elongasome and divisome proteins indicates that these complexes are simultaneously present at mid-cell for approximately 40% of the cell division cycle, and that the peptidoglycan synthesis enzymes from each complex physically interact prior to visible cell constriction (31). This organization would permit transduction of the signal between the peptidoglycan synthesis proteins in the periplasm (namely penicillin binding protein 2 [PBP2] of the elongasome and FtsI [PBP3] of the divisome), but would not account for constriction initiation by the Z-ring.

Based on the bifunctional nature of FtsK, it has been well established that it may serve as a critical checkpoint of cell division in *E. coli* (121, 134, 156, 165, 190). However, until recently, this checkpoint function was thought to merely signal completion of chromosome segregation prior to septation. With the new evidence presented in Chapter 2 regarding a novel functional periplasmic loop of FtsKN, a revised role for FtsKN during division has been proposed that involves the regulation of cell envelope remodeling events (163). The uncoupling of cytoplasmic constriction from peptidoglycan and outer membrane invagination caused by mutation of periplasmic residues of FtsKN suggests that FtsKN might link the Z-ring to the periplasmic peptidoglycan synthesis machinery. While sufficient biochemical evidence suggests that FtsKN interacts with the Z-ring during division (121, 157, 159, 160), interaction between FtsK and proteins involved in peptidoglycan synthesis (e.g., FtsI) has not been unequivocally verified (121, 157, 160).

In the following study, the periplasmic loops of FtsKN were probed by *in vivo* site-specific incorporation of an unnatural photoactivatable cross-linking residue to identify novel protein
interaction partners of FtsK that may contribute to its checkpoint function. We identified 63 unique proteins as potential FtsK_N interaction partners, including cell division proteins FtsN and DamX, and the elongasome peptidoglycan synthase PBP1a. Interestingly, an outer membrane lipoprotein of unknown function in *E. coli*, rare lipoprotein A (RlpA), was identified in all cross-linked samples within the functional periplasmic loop of FtsK_N (i.e., residues D135, D136 and Y139). RlpA is one of four SPOR-domain containing proteins in *E. coli* (including FtsN, DedA and DamX) that bind peptidoglycan and are targeted to the septum during division (95, 97, 194). Recently, RlpA was shown to function as a lytic transglycosylase in *P. aeruginosa* (195). Deletion of *rlpA* in *P. aeruginosa* caused slow growth and chaining of cells when grown in a low osmotic strength medium, suggesting a role for RlpA in cell-cell separation and rod shape maintenance (195). Despite considerable effort by several groups to determine the function of RlpA in *E. coli*, null mutants of *rlpA* in this species have yielded no morphological defects, nor has purified *E. coli* RlpA shown any enzymatic activity towards peptidoglycan (95, 97, 195). Here, we show that *E. coli* RlpA directly interacts with divisome protein FtsK_N in vitro, and that deletion of *rlpA* partially bypasses the requirement for functional FtsK, as seen by growth and morphological analysis of an *rlpA* knockout strain.

### 3.3 Experimental Procedures

3.3.1 *Bacterial Strains, Plasmids, and Growth Conditions*

Bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* W3110, DH5α and Lemo21 cultures were grown at 37°C in lysogeny broth (LB) (BD Biosciences) in a rotary shaker at 200 rpm. Media were supplemented with 150 µg/mL ampicillin, 30 µg/mL
## Table 3.1. Bacterial strains and plasmids

The abbreviations used are as follows: Amp, ampicillin; Cam, chloramphenicol; Kan, kanamycin.

<table>
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<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<td><strong>E. coli strain</strong></td>
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<td>W3110</td>
<td>rph-1IN (rrnD-rrnE)</td>
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<td>Lemo21 (DE3)</td>
<td>fhuA2 [lon] ompT gal (iDE3) [dem] ΔhsdS' pLemo(Cam&lt;sup&gt;R&lt;/sup&gt;)</td>
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<td>λ DE3 = λ sBamHlo ΔEcoRI-Blac(int::PlacUV5::T7gene1) i21 Δnin5 pLemo = pACYC184-PrhaBAD-lysY</td>
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<td>DH5α</td>
<td>F-Φ80lacZAM15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ-thi-1 gyrA96 relA1</td>
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<td>DP11-1</td>
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<td>Expression vector encoding tRNA/aminocacyl-tRNA synthetase pair derived from Methanococcus jannaschii for in vivo incorporation of the photo-crosslinker p-benzoyl-L-phenylalanine (pBpa) into proteins in E. coli; Cam&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pKD4</td>
<td>Template plasmid for λ red mediated gene deletion; Kan&lt;sup&gt;R&lt;/sup&gt;; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pKD46</td>
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<td>Addgene</td>
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<td>pWQ743</td>
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<td>(198)</td>
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<td>pET28a</td>
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<td>pWQ572</td>
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<td>pBAD24 derivative encoding amino acids 1-220 of FtsK (His&lt;sub&gt;10&lt;/sub&gt;-FtsK&lt;sub&gt;220&lt;/sub&gt;) from E. coli</td>
<td>(163)</td>
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<td>pAB008-1</td>
<td>pWQ743 derivative encoding amino acids 1-220 of FtsK (His&lt;sub&gt;10&lt;/sub&gt;-FtsK&lt;sub&gt;220&lt;/sub&gt;) from E. coli</td>
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<td>pSG001-1</td>
<td>pET28a derivative encoding amino acids 18-362 of RlpA with an N-terminal FLAG-tag (FLAG-RlpA) from E. coli</td>
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<td>pSG002-1</td>
<td>pWQ572 derivative encoding amino acids 18-362 of RlpA with an N-terminal FLAG-tag (FLAG-RlpA) from E. coli</td>
<td>This study</td>
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</table>

Chloramphenicol or 50 µg/mL kanamycin where appropriate for plasmid-carrying strains. *E. coli* LP11-1 cultures were grown in Complementation Media (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl [Fisher]) supplemented with 15 µg/mL tetracycline, as well as appropriate antibiotics for plasmid selection where applicable. Cultures were grown at 30°C or 42°C, as described in Section 2.3.4 (163). Maintenance of the kanamycin resistance cassette in the ΔrlpA knockout strains was achieved by the addition of 25 µg/mL kanamycin to all overnight cultures. To assess the impact of deletion of *rlpA* on cell growth and morphology, overnight
cultures of LP11-1 ΔrlpA and W3110 ΔrlpA were each diluted to an \( A_{600} \) of 0.1 (SmartSpec™ Plus Spectrophotometer; Bio-Rad) in two separate 25-mL aliquots of fresh Complementation Media and grown at 30°C or 42°C for 8 h \( (n = 3 \) per strain per temperature). The \( A_{600} \) of two technical replicates of each culture were sampled every hour and cells were imaged by phase contrast microscopy (Leica DM2000 LED, ProgRes CT3 camera; Jenoptik AG). Cell length was assessed for 150 random cells from each culture using the ImageJ program (version 1.46r, National Institutes of Health) and is reported as mean length ± S.E. Statistical analysis for cell growth \( (A_{600}) \) and mean length were completed using one-way analysis of variance with Tukey-Kramer multiple comparison post-tests by Prism 5 software (GraphPad Software, Inc.) using a level of significance of \( \alpha = 0.05 \) for all tests. \textit{E. coli} K12 W3110 and LP11-1 were also grown at 30°C and 42°C as described above as wild-type (WT) controls. Deletion of \textit{rlpA} was complemented in \textit{trans} by expression of \textit{FLAG-rlpA} from plasmid pSG002-1 in strain LP11-1 ΔrlpA using 80 ng/mL anhydrotetracycline (ATc).

3.3.2 Plasmid Construction

An oligonucleotide encoding the N-terminal 220 amino acids of FtsK (FtsK\textsubscript{N(220)}) with an N-terminal decahistidine tag was cloned into the ATc-inducible expression vector pWQ743 to produce pAB008-1, as described in Section 2.3.2 (163). For over-expression and purification of RlpA, an oligonucleotide encoding a soluble derivative of RlpA containing an N-terminal FLAG\textsuperscript{®}-tag in place of its type II signal sequence (FLAG-RlpA) was cloned into expression vector pET28a following the protocol described in Section 2.3.2 (163), using custom primers AMB006Fa (5’-TGGACCATGGATTATAAAGATGATGATGATAAATCCAGTACAAGCGATGATGGTCAGC-3’) and AMB006R (5’-AAGGTCAAGCTTACTTTACTGCGCGGTAGTAA-TAAATGACTGTAAATTGGGCTTC-3’) to produce pSG001-1. Similarly, for complementation
of the ΔrlpA knockout strains, the oligonucleotide encoding FLAG-RlpA was also cloned into the ATc-inducible expression vector pWQ572 using the above primers, producing pSG002-1. All constructs were verified by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph).

### 3.3.3 Site-directed Mutagenesis

To capture FtsK<sub>N</sub> protein interaction partners, five site-specific amber stop codon variants of FtsK<sub>N</sub> (FtsK<sub>N</sub>*) were generated by site-directed mutagenesis. A single amber codon (TAG) was introduced into the sequence of ftsK<sub>N</sub> corresponding to the periplasmic amino acid positions Trp-51, Asp-135, Asp-136, Tyr-139 or Leu-158 using a QuikChange Lightning site-directed mutagenesis kit (Stratagene). All primers used for mutagenesis and resulting plasmids are shown in Table 3.2. All single amber codon variants were confirmed by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph).

<table>
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<tr>
<th>Mutation</th>
<th>Sequence of mutagenic oligonucleotide (5′ to 3′)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>W51*</td>
<td>CGGACCCCAGCTAGTCGCAAACGGC</td>
<td>pAB008-W51*</td>
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<td>GCCGTTTGCAGCTAGGCTGGGGTCCG</td>
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<tr>
<td>D135*</td>
<td>CTGGCGGCAATCAACGCTTAGGATATCTGGTATTTTCGG</td>
<td>pAB008-D135*</td>
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<tr>
<td></td>
<td>GGCAAAATACCACTAGATTTTCGCTGAGGTCGAGGGCAG</td>
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<tr>
<td>D136*</td>
<td>GCGGCCAATCAACGCTGAGATCTGGTATTTTCGCCCTCC</td>
<td>pAB008-D136*</td>
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<td>GGAGGCCAAAATACCACTAGAGTCCAGCGTGTAGTGGGCAGCCGC</td>
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<tr>
<td>Y139*</td>
<td>CTGACGATATCTGGTAGTTGCTCCGGTGGGCG</td>
<td>pAB008-Y139*</td>
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<tr>
<td></td>
<td>CGCCACCGGAGCAGCAACTCCCTAGCTGTATCGTACAG</td>
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<tr>
<td>L158*</td>
<td>CACTACGCTACAACCTAGGTCGACAGTAGCGGGGGACTA</td>
<td>pAB008-L158*</td>
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<tr>
<td></td>
<td>TAGTTCCCGCCTACTGTGCTACAGTGTTGTAGCGTAGTG</td>
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<sup>a</sup> Base changes are underlined and in boldface.
3.3.4 In vivo UV Cross-linking

For in vivo cross-linking of FtsK_N interacting proteins, the photoactivatable amino acid \( p \)-benzoyl-L-phenylalanine (\( p \)Bpa) was incorporated into FtsK_N by a mutant tRNA/tRNA synthetase pair from \textit{Methanococcus jannaschii} that had been engineered to recognize the amber stop codon (TAG)(196). Cross-linking of whole bacterial cells expressing site-specific amber stop codon variants of FtsK_N (FtsK_N*) in the presence of \( p \)Bpa and the mutant tRNA/tRNA synthetase (encoded on plasmid pEVOL-pBpF) was performed as described by Nickerson \textit{et al.} (198), with minor modifications. Briefly, overnight cultures of \textit{E. coli} LP11-1 strains carrying FtsK_N* variants were diluted to an \( A_{600} \) of 0.1 (SmartSpec™ Plus Spectrophotometer; Bio-Rad) in 400 mL Complementation Media supplemented with ampicillin and chloramphenicol (75 \( \mu \)g/mL and 15 \( \mu \)g/mL, respectively), 1 mM \( p \)Bpa (Bachem), as well as 0.02% (w/v) \( L \)-arabinose to induce synthesis of the tRNA/tRNA synthetase pair from pEVOL-pBpF. FtsK_N* variants were induced with 40 ng/mL ATc. Cultures were incubated under reduced lighting at 42°C for 2 h in a rotary shaker at 200 rpm. Identical cultures grown in the absence of 1 mM \( p \)Bpa were prepared to assess the efficiency of \( p \)Bpa incorporation into full-length FtsK_N. One-millilitre aliquots of cultures grown in the presence and absence of \( p \)Bpa were prepared to assess the efficiency of \( p \)Bpa incorporation into full-length FtsK_N. One-millilitre aliquots of cultures grown in the presence and absence of \( p \)Bpa were concentrated 1:100, mixed with 20 \( \mu \)L of 5× Loading Buffer (250 mM Tris-HCl, pH 6.8, 10% [w/v] SDS, 30% [v/v] glycerol, 5% [v/v] \( \beta \)-mercaptoethanol, 0.1% [w/v] bromophenol blue), and boiled for 10 min in a covered beaker. Samples were analyzed by SDS-PAGE and Western blotting as described in Section 3.3.9.

To induce in vivo cross-linking of FtsK_N* variants, cultures grown in the presence of \( p \)Bpa were harvested by centrifugation and washed twice in 10 mL sterile PBS. The resulting cell pellets were suspended into 10 mL phosphate-buffered saline (PBS) for every \( A_{600} \) 0.1 of the original culture (e.g., a culture with an \( A_{600} \) of 0.5 was suspended in 50 mL PBS) before splitting.
into two equal aliquots. Aliquot #1 was placed into a 6-well tissue culture plate on ice (5 mL suspension per well) and irradiated at 365nm with a handheld UV lamp (Spectroline Model EN-180L; 2.5 cm from surface of the cell suspension) for 15 min. Aliquot #2 was covered in tinfoil and incubated on ice for 15 min as a non-crosslinked control. Both aliquots were harvested by centrifugation and the pellets were stored at –20°C. All samples were protected from additional light exposure, where possible, during storage and purification of FtsK<sub>N</sub>. All samples were prepared in duplicate from biological replicate cultures. An additional culture expressing WT FtsK<sub>N(220)</sub> was harvested and processed in parallel with cross-linked and non-crosslinked control cells below to account for contaminant proteins inherent to the purification of FtsK<sub>N</sub>.

Cross-linked (aliquot #1) and non-crosslinked control cells (aliquot #2) were thawed in 3 mL SDS Lysis Buffer (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1% [w/v] SDS) and sonicated on ice for 1 min (10 s on, 10 s off; Misonix Ultrasonic Processor XL2020, Misonix, Inc.) to complete cell lysis. Samples were then diluted with 27 mL Purification Buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 25 mM imidazole) to reach a final SDS concentration of 0.1% (w/v). Profinity Ni<sup>2+</sup>-charged immobilized metal affinity chromatography (IMAC) resin (Bio-Rad) was added to each sample at a bed volume of 100 µL and then incubated at 4°C for 1.5 h with rocking. Cell lysate/resin mixtures were collected by centrifugation at 4,500 × g for 5 min at 4°C and washed five times with 1 mL of Purification Buffer containing 0.1% (w/v) SDS before performing on-bead digestion for protein analysis.

3.3.5 On-bead Digestion and Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS)

WT FtsK<sub>N</sub> and FtsK<sub>N</sub>* from cross-linked and non-crosslinked controls were subjected to on-bead proteolytic digestion with chymotrypsin as described by Park <i>et al.</i> (199). Briefly, resin mixtures containing FtsK<sub>N</sub> complexes were washed five times in 1 mL freshly prepared 50 mM
ammonium bicarbonate (ABC) buffer (pH 8.0). Beads were then suspended in Denaturation Buffer (6 M urea/2 M thiourea in 10 mM HEPES, pH 8.0) and proteins were subsequently reduced by incubation at 50°C for 30 min in Reduction Buffer (10 mM dithiothreitol in ABC buffer). Samples were then incubated in Alkylation Buffer (55 mM iodoacetamide in ABC buffer) for 30 min at room temperature in the dark. Reduced and alkylated proteins were diluted with ABC buffer and digested with 1 µg chymotrypsin (Princeton Separations) in the presence of 0.01% (w/v) ProteaseMAX surfactant (Promega) at 30°C overnight. The IMAC resin was removed by centrifugation and digested peptides in the supernatant were lyophilized using a speed-vacuum concentrator in order to accommodate ambient-temperature shipment. Samples were reconstituted in 1% trifluoroacetic acid in water and passed through a C18 stage tip to remove any trace detergent and salt from the purification process. Purified samples were lyophilized and resuspended in 0.1% formic acid prior to analysis by liquid chromatography tandem-mass spectrometry (LC-MS/MS) to identify cross-linked proteins.

Samples were analyzed on an Orbitrap analyzer (Q-Exactive, ThermoFisher) outfitted with a nanospray source and EASY-nLC nano-LC system (ThermoFisher). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm × 50 cm PepMax RSLC EASY-Spray column filled with 2 µm C18 beads (ThermoFisher) at a pressure of 800 Bar heated to 60°C. Peptides were eluted over 90 min at a rate of 250 nL/min using a 0 to 40% Solution A to Solution B gradient (Solution A: 0.1% formic acid; Solution B: 80% acetonitrile, 0.1% formic acid).

Peptides were introduced by nano-electrospray into the Q-Exactive mass spectrometer (ThermoFisher). The instrument method consisted of one MS full scan (400-1500 m/z) in the Orbitrap mass analyzer with an automatic gain control (AGC) target of 1×10⁶, maximum ion
injection time of 120 ms and a resolution of 70,000, followed by 10 data-dependent MS/MS scans with a resolution of 17,500, an AGC target of $1 \times 10^6$, maximum ion time of 120 ms, and one microscan. The intensity threshold to trigger an MS/MS scan was set to $6.7 \times 10^4$. Fragmentation occurred in the higher-energy collisional dissociation (HCD) trap with normalized collision energy set to 27. The dynamic exclusion was applied using a setting of 10 seconds.

Raw data files were loaded into PEAKS 7 software (Bioinformatics Solutions, Inc.) for peptide and protein analysis using the UniProtKB *E. coli* K12 database. Proteins identified with protein and peptide thresholds of $>95\%$ probability and a minimum of 2 unique peptides in both biological replicates were accepted as positive identifications. Proteins detected in the WT preparation of FtsK$_N$ and non-crosslinked negative controls were used as an exclusion list for proteins found in the cross-linked samples. Proteins conclusively reported to have a cytoplasmic cellular localization were also excluded. Protein-protein interaction networks of all cross-linked proteins were built using STRING version 10.0 (200) using all available prediction methods and a minimum confidence level of 0.4.

### 3.3.6 Expression and Purification of His$_{10}$-FtsK$_N$(220)

Overnight cultures of *E. coli* Lemo21 cells carrying WT His$_{10}$-FtsK$_N$(220) encoded by a pBAD24 derivative (plasmid pAB006-2) were diluted 1:50 into 250 mL LB media and grown at 37°C for 1.5 h. L-arabinose was added to a final concentration of 0.2% (w/v) and incubated for another hour. Following induction, the entire culture was harvested by centrifugation ($8,000 \times g$, 10 min, 4°C) and cells were resuspended in 5 mL of Lysis Buffer (20 mM Tris-HCl pH 7.0, 100 mM NaCl, 4 mM EDTA tetrasodium dihydrate, 40 µg/mL DNaseI, 40 µg/mL RNase A, and 300 µg/mL lysozyme). Samples were incubated at room temperature for 10 min with rocking, then sonicated for 1 min (10 s on, 10 s off) to complete cell lysis. The total membrane fraction was
collected by ultracentrifugation at 120,000 × g for 1 h at 4°C (Beckman L8-55 M ultracentrifuge, Ti70 rotor). Membranes were suspended in 3 mL 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 100 µL of Profinity Ni²⁺-charged IMAC resin (Bio-Rad) and were incubated at 4°C for 1.5 h with rocking. Solubilized membrane/resin mixtures were collected by centrifugation at 14,000 × g for 1 min at 4°C in microcentrifuge tubes and washed ten times with 1 mL of Purification Buffer containing 0.05% LDAO. Purified, resin-bound FtsK_N was then used during in vitro characterization of protein interaction between FtsK and RlpA by pull-down assay (Section 3.3.8).

3.3.7 Expression and Purification of FLAG-RlpA

Overnight cultures of E. coli Lemo21 cells carrying soluble FLAG-RlpA encoded by a pET28a derivative (plasmid pSG001-1) were diluted 1:100 into 1 L LB media and grown at 37°C for 1 h before transferring to 30°C for an additional hour of growth. Isopropyl-β-D-thiogalactoside (IPTG) was added to 1 mM and the culture was incubated for an additional 3 h at 30°C. Following induction, the entire culture was harvested by centrifugation (8,000 × g, 10 min, 4°C) and resuspended in 25 mL of Lysis Buffer before lysing by three passes through a French pressure cell (operating at 1000 psi). 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 remove the membrane fraction. The resulting supernatant containing soluble FLAG-RlpA was added to 300 µL of anti-FLAG® M2 affinity gel (Sigma-Aldrich) and incubated overnight at 4°C with rocking. FLAG-RlpA/resin mixtures were collected by centrifugation at 100 × g for 2 min at 4°C and
washed five times with 1 mL of Wash Buffer (20 mM Tris-HCl pH 7.0, 100 mM NaCl). FLAG-RlpA was successively eluted in four washes of 250 µL Wash Buffer containing 100 µg/mL FLAG® peptide (Sigma-Aldrich). Eluted FLAG-RlpA was pooled and the total protein concentration was determined by a bicinchoninic acid protein assay as per manufacturer’s instructions (Thermo Scientific) using bovine serum albumin (BSA) as a standard.

3.3.8 Pull-Down Assays

To assess the interaction between FtsK and RlpA, 320 µg of purified FLAG-RlpA was incubated with 100 µL of IMAC resin-bound His10-FtsK_N at 4°C overnight with rocking. The beads were collected by centrifugation and washed three times in 1 mL of Wash Buffer containing 25 mM imidazole. Bound proteins were eluted in 240 µL of Wash Buffer containing 1 M imidazole, mixed with 60 µL of 5× Loading Buffer (250 mM Tris-HCl, pH 6.8, 10% [w/v] SDS, 30% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.1% [w/v] bromophenol blue) and boiled for 10 min. The presence of both proteins was verified by loading 15-µL aliquots onto duplicate 13% SDS-polyacrylamide gels (29:1 acrylamide:bisacrylamide) followed by Western blotting (Section 3.3.9). Unbound FLAG-RlpA in the flow through was diluted 1:20 before loading. Purified FLAG-RlpA was also incubated with 100 µL of unbound IMAC resin and washed as above to verify the absence of non-specific binding of FLAG-RlpA to the resin itself. Pull-down assays were also performed with IMAC resin-bound His10-FtsK_N and 250 µg of purified FtsZ (purified as in Ref. 201) as a positive interaction control. All experiments were conducted in duplicate.
3.3.9 Western Blotting

Expression of each FtsK_N* construct was verified by preparing whole cell lysates for SDS-PAGE and Western blotting. FtsK_N* variants expressed in the presence and absence of pBpa were diluted to an $A_{600}$ of 0.25, equal volumes separated using a 13% SDS-polyacrylamide gel (29:1 acrylamide: bisacrylamide) and transferred onto a nitrocellulose membrane (Bio-Rad) 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$_6$ (Clontech) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) (Bio-Rad), respectively. Chemiluminescence was detected using 1 mL of HRP Detection Buffer (100 mM Tris-HCl pH 8.8, 1.25 mM luminol, 2 mM 4-iodophenylboronic acid, 5.3 mM hydrogen peroxide) with long exposure (5 min) in a Bio-Rad Gel Doc™ XR imaging system.

To verify the presence of FtsK_N, RlpA and FtsZ in the pull-down assays, samples were separated using 13% SDS-polyacrylamide gels (29:1 acrylamide: bisacrylamide) and transferred onto nitrocellulose membranes that were then blocked in 5% (w/v) skim milk in Tris-buffered saline (TBS) at 4°C overnight. Mouse anti-His$_6$ (Clontech) and rabbit anti-FtsZ (Cedarlane) primary antibodies were used for the detection of FtsK_N and FtsZ, respectively, in conjunction with the secondary antibodies alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Sigma) and alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) (Sigma). FtsK_N and FtsZ blots were developed using a solution of 3.3 mg of nitro blue tetrazolium and 1.7 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$_2$). RlpA was probed directly using an HRP-
conjugated mouse anti-FLAG® (Sigma-Aldrich) antibody and chemiluminescence was detected using 1 mL of HRP Detection Buffer in a Bio-Rad Gel Doc™ XR imaging system.

3.3.10 Lambda Red Deletion of rlpA

Deletion of rlpA from E. coli LP11-1 and W3110 was achieved using the Lambda red recombinase method described by Datsenko and Wanner (197). Briefly, a PCR fragment containing a kanamycin resistance cassette flanked by 50 bp homology extensions of the upstream and downstream regions of rlpA was generated from template pKD4 using primers rlpA_P1 (5′-AATCCACACCCACAGGAAAATGTTGTCGAAAAAGCGTGAAGAGGTGC-G-CAGTGTAGGCTGGAGCTGCTTC-3′) and rlpA_P2 (5′-TTTGTTAACGTCATTTACAGAAA-TTGACACATCAGCTCGCTTTAGATGAATATCCTCCCTTAG-3′). An overnight culture of E. coli LP11-1 or W3110 carrying the Lambda red helper plasmid pKD46 was diluted 1:100 into 20 mL Complementation Media supplemented with 150 µg/mL ampicillin and 10mM L-arabinose, and grown for 2h at 30°C. Cells were made electrocompetent by washing three times in ice-cold filtered Milli-Q H2O and suspended in 200 µL ice-cold filtered Milli-Q H2O (100-fold concentration). Fifty-microlitres of competent cells and 100 ng of PCR product were added to a 1 mm gap electroporation cuvette and incubated on ice for 5 min. Electroporation was done using a Gene Pulser Xcell™ electroporation system (Bio-Rad) using the following parameters: 1800 V, 25 μF, 200 Ω. Shocked cells were recovered for 1 h at 30°C in 1 mL Super Optimal Broth with catabolite repression (SOC) medium (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose [Fisher]), then allowed to stand overnight at room temperature before spreading onto LB agar containing 25 µg/mL kanamycin to select for kanamycin resistant transformants. Deletion of rlpA and insertion of the kanamycin resistance cassette in each strain was verified by colony PCR using primers homologous to a region 500 bp
upstream of rlpA (ΔrlpAFor; 5′-CCAGCGCGTAATGATGCTCCTGGAC-3′) and within the kanamycin resistance cassette (kl; 5′-CAGTCATAGCCGAATAGCCT-3′)(197), as well as primers homologous to regions 50 bp upstream (ΔrlpAUp; 5′-CAATCCACACCCACAG-GAAAATGTTCGTC-3′) and 50 bp downstream of rlpA (ΔrlpADown; 5′-ACTTTGTTA-ACGTCATTTACAGAAATTTGACACA-3′). PCR verification of rlpA deletion in each strain was completed in duplicate along with control colonies of WT E. coli W3110 and LP11-1.

3.4 Results

3.4.1 In Vivo UV Cross-Linking Approach

To identify FtsK_N periplasmic interaction partners, a site-specific in vivo UV crosslinking approach was used (Figure 3.1). This technique has been successfully used in E. coli to probe various protein interaction surfaces, including mapping of the SecA dimer interface and its interaction with the Sec translocon (203, 204), transmembrane translocation by the Tat-pathway (205), capsular polysaccharide export by Wza (198) and formation of the essential divisome subcomplex FtsQ/B/L (39). An exogenous photoactivatable amino acid, p-benzoyl-L-phenylalanine (pBpa), is incorporated into the amino acid sequence of FtsK_N by a mutant aminoacyl-tRNA synthetase/tRNA pair derived from Methanococcus jannaschii (196, 206). This suppresses an amber codon (TAG) engineered within the sequence of ftsK_N by incorporating the pBpa residue, and generates a photo-modified protein variant (FtsK_N*) (Figure 3.1A). When cells expressing the photo-modified variants are exposed to long wavelength UV light, a site-specific covalent link between FtsK_N* and any interacting protein within 3.1Å is formed (Figure 3.1, B and C)(207). Given that residues in the second periplasmic loop of FtsK_N were previously found to be
Figure 3.1. In vivo UV cross-linking with \( p \)-benzoyl-L-phenylalanine (\( p \)Bpa). A, for in vivo cross-linking of FtsK\( _N \) interacting proteins, the photo-activatable amino acid \( p \)-benzoyl-L-phenylalanine (\( p \)Bpa; top panel) is incorporated into FtsK\( _N \) by a mutant tRNA/tRNA synthetase pair engineered to recognize the amber stop codon, TAG (bottom panel). Site-directed mutagenesis is used to introduce single amber codons at specific sites within \( ftsK_N \). When each \( ftsK_N^* \) amber mutant is expressed in the presence of \( p \)Bpa, \( p \)Bpa is incorporated by the mutant tRNA to produce full-length, photo-modified FtsKN\( ^* \). Asterisk (*) in the \( p \)Bpa structure indicates the carbonyl group that forms a triplet state diradical upon UV excitation. B, to induce in vivo cross-linking, cultures expressing photo-modified FtsKN\( ^* \) variants are exposed to UV light. Upon exposure, the \( p \)Bpa residue forms a covalent bond with any interacting residue within 3.1Å. C, example of the cross-linking reaction initiated upon exposure to UV light between \( p \)Bpa and the amino acid glutamate (Glu). Panels A and B are adapted from Ref. 208. Panel C is redrawn from Ref. 209.
critical for function (163), two functionally important residues (D135 and D136) were chosen for substitution with \( p\text{Bpa} \). Further, three additional residues (W51, Y139 and L158) were also chosen for substitution. Each position was selected to spread coverage over the entire FtsK\(_N\) periplasmic surface and verify the presence of additional protein binding sites at locations distinct from the functional region identified previously (163). Large/aromatic amino acids were preferentially chosen for replacement to minimize disruption of protein contacts by incorporation of the bulky \( p\text{Bpa} \) residue.

The goal of our cross-linking strategy was to capture endogenous interacting proteins. It was therefore critical to achieve near physiological expression of each FtsK\(_N^*\) variant. Using a highly tunable ATc-inducible expression system (pWQ572; construction of this expression plasmid is described in the Supporting Information Materials and Methods of Ref. 198), expression of each FtsK\(_N^*\) variant in the presence and absence of \( p\text{Bpa} \) was assessed by SDS-PAGE and Western blotting. This also verified production of full-length FtsK\(_N\). Full-length FtsK\(_N\) could be detected in all samples only when \( p\text{Bpa} \) was added to the culture medium (Figure 3.2). In the absence of \( p\text{Bpa} \), truncated versions of FtsK\(_N\) could be detected (with the exception of W51*), highlighting the specificity of the mutant tRNA/tRNA synthetase pair and proper incorporation of the amber stop codon into the sequence of \( ftsK_N \). All FtsK\(_N\) constructs were only detectable in long exposures of the Western blot (5 min), indicating very low levels of expression were achieved.

Initially, we attempted to identify FtsK\(_N\) periplasmic interaction partners by affinity-purifying FtsK\(_N\) cross-linked products, separating the adducts by SDS-PAGE and identifying the resulting protein complex by mass spectrometry following in-gel digestion of the appropriate protein (as verified by Western blotting). However, given the low-level expression of the FtsK\(_N^*\)
Figure 3.2. Growth of FtsK\textsubscript{N} amber mutants (FtsK\textsubscript{N*}) in the presence of pBpa restores expression of full-length FtsK\textsubscript{N}. Western blot analysis of FtsK\textsubscript{N} amber mutant expression. Temperature-sensitive \textit{E. coli} strain LP11-1 (ftsK44) carrying WT FtsK\textsubscript{N} or each amber mutant was cultured at 42°C for 2h with (+) or without (−) 1 mM pBpa. Concentrated whole cell lysates were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and subsequently probed with mouse anti-His\textsubscript{6} antibodies, followed by horseradish peroxidase-conjugated goat anti-mouse IgG. tRNA and pWQ743 represent control cells harbouring the mutant tRNA/tRNA synthetase and empty vector, respectively. Asterisks (*) indicated the presence of truncated FtsK\textsubscript{N} in cultures without pBpa.

variants and limited abundance (50 to 900 copies per cell) of most endogenous essential cell division proteins (with the exception of FtsZ and ZipA)(9, 158, 210), repeated attempts using this process resulted in unreliable identification of adducts containing FtsK\textsubscript{N}. Therefore, we decided to use a broader approach consisting of on-bead digestion of purified FtsK\textsubscript{N} cross-linked products followed by liquid chromatography tandem-mass spectrometry (LC-MS/MS) identification of the total FtsK\textsubscript{N} periplasmic interactome (Figure 3.3). This unbiased approach allowed us to identify a diverse interaction network of potential FtsK\textsubscript{N} interaction partners, as described below.

3.4.2 \textit{In Vivo} UV Cross-Linking Reveals Network of FtsK\textsubscript{N} Periplasmic Interaction Partners

Cells expressing each of the five FtsK\textsubscript{N*} variants were irradiated with long wavelength UV light to capture endogenous protein interaction partners \textit{in vivo}. The irradiated cells were
Figure 3.3. In vivo UV cross-linking experimental workflow. Following in vivo cross-linking of FtsK\textsubscript{N}* variants (as described in Figure 3.1), FtsK\textsubscript{N}* cross-linked complexes were purified by immobilized metal affinity chromatography (IMAC) and subjected to in-solution protein digestion. Digested peptides were then identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to generate a total FtsK\textsubscript{N} periplasmic interactome.

lysed and the protein complexes were purified from the membrane fraction under stringent conditions by immobilized metal affinity chromatography (IMAC), as described in Section 3.3.4. FtsK\textsubscript{N} and corresponding periplasmic interaction partners in each sample were then identified by LC-MS/MS. In total, 63 different proteins were identified, distributed across all five cross-linked samples (Figure 3.4, Appendix Table A1.1). All proteins were detected in UV-treated samples only and were absent from untreated and WT controls. Of the proteins identified, 92% were found in at least one cross-linked sample at or near the functional loop of FtsK\textsubscript{N} (residues D135, D136 or Y139), suggesting this region may be critical for the maintenance of multiple protein contacts during growth and division.

Our network of potential FtsK\textsubscript{N} interaction partners includes multiple protein groups of highly related function, including cell division proteins FtsN and DamX, and elongasome protein PBP1a (Figure 3.5). Interestingly, a large proportion of the proteins detected are involved in cell wall organization and modification. As shown previously, disruption of the functional
Figure 3.4. Distribution of protein groups detected by mass spectrometry of potential FtsK\textsubscript{N} interaction partners. FtsK\textsubscript{N}* variants W51*, D135*, D136*, Y139* and L158* were purified from cultures treated with (+) and without (–) UV light, and digested with chymotrypsin. The resulting peptides were analysed by LC-MS/MS on a Q-Exact Orbitrap mass spectrometer to detect cross-linked proteins. Numbers indicate proteins exclusively detected in UV-treated samples (absent in untreated and WT controls), and identified with a minimum of 2 unique peptides at >95% probability in all biological replicates processed.
Figure 3.5. STRING protein interaction network of potential FtsK_N interaction partners identified by mass spectrometry. Proteins shown were identified exclusively in UV treated samples of FtsK_N* variants W51*, D135*, D136*, Y139* and L158* (absent in untreated and WT controls). Interactions were determined using STRING 10.0 (200). Lines indicate known or predicted protein-protein interactions, with thicker lines indicating higher levels of confidence (minimum interaction score, 0.4). Models of known protein structures are shown inside the corresponding protein spheres. Blue text highlights proteins involved in elongation and cell division, red text indicates proteins involved in general peptidoglycan modification. FtsK is circled and highlighted in yellow.
periplasmic loop of FtsK_{N} leads to uncoupling of septation events, and improper invagination of the peptidoglycan and outer membrane layers of the cell envelope (163). This suggests that the function of FtsK in division might be to couple cell envelope septation events and transition the cell from elongation to septation. Therefore, to carry out its role as this essential checkpoint, it stands to reason that the periplasmic loops of FtsK_{N} would be in direct contact with the peptidoglycan synthesis machinery.

The use of mass spectrometry to identify purified protein complexes has notable limitations with respect to the specificity and sensitivity of the data provided. While affinity chromatography affords us the ability to purify protein complexes that contain FtsK_{N}, it cannot guarantee complete removal of other proteins or native complexes in tight association with the FtsK_{N} cross-linked adduct. In conjunction with the attomolar sensitivity common with mass spectrometry analysis (211), identification of even the smallest amount of contaminating proteins is unavoidable. Taken together with such a diverse network of proteins isolated, it is unlikely that all 63 proteins identified truly interact with FtsK \textit{in vivo}. As such, further biochemical and \textit{in vivo} verification of the potential FtsK_{N} interaction partners is required.

To begin validation, we narrowed our list of candidate proteins by several criteria (Appendix Table A1.1). First, proteins were ranked based on the average spectrum count and total number of unique peptides detected across all samples processed by LC-MS/MS to give a basic measure of protein abundance. While the LC-MS/MS method used was not quantitative with respect to absolute protein abundance, spectral counting, which counts and compares the number of fragment spectra identifying peptides of a given protein, can be used as a semi-quantitative, label free method for estimating protein abundance (212). Ranking the protein list by the total number of unique peptides identified (i.e., the number of different amino acid sequences that are attributed to a single protein) also allowed us to roughly filter the putative
FtsK\textsubscript{N} interaction partners by the confidence of identification. By this parameter, a greater number of unique peptides would denote increased confidence that the protein has been correctly identified in the sample. Second, proteins were also classified based on the total number of samples in which they were detected. Finally, to identify partners critical for the checkpoint function of FtsK\textsubscript{N}, we focused on proteins identified in all cross-linked samples at or near the functional periplasmic region of FtsK (residues D135, D136 and Y139). Following these criteria, the putative acyl-CoA thioester hydrolase YbhC and septal peptidoglycan binding protein RlpA were chosen for further analysis because they had the highest average spectrum counts and most unique peptides among proteins detected in 4 of the 5 cross-linked samples. Appendix A2 describes \textit{in vitro} and \textit{in vivo} analysis of the interaction between FtsK\textsubscript{N} and YbhC. Briefly, we verified that YbhC does not interact with FtsK\textsubscript{N} by an \textit{in vitro} pull-down assay (\textit{Appendix Figure A2.1}). In addition, generation of an \textit{E. coli} LP11-1 \textit{ybhC} deletion strain and subsequent growth analysis showed no significant change in cell growth or morphology compared to LP11-1 (\textit{Appendix Figure A2.2}). YbhC was therefore excluded as a putative FtsK\textsubscript{N} interactor. Evaluation of RlpA as a potential FtsK\textsubscript{N} interaction partner is described in detail below.

\textit{3.4.3 In Vitro Analysis of Novel Protein Interaction Between FtsK\textsubscript{N} and RlpA}

Confirmation of direct interaction between FtsK\textsubscript{N} and RlpA was achieved by an \textit{in vitro} pull-down assay using His\textsubscript{10}-FtsK\textsubscript{N(220)} and a FLAG\textsuperscript{R}-tagged soluble derivative of RlpA (FLAG-RlpA). Purified FLAG-RlpA was incubated with purified, IMAC resin-bound FtsK\textsubscript{N} and successively washed to remove unbound protein. Upon elution of His\textsubscript{10}-FtsK\textsubscript{N(220)}, RlpA and FtsK\textsubscript{N} were detected in the elution fraction by Western blot analysis (\textit{Figure 3.6A}). To verify accuracy of the pull-down assay, RlpA was also incubated with IMAC resin in the absence of
Figure 3.6. In vitro analysis of interaction between FtsK_N and RlpA. A, representative Western blots of pull-down assay between FLAG-RlpA (left panel) and His_{10}-FtsK_{N(220)} (right panel). B, Western blot of FLAG-RlpA incubated with empty IMAC resin (negative control). C, representative Western blot of pull-down assay between FtsZ (left panel) and His_{10}-FtsK_{N(220)} (right panel). *Bead* lane represents the elution fraction of FtsZ incubated with empty IMAC resin (negative control). For all blots, the primary antibodies are indicated in the bottom right corner. In all panels: ‘FT’ – flow through; ‘W1, W2, W3’ – wash fractions 1, 2, and 3; ‘Elution’ – elution with 1 M imidazole.
FtsK\textsubscript{N} as a negative control and was not detected in the elution fraction (Figure 3.6B). Thus, this assay indicated direct interaction between FtsK\textsubscript{N} and RlpA. Additionally, purified FtsZ was used as a positive control of a known FtsK\textsubscript{N} interaction partner. FtsZ was successfully detected in the elution fraction at an increased proportion when FtsK\textsubscript{N} was bound to the IMAC resin (Figure 3.6C, Elution), in comparison to the empty bead control (Figure 3.6C, Bead), further verifying that the assay used can accurately detect the presence of direct protein interaction with FtsK\textsubscript{N}, and supports identification of a novel protein interaction between FtsK\textsubscript{N} and RlpA \textit{in vitro}.

3.4.4 Deletion of \textit{rlpA} from LP11-1 (ftsK44) Partially Suppresses Cellular Filamentation

Although recent evidence has suggested that the septal peptidoglycan binding protein RlpA is a lytic transglycosylase involved in cell-cell separation in \textit{P. aeruginosa} (195), the exact function of RlpA in \textit{E. coli} remains elusive. While several groups have attempted to uncover the role of RlpA in \textit{E. coli} through the use of gene deletion mutants and enzymatic assays to probe the impact of purified RlpA on peptidoglycan degradation (95, 97, 195), no study to date has been able to visualize any division-related phenotype for ΔrlpA mutants nor show enzymatic activity of the protein. In \textit{E. coli}, \textit{rlpA} is immediately upstream of the gene encoding penicillin binding protein 5 (\textit{dacA})(213). Given deletion of \textit{dacA} has been previously shown to suppress the loss of functional FtsK in temperature-sensitive \textit{ftsK44} strains of \textit{E. coli} (112), we reasoned that a unique division-related phenotype might also be observed if \textit{rlpA} was deleted in conjunction with the loss of FtsK. Therefore, we deleted \textit{rlpA} in the temperature-sensitive \textit{E. coli} strain LP11-1 (\textit{ftsK44}) to generate LP11-1 ΔrlpA (Figure 3.7), and assessed the impact of this deletion on cell growth and morphology. We also created an \textit{rlpA} knockout in the \textit{E. coli} strain W3110 to compare the impact of \textit{rlpA} deletion in an otherwise WT background. In both cases, the gene
Figure 3.7. PCR verification of rlpA deletion. A, genomic arrangement, and B, colony PCR of the rlpA deletions to verify insertion of the kanamycin resistance cassette (KanR). Boundaries of the KanR insert are denoted by the yellow triangles and grey line. Red and blue arrows indicate binding positions of the primers used to verify insertion of KanR. ΔrlpAFOR binds 500 bp upstream of rlpA and K1 binds 499 bp downstream of the 5′ boundary of the KanR insert, resulting in a PCR amplicon of 999 bp. ΔrlpAUP and ΔrlpADOWN bind 50 bp upstream and downstream of rlpA, respectively, resulting in a PCR amplicon of 1189 bp in the parental strains (noted above each gel) and 1574 bp in the deletion strains. PCR verification of rlpA deletion was completed in duplicate along with colonies of WT E. coli W3110 and LP11-1 as negative controls.

deletion was created within the boundaries of the rlpA open reading frame to minimize disruption of the surrounding genes or potential promoter sequences and minimize polar effects.

To probe the impact of rlpA deletion in both the presence and absence of functional FtsK, we completed growth and morphology analysis of the LP11-1 ΔrlpA and W3110 ΔrlpA strains at
both permissive (30°C) and nonpermissive (42°C) temperatures, respectively (Figures 3.8 and 3.9). Although the WT *E. coli* strain W3110 grew more rapidly and to a higher absorbance than the temperature-sensitive strain LP11-1 (*p < 0.05*) at both temperatures, deletion of *rlpA* only had a significant impact on growth in the absence of functional FtsK (i.e., in the LP11-1 background during growth at 42°C) (Figure 3.8). In this case, *rlpA* deletion significantly alleviated the severe growth defect caused by the loss of FtsK, and partially suppressed the cellular filamentation typically associated with the temperature-sensitive *ftsK44* mutation (Figure 3.9). This partial restoration of growth and WT cell morphology could be negated by complementation of the LP11-1 Δ*rlpA* strain with a plasmid-encoded copy of *rlpA* (encoded on plasmid pSG002-1), suggesting that the combined loss of functional FtsK and RlpA was responsible for the observed phenotype.

![Growth curves of Δ*rlpA* knockout (W3110 Δ*rlpA* and LP11-1 Δ*rlpA*) and complemented (LP11-1 Δ*rlpA* pSG002-1) *E. coli* strains grown at permissive (30°C) and nonpermissive (42°C) temperatures.](image)

**Figure 3.8.** Deletion of *rlpA* partially alleviates growth inhibition of the temperature-sensitive *E. coli* strain LP11-1 (*ftsK44*). Growth curves of Δ*rlpA* knockout (W3110 Δ*rlpA* and LP11-1 Δ*rlpA*) and complemented (LP11-1 Δ*rlpA* pSG002-1) *E. coli* strains grown at permissive (30°C) and nonpermissive (42°C) temperatures. In both panels, *E. coli* strains W3110 and LP11-1 were analyzed as WT controls. Pairwise comparison of growth differences was made using an one-way analysis of variance with Tukey-Kramer multiple comparison post-tests (*, *p < 0.05* versus LP11-1 at the same time point).
Figure 3.9. Deletion of \textit{rIpa} restores normal cell morphology of the temperature-sensitive \textit{E. coli} strain LP11-1 (\textit{ftsK44}). \textit{A}, representative phase contrast micrographs, \textit{B}, mean cell length, and \textit{C}, cell length distribution of \textit{ΔrlpA} knockout (W3110 \textit{ΔrlpA} and LP11-1 \textit{ΔrlpA}) and
complemented (LP11-1 ΔarlA pSG002-1) *E. coli* strains grown at 42°C. In all panels, *E. coli* strains W3110 and LP11-1 were analyzed as WT controls. Micrographs depict typical cells of each strain after 0 or 8 h of growth at 42°C. The genotype of each strain is indicated in the upper left corner of each micrograph (bar, 10 µm). Cells were measured for cell length using the ImageJ program and are reported as mean cell length (µm) ± S.E. (*n* = 150 cells per strain per time point). Pairwise comparisons of cell lengths were made using a one-way analysis of variance with Tukey-Kramer multiple comparison post-tests (*p* < 0.001).

### 3.5 Discussion

Despite clear evidence that FtsK is essential during division, the limited amount of biochemical data currently available regarding the protein contacts it makes or its biological function gives only a crude picture of its role during division. With the cross-linking and deletion mutant data presented herein, we have uncovered an FtsK interactome that again points to a role for FtsK in cell envelope remodeling during division.

The use of site-specific cross-linking by the photoactivatable amino acid pBpa allows us to study the putative protein complexes FtsK forms in its natural environment, with minimal alterations to the full-length proteins involved. While this technique allowed us to identify a large number of potential FtsK interaction partners (Figure 3.4, Appendix Table A1.1), limitations regarding the specificity and high sensitivity of our LC-MS/MS approach makes further biochemical investigation of each protein as a direct FtsK interactor necessary. Regardless, the list of proteins identified in our cross-linking analysis provides further insight into the role of FtsK in cell division, as the majority of proteins detected could be grouped into several small functional groups, with a high degree of known interaction among them (Figure 3.5). As expected, we were able to detect several proteins involved in growth and division (e.g., divisome proteins FtsN and DamX, and elongasome protein PBP1a). However, none of the proteins were previously reported in the literature as FtsK interactors. Both FtsN and DamX are SPOR-domain containing proteins that bind peptidoglycan and are targeted to the septum during division (95, 97,
Specifically, FtsN is an essential component of the divisome that is responsible for activation of cell constriction during late stages of division (37, 41, 42). In contrast, while DamX is an early recruit to the septum and interacts with several divisome proteins, it is not essential for division and currently has no known function (95, 97, 214). During elongation, PBP1a acts as a bifunctional transglycosylase/transpeptidase that modifies and synthesizes the growing peptidoglycan layer (28). Given limited knowledge about the functions of both FtsK_N and DamX during division, the significance of an interaction between these two proteins is difficult to interpret. However, the interactions of FtsK with FtsN and PBP1a provide an intriguing link between the early and late stages of division, and the switch from cell elongation to septation in *E. coli*. The formation of a tripartite complex with FtsK_N, FtsN and PBP1a would physically link the elongasome with the divisome in the periplasm, and allow for the direct transmission of a cell signal between these two essential complexes. Completion of cell elongation by the elongasome would be sensed through the interaction between PBP1a and FtsK_N. In turn, this signal could be integrated with a signal for the completion of divisome formation and proper chromosome segregation sensed by FtsK, and then passed to FtsN through direct interaction of these two proteins to trigger constriction. While this report is the first to suggest direct interaction between FtsK and FtsN in *E. coli*, these proteins have been shown to interact in the Gram-negative bacterium *Neisseria gonorrhoeae* (215). Together, these interactions shed light onto a potential mechanism of action for FtsK_N in cell envelope remodeling during both growth and division.

Another interesting potential interaction partner identified in our cross-linking analysis is TolB. In *E. coli*, TolB is a member of the trans-envelope Tol-Pal complex, which localizes to the site of division and helps to coordinate invagination of the OM with both the peptidoglycan and IM cell envelope layers (216). Cells lacking an intact Tol-Pal system show delayed OM invagination during cell constriction (216), which poses an attractive link to the inhibition of OM
and peptidoglycan invagination we first observed upon the expression of non-functional FtsK_N variants (163). Interaction between the periplasmic loops of FtsK_N and the Tol-Pal complex would create a link between the IM and OM that could facilitate coordinated invagination of the entire cell envelope during division. This connection might then account for the cell void phenotype seen with the periplasmic loop mutants described in Chapter 2 (163), as disruption of this interaction would directly uncouple IM division from OM invagination.

Based on prior biochemical evidence, it is surprising to note that essential cell division proteins previously shown to interact with FtsK by bacterial two-hybrid analysis, including IM proteins FtsQ, FtsL, and FtsI (121, 157, 160), were not detected in our cross-linking analysis. Given we have elected to only probe the periplasmic face of FtsK_N, it is possible that these proteins interact with FtsK at locations distinct from our sites of inquiry, namely the transmembrane or cytoplasmic domains of the protein. Alternatively, it is possible that FtsK does not interact directly with these proteins, but rather they are bridged by a third interacting partner that would have confounded previous bacterial two-hybrid results. For example, FtsI has been shown to form a complex with the OM lytic transglycosylases MltA and MltB (217, 218); therefore, our detection of these two proteins by LC-MS/MS (Figure 3.5, Appendix Table A1.1) might explain formation of a tertiary complex between FtsK and FtsI bridged by these lytic transglycosylases.

In addition to the potential FtsK_N interactors described above, this study is the first to report a confirmed, direct interaction between the OM lipoprotein RlpA and an essential member of the divisome (Figure 3.6). Similar to our discussion of DamX above, the implication of this interaction is difficult to interpret in the absence of definitive evidence on the function of RlpA in E. coli. However, general localization data in E. coli (95, 97, 194) and recent biochemical
evidence on the enzymatic activity of RlpA in *P. aeruginosa* (195) highlights the potential significance of this novel interaction.

Of all SPOR-domain containing proteins, RlpA is the most highly conserved across bacterial species (195). This would suggest that RlpA plays an important role in bacteria. Based on its OM localization and ability to preferentially bind septal peptidoglycan (95, 97, 194), one role for RlpA could be to physically link the OM to the peptidoglycan and IM during constriction. In this capacity, the interaction between RlpA and FtsK would function similarly to the Tol-Pal interaction. This functional redundancy might also explain why null mutants of *rlpA* in WT *E. coli* strains have no morphological defects, as the loss of the OM-IM contact made between RlpA and FtsK upon deletion of RlpA could be accommodated by an intact interaction maintained by FtsK with the Tol-Pal complex. However, this scenario does not fully account for the restoration of growth seen in the absence of both RlpA and functional FtsK\(_N\) (*Figure 3.8*), indicating RlpA must play a larger role in division beyond simply bridging all three cell envelope layers.

A second role for RlpA may be in balancing lateral growth of the cell versus septal peptidoglycan synthesis. In addition to its septal localization, RlpA is also found at distinct foci along the cell cylinder (95). Together with the fact that *rlpA* is encoded immediately downstream of two proteins involved in cell elongation, *pbpA* (encoding PBP2) and *rodA* (213) (*Figure 3.10*), it is proposed that RlpA might function as part of the elongation machinery, in addition to the divisome. Interaction between FtsK and RlpA would then represent another link between these two macromolecular complexes, and potentially facilitate the transition between elongation and septation in *E. coli*. The restoration of growth seen upon the loss of both RlpA and functional FtsK suggests that their interaction may be critical in suppressing septation until sufficient elongation and proper assembly of the divisome occurs.
Figure 3.10. Gene arrangement of the $rlpA$ locus in *E. coli*. The gene encoding $rlpA$ is located within the *leuS-dacA* region (15 min) on the *E. coli* chromosome. A 5.4 kb portion of this region (shown) contains the genes *pbpA* and *rodA* (encoding elongasome proteins PBP2 and RodA, respectively), $rlpA$ and *dacA* (encoding PBP5). The length of each open reading frame is indicated below the corresponding gene name.

Perhaps the most intriguing aspect of the interaction between RlpA and FtsK$_N$ is its parallel with the functional interaction between FtsK and the carboxypeptidase DacA (PBP5). As mentioned in Section 1.4.1, DacA catalyzes the removal of the terminal D-alanine from peptidoglycan side chains during cell wall synthesis (7, 120). Interestingly, DacA was also detected as a potential FtsK$_N$ interaction partner in our cross-linking analysis (Figure 3.5, Appendix Table A1.1). Deletion of *dacA* from *E. coli* carrying the temperature-sensitive *ftsK44* allele has been shown to largely suppress filamentation of bacteria grown at a nonpermissive temperature ($42^\circ$C)(112). This is in direct correlation with the phenotype we observe upon the deletion of *rlpA* from the same strain of *E. coli* (Figure 3.9). In addition, and perhaps not surprisingly, *rlpA* also resides on the chromosome immediately upstream of *dacA* (Figure 3.10)(213). Given their proximity and evidence that RlpA is also involved in peptidoglycan remodeling (195), it would not be unreasonable to estimate that the deletion of either of these genes would have a similar impact on the cell in the absence of functional FtsK. In the case of *dacA*, deletion of this gene is only able to compensate for the temperature-sensitive growth defect and not complete loss of FtsK (114), suggesting that under these circumstances cells might be able to restore the function of the mutated FtsK44 protein rather than completely bypass its function. How this occurs is unclear, but we postulate that if DacA and FtsK form a direct protein
interaction, deletion of DacA would free FtsK to form protein contacts with additional proteins that could stabilize its structure and restore its function. In our study, it would be pertinent to note that this may also be the case with the deletion of *rlpA*, and that further investigation into the ability of an *rlpA* deletion to permit the complete deletion of *ftsK* is necessary.

As a whole, the novel periplasmic FtsK\textsubscript{N} interactome and verified interaction with RlpA described in this study provides further evidence for FtsK as a potential regulator of cell envelope remodeling during division. While the identity of the interactor responsible for the shift from cell elongation to septation remains unclear, we detected several proteins that could fulfill this role. In fact, it is most likely that a number of these proteins work in concert to accurately move the bacterium through its growth cycle. As with all of the potential FtsK\textsubscript{N} interaction partners identified, further biochemical verification of the specificity and validity of these interactions is needed to characterize each protein as a true interactor. Further investigation into the impact each interaction with FtsK has on both growth and division will undoubtedly enhance our understanding of both of these essential processes.
CHAPTER 4: FtsA Variant G50E Bypasses the Essential Requirement for FtsK During Bacterial Cell Division in *Escherichia coli*

**Statement of Contributions**

The experiments described below were designed by Alison M. Berezuk, Dr. Reggie Y. Lo and Dr. Cezar M. Khursigara. All experiments were performed by Alison M. Berezuk, with the FtsK<sub>N</sub>-FtsA pull-down assays conducted collaboratively with Dr. Elyse J. Roach. Data analysis, preparation of the manuscript and all accompanying figures was completed by Alison M. Berezuk, with drafting and editorial assistance from Dr. Cezar M. Khursigara.
4.1 Abstract

With the large number of proteins involved in cell division, tight regulation of co-complex formation is necessary to ensure proper and efficient division occurs. In *Escherichia coli*, one key modulator linking early (Z-ring formation) and late (cell envelope remodeling) division complexes is the essential protein FtsK. Although the function of the N-terminal domain of FtsK (FtsKN) during division is unclear, it is proposed that FtsK may modulate septum formation during division through the formation of dynamic and essential protein interactions with both the Z-ring and late-stage division machinery. Here, we identified a G to A transition, leading to a G50E mutation within the Z-ring membrane anchoring protein FtsA, which allows cells to bypass the checkpoint function of FtsK. An *E. coli* strain harbouring a temperature-sensitive chromosomal variant of *ftsK* (*ftsK44*) and plasmid-encoded copy of non-functional FtsKN variant D135C was subjected to random mutagenesis by the chemical mutagen ethyl methanesulfonate (EMS), and subsequently recovered at a nonpermissive growth temperature (42°C) to select for cells that could survive in the absence of functional FtsK. The G50E variant, but not wild-type FtsA, was able to fully complement the temperature-sensitive *ftsK44* mutation of *E. coli* strain LP11-1. In addition, wild-type FtsA and variant G50E were both shown to interact with FtsKN in *vitro*. Given that residue G50 has been mapped to the subunit interface of FtsA, this suggests that the oligomeric state of FtsA, or its interactions through other cell division intermediates, impacts FtsK function. This provides insight into a potential co-complex formed between these components during division.
4.2 Introduction

Broken into discrete steps, bacterial cell division can be considered a relatively simple process. Following proper chromosome segregation and elongation, a macromolecular protein complex forms at mid-cell and constricts to pinch the cell into two. However, with over 30 soluble and membrane-bound proteins involved in formation and stabilization of the *Escherichia coli* division complex (collectively known as the divisome) (12, 32–34), bacterial cell division is one of the most complex and poorly understood processes in microbiology.

The dynamic nature of the *E. coli* divisome complex has played an important role in bacterial survival. As discussed in Section 1.3, complete assembly of the divisome relies on formation of a ring-like structure at mid-cell known as the Z-ring, which is composed of bundled intracellular filaments of the protein FtsZ and is tethered to the cytoplasmic membrane by FtsA and ZipA (48, 72, 78, 82, 101). Following initial formation of this early division complex, remodeling of the cell envelope during septum formation is mediated by the combined action of the remaining essential division proteins (9, 32, 34). Very little overlap in function occurs among these groups of divisome proteins. For example, while either FtsA or ZipA can independently attach FtsZ polymers to the membrane to enable Z-ring formation, both are required for the recruitment of later cell division proteins so that constriction can begin, pointing to independent essential functions of these proteins (82, 83). Therefore, to ensure survival in extreme circumstances where any one divisome protein is unable to perform its essential function (e.g., through mutation or gene deletion), *E. coli* must be able to harness the underlying plasticity of the complex to mitigate this loss.

The ability of certain mutations within essential divisome proteins to bypass the requirement for other proteins in the complex implies an intricate web of interactions
underpinning the organization of the divisome. Most prominently, studies have discovered a multitude of mutations within the Z-ring membrane anchoring protein FtsA that allow *E. coli* to survive the loss of other division proteins, including ZipA, FtsK and FtsN (36, 75, 117–119, 219). The first mutation shown to bypass the loss of an essential divisome protein in *E. coli* was reported by Geissler *et al.* in 2003, who isolated a gain-of-function FtsA variant (FtsA*) harbouring an R286W mutation that could stabilize Z-rings and promote division in the absence of ZipA (219). Subsequently, this mutation was shown to also bypass the requirement for FtsK (117, 118), and an additional 34 mutations have been reported that support the deletion of normally essential cell division genes, three of which allow for the deletion of *ftsK* (*E*124*A*, I143L and the triple missense mutant FtsA*K* [K48R/K117R/E124G])(36, 75, 117–119).

Although we have made great strides to elucidate the function of FtsK in cell division, based on the evidence presented in Chapters 2 and 3, the precise mode of action FtsK employs as a checkpoint of cell envelope remodeling remains unclear. It is apparent that FtsK must modulate septum formation during division through the formation of dynamic and essential protein interactions with both the Z-ring and late-stage division machinery, and that tight regulation of these co-complexes ensures that spatial and temporal cues made during division are followed correctly. However, the identities of all components that might mediate the checkpoint function of FtsK are not currently known. Based on the flexibility of the divisome to compensate for the loss of its essential components described above, we surmised that the isolation of compensatory mutations in other division proteins using a simple genomic mutagenesis approach could highlight proteins whose interactions with FtsK, either directly or indirectly, are essential for its function.

In this study, we used random DNA mutagenesis by the chemical mutagen ethyl methanesulphonate (EMS) to generate suppressor mutations in the temperature-sensitive *E. coli*
strain LP11-1 (ftsK44). This strain also carried a plasmid-encoded copy of non-functional FtsK\textsubscript{N} variant D135C. By using this strain, we aimed to select for mutations that could not only compensate for the temperature-sensitivity of LP11-1, but also the uncoupling of cell envelope septation caused by expression of this variant (163). Together, these suppressor mutations would represent only those that could completely bypass the checkpoint function of FtsK. We recovered a single suppressor strain harbouring a G50E missense mutation within FtsA, designated LP11-1 \textit{ftsA}\textsubscript{G50E}, that permits the loss of functional FtsK during division. In addition, we further investigated the formation of a potential co-complex between FtsK\textsubscript{N} and FtsA by an \textit{in vitro} pull-down assay.

### 4.3 Experimental Procedures

#### 4.3.1 Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 4.1. \textit{E. coli} W3110, Lemo21 and DH5\textalpha\ cultures were grown exactly as described in Section 2.3.1 (163). \textit{E. coli} LP11-1 and LP11-1 \textit{ftsA}\textsubscript{G50E} were grown in Complementation Media (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl [Fisher]) supplemented with 15 \(\mu\)g/mL tetracycline and additionally with 150 \(\mu\)g/mL ampicillin for plasmid-carrying strains. For temperature-sensitive complementation plate assays, cultures were grown at 30°C overnight in 5 mL of Complementation Media. Cultures were diluted to an A\textsubscript{600} of 1.0 (SmartSpec\textsuperscript{™} Spectrophotometer; Bio-Rad) in sterile phosphate-buffered saline (PBS), and then 10-fold serially diluted in PBS before plating 10 \(\mu\)L on duplicate plates of LB agar containing appropriate
Table 4.1. Bacterial strains and plasmids

The abbreviations used are as follows: Amp, ampicillin; Cam, chloramphenicol.

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antibiotics. Plates were incubated at either 30°C or 42°C overnight. To assess the impact of Fts\textsuperscript{A*G50E} on cell morphology, liquid cultures of E. coli LP11-1, LP11-1 *ftsA*G50E, and appropriate controls were grown at 30°C or 42°C, as described in Section 2.3.4 (163). For each strain, mean cell length (± S.E.) and morphology for 75 random cells were assessed by phase contrast microscopy (Leica DM2000 LED, ProgRes CT3 camera; Jenoptik AG). Statistical analysis of mean length was completed using a one-way analysis of variance with Tukey-Kramer multiple comparison post-tests by Prism 5 software (GraphPad Software, Inc.) with a level of significance of α = 0.05 for all tests.
4.3.2 Plasmid Construction

The genes encoding wild-type (WT) FtsA and FtsA variant G50E (FtsAG50E) were amplified by PCR from *E. coli* K12 W3110 and LP11-1 *ftsAG50E* genomic DNA, respectively, using custom primers AMB008F (5′-TGGAGAATTCGATGGATTATAAAGATGATGATGAT-AAATCCAGTATGATCAAGGCGACGGACAGAAAAACTG-3′) and AMB008R (5′-TAATCTTCACTGCAGTTAAAACTCTTTTCGCAAGCCAACACTATTGAGTCGCTTGATCCACG-3′). The custom primers (Operon) were designed to introduce EcoRI and PstI restriction sites and an N-terminal FLAG®-tag to each construct. Sequences were then inserted into the arabinose-inducible expression vector pBAD24 as described in Section 2.3.2 (163). The resulting gene constructs were verified by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph) to produce pAB013-1 (FLAG-FtsA) and pAB014-1 (FLAG-FtsAG50E).

4.3.3 Chemical Mutagenesis

The *E. coli* LP11-1 suppressor strain (LP11-1 *ftsAG50E*) was obtained by treatment of LP11-1 carrying FtsK_N non-functional variant D135C (plasmid pAB006-61) with the chemical mutagen ethyl methanesulfonate (EMS), as described by Parkhomchuk *et al.* (220), with minor modifications. An overnight culture of LP11-1 carrying FtsK_N D135C was diluted to an A600 of 0.1 (SmartSpec™ Spectrophotometer; Bio-Rad) in fresh Complementation Media and cultured at 30°C to an A600 of 0.3. A 2-mL aliquot of cells was washed twice in sterile PBS and suspended in 2 mL PBS containing 35 µL EMS (Sigma-Aldrich) to obtain a final EMS concentration of 1.75% (v/v). Cells were allowed to grow in the PBS/EMS solution for 2 h at 30°C. A 500-µL aliquot of mutagenized cells was diluted 1:10 into fresh Complementation Media and allowed to recover at 30°C for an additional 2 h before plating 100 µL onto LB agar containing 0.02% (w/v)
L-arabinose. The plate was then incubated overnight at 42°C to select for colonies able to grow at the non-permissive temperature and in the presence of a non-functional variant of FtsK\textsubscript{N} (D135C).

Location of the suppressor mutation in colonies obtained above was determined by targeted gene sequencing. Genomic DNA of isolated suppressor strains was obtained using a Maxwell\textsuperscript{®} 16 Cell DNA Purification Kit (Promega) as per manufacturer’s instructions. Target genes and primers used for sequencing are shown in Table 4.2. Presence of the genomic \textit{ftsK44} mutation in all isolated strains was verified first to ensure suppression of the temperature-sensitive phenotype was not caused by reversion to a WT sequence of \textit{ftsK}. Target genes were chosen based on their essential nature in cell division or their presence as a potential interaction partner of FtsK\textsubscript{N} based on the \textit{in vivo} cross-linking study described in Chapter 3. All genes were amplified by PCR using iProof high fidelity DNA polymerase (Bio-Rad) and purified by gel extraction using a GeneJET gel extraction kit (ThermoFisher). Gene sequences were then obtained by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph) using the purified PCR products as a template. Retention of the plasmid-encoded copy of FtsK\textsubscript{N} D135C in suppressor strains was also verified by isolation of pAB006-61 from each strain followed by DNA sequencing of the \textit{his10-ftsK}\textsubscript{N} insert (Genomics Facility, Advanced Analysis Center, University of Guelph).

4.3.4 Expression and Purification of FLAG-FtsA

Expression and purification of FLAG-FtsA and FLAG-FtsA\textsubscript{G50E} were carried out as described in Section 3.3.7, with minor modification to the expression conditions. Overnight cultures of \textit{E. coli} Lemo21 cells carrying either WT FLAG-FtsA (plasmid pAB013-1) or FLAG-FtsA\textsubscript{G50E} (plasmid pAB014-1) were diluted 1:100 into 1 L LB media and grown at 37°C for 1 h. Cells were induced with 0.2% (w/v) L-arabinose and incubated for an additional hour. Cell lysis
and purification using 300 µL of anti-FLAG® M2 affinity gel (Sigma-Aldrich) was performed exactly as described in Section 3.3.7. Total protein concentration of pooled, purified FLAG-FtsA and FLAG-FtsA<sub>G50E</sub> was determined by a bicinchoninic acid protein assay as per manufacturer’s instructions (Thermo Scientific) using BSA as a standard.

Table 4.2. Oligonucleotide pairs used for gene sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of oligonucleotide (5′ to 3′)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>ftsK</td>
<td>TCCGTTTTAGCATCGCAGAAGAACGC</td>
</tr>
<tr>
<td></td>
<td>TCTGTTCGCCGCCCATCG</td>
</tr>
<tr>
<td>ftsZ</td>
<td>GCCGACGATGATTACGCGCAGACGA</td>
</tr>
<tr>
<td></td>
<td>GCCGGCGAGTTTAGACAAAGAGCTCGA</td>
</tr>
<tr>
<td>zipA</td>
<td>CTCTTAGTAGTAAGATTTGTCGCTGCTGCTAAGAGTTAA</td>
</tr>
<tr>
<td>ftsA</td>
<td>GCCGCAAGAGGAATCTACTACGACGAAACAAAC</td>
</tr>
<tr>
<td></td>
<td>CCGATTTCGCTGCTGCCGCTGACGCAAAC</td>
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<tr>
<td>ftsQ</td>
<td>GTATGAGCTCTCGACGTGTAAGATGACGAA</td>
</tr>
<tr>
<td></td>
<td>TCGCGTACCAATTCCAGCTACTACG</td>
</tr>
<tr>
<td>ftsB</td>
<td>AGATCGCTTCCGATGACGATGAGTAAGA</td>
</tr>
<tr>
<td></td>
<td>CACGGCGCAACATCCGAAATGAGTCTG</td>
</tr>
<tr>
<td>ftsL</td>
<td>GAACCTCGTGGCTGAGTAGTTTTG</td>
</tr>
<tr>
<td></td>
<td>CTGAGTTGTTTGGTCTGAGTTTTT</td>
</tr>
<tr>
<td>ftsW</td>
<td>CTTGAAACACGAGGCAAAGTAAGTCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td></td>
<td>CATGTCACCAGTTCCCGCCTGCCATC</td>
</tr>
<tr>
<td>ftsI</td>
<td>GATCCGTCAAAAGAAATATCTGATGACGAA</td>
</tr>
<tr>
<td></td>
<td>GTCAGGTCTATCCTGCGAGTCTGCGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>ftsN</td>
<td>CGTTACTAGTACAGTATGGCGGCGGCTAGACGGAAGGA</td>
</tr>
<tr>
<td></td>
<td>ACAAAATGACGGCGGCAATTATGCAATTATAG</td>
</tr>
<tr>
<td>dacA</td>
<td>GCTATAGTACGCCACTTTTTTTATCTCTGCA</td>
</tr>
<tr>
<td></td>
<td>ACTATGCTTAGTATATGGCGGCGGCTAGACGGAAGGA</td>
</tr>
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<td>rlpA</td>
<td>CAATCCACACCCACAGAAATGATGTC</td>
</tr>
<tr>
<td></td>
<td>ACTTTGTAGCCGATTTTTGAGAATTGAGAAGAAAGAAAGAAAGAAAGAA</td>
</tr>
<tr>
<td>ybhC</td>
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<tr>
<td></td>
<td>CCGTAGGGGCAAATGAGTAATGG</td>
</tr>
<tr>
<td>mltA</td>
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</tr>
<tr>
<td></td>
<td>GTATGCTTACCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>mltB</td>
<td>GTATGCTTACCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td></td>
<td>GAAAAAGCTGATTAGGCGAGGGAAGAAAGCTCA</td>
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<sup>a</sup> All primers are homologous to regions approximately 50 bp upstream and downstream of the target gene

4.3.5 Pull-Down Assays

To assess direct protein interaction between FtsK<sub>N</sub> and FtsA, pull-down assays were performed as described in Section 3.3.8, using 200 µg of purified FtsA or FtsA<sub>G50E</sub> as prey
protein. Purified, resin-bound FtsK<sub>N</sub> was obtained as described in Section 3.3.6. Following analysis of three biological replicates of the pull-down assays by SDS-PAGE and Western immunoblotting, the blots were scanned and band densities were measured using the ImageJ program (version 1.47r, National Institutes of Health).

4.4 Results

4.4.1 Isolation of a Genomic Suppressor for the Loss of Functional FtsK

In order to identify additional interaction partners of FtsK<sub>N</sub> involved in the essential checkpoint function of the protein, we employed a genomic suppressor screen using the chemical mutagen ethyl methanesulfonate (EMS). EMS is a DNA ethylating agent that predominantly attacks guanine nucleotides, generating a modified purine base (221). This modification leads to a mispairing of the ethylated guanine with a thymine nucleotide (Figure 4.1). Subsequent rounds of DNA replication perpetuate this base substitution and generate GC to AT transition mutations (221). We harnessed this simple genetic technique to randomly mutate the genome of the temperature-sensitive <i>E. coli</i> strain LP11-1 (<i>ftsK44</i>) carrying a plasmid-encoded copy of non-functional FtsK<sub>N</sub> variant D135C. As shown in Chapter 2, expression of FtsK<sub>N</sub> D135C in LP11-1 results in the production of cellular voids and an uncoupling of cell envelope septation events (163). By mutating the genome of this strain with EMS and subsequently culturing the mutated strain under conditions normally nonpermissive to growth (42°C in the presence of 0.02% [w/v] l-arabinose to induce expression of FtsK<sub>N</sub> D135C), we hoped to isolate a genomic suppressor strain that could compensate for the loss of functional FtsK. Identification of the location of this suppressor mutation within the genome could then point to genes that play an essential role in the checkpoint function of FtsK.
Figure 4.1. Ethyl methanesulfonate (EMS) mutagenesis. A, structural formula of EMS. B, mispairing of an O\textsuperscript{6} ethylated guanine nucleotide (O\textsuperscript{6}-eG) with a thymine nucleotide. ‘dR’ denotes the position of the deoxyribose sugar. The red dotted lines indicate hydrogen bonds formed between the two bases. C, model of DNA replication that perpetuates the GC to AT transition. EMS-modified guanine nucleotides are shown with a red dot. Panels B and C are redrawn and adapted from Ref. 220.

*E. coli* strain LP11-1 (ftsK44) carrying plasmid pAB006-61 (His\textsubscript{10}-FtsK\textsubscript{N} D135C) was subjected to EMS mutagenesis as described in *Section 4.3.3*. Following recovery of the mutated culture, presence of the genomic *ftsK44* mutation and retention of the plasmid-encoded copy of FtsK\textsubscript{N} D135C in selected colonies were verified first to ensure suppression of the temperature-sensitive phenotype was not caused by reversion to a WT sequence of *ftsK* or loss of the non-functional FtsK\textsubscript{N} variant. Of the colonies tested, a single suppressor strain was found to retain both the temperature-sensitive *ftsK44* chromosomal mutation, as well as the plasmid-encoded
D135C variant. Compared to LP11-1 complemented with a non-functional copy of FtsK$_N$ (D135C or D136C), this recovered suppressor strain was able to partially restore growth at 42°C (Figure 4.2). However, growth was not fully restored to WT levels, as typically seen when LP11-1 is complemented with a functional copy of FtsK$_N$ (e.g., WT FtsK$_N$ or Y139C). This would suggest that either the suppressor mutation does not fully compensate for the loss of FtsK, or that a dominant negative phenotype associated with the expression of non-functional variant D135C impedes growth of the mutated strain even in the presence of a compensatory mutation.

Figure 4.2. Complementation analysis of WT FtsK$_N$ and single cysteine variants compared to the recovered suppressor strain. Overnight cultures of the temperature-sensitive *E. coli* strain LP11-1 (*ftsK44*) carrying plasmid-encoded WT FtsK$_N$, functional (Y139C) or non-functional (D135C, D136C) single cysteine FtsK$_N$ variants, as well as the recovered suppressor strain were diluted to an $A_{600}$ of 1.0, and then 10-fold serially diluted in PBS. Ten-microlitres of each serial dilution were spotted onto LB agar plates (supplemented with 15 µg/mL tetracycline and 150 µg/mL ampicillin), and incubated at the indicated temperature for 18 h. LP11-1 carrying empty pBAD24 was used as a negative control for the LP11-1 growth defect. Plasmids encoding WT FtsK$_N$ and variants D135C, D136C and Y139C (i.e., plasmids pAB006-2, pAB006-61, pAB006-162, and pAB006-211) are denoted pFtsK$_N$, pFtsK$_N$ D135C, pFtsK$_N$ D136C, and pFtsK$_N$ Y139C, respectively.

To identify the position of the suppressor mutation responsible for the restoration of growth, we used a targeted gene sequencing approach. Given the selection pressure for isolation of the suppressor strain was within an essential cell division gene, all ten essential cell division
genes were targeted for DNA sequencing, along with select genes identified during the in vivo cross-linking analysis completed in Chapter 3 (Table 4.2). Of the 14 genes sequenced, it was determined that all except essential cell division gene \( \text{ftsA} \) retained a WT sequence. The recovered suppressor strain harbours a G to A transition at position 149 of \( \text{ftsA} \), leading to a G50E missense mutation in the resulting protein (Figure 4.3). As such, the recovered suppressor strain will be designated ‘LP11-1 \( \text{ftsA}_{G50E} \)’ throughout the remainder of this chapter.

![Diagram of DNA sequencing chromatogram and amino acid sequence alignment](image)

**Figure 4.3.** Recovered suppressor strain of LP11-1 harbours a G to A transition in the essential cell division gene \( \text{ftsA} \). DNA sequencing chromatogram (top panel) of WT \( \text{ftsA} \) and the suppressor strain, and their corresponding amino acid sequence alignment (bottom panel). Location of the G to A transition is highlighted in yellow and the G50E missense mutation in the protein sequence is indicated by the yellow box. DNA sequencing chromatogram and amino acid sequence alignment were created using Geneious Pro™ (version 5.5.9; Biomatters, Ltd.).

4.4.2 \( \text{ftsA}_{G50E} \) Fully Bypasses the Essential Requirement for FtsK

The ability for certain FtsA mutations to bypass the requirement for other essential cell division proteins, including FtsK, has been well established in the literature (36, 75, 117–119, 219). In particular, the \( \text{ftsA}_{G50E} \) variant identified above was also recently isolated as an intragenic suppressor of a temperature-sensitive variant of FtsA (\( \text{ftsA}_{27} \)). In this study by
Herricks et al., the G50E missense mutation was able to bypass the requirement for the essential cell division protein ZipA (75), but has not been shown to allow for the loss of FtsK in *E. coli*.

Since it was necessary to isolate our recovered suppressor strain under two selection pressures to ensure complete loss of FtsK and uncoupling of cell envelope septation (i.e., growth at 42°C and in the presence of non-functional FtsK<sub>N</sub> D135C), it is possible that the *ftsA<sub>G50E</sub>* mutation simply suppresses the chromosomal temperature-sensitive *ftsK<sub>44</sub>* mutation and not the cell envelope uncoupling caused by expression of FtsK<sub>N</sub> D135C. To test whether FtsA<sub>G50E</sub> counteracts the temperature-sensitivity of the *ftsK<sub>44</sub>* allele alone, we complemented the LP11-1 (*ftsK<sub>44</sub>* strain) with both WT FtsA and variant G50E expressed from the arabinose-inducible expression vector pBAD24. Given over-expression of FtsA has been shown to be lethal in *E. coli* (222, 223), leaky expression from the P<sub>ara</sub> promoter in the absence of L-arabinose was used to ensure non-toxic levels of expression were achieved. FtsA<sub>G50E</sub>, but not WT FtsA, eliminated the temperature-sensitivity of strain LP11-1 (*Figure 4.4*). This complementation was further corroborated by morphological analysis, which showed that FtsA<sub>G50E</sub> restored both normal cell length and morphology to the LP11-1 strain at 42°C (*Figure 4.4, B and C*). Although the morphology of LP11-1 with exogenous FtsA<sub>G50E</sub> was similar to that of LP11-1 *ftsA<sub>G50E</sub>* strain, it is interesting to note that plasmid-encoded *ftsA<sub>G50E</sub>* restored WT levels of growth in the plate assay (*Figure 4.4A*), while chromosomally-encoded *ftsA<sub>G50E</sub>* did not (*Figure 4.2*). There are several explanations for this discrepancy, including that the G50E mutation in FtsA only suppresses the temperature-sensitivity of the chromosomal *ftsK<sub>44</sub>* allele and does not compensate for the inhibition of growth caused by non-functional FtsK<sub>N</sub> D135C in the recovered strain. Alternatively, LP11-1 *ftsA<sub>G50E</sub>* may contain additional mutations in the genome, not accounted for in our targeted gene sequencing approach, that impede its growth.
Figure 4.4. FtsA<sub>G50E</sub>, but not WT FtsA, fully complements the temperature-sensitive <i>E. coli</i> strain LP11-1 (<i>ftsK44</i>). <i>A</i>, complementation, and <i>B</i>, mean cell length analysis of LP11-1 complemented with plasmid-encoded WT FtsA (pAB0013-1, denoted pFtsA) and FtsA<sub>G50E</sub> (pAB014-1, denoted pFtsA<sub>G50E</sub>). Complementation analysis was completed as described in Section 4.3.1. For cell length measurements, overnight cultures of each strain were diluted to an A<sub>600</sub> of 0.1 in Complementation Media and incubated at the indicated temperatures for 2 h. Cell lengths were measured using ImageJ and are reported as mean cell length (µm) ± S.E. (n = 75 cells per strain per condition). *, p < 0.001 versus LP11-1 complemented with plasmid-encoded
WT FtsKN (pAB006-2, denoted pFtsKN) grown at the same temperature. C, representative phase contrast micrographs of strain LP11-1 complemented with plasmid-encoded FtsKN and FtsA variants, as well as the recovered suppressor strain (LP11-1 ftsA<sub>G50E</sub>). In panels B and C, LP11-1 carrying empty pBAD24 are included as a vector control. Bar, 5 µm.

4.4.3 In Vitro Analysis of Novel Protein Interaction Between FtsKN and FtsA

Physical interaction between <i>E. coli</i> FtsK and FtsA has not been previously reported in <i>E. coli</i>. We therefore determined whether the restoration of growth seen in LP11-1 ftsA<sub>G50E</sub> could be explained by a gain of interaction between FtsKN and FtsA<sub>G50E</sub> during division. In this scenario, FtsA<sub>G50E</sub> would be able to interact with FtsK to stabilize the divisome and recruit downstream division proteins under normally nonpermissive conditions, whereas WT FtsA would not. Interactions of FtsKN with either WT FtsA or variant G50E were probed by <i>in vitro</i> pull-down assays using His<sub>10</sub>-FtsK<sub>N(220)</sub> and FLAG<sup>®</sup>-tagged derivatives of each FtsA protein. Following incubation of purified, IMAC resin-bound FtsKN with purified FLAG-FtsA or FLAG-FtsA<sub>G50E</sub>, and successive washing to remove unbound protein, FtsKN was eluted and all proteins detected by Western blot analysis. In both cases, FtsA was successfully detected in the elution fraction (<b>Figure 4.5, A and B</b>), indicating direct interaction between these proteins. When normalized to the detected FtsK<sub>N</sub>, there was no significant difference in the amount of WT FtsA and FtsA<sub>G50E</sub> pulled down by FtsK<sub>N</sub>. To verify accuracy of the pull-down assay, WT FtsA and FtsA<sub>G50E</sub> were also incubated with IMAC resin in the absence of FtsK<sub>N</sub> as a negative control. While WT FtsA was not detected in the elution fraction (<b>Figure 4.5C, left panel</b>), minimal amounts of FtsA<sub>G50E</sub> were retained by the IMAC beads (<b>Figure 4.5C, right panel</b>), indicating an increase in non-specific interaction with the protein support. However, based on densitometry analysis, incubation of FtsA<sub>G50E</sub> with FtsK<sub>N</sub> retained 1.58x ± 0.29 (S.E.) more protein compared to the empty IMAC beads. This suggests that this variant does interact with FtsK<sub>N</sub>, but possibly with a reduced affinity.
Figure 4.5. *In vitro* analysis of interaction between FtsK\textsubscript{N} and FtsA. Representative Western blots of pull-down assays between, A, WT FLAG-FtsA and His\textsubscript{16}-FtsK\textsubscript{N(220)}, and B, FLAG-FtsA\textsubscript{G50E} and His\textsubscript{16}-FtsK\textsubscript{N(220)}. C, Western blots of WT FLAG-FtsA (left panel) and FLAG-FtsA\textsubscript{G50E} (right panel) incubated with empty IMAC resin (negative control). For all blots, the primary antibodies are indicated in the bottom right corner. In all panels: ‘FT’ – flow through; ‘W1, W2, W3’ – wash fractions 1, 2, and 3; ‘Elution’ – elution with 1 M imidazole.
4.5 Discussion

Identifying the protein contacts FtsK makes during division is a critical step in unraveling its essential role during division. We employed a genetic suppressor screen to identify genes that, when mutated, allow cells to compensate for the loss of FtsK. With this approach, we could highlight proteins whose interaction with FtsK, either directly or indirectly, would normally be essential for its proposed checkpoint function.

We report here the first evidence that mutation of FtsA residue G50 allows cells to bypass the requirement for FtsK during bacterial cell division in *E. coli* (**Figure 4.4**). In addition, perhaps the most surprising result of our study was the detection of a direct protein-protein interaction between FtsK and WT FtsA *in vitro* (**Figure 4.5**). While the ability for certain FtsA mutations to bypass the requirement for FtsK has been well established in the literature (117–119), to date no interaction between FtsA and FtsK in *E. coli* has been reported. Despite this, interaction between these two proteins would not be unreasonable to envision, as FtsK has been shown to interact with FtsA in other bacterial species, such as *Neisseria gonorrhoeae* (215) and *Streptococcus pneumoniae* (224). In fact, a complex formed between FtsK and FtsA makes an intriguing addition to the putative signal transduction pathway that may modulate the transition between cell elongation and septation.

Recently, FtsA has been implicated as a key player in initiating cell constriction through its interaction with the essential cell division protein FtsN (36, 37, 41, 42, 225). In Chapter 3, we describe the potential formation of a tripartite complex including FtsK, FtsN and PBP1a. Together, these proteins possess the ability to sense both sufficient cell elongation by the elongasome and proper formation of the divisome at mid-cell. With our report that FtsK and FtsA form a direct protein contact, we propose an extended model for both the initiation of cell
constriction in *E. coli* and the bypass of FtsK by point mutations in FtsA (Figure 4.6). This model, first reported by Pichoff *et al.* in 2015, relies on the interaction between FtsA, FtsN and at least one other divisome protein, as well as modulation of the oligomeric state of FtsA (37). We postulate that one of the divisome proteins responsible for coordinating these interactions is FtsK. Based on the domain architecture of FtsA, protein recruitment and self-interaction of FtsA monomers is mediated by a single domain (1C)(36, 68–70). As such, it is proposed that FtsA switches between two forms; a polymeric form that is unable to interact with downstream proteins, and a monomeric form that is active in recruitment (36, 164). Lowering FtsA’s ability to self-interact, typically through its interaction with ZipA (36, 37), is thought to increase its ability to recruit one or more later cell division proteins. The direct recruitment of FtsK by FtsA would then prime the divisome to sense the growth stage of the bacterial cell, perhaps through RlpA or PBP1a as described in *Chapter 3* (Figure 4.6A). FtsK could participate in delaying the recruitment of FtsN to the divisome until elongation is complete and septation is ready to proceed. The addition of FtsA to this complex has several implications, as it could increase the efficiency of signal transduction by the complex or enhance recruitment of FtsN to the divisome. Not only would it allow FtsK to more effectively sense proper formation of the Z-ring in the cytoplasm, but it may also allow for signal amplification, as complete assembly of the divisome could be relayed to FtsN by direct interaction with both FtsK and FtsA.

With regard to the bypass phenotype seen upon the expression of FtsA variant G50E (i.e., restoration of growth to *E. coli* strain LP11-1 under typically nonpermissive growth conditions), this phenotype is likely driven by artificial changes in the oligomeric state of FtsA. Residue G50 of FtsA is located in domain 1A along the FtsA-FtsA interface (Figure 1.5), and has been shown
Figure 4.6. Model for the bypass of FtsK by FtsA_{G50E}. A, under normal growth conditions, increased self-interaction typical of WT FtsA suppresses full assembly of the divisome. Decreased self-interaction of FtsA modulated by ZipA allows for the recruitment of downstream division proteins, namely FtsK. Once an interaction between FtsA and FtsK has been established at mid-cell, the functional periplasmic loops of FtsK_N could sense the status of cell elongation, either through direct or indirect interaction with the elongation machinery. A corresponding signal would then be transmitted to FtsN to initiate septation. B, to bypass the requirement for FtsK, the intrinsic decrease in self-interaction exhibited by FtsA_{G50E} would allow for FtsA to directly recruit FtsN to the divisome, thereby initiating septation in the absence of an additional stimulus from FtsK and other divisome proteins, such as ZipA. Adapted from Refs. 37, 42.
to disrupt the ability of FtsA to self-interact in vivo (67, 75). This intrinsic decrease in self-interaction would inherently shift FtsA to its active monomeric state without the need for either FtsK or ZipA, and allow for the direct recruitment of downstream division proteins, such as FtsN (Figure 4.6B). Given all FtsA mutations that decrease self-interaction are also able to bypass ZipA (75), it is likely that this mechanism plays a predominant role in the bypass of FtsK seen here; however, the precise impact of changes to the oligomeric state of FtsA on the cell is still unclear. In particular, it would be interesting to determine whether mutations in FtsA that are impaired in self-interaction, such as FtsAG50E, display increased interaction with FtsN compared to WT FtsA, or if they are simply recruited more efficiently. If FtsAG50E were to exhibit increased affinity for FtsN, this may also explain why the overproduction of FtsN also makes FtsK dispensable for division (116, 117). In addition to direct recruitment of FtsN, it is also suggested that the bypass ability of certain mutations in FtsA may be caused by the increased affinity of these variants for the Z-ring (36, 75). In this instance, decreased self-interaction of FtsAG50E would not only increase the availability of domain 1C for interaction with downstream divisome proteins, but also increase the availability of domain 2B to interact with FtsZ. In support of this scenario, FtsAG50E has been shown to enhance ATP binding and hydrolysis in vitro, which are both properties required for interaction with FtsZ, and can restore FtsZ binding activity to a FtsZ binding deficient FtsA variant (R300E) in vivo (75). Undoubtedly, further experimentation is needed to pinpoint which of the above scenarios, or combination thereof, is responsible for the bypass of FtsK observed here.

The fact that early stage (ZipA), middle (FtsK) and late stage (FtsN) cell division proteins can be bypassed by single point mutations in FtsA displays how FtsA can exert multiple effects on the divisome at different stages. Based on the number of mutations of FtsA that are able to alleviate the requirement for FtsK during division, it is likely that its interaction with FtsK plays a
key role in regulating proper formation of the divisome under normal growth conditions. The novel interaction between FtsA and FtsK reported in our study not only provides an additional link between different stages of division, but also helps to unify our current understanding of how FtsK may perform its role as a cell division checkpoint.
CHAPTER 5: Conclusions and Future Directions

Prior to the start of our study, limited knowledge with respect to both the overall structure of FtsK and the protein contacts it makes during division severely hindered investigation into the precise function of this protein. We hypothesized that FtsK modulates septum formation during bacterial cell division through the formation of dynamic and essential protein-protein interactions with both the Z-ring and peptidoglycan synthesis machinery. Using three different experimental workflows, this thesis has not only refined our understanding of the structural topology of FtsK, but also uncovered a novel functional region in the protein, and confirmed interaction of FtsK with the Z-ring and peptidoglycan synthesis machinery via two novel protein interaction partners (RlpA and FtsA). Along with the isolation of a diverse periplasmic interactome involving FtsK, we highlight a key role for this protein in mediating cell envelope remodeling during division.

In Chapter 2, we report the use of site-directed fluorescence labeling to refine the membrane topology of FtsK. In doing so, we confirmed the presence of four transmembrane segments that anchor FtsK into the IM; however, most importantly, we identified a large shift in the topology of transmembrane segments 3 and 4 that revealed a novel periplasmic loop of FtsK that is critical for the protein’s function. Using fluorescence microscopy and high-resolution TEM, we demonstrated that mutations to the second periplasmic loop of FtsK (specifically residues 135–138) uncouple cell envelope septation events and produce large, asymmetric cellular voids. In these cells, we observed that the IM completely divides in the absence of clear invagination of either the OM or peptidoglycan layer, a phenomenon that has not been previously reported in the literature. As mentioned in Section 1.3.2, there is considerable debate over how cells generate the force necessary for constriction, whether it is through pulling on the IM by
constriction of the Z-ring, pushing on the IM by inward growth of the peptidoglycan layer, or a combination of both processes (101, 102). The production of the cellular voids caused by mutation of FtsK_N represents a significant advancement in this field, as it suggests that the Z-ring can generate sufficient force to completely divide the cytoplasm in the absence of septal cell wall synthesis, and is the first \textit{in vivo} evidence in support of this model.

In addition to its implication on constrictive force generation, the cellular void phenotype is also the first evidence that FtsK_N plays a significant role during cell envelope remodeling, and does not only act as a mechanism to sense the completion of chromosome segregation prior to septation. We suggest that mutation of the functional periplasmic loop of FtsK_N uncouples the switch necessary for the cell to complete septation, and that the FtsK checkpoint function might be specific to the transmission of the cell signal to shift from cell elongation to septation in \textit{E. coli}. In support of this, the FtsK interaction partners identified by our site-specific UV cross-linking (Chapter 3) and genomic suppressor screen (Chapter 4) provide several connections between FtsK and numerous components of the macromolecular protein complexes involved in elongation and division. For example, we detected a potential contact between FtsK_N and the bifunctional transglycosylase/transpeptidase PBP1a that is involved in lateral cell growth. In addition, we verified a direct interaction between FtsK_N and OM lipoprotein RlpA by an \textit{in vitro} pull-down assay. While the function of RlpA in \textit{E. coli} in not entirely clear, evidence on its enzymatic activity in \textit{P. aeruginosa} (195), and its localization and genomic arrangement in \textit{E. coli} (95, 97, 194, 213), support a role for RlpA in linking elongation and division. Significantly, we also observed partial restoration of growth and normal cell morphology upon the deletion of \textit{rlpA} from the temperature-sensitive \textit{E. coli} strain LP11-1 (ftsK44). These observations mark the first account of a phenotypic change caused by the deletion of \textit{rlpA} in \textit{E. coli}. Together, PBP1a and RlpA represent novel FtsK_N interactors and provide the first reported links between the
elongasome and divisome mediated by FtsK. Such a link would be essential for FtsK to function as a checkpoint between cell elongation and septation in \textit{E. coli}. However, whether the signal transmitted to FtsK regarding the state of elongation is facilitated through interaction with RlpA, PBP1a, both proteins or another protein altogether, requires further investigation.

Our examination of FtsK$_N$ protein interactors critical for FtsK$_N$ function also uncovered two novel interactions with essential cell division proteins. The \textit{in vitro} UV cross-linking analysis described in \textit{Chapter 3} revealed a potential contact between FtsK$_N$ and FtsN, while our genomic suppressor screen and \textit{in vitro} pull-down assay described in \textit{Chapter 4} showed direct interaction between FtsK$_N$ and FtsA. Together, these interactions with FtsK provide direct links between the early and late stages of division, and give insight into how FtsK$_N$ might sense proper formation of the Z-ring in the cytoplasm and subsequently trigger cell constriction. While interaction between FtsK$_N$ and both FtsA and FtsN have been reported in other bacterial species \cite{215,224}, we have provided the first evidence that FtsK$_N$ interacts with these essential proteins in \textit{E. coli}.

Based on the evidence reported in this thesis, we can only speculate on how each of these interactions might modulate the function of FtsK$_N$. Further investigation into the validity of each protein interaction detected by our cross-linking analysis is undoubtedly required. For those proteins that we have shown directly interact with FtsK$_N$ \textit{in vitro} (i.e., RlpA and FtsA), verification of their interaction \textit{in vivo} is also necessary to ensure each contact is physiologically relevant. In all cases, the use of standard biochemical techniques in conjunction with newly advanced imaging technologies will be central in determining the architecture of the protein complexes involved in cell growth and division. Significant increases in lateral resolution seen with super-resolution fluorescence microscopy (down to 10–20 nm \cite{226}) would aid in narrowing the precise localization of each protein during division, and provide further evidence for their interaction with FtsK$_N$ based on this higher-resolution co-localization data. In addition,
identification of the precise residues required for each interaction, for example by site-directed mutagenesis or Förster resonance energy transfer (FRET), would not only aid in understanding how FtsK may modulate multiple protein interactions, but would also benefit the development of novel cell division inhibitors that target these critical protein-protein contacts.

The exploitation of bacterial cell division as a potential target for the development of novel antibiotics has garnered much attention as pathogenic bacteria become increasingly resistant to the majority of current drugs. It represents an area of untapped potential, not only since division is essential for the survival of all living cells, but also due to the broad applicability they would have over a wide range of bacterial species. The various proteins involved in cell division are highly conserved throughout both Gram-negative and Gram-positive bacterial species, and are distinct from those involved in mammalian cell division (33, 80, 227, 228). With respect to FtsK as a potential target, most bacteria also have at least one member of the FtsK/SpoIIIE family of proteins (129). In addition, FtsK makes an even better target simply given the fact that it is a membrane protein. Membrane proteins are excellent targets for the development of novel antimicrobials, as a large number of functional regions are found in loops external to the cytoplasm, which makes them more accessible than intracellular targets (33). This location also circumvents issues surrounding drug-efflux pumps in some organisms, a major roadblock during the development of antibiotic resistance (33). As such, more than half of currently marketed drugs target membrane proteins, despite accounting for less than 1% of the structures in the Protein Data Bank (229, 230). The main focus of cell division inhibitors has been on the modulation of FtsZ polymerization and formation of the Z-ring (227). However, the interaction of FtsK with the proteins identified in this thesis also provide intriguing targets, especially those involving essential division proteins. Disruption of these interactions with a novel cell division inhibitor directed against FtsK could prevent bacterial cells from physically
dividing, and effectively limit harmful bacterial populations in the body. While our identification of a specific region of FtsK$_N$ critical for its function provides an excellent starting point for drug design, an atomic resolution structure of FtsK$_N$ would significantly aid in novel drug development.

Despite the fact that bacterial cell division is an essential process that has been intensely studied for many decades, we still know very little about the molecular mechanisms and protein interactions that govern formation and activity of the divisome. The findings of this thesis provide critical evidence on both the structure and function of FtsK$_N$, and advance our knowledge of the protein interactions that may influence its role as an essential checkpoint of bacterial cell division in \textit{E. coli}. With this, a greater understanding of what is necessary for bacteria to replicate and survive may not only lead to the development of novel therapeutic agents directed against pathogenic bacteria, but will continue to serve as a valuable platform for basic research.
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Identification of SPOR domain amino acids important for septal localization,


Appendix A1

Table A1.1. Protein groups detected by mass spectrometry of potential FtsK\textsubscript{N} interaction partners. Listed proteins were exclusively detected in at least one UV treated sample (absent in untreated and WT controls) of FtsK\textsubscript{N}* variants W51*, D135*, D136*, Y139* and L158*. Proteins which yielded a minimum of 2 unique peptides at >95% probability in all biological replicates are listed.

The abbreviations used are as follows: GO, gene ontology; IM, inner membrane; OM, outer membrane; PG, peptidoglycan; LPS, lipopolysaccharide.

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<th>Total Unique Peptides\textsuperscript{b}</th>
<th>GO Biological Process\textsuperscript{c}</th>
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<sup>a</sup> Calculated using the spectrum count detected across all samples.
<sup>b</sup> Sum of all unique peptides detected across all samples.
<sup>c</sup> Gene ontology biological process, as reported in the UniProtKB *E. coli* K12 database.
<sup>d</sup> GO biological process annotation not provided, term determined based on molecular function.
Appendix A2

A2.1 Experimental Procedures

A2.1.1 Lambda Red Deletion of ybhC

Deletion of \( ybhC \) from \( E. coli \) LP11-1 was achieved using the Lambda red recombinase method described by Datsenko and Wanner (197). Briefly, a PCR fragment containing a kanamycin resistance cassette flanked by 50 bp homology extensions of the upstream and downstream regions of \( ybhC \) was generated from template pKD4 using primers \( ybhC\_P1 \) (5′-AAGCAGGCTTCAACGGATTCATTTCATTTTCTATTTCCATAGCCGGAGCAACCTGTGAGGCTGGAGCTGCTTC-3′) and \( ybhC\_P2 \) (5′-GTAGGCCGGATAAGCGTTTACGCCGCATCCGGCAAATGTAAATTGCTCATGAGATCTCCTCCTTAG-3′). Transformation of the homologous PCR product into \( E. coli \) LP11-1 was achieved by electroporation as described in Section 3.3.10. Deletion of \( ybhC \) and insertion of the kanamycin resistance cassette was verified by colony PCR using primers homologous to a region 500 bp upstream of \( ybhC \) (\( \Delta ybhC \) For; 5′-GAAAAGTTTTGTTGTCACCTGCTACG-3′) and within the kanamycin resistance cassette (k1; 5′-CAGTCATAGCCGAATAGCCT-3′)(197). PCR verification of \( ybhC \) deletion was completed in duplicate along with control colonies of \( E. coli \) LP11-1.

A2.1.2 Growth Analysis of \( E. coli \) LP11-1 \( \Delta ybhC \)

To assess the impact \( ybhC \) deletion on cell growth and morphology, overnight cultures of LP11-1 \( \Delta ybhC \) were diluted to an A\(_{600}\) of 0.1 (SmartSpec™ Plus Spectrophotometer; Bio-Rad) in two separate 25-mL aliquots of fresh Complementation Media supplemented with 15 \( \mu \)g/mL tetracycline, and grown at 30°C or 42°C for 8 h (\( n = 3 \) per strain per temperature). The A\(_{600}\) of two technical replicates of each culture were sampled every hour and cells were imaged by phase contrast microscopy (Leica DM2000 LED, ProgRes CT3 camera; Jenoptik AG). Cell length was
assessed for 75 random cells using the ImageJ program (version 1.46r, National Institutes of Health) and is reported as mean length ± S.E. Statistical analysis for cell growth (A600) and mean length were completed using one-way analysis of variance with Tukey-Kramer multiple comparison post-tests by Prism 5 software (GraphPad Software, Inc.) using a level of significance of α = 0.05 for all tests. *E. coli* LP11-1 was also grown at 30°C and 42°C as described above for comparison.

**A2.1.3 Plasmid Construction**

For over-expression and purification of YbhC, an oligonucleotide encoding a soluble derivative of YbhC (amino acids 29 to 427) was cloned into expression vector pET28a following the protocol described in *Section 2.3.2* (163), using custom primers AMB007Fa (5′-TGGACCA-TGGATTATAAAGATGATGATGATAAATCCAGTCAACGTCCTTCTGATCAAAC-3′) and AMB007R (5′-AAGGTCAAGCTTACTTTACTTCTCGCTCTGCAACCACCTTACTACC-3′). The custom primers (Operon) were designed to introduce NcoI and HindIII restriction sites and an N-terminal FLAG®-tag to each construct. The resulting gene product was verified by DNA sequencing (Genomics Facility, Advanced Analysis Center, University of Guelph) to produce pSG003-1.

**A2.1.4 Expression and Purification of FLAG-YbhC**

Expression and purification of FLAG-YbhC was carried out as described in *Section 3.3.7*, with minor modification to the expression conditions. An overnight culture of *E. coli* Lemo21 cells carrying FLAG-YbhC (plasmid pSG003-1) was diluted 1:100 into 1 L LB media and grown at 37°C in a rotary shaker (200 rpm) for 1.5 h (A600 ~ 0.6). L-arabinose was added to a final concentration of 0.2% (w/v) and the culture was incubated for an additional 3.5 h at 37°C with
shaking. Cell lysis and purification using 300 µL of anti-FLAG® M2 affinity gel (Sigma-Aldrich) was performed exactly as described in Section 3.3.7. Total protein concentration of pooled, purified FLAG-YbhC was determined by a bicinchoninic acid protein assay as per manufacturer’s instructions (Thermo Scientific) using BSA as a standard.

A2.1.5 Pull-Down Assays

To assess direct protein interaction between FtsK_N and YbhC, pull-down assays were performed as described in Section 3.3.8, using 125 µg of purified YbhC as prey protein. Purified, resin-bound FtsK_N was obtained as described in Section 3.3.6. SDS-PAGE and Western immunoblotting of the flow through, wash and elution fractions was performed as described in Section 3.3.9.

A2.2 Results

Direct interaction between FtsK_N and YbhC was first assessed by an in vitro pull-down assay using His10-FtsK_N(220) and a FLAG®-tagged soluble derivative of YbhC (FLAG-YbhC). Purified FLAG-YbhC was incubated with purified, IMAC resin-bound FtsK_N and successively washed to remove unbound protein. Upon elution of His10-FtsK_N(220), YbhC was not detected in the elution fraction by Western blot analysis (Appendix Figure A2.1). To assess whether YbhC might have an indirect impact on FtsK function, we deleted ybhC in the temperature-sensitive E. coli strain LP11-1 (ftsK44) to generate the strain LP11-1 Δybhc, and assessed the impact of this deletion on cell growth and morphology in both the presence and absence of functional FtsK (i.e., at 30°C and 42°C, respectively). Deletion of ybhC from E. coli LP11-1 did not have a significant impact on either cell growth or morphology at either the permissive (30°C) or nonpermissive
(42°C) temperature (Appendix Figure A2.2). Taken together, these results suggest that YbhC is not a putative FtsK<sub>N</sub> interacting protein.

**Figure A2.1. In vitro analysis of interaction between FtsK<sub>N</sub> and YbhC.** Western blots of pull-down assays between FLAG-YbhC (left panel) and His<sub>10</sub>-FtsK<sub>N(220)</sub> (right panel). For both blots, the primary antibodies used are indicated in the bottom right corner. In all panels: ‘FT’ – flow through; ‘W1, W2, W3’ – wash fractions 1, 2, and 3; ‘Elution’ – elution with 1 M imidazole.
Figure A2.2. Cell growth and morphology analysis of E. coli strain LP11-1 Δybhc. A, growth curves, B, representative phase contrast micrographs, C, mean cell lengths, and D, cell length distribution of E. coli LP11-1 and the corresponding ybhc knockout strain (LP11-1 Δybhc). For growth curve analysis, overnight cultures were diluted to an $A_{600}$ of 0.1 in 25 mL Complementation Media supplemented with 15 µg/mL tetracycline and grown at permissive
(30°C) or nonpermissive (42°C) temperatures for 8 h. Micrographs depict typical cells of each strain after 0 or 8 h of growth at 42°C. The genotype of each strain is indicated in the upper left corner of each micrograph (bar, 10 µm). Cells were measured for cell length after 0 and 8 h of growth at 42°C using the ImageJ program and are reported as mean cell length (µm) ± S.E. (n = 75 cells per strain per temperature).