CHARACTERIZATION OF FTSA-FTSN INTERACTION DURING ESCHERICHIA COLI CELL DIVISION

kimberly.busiek@gmail.com K. Busiek

Follow this and additional works at: http://digitalcommons.library.tmc.edu/utgsbs_dissertations

Part of the Bacteria Commons, Bacteriology Commons, Biochemistry Commons, Biology Commons, Cell Biology Commons, Developmental Biology Commons, Microbial Physiology Commons, Molecular Biology Commons, Molecular Genetics Commons, and the Organismal Biological Physiology Commons

Recommended Citation
Busiek, kimberly.busiek@gmail.com K., "CHARACTERIZATION OF FTSA-FTSN INTERACTION DURING ESCHERICHIA COLI CELL DIVISION" (2014). UT GSBS Dissertations and Theses (Open Access). 454.
http://digitalcommons.library.tmc.edu/utgsbs_dissertations/454

This Dissertation (PhD) is brought to you for free and open access by the Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in UT GSBS Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact laurel.sanders@library.tmc.edu.
CHARACTERIZATION OF FTSA-FTSN INTERACTION
DURING ESCHERICHIA COLI CELL DIVISION

By

Kimberly Kennett Busiek, B.S.

APPROVED:

Supervisory Professor
William Margolin, Ph.D.

Peter Christie, Ph.D.

Carmen Dessauer, Ph.D.

Michael Lorenz, Ph.D.

Kevin Morano, Ph.D.

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston
CHARACTERIZATION OF FTSA-FTSN INTERACTION
DURING ESCHERICHIA COLI CELL DIVISION

A
Dissertation

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

By
Kimberly Kennett Busiek, B.S.
Houston, TX
May, 2014
Acknowledgements

I would like to thank my graduate advisor and mentor, William Margolin, for his guidance and unwavering support since I joined his laboratory in 2009. Bill has an admirable and relentless work ethic that has served as an excellent example for me throughout graduate school. His kindness and patience have always made me feel welcome to ask questions and explore new opportunities, which have ultimately helped me become a more critical, independent, and well-rounded scientist.

I would also like to thank past and current members of the Margolin laboratory for their insightful comments and technical assistance. Jennifer Herricks persuaded me to join the laboratory after serving as a patient and helpful mentor during my rotation. She has always been willing to discuss unexpected results and to provide feedback on my presentations and written work. Veronica Rowlett has been my pint-sized cheerleader throughout our time together in the laboratory, celebrating accomplishments and encouraging me when I encountered challenges. I would also like to acknowledge Yipeng Wang and Jesus Eraso for contributing bacterial two-hybrid and Far-Western experiments, respectively, to the third chapter of this thesis.

The advice and guidance of my advisory and supervisory committees have been critical to the development of this work. I would like to thank my current supervisory committee members who include Peter Christie, Carmen Dessauer, Mike Lorenz, and Kevin Morano. The constructive and thoughtful criticisms brought forth by the committee have helped drive this work to completion in a timely manner. I am also very appreciative of the countless letters of recommendation written by Mike and Kevin for scholarship and award applications. Their support also extended beyond these letters, and I am eternally grateful for their encouragement.
During my time as a GSBS student, I have interacted with a number of faculty, administrators, and students who have enhanced my graduate school experience. First, I am thankful to Terri Koehler, Chris Mackenzie, Ambro van Hoof, and Peter Christie for creating an opportunity for me to teach sections of the medical microbiology laboratory course. The teaching and presentation skills I gained during that time have helped me become a better speaker and mentor to rotating and undergraduate students. Second, I am indebted to Dean Andrew Bean and Ale Klauer for contributing significantly to my search for a career path and for opening doors when opportunities did arise. Third, I appreciate the tremendous work by administrators Lyz Morrison and Kim Campbell who supported my efforts to create promotional materials for the Microbiology and Molecular Genetics (MMG) program and Graduate Student Education Committee (GSEC). Last but not least, I would like to thank former and current MMG students who have shared ideas, know-how, and friendship with me over the last six years. I am particularly obliged to Jenny Dale, who has served as a knowledgeable and supportive mentor to me throughout her tenure at MMG and beyond.

I would like to dedicate this thesis to my family who has supported me in countless ways prior to and during my time at The University of Texas. My mother, Deborah Kennett, instilled a strong work ethic that has served me well, often repeating that I could “do anything if I worked hard enough” when I was young enough to believe it. I would also like to thank my sister, Dayna Kennett, and brother, Matt Kennett, who entertained (and luckily, survived) my earliest quests to investigate the world around us. They greatly fostered the curiosity required for legitimate scientific training. Finally, I would like to thank my husband, Jason Busiek, and his incredibly sweet and supportive parents. Jason has been my “biggest fan” and has made numerous sacrifices to support my educational pursuits. I am extremely fortunate to have him by my side, and I hope that I have made him proud.
CHARACTERIZATION OF FTSA-FTSN INTERACTION
DURING ESCHERICHIA COLI CELL DIVISION

Kimberly Kennett Busiek, B.S.

Supervisory Professor: William Margolin, Ph.D.

Division of a bacterial cell into two equal daughter cells requires precise assembly and constriction of the division machinery, or divisome. The *Escherichia coli* divisome includes nearly a dozen essential cell division proteins that assemble at midcell between segregating sister chromosomes. FtsZ, a homolog of eukaryotic tubulin, is the first essential cell division protein to localize at midcell where it polymerizes into a ring-shaped scaffold (Z ring). Establishment of the Z ring is required for recruitment of downstream cell division proteins including FtsA, a cytoplasmic protein that tethers the Z ring to the inner membrane. Following localization of FtsA and other early cell division proteins, a number of additional cell division proteins are recruited to midcell. The last essential cell division protein to be recruited to the divisome is FtsN, a transmembrane protein with a small cytoplasmic tail and a large periplasmic domain. Although the temporal order of divisome assembly is well established, the essential functions of the majority of the divisome proteins are not known. Additionally, many interactions among the cell division proteins have been suggested using *in vivo* assays, but few have been demonstrated biochemically. Identifying these functions and interactions is vital to understanding the mechanisms that drive bacterial cell division.

Although FtsA localizes to division sites early, previous data suggested that FtsA interacts with the late cell division protein FtsN. To confirm the FtsA-FtsN interaction observed *in vivo*, protein-protein interaction assays were performed *in vitro*. These assays showed that the interaction between FtsA and FtsN was direct, and that the cytoplasmic
and transmembrane domains of FtsN (FtsN<sub>Cyto-TM</sub>) were sufficient for interaction with FtsA. Suprisingly, FtsN<sub>Cyto-TM</sub> localized to midcell in an FtsA-dependent manner, independent of FtsN’s periplasmic localization domain (SPOR). I discovered that both the cytoplasmic and SPOR domains facilitate midcell localization of FtsN and that loss of both domains abolishes recruitment to the divisome. These results suggest that one role of FtsA-FtsN interaction is to localize FtsN more efficiently to midcell. Together, these data provide a better understanding of how and why <i>E. coli</i> cell division proteins interact to ensure faithful and robust operation of the divisome.
Table of Contents

Approval Sheet i
Title Page ii
Acknowledgements iii
Abstract v
Table of Contents vii
List of Figures ix
List of Tables xii

Chapter I: Introduction 1
  Chromosome replication 2
  Chromosome segregation 4
  Division site selection 6
  Divisome assembly 10
  Divisome constriction 15
  Gaps in knowledge and significance of research 17

Chapter II: Materials and Methods 21

Chapter III: The amino terminus of FtsN is required and sufficient for interaction 45
  with FtsA
  Introduction 46
  Results 49
  Discussion 70

Chapter IV: FtsA-FtsN interaction initiates localization of FtsN to division sites 74
  Introduction 75
### Chapter V: Overproduction of \( \text{FtsN}_{\text{cyto-M}} \) causes dominant negative effects in rich and minimal media

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>109</td>
</tr>
<tr>
<td>Results</td>
<td>110</td>
</tr>
<tr>
<td>Discussion</td>
<td>126</td>
</tr>
</tbody>
</table>

### Chapter VI: Discussion and Future Directions

- Structure of the FtsA-FtsN interaction interface
- Putative interactions among other early and late cell division proteins
- Suppression of the essential activity of FtsN by FtsA-E124A
- Additional roles for FtsA-FtsN interaction
- Relationship between FtsN and chromosome replication and segregation

### Bibliography

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
</tr>
</tbody>
</table>

### Vita

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>164</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1-1. Negative regulation of FtsZ polymerization in *E. coli.* 7

Figure 1-2. *E. coli* cell division, constriction, and separation proteins 11

Figure 2-1. DNA sequence of His$_6$-FtsN$_{Cyto}$-ParLeu-FLAG, His$_6$-FtsN$_{Cyto}$ AntiLeu-FLAG and His$_6$-GluGlu-FtsA 31

Figure 3-1. Co-affinity purification of FtsA and FtsN from cobalt resin suggests an *in vitro* interaction 50

Figure 3-2. The periplasmic region of FtsN is not required for interaction with FtsA in BACTH assays 52

Figure 3-3. Co-affinity purification of FLAG-FtsA and His$_6$-FtsN$_{Cyto}$ 54

Figure 3-4. Binding of purified FtsA to purified derivatives of FtsN and FtsN$_{Cyto}$ 57

Figure 3-5. BACTH assay showing positive interaction between N$_1$-54'PhoA and FtsN 60

Figure 3-6. Co-affinity purification of His$_6$-FtsA-1c and FtsN 64

Figure 3-7. FtsA1c-83-176 binding to FtsN and to FtsN$_{Cyto}$ 65

Figure 3-8. Overproduction phenotypes of FtsN and FtsN$_{Cyto}$ 68

Figure 4-1. The cytoplasmic domain of FtsN contributes to midcell localization independently of native FtsN 78

Figure 4-2. FtsA12(Ts) delocalizes from division sites within 5 minutes at 42°C 81

Figure 4-3. Localization of GFP-FtsN$_{Cyto}$ to midcell is dependent on FtsA 83

Figure 4-4. Localization of GFP- and TT$m$Cherry-tagged constructs is not disrupted at 42°C in wild-type cells 85

Figure 4-5. Localization of GFP-tagged FtsN$_{Cyto}$ and FtsN is not dependent
Figure 4-6. Localization of GFP-FtsN<sub>Cyto-TM</sub> in <i>ftsQ</i>(Ts) and <i>ftsI</i>(Ts) strains on FtsI activity 86

Figure 4-7. DivIVA-FtsA and DivIVA-FtsA-E124A recruit GFP-FtsN<sub>Cyto-TM</sub> to poles 90

Figure 4-8. Removal of both cytoplasmic and SPOR domains of FtsN abolishes midcell localization 92

Figure 4-9. GFP-FtsN<sub>Cyto-TM</sub> localizes to division sites prior to <sup>TT</sup>mCherry-FtsN<sub>SPOR</sub> 95

Figure 4-10. The cytoplasmic and SPOR domains are both needed for full FtsN function in cell division 98

Figure 4-11. Substitution and/or deletion of the cytoplasmic and SPOR domains of FtsN reduce viability in an <i>ftsN</i> depletion strain 102

Figure 4-12. Proposed role of FtsA-FtsN interactions in cell division 103

Figure 5-1. Overproduction of FtsN<sub>Cyto-TM</sub>, but not FtsI<sub>Cyto-TM</sub>, causes a cell division delay in rich medium 111

Figure 5-2. Overproduction of FtsN<sub>Cyto-TM</sub> causes a decrease in native FtsZ levels 114

Figure 5-3. Deletion of FtsZ protease components ClpP or ClpX restores FtsZ levels when FtsN<sub>Cyto-TM</sub> is overproduced but does not prevent delayed cell division 116

Figure 5-4. Overproduction of FtsN<sub>Cyto-TM</sub> causes cell shape and slow growth phenotypes when grown in minimal medium 118

Figure 5-5. Cells overproducing FtsN<sub>Cyto-TM</sub> often contain nucleoids trapped at midcell that occlude FtsZ 120

Figure 5-6. GFP-FtsN<sub>Cyto-TM</sub> localizes at midcell in cells with trapped nucleoids 121

Figure 5-7. Trapped nucleoids contain multiple replication initiation sites 123
Figure 5-8. Overproduction of $\text{FtsN}_{\text{Cyto-TM}}$ induces expression of the SOS reporter $\text{sulA-gfp}$

Figure 6-1. The hypothesized effect of early and late cell division proteins on stability of the Z ring

Figure 6-2. $\text{ftsN}$ loci of *Caulobacter crescentus* and *Escherichia coli*
List of Tables

Table 2-1. Strains and plasmids used in described studies 24
Table 2-2. Primers used in described studies 29
Table 4-1. Frequency of division site localization of GFP-tagged and TTmCherry-tagged division proteins in the ftsA12(Ts) strain 88
Table 4-2. Frequency of division site localization of GFP-tagged FtsN variants 93
Table 4-3. Distinct localization biases of two FtsN domains 96
Cell division is a fundamental process shared among all domains of life. For most prokaryotic organisms, splitting of a mother cell into two genetically identical daughter cells is accomplished by binary fission, a method of asexual reproduction. Binary fission must be tightly regulated to prevent untimely division of the mother cell, which requires coordination of chromosome replication and segregation with proper placement, assembly, and constriction of the cell division machinery, or divisome. This introduction provides a brief overview of the known factors involved in temporal and spatial control of cell division, focusing on the Gram negative bacterium, *Escherichia coli*. Current lapses in our understanding of bacterial cell division are also discussed.

1.1 Chromosome replication

1.1.1 Initiation

To generate genetically equal daughter cells, a bacterial mother cell must replicate and segregate its chromosomes prior to cytokinesis. Copying and partitioning the chromosomes occurs in three well studied steps: initiation, elongation, and termination. Initiation of chromosome replication is triggered by binding of *E. coli* protein DnaA to the origin of replication, or oriC (Fuller et al., 1984, Mott & Berger, 2007, Zyskind et al., 1977). DnaA is a highly conserved bacterial ATPase that, when bound to ATP, assembles at the oriC site (Katayama et al., 2010, Sekimizu et al., 1987). Assembly of DnaA-ATP at oriC separates complementary DNA strands and allows entrance of additional DNA replication machine or “replisome” components (Kaguni, 2011, Funnell et al., 1987). Specifically, helicase DnaB is recruited by DnaA-ATP and loaded onto single-stranded DNA at oriC by its cognate loader, DnaC (Wahle et al., 1989, Wickner & Hurwitz, 1975). DNA gyrase is also recruited to introduce negative supercoiling to counteract the positive supercoils formed
during DNA unwinding (Gellert et al., 1976). The helicase activity of DnaB and the modification of adenines at “GATC” sequences by DNA adenine methylase (Dam) aid separation of the duplex DNA while the reannealing of parental DNA strands is prevented by decoration of separated DNA strands by single stranded binding proteins (Smith et al., 1985, Meyer et al., 1979).

To prevent erroneous re-initiation of chromosome replication, *E. coli* protein SeqA binds hemimethylated GATC sites to preclude binding of DnaA-ATP (Lu et al., 1994). In *Bacillus subtilis* and other gram-positive organisms, YabA is a negative regulator of DNA replication initiation that inhibits assembly of DnaA-ATP (Noirot-Gros et al., 2006, Scholefield & Murray, 2013). In addition to SeqA and YabA, premature re-initiation is also prevented by the transcriptional control of dnaA as well as its slow exchange of ADP for ATP (Kawakami et al., 2006). Together, these methods ensure that the availability of DnaA-ATP peaks only once per cell cycle (Kaguni, 2006). In the event of stalls or DNA breaks that inhibit the progression of DNA replication, initiation of chromosome replication can be restarted by helicase PriA, which reloads the DnaBC complex (Zavitz & Marians, 1991).

### 1.1.2 Elongation

Following initiation, additional DNA replication proteins are loaded at oriC to replicate the circular, chromosomal DNA of *E. coli* in a bidirectional manner (Prescott & Kuempel, 1972). Primase DnaG dislodges DnaC, the DnaB loader, and synthesizes an RNA primer that is complementary to the DNA template (Rowen & Kornberg, 1978). The RNA-DNA duplex serves as the site of loading for the β sliding clamp, a ring-shaped processivity factor of DNA polymerase III (PolIII) (Stukenberg et al., 1991). PolIII then synthesizes a nascent DNA strand from the leading strand template by continuous strand synthesis in the 5’ to 3’ direction (Gefter et al., 1971). Because the lagging strand must also be synthesized in the 5’ to 3’ direction, the lagging strand is replicated in short Okazaki
fragments between multiple RNA primers synthesized by DnaG (Yudelevich et al., 1968). RNA primers are removed by the exonuclease activity of DNA polymerase I and replaced with DNA, after which the DNA fragments are enzymatically fused by ligase to form a single DNA strand (Lehman, 1974, Konrad & Lehman, 1974).

1.1.3 Termination

Without a dedicated means of replication termination, the two bidirectional replisomes would eventually collide or bypass one another. To circumvent this problem, the region of the circular chromosome opposite of the oriC contains multiple termination or “ter” sites (Kobayashi et al., 1989). The replication terminator protein, Tus, binds the chromosomal ter sites, preventing advancement of replisomes as the two replication forks meet (Hill et al., 1989, Kuempel et al., 1989). Once replication is complete, the sister chromosomes cannot completely segregate from one another until the linking or catenation of the DNA molecules is resolved. The unwinding activity of DNA gyrase prevents the majority of potential decatenation events (Steck & Drlica, 1984). However, when interlinking of the chromosomes cannot be prevented, topoisomerase IV, comprised of ParC and ParE subunits, decatenates the affected chromosomes (Kato et al., 1990). Without the decatenation activity of ParC, the replicated chromosomes would persist at midcell instead of segregating toward cell poles (Kato et al., 1988).

1.2 Chromosome segregation

Although entropic forces may play a passive role in chromosome segregation, the essential cell division protein FtsK (Filamentous temperature sensitive protein K) has been identified as an active participant in separation of sister chromosomes (Jun & Wright, 2010, Bigot et al., 2007). FtsK is a conserved bacterial DNA translocase that localizes to cell division sites at midcell (Begg et al., 1995, Yu et al., 1998a, Liu et al., 1998, Yu et al.,
Here, FtsK can pump DNA away from the closing division septum to prevent guillotining of chromosomes. In addition to its translocase activity, FtsK also resolves chromosomal dimers formed during DNA replication to assist sister chromosome separation (Steiner et al., 1999). To facilitate the activity of FtsK, E. coli structural maintenance of chromosome (SMC) proteins MukBEF are thought to correctly position the oriCs within the cell and condense replicating chromosomes (Case et al., 2004, Danilova et al., 2007). Diverse bacteria, including B. subtilis and the Gram-negative alpha-proteobacterium Caulobacter crescentus, also rely on SMC proteins for proper chromosome segregation. B. subtilis also produces ScpA, a member of the kleisin family of SMC protein partners, and ScpB, an ScpA-interacting protein. Together, ScpA and ScpB form a complex with SMC to regulate the activity of SMC on chromosome segregation. C. crescentus encodes predicted homologs of ScpA (CC_2005) and ScpB (CC_2004), although neither homolog has a demonstrated role in chromosome segregation in Caulobacter (Schwartz & Shapiro, 2011). Regardless, the broad use of SMC proteins suggests that precise orientation of chromosomes is a key step among a variety of organisms (Britton et al., 1998, Jensen & Shapiro, 1999).

In addition to FtsK and SMC proteins, B. subtilis and C. crescentus also utilize ParAB proteins to properly position chromosomes during sporulation and cell differentiation, respectively (Gruber & Errington, 2009, Ptacin et al., 2010). Until recently, a similar mechanism of chromosome segregation had not been reported in E. coli cells. However, a homolog of ParA, MinD, was shown in 2013 to bind and attach DNA to the inner membrane of E. coli cells, suggesting that MinD may participate in chromosome segregation (Di Ventura et al., 2013). MinD has an established role in division site selection (see section 1.3.1), which implies that MinD may function in coordinating chromosome segregation with proper placement of the divisome (Hu & Lutkenhaus, 2001).
1.3 Division site selection

1.3.1 The Min System

As replicated chromosomes segregate to cell poles, the division machinery begins to assemble at the middle of rod-shaped cells. In cocci-shaped bacterial cells such as streptococci, the divisome is built at the site of greatest width within the cell (Zapun et al., 2008). Two major mechanisms direct proper placement of the divisome in E. coli cells: the Min system and nucleoid occlusion (Fig. 1-1) (Margolin, 2006). The Min system consists of three proteins, MinCDE, which direct divisome assembly away from the poles of the cell (Hu & Lutkenhaus, 1999). MinC exerts its negative regulation of cell division by inhibiting the polymerization of FtsZ (Dajkovic et al., 2008). FtsZ is a scaffolding protein and tubulin homolog that polymerizes into a ring structure at division sites and is required for further assembly of the divisome (Erickson, 1997, Bi & Lutkenhaus, 1991, Ma et al., 1996).

To concentrate cytoplasmic MinC at the poles, where it can prevent aberrant FtsZ assembly, MinC binds MinD, an ATPase with a membrane targeting sequence that oscillates from one cell pole to the other (de Boer et al., 1991). Oscillation of MinCD is controlled by the cytoplasmic protein MinE. More specifically, MinE stimulates the ATP hydrolysis activity of membrane-bound MinD, causing MinCD to dissociate from the inner membrane and diffuse away from MinE (Fu et al., 2001). The diffusion of MinCD away from MinE results in a gradient of the FtsZ inhibitor MinC that is highest at cell poles and lowest at midcell, where FtsZ can begin assembling the divisome scaffold. As division proceeds, MinCD continues to oscillate to cell poles, but also pauses on either side of the division septum through an unknown mechanism (Juarez & Margolin, 2010). One purpose of the septal pausing of MinCD may be to stimulate the constriction of the Z ring during the later stages of division (Juarez & Margolin, 2010).

MinC, MinD, and MinE are not essential proteins, but loss of the Min system allows
Figure 1-1. Negative regulation of FtsZ polymerization in *E. coli*. As part of the Min system, MinC (green) prevents polymerization of FtsZ near the poles of *E. coli* cells. The nucleoid occlusion effector, SlmA, binds multiple sites on the sister chromosomes, where it inhibits Z ring formation over nucleoids (maroon ovals). Following DNA damage, SulA is produced as part of the SOS response to halt polymerization of FtsZ, including at potential division sites (grey). OpgH binds UDP-glucose when cells are grown in nutrient-rich conditions, preventing Z ring formation until cells become a larger size. ClpX also inhibits FtsZ polymerization, although the factors regulating its effect on FtsZ are not understood.
division septa to form near cell poles, producing DNA-free minicells (Zusman & Krotoski, 1974). Despite its non-essential role, the Min system is well conserved among bacteria. For example, some coccoid bacteria, including the human pathogen *Neisseria gonorrhoeae*, also utilize MinCDE to select division sites (Szeto *et al.*, 2001). Although *B. subtilis* contains MinC and MinD proteins, MinE is not present. Instead, *B. subtilis* utilizes DivIVA to anchor MinCD at sites of membrane curvature including cell poles and division septa (Harry & Lewis, 2003). The localization of DivIVA and MinCD to both poles and nascent septa of *B. subtilis* is quite similar to the localization pattern of MinCD at *E. coli* cell poles and (transiently) at midcell (Juarez & Margolin, 2010).

### 1.3.2 Nucleoid Occlusion

While the Min system prevents divisome assembly at cell poles, nucleoid occlusion inhibits aberrant divisome assembly over chromosomes or “nucleoids” (Wu & Errington, 2011). Like the Min system, nucleoid occlusion exacts its regulation through negative regulation of FtsZ polymerization (Tonthat *et al.*, 2013). The effector of nucleoid occlusion in *E. coli* is SlmA, a tetR repressor-like protein that binds SlmA-binding sequences (SBSs) located throughout the chromosome (Bernhardt & de Boer, 2005, Tonthat *et al.*, 2011, Cho *et al.*, 2011). Interestingly, the inhibitory activity of SlmA on FtsZ polymer formation is enhanced by binding of SlmA to SBSs, providing a mechanistic understanding of SlmA regulation (Cho *et al.*, 2011). *E. coli* cells lacking SlmA appear relatively normal, but removal of both MinCDE and SlmA frequently yields cells with divisomes forming at poles and on top of nucleoids (Bernhardt & de Boer, 2005). Despite frequent aberrant divisome formation, the majority of cells lacking the Min system and SlmA contain properly positioned divisomes, indicating that other regulators of FtsZ polymerization must be present (Cambridge *et al.*, 2014). Negative regulation of divisome assembly over nucleoids is not limited to *E. coli*. *B. subtilis*, for instance, also utilizes nucleoid occlusion to prevent
polymerization of FtsZ on top of nucleoids, although an unrelated DNA-binding protein, Noc, is used for this purpose (Wu & Errington, 2004). *C. crescentus*, on the other hand, utilizes a single protein, MipZ, to perform the functions of the Min system and nucleoid occlusion (Thanbichler & Shapiro, 2006).

### 1.3.3 Other negative regulators of FtsZ assembly

In addition to the Min system and nucleoid occlusion, *E. coli* cells utilize other proteins to exert negative regulation on divisome assembly. SulA, for example, is a negative regulator produced as part of the global SOS response to DNA damage (Schoemaker *et al.*, 1984). SulA sequesters FtsZ monomers to prevent polymerization of FtsZ, halting cell division until the DNA damage can be repaired (Chen *et al.*, 2012). Although SulA is not a well-conserved protein, *B. subtilis* protein YneA and *C. crescentus* protein SidA are similarly produced during the SOS response to prevent cell division (Kawai *et al.*, 2003, Modell *et al.*, 2011). YneA and SidA, however, affect later steps in divisome assembly.

Unlike SulA, ClpX is a well conserved negative regulator of FtsZ assembly. ClpX is an ATPase that can form a complex with the serine protease ClpP to degrade protein substrates (Wojtkowiak *et al.*, 1993). Deletion of *clpX* and *clpP* increases the abundance of temperature sensitive FtsZ (FtsZ84) in an *ftsZ84* strain, suggesting that ClpXP regulates FtsZ protein levels, likely through proteolysis (Camberg *et al.*, 2011). Despite its association with the proteolytic subunit ClpP, ClpX can also inhibit *in vitro* polymerization of *E. coli* and *B. subtilis* FtsZ in a ClpP-independent manner (Weart *et al.*, 2005, Camberg *et al.*, 2009). Additionally, overproduction of ClpX in either species induces a cell filamentation phenotype, indicating that cell division can be negatively regulated by ClpX *in vivo* (Camberg *et al.*, 2011).
Negative regulation of FtsZ assembly also occurs in response to environmental conditions. Specifically, FtsZ assembly is coordinated with nutrient availability by glucosyltransferases OpgH (*E. coli*) and UgtP (*B. subtilis*) (Hill *et al*., 2013, Weart *et al*., 2007). Expression of the genes encoding both proteins is upregulated during growth in nutrient-rich media, allowing more OpgH and UgtP molecules to bind UDP-glucose, an indicator of nutrient availability within the cell. The increase in OpgH and UgtP bound to UDP-glucose prevents cell division through negative regulation of FtsZ polymerization or bundling. This temporary ban on cell division allows cells to grow to a larger size prior to cytokinesis and accommodate many replicating copies of the bacterial chromosome.

### 1.4 Divisome assembly

#### 1.4.1 Early cell division proteins

Following chromosome replication and segregation, negative regulation of FtsZ by the Min system and nucleoid occlusion directs assembly of the division machinery toward the middle of the cell. In addition to FtsZ, the divisome is comprised of over a dozen essential and non-essential proteins that are recruited to the midcell Z ring in a temporal order (Fig. 1-2) (Vicente & Rico, 2006, Goehring & Beckwith, 2005). Loss of any essential cell division protein results in cell filamentation and eventual death (Ricard & Hirota, 1973). Assembly of all divisome components is dependent upon proper localization and polymerization of the first essential cell division protein to migrate to midcell, FtsZ (Bi & Lutkenhaus, 1991, Lutkenhaus *et al*., 1980, Ricard & Hirota, 1973). As noted above (see section 1.3.1), FtsZ is a homolog of eukaryotic tubulin that shares the ability to bind and hydrolyze GTP (de Boer *et al*., 1992a, Raychaudhuri & Park, 1992). Like tubulin, GTP-binding is required for polymerization of FtsZ into head-to-tail arranged protofilaments. Unlike tubulin, however, FtsZ protofilaments do not arrange into tube-like microtubule
Figure 1-2. *E. coli* cell division, constriction, and separation proteins. (A) The order of essential cell division protein recruitment. Proteins localizing to the midcell Z ring (B; top graphic) are drawn as topologically-relevant cartoons (B; bottom graphic). Early-localizing proteins (FtsZ, FtsA, ZipA, Zap) are depicted on the left side of the figure. Late-localizing proteins (FtsK, FtsQBL, FtsW, FtsI, FtsN, Ami [AmiBC], Tol-Pal complex [TolQARB and Pal]) are grouped on the right.
structures (Romberg et al., 2001). Instead, protofilaments interact laterally to bundle FtsZ into other configurations, including sheets (Erickson et al., 1996). These laterally-interacting protofilaments congregate at midcell, escaping the negative regulatory effects of the Min system and nucleoid occlusion (Margolin, 2006). Here, FtsZ polymerizes into a ring-shaped structure or “Z ring” that encircles the midcell between segregating chromosomes (Bi & Lutkenhaus, 1991). FtsZ is highly conserved among prokaryotes, including some members of the archaea that also rely on Z ring formation for cell division (Busiek & Margolin, 2011). FtsZ is also found among plants and protists that utilize Z rings for fission of chloroplasts and mitochondria, respectively, but is not present in animal organelles (Osteryoung & Vierling, 1995).

Because FtsZ is a cytoplasmic protein with no affinity for phospholipids, anchoring the Z ring to the inner membrane requires interaction between FtsZ and membrane associated proteins. In *E. coli* cells, two essential cell division proteins have overlapping roles in tethering the Z ring to the membrane: ZipA and FtsA (Ma et al., 1996, Raychaudhuri, 1999, Pichoff & Lutkenhaus, 2002, Pichoff & Lutkenhaus, 2005). ZipA (FtsZ interacting protein A) is a transmembrane protein with a short, periplasmic amino terminus, a single pass transmembrane region, and a long, carboxy-terminal cytoplasmic domain (Hale & de Boer, 1997). Unlike ZipA, FtsA is a homolog of eukaryotic actin and does not traverse the inner membrane (Ricard & Hirota, 1973, Bork et al., 1992). Instead, FtsA contains an amphipathic helix at its carboxy-terminus that allows FtsA to associate with the membrane (Pichoff & Lutkenhaus, 2005). This membrane targeting sequence (MTS) is required for proper tethering of the Z ring by FtsA.

Although ZipA and FtsA serve seemingly redundant functions to anchor the Z ring, FtsA appears to have a more dominant role in cell division. Not only is FtsA more widely conserved than ZipA, but a point mutation in FtsA (R286W, FtsA*) can bypass the need for ZipA (Geissler et al., 2003). Additionally, FtsA has a suspected role in both assembly and
disassembly of the Z ring. These contradictory activities are supported by in vitro evidence that FtsA* can shorten and curve the shape of FtsZ protofilaments, consistent with a role in Z ring disassembly (Beuria et al., 2009). Furthermore, FtsA promotes dynamic patterns of FtsZ filament formation and subunit exchange when both proteins are added with GTP and an adenosine nucleotide to supported lipid bilayers (Loose & Mitchison, 2014). Conversely, FtsZ dynamics were much slower when FtsA was replaced with ZipA. FtsA may therefore serve dual functions during cell division that include tethering the Z ring at midcell during the early stages of division and controlling disassembly of FtsZ protofilaments during the late stages of division.

In addition to ZipA and FtsA, the non-essential Zap proteins (FtsZ associated proteins) also contribute to assembly of the Z ring. Zap proteins ZapABCD co-localize with FtsZ at midcell (Low et al., 2004, Galli & Gerdes, 2010, Durand-Heredia et al., 2011), where all four Zap proteins act as positive regulators of FtsZ assembly, redundantly promoting bundling to increase the integrity of the Z ring during the early steps of divisome assembly (Monahan et al., 2009). Although non-essential, cells lacking ZapA are moderately filamentous, consistent with a cell division defect (Mohammadi et al., 2009). ZapA is well conserved among divergent bacteria as well as mitochondria of some eukaryotic cells, suggesting that the Zap proteins play important fine-tuning roles during assembly of the divisome (Gueiros-Filho & Losick, 2002).

1.4.2 Late cell division proteins

Once the early-localizing components of the divisome are in place, the late-localizing division proteins are recruited to midcell (Aarsman et al., 2005). A sequential order of recruitment dependency has been established based upon earlier studies. These studies utilized E. coli strains with conditional mutations in essential cell division proteins (reviewed in (Goehring & Beckwith, 2005)). Under non-permissive conditions, the
localization of “downstream” cell division proteins was hampered, thus allowing researchers to outline the order of divisome protein recruitment.

The first essential cell division protein to localize after establishment of the Z ring (and its associated membrane anchors) is FtsK (Begg et al., 1995, Yu et al., 1998a). FtsK is a multi-pass membrane protein with a demonstrated role in translocation of DNA away from the division site (see section 1.2) (Liu et al., 1998). The essential cell division function of FtsK, however, has not been identified (Yu et al., 1998b). Following recruitment of FtsK, essential cell division proteins FtsQBL join the divisome as a heterotrimer (Buddelmeijer & Beckwith, 2004). Like FtsK and most of the essential cell division proteins, the essential roles of FtsQ, FtsB, and FtsL are unknown. All three members of the trimeric complex are bitopic membrane proteins and each forms multiple interactions with cell division proteins outside of the FtsQBL trimer (Grenga et al., 2013, van den Berg van Saparoea et al., 2013). For this reason, the FtsQBL complex has a hypothesized role in “bridging” early and late cell division proteins.

The last three essential cell division proteins to localize to midcell are FtsW, FtsI, and FtsN (Dai et al., 1993, Wang et al., 1998). FtsW is recruited to midcell prior to FtsW and FtsI and shares a multi-pass membrane topology with FtsK (Boyle et al., 1997). The topological organization of FtsW is consistent with its flippase activity, which involves transportation of peptidoglycan intermediate Lipid II from the cytoplasm, across the inner membrane, and into the periplasm. Once Lipid II is flipped into the periplasm, it can be incorporated into nascent peptidoglycan by pencillin-binding proteins (PBPs) such as FtsI (Mohammadi et al., 2011, Fraipont et al., 2011). FtsI, or PBP3, is a transpeptidase enzyme that forms peptide cross-links between nearby glycan strands of the peptidoglycan layer, reinforcing the rigid structure (Botta & Park, 1981). Together, FtsW and FtsI contribute to septal peptidoglycan synthesis during E. coli cell division (Fraipont et al., 2011).
FtsN is the last essential cell division protein to localize to division sites in *E. coli* cells (Addinall *et al.*, 1997). FtsN is conserved among members of the γ, β, δ, and α-proteobacteria, but is not present in gram-positive organisms including *B. subtilis* (Moll & Thanbichler, 2009). Like FtsI, FtsN is a bitopic membrane protein with a short cytoplasmic tail and much longer periplasmic region (Dai *et al.*, 1996). The periplasmic region of FtsN contains a carboxy-terminal SPOR domain which helps target FtsN to the divisome (Gerding *et al.*, 2009). The essential role of FtsN is unknown, but FtsN has a putative role in the stimulation of FtsI’s transpeptidase activity and divisome stabilization (Rico *et al.*, 2010, Muller *et al.*, 2007). FtsN may also coordinate septal peptidoglycan synthesis with peptidoglycan splitting and outer membrane invagination (see section 1.5). Because of its very late recruitment to the divisome, FtsN is well-positioned to integrate the final steps of cell division including peptidoglycan building and shaping as well as constriction of the outermost layer of the cell envelope (Lutkenhaus, 2009).

### 1.5 Divisome constriction

Upon completion of divisome assembly, the cell is poised to constrict and separate all three layers of the *E. coli* cell envelope: the inner membrane, the peptidoglycan layer, and the outer membrane. The factor(s) required to trigger the constriction phase of *E. coli* cell division is not known. Although still controversial, the membrane-bound Z ring has been implicated as the driving force that invaginates the inner membrane (Hsin *et al.*, 2012). This conclusion is based largely on the ability of membrane-targeted FtsZ to pinch liposomes, or laboratory-created vesicles composed of a lipid bilayer (Osawa & Erickson, 2013). Protofilaments of FtsZ are capable of shortening and curling, which may provide the force needed to deform membranes (Erickson *et al.*, 2010). Because other essential members of the divisome are peripherally or integrally associated with the inner membrane, constriction
of the Z ring would also affect the invagination of other membrane-associated cell division proteins.

FtsI is an important factor in building peptidoglycan at the division site. Septal peptidoglycan synthesis, however, requires the activity of FtsI and at least one additional transpeptidase: PBP1A, PBP1B, or Mtg (Botta & Park, 1981). Additionally, inward growth of septal peptidoglycan on the inner membrane-proximal side of the periplasm must be counteracted with splitting of peptidoglycan on the outer membrane-proximal edge of the peptidoglycan layer. Concurrent building and splitting of peptidoglycan at division sites allows the inner membrane, peptidoglycan layer, and outer membrane to constrict together, forming rounded *E. coli* cell poles. In *B. subtilis* cells, the septal peptidoglycan layer is synthesized prior to peptidoglycan splitting, forming chains of cells with blunt poles (Adams & Errington, 2009).

The splitting of the peptidoglycan layer is orchestrated by murein amidases AmiA, AmiB, and AmiC (Heidrich *et al*., 2001). AmiB and AmiC localize to division sites in an FtsN-dependent manner (Peters *et al*., 2011). Conversely, AmiA does not localize to division sites but peripherally along the periplasm (Cormack *et al*., 1996). The peptidoglycan hydrolysis activity of all three amidases is controlled by their cognate activators: EnvC and NlpD (Uehara *et al*., 2009). AmiA and AmiB are activated by protein EnvC, while AmiC is activated by NlpD. Like AmiBC, EnvC and NlpD localize to division sites (Peters *et al*., 2011). The midcell recruitment of NlpD is dependent upon proper localization of FtsN, while the factor responsible for division site localization of EnvC is unknown (Peters *et al*., 2011).

The constriction of the outer membrane was previously considered a passive process in *E. coli* cells, dictated by invagination of the associated inner membrane and peptidoglycan layer (de Boer, 2010). Current data, however, suggest that the Tol-Pal complex plays a major role in outer membrane invagination (Gerding *et al*., 2007). The Tol-
Pal complex includes three inner membrane proteins (Tol proteins TolQAR), one periplasmic protein (TolB), and a single outer membrane protein (Pal) that, together, span the entire cell envelope. Communication between the Tol-Pal complex and the divisome appears to occur through FtsN, as recruitment of TolA to midcell requires the essential activity of FtsN.

1.6 Gaps in knowledge and significance of research

Molecular, genetic, and biochemical assays have identified a number of proteins and mechanisms involved in bacterial chromosome replication and segregation as well as subsequent division of the mother cell. Despite these advances, the essential functions of many divisome proteins remain unknown (de Boer, 2010). Additionally, putative interactions among many essential cell division proteins have been suggested, but few have been demonstrated biochemically (Goehring et al., 2005, Karimova et al., 2005, Goehring et al., 2006). These key data must be obtained in order to better understand the fundamental process of prokaryotic cell division. Furthermore, deciphering the web of interactions that govern bacterial cytokinesis could help identify novel targets for new antimicrobial therapies (Singh & Panda, 2010, Haydon et al., 2008).

For this study, the putative interaction between essential cell division proteins FtsA and FtsN was investigated in detail. The essential functions of FtsA and FtsN are not known, but several nonessential functions of both proteins have been ascribed. As mentioned in section 1.4, FtsA localizes to division sites early, where it aids membrane anchoring of the Z ring, but may also promote disassembly of the Z ring during the later stages of division (Pichoff & Lutkenhaus, 2005, Beuria et al., 2009). FtsN, on the other hand, is a late-localizing cell division protein that likely coordinates peptidoglycan synthesis and degradation as well as outer membrane invagination (Peters et al., 2011, Addinall et al., 1997, Muller et al., 2007, Gerding et al., 2009, Bernard et al., 2007). Despite the
temporal differences in FtsA and FtsN localization, multiple in vivo assays have suggested that FtsA and FtsN interact (see section 3.1 for details) (Corbin et al., 2004, Rico et al., 2004, Karimova et al., 2005). A genetic interaction between ftsA and ftsN is apparent from the manner in which ftsN was identified. The ftsN gene was discovered during a screen for multi-copy suppressors of an FtsA temperature sensitive mutant (Dai et al., 1993). Likewise, FtsA mutants FtsA* and FtsA-E124A can partially or completely bypass the need for FtsN (Bernard et al., 2007). FtsA also affects midcell recruitment of FtsN; in cells lacking FtsA, a ZapA-FtsQ fusion protein recruits all essential late proteins to the divisome except FtsN (Goehring et al., 2005). It is unclear how FtsA assists recruitment of FtsN to the divisome and if this recruitment is direct or indirect.

Describing both in vivo and in vitro approaches, I developed a research plan to determine 1) if FtsA and FtsN interact directly and 2) how and why they interact. Chapter Three details the bacterial two-hybrid, co-affinity purification, and Far Western methodologies utilized to confirm FtsA-FtsN interaction. The domains of each protein involved in FtsA-FtsN interaction were also verified. The 1C subdomain of FtsA, unique among actin homologs, was sufficient for interaction with FtsN. Although the cytoplasmic domain of FtsN was required for interaction with FtsA, it was not sufficient. Instead, the cytoplasmic domain required fusion to the transmembrane domain of FtsN or a parallel leucine zipper to maintain interaction with FtsA. These data suggested that self-interaction of FtsN is required for FtsA-FtsN interaction.

After confirming interaction between FtsA and FtsN, I focused on the physiological role(s) of FtsA-FtsN interaction during E. coli cell division. I had originally hypothesized that FtsN transduced a signal to FtsA, indicating that assembly of the divisome was complete. I also proposed that this signaling event triggered the FtsZ disassembly activity of FtsA during late cell division (Beuria et al., 2009). While testing this hypothesis, however, I confirmed earlier observations that the interaction between FtsA and FtsN is required for
efficient localization of FtsN, as detailed in Chapter Four. Removal of either the FtsA-interacting portion of FtsN or its other localization determinant, the periplasmic SPOR domain, did not abrogate division site localization, but removal of both domains completely abolished midcell localization of FtsN. Importantly, both single mutants survived the deletion of native \textit{ftsN} when they were overproduced, but the double mutant did not survive the loss of \textit{ftsN} at any level of induction. Together, these data strongly suggest that one physiological role of FtsA-FtsN interaction is efficient recruitment of FtsN to the divisome.

In addition to determining if and how FtsA and FtsN interact, I also showed in Chapter 3 that overproduction of the cytoplasmic and transmembrane domains of FtsN (FtsN$_{\text{Cyto-TM}}$) caused moderate filamentation of cells. I investigated this dominant negative phenotype further, hoping to glean information about other possible roles of FtsN. These experiments are described in Chapter 5. Briefly, I observed that Z rings continued to form in cells overproducing FtsN$_{\text{Cyto-TM}}$, but that the overall level of cellular FtsZ decreased. Additional experimentation, however, showed that the drop in FtsZ was not correlated with the cell filamentation phenotype. Because filamentation only occurred as cells were grown to late logarithmic or stationary phase, I hypothesized that this phenotype was linked to poor nutrient availability. To test this idea, I overproduced FtsN$_{\text{Cyto-TM}}$ in cells growing in a poor nutrient source (minimal medium) in order to exacerbate the dominant negative phenotype. Indeed, these cells exhibited more severe phenotypes, including a curved cell shape with nucleoids trapped at midcell and Z rings flanking either side of the DNA. The chromosomes of these cells appeared to undergo replication, but segregation was hampered. Inhibited chromosome segregation likely explains the DNA damage and slow growth that was also observed with these cells. Because these results rely on overproduction of FtsN$_{\text{Cyto-TM}}$, additional experiments will be required to understand how FtsN affects the segregation of sister chromosomes and if these effects involve interaction with FtsA.
In summary, the results presented herein contribute to our understanding of how FtsA and FtsN interact and why this interaction is important to *E. coli* cell division. This work also embodies the first biochemical demonstration of interaction between early and late essential cell division proteins in *E. coli*. Because FtsA and FtsN are conserved among many classes of bacteria including the proteobacteria classes, their interaction may serve similar roles in other organisms (Moll & Thanbichler, 2009). Finally, the unexpected phenotypes observed upon overproduction of the FtsA-interacting portion of FtsN suggest that the interaction between FtsA and FtsN could have additional physiological effects.
Chapter II

Materials and Methods

2.1 Strains and growth media

All E. coli strains were grown in Luria-Bertani (LB) medium or M9 minimal medium containing 1% glycerol at 37°C, unless otherwise indicated. Media were supplemented with ampicillin (Fisher Scientific; 50-100 µg ml⁻¹), kanamycin (Sigma-Aldrich; 50 µg ml⁻¹), chloramphenicol (Acros Organics; 10-20 µg ml⁻¹), glycerol (Fisher Scientific; 1%) and glucose (Sigma-Aldrich; 1%) as needed. To inhibit FtsI activity (Fig. 4-3), cultures were treated with cephalexin (Sigma-Aldrich) at a final concentration of 200 µg ml⁻¹ for 30 or 60 minutes. Gene expression from pET28a, pKT25F, pUT18c, pRR48, pDSW207, and pDSW210 vectors was induced with isopropyl-β-D-galactopyranoside (IPTG) at concentrations indicated. The araBAD promoter in pBAD30 and pBAD33 vectors was induced at a final concentration of 0.2% L-arabinose and the nahR promoter in pKG116 vectors was induced with 1.0 µM sodium salicylate. JM109 (DE3) and BL21 (DE3) strains were used for expression from pET28a and its derivatives. All vectors were transformed into XLI, DHMI, DH5α, WM1115 [ftsA12(Ts)], WM2739 (sulA-gfp), WM3114 (clpX),
WM4007 (clpP), WM4049 (1074/pBAD33), WM4050 (1074/pBAD33-ftsN<sub>Cyto-TM</sub>), or wild-type strains W3110 or WM1074. Strains and plasmids are listed in Table 2-1.

The ftsN depletion strain WM4028 was created by transducing strain WM1074 carrying pWM2964 (a temperature sensitive depletion plasmid encoding ftsN) with P1 phage carrying ∆ftsN::kan, selecting for Kan<sup>R</sup> colonies, and screening for colonies that could grow at 30°C but not at 42°C. Similarly, strain WM3302 was constructed by transducing strain WM2935 (W3110 Tn10 ftsA-124A) with ∆ftsN::kan P1 lysate and selecting for Kan<sup>R</sup> survivors.

Strains were observed using an Olympus BX60 microscope and UPlanSApo 100X oil immersion objective. Differential interference contrast (DIC) and fluorescence images were captured on a Hamamatsu Digital Camera (Model C8484) using HCImageLive (Hamamatsu) software. Cellular membranes were stained as needed to assist visualization by incubating 1 mL cultures with 0.5 µg mL<sup>-1</sup> FM4-64 stain for 15 minutes at room temperature.

2.2 DNA and protein manipulation and analysis

Standard protocols or manufacturer's instructions were used to isolate plasmid DNA, as well as for restriction endonuclease, DNA ligase, PCR, and other enzymatic treatments of plasmids and DNA fragments. Phusion high-fidelity DNA polymerase New England Biolabs, Inc. (NEB; Beverly, MA) and KAPA HiFi DNA polymerase from VWR (Radnor, PA) were used as the high-fidelity DNA polymerases. Oligonucleotides were purchased from Sigma Aldrich and Integrated DNA Technologies (IDT; Coralville, IA). Restriction endonucleases and T4 DNA ligase enzymes were purchased from NEB and Invitrogen (Carlsbad, CA). Plasmid DNA was purified using the Wizard SV miniprep and PCR clean-up kits from Promega (Madison, WI.). The final versions of all relevant clones were sequenced by Genewiz (South Plainfield, NJ) to verify their construction. Protein
Table 2-1. Strains and plasmids used in described studies.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>Wild-type strain</td>
<td></td>
</tr>
<tr>
<td>WM1074</td>
<td>MG1655 <em>lacU169</em> (TX3772)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>WM1115</td>
<td>TX3772 <em>leu::Tn10ftsA12(Ts)</em></td>
<td>Geissler <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>WM1260</td>
<td>BL21(DE3)/pWM1260</td>
<td>Beuria <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>WM1835</td>
<td>BL21(DE3)/pWM1833</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>WM2193</td>
<td><em>ftsQ</em>(Ts) strain TOE1, K12 parent strain AB2497</td>
<td>Begg <em>et al.</em> (1980)</td>
</tr>
<tr>
<td>WM2348</td>
<td><em>ftsI</em>(Ts) strain AX655, K12 parent strain AB1157</td>
<td>Allen <em>et al.</em> (1974)</td>
</tr>
<tr>
<td>WM2355</td>
<td>FtsN depletion strain (<em>ΔftsN::cat</em>) with <em>ftsN</em> expressed from a plasmid with a Ts replication origin</td>
<td>Bernard <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>WM2700</td>
<td>W3110/pWM2700</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>WM2739</td>
<td>TX3772 chromosomal <em>sulA-gfp</em> reporter</td>
<td>Geissler <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>WM2935</td>
<td>W3110 (WT strain) <em>leu::Tn10ftsA-E124A</em></td>
<td>Shiomi and Margolin (2008)</td>
</tr>
<tr>
<td>WM2964</td>
<td>FtsN depletion strain JKD42 (W3110 <em>srt::Tn10 recA56ΔftsN::kan</em>); <em>ftsN</em> expressed from a plasmid with a Ts replication origin (pKD123)</td>
<td>Dai &amp; Lutkenhaus (1993)</td>
</tr>
<tr>
<td>WM3114</td>
<td>BW25113 (K-12 strain) <em>clpX::kan</em></td>
<td>Baba <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>WM3302</td>
<td>WM2935 <em>ΔftsN::kan</em></td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>WM3428</td>
<td>BL21(DE3)/pWM2254</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>WM3616</td>
<td>BL21(DE3)/pWM3616</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>WM4007</td>
<td>BW25113 (K-12 strain) <em>clpP::kan</em></td>
<td>Baba <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>WM4028</td>
<td>WM1074 <em>ΔftsN::kan</em> pWM2964</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>WM4031</td>
<td>WM2964/pDSW210</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>WM4033</td>
<td>WM2964/pWM4032</td>
<td>Busiek <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>WM4035</td>
<td>WM2964/pWM4034</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>WM4049</td>
<td>WM1074/pBAD33</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>WM4050</td>
<td>WM1074/pWM4050</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>WM4051</td>
<td>WM1074/pWM4051</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>WM4228</td>
<td>WM1074/pWM4228</td>
<td>This work</td>
</tr>
<tr>
<td>WM4240</td>
<td>WM4007/pBAD33</td>
<td>This work</td>
</tr>
<tr>
<td>WM4241</td>
<td>WM4007/pWM4050</td>
<td>This work</td>
</tr>
<tr>
<td>WM4243</td>
<td>WM3114/pBAD33</td>
<td>This work</td>
</tr>
<tr>
<td>WM4244</td>
<td>WM3114/pWM4050</td>
<td>This work</td>
</tr>
<tr>
<td>WM4279</td>
<td>WM2739/pBAD33</td>
<td>This work</td>
</tr>
<tr>
<td>WM4280</td>
<td>WM2739/pWM4050</td>
<td>This work</td>
</tr>
<tr>
<td>WM4282</td>
<td>WM2739/pWM4228</td>
<td>This work</td>
</tr>
<tr>
<td>WM4359</td>
<td>WM4049/pWM1492</td>
<td>This work</td>
</tr>
<tr>
<td>WM4360</td>
<td>WM4050/pWM1492</td>
<td>This work</td>
</tr>
<tr>
<td>WM4362</td>
<td>WM4228/pWM1492</td>
<td>This work</td>
</tr>
<tr>
<td>WM4558</td>
<td>WM4050/pWM4528</td>
<td>This work</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[Fr-] proAB lacF ΔM15] Tn10</td>
<td>Stratogene</td>
</tr>
<tr>
<td></td>
<td>FhuA2 [Jon] ompT gal(DE3) [dcm] ΔhsdS</td>
<td>Novagen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>FhuA2 Δ(argF-lacZ)U169 phoA glnV44 Δ(lacZ) M15 gyrA96 recA1 relA1 endA1 thy-1 hsdR17</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α</td>
<td>F- glnV44(AS) recA1 endA gyrA96 thi-1 hsdR17 spoT1 rfbD1 cya-854</td>
<td>Karimova et al. (2005)</td>
</tr>
<tr>
<td>DHMI</td>
<td>endA1 recA1 gyrA96 thi hsdR17(rv- mcv-) relA1 supE44 Δ(lac-proAB) [Fr- traD36 proAB lacF ΔM15]</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17(rv- mcv-) relA1 supE44 Δ(lac-proAB) [Fr- traD36 proAB lacF ΔM15]</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td></td>
<td>Plasmids</td>
<td></td>
</tr>
<tr>
<td>pBAD30</td>
<td>Derivative of pDHB60 with araBAD promoter</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Derivative of pDHB60 with araBAD promoter</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pDSW207</td>
<td>P_{trc-gfp} pBR322 derivative with strong promoter</td>
<td>Weiss et al. (1999)</td>
</tr>
<tr>
<td>pDSW210</td>
<td>P_{trc-gfp} pBR322 derivative with weak promoter</td>
<td>Weiss et al. (1999)</td>
</tr>
<tr>
<td>pET28a</td>
<td>Expression vector with PT7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pKG116</td>
<td>pACYC184 derivative containing the nahG promoter</td>
<td>J.S. Parkinson</td>
</tr>
<tr>
<td>pKT25F</td>
<td>T25 fragment of B. pertussis cyaA (residues 1 to 224) in pSU40; FLAG sequence at N terminus of T25</td>
<td>Modified from Karimova et al. (2005)</td>
</tr>
<tr>
<td>pRR48</td>
<td>Derivative of pBR322 with P_{lac} promoter and ideal lacO</td>
<td>J.S. Parkinson</td>
</tr>
<tr>
<td>pUT18c</td>
<td>T18 fragment of B. pertussis cyaA (residues 225 to 399) in</td>
<td>Karimova et al. (2005)</td>
</tr>
</tbody>
</table>

**Plasmids**

- **pBAD30**: Derivative of pDHB60 with *araBAD* promoter [Guzman et al. (1995)]
- **pBAD33**: Derivative of pDHB60 with *araBAD* promoter [Guzman et al. (1995)]
- **pDSW207**: *P_{trc-gfp} pBR322* derivative with strong promoter [Weiss et al. (1999)]
- **pDSW210**: *P_{trc-gfp} pBR322* derivative with weak promoter [Weiss et al. (1999)]
- **pET28a**: Expression vector with PT7 promoter [Novagen]
- **pKG116**: pACYC184 derivative containing the *nahG* promoter [J.S. Parkinson]
- **pKT25F**: T25 fragment of *B. pertussis cyaA* (residues 1 to 224) in pSU40; FLAG sequence at N terminus of T25 [Modified from Karimova et al. (2005)]
- **pRR48**: Derivative of pBR322 with *P_{lac} promoter* and ideal *lacO* [J.S. Parkinson]
- **pUT18c**: T18 fragment of *B. pertussis cyaA* (residues 225 to 399) in [Karimova et al. (2005)]
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19; compatible with pKT25F</td>
<td>Weiss et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>pWM1088</td>
<td>gfp in pDSW207</td>
<td>Corbin et al. (1999)</td>
</tr>
<tr>
<td>pWM1152</td>
<td>gfp-ftsN in pDSW207</td>
<td>Corbin et al. (2004)</td>
</tr>
<tr>
<td>pWM1492</td>
<td>gfp-seqA in pDSW209</td>
<td>Brendler et al. (2009)</td>
</tr>
<tr>
<td>pWM1806</td>
<td>divIVA-ftsA in pBAD33</td>
<td>Corbin et al. (2004)</td>
</tr>
<tr>
<td>pWM2964</td>
<td>pKD123 (Ts ftsN plasmid)</td>
<td>Dai et al. (1993)</td>
</tr>
<tr>
<td>pWM3021</td>
<td>T18-ftsA in pUT18c</td>
<td>Shiomi and Margolin (2007)</td>
</tr>
<tr>
<td>pWM3451</td>
<td>T25-ftsN1-54-'phoA in pKT25F (residues 1 to 54 of FtsN plus residues 63 to 471 of PhoA)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3616</td>
<td>his-e'ftsN_Cyto-TM in pET28a (residues 1 to 55 of FtsN)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3775</td>
<td>gfp-ftsZ in pDSW207</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM3866</td>
<td>T25-virB101-60-'phoA in pKT25F (residues 1 to 60 of VirB10 plus residues 63 to 471 of PhoA)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3947</td>
<td>his-e'ftsN_Cyto-AntiLeu-flag in pET28a (residues 1 to 33 of FtsN plus the anti-parallel leucine zipper of B. subtilis MtaN)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3948</td>
<td>his-e'ftsN_Cyto-flag in pET28a (residues 1 to 33 of FtsN)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3949</td>
<td>his-e'ftsN_Cyto-ParLeu-flag in pET28a (residues 1 to 33 of FtsN plus the parallel leucine zipper of S. cerevisiae GCN4)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3950</td>
<td>his-e'ftsN-flag in pET28a</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM3951</td>
<td>his-e'ftsN_Cyto-DDEE-ParLeu-flag in pET28a (residues 1 to 33 of FtsN containing the RRKK&gt;DDEE mutation at residues 16-19 plus the parallel leucine zipper)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4032</td>
<td>ftsN-flag in pDSW210</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4034</td>
<td>ftsN_DDDEE-flag in pDSW210 (residues 1 to 319 of FtsN containing the RRK&gt;DDEE mutation at residues 16-19)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4050</td>
<td>his &lt;sup&gt;e&lt;/sup&gt;-ftsN&lt;sub&gt;Cyto-TM&lt;/sub&gt; in pBAD33 (residues 1 to 55 of FtsN)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>pWM4051</td>
<td>his &lt;sup&gt;e&lt;/sup&gt;-ftsN in pBAD33</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4093</td>
<td>his &lt;sup&gt;e&lt;/sup&gt;-gluglu-ftsA in pET28a</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4094</td>
<td>his &lt;sup&gt;e&lt;/sup&gt;-gluglu-ftsA-1c in pET28a (residues 81 to 140 of FtsA)</td>
<td>Busiek et al. (2012)</td>
</tr>
<tr>
<td>pWM4228</td>
<td>his &lt;sup&gt;e&lt;/sup&gt;-ftsI&lt;sub&gt;Cyto-TM&lt;/sub&gt; in pBAD33 (residues 2 to 40 of FtsI)</td>
<td>This work</td>
</tr>
<tr>
<td>pWM4528</td>
<td>gfp-ftsN&lt;sub&gt;Cyto-TM&lt;/sub&gt; in pDSW207 (residues 2 to 55 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4582</td>
<td>flag-virB10&lt;sub&gt;Cyto-ftsN&lt;sub&gt;TM-Per&lt;/sub&gt;&lt;/sub&gt; in pDSW210 (residues 2 to 32 of VirB10 plus residues 29 to 319 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4610</td>
<td>gfp-virB10&lt;sub&gt;Cyto-ftsN&lt;sub&gt;TM&lt;/sub&gt;&lt;/sub&gt; in pDSW207 (residues 2 to 32 of VirB10 plus residues 29 to 55 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4611</td>
<td>gfp-ftsN&lt;sub&gt;Cyto-virB10&lt;sub&gt;TM&lt;/sub&gt;&lt;/sub&gt; in pDSW207 (residues 2 to 33 of FtsN plus residues 33 to 50 of VirB10)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4612</td>
<td>flag-virB10&lt;sub&gt;Cyto-ftsN&lt;sub&gt;TM-PerΔSPOR&lt;/sub&gt;&lt;/sub&gt; in pDSW210 (residues 2 to 32 of VirB10 plus residues 29 to 242 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4613</td>
<td>flag-ftsN&lt;sub&gt;ΔSPOR&lt;/sub&gt; in pDSW210 (residues 2 to 242 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4637</td>
<td>divIVA-ftsA-E124A in pBAD33</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4693</td>
<td>gfp-ftsN&lt;sub&gt;ΔSPOR&lt;/sub&gt; in pDSW207 (residues 2 to 242 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4694</td>
<td>gfp-virB10&lt;sub&gt;Cyto-ftsN&lt;sub&gt;TM-Per0,SPOR&lt;/sub&gt;&lt;/sub&gt; in pDSW207 (residues 2 to 32 of VirB10 plus residues 29 to 242 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4696</td>
<td>gfp-virB10&lt;sub&gt;Cyto-ftsN&lt;sub&gt;TM-Per&lt;/sub&gt;&lt;/sub&gt; in pDSW207 (residues 2 to 32 of VirB10 plus residues 29 to 319 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
<tr>
<td>pWM4740</td>
<td>&quot;torA signal sequence&quot;mCherry-ftsN&lt;sub&gt;SPOR&lt;/sub&gt; in pKG116 (residues 241 to 319 of FtsN)</td>
<td>Busiek and Margolin (2014)</td>
</tr>
</tbody>
</table>
concentrations were determined using the bicinchoninic acid (BCA) assay from Thermo scientific (Rockford, IL).

2.3 Plasmid constructions

All plasmids and primers used in this work are listed in Tables 2-1 and 2-2, respectively. DNA sequences of leucine zipper chimeras and GluGlu-tagged FtsA are shown in Fig. 2-1. The plasmid template used for *ftsN* constructions was pWM3157, which carries *ftsN* in pDSW210F as an *XbaI*-*PstI* fragment cloned by PCR-amplifying the *E. coli ftsN* gene with primers 1115 and 1116. Constructions for overexpressing full-length or truncated *ftsN* fragments with epitope tags were cloned into pET28a (Novagen, EMD4Biosciences, USA) between *EcoRI* and *HindIII* sites. All contain carboxy-terminal FLAG tags, except for pWM2254 and pWM3616; pWM2254 was made using primers 112 and 113 to PCR amplify *ftsN*. FLAG tags were added to the 3’ ends of the PCR-amplified *ftsN* fragments by additional PCR amplification as described below.

Leucine zippers were added by combinatorial PCR (Eraso & Kaplan, 2002). Primer 1520 was used as the forward primer in the following PCR reactions to clone *ftsN*. Plasmid pWM3950 contains full-length *ftsN* in pET28a, with an N-terminal *His6* tag and C-terminal FLAG tag; it was amplified using the 1520 forward primer in three sequential reactions using the partially overlapping primers 1562, 1565 and 1520, in that order, as the reverse primers. Plasmids pWM3616 and pWM3948 contain N-terminally *His6*-tagged derivatives of either the first 55 codons of *ftsN* (encoding FtsN<sub>Cyto-TM</sub>) or the first 33 codons (encoding FtsN<sub>Cyto</sub>-FLAG), respectively. The partially overlapping reverse primers 1564, 1566 and 1568, in that order, were used to make FtsN<sub>Cyto</sub>-FLAG.

Plasmids pWM3949 and pWM3947 (Fig. 2-1, A-B) contain the first 33 codons (encoding FtsN<sub>Cyto</sub>), followed by a parallel or an anti-parallel leucine zipper motif, respectively, immediately before the FLAG tags. The parallel and antiparallel leucine
Table 2-2. Primers used in described studies.

<table>
<thead>
<tr>
<th>Primer Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>CGAGAATTCAACAACACGCACAACGAGATTATG</td>
</tr>
<tr>
<td>112</td>
<td>ACGAAGCTTCTCAAAAAACCACCACGCCGAG</td>
</tr>
<tr>
<td>1115</td>
<td>GCTCTAGAGCAACACGAGATTATGTAGCCGC</td>
</tr>
<tr>
<td>1116</td>
<td>GCCCTGAGCTCAACCCCCGGCGCGAGCCCAGAACATGC</td>
</tr>
<tr>
<td>1161</td>
<td>GGCTGCAGGGGACTCAAGGAGCGAGATGACAAGGCGACTCTAGAGGATGCGGCCGGGT</td>
</tr>
<tr>
<td>1162</td>
<td>TCAAGATCTGTATCAAGAGACAGGA</td>
</tr>
<tr>
<td>1191</td>
<td>CCGTCTAGAGGTGGCAACACAGAGATTATGTAGC</td>
</tr>
<tr>
<td>1194</td>
<td>CCGGGGTACCCGTCAACCCCCCGCGCGAGCGAATGCAGTT</td>
</tr>
<tr>
<td>1214</td>
<td>CCGGGTGCTACGCTCAATCGCCATTGTGTTCAAGAG</td>
</tr>
<tr>
<td>1215</td>
<td>CCGGGGTACCCGTCAATTTCTCAGCGGCTGTTTGGCGC</td>
</tr>
<tr>
<td>1216</td>
<td>CTTCTTGAACAATGACAGGCTGTAAAGACAACCGAGCGCTGGGTAGGCTG</td>
</tr>
<tr>
<td>1217</td>
<td>CACCATCGAGCGGCTTGCATTATCTACGCGATTATGTGCAAGAAGAAGGTTGGGCTG</td>
</tr>
<tr>
<td>1239</td>
<td>TTCATTAGCATCAACAGAAGAAGAAGGCTCCAGAGACCGAGCTGATTGAAAGCTG</td>
</tr>
<tr>
<td>1240</td>
<td>CGGAACCTTCACACCAGCTGCTGCTGCTGGGACTCTTCTTCTTTTGTAGTGTCGTAATGAAAGGCATTG</td>
</tr>
<tr>
<td>1262</td>
<td>AATTAATACGAACCTCAATCAGG</td>
</tr>
<tr>
<td>1263</td>
<td>ATGTGATTGATTCGCTACGCC</td>
</tr>
<tr>
<td>1280</td>
<td>CCGTCTAGAVAAATACGATAGTCAGCAACGGGCACATGAG</td>
</tr>
<tr>
<td>1302</td>
<td>ATCGAATTCTGTGGACCACACGAAGATTATGTACGCCGCAGC</td>
</tr>
<tr>
<td>1303</td>
<td>CCGCCCGGGCAATGAAGTAGTACAGACCCGATAAAAGTCAC</td>
</tr>
<tr>
<td>1305</td>
<td>CCGGGGTACCCGTATTTCTCCAGGCCAGGCTGTTTATCGTGTCATTTTGCAAGATG</td>
</tr>
<tr>
<td>1319</td>
<td>CCGGATACGTGAATGAAACGATAGTCAAGCGAAGCGCATG</td>
</tr>
<tr>
<td>1320</td>
<td>AATCGGAGGCAGTCGTTGTCATTTGCCAATTCTTCTTGTG</td>
</tr>
<tr>
<td>1520</td>
<td>CCGGAAATTCGTTGGCCACAACAGAGATTATGTACGCC</td>
</tr>
<tr>
<td>1542</td>
<td>CGAANATCGTGCTGGCTGTTTCTATGCTGATCAGCTCAATTTTGGACCGG</td>
</tr>
<tr>
<td>1543</td>
<td>GTCAAAATCCGAGATCGACATAGAACCACCCAGGCGAGAATTTCGCC</td>
</tr>
<tr>
<td>1547</td>
<td>CATCCAAAGCTTTTCACTTTGCTATCAGCTGCTCTTCTTGTAGTACGCTACGCTGGATGACGAGATG</td>
</tr>
<tr>
<td>1552</td>
<td>CGAAGATCTGTGGCTGTTTCTATCCACGCATTAAAGACGCTTGGAAGAC</td>
</tr>
<tr>
<td>1553</td>
<td>TCCAGGCTTTTTCACTACGCGATAGAAACCGCAGGAGATTTTTCGGCGCG</td>
</tr>
<tr>
<td>1562</td>
<td>CTTGTAGTCATGCAGCTGATGACGCAGCTCAGGATTTTCGCCAATGTC</td>
</tr>
<tr>
<td>1563</td>
<td>CTTGTAGTCATGCAGCTGATGACGCAGCTCAGGATTTTCGCCAATGTC</td>
</tr>
<tr>
<td>1565</td>
<td>TCATTGTACAGGCTGCTTTGTAGTACGCTACGCCCTTGTGACGATACG</td>
</tr>
<tr>
<td>1566</td>
<td>TCAATGTACGCTGCTTTGTAGTACGCTACGCCCTTGTGACGATACG</td>
</tr>
<tr>
<td>1567</td>
<td>TCAATGTACGCTGCTTTGTAGTACGCTACGCCCTTGTGACGATACG</td>
</tr>
<tr>
<td>1568</td>
<td>CATCCAAAGCTTTTCACTTGTCATCGCTGCCTTTTGAG</td>
</tr>
<tr>
<td></td>
<td>DNA Sequence</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------</td>
</tr>
<tr>
<td>1579</td>
<td>AAGGGAATCTCATATGGACTACAAGGACGACGATG</td>
</tr>
<tr>
<td>1582</td>
<td>CATGCTCGAGTTAAACTCTTTTCGCAGCAACTATTTG</td>
</tr>
<tr>
<td>1803</td>
<td>GTCTTTTCGGGGTCTCAGGAACGCTTCGCGTCTCCGCG</td>
</tr>
<tr>
<td>1804</td>
<td>GCGGGAGAACAACCCGAGCCAGCTTTTCTGAGACCCCGAAAGAC</td>
</tr>
<tr>
<td>1809</td>
<td>GGTCTAGAAAATAACGATAGTCAGCAAG</td>
</tr>
<tr>
<td>2003</td>
<td>ACGAAGCTTTTCAGTAATGAAGTACAG</td>
</tr>
<tr>
<td>2047</td>
<td>ACGAAGCTTTCAACCTAGCCCAAATGAGG</td>
</tr>
<tr>
<td>2048</td>
<td>CGAGAATTCAAAACAACAAATAACGATAGTCAGC</td>
</tr>
<tr>
<td>2049</td>
<td>GCCTGCACTATTCTCGCCGTCGTTTTGCG</td>
</tr>
<tr>
<td>2051</td>
<td>AATCTAGAATGATCAAGGCGACGGACAG</td>
</tr>
<tr>
<td>2052</td>
<td>TTCTGCACTCTCCGATTTCCTGCTGTC</td>
</tr>
<tr>
<td>2054</td>
<td>CGACTGCGGCGCAAGCGCGATGGTGAGCAAGGAGCGAGG</td>
</tr>
<tr>
<td>2055</td>
<td>CCTCGCCCTTTCGCTACCATGCGCCGCTCGCAGCAGTCG</td>
</tr>
<tr>
<td>2056</td>
<td>GCGGAATTCATGGCGAACAATAACGATC</td>
</tr>
<tr>
<td>2057</td>
<td>CGCGGATCCCGCTTTGTACAGCTCGTCAGTCAGC</td>
</tr>
<tr>
<td>2058</td>
<td>GCGGGATCCCGAGAAAAAGACGAACGC</td>
</tr>
<tr>
<td>2059</td>
<td>CGCTCTAGATCAACCCCGGCGGCGAGC</td>
</tr>
<tr>
<td>2060</td>
<td>ACGAAGCTTTTCATTTCTCCGCGTCGTTTTGGC</td>
</tr>
<tr>
<td>2063</td>
<td>GCGATGCAATGCGGGAACAATAACGATC</td>
</tr>
</tbody>
</table>
Figure 2-1. DNA sequence of His\textsubscript{6}-FtsN\textsubscript{Cyto-ParLeu-FLAG}, His\textsubscript{6}-FtsN\textsubscript{Cyto-AntiLeu-FLAG} and His\textsubscript{6}-GluGlu-FtsA. The regulatory region of the expression vector pET28a is shown in all cases. Relevant restriction enzymes are indicated. (A) His\textsubscript{6}-FtsN\textsubscript{Cyto-ParLeu-FLAG} and (B) His\textsubscript{6}-FtsN\textsubscript{Cyto-AntiLeu-FLAG}. DNA regions encoding the first 33 amino acids of FtsN are highlighted inside green-shaded boxes. Pink-shaded boxes denote the parallel leucine zipper (A) and the anti-parallel leucine zipper (B). FLAG tags are highlighted in yellow. (C) His\textsubscript{6}-GluGlu-FtsA. The GluGlu tag is highlighted in yellow. The ftsA reading frame is highlighted in green, with only the initiation and stop codons shown.
zippers were amplified from yeast GCN4 (Hu et al., 1990), encoded on plasmid pUTE1019-GCN4-zip, using the forward primer 1552 in three sequential reactions using the partially overlapping reverse primers 1563, 1567 and 1568, in that order. The antiparallel leucine zipper was amplified from *B. subtilis* mtaN (Godsey et al., 2001), encoded on plasmid pUTE1019-MtaN-CC, using 1542 and 1547 as the forward and reverse primers, respectively. Both pUTE1019-GCN4-zip and pUTE1019-MtaN-CC were generously provided by the T. Koehler laboratory. FtsN<sub>Cyto</sub>-ParLeu-FLAG and FtsN<sub>Cyto</sub>-AntiLeu-FLAG were made first by PCR-amplifying the FtsN<sub>Cyto</sub> fragment with primers 1520 plus 1553 and 1520 plus 1543, respectively. Combinatorial PCR reactions were subsequently performed using a mixture of appropriate PCR products in approximately equimolar amounts, and the outside primers 1520 plus 1568 or 1520 plus 1547, respectively. The RRKK residues at positions 16-19 in the FtsN<sub>Cyto</sub> portion in pWM3949 were mutated by site-directed mutagenesis to DDEE, using primers 1588 (forward primer) and 1589 (reverse primer), respectively, to create plasmid pWM3951, which encodes the His<sub>6</sub>-FtsN<sub>Cyto</sub>-DDEE-ParLeu-FLAG mutant protein.

For the *in vivo* overproduction or complementation experiments, *his<sub>6</sub>-ftsN* or *his<sub>6</sub>-ftsN<sub>Cyto-TM</sub>* along with the strong ribosome binding site of pET28a were cloned into pBAD33 directly from pWM2254 or pWM3616 as *Xbal*-HindIII fragments, respectively, to make pWM4051 and pWM4050. To construct pWM4228, the cytoplasmic and transmembrane domains of *ftsI* (encoding residues 2-40) and the ribosome binding site of pET28a were amplified from plasmid pET28a-*his<sub>6</sub>-ftsI<sub>Cyto-TM</sub>* using primers 1262 and 1263. The fragment was digested at *Xbal* and *HindIII* sites and ligated with pBAD33. For *ftsN* complementation experiments, *ftsN-flag* and *ftsN* with the DDEE replacement between residues 16-19 were cloned into pDSW210 (Weiss et al., 1999) between the *EcoRI* and *Xbal* sites under the control of the weakened *trc* promoter to make pWM4032 and pWM4034, respectively.
For BACTH assays, an N-terminal FLAG tag was inserted between the PstI and BglII sites of pKT25 using primers 1161 and 1162 to make pKT25F. To construct pKT25F-ftsN, pKT25F-ftsN1-128, and pKT25F-ftsN1-242, ftsN constructs were amplified and cloned into pKT25F as XbaI-KpnI fragments. pKT25F-ftsN∆63-130 was created using primers 1191 and 1194 in a combinatorial PCR reaction that fused ftsN1-62 (generated by primers 1191 and 1240) and ftsN131-319 (generated by primers 1239 and 1194). The combinatorial PCR product was digested with XbaI and KpnI and subsequently ligated to pKT25F. Using pKT25F-ftsN∆63-130 as a template, ftsN∆63-130∆243-319 was constructed by using primers 1191 and 1215 and cloned as above to further truncate the ftsN construct. Plasmid pKT25F-ftsN∆129-242 was constructed by using primers 1191 plus 1216 and 1217 plus 1194 and cloning with XbaI and KpnI. To create the N1-54'phoA chimera, ftsN1-54 was amplified using primers 1302 and 1303, and cloned as a blunt-Smal fragment into a Smal-cutt Bluescript II derivative, pUI1160, containing 'phoA. The ftsN1-54 and 'phoA in this fusion were joined by a peptide linker, ARGIDPR. The fusion was then amplified with primers 1191 plus 1305 and inserted into pKT25F as an XbaI-KpnI fragment in frame with T25. The virB101-60'phoA fusion was created by amplifying A. tumefaciens virB101-60 (a gift from P. Christie) with primers 1319 and 1320, which added EcoRV and PstI restriction sites, and cloned into pUI1160 to fuse it to 'phoA. This chimera was then subcloned as an XbaI-KpnI fragment into pKT25F using primers 1280 and 1305. The virB101-60 and 'phoA fragments in this fusion were connected by a peptide linker, PAARGIDPR.

The GluGlu tag was used as an additional tag for Far-Western analysis. The sequence encoding the GluGlu epitope (EEEVMPME) (Grussenmeyer et al., 1985) was added using primers 1579 and 1582 to the 5'-ends of the PCR-amplified fragments containing ftsA (Fig. 2-1C). The ftsA fragments were amplified from pWM2785 (see Table 2-1). Plasmid pWM4093 contains full-length ftsA and pWM4094 (encoding His6-GluGlu-FtsA-1c) contains the shortened 1c subdomain from codon 81 to 140, preceded by an ATG.
and immediately followed by a stop codon. The shortened 1c (81-140) was amplified using primers 1615 and 1616, and its amino-terminal GluGlu tag was amplified from DNA encoding the full-length GluGlu-FtsA protein with primers 1613 and 1614, followed by combinatorial PCR using the outside primers 1613 and 1616. The PCR products containing gluglu-tagged full-length or truncated ftsA fragments were cloned into pET28a between its Ndel and Xhol sites. The 1c subdomain, encoding residues 83-176 of FtsA but lacking a GluGlu tag, was cloned into pET28a between the Ndel and EcoRI sites to make pWM1833, which expresses subdomain 1c with an N-terminal His6-tag. Finally, the flag-ftsA overexpression plasmid pWM2700 was constructed by cloning flag-ftsA into a pRR48 derivative as described for other ftsA derivatives (Bernard et al., 2007).

The polar recruitment assay vector pWM4637 (pBAD33-divIVA-ftsA-E124A) was constructed by amplifying the ftsA-E124A gene from template plasmid pWM2702 (pRR48-flag-ftsA-E124A) with primers 2051 and 2052. The PCR product and recipient vector pBAD33-divIVA were then digested with XbaI and PstI and ligated.

To create pWM4740 (pKG116-TTmCherry-ftsNSPOR), TTmCherry-ftsNSPOR was amplified from template pWM4682 (pDSW208-TTmCherry-ftsNSPOR) using primers 2053 and 2059. The insert was then cloned into vector pKG116 as an Nsil-XbaI fragment. To construct the original template plasmid pWM4682, the signal sequence of torA was amplified with primers 2056 and 2055 from pWM1487 (pBAD24-TTgfp) and the mCherry sequence was amplified with primers 2054 and 2057 from pWM4367 (pDSW208-ftsZ1-176-mCherry-ftsZ177-383). The torA signal sequence and mCherry were then fused using combinatorial PCR (primers 2056 and 2057) to create a TTmCherry fragment that was cloned into pDSW208 using EcoRI and BamHI restriction sites. The SPOR domain of ftsN was then amplified from template pWM1152 (pDSW207-ftsN) with primers 2058 and 2059 and cloned into pDSW208-TTmCherry as a BamHI-XbaI fragment.
All pWM2784 (pDSW210-*flag*) derivatives engineered for this study were cloned as XbaI-PstI fragments. To create plasmid pWM3157 (pDSW210-*flag-ftSN*), the *ftsN* insert was amplified with primers 1115 and 1116. Plasmid pWM4582 (pDSW210-*flag-virB10CytoN\textsubscript{TM-Peri}*) was created by amplifying the sequences encoding the cytoplasmic domain of *virB10* with primers 1809 and 1804 and the transmembrane and periplasmic domains of *ftsN* with primers 1803 and 1116. The PCR products were then joined in a combinatorial PCR reaction with primers 1809 and 1116. To create plasmid pWM4612 (pDSW210-*flag-virB10CytoN\textsubscript{TM-Peri}\Delta\text{SPOR}*), the *virB10CytoN\textsubscript{TM-Peri}\Delta\text{SPOR}* sequence was amplified from template pWM4582 (pDSW210-*flag-virB10CytoN\textsubscript{TM-Peri}*), using forward primer 1809 and reverse primer 2049 to omit the SPOR sequence from the PCR product. The pDSW210-*flag-ftSN\Delta\text{SPOR}* plasmid pWM4613 was constructed by amplifying *ftsN\Delta\text{SPOR}* from template pWM3157 (pDSW210-*flag-ftSN*), using primers 1115 and 2049 to also omit the SPOR sequence.

Insertion of DNA sequences into the multiple cloning site of vector pDSW207 fuses GFP to the amino terminus of encoded proteins. The GFP-FtsZ fusion produced from pWM3775 (pDSW207-*gfp-ftsZ*) was created by subcloning *ftsZ* from pDSW209-*gfp-ftsZ* (pWM3439) into vector pDSW207 using *SacI* and *HindIII* restriction sites. All other pDSW207 plasmids were constructed using *EcoRI* and *HindIII* sites and all forward primers included a sequence to create an Asn-Asn-Asn linker between GFP and the protein of interest. pWM4528 (pDSW207-*gfp-ftSN\textsubscript{Cyto-TM}*), was created by amplifying the cytoplasmic and transmembrane regions of *ftsN* from template pWM1152 (pDSW207-*gfp-ftSN*) with primers 112 and 2003. To construct pWM4610 (pDSW207-*gfp-virB10CytoN_{TM}*), *virB10CytoN_{TM}* was amplified from template pWM4582 (pDSW210-*flag-virB10CytoN_{TM-Peri}*), using primers 2048 and 2003. pWM4611 (pDSW207-*gfp-ftSN_{Cyto-virB10_{TM}}*) was cloned by amplifying the cytoplasmic and transmembrane domains of pWM4432 (pKT25-*flag-ftSN_{Cyto-virB10_{TM}}^{phoA}*), using primers 112 and 2047. For pWM4696 (pDSW207-*gfp-virB10CytoN_{TM-Peri}*), the *virB10CytoN_{TM-Peri}* sequence was amplified from pWM4582 (pDSW210-*flag-virB10CytoN_{TM-Peri}*).
using primers 2048 and 111. To create pDSW207-gfp-ftsN\textsubscript{ΔSPOR} (pWM4693), \textit{ftsN}\textsubscript{ΔSPOR} was amplified from pWM4613 (pDSW210-\textit{flag-ftsN}) using primers 112 and 2060. Finally, pWM4694 (pDSW207-gfp-virB10\textsubscript{Cyto}N\textsubscript{TM-Perl}\textsubscript{ΔSPOR}) was cloned by amplifying \textit{virB10}\textsubscript{Cyto}N\textsubscript{TM-Perl}\textsubscript{ΔSPOR} from pWM4612 (210-\textit{flag-virB10}\textsubscript{Cyto}N\textsubscript{TM-Perl}\textsubscript{ΔSPOR}) using primers 2048 and 2060.

**2.4 Protein purification**

Typically, His\textsubscript{6}-GluGlu-FtsA, His\textsubscript{6}-GluGlu-FtsA-1c-81-140, and His\textsubscript{6}-FtsA1c-83-176 were purified from four liters of JM109 (DE3) containing plasmid pWM4093, encoding the full-length FtsA, or BL21 (DE3) containing plasmids pWM4094 and pWM1835 encoding the 1c subdomain portions 81-140 or 83-176, respectively, grown at 30°C. The FtsA lysis buffer contained 25 mM Tris (pH 7.5), 50 mM KCl, 25 mM potassium glutamate, and 5 mM MgCl\textsubscript{2}. Phenylmethylsulfonyl fluoride (PMSF) was used at 1mM final concentration. Cells were lysed by three passages through a French pressure cell press (SLM Aminco, Rochester, NY). The cell lysates were clarified by centrifugation at 11,000x\textit{g} for 20 min at 4°C. TALON metal affinity resin (Clontech, Mountain View, CA) was used for purifications, following manufacturer's instructions. Crude extracts were incubated with the resin for 2 hours at 4°C prior to column purification. After washes in FtsA lysis buffer containing 5, 20, and 30 mM imidazole, and elution in FtsA lysis buffer with 200 mM imidazole, the proteins were dialyzed three times at 4 °C over a period of approximately 18 h against buffer containing 25 mM Tris (pH 7.5), 50 mM KCl, 25 mM potassium glutamate, 5 mM MgCl\textsubscript{2}, 2 mM DTT and 20% glycerol. The purified proteins were distributed in 100 µl aliquots and quick-frozen in a dry-ice/ethanol bath prior to storage at -80°C. The purification procedure yielded proteins that were ≥ 95% pure (data not shown).

His\textsubscript{6}-FtsN-FLAG and His\textsubscript{6}-FtsN\textsubscript{Cyto-TM-FLAG} were purified from 12 liters of BL21 (DE3) containing plasmids pWM3950 and pWM3616, respectively, grown at 30°C. The protocol was similar to that used for purification of FtsA, with the following modifications.
The FtsN lysis buffer contained 20 mM Tris (pH 7.5), 50 mM KCl, 200 mM NaCl, 5 mM MgCl$_2$ and 1 mM PMSF. The supernatant after clarification, which contains FtsN-loaded inner membranes, was centrifuged at 150,000$x$g for 1 h at 4°C. The inner-membrane pellet was solubilized in FtsN lysis buffer containing 1% Tween-20 using a Pyrex tissue grinder. The solubilized membranes were shaken in an orbital shaker for 2 h at 4°C and diluted at least 10-fold in FtsN buffer prior to incubation with the TALON resin. After washes in FtsN lysis buffer containing 5, 20 and 30 mM imidazole, and elution in FtsN lysis buffer with 200 mM imidazole, the proteins were dialyzed three times at 4 °C over a period of approximately 18 h against buffer containing 25 mM Tris (pH 7.5), 50 mM KCl, 25 mM potassium glutamate, 5 mM MgCl$_2$, 1% Tween-20, and 20% glycerol.

His$_6$-FtsN$_{Cyto}$-ParLeu-FLAG, His$_6$-FtsN$_{Cyto}$-FLAG, His$_6$-FtsN$_{Cyto}$-DDEE-ParLeu-FLAG, and His$_6$-FtsN$_{Cyto}$-AntiLeu-FLAG were purified from four liters of BL21 (DE3) containing plasmids pWM3949, pWM3948, pWM3951, and pWM3947, respectively, grown at 30°C, following the same protocol used for the purification of FtsA, but omitting addition of DTT in the dialysis step. The BCA assay (Thermo Scientific, Rockford, IL) was used to determine protein concentration.

2.5 Immunoblot analysis

For Chapter 3 experiments, crude extracts, eluates, and purified protein were resuspended in SDS loading buffer, boiled for 10 minutes, and separated by SDS-PAGE in Mini Protean 3 cells (BIO-RAD, Hercules, CA), using 12% or 18% gels made with a 40% stock of 29:1, acrylamide: bis-acrylamide. Proteins were transferred to nitrocellulose membranes using a wet apparatus. Mouse monoclonal anti-FLAG and anti-His primary antibodies were purchased from Sigma Aldrich (St. Louis, MO), and monoclonal anti-GluGlu antibodies were purchased from Covance Research Products, Inc. (Emeryville, CA). Anti-FtsA and Anti-FtsN primary antibodies were produced in rabbits. A 1:2000 dilution of
primary antibody was used for detection of FLAG-FtsA in the eluate immunoblot shown in Fig. 3-3 and for detection of His-1c in the eluate immunoblot shown in Fig. 3-6. All other immunoblotting was done at primary antibody dilutions of either 1:5000 or 1:10,000. Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were used at 1:10,000 dilutions. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and Amersham ECL Plus Western Blotting Detection System (GE) were used as substrates for HRP detection.

For Chapter 4 immunoblot experiments, samples from the FtsN depletion strain were collected after normalization, separated by SDS-PAGE, and transferred onto nitrocellulose membrane. The transferred FLAG-tagged proteins were then stained with Swift membrane stain (G-Biosciences; St. Louis, MO) to show that similar levels of proteins in all lanes were loaded and transferred. The membrane was immunoblotted with mouse monoclonal anti-FLAG antibody (1:5,000) and goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP; 1:10,000). HRP was detected using Western Lightning ECL Pro (PerkinElmer, Inc., Waltham, MA) and quantified using Image Studio Software (LI-COR; Lincoln, NE). Samples immunoblotted in Chapter 5 experiments were similarly normalized, separated, transferred, and stained. His$_6$-tagged proteins were detected using mouse monoclonal anti-His antibody (1:10,000) and the same goat anti-mouse antibody and concentration noted above. FtsZ and SulA-GFP proteins were detected using 1:5,000 anti-FtsZ and 1:2,500 anti-GFP concentrations of rabbit primary antibodies, respectively, and 1:5,000 goat anti-rabbit secondary antibody conjugated to horseradish peroxidase.

2.6 Co-affinity purification

Flasks containing 600 ml of LB were inoculated at a dilution of 1:100 from overnight cultures and grown in appropriate antibiotics to late logarithmic phase (OD$_{600}$=0.6-0.8).
During this phase, cultures were induced with 1 mM IPTG or 0.2% arabinose for 1.5 h, pelleted in a Beckman Model J2-21 centrifuge with a JA-10 rotor at 9K (14,300xg), and stored at -80°C. Each protein of interest was overproduced from a separate strain, not co-cultured. Cell pellets were resuspended in low ionic strength buffer (50 mM KCl, 20 mM Tris pH 7.5, 5 mM MgCl₂) and lysed using a French press. Lysates were subjected to centrifugation with a JA-17 rotor at 9K (11,200xg) for 10 min at 4°C to pellet cellular debris. Crude extracts expressing different proteins were combined as indicated and an aliquot was saved for detection of total protein levels prior to co-affinity purification. The remaining extracts were added to 5 ml of TALON metal affinity resin that was pre-washed with low ionic strength buffer containing 5 mM imidazole to reduce non-specific binding. Resin/crude extract mixtures were rocked at 4°C for 2 hours before adding each to gravity flow columns. The resin was washed repeatedly with low ionic strength buffer containing 5 mM imidazole and 20 mM imidazole to remove non-specifically bound proteins. His₆-tagged proteins, as well as associated proteins, were eluted with low ionic strength buffer containing 150 mM imidazole and subsequently concentrated 8-fold in Nanosep centrifugal devices (Pall Life Sciences). Crude extract aliquots and concentrated eluates were immunoblotted as described above. In the experiment to test specificity of the co-affinity purification of His-FtsN and FLAG-FtsA, 50, 100, 150, and 200 mM KCl concentrations were used in the wash buffers.

2.7 Bacterial adenylate cyclase two-hybrid (BACTH) experiments

For BACTH experiments, FtsA was fused to the carboxy-terminus of T18 using the BACTH plasmid pUT18C. FtsN variants were fused to the carboxy-terminus of T25 using a modified version of the BACTH plasmid (referred to as pKT25F) that contains a FLAG sequence between the T25 fragment and the fused protein. Plasmids were heat shock transformed sequentially into competent DHM1 cells and grown at 30°C. Strains were
streaked onto media containing 50 µg ml\(^{-1}\) X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Gold Biotechnology), 0.5 mM IPTG, and antibiotics. Colonies were screened for color change following two days of incubation at 30˚C. Proper insertion of T25F-N\(_{1-54}\)PhoA and T25F-VirB10\(_{1-60}\)PhoA into the cytoplasmic membrane was verified using 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma-Aldrich). Specifically, each construct was heat shock transformed into the phoA- strain DH5\(\alpha\) and streaked onto media containing 50 µg ml\(^{-1}\) BCIP and appropriate antibiotics.

**2.8 Far-Western analysis**

Far-Western analysis was performed using purified His\(_6\)-GluGlu-FtsA, His\(_6\)-GluGlu-FtsA1c-81-140, His\(_6\)-FtsA1c-83-176, His\(_6\)-FtsN-FLAG, His\(_6\)-FtsN\(_{\text{Cyto}}\)-ParLeu-FLAG, His\(_6\)-FtsN\(_{\text{Cyto}}\)-FLAG, His\(_6\)-FtsN\(_{\text{Cyto-DEE}}\)-ParLeu-FLAG, and FtsN\(_{\text{Cyto}}\)-AntiLeu-FLAG. Typically 1 to 3 µg of the purified proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. FtsA buffer (see protein purification section) was used for all incubations and washes. After transfer the membranes were blocked overnight (typically 16 h) in FtsA buffer/5% nonfat dry milk. After blocking, the membranes used in the Far-Western experiment were further incubated for 1 h at room temperature in fresh FtsA lysis buffer/5% dry milk/1% Tween-20, containing either native His\(_6\)-GluGlu-FtsA, His\(_6\)-GluGlu-FtsA1c-81-140 or His\(_6\)-FtsA1c-83-176, at a concentration 6- to 10-fold higher than the amount loaded in the gels. After incubation with the appropriate proteins the Far-Western membranes were washed 3X for 2 min with FtsA buffer, and all membranes were incubated in FtsA buffer/5% dry milk containing the appropriate primary antibodies, followed by the typical immunoblotting protocol.

For measuring the effects of overproduction of FtsN or FtsN\(_{\text{Cyto-TM}}\) on cell division, wild-type (WM1074) strains containing pBAD33, pWM4051 or pWM4052 were grown in LB
plus chloramphenicol at 37˚C to an OD\textsubscript{600} of approximately 0.1, then induced with or without arabinose for 1.5-2 h before imaging cells and measuring cell lengths.

2.9 FtsN depletion experiments

To obtain transductants harboring the \(\Delta\text{ftsN::kan}\) allele, wild-type cells containing plasmid pWM2784 (pDSW210-\textit{flag}), pWM4612 (pDSW210-\textit{flag-virB10\textit{CytoftsN}\textsubscript{TM-Pen\Delta\textit{SPOR}}}), pWM4582 (pDSW210-\textit{flag-virB10\textit{CytoftsN}\textsubscript{TM-Pen}}), pWM4613 (pDSW210-\textit{flag-ftsN\textsubscript{ASPOR}}), or pWM3643 (pDSW210-\textit{flag-ftsN}) were grown to low OD\textsubscript{600} (~0.2) and transduced with a P1 phage lysate derived from \(\Delta\text{ftsN::kan}\) strain WM3303, which also carries the \textit{ftsA-E124A} allele to permit survival in the absence of FtsN. Following a one hour outgrowth in media containing 10 mM sodium citrate, cells were plated onto media containing kanamycin and ampicillin to select for the plasmids and \(\Delta\text{ftsN::kan}\), and with 0, 10, or 100 \(\mu\text{M}\) IPTG to induce expression from the pDSW210 vector. Survivors were picked from the plates containing 100 \(\mu\text{M}\) IPTG, grown at 37˚C to an OD\textsubscript{600} \(\leq\)0.5 in selective medium containing 100 \(\mu\text{M}\) IPTG, and visualized by DIC microscopy. Cells were then washed three times to remove IPTG and grown in the absence of inducer to decrease expression from the pDSW210 vector. Cultures were back-diluted as needed to maintain a low-mid OD\textsubscript{600}. Cells were visualized every hour following exposure to depletion conditions for a total of 4 hours.

To obtain samples for immunoblot analysis, plasmids pWM4612 (pDSW210-\textit{flag-virB10\textit{CytoftsN}\textsubscript{TM-Pen\Delta\textit{SPOR}}}), pWM4582 (pDSW210-\textit{flag-virB10\textit{CytoftsN}\textsubscript{TM-Pen}}), pWM4613 (pDSW210-\textit{flag-ftsN\textsubscript{ASPOR}}), and pWM3643 (pDSW210-\textit{flag-ftsN}) were transformed into \textit{ftsN} depletion strain WM4028, grown at the permissive temperature of 30˚C to low-mid OD\textsubscript{600} in the absence of inducer, and shifted to 42˚C in the presence of 10 or 100 \(\mu\text{M}\) IPTG for 4 hours. Cultures were continually back-diluted throughout the experiment to remain in logarithmic phase. Cultures were then normalized based on OD\textsubscript{600} and boiled in sodium dodecyl sulfate (SDS) dye for 10 minutes prior to storage at -20˚C.
2.10 Temperature-shift experiments with ftsA12(Ts)

Cultures of ftsA12(Ts) strain WM1115 expressing gfp (pWM1088), gfp-ftsN_{Cyto-TM} (pWM4528), gfp-ftsN (pWM1152), gfp-ftsZ (pWM3775), or TTmCherry-ftsN_{SPOR} (pWM4740) were grown at 30˚C to an optical density at 600nm (OD_{600}) of 0.3-0.4 prior to temperature shift. Strains expressing gfp fusions from vector pDSW207 remained uninduced throughout the experiment, whereas the strain expressing TTmCherry-ftsN_{SPOR} from pKG116 was grown in the presence of 1 µM sodium salicylate. Cultures were shifted to 42˚C for 5 minutes. DIC and fluorescence images were obtained at each time point.

2.11 Polar recruitment assay

Cultures of strains carrying the pBAD33-divIVA-ftsA (pWM1806) or pBAD33-divIVA-ftsA-E124A (pWM4637) vector and pDSW207 plasmids expressing gfp (pWM1088), gfp-ftsN_{Cyto-TM} (pWM4528), or gfp-ftsN (pWM1152) were grown and induced as described previously (Corbin et al., 2004). Briefly, cultures were grown at 30˚C to a low OD_{600} (~0.1) and induced for 1.5-2 hours with 40 µM IPTG only (inducing expression from pDSW207 plasmids) or 40 µM IPTG plus 0.2% arabinose (inducing expression from pDSW207 and pBAD33 plasmids).

2.12 Immunofluorescence microscopy

Cells of ftsA12(Ts) strain WM1115 expressing gfp (pWM1088) were fixed using paraformaldehyde/glutaraldehyde fixative and stained as described previously (Levin, 2002). Rabbit α-His_{6}-FtsA antibody (Capralogics, Inc.; Hardwick, MA) was affinity purified (Levin, 2002) and applied at a final concentration of 1:500. Goat α-rabbit Alexa Fluor 488-conjugated antibody (Molecular Probes; Eugene, OR) was applied at a final concentration of 1:200. DNA was stained using 4’,6-diamidino-2-phenylindole (DAPI) at 0.5mg ml^{-1}. Anti-FtsZ immunofluorescence microscopy (IFM) was performed using methanol fixed strains,
as described previously (Levin, 2002). Rabbit α-FtsZ antibody was applied at a concentration of 1:500 and goat α-rabbit Alexa Fluor 488-conjugated antibody was used at a final concentration of 1:100. DNA was stained as described above.

2.13 Temperature-shift experiments with ftsQ TOE1 and ftsI AX655 temperature sensitive strains

Cultures of ftsQ(Ts) strain WM2193 and ftsI(Ts) strain WM2348 expressing gfp (pWM1088), gfp-ftsN<sub>Cyto-TM</sub> (pWM4528), gfp-ftsN (pWM1152), or gfp-ftsZ (pWM3775), or<sup>TT</sup>mCherry-ftsN<sub>SPOR</sub> (pWM4740) were grown at 30˚C without inducer to an OD<sub>600</sub> of 0.3-0.4 prior to heat shock. Thymine was added to WM2193, which is thyA-. Cultures were shifted to 42˚C for 30 minutes. DIC and fluorescence images were obtained immediately before and after shifting cultures to the non-permissive temperature.

2.14 Spot dilution assays

Spot dilution assays of ftsN depletion strain WM4028 harboring pWM2784 (pDSW210-<i>flag</i>), pWM4612 (pDSW210-<i>flag-virB10</i><sub>CytoN<sub>TM</sub>-PeriΔSPOR</sub>), pWM4582 (pDSW210-<i>flag-virB10</i><sub>CytoN<sub>TM</sub>-Peri</sub>), pWM4613 (pDSW210-<i>flag-ftsNΔSPOR</i>), or pWM3157 (pDSW210-<i>flag-ftsN</i>) were performed using cultures grown without IPTG to low OD<sub>600</sub> at 30˚C. Cultures were spot diluted (10<sup>-3</sup>-10<sup>-6</sup>) onto LB plates containing 0, 10, or 100 µM IPTG. Plates were incubated at 30˚C and 42˚C. Strains WM4049, WM4050, and WM4228 containing plasmids pBAD33, pBAD33-<i>ftsN</i><sub>Cyto-TM</sub>, and pBAD33-<i>ftsI</i><sub>Cyto-TM</sub>, respectively, were spot diluted onto LB agar or M9 plus 1% glycerol agar plates supplemented with 0% or 0.2% arabinose. Prior to spot dilution, cultures were treated as described in the text and diluted at 10<sup>-1</sup>-10<sup>-6</sup>.
Chapter III

The amino terminus of FtsN is required and sufficient for interaction with FtsA

3.1 Introduction

Although a linear dependency order of division protein recruitment has been established in *E. coli*, additional data suggest that many of the proteins interact outside of this simple framework (Di Lallo *et al.*, 2003, Goehring *et al.*, 2006, Goehring *et al.*, 2005, Karimova *et al.*, 2005, Alexeeva *et al.*, 2010, Corbin *et al.*, 2004). For example, *in vivo* approaches have detected a significant interaction between the early division protein FtsA and two late essential division proteins, FtsI and FtsN (Corbin *et al.*, 2004, Karimova *et al.*, 2005). These interactions involve the 1c subdomain of FtsA, which encompasses residues 87-164 of the *E. coli* protein (van Den Ent & Lowe, 2000), because a fusion of the polarly localized protein DivIVA with FtsA or only the 1c subdomain was sufficient to drive GFP fusions of FtsN or FtsI to the *E. coli* cell poles. Interactions between FtsA and other divisome proteins that require FtsA for their localization, such as FtsQ, were not detected by this assay. This interaction between FtsA and FtsN or FtsI was intriguing because the latter two proteins have bitopic topology, with only the small N-terminal cytoplasmic and transmembrane domains that would be able to interact directly with the cytoplasmic FtsA protein, which is anchored peripherally to the inner membrane via its membrane targeting sequence (MTS) (Pichoff & Lutkenhaus, 2005, Yang *et al.*, 2004). However, the ability to replace the MTS with a transmembrane segment from another protein and retain its
function (Shiomi & Margolin, 2008) argues against the MTS having any essential role in interactions with FtsI, FtsN, or other divisome proteins.

In addition to in vivo protein-protein interaction assays, a relationship between FtsA and FtsN is also suggested by genetic evidence. As mentioned in section 1.6, FtsN was originally identified as a multicopy suppressor of the temperature sensitive mutant ftsA12 (Dai et al., 1993). Moreover, an unbiased search for bypass suppressors of FtsN led to the isolation of a point mutation in subdomain 1c of FtsA that allows modest cell division activity in the absence of FtsN (Bernard et al., 2007). Finally, when ftsA is inactivated, premature targeting of later divisome proteins to the Z ring via fusions to the FtsZ-binding protein ZapA resulted in recruitment of all downstream divisome proteins except for FtsN (Goehring et al., 2005, Goehring et al., 2006). This suggests that FtsN needs not only its predecessor FtsI but also at least FtsA to localize to the divisome.

A recent study showed that FtsN uses two of its own periplasmic domains, a nonessential SPOR domain near the carboxy terminus and an essential 3-helix domain closer to the membrane, to self-enhance its proper septal localization (Gerding et al., 2009). Other proteins needed for proper FtsN localization to the septum include FtsI (Wissel & Weiss, 2004) and at least one of the three amidases, AmiA, AmiB or AmiC (Gerding et al., 2009). Other proteins implicated in genetic and/or physical interactions with FtsN include DamX and DedD, which, like FtsN, contain a SPOR domain and localize to the divisome (Arends et al., 2010, Gerding et al., 2009).

The previous evidence for FtsA-FtsN interaction suggested that early and late proteins could potentially interact as part of a feedback mechanism to regulate Z ring constriction in response to septum synthesis. However, because the previous in vivo assays were done in E. coli and were dependent on protein overproduction to generate the output signal, it was difficult to rule out the possibility that the observed interactions resulted from indirect interactions with other divisome proteins. Therefore, in this study, both in vivo
and in vitro approaches were undertaken to provide the first biochemical evidence for direct interaction between early and late essential divisome proteins in *E. coli*. The regions of FtsA and FtsN that are sufficient for their protein-protein interaction were also identified, and suggest that self-association of FtsN positively influences interaction with FtsA.
3.2 Results

3.2.1 FtsA and FtsN interact in a co-affinity purification assay.

Interaction between FtsA and FtsN was suggested previously using the DivIVA polar recruitment assay and the bacterial adenylate cyclase two-hybrid (BACTH) method (Karimova et al., 2005, Corbin et al., 2004). To confirm the interaction detected by these *in vivo* methods, I utilized co-affinity purification. Briefly, cell extracts containing His$_6$-FtsN produced from WM3428 and FLAG-FtsA produced from WM2700 were combined and added to cobalt resin to immobilize His$_6$-tagged FtsN and associated proteins. Following repeated washes, His$_6$-FtsN was eluted with imidazole. Western blot analysis of the eluates detected a strong signal corresponding to FLAG-FtsA in the presence of His$_6$-FtsN and a much weaker signal in the absence of His$_6$-FtsN (Fig. 3-1A). This interaction was not affected by modest increases in ionic strength (up to 200 mM KCl) in the wash buffers (data not shown).

To further demonstrate FtsA-FtsN interaction by this method, the experiment was repeated with reverse tagging of the proteins (i.e. His$_6$-FtsA and FLAG-FtsN). However, FLAG-FtsN was not detected in the eluate in the presence or absence of His$_6$-FtsA (data not shown). Because the amino-terminal fusion of the FLAG-tag to FtsN may have disrupted its interaction with His$_6$-FtsA, I tested the interaction between an untagged variant of FtsN expressed from a plasmid (pWM2022) and His$_6$-FtsA overproduced from pWM1260. Western blot analysis using anti-FtsN antibody detected co-affinity purification of untagged FtsN when His-FtsA was present, but not when His-FtsA was absent (Fig. 3-1B). Therefore, co-purification of FLAG-FtsA along with His$_6$-FtsN, as well as untagged FtsN with His$_6$-FtsA, provides the first *in vitro* evidence of FtsA-FtsN interaction. In addition, the inability of FLAG-FtsN and the ability of untagged FtsN to interact with His$_6$-FtsA rules out an artifactual interaction between the His$_6$ and FLAG tags.
Figure 3-1. Co-affinity purification of FtsA and FtsN from cobalt resin suggests an *in vitro* interaction. (A) Cells expressing His$_6$-FtsN (WM3428) or FLAG-FtsA (WM2700) from plasmids were induced, pelleted, and lysed. Resulting crude extracts were mixed together and incubated with TALON cobalt resin. The resin was washed and His$_6$-FtsN was eluted with 150 mM imidazole. Crude extracts and eluates were immunoblotted with anti-His and anti-FLAG antibodies. (B) The protocol was repeated using crude extracts of His$_6$-FtsA (WM1260) and untagged FtsN (WM2022) expressed from plasmids. Anti-FtsN antibody was used to detect untagged FtsN.
3.2.2 The periplasmic region of FtsN is not required for interaction with FtsA.

To screen for the region(s) of FtsN required for FtsA-FtsN interaction, I used the BACTH assay to measure in vivo protein-protein interactions (Karimova et al., 1998). In this assay, each protein of interest is fused to a complementary fragment of adenylate cyclase from Bordetella pertussis (T18 or T25) and expressed from two separate plasmids in a cya deficient strain, in this case DHM1. Interaction between the proteins of interest forces interaction of the T18 and T25 fragments, reconstituting adenylate cyclase. This, in turn, results in cAMP-CRP-dependent expression of lacZ, which can be detected as blue colonies on LB plates containing both X-Gal and IPTG.

Cells were engineered to co-produce wild-type FtsA from the BACTH plasmid pUT18c and variants of FtsN produced from the BACTH plasmid pKT25F (Fig. 3-2). As expected, full length FtsN gave a positive interaction with FtsA (Fig. 3-2, A-B). Various deletions of the periplasmic domain maintained a positive interaction with FtsA: for example, removal of the SPOR domain (N1-242), both SPOR and Q-rich domains (N1-128) or both SPOR and the essential 3-helices domain (NΔ63-130/Δ243-319) all retained interaction with FtsA. Consistent with previously published results (Dai et al., 1996, Yang et al., 2004, Gerding et al., 2009), T18 or T25 fusions of FtsN could complement an ftsN null mutant only if the 3 helices domain was present (Fig. 3-2, A-B). Notably, however, FtsN lacking this domain or any other segment of the periplasmic domain continued to interact with FtsA.

As all three periplasmic domains of FtsN were dispensable for the interaction with FtsA, the remaining portion of FtsN, which included the cytoplasmic and transmembrane segments, was tested to understand if these regions were sufficient for the interaction. Therefore, the entire periplasmic region of FtsN was replaced with the secreted form of alkaline phosphatase ('PhoA), which has not been reported to interact with the divisome. Strikingly, this N1-54'PhoA chimera still strongly interacted with FtsA in the BACTH assay (Fig. 3-2, A-B).
Figure 3-2. The periplasmic region of FtsN is not required for interaction with FtsA in BACTH assays. (A) FtsN variants were constructed in the BACTH plasmid pKT25F and co-transformed into the cya strain DHM1 with pUT18c-ftsA (pWM3021). Chimeras constructed with the secreted form of alkaline phosphatase (PhoA) and the cytoplasmic and transmembrane regions of the *Agrobacterium tumefaciens* protein VirB10 are also shown. Positive interaction between the FtsA and FtsN variants is indicated by a (+) symbol. Complementation of an FtsN depletion strain (WM2355) by the FtsN variants and chimeras is also shown. ND, not determined. (B) Interaction between the chimeras and FtsA was determined using media containing X-Gal (50 μg ml⁻¹) and IPTG (0.5 mM IPTG). All strains shown are in a DHM1 background, transformed with pUT18c-ftsA (pWM3021) and pKT25F-ftsN (pWM3772) (1), pKT25F-ftsN₁₋₁₂₈ (pWM3861) (2), pKT25F-ftsN₁₋₂₄₂ (3), pKT25F-ftsN₁₋₆₉ (4), pKT25F-ftsN₁₋₁₅₄ (5), pKT25F-ftsN₁₋₂₄₂ (6), pKT25F-ftsN₁₋₆₉/Δ₂₄₃₋₃₁₉ (7), and pKT25F-ftsN₁₋₁₅₄/Δ₂₄₃₋₃₁₉ (8).
(pWM3862) (3), pKT25F-ftsNΔ63-130 (pWM3863) (4), pKT25F-ftsNΔ63-130/243-319 (pWM3864) (5), pKT25F-ftsN129-242 (pWM3865) (6), pKT25F-N1-54’phoA (pWM3451) (7), pKT25F-virB101-60’phoA (pWM3866) (8), and pKT25F empty vector (EV). (C) Successful translocation of N1-54’PhoA and VirB101-60’PhoA was confirmed in the phoA strain DH5α using BCIP media (50 μg ml⁻¹). **Note: BACTH constructs and experiments described in Fig. 3-2 were contributed by Yipeng Wang and Kimberly Busiek.**
To rule out the possibility of an interaction between 'PhoA and FtsA, 'PhoA was fused to the cytoplasmic and transmembrane regions of the unrelated *Agrobacterium tumefaciens* protein VirB10 (VirB10_{1-60}'PhoA) and tested for interaction with FtsA. No interaction was detected between VirB10_{1-60}'PhoA and FtsA, suggesting that 'PhoA does not facilitate the interaction between FtsA and N_{1-54}'PhoA (Fig. 3-2, A-B). To ensure that the VirB10_{1-60}'PhoA fusion was expressed and translocated properly, I tested if the 'PhoA moiety of this fusion was active in the periplasm using the *phoA* strain DH5α to express N_{1-54}'PhoA and VirB10_{1-60}'PhoA. Both strains produced blue colonies on BCIP media, confirming that 'PhoA was secreted into the periplasm (Fig. 3-2C). Together, these results strongly suggest that the periplasmic region of FtsN is dispensable for FtsA-FtsN interaction.

3.2.3 The cytoplasmic and transmembrane regions of FtsN are sufficient for interaction with FtsA.

To determine if the remaining regions of FtsN are sufficient for interaction with FtsA, I asked whether the cytoplasmic and transmembrane regions of FtsN encompassing residues 1-55 (His$_6$-FtsN$_{Cyto-TM}$; pWM3616) were sufficient to interact with FLAG-FtsA (produced from pWM2700) in a co-affinity purification experiment. Although His$_6$-FtsN$_{Cyto-TM}$ did not seem to bind and/or elute from the cobalt resin as well as full length His$_6$-FtsN (lane 2 vs. lane 3, eluate), FLAG-FtsA detectably co-eluted in the presence of His$_6$-FtsN$_{Cyto-TM}$ as shown on Western blots, suggesting that FLAG-FtsA interacts specifically with His$_6$-FtsN$_{Cyto-TM}$ (Fig. 3-3). This result therefore suggests that the predicted cytoplasmic and transmembrane domains of FtsN are sufficient to interact with FtsA, in support of the BACTH results.

3.2.4 Purified FtsA and FtsN interact directly in vitro.
Figure 3-3. Co-affinity purification of FLAG-FtsA and His$_6$-FtsN$_{Cyto-TM}$. Crude extracts containing overexpressed FLAG-FtsA (WM2700) were combined with those containing His$_6$-FtsN$_{Cyto-TM}$ (WM3616) or His$_6$-FtsN (WM3428) and treated as described in the legend to Fig. 3-1.
A positive result in the co-affinity purification assay suggested that FtsA and FtsN proteins interact directly. One concern with this assay, however, is that other divisome proteins, which are probably also present in the extracts, may bridge an indirect FtsA-FtsN interaction, which would give a false positive result. Some proteins such as FtsI can be ruled out as bridging proteins because of their low abundance (~50 molecules/cell for FtsI (Weiss *et al*., 1997)) relative to the overproduced levels of FtsA and FtsN that gave a positive interaction signal.

To prove direct interaction more rigorously, a Far-Western experiment was performed to show that purified proteins could interact in the absence of a cell lysate. Two strains were engineered to perform this experiment. The first overproduced His$_6$-GluGlu-FtsA, which could be affinity purified on a cobalt resin and detected with a monoclonal antibody directed against the GluGlu epitope. The His$_6$-GluGlu-FtsA fusion was fully functional, as it was able to complement an ftsA12(ts) strain (WM1115) as efficiently as wild-type FtsA (data not shown). The second strain overproduced His$_6$-FtsN-FLAG, with FLAG at the periplasmic C terminus and thus unlikely to interfere with binding to FtsA. This tagged FtsN could fully complement the FtsN depletion strain WM2964 (data not shown). Both His$_6$-GluGlu-FtsA (Fig. 3-4B, last lane) and His$_6$-FtsN-FLAG (Fig. 3-4, A-B, lane 1) were purified to near homogeneity.

The purified proteins were then used to test the interaction between FLAG-tagged FtsN and GluGlu tagged FtsA with protein overlay (Far-Western) blots (Datta *et al*., 2002). His$_6$-FtsN-FLAG from SDS-PAGE was transferred to a nitrocellulose membrane, allowing it to renature (Burgess *et al*., 2000). The membrane was then overlaid with a solution containing purified His$_6$-GluGlu-FtsA, and immunoblotted with anti-GluGlu antibody. His-GluGlu-FtsA from SDS-PAGE was used as a positive control (Fig. 3-4, C-E, last lanes). Importantly, His$_6$-GluGlu-FtsA was strongly detected on the anti-GluGlu immunoblot on top of the band corresponding to purified His$_6$-FtsN-FLAG (Fig. 3-4E; lane 1). This result
Figure 3-4. Binding of purified FtsA to purified derivatives of FtsN and FtsN\textsubscript{Cyto}. Panel (A) shows cartoon diagrams of the constructions used in this assay. The cylinders representing the different portions are color-coordinated, and refer to: (i) His\textsubscript{6}-tag (red); (ii) full-length FtsN or FtsN\textsubscript{Cyto} (blue); (iii) FLAG-tag (yellow); (iv) GCN4 or MtaN leucine zippers.
(black); and (v) FtsN_{Cyto} with DDEE replacing RRKK_{16-19} (FtsN_{Cyto-DDEE}, orange). The parallel or anti-parallel leucine zippers are denoted by convergent or divergent arrows, respectively. The basic nature of the RRKK region is indicated by (+) signs. Combined Western (panels C and D) and Far-Western (panel E) results showing binding of purified His\textsubscript{6}-GluGlu-FtsA probe to His\textsubscript{6}-FtsN-FLAG and His\textsubscript{6}-FtsN\textsubscript{Cyto}-ParLeu-FLAG (lanes 1, 2), but not to His\textsubscript{6}-FtsN\textsubscript{Cyto}-FLAG, His\textsubscript{6}-FtsN\textsubscript{Cyto-DDEE}-ParLeu-FLAG, or His\textsubscript{6}-FtsN\textsubscript{Cyto-AntiLeu}-FLAG (lanes 3, 4, and 5, respectively). The purified proteins were subjected to SDS-PAGE, and both loading and protein purity controls are shown in panel B by staining with Coomassie blue. His\textsubscript{6}-GluGlu-FtsA, loaded to the right of lane 5, was used as a negative control for anti-FLAG and a positive control for anti-GluGlu. The membrane in panel C was incubated with anti-FLAG antibody, and those in panels D and E with anti-GluGlu. The four gels were loaded and run identically. The reaction conditions are described in the Materials and Methods. Panel F shows an alignment of FtsN cytoplasmic and transmembrane domains from the species indicated. The conservation of the basic patch of residues corresponding to residues 16-19 in \textit{E. coli} is highlighted in red. \textbf{Note: Protein purification and Far-Western experiments required for Fig. 3-4 were performed by Jesus Eraso.}
strongly suggests that the interaction between FtsA and FtsN is direct. Western blot analysis of purified Histagged FtsN-FLAG with anti-GluGlu antibody detected no signal prior to incubation with Histagged GluGlu-FtsA, eliminating the possibility of cross-reactivity between the anti-GluGlu antibody and Histagged FtsN-FLAG (Fig. 3-4D; lane 1).

3.2.5 FtsA interacts preferentially with a dimerized FtsN cytoplasmic domain.

BACTH analysis and co-affinity purification suggested that the cytoplasmic and transmembrane regions of FtsN are necessary and sufficient for interaction with FtsA (Fig. 3-2, Fig. 3-3). To further narrow down the region of FtsN involved in FtsA-FtsN interaction, the cytoplasmic portion of FtsN was purified using the same amino-terminal Histagged tag as the full-length protein, also with a carboxy-terminal FLAG tag used for immunodetection (Fig. 3-4, A-B, lane 3). As shown in lane 3 of Fig. 3-4E, Histagged GluGlu-FtsA did not interact with Histagged FtsNCyto-FLAG (called FtsNCyto-FLAG hereafter for brevity) on the Far-Western blot, suggesting that the cytoplasmic domain of FtsN is not sufficient to interact with FtsA.

Because the cytoplasmic region of FtsN has a large number and proportion of basic residues, I hypothesized that the molecules of FtsNCyto-FLAG, even with the acidic FLAG residues present, might electrostatically repel each other. This putative lack of interaction between the FtsN constructs could in turn affect FtsA-FtsN interaction, as previous evidence suggests that FtsN interacts strongly with itself in vivo (Karimova et al., 2005, Alexeeva et al., 2010). Indeed, FtsN self-interaction may be mediated by the transmembrane domains, because the N1-54'PhoA chimera interacted with full length FtsN in the BACTH assay (Fig. 3-5). Forcing the FtsNCyto domains to interact in parallel might mimic the function of the potentially interacting transmembrane domains and restore the ability of FtsNCyto to bind to FtsA.

To force dimerization of the cytoplasmic region of FtsN independently of the transmembrane domain, a parallel leucine zipper from the budding yeast transcription factor
Figure 3-5. BACTH assay showing positive interaction between $N_{1-54}$ PhoA and FtsN.

Duplicate colonies were streaked on LB agar plates containing 0.5 mM IPTG and 50 µg ml$^{-1}$ X-Gal for each of the three samples.
GCN4, with a FLAG tag at its carboxy terminus, was fused to the carboxy-end of FtsN_{Cyto} (FtsN_{Cyto}-ParLeu-FLAG, Fig. 3-4A, lane 2). ParLeu-FLAG was placed at the carboxy terminus of FtsN_{Cyto} instead of the amino terminus with the idea that the artificial dimerization domain would functionally substitute for the FtsN transmembrane domain, which could potentially drive FtsN_{Cyto} dimerization (Fig. 2-1). As a control, an anti-parallel leucine zipper was also fused to the cytoplasmic domain to force neighboring cytoplasmic domains in opposing and aberrant orientations (FtsN_{Cyto}-AntiLeu-FLAG, Fig. 3-4A, lane 5; Fig. 2-1).

Similar quantities of these purified protein chimeras were separated by SDS-PAGE and tested their ability to interact with purified His$_6$-GluGlu-FtsA in Far-Western assays. Notably, His$_6$-GluGlu-FtsA strongly interacted with a band corresponding to the monomer size of FtsN_{Cyto}-ParLeu-FLAG (Fig. 3-4E; lane 2). In contrast, His$_6$-GluGlu-FtsA was only weakly detected in the lane corresponding to FtsN_{Cyto}-AntiLeu-FLAG (Fig. 3-4E, lane 5), again at the monomer size. Although likely denatured by the SDS in the gel (Meng et al., 2001), these proteins probably renature on the nitrocellulose membrane after transfer. This could explain how a protein that should dimerize strongly would migrate as a monomer on SDS-PAGE yet act as a dimer (or oligomer) in the Far-Western blot. These results suggest that FtsN_{Cyto} is necessary and sufficient for interaction with FtsA, but only if FtsN_{Cyto} is in a parallel homodimer.

### 3.2.6 Role of a conserved patch of basic residues within the cytoplasmic domain of FtsN in interaction with FtsA.

After defining the FtsN_{Cyto} domain as sufficient for FtsA-FtsN interaction, specific residues within this domain important for binary interaction were identified. Amidst the generally basic nature of the cytoplasmic domain of FtsN, there is a conserved patch of basic residues (RRKK) at amino acid positions 16 through 19 (Fig. 3-4F, highlighted in red)
that were sufficiently far from the predicted transmembrane domain to potentially be involved in a direct contact with FtsA. To investigate the role of these residues in FtsA-FtsN interaction, the basic RRKK residues were mutated to acidic DDEE residues in the FtsN<sub>Cyto</sub>-ParLeu-FLAG construct to create FtsN<sub>Cyto</sub>-DDEE-ParLeu-FLAG (Fig. 3-4A, lane 4).

When His<sub>6</sub>-GluGlu-FtsA was used to probe FtsN<sub>Cyto</sub>-DDEE-ParLeu-FLAG, which was on the same blot as the positively interacting FtsN<sub>Cyto</sub>-ParLeu-FLAG, only the latter band was detectable (Fig. 3-4, B-E, compare lane 4 with lane 2). This indicated that mutation of residues 16-19 abolished interaction, and suggests that the basic patch in FtsN<sub>Cyto</sub> is important for FtsA-FtsN interaction. To test this hypothesis in the context of full length FtsN in vivo, the abilities of pDSW210-FtsN<sub>DDEE</sub>-FLAG (pWM4034) and pDSW210-FtsN-FLAG (pWM4032) to complement an FtsN depletion strain (WM2964) were compared. Under depletion conditions in WM2964 at 42˚C in LB medium, FtsN-FLAG and FtsN<sub>DDEE</sub>-FLAG each failed to complement at 0 mM IPTG but they both complemented fully at 1 mM IPTG (data not shown), indicating that the DDEE mutation has no significant deleterious effect on FtsN function in vivo. This is consistent with the ability to replace the cytoplasmic domain of FtsN with MalF as shown previously (Goehring et al., 2007b), and suggests that the DDEE mutation does not grossly perturb protein structure or insertion into the membrane.

### 3.2.7 The 1c subdomain of FtsA is sufficient for interaction with FtsN.

Previous results from the Margolin laboratory suggested that the 1c subdomain of FtsA is sufficient for recruitment of GFP-FtsN to the cell poles in an in vivo DivIVA recruitment assay (Corbin et al., 2004). However, this effect could have been indirect. To verify the role of FtsA-1c in direct FtsA-FtsN interaction, the ability of His<sub>6</sub>-tagged FtsA-1c produced from pWM1835 to interact with untagged FtsN produced from pWM2022 in co-affinity purifications from cell lysates was tested. Four cultures expressing His<sub>6</sub>-FtsA-1c were induced at increasing levels of IPTG (0, 0.01, 0.1, and 1 mM) to create a gradient of
His<sub>6</sub>-FtsA-1c. Crude extracts containing His<sub>6</sub>-FtsA-1c were combined with crude extracts of cells overproducing a stable level of untagged FtsN. As shown in Fig. 3-6, untagged FtsN was detected in the eluate when His<sub>6</sub>-FtsA-1c was at high levels after being induced at 0.1 or 1 mM IPTG, but not when His<sub>6</sub>-FtsA-1c was at lower levels (0 or 0.01 mM IPTG). Although His<sub>6</sub>-FtsA-1c was difficult to detect in the lysates, it was significantly enriched in the eluates when induced at 0.1 or 1 mM IPTG. The co-affinity purification of untagged FtsN with His<sub>6</sub>-FtsA-1c suggests that the 1c subdomain of FtsA specifically interacts with FtsN and is sufficient for FtsA-FtsN interaction.

To confirm that this interaction is direct, His<sub>6</sub>-FtsA-1c containing residues 83-176 was purified to homogeneity (Fig. 3-7A, lane 4) and used to probe blots containing FtsN or FtsN<sub>Cyto</sub> derivatives in Far-Western experiments. As shown in Fig. 3-7, A-D, His<sub>6</sub>-FtsA-1c-83-176 interacted with FtsN<sub>Cyto</sub>-ParLeu-FLAG (lane 2), but only weakly with an equivalent amount of FtsN<sub>Cyto</sub>-AntiLeu-FLAG (lane 3). Although the interaction with full-length His<sub>6</sub>-FtsN was variable (lane 1) for reasons that are not clear, the data suggest that the 1c domain, like full length FtsA, is sufficient to bind to a dimerized FtsN<sub>Cyto</sub>.

As the 1c domain is fairly large, with one alpha helix (H2) and three beta strands (S5, S6 and S7) as determined from the T. maritima crystal structure (van Den Ent & Lowe, 2000) (Fig. 3-7E), a truncated version that contained only residues 81-140 (His<sub>6</sub>-GluGlu-FtsA-1c-81-140) which removes 36 residues at the C-terminal portion of the domain including the S7 beta strand and the loop connecting it to the S6 beta strand, was purified. This truncated domain, like the full-length domain, interacted with FtsN, FtsN<sub>Cyto</sub>-ParLeu-FLAG, but again, only weakly with FtsN<sub>Cyto</sub>-AntiLeu-FLAG (data not shown). This confirms that this interaction is reproducible, suggests that only 60 amino acids (residues 81 to 140) of the FtsA 1c subdomain are needed for FtsA to bind to the cytoplasmic domain of FtsN, and supports the binding data for the longer version of subdomain 1c.
Figure 3-6. Co-affinity purification of His$_6$-FtsA-1c and FtsN. Crude extracts containing untagged FtsN (WM2022) were combined with those containing His$_6$-FtsA-1c-83-176 (WM1835) induced at various IPTG concentrations (0, 0.01, 0.10, and 1.00 mM). Untagged FtsN was detected with anti-FtsN antibody.
Figure 3-7. FtsA1c-83-176 binding to FtsN and to FtsN\textsubscript{Cyto}. Combined Western (panels B and C) and Far-Western (panel D) results showing binding of purified His\textsubscript{6}-FtsA1c-83-176 used to probe membranes containing His\textsubscript{6}-FtsN-FLAG (lane 1, upper arrow in panel B); His\textsubscript{6}-FtsN\textsubscript{Cyto}-ParLeu-FLAG (lane 2, lower arrow in panel B); His\textsubscript{6}-FtsN\textsubscript{Cyto}-AntiLeu-FLAG (lane 3); or His\textsubscript{6}-FtsA1c-83-176 (lane 4) transferred from SDS-PAGE. The lower band in panel B, lane 1 is a degradation product also visible in panel A, lane 1. Panel A shows loading and protein purity controls after SDS-PAGE and Coomassie blue staining. Molecular weight markers are shown to the left in each panel. The membrane in panel B was incubated with anti-His antibody, and those in panels C and D with anti-FtsA. The upper band in lane 4, panels C and D may be a trace contaminant in the FtsA1c-83-176 preparation recognized only by the anti-FtsA antibody. The four gels were loaded and run identically, and the reaction conditions are described in the Materials and Methods. Panel E shows a cartoon diagram of the FtsA1c sub-domain (83-176) containing alpha helix H2 and
beta strands S5, S6 and S7 from the *T. maritima* structure (van Den Ent & Lowe, 2000).

*Note: Protein purification required for Fig. 3-7 was performed by Jesus Eraso and Kimberly Busiek; Far-Western experiments were performed by Jesus Eraso.*
3.2.8 Excess His$_6$-FtsN$_{Cyto-TM}$ inhibits cell division.

Although FtsN$_{Cyto-TM}$ is dispensable for FtsN function in vivo, I reasoned that overproduction of this segment of FtsN might exert a dominant negative effect on cell division. I cloned His$_6$-FtsN$_{Cyto-TM}$ in pBAD33, which provides induction of gene expression by arabinose. Uninduced wild-type WM1074 cells containing pBAD33- His$_6$-FtsN$_{Cyto-TM}$ or pBAD33-His$_6$-FtsN were short and indistinguishable from cells with pBAD33 or no plasmid. However, after 0.2% arabinose induction for several generations, cells expressing His$_6$-FtsN$_{Cyto-TM}$ or His$_6$-FtsN became moderately filamentous to approximately the same degree (Fig. 3-8, A-D). Although strains with pBAD33, pBAD33- His$_6$-FtsN$_{Cyto-TM}$ or pBAD33-His$_6$-FtsN all formed colonies on LB agar plates supplemented with 0.2% arabinose, colonies expressing His$_6$-FtsN$_{Cyto-TM}$ were wrinkled (Fig. 3-8F). This morphotype indicates that a colony contains a significant proportion of nondividing cells (Goehring et al., 2007a). Colonies expressing full length FtsN did not appear significantly different from control colonies (Fig. 3-8, E and G), perhaps because the effect of full length FtsN was less severe on solid media. Taking into account the cell length data, these results indicate that cell division is modestly inhibited by overproduction of full-length FtsN, consistent with previous data indicating that overproduction of FtsN delays divisome constriction (Aarsman et al., 2005), and suggest that this effect is largely mediated by FtsN$_{Cyto-TM}$ itself.
Figure 3-8. Overproduction phenotypes of FtsN and FtsN\textsubscript{Cyto-TM}. Panel A shows a histogram of length distributions of cells with pBAD33 (WM4049), pBAD33-FtsN\textsubscript{Cyto-TM}
(WM4050), or pBAD33-FtsN (WM4051) after induction of expression with 0.2% arabinose for 2h. Data was obtained from two independent experiments. Panels B (pBAD33 vector; WM4049), C (FtsN_{Cyto-TM}; WM4050), and D (FtsN; WM4051) are representative micrographs obtained from the cells quantified in Panel A; scale bar = 5 μm. Colony morphotypes shown in panels E, F, and G depict the typical phenotype observed from the strains represented in panels B, C, and D, respectively, when cells were spot diluted onto LB agar plates containing 0.2% arabinose.
3.3 Discussion

These results indicate that the early divisome protein FtsA and the last essential divisome protein to localize to midcell, FtsN, interact directly. The Margolin laboratory initially provided in vivo evidence of FtsA-FtsN interaction using the DivIVA polar recruitment assay (Corbin et al., 2004). Using this method, they observed polar recruitment of GFP-FtsN by molecules of FtsA that were fused to DivIVA, a Bacillus subtilis protein that localizes to E. coli cell poles. In vivo interaction between FtsA and FtsN was further supported by BACTH studies (Karimova et al., 2005). Because the DivIVA and BACTH assays are unable to differentiate between direct and indirect protein-protein interactions, in vitro techniques were utilized herein to investigate FtsA-FtsN interaction further.

The involvement of the cytoplasmic domain of FtsN in FtsA-FtsN interaction is entirely consistent with the topology of each protein. FtsA is located exclusively in the cytoplasm, associating only peripherally with the inner membrane via its MTS. FtsN, on the other hand, resides mostly in the periplasm, exposing only a small portion of the protein to the cytoplasm of the cell. To my knowledge, this is the first biochemical evidence of an interaction between the cytoplasmic domain of a bitopic divisome protein and another divisome protein.

These data also suggest that the cytoplasmic domain of FtsN must dimerize or multimerize to interact with FtsA. FtsN self-interaction has been suggested by BACTH analysis and FRET activity in previous studies (Karimova et al., 2005, Alexeeva et al., 2010). Although FtsN\textsubscript{Cyto} did not interact with FtsA unless it was fused to a parallel leucine zipper, FtsN\textsubscript{Cyto-TM} did interact with FtsA in membrane-containing crude extracts. This, along with the positive interaction between N\textsubscript{1-54-PhoA} and FtsN in BACTH assays (Fig. 3-5), suggests that the transmembrane domain of FtsN helps to mediate FtsN self-interaction.
I also showed that the 1c subdomain of FtsA is sufficient to bind to FtsN. The 1c subdomain was implicated in FtsA-FtsN interaction previously using the DivIVA polar recruitment assay, because GFP-FtsN could be recruited to cell poles by fusion of 1c directly to DivIVA (Corbin et al., 2004). Like FtsN, FtsA has been shown to interact with itself (Yim et al., 2000, Rico et al., 2004, Karimova et al., 2005, Shiomi & Margolin, 2007, Pichoff & Lutkenhaus, 2005). It has been postulated that the 1c subdomain of FtsA constitutes a portion of the FtsA-FtsA interface (Carettoni et al., 2003). If the 1c subdomain of FtsA is indeed involved in FtsA self-interaction, then it is possible that FtsN binding to 1c could compete with this self-interaction. Previous results suggest that decreased FtsA-FtsA interaction correlates with increased cell division inhibition by FtsA (Shiomi & Margolin, 2007). Therefore, one possibility is that overproduced FtsN\textsubscript{Cyto-TM} may inhibit FtsA self-interaction mediated by the 1c subdomain. This would inhibit cell division and make FtsA more toxic (i.e., mimic an FtsA mutant defective in self-interaction). However, other explanations are also possible, including altering the interactions of other late divisome proteins such as FtsI with the 1c subdomain, which could alter FtsA activity.

A conserved patch of basic residues is located between positions 16 through 19 of FtsN (Fig. 3-4E). Because replacement of these residues with acidic amino acids greatly reduced interaction with FtsA, I speculate that this conserved basic patch in FtsN is crucial for direct interaction with a segment of subdomain 1c of FtsA. Although it is tempting to speculate that FtsN and FtsA interact electrostatically, I cannot rule out the possibility that mutation of the basic patch in FtsN disturbs a secondary structure of FtsN that is required for FtsA-FtsN interaction.

PilM, a component of type IV pili, is a structural homolog of FtsA, and like FtsA is a cytoplasmic protein associated with the inner membrane as part of a complex with transmembrane proteins (Karuppiah & Derrick, 2011). One of these transmembrane proteins, PilN, inserts its short cytoplasmic domain in the cleft between domains 1A and 1C.
of PilM as shown by NMR studies (Karuppiah & Derrick, 2011). As the E124A mutation of FtsA suppresses the loss of FtsN and is located at the analogous cleft in FtsA, I speculate that the cytoplasmic domain of FtsN, possibly involving the RRKK motif, may contact subdomain 1c at or near the cleft. Defining the binding interface between FtsN and FtsA will clearly require detailed structural studies.

It has been hypothesized that FtsN helps to trigger septation in a dividing *E. coli* cell (Lutkenhaus, 2009, Corbin et al., 2004). FtsN is well positioned to serve this role because it is the last essential divisome protein to localize to the site of division, requiring a number of proteins downstream of FtsA in the pathway for its recruitment. In addition, FtsN is required to recruit amidases that commence degradation of peptidoglycan at midcell (Addinall et al., 1997, Bernhardt & de Boer, 2003, Uehara *et al.*, Peters et al., 2011). FtsN also indirectly recruits the Tol-Pal complex, which is involved in invagination of the outer membrane (Gerding *et al.*, 2007, Bernard et al., 2007). Like FtsN, FtsA may also act as a trigger for septation as it has been shown to influence both the assembly and disassembly of FtsZ polymers (Geissler *et al.*, 2007, Jensen *et al.*, 2005, Beuria *et al.*, 2009), which would potentially allow FtsA-mediated constriction of the Z ring following interaction with FtsN.

The working model, then, is that after FtsN is recruited to the divisome by multiple proteins and by self-enhanced localization (Gerding *et al.*, 2009), FtsN dimers are formed, which then can bind directly to subdomain 1c of FtsA. Other divisome proteins such as FtsI may also bind to this portion of FtsA (Corbin *et al.*, 2004), and possibly compete for binding. As FtsA binds directly to FtsZ, probably via FtsA’s 2B subdomain (Pichoff & Lutkenhaus, 2007), I postulate that this interaction then helps transduce a signal to FtsZ polymers that coordinates Z ring constriction with septal murein synthesis activities. The cytoplasmic and transmembrane domains of FtsN can be replaced by heterologous cytoplasmic and transmembrane domains, although FtsNCyto may have a role in restoring some cell division activity in the absence of FtsK (Goehring *et al.*, 2007b, Geissler & Margolin, 2005, Draper *et
To potentially explain why the FtsA-FtsN interaction seems to be dispensable for cell division, I favor the idea that the interaction between FtsA and FtsN provides one of many redundant Z ring constriction signals (Peters et al., 2011). Indeed, recent in vivo data implicate an interaction between FtsN and ZapA (Rico et al., 2010, Alexeeva et al., 2010) and between amidases and FtsZ via FtsEX (Yang et al., 2011, Corbin et al., 2007). Such redundant triggers would ensure robust and timely constriction of the Z ring.
Chapter IV

FtsA-FtsN interaction initiates localization of FtsN to division sites
This chapter is based upon “Busiek, K.K. and Margolin, W. (2014) A role for FtsA in SPOR-independent localization of the essential Escherichia coli cell division protein FtsN. Molecular Microbiology [Epub ahead of print]” with permission from John Wiley and Sons (Copyright © John Wiley and Sons, Molecular Microbiology, Epub ahead of print 2014 Apr 21, 10.1111/MMI.12623).

4.1 Introduction

Recent studies have suggested that recruitment of FtsN to division sites requires more than prior localization of FtsI (Fig. 1–2). For instance, FtsN fails to localize to division sites in the absence of FtsA even after all other essential cell division proteins, including FtsI, are artificially targeted to midcell (Goehring et al., 2005). FtsN also appears to have a role in divisome stabilization, as removal of FtsN leads to disassembly of divisome components (Rico et al., 2010).

In addition to its requirement for proper localization of FtsA, FtsN also depends on its periplasmic SPOR domain for recruitment to the divisome. The SPOR domain is named after a sporulation protein in B. subtilis, CwlC, which contains a similar RNP-like fold (Dai et al., 1996). Like CwlC, the SPOR domain of FtsN preferentially binds peptidoglycan that has been treated with amidases to remove peptide crosslinks between glycan strands (Ursinus et al., 2004). SPOR-dependent localization of FtsN to division sites requires three activities: the peptidoglycan synthesis activity of penicillin binding protein FtsI (PBP3); the essential activity of FtsN; and the activity of at least one periplasmic murein amidase (Gerding et al., 2009). Although SPOR-mediated localization of FtsN is dependent upon prior activation of these proteins, FtsN has a demonstrated role in the recruitment of amidases and an implied role in FtsI stimulation, suggesting that its targeting to the divisome is self-reinforced. Specifically, the recruitment of amidases AmiB and AmiC to division sites is dependent on FtsN, as is the localization of NlpD, the cognate activator of AmiC (Peters et al., 2011).
FtsN has also been shown to activate PBP1b and is suspected of activating FtsI (Muller et al., 2007). If FtsN is involved in the initial localization and stimulation of these proteins, how does FtsN get to the divisome in the first place?

Here, I provide evidence that the previously established interaction between FtsA and FtsN (Karimova et al., 2005, Corbin et al., 2004, Busiek et al., 2012) facilitates midcell localization of FtsN. I also show that this SPOR-independent means of FtsN localization precedes SPOR-dependent midcell recruitment. Together, these results provide a mechanism for the initial localization of FtsN that is needed for recruitment or stimulation of FtsI, FtsN, and amidase activities and subsequent SPOR-dependent localization of FtsN.
4.2 Results

4.2.1 The cytoplasmic domain of FtsN contributes to its localization to the divisome independently of native FtsN.

Previously, I provided *in vitro* evidence that proteins FtsA and FtsN interact, and that the first 55 residues of FtsN, including the short cytoplasmic tail and transmembrane domain (FtsN<sub>Cyto-TM</sub>) are sufficient for this interaction (Busiek et al., 2012). I also found that gross overproduction of FtsN<sub>Cyto-TM</sub> caused moderate filamentation of cells (Fig. 3-8), which prompted me to ask if the cytoplasmic and transmembrane domains of FtsN can localize to division sites without the aid of the known divisome targeting determinants in the periplasmic domain. To observe the localization of FtsN<sub>Cyto-TM</sub>, I fused green fluorescent protein (GFP) to the amino terminus of FtsN<sub>Cyto-TM</sub> (Fig. 4-1A) and expressed the fusion at uninduced levels from plasmid pDSW207, which has a weakened trc promoter with leaky expression. Although GFP itself localized diffusely throughout the cell (Fig. 4-1B), GFP-FtsN<sub>Cyto-TM</sub> localized specifically to division sites and the membrane (Fig. 4-1C). The ability of FtsN to localize weakly in the absence of the SPOR domain was also noted when observing GFP fusions to FtsN<sub>1-243</sub>, FtsN<sub>1-105</sub>, and FtsN<sub>1-90</sub> (Gerding et al., 2009).

To narrow down the segment of FtsN<sub>Cyto-TM</sub> required for midcell localization, I replaced the cytoplasmic or transmembrane segments of FtsN<sub>Cyto-TM</sub> with the corresponding domains of the unrelated *Agrobacterium tumefaciens* protein VirB10, which has similar bitopic membrane topology. GFP-VirB10<sub>Cyto</sub>N<sub>TM</sub> localized uniformly around the membrane but failed to localize to division sites (Fig. 4-1D), whereas GFP-FtsN<sub>Cyto</sub>VirB10<sub>TM</sub> localized clearly to midcell (Fig. 4-1E). These results indicate that the FtsN cytoplasmic domain is sufficient to promote midcell localization of GFP-FtsN<sub>Cyto-TM</sub>. However, GFP-FtsN<sub>Cyto</sub> alone did not localize to division sites (data not shown), suggesting that the transmembrane domain of VirB10 facilitates midcell localization of GFP-FtsN<sub>Cyto</sub>VirB10<sub>TM</sub>.
Figure 4-1. The cytoplasmic domain of FtsN contributes to midcell localization independently of native FtsN. (A) Domain organization of FtsN and engineered constructs used in this study. Midcell localization of each GFP-tagged construct in a wild-type strain is indicated by a (+) symbol; diffuse or peripheral membrane localization is noted by a (-) symbol. The essential region of FtsN (aa71-105; Gerding et al. 2009) is represented by a
black star. (B-F) DIC (left) and fluorescence (right) images of cells grown to low OD$_{600}$ at 37˚C in the absence of IPTG. Cells shown in panels B-E are wild-type WM1074 harboring plasmids pWM1088 (pDSW207-gfp) (B), pWM4528 (pDSW207-gfp-ftsN$_{Cyto-TM}$) (C), pWM4610 (pDSW207-gfp-virB10$_{CytoN_TM}$) (D), or pWM4611 (pDSW207-gfp-ftsN$_{Cyto}$virB10$_{TM}$) (E). Cells shown in (F) lack the native ftsN allele but harbor a single chromosomal copy of ftsA-E124A (WM3303 background) as well as plasmid pWM4528 (pDSW207-gfp-ftsN$_{Cyto-TM}$). Scale bar = 4 µm.
possibly through the weak dimerization or membrane association of VirB10<sub>TM</sub> (Garza & Christie, 2013). Consistent with this idea, the cytoplasmic domain of FtsN alone fails to interact with FtsA unless it is fused to a dimerization motif, such as a leucine zipper (Busiek et al., 2012).

Because self-interaction of FtsN was previously reported (Karimova et al., 2005, Alexeeva et al., 2010), I wanted to rule out the possibility that GFP-FtsN<sub>Cyto-TM</sub> localizes to division sites through its interaction with native FtsN. If midcell localization of GFP-FtsN<sub>Cyto-TM</sub> is indeed dependent on interaction with previously localized FtsN, then loss of FtsN should result in delocalization of GFP-FtsN<sub>Cyto-TM</sub>. Although FtsN is an essential cell division protein, cells can survive the loss of ftsN when a single amino acid mutation in FtsA (FtsA-E124A) is present (Bernard et al., 2007). Using an ftsN deletion strain carrying a chromosomal ftsA-E124A allele (WM3302), I observed localization of GFP-FtsN<sub>Cyto-TM</sub> at division sites in 87% of cells, indicating that GFP-FtsN<sub>Cyto-TM</sub> is efficiently recruited to the divisome independently of FtsN (Fig. 4-1F).

4.2.2 Localization of GFP-FtsN<sub>Cyto-TM</sub> to midcell is dependent on FtsA.

Because the amino terminus of FtsN interacts with cell division protein FtsA, I hypothesized that FtsA recruits GFP-FtsN<sub>Cyto-TM</sub> directly to division sites. Using the ftsA12(Ts) strain WM1115, I compared the localization of GFP-FtsN<sub>Cyto-TM</sub> at the permissive temperature of 30°C to the pattern of localization at the non-permissive temperature of 42°C. Because FtsA12 delocalizes from division sites within 5 minutes at 42°C (Fig. 4-2), I visualized cells 5 minutes after the temperature shift. GFP-FtsN<sub>Cyto-TM</sub> formed midcell rings at the permissive temperature as expected, but delocalized within the 5 minute temperature shift (Fig. 4-3). Similar results were seen when I monitored the localization of GFP-tagged FtsN<sub>ΔSPOR</sub>, a construct that retains the cytoplasmic and transmembrane domains of FtsN but lacks the SPOR domain (Fig. 4-1A and data not shown). It is unlikely that thermal
Figure 4-2. FtsA12(Ts) delocalizes from division sites within 5 minutes at 42°C. DIC (left), DAPI (middle), and α-FtsA (right) images of ftsA12(Ts) (WM1115) strain harboring plasmid pWM1088 (pDSW207-gfp). Cells were grown to low OD$_{600}$ without IPTG at 30°C then shifted to 42°C for 5 and 30 minutes. Cells were fixed before staining DNA with DAPI.
and detecting FtsA with α-FtsA primary and Alexa Fluor 488-conjugated secondary antibodies. Scale bar = 4 µm.
Figure 4-3. Localization of GFP-FtsN_{Cyto-TM} to midcell is dependent on FtsA. DIC (left) and fluorescence (right) images of *ftsA12*(Ts) strains harboring plasmids pWM1088 (pDSW207-*gfp*), pWM4528 (pDSW207-*gfp-ftsN_{Cyto-TM}*), pWM1152 (pDSW207-*gfp-ftsN*), pWM3775 (pDSW207-*gfp-ftsZ*), or pWM4740 (pKG116-\textsuperscript{TT}*mCherry-ftsN_{SPOR}*). Cells were grown to low OD\textsubscript{600} without IPTG (strains containing pDSW207 derivatives) or with 1 µM sodium salicylate (strain containing pKG116 derivative) at 30°C then shifted to 42°C for 5 minutes. Scale bar = 4 µm.
instability or other non-specific factors caused GFP-FtsN_{Cyto-TM} to delocalize, as GFP-FtsN_{Cyto-TM} continued to form fluorescent rings at midcell after 5 or 30 minutes at 42°C in WM1074, the wild-type parent of WM1115 (Fig. 4-4). Moreover, GFP-FtsN_{Cyto-TM} continued to localize at potential division sites after deactivation of FtsI by cephalexin treatment (Fig. 4-5) and after therminactivation of ftsI(Ts) or ftsQ(Ts) at 42°C (Fig. 4-6). Although rapid cell lysis has been described during cephalexin treatment of *E. coli* cells under specific conditions (Chung et al., 2009), I did not observe lysis using standard growth conditions.

I then asked whether full length FtsN depended on FtsA for its localization. Like GFP-FtsN_{Cyto-TM}, GFP-FtsN localized at the permissive temperature in the *ftsA*{12}(Ts) strain (Fig. 4-3). Localization of GFP-FtsN at midcell decreased in frequency after therminactivation at 42°C (Table 4-1), but weak fluorescent rings could still be observed at division sites at 42°C, perhaps via SPOR-dependent localization. To test this hypothesis, I tagged the SPOR domain of FtsN with Tat-targeted mCherry (TTmCherry-FtsN_{SPOR}). TTmCherry harbors the signal sequence of TorA, a Tat-targeted substrate that allows efficient export of FtsN_{SPOR} to the periplasm (Thomas et al., 2001). TTmCherry-FtsN_{SPOR} remained at midcell after therminactivation of *ftsA*{12}(Ts) (Fig. 4-3), supporting the idea that the SPOR domain of FtsN localizes to the divisome independently of FtsA. Not surprisingly, GFP-tagged FtsZ, which does not depend solely on FtsA for localization (Pichoff & Lutkenhaus, 2002), remained largely unaffected by the temperature shift, forming fluorescent rings at 42°C. Overall, the failure of GFP-FtsN_{Cyto-TM} to form fluorescent rings in the *ftsA*{12}(Ts) strain at the non-permissive temperature suggests that localization of GFP-FtsN_{Cyto-TM} to division sites is dependent on proper localization of FtsA.

To test independently whether FtsA is sufficient to recruit FtsN_{Cyto-TM}, I turned to the polar recruitment assay, which had shown for the first time that FtsA and full-length FtsN could interact (Corbin et al., 2004). Briefly, a bait protein is fused to the *Bacillus subtilis* protein DivIVA that preferentially localizes to areas of curvature within any cell, which in *E.
Figure 4-4. Localization of GFP- and TTmCherry-tagged constructs is not disrupted at 42˚C in wild-type cells. DIC (left) and fluorescence (right) images of wild-type (WM1074) strains harboring plasmids pWM1088 (pDSW207-gfp), pWM4528 (pDSW207-gfp-ftsN<sub>Cyo</sub>-<sub>TM</sub>), pWM1152 (pDSW207-gfp-ftsN), or pWM4740 (pKG116-T<sup>T</sup>TmCherry-ftsN<sub>SPOR</sub>). Cells were grown to low OD<sub>600</sub> without IPTG (pDSW207 strains) or with 1 µM sodium salicylate (pKG116 strain) at 30˚C then shifted to 42˚C for 5 and 30 minutes. Scale bar = 4 µm.
Figure 4-5. Localization of GFP-tagged FtsN_{Cyto-TM} and FtsN is not dependent on FtsI activity. DIC (left) and fluorescence (right) images of wild-type (WM1074) strains harboring plasmids pWM4528 (pDSW207-gfp-ftsN_{Cyto-TM}), pWM1152 (pDSW207-gfp-ftsN), or pWM4740 (pKG116-\textsuperscript{TmCherry-ftsN}_{SPOR}). Cells were grown to low OD_{600} without IPTG (strains containing pDSW207 derivatives) or with 1 µM sodium salicylate (strain containing pKG116 derivative) at 37°C. Cultures were back-diluted 1:1 and imaged at 30 minutes and 60 minutes after addition of cephalexin to inhibit FtsI activity. Cultures were also back-diluted after the 30-minute time point to maintain a low OD_{600}. Scale bar = 4 µm.
Figure 4-6. Localization of GFP-FtsN_{Cyto-TM} in *ftsQ*(Ts) and *ftsI*(Ts) strains. DIC (left) and fluorescence (right) images of *ftsQ*(Ts) and *ftsI*(Ts) strains harboring plasmids pWM1088 (pDSW207-*gfp*), pWM4528 (pDSW207-*gfp*-ftsN_{Cyto-TM}), pWM1152 (pDSW207-*gfp*-ftsN), or pWM3775 (pDSW207-*gfp*-ftsZ). Cells were grown to low OD$_{600}$ without IPTG at 30°C then shifted to 42°C for 30 minutes. Scale bar = 4 µm.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fusion</th>
<th>Total # cells</th>
<th>% Cells with rings at 30°C</th>
<th>% Cells with rings after 5 minutes at 42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWM1088</td>
<td>GFP</td>
<td>233</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pWM4528</td>
<td>GFP- FtsN&lt;sub&gt;Cyto-TM&lt;/sub&gt;</td>
<td>211</td>
<td>63</td>
<td>0</td>
</tr>
<tr>
<td>pWM1152</td>
<td>GFP- FtsN</td>
<td>595</td>
<td>67</td>
<td>15</td>
</tr>
<tr>
<td>pWM3775</td>
<td>GFP- FtsZ</td>
<td>241</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>pWM4740</td>
<td>TT&lt;sub&gt;mCherry&lt;/sub&gt;- FtsN&lt;sub&gt;SPOR&lt;/sub&gt;</td>
<td>408</td>
<td>88</td>
<td>39</td>
</tr>
</tbody>
</table>

**TABLE 4-1.** Frequency of division site localization of GFP-tagged and TT<sub>mCherry</sub>-tagged division proteins in the *ftsA12(Ts)* strain. Cells were grown as described in Fig. 4-3 and imaged live.
coli are the cell poles and division septa (Edwards et al., 2000). If the co-produced GFP-tagged prey protein is a divisome protein and interacts with the bait protein, the prey will relocalize from solely the septum to the cell poles as well as the septum. This change in localization is easily detected using fluorescence microscopy (Ding et al., 2002). For the current experiment, bait protein FtsA was fused to DivIVA (pBAD33-divIVA-ftsA) and prey protein FtsN<sub>Cyto-TM</sub> was fused to GFP (pDSW207-gfp-ftsN<sub>Cyto-TM</sub>). Upon induction of gfp-ftsN<sub>Cyto-TM</sub> expression only, GFP-FtsN<sub>Cyto-TM</sub> localized to cell division sites but did not accumulate at poles (Fig. 4-7A, top middle panel). When expression of both gfp-ftsN<sub>Cyto-TM</sub> and divIVA-ftsA was induced, however, GFP-FtsN<sub>Cyto-TM</sub> localized not only at division sites, but also the cell poles, indicating that DivIVA-FtsA can efficiently recruit GFP-FtsN<sub>Cyto-TM</sub> to cell poles (Fig. 4-7A, bottom middle panel, arrow). Additionally, I confirmed that DivIVA-FtsA does not affect the diffuse localization of unfused GFP but does recruit GFP-FtsN to cell poles as reported in Corbin et al. (2004) (Fig. 4-7A, left and right panels, arrow).

Because GFP-FtsN<sub>Cyto-TM</sub> localized to midcell in a ΔftsN ftsA-E124A strain that lacks the wild-type ftsA allele (Fig. 4-1F), I also examined whether DivIVA-FtsA-E124A could recruit GFP-FtsN<sub>Cyto-TM</sub> to cell poles. Like DivIVA-FtsA, DivIVA-FtsA-E124A recruited both GFP-FtsN<sub>Cyto-TM</sub> and GFP-FtsN to sites of cell curvature (Fig. 4-7B, arrows). Based on the delocalization of GFP-FtsN<sub>Cyto-TM</sub> upon thermonactivation of FtsA12(Ts) (Fig. 4-3) and the polar recruitment of GFP-FtsN<sub>Cyto-TM</sub> by DivIVA-FtsA and DivIVA-FtsA-E124A (Fig. 4-7), I conclude that FtsA is likely necessary and sufficient for recruitment of GFP-FtsN<sub>Cyto-TM</sub> to division sites.

4.2.3 The cytoplasmic and SPOR domains of FtsN share overlapping roles in localization of FtsN.

Although the FtsA-FtsN<sub>Cyto-TM</sub> interaction is capable of recruiting FtsN<sub>Cyto-TM</sub> to specific cellular locations, the periplasmic SPOR domain is thought to be the major
**Figure 4-7. DivIVA-FtsA and DivIVA-FtsA-E124A recruit GFP-FtsNCyto-TM to poles.** Images are of wild-type cells containing pWM1088 (pDSW207-gfp), pWM4528 (pDSW207-gfp-ftsNCyto-TM), or pWM1152 (pDSW207-gfp-ftsN) plus either pWM1806 (pBAD33-divIVA-ftsA) (A) or pWM4637 (pBAD33-divIVA-ftsA-E124A) (B). Cells were grown to low OD$_{600}$ at 30˚C and then induced for two hours with either 40 µM IPTG only (to induce expression from pDSW207 plasmids) or 40 µM IPTG plus 0.2% arabinose (to induce expression from both plasmids). White arrows indicate examples of polar localization of GFP-tagged constructs. Scale bar = 4 µm.
localization determinant of FtsN (Gerding et al., 2009). To elucidate the relative contributions of the cytoplasmic and SPOR domains to midcell localization of FtsN, I visualized cells producing GFP fusions to FtsN mutants that lacked the native cytoplasmic domain (VirB10_{Cyto}N_{TM-Peri}), the SPOR domain (FtsN_{ΔSPOR}), or both domains (VirB10_{Cyto}N_{TM-PeriΔSPOR}) and quantified the occurrence of midcell localization at non-constricting and constricting sites. I had also attempted to truncate the first 20 residues of the cytoplasmic domain of FtsN (FtsN_{Δ1-20}), but the construct was unstable (data not shown). Additionally, I mutated residues 16-19 that are involved in FtsA-FtsN interaction in vitro (Busiek et al., 2012) but, like FtsN_{Δ1-20}, this mutant was also unstable (data not shown). Although GFP-VirB10_{Cyto}N_{TM-Peri} and GFP-FtsN_{ΔSPOR} localized to potential division sites, the fusion lacking both domains (GFP-VirB10_{Cyto}N_{TM-PeriΔSPOR}) failed to localize (Fig. 4-8).

These results indicate that localization of FtsN requires either the cytoplasmic or SPOR domain for localization to potential division sites and that loss of both domains prevents recruitment of FtsN. Furthermore, the majority of fluorescent GFP-VirB10_{Cyto}N_{TM-Peri} rings were located at division sites that were visibly constricting (64%), whereas the majority of fluorescent GFP-FtsN_{ΔSPOR} rings were located at non-constricting sites (62%; Table 4-2). These results imply that on average, GFP-VirB10_{Cyto}N_{TM-Peri} localizes to midcell during a later stage of cytokinesis than GFP-FtsN_{ΔSPOR}.

4.2.4 GFP-FtsN_{Cyto-TM} localizes to midcell prior to TTmCherry-FtsN_{SPOR}

To test directly the hypothesis that SPOR-independent localization of FtsN precedes SPOR-dependent localization, I co-produced GFP-FtsN_{Cyto-TM} and Tat-targeted mCherry (TTmCherry) fused to FtsN_{SPOR} in the same cell and compared their localization. The Tat-targeting sequence was fused to the mCherry-FtsN_{SPOR} chimera to facilitate export of the construct to the periplasm (Thomas et al., 2001). Although I observed frequent co-localization of GFP-FtsN_{Cyto-TM} and TTmCherry-FtsN_{SPOR} as expected, GFP-FtsN_{Cyto-TM} and
Figure 4-8. Removal of both cytoplasmic and SPOR domains of FtsN abolishes midcell localization. DIC (left) and fluorescence (right) images of wild-type strains containing plasmid pWM1088 (pDSW207-gfp) (A), pWM4528 (pDSW207-gfp-ftsN_Cyto-TM) (B), pWM1152 (pDSW207-gfp-ftsN) (C), pWM4694 (pDSW207-gfp-virB10_CytoN_TM-PeriΔSPOR) (D), pWM4696 (pDSW207-gfp-virB10_CytoN_TM-Peri) (E), or pWM4693 (pDSW207-gfp-ftsNΔSPOR) (F). Cultures were grown to low OD$_{600}$ at 30˚C without inducer before visualization. Scale bar = 4 µm.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fusion</th>
<th>Total # cells</th>
<th>% Cells with rings</th>
<th>% Cells without rings</th>
<th>% Rings at no constrictions</th>
<th>% Rings at constrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWM1152</td>
<td>GFP-FtsN</td>
<td>130</td>
<td>72</td>
<td>28</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>pWM4528</td>
<td>GFP-FtsN&lt;sub&gt;Cyto-TM&lt;/sub&gt;</td>
<td>165</td>
<td>56</td>
<td>44</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>pWM4693</td>
<td>GFP-FtsN&lt;sub&gt;ΔSPOR&lt;/sub&gt;</td>
<td>139</td>
<td>60</td>
<td>40</td>
<td>62</td>
<td>38</td>
</tr>
<tr>
<td>pWM4696</td>
<td>GFP-VirB10&lt;sub&gt;Cyto N&lt;sub&gt;TM-Peri&lt;/sub&gt;</td>
<td>130</td>
<td>64</td>
<td>36</td>
<td>36</td>
<td>64</td>
</tr>
<tr>
<td>pWM4694</td>
<td>GFP-VirB10&lt;sub&gt;Cyto N&lt;sub&gt;TM-PeriΔSPOR&lt;/sub&gt;</td>
<td>142</td>
<td>0</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**TABLE 4-2. Frequency of division site localization of GFP-tagged FtsN variants.** Cells were grown as described in Fig. 4-7 and imaged live. Visible constriction of cells was determined using DIC images and FM4-64 staining of cell membrane.
TTmCherry-FtsN_{SPOR} also localized to division sites independently of one another. Specifically, rings of GFP-FtsN_{Cyto-TM} were present at quarter sites of cells in the late stages of cytokinesis (denoted by arrows in Fig. 4-9), consistent with GFP-FtsN_{Cyto-TM} localizing to nascent division sites. Conversely, TTmCherry-FtsN_{SPOR} lingered at deep constrictions (denoted by asterisks in Fig. 4-9) and was often not detectable at the nascent division sites that contained GFP-FtsN_{Cyto-TM} (arrows).

I quantified the difference in localization between the constructs by measuring the frequency of division site localization at constricting and non-constricting sites. In cells co-producing both fusions, the vast majority of GFP-FtsN_{Cyto-TM} rings (83%) were located at non-constricting sites, whereas the majority of TTmCherry-FtsN_{SPOR} rings were located at constrictions (62%; Table 4-3). I observed similar localization patterns when GFP-FtsN was co-produced with TTmCherry-FtsN_{SPOR}, although a larger proportion of GFP-FtsN rings were located at constricting sites. The higher percentage of GFP-FtsN rings at constricting sites compared to GFP-FtsN_{Cyto-TM} is expected, as GFP-FtsN also contains the SPOR domain that, according to the above data, localizes preferentially to constricting sites. Together, these data support the hypothesis that SPOR-independent localization of FtsN precedes SPOR-dependent localization.

4.2.5 Inactivation of cytoplasmic or SPOR domains inhibits the ability of FtsN to function in cell division.

To explore the relative physiological importance of SPOR-independent and SPOR-dependent means of FtsN localization, I attempted to create strains carrying a single ftsN allele that lacked the cytoplasmic, SPOR, or both domains. To engineer these strains, I introduced the ΔftsN::kan allele by P1 phage transduction into wild-type cells harboring plasmids pDSW210-flag-virB10_{CytoN_{TM-Peri}}-ftsN_{ΔSPOR}, or -virB10_{CytoN_{TM-PeriΔSPOR}}. Strains carrying pDSW210-flag or pDSW210-flag-ftsN were transduced as negative and positive
Figure 4-9. GFP-FtsN\textsubscript{Cyto-TM} localizes to division sites prior to $^{TT}\text{mCherry-FtsN}_{SPOR}$. DIC (left), GFP (middle), and mCherry (right) filter sets were used to capture images of cells grown to low \(OD_{600}\) at 30°C in the absence of inducers. All strains were wild-type background and contained plasmid pWM4740 (pKG116-$^{TT}\text{mCherry-ftsN}_{SPOR}$) plus either plasmid pWM1088 (pDSW207-gfp) (A), pWM4528 (pDSW207-gfp-ftsN\textsubscript{Cyto-TM}) (B), or pWM1152 (pDSW207-gfp-ftsN) (C). Arrows denote division site localization of GFP-tagged proteins only and asterisks mark localization of $^{TT}\text{mCherry-FtsN}_{SPOR}$ only. Scale bar = 4 \(\mu\)m.
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Fusion</th>
<th>Total # cells</th>
<th>% GFP rings at no constrictions</th>
<th>% GFP rings at constrictions</th>
<th>% mCherry rings at no constrictions</th>
<th>% mCherry rings at constrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWM4528 + pWM4740</td>
<td>GFP-FtsN&lt;sub&gt;Cyto-TM&lt;/sub&gt; + mCherry-FtsN&lt;sub&gt;SPOR&lt;/sub&gt;TT</td>
<td>67</td>
<td>83</td>
<td>16</td>
<td>38</td>
<td>62</td>
</tr>
<tr>
<td>pWM1152 + pWM4740</td>
<td>GFP-FtsN&lt;sub&gt;TT&lt;/sub&gt; + mCherry-FtsN&lt;sub&gt;SPOR&lt;/sub&gt;</td>
<td>70</td>
<td>59</td>
<td>41</td>
<td>35</td>
<td>65</td>
</tr>
</tbody>
</table>

**TABLE 4-3. Distinct localization biases of two FtsN domains.** Cells were grown as described in Fig. 4-8 and imaged live. Visible constriction of cells was determined using DIC images of each cell.
controls, respectively. In the absence of inducer, leaky expression of *ftsN* from the weakened *trc* promoter of pDSW210 complemented the loss of the chromosomal *ftsN* allele (Fig. 4-10A, right column), whereas no transductants were found with empty pDSW210 vector at any IPTG concentration (Fig. 4-10A, left column). Induced expression of *flag-ftsN* also conferred survival in the absence of chromosomal *ftsN* at 10 µM and 100 µM IPTG, suggesting that higher levels of *ftsN* expression did not inhibit growth significantly as has been shown previously (Aarsman et al., 2005).

The mutant *ftsN* constructs were similarly tested for growth in the absence of chromosomal *ftsN*. None of the mutants could complement the loss of the native *ftsN* allele in the absence of inducer. However, in the presence of 10 µM IPTG, FLAG-FtsNΔSPOR partially complemented the loss of the native allele as evidenced by the small colonies formed on this plate. FLAG-FtsNΔSPOR completely complemented upon full induction of the plasmid (100µM IPTG). Although VirB10CytoN_{TM-Peri} did not complement at 0 µM or 10 µM IPTG, full induction of the mutant allele conferred survival. FLAG-VirB10CytoN_{TM-PeriΔSPOR} was unable to complement the loss of *ftsN* at any level of induction, consistent with the fluorescence microscopy results that showed no midcell localization of this mutant (Fig. 4-8). The lack of complementation by FLAG-VirB10CytoN_{TM-PeriΔSPOR} was not due to instability of the protein, because it was present at equal or greater levels than FLAG-FtsNΔSPOR or FLAG-FtsN, both of which were capable of complementing the loss of native FtsN (Fig. 4-10B). Notably, FLAG-VirB10CytoN_{TM-Peri} failed to survive the deletion of *ftsN* at levels of protein equivalent to or higher than FLAG-FtsNΔSPOR (Fig. 4-10A, 10 µM IPTG; compare protein levels at 10 and 100 µM IPTG in Fig. 4-10B), supporting the idea that SPOR-independent localization of FtsN plays an important role in proper localization of FtsN.

To confirm the transduction data, I also tested the effects of depleting FtsN in a strain carrying the same plasmids used in the transduction experiments. Consistent with the transduction data, FLAG-VirB10CytoN_{TM-PeriΔSPOR} failed to complement the loss of *ftsN* at any
Figure 4-10. The cytoplasmic and SPOR domains are both needed for full FtsN function in cell division. (A) ΔftsN::kan P1 transduction of wild-type strains containing pWM2784 (pDSW210-flag), pWM4582 (pDSW210-flag-virB10CytoN_{TM-Peri}), or pWM3157
(pDSW210-\textit{flag-ftsN}). Transductants were plated onto LB medium containing 0, 10, or 100 µM IPTG. (B) \(\alpha\)-FLAG Western blot analysis of FLAG-VirB10\textsubscript{CytoN\textsubscript{TM}}-PeriΔSPOR\(_{\text{C}}\), FLAG-VirB10\textsubscript{CytoN\textsubscript{TM}}-Peri, FLAG-FtsNΔSPOR, and FLAG-FtsN produced from \textit{ftsN}(Ts) strain WM4028. Samples were collected after strains were grown without IPTG to low OD\(_{600}\) at 30°C and subsequently shifted to 42°C at various levels of IPTG induction for 4 hours. The ability of the FtsN constructs to survive P1 transduction at the various levels of IPTG induction are represented by (+) and (-) symbols. (C) DIC images of transductants isolated in (A). Cultures were grown in the presence of 100 µM IPTG to low-mid OD\(_{600}\) at 37°C and visualized (\(T = 0\) hours). Cells were then washed and grown in the absence of IPTG for four hours (\(T = 1, 2, 3,\) and 4 hours) and back-diluted as needed to prevent entry into stationary phase. Scale bar = 12 µm.
level of induction and FLAG-FtsN$_{\Delta SPOR}$ began complementing at a lower level of induction than FLAG-VirB10$_{\text{CytoN}_{\text{TM-Peri}}}$ (Fig. 4-11). Also consistent with the transduction data, FLAG-FtsN complemented both in the absence and presence of inducer. Although substitution of the cytoplasmic and transmembrane regions or deletion of the SPOR domain did not result in cell death in previous publications (Dai et al., 1996, Addinall et al., 1997, Goehring et al., 2007a, Gerding et al., 2009), all of these studies relied on overproduction of the mutated constructs. These results are consistent with these previous results in that FLAG-VirB10$_{\text{CytoN}_{\text{TM-Peri}}}$ and FLAG-FtsN$_{\Delta SPOR}$ could complement the loss of ftsN, but only when overproduced.

To ensure that the cell viability defects of the mutant FtsN constructs were caused by inhibition of cell division and not a general growth defect, I observed the morphology of the transduction survivors upon full IPTG induction of the plasmids and for several generations after depletion of inducer. As expected, ∆ftsN::kan cells producing FLAG-FtsN did not form filaments prior to or after depletion of IPTG (Fig. 4-10C, right panels). Transductants lacking chromosomal ftsN but producing FLAG-FtsN$_{\Delta SPOR}$, on the other hand, formed moderate filaments upon depletion of the inducer (Fig. 4-10C, middle panels), consistent with the results in Fig. 4-10, A-B and Fig. 4-11. Also consistent with the transduction and spot dilution data, ∆ftsN::kan cells producing FLAG-VirB10$_{\text{CytoN}_{\text{TM-Peri}}}$ were more affected by the lack of IPTG, forming long filaments, inferring that cell division was severely compromised. The impacts on cell division among the mutant FtsN strains at low levels of induction are compatible with the idea that these mutant proteins localize poorly. These results suggest that although the cytoplasmic and SPOR domains have overlapping roles in localization, both domains are needed for efficient localization of FtsN to the divisome.
Figure 4-11. Substitution and/or deletion of the cytoplasmic and SPOR domains of FtsN reduce viability in an ftsN depletion strain. Spot dilution assays of ftsN depletion strain WM4028 harboring pWM2784 (pDSW210-\textit{flag}), pWM4612 (pDSW210-\textit{flag-virB10\textsubscript{Cyto}\textsubscript{N}\textsubscript{TM-Peri}\textsubscript{ΔSPOR}}), pWM4582 (pDSW210-\textit{flag-virB10\textsubscript{Cyto}\textsubscript{N}\textsubscript{TM-Peri}}), pWM4613 (pDSW210-\textit{flag-ftsN\textsubscript{ΔSPOR}}), or pWM3157 (pDSW210-\textit{flag-ftsN}). Cultures were grown without IPTG to low OD\textsubscript{600} at 30°C. Cultures were diluted to $10^{-3}$-$10^{-6}$ concentrations and spotted onto LB plates containing 0, 10, or 100 µM IPTG and incubated at 30°C and 42°C.
Figure 4-12. Proposed role of FtsA-FtsN interactions in cell division. Following midcell localization of FtsA (A), interaction between FtsA and the amino-terminus of FtsN weakly recruits FtsN to midcell in a SPOR-independent manner (B; black asterisks represent recruitment). The small fraction of FtsN localized at midcell recruits amidases AmiB and AmiC and stimulates the peptidoglycan synthesis activity of FtsI. Activities of amidases AmiB and AmiC are stimulated by their cognate activators including FtsN-dependent NlpD (not shown) to degrade peptidoglycan (C; lightning bolt graphic represents stimulation of activity). Peptidoglycan remodeling at midcell exposes denuded glycan strands which, in turn, promote SPOR-dependent localization of FtsN (D). Additional FtsN molecules at the division site stimulate further peptidoglycan remodeling in a positive feedback loop, represented by a “+” symbol. OM=outer membrane, PG=peptidoglycan, IM=inner membrane.
4.3 Discussion

The data presented here support a model in which interaction between FtsA and FtsN weakly recruits FtsN to midcell by its amino-terminus following incorporation of FtsA into the divisome (Fig. 4-12). After its recruitment to the divisome, FtsN is poised to recruit amidases and stimulate the activity of FtsI. The activity of FtsN, amidases, and FtsI attracts additional FtsN molecules via the SPOR domain and reinforces a positive feedback loop. A similar model of self-enhanced recruitment of FtsN was first proposed by Gerding et al. (2009), but the mechanism of initial FtsN localization was unknown at the time.

SPOR-dependent localization of cell division proteins appears to be a common mechanism for division site recruitment: the SPOR domain of *E. coli* cell division proteins FtsN, DamX, and DedD directs all three of these proteins to midcell (Gerding et al., 2009, Arends et al., 2010). Although SPOR-dependent localization of FtsN is supported here, I also assert that the amino-terminus of FtsN contributes to midcell recruitment. The need for both cytoplasmic and periplasmic domains for midcell localization has already been shown for the essential *E. coli* cell division protein FtsQ. Similar to FtsN, the cytoplasmic domain of FtsQ was originally described as a dispensable portion of the protein (Goehring et al., 2007a). However, as with the experiments performed with FtsN proteins that lacked the cytoplasmic domain, these studies were performed at induced levels of the mutant protein (Guzman et al., 1997). It was later shown that the cytoplasmic domain of FtsQ is not dispensable and, in addition to its periplasmic localization determinant, has a role in midcell localization of the protein (Goehring et al., 2007a). The cytoplasmic and periplasmic domains are not the only domains of the bitopic division proteins that contribute to localization. The localization of FtsI, for example, requires its transmembrane region (Wissel et al., 2005). Although the transmembrane domain of FtsN is not directly involved in SPOR-independent localization (Fig. 4-1), the results suggest that putative dimerization of
this domain (Busiek et al., 2012) is important for its interaction with FtsA and, hence, FtsA-dependent recruitment of FtsN.

The immediate delocalization of GFP-FtsNCyto-TM from division sites upon shifting the \(ftsA12(Ts)\) strain to the non-permissive temperature (Fig. 4-3) strongly suggests that SPOR-independent localization of GFP-FtsNCyto-TM is mediated by its interaction with FtsA. This conclusion is further supported by the efficient recruitment of GFP-FtsNCyto-TM to poles by DivI-FAFtsA and DivI-FAFtsA-E124A (Fig. 4-7) as well as previously published evidence that FtsA and FtsN interact directly (Busiek et al., 2012). FtsA-dependent localization of FtsN may also explain why FtsN fails to localize to midcell in the absence of FtsA, despite the recruitment of all other essential cell division proteins by a ZapA-FtsQ fusion (Goehring et al., 2005).

The involvement of FtsA in the localization of FtsN also provides a potential explanation for the ability of FtsN to suppress the \(ftsA12(Ts)\) strain at 37°C (but not at 42°C) when present in a multicopy pBR322 derivative plasmid (Dai et al., 1993). Overproduction of FtsN from the plasmid might partially overcome the need for SPOR-independent localization of FtsN, similar to how overproduction of VirB10\text{Cyto}\text{N}_{TM-Peri} is capable of complementing the lack of wild-type FtsN (Fig. 4-10A, 4-10C, and 4-11). Overproduction of FtsN or VirB10\text{Cyto}\text{N}_{TM-Peri} enriches these proteins in the membrane and likely increases the probability that these proteins will interact with the divisome non-specifically. Although I believe that multicopy expression of \(ftsN\) may bypass the need for FtsA-mediated localization, I do not yet understand how the cytoplasmic and transmembrane domains of FtsN can partially suppress the loss of \(ftsK\) or \(ftsQ\) (Geissler & Margolin, 2005, Goehring et al., 2007a).

The disparate enrichment of GFP-FtsN\(_{\Delta SPOR}\) at non-constricting sites and GFP-VirB10Cyto\text{N}_{TM-Peri} at constricting sites initially suggested to us that SPOR-independent localization of FtsN precedes SPOR-dependent localization of FtsN (Table 4-2). I confirmed
this timing by observing cells that co-produced GFP-tagged FtsNCyto-TM and TTmCherry-tagged FtsNSPOR (Fig. 4-9). Intriguingly, while TTmCherry-FtsNSPOR seemed to persist at deep constrictions, GFP-FtsNCyto-TM appeared to abandon these sites for nascent division sites (as shown by the loss of GFP-FtsNCyto-TM fluorescence at deep constrictions in Fig. 4-9). This observation suggests that while the amino-terminus of FtsN facilitates initial localization of FtsN, this interaction may weaken despite the continuous presence of FtsA at the septum until later stages of cytokinesis. This may be because at later stages, FtsNCyto-TM now faces increasing competition with another protein for its binding site within subdomain 1c of FtsA, including another molecule of FtsA (Pichoff et al., 2012). Another possibility is that FtsA itself begins to leave the divisome prior to septum completion, although a very recent study suggests that FtsA persists until septum closure (Soderstrom et al.). In any case, in this model, the main responsibility of maintaining FtsN at the growing septum until completion would then fall to the SPOR domain. The anchoring of FtsN to old division sites by the SPOR domain would prevent premature abandonment by FtsN as FtsA redeployed to newly assembled Z rings. This built-in regulation of FtsN localization is likely an important feature of FtsN, as substitution or deletion of either localization determinant resulted in reduced cell division and viability (Fig. 4-10A, 4-10C, and 4-11).

While two previous publications from the Margolin laboratory have explored if and how FtsA and FtsN interact (Corbin et al., 2004, Busiek et al., 2012), this study addresses the more interesting question of why these early and late proteins contact one another. At least one physiological role of FtsA-FtsN interaction is efficient recruitment of FtsN to the divisome, although additional functions of this interaction may also be uncovered. One interesting possibility is that once recruited, FtsN may transduce a signal to FtsA that triggers initiation of Z ring constriction, but further studies will be needed to address this prospect (Lutkenhaus, 2009, Busiek et al., 2012, Corbin et al., 2004). I also speculate that the role of other early-late cell division protein interactions (Corbin et al., 2004, Karimova et
al., 2005) could be to facilitate efficient recruitment of late cell division proteins to the divisome. Regardless, the current study provides the first evidence for a physiological role of FtsA-FtsN interaction under physiological conditions.
Chapter V

Overproduction of FtsN$_{\text{Cyto-TM}}$ causes dominant negative effects in rich and minimal media
5.1 Introduction

The data presented in Chapters 3 and 4 demonstrate that the cytoplasmic and transmembrane domains of FtsN (FtsN\textsubscript{Cyto-TM}) do not simply serve as a membrane anchor for the periplasmic domain. Instead, these regions are required and sufficient for the interaction of FtsN with the cytoplasmic divisome protein FtsA (Fig. 3-3). Furthermore, FtsA-FtsN interaction via the amino-terminal region of FtsN appeared to play an important function during \textit{E. coli} cell division because removal of FtsN’s cytoplasmic domain inhibited cell growth (Fig. 4-10). Indeed, a physiological role of FtsA’s interaction with FtsN\textsubscript{Cyto-TM} was identified and involved the efficient localization of FtsN to division sites (Fig. 4-3 and Fig. 4-7).

One remaining question regarding the activity of FtsN\textsubscript{Cyto-TM} was its dominant negative effect upon overproduction. Specifically, cells overproducing FtsN\textsubscript{Cyto-TM} became moderately filamentous, indicating a delay in cell division (Fig. 3-8C). Overproduction of FtsN\textsubscript{Cyto-TM} also caused a wrinkled colony morphotype when cells were induced on rich media agar plates (Fig. 3-8F). Here, I utilized a number of techniques to investigate the dominant negative phenotypes further. During this exploration, I discovered additional effects of FtsN\textsubscript{Cyto-TM} overproduction, including unexpected changes in cell shape, FtsZ localization, and chromosome segregation. I also determined that these effects were specific to FtsN, ruling out the possibility that overproduction of unrelated cytoplasmic and transmembrane domains causes a general effect on cell division and nucleoids.
5.2 Results

5.2.1 Overproduction of FtsI_{Cyto-TM} does not cause moderate filamentation of cells.

The first 55 residues of FtsN (FtsN_{Cyto-TM}) are 1) sufficient for interaction with FtsA (Fig. 3-3), 2) cause moderate filamentation of cells when overproduced (Fig. 3-8), and 3) localize to division sites (Fig. 4-1). To investigate the moderate filamentation phenotype further, I first determined if the average increase in cell length was specific to overproduction of FtsN_{Cyto-TM} or a non-specific effect caused by overproduction of the cytoplasmic and transmembrane domains from similarly organized *E. coli* cell division proteins. I tested this idea by overproducing the cytoplasmic and transmembrane domains of FtsI (FtsI_{Cyto-TM}). Like FtsN, FtsI is an essential bitopic membrane protein with a small cytoplasmic tail, a single pass transmembrane region, and a much larger periplasmic domain. FtsI also localizes to division sites late during cell division although, unlike FtsN, the localization of FtsI is dependent on its transmembrane domain (Wissel et al., 2005).

Despite the similarities in temporal localization and domain organization between FtsI and FtsN, FtsI_{Cyto-TM} did not cause a moderate cell division delay when overproduced (Fig. 5-1A). Instead, cells containing empty vector or overproducing FtsI_{Cyto-TM} became only slightly longer upon induction with the sugar arabinose, as has been noted previously (unpublished data). The lack of filamentation was not due to insufficient protein levels of FtsI_{Cyto-TM}, as shown by Western blot analysis (Fig. 5-1B). These results strongly suggest that the moderate filamentation phenotype observed among cells overproducing FtsN_{Cyto-TM} is specific to the cytoplasmic and transmembrane regions of FtsN. Although overproduction of FtsN_{Cyto-TM} caused moderate filamentation of cells, overproduction of FtsN_{Cyto-TM} or FtsI_{Cyto-TM} did not significantly affect cell viability in spot dilution assays (Fig. 5-1C).

5.2.2 Overproduction of FtsN_{Cyto-TM} negatively affects cellular levels of FtsZ.
Figure 5-1. Overproduction of FtsNCyto-TM, but not FtsICyto-TM, causes a cell division delay in rich medium. Panel A shows representative micrographs obtained from cells of strains WM4049 (EV), WM4050 (FtsNCyto-TM), and WM4228 (FtsICyto-TM) that were grown to low OD$_{600}$ at 37°C in LB broth and either induced at 0.2% arabinose (I) or uninduced (U) for 2 hours. (B) α-His Western blot analysis of strains grown and treated as described in (A). Panel C shows a spot dilution assay of the strains described above. Cultures were grown without inducer prior to spotting onto LB agar plates containing 0% arabinose (uninduced) or 0.2% arabinose (induced).
To understand the molecular mechanism of the cell division delay observed among cells overproducing FtsN\textsubscript{Cyto-TM}, I first checked if the scaffold of the divisome, the Z ring, was affected. Although Z rings continued to form in cells overproducing FtsN\textsubscript{Cyto-TM} (data not shown), cellular levels of FtsZ decreased (5-2, A-B). Based on this result, I hypothesized that the observed cell division delay was a consequence of a diminished cellular pool of FtsZ. If low FtsZ protein levels were responsible for moderate filamentation of cells overproducing FtsN\textsubscript{Cyto-TM}, then restoration of FtsZ to native levels should suppress cell filamentation. To restore native levels of FtsZ, I utilized two strains, each lacking a component of the FtsZ protease ClpXP (Camberg \textit{et al.}, 2009). Western blot analysis revealed that deletion of \textit{clpX} (encoding the ATPase subunit of ClpXP) or \textit{clpP} (encoding the proteolytic subunit of ClpXP) prevented the reduction in FtsZ levels typically observed upon overproduction of FtsN\textsubscript{Cyto-TM} (Fig. 5-3A). Contrary to my hypothesis, restoration of FtsZ levels did not suppress the moderate cell filamentation phenotype (Fig. 5-3B), suggesting that a decrease in FtsZ levels upon overproduction of FtsN\textsubscript{Cyto-TM} was not responsible for the cell filamentation phenotype.

\textbf{5.2.3 Overproduction of FtsN\textsubscript{Cyto-TM} causes a change in cell shape and a slow growth phenotype when cells are cultured in minimal media}

I had previously determined that overproduction of FtsN\textsubscript{Cyto-TM} caused moderate filamentation of cells only if the cell culture entered the late logarithmic/early stationary phase of growth (data not shown). This observation led me to hypothesize that the cell filamentation phenotype was associated with starvation or low nutrient availability. To test this idea, cells overproducing FtsN\textsubscript{Cyto-TM} were grown in media containing a poor carbon source and lacking amino acids (M9 minimal medium broth plus 1% glycerol) to mimic the late logarithmic/early stationary phase of growth. Surprisingly, overproduction of FtsN\textsubscript{Cyto-TM} in minimal media cultures caused a change in cell morphology from a typical rod shape to
Figure 5-2. Overproduction of FtsN_{Cyto-TM} causes a decrease in native FtsZ levels.

Panel A shows $\alpha$-FtsZ Western blot analysis of strains WM4049 (EV), WM4050 (FtsN_{Cyto-}
and WM4228 (FtsI<sub>Cyo-TM</sub>) that were grown to low OD<sub>600</sub> at 37°C in LB broth and either induced at 0.2% arabinose (I) or uninduced (U) for 2 hours. Quantitation of relative FtsZ protein levels from panel A is displayed in (B).
Figure 5-3. Deletion of FtsZ protease components ClpP or ClpX restores FtsZ levels when FtsN_{Cyto-TM} is overproduced but does not prevent delayed cell division. (A) α-FtsZ Western blot analysis of ClpP− strains WM4240 (EV) and WM4241 (FtsN_{Cyto-TM}) and ClpX− strains WM4243 (EV) and WM4244 (FtsN_{Cyto-TM}). Cultures were grown to low OD_{600} at 37°C in LB broth and either induced at 0.2% arabinose (I) or uninduced (U) for 2 hours. Samples were visualized before collection and normalization (B).
an atypical curved shape (Fig. 5-4A). The effect of FtsN\textsubscript{Cyto-TM} overproduction appeared to be specific, as overproduction of FtsI\textsubscript{Cyto-TM} did not affect cell shape (Fig. 5-4A).

Because cell shape was markedly affected by overproduction of FtsN\textsubscript{Cyto-TM}, I tested if cell viability was also affected during growth on minimal media. Spot dilution assays showed that overproduction of FtsN\textsubscript{Cyto-TM} on minimal medium plates caused a significant delay in growth, yielding weak colonies after several days of incubation at 37˚C (Fig. 5-4, B-C). Overproduction of FtsI\textsubscript{Cyto-TM} did not inhibit growth (Fig. 5-4B). These data indicate that overproduction of FtsN\textsubscript{Cyto-TM}, although not lethal, strongly affects cell shape and cell growth in minimal media.

5.2.4 Overproduction of FtsN\textsubscript{Cyto-TM} affects Z ring and nucleoid placement among cells grown in minimal media.

Although FtsN\textsubscript{Cyto-TM} overproduction had no observable effects on Z ring formation when cells were grown in rich media, the impressive change in cell shape and inhibited growth on minimal media prompted me to ask if Z ring formation was affected in cells overproducing FtsN\textsubscript{Cyto-TM} in minimal medium. Using α-FtsZ IFM, I observed infrequent localization of FtsZ at midcell. Instead, FtsZ often localized near cell poles (Fig. 5-5, right image). DAPI-staining of chromosomes revealed that nucleoids were often present at midcell, not segregated to cell poles as would be expected (Fig. 5-5, right image; Fig. 5-6, DAPI image). In longer cells containing segregated nucleoids, FtsZ was no longer located on either side of the nucleoid; instead, FtsZ localized between the nucleoids at midcell where it formed V-shaped or globular structures (Fig. 5-5, right image). These localization patterns differed significantly from the sharp midcell Z rings formed in cells containing empty vector only (Fig. 5-5, left image). Together, these observations suggest that Z ring formation is negatively affected by overproduction of FtsN\textsubscript{Cyto-TM} in minimal medium, likely as a consequence of DNA occluding FtsZ from midcell by nucleoid occlusion.
Figure 5-4. Overproduction of $\text{FtsN}_{\text{Cyto-TM}}$ causes cell shape and slow growth phenotypes when grown in minimal medium. Panel A shows representative micrographs obtained from cells of strains WM4049 (EV), WM4050 ($\text{FtsN}_{\text{Cyto-TM}}$), and WM4228 ($\text{FtsI}_{\text{Cyto-TM}}$) that were grown to low OD$_{600}$ at 37°C in M9 broth containing 1% glycerol and either induced at 0.2% arabinose (I) or uninduced (U) for 2 hours. Panels B-C show a spot dilution assay of the strains described above. Cultures were grown without
inducer prior to spotting onto M9 agar plates containing 1% glycerol and either 0% arabinose (uninduced) or 0.2% arabinose (induced). Plates were incubated overnight (B) or 5 days (C) at 37°C.
Figure 5-5. Cells overproducing $FtsN_{Cyto-TM}$ often contain nucleoids trapped at midcell that occlude FtsZ. Overlay of $\alpha$-FtsZ (false-colored green) and DAPI (false-colored red) images of strains WM4049 (EV) and WM4050 ($FtsN_{Cyto-TM}$). Cultures were grown to low OD$_{600}$ at 37˚C in M9 minimal medium containing 1% glycerol but lacking arabinose. A final concentration of 0.2% arabinose was then added to cultures for 2 hours prior to methanol fixation. DNA was stained with DAPI and native FtsZ was detected with $\alpha$-FtsZ primary and Alexa Fluor 488-conjugated secondary antibodies.
**Figure 5-6.** GFP-FtsN$_{\text{Cyto-TM}}$ localizes at midcell in cells with trapped nucleoids. DIC (upper left), GFP (upper right; false-colored green), DAPI (lower left; false-colored red), and GFP/DAPI overlay (lower right) images of strain WM4558. The culture of WM4558 was grown to low OD$_{600}$ at 37˚C in M9 minimal medium containing 1% glycerol but lacking arabinose. At a low OD$_{600}$, expression of ftsN$_{\text{Cyto-TM}}$ from plasmid pBAD33 was induced at 0.2% arabinose while expression of gfp-ftsN$_{\text{Cyto-TM}}$ from plasmid pDSW207 remained uninduced.
Because the localization of FtsZ and nucleoids was perturbed upon overproduction of FtsN$_{Cyt0-TM}$ in minimal medium (Fig. 5-5, right image), I determined if localization of GFP-tagged FtsN$_{Cyt0-TM}$ was affected in these cells. When grown in rich medium, GFP-tagged FtsN$_{Cyt0-TM}$ had localized to midcell at future sites of division (Fig. 4-1). When grown in minimal medium, however, GFP-FtsN$_{Cyt0-TM}$ no longer co-localized with FtsZ when FtsN$_{Cyt0-TM}$ was overproduced. Instead, GFP-FtsN$_{Cyt0-TM}$ localized as puncta at midcell (Fig. 5-6, upper right image). These puncta of GFP-FtsN$_{Cyt0-TM}$, in turn, co-localized with a portion of the DAPI-stained nucleoids (Fig. 5-6, lower right image). Although more rigorous testing is needed, the co-localization of GFP-FtsN$_{Cyt0-TM}$ with nucleoids offers the possibility that FtsN$_{Cyt0-TM}$ interacts with DNA, either directly or indirectly.

5.2.5 Midcell nucleoids initiate DNA replication but are not efficiently segregated.

The trapping of nucleoids at midcell is likely due to a chromosome replication or segregation problem. To investigate the first possibility, I co-produced GFP-tagged SeqA with either FtsN$_{Cyt0-TM}$ or, as a negative control, FtsI$_{Cyt0-TM}$. SeqA is a negative regulator of DNA replication initiation that binds newly replicated origin of replication sites (oriC) to prevent premature re-initiation of replication (Lu et al., 1994). Binding of GFP-SeqA to oriC therefore labels origins of replication, allowing an approximate number of chromosome origins of replication to be enumerated. In slowly growing E. coli cells, such as those grown in minimal medium, 1-2 GFP-SeqA foci are typically observed. One or two foci per cell indicate that a cell contains either one nucleoid or two replicating and segregating nucleoids, respectively. Cells containing an empty vector (Fig. 5-7, left image) or overproducing FtsI$_{Cyt0-TM}$ (Fig. 5-7, right image) contained 1-2 GFP-SeqA foci per cell, indicating that DNA replication and segregation proceeded normally. In contrast, curved cells that overproduced FtsN$_{Cyt0-TM}$ often contained a cluster of >2 GFP-SeqA foci near...
Figure 5-7. Trapped nucleoids contain multiple replication initiation sites.

Fluorescence images of GFP-SeqA strains WM4359 (EV), WM4360 (FtsN<sub>Cyto-TM</sub>), and WM4362 (FtsI<sub>Cyto-TM</sub>). Cultures of each strain were grown at 37°C in M9 minimal medium containing 1% glycerol but lacking arabinose and IPTG. At a low OD<sub>600</sub>, expression from plasmid pBAD33 (EV, FtsN<sub>Cyto-TM</sub>, or FtsI<sub>Cyto-TM</sub>) was induced at 0.2% arabinose and expression from plasmid pDSW209 (GFP-SeqA) was induced at 0.01 mM IPTG. Cells were imaged 2 hours following arabinose and IPTG induction.
midcell (Fig. 5-7, middle image), suggesting that nucleoids continued to initiate replication, but did not segregate properly.

To further explore a possible chromosome segregation problem amongst the curved shaped cells, I utilized a SulA-GFP reporter strain. Expression of sulA is induced as part of the SOS response to DNA damage (Little & Mount, 1982). One recently reported source of DNA damage is improper segregation of newly replicated chromosomes (Janssen et al., 2011). Therefore, I utilized the SulA-GFP reporter strain as another indicator of faulty chromosome segregation. Cells overproducing FtsN_{Cyto-TM} in minimal medium cultures were collected and subjected to anti-GFP Western blot analysis to detect SulA-GFP. Western blot analysis detected an elevated level of SulA-GFP when FtsN_{Cyto-TM} was overproduced in minimal media (Fig. 5-8A, middle panel) but not when FtsI_{Cyto-TM} was overproduced (Fig. 5-8A, right panel). Levels of SulA-GFP differed between the two strains despite robust production of FtsI_{Cyto-TM} (Fig. 5-8B, right panel). These data indicate that cells overproducing FtsN_{Cyto-TM} in minimal medium cultures encounter DNA damage, possibly due to defective chromosome segregation.
Figure 5-8. Overproduction of FtsN<sub>Cyto-TM</sub> induces expression of the SOS reporter <i>sulA-gfp</i>. (A) α-GFP Western blot analysis of SulA-GFP reporter strains WM4279 (EV), WM4280 (FtsN<sub>Cyto-TM</sub>), and WM4282 (FtsI<sub>Cyto-TM</sub>). Cultures of each strain were grown to low OD<sub>600</sub> at 37˚C in M9 minimal medium containing 1% glycerol but lacking arabinose. At a low OD<sub>600</sub>, expression from plasmid pBAD33 (EV, FtsN<sub>Cyto-TM</sub>, or FtsI<sub>Cyto-TM</sub>) was induced at 0.2% arabinose for 2 hours. (B) α-His Western blot analysis of induced strains grown and treated as described in (A) was performed to detect His<sub>6</sub>-tagged FtsN<sub>Cyto-TM</sub> and FtsI<sub>Cyto-TM</sub>. 
5.3 Discussion

The data presented here provide clues regarding the mechanism of cell division delay among cells overproducing $F_{tsN}$Cyto-TM. Although overproduction of $F_{tsN}$Cyto-TM caused cellular FtsZ levels to decrease (Fig. 5-2), I was able to separate the effect of reduced FtsZ levels from the moderate filamentation phenotype. This was accomplished by restoring normal FtsZ levels using clpXP mutant strains that overproduced $F_{tsN}$Cyto-TM (Fig. 5-3). The mechanism of FtsZ reduction during overproduction of $F_{tsN}$Cyto-TM is unclear. However, the ability to restore normal FtsZ levels by disruption of a known FtsZ protease (ClpXP) suggests that FtsZ levels are affected at the protein level, not at the transcriptional level. Curiously, samples containing overproduced $F_{tsN}$Cyto-TM and $F_{tsI}$Cyto-TM formed doublet bands during Western blot analysis, suggesting that both proteins may be modified (Fig. 5-1B). It is unknown if or how putative modification of $F_{tsN}$Cyto-TM could contribute to the reduction of cellular FtsZ.

In addition to the moderate filamentation phenotype, overproduction of $F_{tsN}$Cyto-TM in minimal medium cultures also caused a number of unexpected defects in cell shape and chromosome segregation. Changes in E. coli cell shape are often associated with perturbation of cell wall synthesis and degradation enzymes, including penicillin binding proteins such as the FtsN-interacting PBP, FtsI (Young, 2013, Karimova et al., 2005). But cell shape abnormalities can also arise from mutation of cytoplasmic proteins, including FtsA. An FtsA mutant that lacks its membrane targeting sequence, or MTS, forms long, filamentous structures of the mutant protein at midcell and causes cells to curve into a “C” shape (Gayda et al., 1992). Interestingly, GFP-$F_{tsN}$Cyto-TM also formed aggregates at midcell when $F_{tsN}$Cyto-TM was overproduced, although long filaments of GFP-$F_{tsN}$Cyto-TM were not seen (Fig. 5-6). Another possible means of cell shape change elicited by overproduction of $F_{tsN}$Cyto-TM is through the interaction between FtsN and MreB (Fenton & Gerdes, 2013). MreB, like FtsA, is an actin homolog involved in cell shape maintenance (Jones et al.,
The trapping of nucleoids at midcell is an unlikely cause of the curved cell shape, as cell shape changes are not a common feature of chromosome segregation mutants (Yu & Margolin, 1999). Although it is tempting to speculate that the curved cell shape is associated with the interaction between FtsN_{Cyto-TM} and FtsA, more experimentation will be needed to address other possible means of cell shape change.

The trapping of nucleoids at the bend of curve-shaped cells (Fig. 5-6) appears to be a chromosome segregation problem, rather than a DNA replication issue. This conclusion is based in part on the striking similarity between cells overproducing FtsN_{Cyto-TM} in minimal media and parC mutants. ParC is a subunit of topoisomerase IV responsible for decatenating sister chromosomes following DNA replication (Kato et al., 1988). Mutations in parC can prevent its decatenase activity, inhibiting chromosome segregation and yielding cells with midcell nucleoids and aberrant FtsZ localization (Yu & Margolin, 1999). A chromosome segregation problem is also suggested by the clustering of multiple GFP-SeqA foci in curved cells (Fig. 5-7) which represents multiple origins of replication and, presumably, multiple nucleoids. Although many cells overproducing FtsN_{Cyto-TM} in minimal media contained a single, midcell region of DAPI-stained DNA, longer cells within the population had two regions of DAPI-stained DNA, suggesting that chromosome segregation is not completely prevented, but is delayed (Fig. 5-5). This result is consistent with the slow growth phenotype observed by spot dilution assay (Fig. 5-4). Finally, cells overproducing FtsN_{Cyto-TM} in minimal media cultures contained high levels of SulA-GFP, indicating that DNA damage had occurred (Fig. 5-8). One recently identified cause of DNA damage is the improper segregation of newly replicated chromosomes (Janssen et al., 2011). Although other sources of DNA damage are possible, including “guillotining” of trapped nucleoids during closing of the division septum, the similarities between the curved cells and the chromosome segregation parC mutant as well as the clustering of GFP-SeqA foci at midcell
suggest that a chromosome segregation problem prompted expression of the DNA damage reporter *sulA-gfp*.

The mechanism driving delayed chromosome segregation during overproduction of FtsN_Cyto-TM in minimal media cultures is not clear. One possibility is that midcell aggregates of GFP-FtsN_Cyto-TM (Fig. 5-6) directly bind DNA and tether it to the membrane. The conserved, basic patch of residues within the cytoplasmic domain of FtsN (Fig. 3-4F) could facilitate direct DNA binding, although FtsN has no previously demonstrated role in DNA-binding. Another possible source of deficient chromosome segregation involves the genetic interaction between *ftsN* and *minD*, as *ftsN* was isolated during a screen for synthetically lethal with a defective Min system (*slm*) mutants (Goehring et al., 2007b). MinD, in concert with MinC and MinE, comprise the Min system that regulates proper, midcell placement of Z rings in *E. coli* (de Boer et al., 1992b). In addition to its role in Z ring placement, recent evidence suggests that the Min system is also involved in active segregation of *E. coli* chromosomes via binding of chromosomal DNA to the membrane-bound gradient of MinD (Di Ventura et al., 2013). Yet another possible mechanism of deficient chromosome segregation is that overproduction of FtsN_Cyto-TM affects the chromosome segregation activity of the essential cell division protein and DNA translocase, FtsK (Aussel et al., 2002, Begg et al., 1995). FtsN is a multicopy suppressor of FtsK, dependent on FtsN_Cyto-TM (Draper et al., 1998, Goehring et al., 2007b). Finally, overproduction of FtsN_Cyto-TM could compete wild-type FtsN away from the divisome, mimicking an FtsN depletion strain. However, no chromosome segregation defects have been reported upon FtsN depletion (Addinall et al., 1997). Because one or more possible mechanisms for defective chromosome segregation exist, further testing will be needed to identify the responsible factors.

Why do cells overproducing FtsN_Cyto-TM exhibit different phenotypes in rich media cultures compared to poor media cultures? Culturing cells in poor media might be expected
to alleviate some non-lethal division defects because slowed cellular growth could allow 
defective machinery to operate at a slower pace. Then again, starvation conditions present
an additional stress that could exacerbate some cell division problems. It is known, for
instance, that cells producing mutated FtsN<sub>slm117</sub> (residues 1-118) in place of wild-type FtsN
exhibit more severe division phenotypes upon growth in minimal media than rich media
(Gerding et al., 2009). In any case, the data presented here clearly demonstrate that
overproduction of FtsN<sub>Cyto-TM</sub> in rich and poor media produces a number of interesting
phenotypes. Understanding the underlying mechanism(s) of these phenotypes will require
further investigation into the causes of FtsZ degradation, cell shape changes, and nucleoid
trapping.
Chapter VI

Discussion and Future Directions
Efficient and accurate division of a bacterial cell requires 1) faithful replication and segregation of the mother cell chromosome and 2) precise placement, assembly, and function of the divisome. Assembly of the divisome occurs in a temporal manner, with some cell division proteins localizing soon after the initial segregation of the replicated chromosomes and others localizing late (Fig. 1-2) (Aarsman et al., 2005). A linear order of recruitment dependency has been established for the essential \textit{E. coli} cell division proteins (FtsZ$\rightarrow$FtsA/ZipA$\rightarrow$FtsK$\rightarrow$FtsQBL$\rightarrow$FtsW$\rightarrow$FtsI$\rightarrow$FtsN) whereby upstream proteins must be localized at midcell in order for downstream proteins to be recruited (Goehring & Beckwith, 2005). \textit{In vivo} protein-protein interaction studies, however, have suggested that a complex network of interactions occur outside of the linear recruitment order (Karimova et al., 2005, Alexeeva et al., 2010, Goehring et al., 2005). A proportion of these interactions may be indirect and a consequence of proximity of cell division proteins within the assembled division machinery. Thus, biochemical evidence is required to delineate which interactions are both genuine and specific. These studies, however, are complicated by difficulties encountered during purification of several essential cell division proteins, including FtsA (Martos \textit{et al.}, 2012).

6.1 Structure of the FtsA-FtsN interaction interface

This work focuses on the interaction between essential cell division proteins FtsA and FtsN, an interaction that was originally identified using \textit{in vivo} protein-protein interaction assays (Corbin \textit{et al.}, 2004, Rico \textit{et al.}, 2004, Karimova \textit{et al.}, 2005). Co-affinity purification and Far Western experiments confirmed that the interaction was direct and specific (Chapter 3). Although the subdomains of FtsA and FtsN required for this interaction were identified, a structural understanding of how the dimerized cytoplasmic domain of FtsN interfaces with the 1c subdomain of FtsA was not obtained. Future NMR studies observing both subdomains in solution may provide much needed clues regarding the specific
contacts made between FtsA and FtsN and why dimerization of FtsN is required. Such studies may also assist the development of antimicrobial inhibitors of FtsA-FtsN interaction. For instance, X-ray crystal and NMR solution structures describing the ZipA-FtsZ interaction interface were used to identify two novel small molecule inhibitors of their interaction (Moy et al., 2000, Mosyak et al., 2000, Vollmer, 2006).

6.2 Putative interactions among other early and late cell division proteins

In addition to FtsA and FtsN, interaction between other early and late cell division proteins has also been suggested. FtsA, for instance, interacts with the bitopic membrane protein FtsI in BACTH and DivIVA polar recruitment assays (Corbin et al., 2004, Karimova et al., 2005). There is no biochemical evidence supporting an interaction between FtsA and FtsI. However, it is likely that cytoplasmic FtsA interacts with the short, cytoplasmic tail of FtsI, much like FtsN. Also similar to FtsN, FtsA-FtsI interaction may involve self-interaction of FtsI via its transmembrane leucine zipper (Garza & Christie, 2013). Unlike FtsN, the putative interaction between FtsA and FtsI is not likely to facilitate localization of FtsI. This prediction is based on the ability of FtsI to localize to midcell in cells that are producing a ZapA-FtsQ fusion but lacking FtsA (Goehring et al., 2006), suggesting that localization of FtsI is FtsA-independent. FtsA-FtsI interaction could, however, facilitate a different function than FtsI recruitment.

Besides FtsI, FtsA also interacts with the late cell division protein FtsQ in the BACTH assay (Karimova et al., 2005). FtsQ is encoded within the same co-transcribed locus as ftsA and ftsZ, and the ftsQ stop codon overlaps the start of ftsA, further suggesting that FtsA and FtsQ may interact (Begg et al., 1980). Although FtsA and FtsQ interacted in the BACTH assay, DivIVA-FtsA was unable to recruit GFP-FtsQ to cell poles in the polar recruitment assay—possibly due to steric hindrance by the bulky, N-terminal GFP tag (Corbin et al., 2004). Furthermore, FtsQ continued to localize to the divisome in cells
producing a ZapA-FtsI or ZapA-FtsW fusion but lacking FtsA (Goehring et al., 2006). Together, these data suggest that FtsA and FtsQ may share an interaction, but, similar to FtsI, the interaction is unlikely to serve the same recruitment role as FtsA-FtsN interaction.

Just as FtsA shares putative interactions with other late cell division proteins, FtsN may also interact with other early cell division proteins. Using Förster Resonance Energy Transfer, or FRET, the den Blaauwen laboratory showed that FtsN and FtsI interact with the early, FtsZ-bundling protein, ZapA (Alexeeva et al., 2010). In this assay, ZapA did not interact with the other late cell division proteins tested, including FtsQ and FtsW, which suggests that ZapA’s interaction with FtsN and FtsI may be specific. One possible role of these interactions could be competition for binding between ZapA-FtsZ and ZapA-FtsI/FtsN. This competition would titrate ZapA away from FtsZ during late cell division, facilitating the debundling of FtsZ during disassembly of the Z ring (Gueiros-Filho & Losick, 2002). Because ZapA is a cytoplasmic protein, the cytoplasmic domains of FtsI and FtsN are likely involved in their interaction with ZapA. Intriguingly, ZapA-FtsN interaction could explain why overproduction of the cytoplasmic and transmembrane domains of FtsN (FtsN_{Cyto-TM}) caused an overall decrease in FtsZ protein levels (Fig. 5-2). ZipA, like ZapA, promotes bundling of FtsZ protofilaments and was recently shown to protect FtsZ from ClpXP-mediated degradation (Pazos et al., 2013). It is possible that FtsZ bundling by ZapA serves a similar role and that titration of ZapA away from FtsZ by FtsN_{Cyto-TM} promotes degradation of FtsZ. In conflict with this hypothesis, cells overproducing FtsI_{Cyto-TM} did not experience a drop in FtsZ levels, as would be expected if FtsI also titrates ZapA away from FtsZ (Fig. 5-2). It is possible, however, that the interaction between FtsI and ZapA is weaker than that between FtsN and ZapA, causing less titration of ZapA away from FtsZ and thus preserving FtsZ levels. Regardless, the common interactions shared by FtsI and FtsN warrant further investigation to fully appreciate the potentially different roles of early-late cell division protein interactions.
6.3 Suppression of the essential activity of FtsN by FtsA-E124A

In chapter 4, I ascribed a physiological role to FtsA-FtsN interaction. I showed that the initial recruitment of FtsN to division sites was dependent on its interaction with FtsA. This functionality likely explains how FtsN can at least partially suppress the loss of FtsA when present in multiple copies (Dai et al., 1993); overproduced FtsN becomes less dependent on FtsA for its recruitment as it accumulates in the cell membrane and makes non-specific contacts with the divisome. Overproduction of FtsN could not completely suppress the loss of FtsA, however, verifying that FtsA performs other roles during cell division, including tethering FtsZ to the membrane (Dai et al., 1993, Pichoff & Lutkenhaus, 2005). Similarly, FtsN serves several functions during cell division, including a possible role in FtsI stimulation, recruitment of amidases (and at least one amidase activator), and recruitment of the Tol-Pal complex (Muller et al., 2007, Peters et al., 2011, Gerding et al., 2007). Because these cellular tasks involve the periplasmic region of FtsN, it is unclear how a single point mutation in cytoplasmic FtsA can suppress the deletion of ftsN (Bernard et al., 2007). The suppression of ΔftsN by FtsA-E124A, however, is not complete, as this strain can form chains of unseparated daughter cells (Fig. 4-1). A cell chaining phenotype is consistent with inadequate splitting of the peptidoglycan layer, which is normally performed by amidases AmiABC (Heidrich et al., 2001). Although recruitment of AmiB and AmiC to the division site is dependent on FtsN, AmiA does not localize to division sites and is therefore independent of FtsN (Peters et al., 2011). It is possible, then, that AmiA performs some of the functions of delocalized AmiBC when FtsN is absent, thus allowing some cells to completely separate their peptidoglycan layers from one another. Cell chaining has also been associated with inadequate tethering of the outer membrane to the constricting inner membrane and peptidoglycan layers, as in a tol-pal mutant (Gerding et al., 2007). Unlike C. crescentus, however, the Tol-Pal complex is not essential in E. coli cells and can therefore survive without this FtsN-dependent function (Yeh et al., 2010).
If cells can survive without the periplasmic amidase activity of AmiBC and the outer membrane invagination orchestrated by Tol-Pal, what is the essential activity of FtsN that can be bypassed by FtsA-E124A? It is likely that the essential function of FtsN is stimulation of septal peptidoglycan synthesis activity (Muller et al., 2007). The building of septal peptidoglycan requires the combined activities of FtsI and PBPs 1A (MrcA), 1B (MrcB), or MtgA (Gerding et al., 2007). FtsN interacts in vivo with all four of these enzymes and has a demonstrated role in stimulating the peptidoglycan synthesis activity of at least one PBP (PBP1B) (Muller et al., 2007, Karimova et al., 2005). It is unclear, however, how FtsA-E124A could bypass the stimulatory activity of FtsN on PBPs. FtsA does share a putative interaction with FtsI, but FtsA is not known to interact with other septal PBPs (Karimova et al., 2005). Furthermore, FtsA has no demonstrated role in activation of FtsI. It is possible that FtsA or FtsA-E124A does not directly stimulate peptidoglycan synthesis activity, but instead directs the activity of a PBP-activating protein(s), thus bypassing the need for FtsN-mediated stimulation of PBPs. In addition to FtsN, outer membrane lipoproteins LpoA and LpoB have also been shown to stimulate the peptidoglycan synthesis activity of PBPs, but neither protein contacts the cytoplasm where FtsA resides (Paradis-Bleau et al., 2010). Of course, to better understand how FtsA-E124A bypasses the need for FtsN, the essential role of FtsN will need to be verified.

### 6.4 Additional roles for FtsA-FtsN interaction

In addition to its proper recruitment of FtsN to the divisome, FtsA-FtsN interaction may also serve other functions during cell division. The 1c subdomain of FtsA is sufficient for FtsA-FtsN interaction but also comprises a portion of the FtsA-FtsA binding interface (Fig. 3-6 and Fig. 3-7). It is possible, therefore, that FtsA-FtsN interaction disrupts FtsA self-interaction. The disruption of FtsA-FtsA interaction may, in turn, promote disassembly of FtsZ protofilaments during the late stages of cell division, when FtsN arrives at midcell. This
hypothesis is based on published work from the Margolin and Lutkenhaus laboratories that show that FtsA*, which is more monomeric than wild-type FtsA, shortens and curves FtsZ protofilaments in vitro (Beuria et al., 2009, Pichoff et al., 2012). Wild-type FtsA did not have any activity on the FtsZ protofilaments, suggesting that FtsA*, a partial bypass mutant of FtsN, exhibits a gain-of-function activity. Future in vitro experiments should combine wild-type FtsA, FtsZ, and FtsN to test if FtsN is needed to stimulate the depolymerization activity of FtsA, possibly through increased monomerization of FtsA.

The demonstrated interaction between FtsA and FtsN, the putative interactions between other early and late cell division proteins (see section 6.2), and the effect of monomeric FtsA on FtsZ protofilaments in vitro support an overall model of Z ring assembly and constriction shown in Figure 6-1 (Beuria et al., 2009). During assembly of the divisome, early cell division proteins FtsA and ZipA promote the integrity of the Z ring by tethering the cytoskeletal structure to the inner membrane (Pichoff & Lutkenhaus, 2005, Ma et al., 1996, Raychaudhuri, 1999, Pichoff & Lutkenhaus, 2002). Additionally, ZipA and Zap proteins promote bundling of FtsZ protofilaments (shown as green spheres) (Low et al., 2004, Galli & Gerdes, 2010, Durand-Heredia et al., 2011). Although FtsA is likely in a multimeric configuration during early assembly of the divisome, interaction between FtsA and FtsQ may promote FtsA monomerization following the late arrival of FtsQ (Karimova et al., 2005). Monomerization of FtsA, in turn, would promote the initial shortening and curving of FtsZ protofilaments (Beuria et al., 2009). Because FtsQ is less abundant than FtsA (22 and 200-740 molecules per E. coli cell, respectively), further monomerization of FtsA would require an additional factor(s) (Raychaudhuri & Park, 1992, Carson et al., 1991, Rueda et al., 2003). One of these factors may include FtsI, which is recruited to division sites following localization of the FtsQBL complex and FtsW (Mercer & Weiss, 2002). Here, FtsI may also disrupt FtsA self interaction by direct interaction with FtsA (Karimova et al., 2005, Corbin et al., 2004). Like FtsQ, FtsI is also present at low cellular levels (100 molecules per cell).
Figure 6-1. The hypothesized effect of early and late cell division proteins on stability of the Z ring. See section 6.4 for a detailed explanation of this model.
(Weiss et al., 1997). Therefore, localization of the last essential cell division protein to arrive at midcell, FtsN, could disrupt any remaining FtsA multimers (Addinall et al., 1997). Disruption of FtsA multimers by FtsQ and FtsI may also facilitate FtsA-FtsN interaction during the late stages of division by exposing the 1c subdomain of FtsA. The high abundance of FtsN (3000-6000 molecules per cell) likely promotes the dimerization required for FtsA-FtsN interaction (Ursinus et al., 2004). In addition to FtsA-FtsN interactions, interaction between ZapA and FtsN may also promote Z ring constriction by titrating ZapA away from FtsZ and decreasing FtsZ protofilament bundling (Alexeeva et al., 2010). SPOR-dependent localization of FtsN would promote self-accumulation of FtsN during the latest stages of division, thus promoting FtsA-FtsN and ZapA-FtsN interactions after stimulation of septal peptidoglycan synthesis and degradation activities. The progressive disruption of FtsA multimers by FtsQ, FtsI, and finally FtsN would provide temporal control of Z ring disassembly as FtsA-interacting proteins continue to accumulate at division sites during the latest stages of divisome assembly. This mechanism would ensure that aggressive disassembly of the Z ring only occurred after all divisome and peptidoglycan remodeling proteins were in place.

6.5 Relationship between FtsN and chromosome replication and segregation

While searching for other possible roles of FtsA-FtsN interaction, I discovered that overproduction of the FtsA-interacting portion of FtsN, FtsN_{Cyto-TM}, produced many phenotypes in both rich and poor media sources (Chapter 5). The most striking phenotypes were observed when FtsN_{Cyto-TM} was overproduced in cells grown under starvation conditions (minimal medium) and included changes in cell shape and trapping of nucleoids at midcell (Fig. 5-5 and Fig. 5-6). The trapping of nucleoids at midcell was attributed to a chromosome segregation problem based on several observations. First, these cells contained multiple GFP-SeqA foci (Fig. 5-7), indicating that chromosome replication was
not inhibited. Second, the arrangement of midcell nucleoids and flanking Z rings closely resembled chromosome segregation mutants (parC) (Fig. 5-5) (Kato et al., 1988). Third, induction of a sulA-gfp reporter (Fig. 5-6) indicated that DNA damage occurred, consistent with a chromosome segregation problem (Janssen et al., 2011).

The effects of FtsN<sub>Cyto-TM</sub> overproduction on chromosome segregation were unexpected, as FtsN is typically associated with the late stages of cell division, including septal peptidoglycan synthesis and degradation (Muller et al., 2007, Addinall et al., 1997, Peters et al., 2011). There are, however, several known genetic interactions between FtsN and chromosome replication, repair, and segregation proteins. For example, FtsN was identified during an unbiased screen for mutations that were synthetically lethal with the loss of the Min system (Gerding et al., 2009). The Min system participates in division site selection, but MinD, a homolog of chromosome partitioning protein ParA, was also recently identified as a component of the <i>E. coli</i> chromosome segregation machinery (Di Ventura et al., 2013). Additionally, FtsN overproduction in <i>E. coli</i> cells can weakly suppress the depletion or deletion of <i>ftsK</i>, a septal DNA translocase, dependent on the cytoplasmic and transmembrane domains of FtsN (Goehring et al., 2007b). In <i>C. crescentus</i>, the FtsN homologue is encoded by gene cc_2007 and is predicted to form a single, co-transcribed transcript with three upstream genes (Fig. 6-2) (Keseler et al., 2010). Two of these genes encode ScpA and ScpB homologues that together, as discussed in section 1.2, dictate the interaction between SMC proteins and DNA to segregate chromosomes (Schwartz & Shapiro, 2011). In <i>E. coli</i>, <i>ftsN</i> is located within the same locus as <i>hslU</i> and <i>priA</i>, although the genes are not predicted to be co-transcribed (Keseler et al., 2010). HslU is the ATPase subunit of the highly conserved HsIVU protease (Chuang et al., 1993). In the mitochondria of the eukaryote <i>Trypanosoma brucei</i>, knockdown of <i>hslVU</i> caused over-replication of
Figure 6-2. *ftsN* loci of *Caulobacter crescentus* and *Escherichia coli*. Proteins encoded by the red-shaded genes have a demonstrated role in DNA replication or segregation in *E. coli* or other organisms. Known or predicted transcriptional units are drawn together, while independent transcripts are separated by a horizontal line. Predicted transcriptional units were obtained from EcoCyc (Keseler *et al.*, 2010).
mitochondrial DNA and subsequent failure to segregate the replicated DNA (Li et al., 2008). Intriguingly, PriA is also involved in initiating DNA replication in E. coli, although its role is usually limited to sites of double-stranded DNA breaks (Zavitz & Marians, 1991). It is possible that the multiple foci of GFP-SeqA observed in Fig. 5-7 resulted from aberrant re-initiation of DNA replication, and that chromosome segregation was affected as a consequence. Further testing of the strains overproducing FtsN<sub>Cyto-TM</sub> should include careful quantitation of GFP-SeqA foci over time to determine if chromosomes are being over-replicated. If chromosome replication initiation is being “misfired,” additional studies should determine if FtsN has a direct or indirect role.

In summary, these data provide a greater understanding of how two essential cell division proteins interact, providing the first biochemical evidence of early-late cell division protein interactions in E. coli. I also characterized a physiological role of FtsA-FtsN interaction and proposed additional functions that this interaction may serve. Finally, through overproduction studies, I obtained preliminary evidence that FtsN may serve a role in chromosome replication and/or segregation prior to cell division. Together, these data advance our understanding of the complex network of protein-protein interactions that guide bacterial cell division.
Bibliography


FtsZ determines the magnitude of the FtsZ polymer bundling by ZapA in vitro.  
*Biochemistry* 48: 11056-11066.


Vita

Kimberly Kennett Busiek was born in Belleville, Illinois on January 9, 1982, the daughter of Deborah Kennett. After completing her work at Althoff Catholic High School, Belleville, Illinois in 2000, she entered Truman State University in Kirksville, Missouri. She received the degree of Bachelor of Sciences with a double major in biology and health science from Truman in May, 2005. For the next two and one half years, she worked as a research assistant, quality analyst, and associate scientist at Saint Louis University School of Medicine (Saint Louis, Missouri), Mallinckrodt Inc. (Greenville, Illinois), and STERIS Corporation (Saint Louis, Missouri), respectively. In August of 2008 she entered The University of Texas Graduate School of Biomedical Sciences at Houston.

Permanent address:

17018 Tallgrass Prairie Lane

Humble, TX  77346