The C Terminus of FtsZ Regulates FtsZ Assembly Dynamics and Is Required for Bacillus Subtilis Cell Division

PJ Buske
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/etd

Recommended Citation
https://openscholarship.wustl.edu/etd/1125

This Dissertation is brought to you for free and open access by Washington University Open Scholarship. It has been accepted for inclusion in All Theses and Dissertations (ETDs) by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Biochemistry

Dissertation Examination Committee:
Petra A. Levin, Chair
John A. Cooper
Ram Dixit
Elliot L. Elson
Joseph M. Jez
L. David Sibley

The C Terminus of FtsZ Regulates FtsZ Assembly Dynamics and Is Required for *Bacillus Subtilis* Cell Division

by

Paul J. “PJ” Buske

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in St. Louis in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013

St. Louis, Missouri
# Table of Contents

List of Tables viii  
List of Figures ix  
Acknowledgements xii  
Dedication xiv  
Abstract of the Dissertation xv  
Epigraph 1  
Chapter 1: Introduction 2  
  Bacterial Cell Division and the Prokaryotic Tubulin Homolog FtsZ 3  
  FtsZ Structure 5  
  FtsZ Assembly Dynamics In Vitro 7  
    GTP Hydrolysis 8  
    Cooperative Assembly of FtsZ Filaments 11  
    Lateral FtsZ Filament Interactions 12  
  The FtsZ Ring In Vivo 14  
  FtsZ and the Generation of Constrictive Force 17  
  Factors That Regulate FtsZ Assembly 21  
    Inhibitors of FtsZ Assembly 21  
    FtsZ Stabilizers 25  
  Toward a Unified Model of FtsZ Assembly 28  
  Significance, Summary, and Scope of Dissertation 31  
  References 34  
Chapter 2: The extreme C-terminus of the bacterial cytoskeletal protein 51
FtsZ plays a fundamental role in assembly independent of modulatory proteins

Abstract

Introduction

Results

FtsZ from both B. subtilis and E. coli display distinct assembly properties

Deletion of the conserved C-terminal tail eliminates B. subtilis FtsZ lateral interactions

The B. subtilis FtsZ CTV is sufficient to induce lateral interactions between FtsZ protofilaments

CTV charge plays a critical role in lateral interaction potential

Defects in lateral interaction potential impair cell division in B. subtilis

Discussion

Electrostatic forces as the primary determinant of lateral interaction potential

Filament length appears to be independent of lateral interaction potential

Changes in lateral interaction potential in vitro impact FtsZ ring stability in vivo

A role for FtsZ’s C-terminus in mediating lateral interactions between FtsZ protofilaments

Experimental Procedures

References

Acknowledgements
Chapter 3: A flexible C-terminal linker is required for proper FtsZ assembly

\textit{in vitro} and cytokinetic ring formation \textit{in vivo}

Abstract

Introduction

Results

The FtsZ linker is required for normal function in \textit{B. subtilis}

FtsZ function is independent of its linker sequence

Linker length is an important determinant for normal FtsZ function

The FtsZ linker must be flexible and unstructured

Discussion

An intrinsically disordered linker is essential for FtsZ assembly and Z-ring formation

Conservation of an intrinsically disordered peptide

FtsZ linker charge impacts lateral filament interactions

The FtsZ linker is a flexible tether

Experimental Procedures

References

Acknowledgements

List of Abbreviations
### Tables and Figures

- Supplemental Tables and Figures
- Addendum

### Chapter 4: Summary, discussion, and future directions

#### Summary

- The FtsZ CTV Mediates Lateral Filament Interactions
- The FtsZ CTL Plays an Important Role in Protofilament Assembly and Z ring Stability

#### Discussion

- FtsZ Bundling In Vivo: Is It Important?
- Role of the Linker in FtsZ Protofilament Assembly
- Conformational Flexibility of the FtsZ Linker
- FtsZ CTL and CTV Chimeras as Tools for Understanding FtsZ-Modulatory Protein Interactions

#### Future Directions

- Concluding Remarks

#### References

### Appendix: A moonlighting enzyme links *Escherichia coli* cell size with central metabolism

- Abstract
- Author Summary
- Introduction
- Results
*E. coli* utilizes UDP-glucose to couple cell size with nutrient availability

Defects in UDP-glucose biosynthesis increase the frequency of FtsZ rings over incompletely segregated nucleoids

Defects in UDP-glucose biosynthesis stabilize FtsZ assembly at midcell

Defects in the glucosyltransferase OpgH reduce *E. coli* cell size

OpgH inhibits division in a UDP-glucose-dependent fashion

OpgH regulates cell size independent from its role in OPG synthesis

OpgH localizes to the division ring only in nutrient-rich conditions, yet independent of UDP-glucose

The N-terminus of OpgH is both necessary and sufficient for division inhibition

OpgH inhibits division by blocking FtsZ ring formation

Genetic data suggests that OpgH reduces the pool of FtsZ available for assembly into the cytokinetic ring

OpgH\(^N\) interacts directly with FtsZ to inhibit assembly

OpgH raises the apparent critical concentration of FtsZ required for GTP hydrolysis

**Discussion**

**Experimental Procedures**

**References**

**Figures**

**Supplemental Text**

**Supplemental Text References**
Supplemental Tables and Figures 249
Addendum 278
List of Tables

Chapter 2:

Table 1. Bacterial strains and plasmids used in this study. 87
Table 2. FtsZ GTPase turnover rates 89
Supplemental Table 1. FtsZ point mutants that abolish modulator or lateral interactions 100
Supplemental Table 2. Primers used in strain construction 101

Chapter 3:

Table 1. FtsZ GTPase turnover rates and critical concentrations 145
Table 2. Bacterial strains and plasmids used in this work 146
Supplemental Table 1. Primers used in strain construction 161

Appendix

Supplemental Table 1. Phenotypes of combining defects in UDP-glucose synthesis (Δpdm) or ΔopgH with inactivating characterized E. coli division inhibitors. 249
Supplemental Table 2. Detailed cell size measurements of mutants associated with UDP-glucose synthesis or utilization cultured in LB-glucose. 250
Supplemental Table 3. Bacterial strains used in this study. 252
Supplemental Table 4. Bacterial plasmids used in this study. 256
List of Figures

Chapter 1:

Figure 1. Cell division in a rod-shaped bacterium. 48
Figure 2. FtsZ assembly dynamics in vitro. 50

Chapter 2:

Figure 1. FtsZ domain structure, C-terminal alignments, and protein constructs. 91
Figure 2. The B. subtilis FtsZ C-terminus is required for lateral interactions in vitro. 93
Figure 3. The B. subtilis CTV is sufficient to promote lateral interactions in vitro. 95
Figure 4. Bs FtsZ CTV charge appears to be the primary determinant of lateral interaction potential. 97
Figure 5. The FtsZ CTV plays a critical role in maintenance of FtsZ ring integrity in vivo. 99

Supplemental Figure 1. Salt affects lateral filament interactions between FtsZ filaments. 104
Supplemental Figure 2. Electron microscopy measurements. 106
Supplemental Figure 3. Expression Ec FtsZ and Ec FtsZ CTVB are able to rescue growth in a temperature sensitive background. 108
Supplemental Figure 4. Quantitative Western blot of Bs ftsZ WT (PL 3188) and Bs ftsZ CTVE (PL 3189) in vivo expression. 110

Chapter 3:

Figure 1. FtsZ Constructs. 148
Figure 2. The FtsZ linker is required for cell division in B. subtilis. 150
Figure 3. FtsZ chimeras and their effect on cell growth. 152
Figure 4. The sequence of the FtsZ linker does not affect protofilament assembly 154
or cell division in *B. subtilis*.

Figure 5. FtsZ linker length does not affect protofilament formation but is important for cell division.

Figure 6. A flexible and disordered linker is essential for FtsZ assembly and cell division.

Figure 7. The FtsZ linker is a flexible tether.

Supplemental Figure 1. Circular dichroism spectra of FtsZ, FtsZ ΔCTL50, and FtsZ CTLH.

Supplemental Figure 2. *B. subtilis* cells that express FtsZ ΔCTL50, FtsZ CTLH, or FtsZ CTLA249 display significant growth deficiencies.

Supplemental Figure 3. Cell wall staining of FtsZs.

Supplemental Figure 4. Deletion of the entire FtsZ C-terminus abolishes ability to form protofilaments.

**Appendix:**

Figure 1. *E. coli* utilizes UDP-glucose to coordinate nutrient availability with cell size.

Figure 2. OpgH acts as a nutrient-dependent division antagonist.

Figure 3. OpgH localizes to midcell in a growth rate- and FtsZ-dependent manner.

Figure 4. The N-terminal cytoplasmic region of OpgH is necessary and sufficient to inhibit cell division.

Figure 5. OpgH\(^N\) is an inhibitor of FtsZ assembly.

Figure 6. OpgH\(^N\) appears to function as an FtsZ monomer sequestering protein.

Figure 7. Glucosyltransferase OpgH couples cell size to nutritional availability and growth rate in *E. coli*.

Supplemental Figure 1. FtsZ84 levels are not elevated in *pgm* null strain.
Supplemental Figure 2. MinD overexpression and FtsZ levels are congruent between in wild type and UDP-glucose pathway mutants.

Supplemental Figure 3. The cell size defect is confined to the UDP-glucose biosynthesis pathway.

Supplemental Figure 4. Thio-OpgH-His expressed to similar levels.

Supplemental Figure 5. opgH-gfp and thio-opgH-his fusion constructs complement for size and glucosyltransferase activity.

Supplemental Figure 6. The ΔopgH cell size defect is independent of osmoregulated periplasmic glucans and Rcs activation.

Supplemental Figure 7. An 18-amino acid peptide of OpgH is sufficient, though unnecessary, to inhibit division and localize to the division ring.

Supplemental Figure 8. UDP-glucose pathway mutants reduce filamentation of ΔminCDE.

Supplemental Figure 9. Heat-treated OpgH^N loses ability to inhibit FtsZ.

Supplemental Figure 10. Phylogenetic tree and alignment of the FtsZ inhibiting domain of OpgH.
Acknowledgements

I would like to first thank the members of the Levin Lab past and present for numerous discussions, technical assistance, advice, and overall making the lab an enjoyable place to work. Dan Haeusser first showed me the ropes around the lab while he was finishing his graduate work and when I first started my rotation. Since he moved on and started his postdoc in Bill Margolin’s lab, Dan has also provided many helpful suggestions on projects and manuscripts and graciously provided materials. The current members of the lab, Bisco Hill, Heidi Arjes, and Adrian Land, have also particularly been instrumental in making this thesis a success, sometimes scientifically and especially in making the lab feel like home away from home.

To that end, Petra has been an incredible advisor and mentor. She has allowed me to grow immensely into a well-rounded scientist. When I first joined the lab, I was interested in pursuing biochemical projects but she emphasized I would benefit from learning bacterial genetics as well. At the time, I was less enthusiastic about doing so, but now taking a multi-disciplinary approach has been a driving force behind my thesis and how I will engage in my research. She has also allowed me the freedom to answer the questions that interest me and let me guide the direction of my research. Perhaps most importantly, she has taught me the value of being an effective communicator, both writing about and presenting my research and helped me improve greatly in those areas.

I would also like to thank the members of my committee for being an important guiding influence on my work and providing helpful advice and criticism. The conversations I have had with them, scientifically related or not, have been thoroughly beneficial and enjoyable. Other members of the Biochemistry and Biophysics community at Washington University who have taught classes and served on preliminary exam committees deserve special thanks as well.
Finally, I would like to thank my friends and family who have provided me with the support and encouragement to take on this journey called grad school.
Dedication

Through all of the ups and downs these last nearly 6 years, my parents and brother, Zach, have been there for me. Though being away from home hasn’t always been easy, I would have never successfully completed this thesis without them. More recently, my girlfriend (currently), Nan, has been by my side for the last two years supporting me as well. I dedicate this work to all of them, my family.
Abstract of the Dissertation

The C Terminus of FtsZ Regulates FtsZ Assembly Dynamics and Is Required for *Bacillus Subtilis* Cell Division

By

PJ Buske

Doctor of Philosophy in Biochemistry

Washington University in St. Louis, 2013

Professor Petra A. Levin, Ph.D., Chair

Bacterial cell division is initiated by the assembly of the tubulin homolog FtsZ into a ring (Z ring) at the nascent division site. Once formed, the Z ring serves as a scaffold for recruitment of the division machinery and helps provide some of the constrictive force for cytokinesis. *In vitro* FtsZ undergoes GTP-dependent assembly where individual subunits form single-stranded protofilaments and laterally-associated filament bundles. How the filamentous FtsZ structures observed *in vitro* translate into the behavior of the Z ring *in vivo* remains a fundamental question. In this dissertation I establish important roles for the previously uncharacterized FtsZ C-terminal domains during both FtsZ assembly and Z ring formation. My work provides significant insight into how FtsZ behavior at the protein level impacts its cellular function.

Structurally, the FtsZ monomer is divided into 5 domains: an unstructured N-terminal peptide, a highly conserved N-terminal globular core, an unstructured C-terminal linker (CTL), a conserved set of ~11 residues referred to here as the C-terminal constant region (CTC), and a small, highly variable group of residues at the extreme C-terminus of FtsZ termed the C-terminal variable region (CTV). For simplicity, the N-terminal peptide and core are treated here as a single unit. The core shows a high degree of sequence conservation amongst bacterial species.
and contains residues required for GTP binding and hydrolysis as well as forming the contacts necessary to make filaments. The entire FtsZ C terminus consists of the CTL, CTC, and CTV. The CTL displays very little conservation between species both in primary sequence and length, is irresolvable by X-ray crystallography, and is presumed to be intrinsically disordered. The CTC and CTV are implicated in interactions between FtsZ and modulatory proteins. To reflect this function the combined domains have been termed the grappling hook peptide (GHP). Prior to this work, the roles that the C-terminal domains had in FtsZ assembly were unknown.

In this dissertation, I demonstrate that these domains do have distinct functions. First I show the CTV is important for regulating lateral interactions between FtsZ protofilaments. *B. subtilis* FtsZ readily forms bundled structures *in vitro*. In contrast, I show *E. coli* FtsZ typically assembles into single-stranded protofilaments. Through deletion analysis and domain swapping, I determine these phenotypes to derive from differences in the CTVs of each species. I also establish that electrostatic interactions are a driving force behind FtsZ bundling. Alterations to the CTV sequence also greatly affect cell division in *B. subtilis* cells, suggesting filament bundling is important for a stable Z ring *in vivo*.

Finally, I demonstrate the FtsZ CTL is essential for FtsZ protofilament assembly and cell division. I determine that a functional CTL must behave as an intrinsically disordered peptide with little primary sequence requirement but must be between 25 and 100 residues in length. These findings lead to a model for FtsZ in which the CTL behaves as a flexible tether anchoring FtsZ filaments to the membrane through interactions between the GHP and FtsZ modulatory proteins like FtsA. The linker can undertake different conformations and allow FtsZ filaments bundle through positioning the CTV near adjacent filaments and to respond to the curvature of the membrane, having implications for how the constrictive force for cytokinesis is generated.
"Science is like a blabbermouth who ruins a movie by telling you how it ends."

-Ned Flanders
Chapter 1:

Introduction
Bacterial Cell Division and the Prokaryotic Tubulin Homolog FtsZ

Cell division presents a conserved theme across nearly all domains of life. As a mother cell divides into daughter cells, the cytoskeleton helps provide the force necessary to split cells and separate the genetic material. In animal and fungi cells, the actomyosin ring generates the contractile force during cytokinesis (Balasubramanian et al., 2012). In plant cells, plate formation is directed by actin and microtubules drive division (Jürgens, 2005). Once thought to be exclusive to eukaryotic cells, bacteria and archaea are also now known to possess their own cytoskeletal proteins. These include homologs of actin (MreB) (Jones et al., 2001; van den Ent et al., 2001), tubulin (FtsZ) (Nogales et al., 1998), and intermediate filaments (CreS) (Ausmees et al., 2003).

First discovered as a temperature sensitive mutant leading to cell filamentation in E. coli (Lutkenhaus et al., 1980), FtsZ (filamentous temperature sensitive mutant Z) is the foremost protein in bacterial cell division. Lending to its importance, FtsZ is highly conserved and found in most bacteria and the Euryarchaeal branch of Archaea (Vaughan et al., 2004). It is also found in eukaryotic cells. Certain protists import nuclear-encoded FtsZ homologs to chloroplasts and mitochondria, while FtsZs in plant chloroplasts have two distinct functions (Osteryoung and Nunnari, 2003; TerBush and Osteryoung, 2012).

In rod-shaped bacteria that undergo binary fission, such as Bacillus subtilis and Escherichia coli, FtsZ assembles into a ring (Z ring) to mark the nascent division site (Bi and Lutkenhaus, 1991). After it assembles, the ring constricts to initiate cytokinesis. The Z ring forms shortly after daughter cells divide and it remains present during most of the cell cycle (den Blaauwen et al., 1999; Weart and Levin, 2003). The resulting mature cytokinetic ring recruits the other proteins responsible for making up the division machinery and guides the formation of a
division septum between the two segregated chromosome masses (nucleoids) (Harry et al., 2006). The Z ring then constricts along this septum and helps provide the contractile force needed to split two daughter cells. At the end of constriction, the ring disassembles and can start over again in the newly formed cell.

The nature of the Z ring directly relates to the behavior of individual FtsZ molecules. As FtsZ is a tubulin homolog, it undergoes GTP-dependent self-assembly into filaments \textit{in vitro} (Romberg and Levin, 2003). Formation of the Z ring \textit{in vivo} also requires assembly of FtsZ, with filaments presumably serving as the scaffold for the more highly-ordered ring structure. Therefore, determining when and where the Z ring forms depends on the tightly controlled regulation of the assembly state of FtsZ (i.e. monomeric vs. polymeric) (Haeusser and Levin, 2008). However, a key question in bacterial cell biology is determining how assembly dynamics observed \textit{in vitro} relate to the actual organization and dynamics of the Z ring seen \textit{in vivo}.

This dissertation sets forth to understand how the molecular details of FtsZ assembly \textit{in vitro} translate into large-scale changes at the cellular level. Notably, I achieve this through the examination of previously disregarded FtsZ structural domains. Chapter 2 identifies a new region of FtsZ important for the ability of individual FtsZ filaments to associate together \textit{in vitro} and how changing these interactions affect Z ring integrity \textit{in vivo}. Work in Chapter 3 describes a domain of FtsZ necessary for regular protofilament assembly and Z ring function during cell division and suggests a mechanism for how FtsZ filaments anchor to the membrane to generate constrictive force. Together this thesis details new roles for once uncharacterized regions of FtsZ and reveal how understanding the synergistic relationship between protein domains is paramount to appreciating cellular function.
**FtsZ Structure**

FtsZ assembly starts at the molecular level where nucleotide binding activates monomers to promote polymerization into filaments and subsequent hydrolysis leads to disassembly of those filaments. Knowing the structure of FtsZ is crucial to understanding these processes. Predictions can then be made as to how different structural domains interact and affect assembly and cellular function. A main achievement of this dissertation is discerning how structural features previously overlooked affect the assembly properties of FtsZ both *in vitro* and *in vivo*.

FtsZ is divided into five structural domains: the unstructured N-terminal peptide, the conserved globular core domain, the unstructured and non-conserved C-terminal linker (CTL), a conserved set of ~11 residues referred to here as the C-terminal constant region (CTC), and a small, highly variable group of residues at the extreme C-terminus of FtsZ termed the C-terminal variable region (CTV) (Buske and Levin, 2012; Vaughan et al., 2004). We have combined the CTC and CTV regions and termed them the grappling hook peptide (GHP) due to their role in binding FtsZ modulatory proteins (Buske and Levin, 2013). Each domain is described in detail below.

The first crystal structure of FtsZ was solved for *Methanocaldococcus jannaschii* and resolved residues 23-356 (total length 364) consisting only of the conserved globular core (Löwe and Amos, 1998). Subsequent crystal structures from other prokaryotes have also been able to determine the structure of only the core domain (Haydon et al., 2008; Leung et al., 2004; Läppchen et al., 2008; Matsui et al., 2012; Oliva et al., 2007; Raymond et al., 2009). The first ~10-30 residues of FtsZ are irresolvable and make up the N-terminal peptide. This domain is not conserved in both sequence and length, and it is presumed to be intrinsically disordered.
The globular core domain is well conserved amongst all FtsZs and consists of two subdomains: the N-terminal domain and the C-terminal domain. In \textit{B. subtilis}, the core domain spans residues 10 – 315. Both subdomains can be expressed separately and fold independently; however, a fully functional protein requires both domains folded continuously (Oliva et al., 2004; Osawa and Erickson, 2005). The N-terminal subdomain contains a Rossman fold common of nucleotide-hydrolyzing enzymes and all of the amino acids required for GTP binding (Erickson et al., 2010). The C-terminal subdomain includes the highly conserved “synergy” T7 loop that contains the sequence NxDFAD (in \textit{B. subtilis}) required for GTP hydrolysis, similar to the NxDxxE sequence found in all \alpha-tubulins (Erickson et al., 2010).

When FtsZ subunits oligomerize, the N-terminal subdomain sits on the lower boundary of the longitudinal bond interface. Crystal structures of FtsZ dimers show the C-terminal T7 loop from an upper adjacent subunit sits near the GTP-binding site of the N-terminal subdomain. Aspartic acid residues in the T7 loop are then positioned to activate nucleophilic attack on the \gamma-phosphate of GTP, creating the FtsZ active site and leading to GTP hydrolysis in the subunit below (Oliva et al., 2007). Mutations to the T7 loop create GTPase-deficient FtsZs (Erickson et al., 1996; Nogales et al., 1998; Redick et al., 2005; Scheffers et al., 2002). Because of the interplay between the GTP-binding site and the T7 loop, FtsZ is a GTPase only when subunits contact each other in an oligomer.

The C-terminal linker follows the globular core domain. The CTL spans residues 316 – 365 in \textit{B. subtilis}. Characterized by the lack of sequence conservation both in composition and length, the CTL has been presumed to behave as an intrinsically disordered peptide (IDP)
Prior to this work, it was shown to be flexible with an average end-to-end distance of 5.2 nm (Ohashi et al., 2007), but whether it was dispensable for FtsZ function remained unknown. In Chapter 3 of this dissertation, the intrinsically disordered nature of the CTL is confirmed and the role of the CTL in FtsZ assembly and function are described.

The final ~10-20 residues of FtsZ comprise the conserved CTC and the variable CTV. The region of FtsZ has been shown to form an extended beta strand followed by an alpha helix in complex with the modulatory proteins ZipA from E. coli and FtsA from Thermotoga maritima (Mosyak et al., 2000; Szwedziak et al., 2012). In addition, the CTC and CTV have been shown to be the site of interaction between FtsZ and other modulatory proteins including MinC in E. coli and ClpX, EzrA, and SepF in B. subtilis (Król et al., 2012; Shen and Lutkenhaus, 2009; Singh et al., 2007; 2008; Sugimoto et al., 2010). Acting as a binding site was presumed to be its only function. However, Chapter 2 of this dissertation sheds new light on the importance of these domains in FtsZ assembly independent of modulatory proteins. Later, I discuss the role of the CTV in modulating FtsZ self-interactions and suggest a possible role in maintaining Z-ring integrity during cell division.

**FtsZ Assembly Dynamics In Vitro**

In the cell, the Z ring is a dynamic structure that must be able to quickly assemble and disassemble in order to respond to the progressing cell cycle and environmental signals. The basis for these dynamics is derived from the behavior of individual FtsZ filaments. Chapter 2 shows how changes to the CTV affect how filaments interact with themselves. Chapter 3
establishes the CTL as necessary for protofilament formation. Other aspects are also discussed below.

**GTP Hydrolysis:**

FtsZ undergoes GTP-dependent stages of assembly and disassembly and readily forms single-stranded protofilaments *in vitro* (Erickson et al., 2010; Mukherjee and Lutkenhaus, 1994; Romberg and Levin, 2003). Assembly into filaments is rapid upon addition of GTP, and steady state addition/loss of subunits onto and off filaments is reached in a matter of seconds (Mukherjee and Lutkenhaus, 1999). Once FtsZ subunits incorporate into a filament, the GTPase active site is formed and GTP hydrolysis occurs (Huecas et al., 2007; Scheffers et al., 2002). GDP-bound FtsZ subunits destabilize the polymer and begin to curve, leading to rapid disassembly of the filament (Bramhill and Thompson, 1994; Erickson et al., 1996; Lu et al., 2000; Mukherjee and Lutkenhaus, 1999; Sossong et al., 1999).

There is a lag between the formation of filaments and GTP hydrolysis, however. FtsZ filaments have a considerable amount of GTP. At steady state, the ratio of GTP to GDP in a protofilament has been measured at 4:1 (Romberg and Mitchison, 2004). This proportion decreases to 1:1 as the external GTP concentration increases due to accelerated hydrolysis rates at elevated GTP levels (Chen and Erickson, 2009). Yet, these data still show a remarkable contrast to microtubules in which subunits are almost entirely GDP bound except for GTP caps at the ends (Desai and Mitchison, 1997).

In filaments, precisely how the exchange of nucleotide occurs has been the matter of some controversy. Some studies propose FtsZ subunits within a protofilament can exchange GDP for GTP in solution (González et al., 2005; Mingorance et al., 2001; Oliva et al., 2004;
A crystal structure of FtsZ dimers from *M. jannaschii* FtsZ show a gap between subunits that would allow GTP to release without hydrolysis (Oliva et al., 2004). Nucleotide has also been shown to incorporate into polymers of apo-FtsZ from *M. jannaschii* (Huecas et al., 2007). However, protofilaments assembled in the presence of nucleotide show slow dissociation of that nucleotide and cannot exchange with free nucleotide in solution. The development of a fluorescence resonance energy transfer (FRET) assay to measure subunit exchange between protofilaments provides new insight to this question (Chen and Erickson, 2005). Chen and Erickson show that, depending on buffer conditions, the half-life of subunit exchange occurs on the order of 3.5 to 35 seconds (Chen and Erickson, 2009). The time required for GTP hydrolysis ranges from 13 to 100 seconds under the same variable conditions, meaning subunit exchange occurs twice as fast as hydrolysis. This suggests two mechanisms for subunit exchange: 1) coupled to GTP hydrolysis and 2) FtsZ-GTP subunits dissociate from protofilament ends without GTP hydrolysis (Chen and Erickson, 2009). Thus, previous observations of nucleotide exchanging within the filament likely stemmed from the pool of subunits dissociating from the filament without hydrolysis undergoing exchange of nucleotide in solution. The likely scenario is that nucleotide exchange occurs only in free monomers or at the ends of filaments when the binding pocket is exposed to solvent.

The nucleotide state of FtsZ subunits within the filament solicits different models for filament dynamics. As subunits hydrolyze GTP to GDP, the FtsZ-GDP at the ends of filaments destabilizes. The dissociating subunits raise the possibility FtsZ can undergo treadmilling like actin. In actin, rate of association at one end equals the rate of dissociation at the other end at steady state and polymerization and depolymerization occur at preferred ends (Wegner, 1976). Indirect evidence for treadmilling comes from examination of FtsZ mutants in which assembly is
blocked at either the top or bottom of the dimer interface, with subunits showing preference to add to the bottom end (Redick et al., 2005).

Subunit association/dissociation from filament ends also denotes the possibility for dynamic instability. Microtubule dynamic instability is characterized by the microtubule ends undergoing different phases of rapid growth and shortening due to GTP hydrolysis (Mitchison and Kirschner, 1984). FtsZ bound to the nonhydrolyzable analog GTPγS stabilizes polymers in the presence of GTP, suggesting a FtsZ-GTP ‘cap’ stabilizing filaments on one end until nucleotide hydrolysis leads to the buildup of FtsZ-GDP that quickly dissociate from the filament (Scheffers et al., 2000). A more direct measurement of FtsZ filament dynamics, perhaps by real-time visualization of filaments using total internal reflection fluorescence (TIRF) microscopy or single-molecule spectroscopy, will be needed to distinguish these mechanisms.

Another mechanism for the dynamic behavior of FtsZ filaments is annealing of filaments (Chen and Erickson, 2009). GTP hydrolysis in FtsZ subunits within the filament is thought to be stochastic. When GTP is hydrolyzed in the middle of a filament, the resulting FtsZ-GDP can destabilize and fragment the filament. The resulting FtsZ-GDP on the filament end can exchange nucleotide for GTP in solution and the new FtsZ-GTP end can anneal to another FtsZ-GTP end. Annealing of protofilaments adhered to a mica surface has been observed by atomic force microscopy AFM (Mingorance et al., 2005), and a tryptophan reporter assay of FtsZ assembly has measured annealing events in solution (Chen et al., 2005). In the Z ring, annealing may play an important role in maintaining the ring structure throughout cytokinesis and constriction (Surovtsev et al., 2008).

FtsZ also assembles into filaments without GTP binding or hydrolysis. Apo-FtsZ from M. jannaschii is able to form filaments (Huecas and Andreu, 2004). FtsZ-GDP can assemble into
protofilaments, though less efficiently than FtsZ-GTP (Huecas et al., 2007; Rivas et al., 2000; Sossong et al., 1999). FtsZ can also form filaments when assembled in the presence of GTP and EDTA, which chelates magnesium in the buffer and prevents nucleotide hydrolysis (Chen et al., 2005). GTP hydrolysis seems to be the key factor in creating dynamic FtsZ filaments.

Cooperative Assembly of FtsZ Filaments:

Cooperative assembly is a characteristic of many cytoskeletal polymers. Actin filaments and microtubules are the best-studied examples of cooperatively assembling filaments. These proteins display the hallmarks of cooperativity: multi-stranded and long filaments, a lag phase associated with a nucleation step, and a critical concentration for assembly (Erickson, 1989; Oosawa and Kasai, 1962; Romberg et al., 2001). In contrast, some self-associating proteins also undergo isodesmic assembly. Isodesmic polymers are typically held together by only one longitudinal bond and fragmentation and end dissociation are thermodynamically equivalent (Erickson, 1989). Filaments are also small and single-stranded and there is no critical concentration for assembly (Romberg et al., 2001).

FtsZ filaments show characteristics of both cooperative and isodesmic polymers. 90° light scattering and fluorescent reporter assays show FtsZ has a critical concentration of ~ 1 μM and apparent lag phase (Chen et al., 2005; Mukherjee and Lutkenhaus, 1999). However, electron microscopy reveals FtsZ assembles into primarily single-stranded filaments typical of being isodesmic (Romberg et al., 2001). Mathematical models have tried to explain these seemingly contradictory observations, suggesting an FtsZ dimer nucleus goes through a conformational change making polymerization favorable (Dajkovic et al., 2008a; Miraldi et al., 2008). However, structural evidence supporting such a change has yet to be observed (Lan et al., 2008).
Lateral FtsZ Filament Interactions:

Individual FtsZ protofilaments are capable of self-interacting through lateral bonds, also termed filament bundling. Lateral filament interactions require that specific contacts be made between subunits of adjacent filaments similar to how polymerized tubulin dimers make a microtubule. This dissertation provides key insight into how these lateral interactions are able to form. As described in Chapter 2, large-scale differences in the inherent bundling abilities between FtsZs from *B. subtilis* and *E. coli* derive from small variations in the CTV. The ability to form lateral interactions without additional divalent cation or crowding agent is mediated by the charged residues providing strong evidence that bundling is an electrostatic event. The possible relevance *in vivo* is considered in Chapter 5.

The *in vitro* study of FtsZ bundles requires careful consideration of buffer conditions. Two important factors in regulating FtsZ bundling are pH and monovalent cation concentration. FtsZ lateral interactions best form at high protein concentration (> 5 μM), low salt (50 mM KCl), and a pH range from 6.0-7.0 (Buske and Levin, 2012; Pacheco-Gomez et al., 2011). Under conditions more closely resembling physiologic (pH 7.4-7.7, 350 mM KCl) FtsZ filaments are primarily single-stranded (Erickson et al., 2010). The monovalent cation present in buffer is essential for FtsZ assembly. Potassium is required for GTP hydrolysis; substituting sodium destabilizes the dimer and increases the critical concentration (Mendieta et al., 2009). Increasing the monovalent cation concentration also reduces FtsZ bundling significantly. Even at lower pH, FtsZ is predominantly in the single-stranded form at sodium or potassium concentrations at or above 100 mM.
Thus, the type of buffer used to measure FtsZ assembly is critical. FtsZ behaves best in the Good’s buffers 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.0-6.5), 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.5-7.5), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0-8.0) at a concentration of 50 mM. While some studies have examined FtsZ assembly using the buffer 1,4-Piperazinediethanesulfonic acid (PIPES) (Jaiswal et al., 2010; Singh et al., 2007), this buffer alone artificially induces bundling and is generally considered a poor buffer to measure FtsZ assembly (Scheffers, 2008).

Divalent cations and molecular crowding agents also have a significant effect on FtsZ bundling. Magnesium is required for the GTPase activity of FtsZ and consequent assembly of FtsZ into filaments (Monterroso et al., 2012). Mg\(^{2+}\) should be used in assembly reactions at a near-physiological free concentration of 1 mM for assembly (Froschauer et al., 2004); however, higher concentrations (>10 mM) of Mg\(^{2+}\) induce long bundles several filaments thick (Erickson et al., 2010). Calcium is another divalent cation shown to induce thicker and longer FtsZ bundles than those induced by Mg\(^{2+}\) (Mukherjee and Lutkenhaus, 1999; Yu and Margolin, 1997). Bundles formed in the presence of both Mg\(^{2+}\) and Ca\(^{2+}\) show slower rates of GTP exchange indicating that subunits trapped within the bundle cannot exchange nucleotide and supporting the notion that exchange only occurs at filament ends (Buske and Levin, 2012; Erickson et al., 2010).

FtsZ lateral interactions formed in the presence of crowding agents such as DEAE-dextran, Ficoll, polyvinyl alcohol, and methyl cellulose form a diverse array of different structures including bundles, sheets, spirals, and torroids (Popp et al., 2009). As the bacterial cytoplasm is a crowded environment (McGuffee and Elcock, 2010), these compounds are meant to mimic what might be occurring in the cell.
The FtsZ Ring *In Vivo*

One of the foremost uncertainties that remains today in the field of bacterial cell division is understanding how the properties of FtsZ observed *in vitro* relate to the behavior of the Z ring *in vivo*. In that vein, the overarching theme of this thesis is to associate changes to the FtsZ subunit to filament assembly and ultimately to its role in cell division.

Understanding the relevance of FtsZ filament dynamics *in vitro* requires a complete picture of the Z ring in the cell. Numerous works have attempted to image FtsZ and the Z ring in bacteria with the goal of recognizing how FtsZ acts during cell division. Using immunoelectron microscopy, Bi and Lutkenhaus provided the first evidence that FtsZ localizes to midcell and to the forming division septum leading the hypothesis that FtsZ arranged itself in a ring around the cell circumference; however, individual filaments were not detected likely due to sample preparation (Bi and Lutkenhaus, 1991). The ring-like arrangement of FtsZ was later confirmed using immunofluorescence microscopy (Levin and Losick, 1996; Wang and Lutkenhaus, 1996). The creation of a functional FtsZ-GFP fusion finally allowed observation of the Z ring in live cells (Ma et al., 1996).

Real-time imaging of the Z ring during the cell cycle shows it undertakes different conformations such as arc, spirals, helices, and rings (Anderson et al., 2004; Peters et al., 2007; Sun and Margolin, 1998; Thanedar and Margolin, 2004). These findings show the Z ring as a dense, well-organized, and continuous structure. Another implication from these works is that the Z ring is highly dynamic. The Z ring has a lifetime of about 8-10 minutes (compared to an approximately 20 minute cell cycle) and intensifies as the cell constricts (Erickson et al., 2010).
The exact mechanism of ring assembly/disassembly and constriction is unknown but likely linked to the dynamic nature of FtsZ filaments.

FtsZ maintains its structure in the Z ring through the constant exchange of individual FtsZ subunits in the cytoplasm with filaments in the ring (Justice et al., 2000). Fluorescence recovery after photobleaching (FRAP) experiments on wild-type FtsZ rings decorated with FtsZ-GFP fusions in both B. subtilis and E. coli indicate subunits in the Z ring are exchanging with those in the cytoplasm on a time scale of 8-12 seconds (Anderson et al., 2004; Geissler et al., 2007; Stricker et al., 2002). This rapid turnover is likely driven by GTP hydrolysis in FtsZ filaments, as Z rings assembled from a GTPase-deficient mutant, FtsZ84, have a 9-fold lower rate in subunit turnover (Stricker et al., 2002).

However, these studies utilizing light microscopy only offer a look at the aggregate ring. A major obstacle in resolving the Z ring structure is that light microscopy is constrained by the diffraction limit of light (~250 nm); objects smaller than this size become much more difficult to resolve (Coltharp and Xiao, 2012). Because FtsZ filaments average 100-200 nm in length, resolving individual protofilaments has not been possible using standard light microscopy.

The recent development of superresolution imaging technology has shed some light on the in vivo structure of the Z ring. The first major breakthrough came with the imaging of FtsZ filaments in C. crescentus using electron cryotomography (ECT) (Li et al., 2007). ECT can achieve single-nanometer resolution and better preserves the cell in a frozen-hydrated state that retains protein structure. However, it cannot accomplish live-cell imaging due to sample preparation. Reconstructed images show short protofilaments loosely arranged with little or no contact scattered near the division site. No complete rings are seen and the authors imply filaments are able to generate a bending force on the membrane (Li et al., 2007). These data have
led to a model of the Z ring in which filaments are arranged in a scattered, staggered, and overlapping orientation along the radial axis of the cell (Erickson et al., 2010).

Further evidence for such a model comes from imaging the Z ring using superresolution fluorescence microscopy. One such technique used to image FtsZ in the cell is photoactivated localization microscopy (PALM). By using photoactivatable fluorophores, single molecules can be detected in live cells at resolutions of ~10-50 nm (Coltharp and Xiao, 2012). Fu et al., revealed the width of the *E. coli* Z ring to be ~110 nm (Fu et al., 2010). Given the average number of FtsZ molecules in a typical *E. coli* cell measures 5000-7000 subunits and that 30% of total FtsZ is in the Z ring, a continuous ring of FtsZ filaments around a cell circumference of ~ 1 μm would only be 2-3 filaments thick (Erickson et al., 2010). A ring of closely-associated filaments would only measure ~12-20 nm in width (each FtsZ subunit is ~4 nm globule), and the authors conclude that Z ring is a loose structure made up of randomly oriented short filaments with space possibly occupied by other proteins (Fu et al., 2010). In support of this model, the width of the *C. crescentus* Z ring has been determined to be 92 nm during division using a similar approach (Biteen et al., 2012). Stimulated emission-depletion (STED) microscopy has also predicts the *B. subtilis* Z ring to be made up of freely arranged filaments (Jennings et al., 2010). With the exception of ECT, these imaging techniques have still yet to detect individual FtsZ filaments in the cell. The precise arrangement of filaments in the Z ring still remains a mystery.

While the exact arrangement of FtsZ is unknown, it appears to be able to form smaller ultrastructures within the ring. A recent examination using 3D-structured illumination microscopy (3D-SIM) shows the Z ring to be discontinuous and made of bead-like ministructures that are dynamic and distributed heterogeneously in both *B. subtilis* and *S. aureus*
These data suggest that local FtsZ concentrations change in the Z ring during cell division. What drives their dynamic behavior is unknown but the cell also contains numerous other protein factors that might interact with FtsZ that affect the arrangement of filaments in the Z ring (discussed below and in Chapter 5).

**FtsZ and the Generation of Constrictive Force**

The discovery that FtsZ forms filaments and is a cytoskeletal protein immediately had implications for its function in the cell. Beyond serving as a recruiter of other division proteins, it now seems clear that FtsZ also serves as a mechanical generator of force as well. Cytoskeletal proteins are well documented in their ability to create force. Actin and myosin are responsible for muscle contraction and cytokinesis. Microtubules are essential for intracellular trafficking and chromosome positioning during mitosis. The basis for these actions is rooted in small-scale changes at the molecular level able to manifest themselves into ordered, large-scale structures (Fletcher and Mullins, 2010). The formation of these higher-ordered structures (i.e. filaments and tubules) allows them to deform and exert long-range mechanical forces (Sun and Jiang, 2011).

This dissertation provides a clear example of how molecular changes affect the mechanics of FtsZ as Chapter 3 illustrates how the FtsZ CTL plays an important role in securing FtsZ to the membrane and helping in force generation.

Unlike eukaryotic cells, prokaryotes lack motor proteins associated with cytoskeletal filaments to drive cytokinesis. The initial discovery that FtsZ follows the invaginating septum in a dividing *E. coli* cell implied FtsZ played a role in this process (Bi and Lutkenhaus, 1991). Subsequent work showed FtsZ as a GTPase able to self-assemble into filaments and that it was
conserved in bacteria lacking other division proteins leading to the “Z-centric hypothesis”
instituting FtsZ as a primary generator of the constrictive force (Erickson, 1997).

Later work provided strong support for such a hypothesis when Z rings were
reconstituted in liposomes (Osawa et al., 2008). Replacing the grappling hook peptide of an
FtsZ-YFP fusion with an amphipathic helix allows FtsZ to bind to the membrane in the absence
of accessory proteins like FtsA or ZipA. When incorporated into tubular liposomes, the
fluorescently-tagged FtsZ assembles into rings around the circumference of the liposome
((Osawa et al., 2008). Noticeable inward deformations of the liposome occur when GTP is added
to the reaction, suggesting nucleotide hydrolysis by FtsZ leads to changes in the filament
curvature that apply a force to the lipid; however, subsequent work has shown that constriction
can still occur without GTP hydrolysis even though deformations were short-lived (Osawa et al.,
2008; Osawa and Erickson, 2011). Even more convincing evidence comes from a recent report
showing that FtsZ and FtsA added to spherical unilamellar liposomes can generate a complete
septum and generate two daughter liposomes in the presence of GTP and ATP (Osawa and
Erickson, 2013). This study clearly suggests cooperation between FtsZ and FtsA to create
contraction, though exactly how the force is exerted on the membrane is unclear.

Numerous physical models have been proposed for force generation via FtsZ filaments
(Allard and Cytrynbaum, 2009; Andrews and Arkin, 2007; Erickson, 2009; Fischer-Friedrich and
Gov, 2011; Fischer-Friedrich et al., 2012; Ghosh and Sain, 2008; Hsin et al., 2012; Hörger et al.,
2008a; Lan et al., 2007; 2009; Shlomovitz and Gov, 2009). One important feature of in modeling
the force generated by FtsZ filaments is their intrinsic bend. Experimental data shows FtsZ
filaments undertake conformations: straight and GTP-bound, intermediately curved and GTP-
bound or highly curved and GDP-bound (Lu et al., 2000). The molecular cause for the different
GTP-bound conformations is unknown but unrelated to GTP hydrolysis. These conformational changes can apply a contractile force which depends crucially on the stiffness of an FtsZ filament, or the persistence length, $l_p$ (Sun and Jiang, 2011). Assuming filaments prefer a straight conformation, two different persistence lengths for FtsZ filaments have been estimated to be 180 nm and 54 nm, respectively (Dajkovic et al., 2008a; Huecas et al., 2008). Using these estimates, the total FtsZ filaments in the Z ring would only generate a force of > 3 pN, well less than the total force estimated to constrict cells at ~8 pN (Lan et al., 2007; 2009). Another study assumes curved filaments are the preferred bend and uses atomic force microscopy to estimate filaments are stiff with an $l_p \sim 4 \mu$m (Hörger et al., 2008b). Perhaps the best estimate comes from a recent study using a more reliable electron cryotomography method to visualize single FtsZ filaments. The authors calculated the $l_p$ to equal 1.15 μm, suggesting FtsZ filaments are rigid but still flexible enough not to impede cell division (Turner et al., 2012). This new value for $l_p$ would significantly change the previous calculation for force generation at > 3 pN to over 17 pN, more than enough force to carry out cytokinesis (Lan et al., 2007; Turner et al., 2012). Another model estimates FtsZ filaments to have rigidity similar to that of Plexiglass and assumes GTP hydrolysis-induced filament bending is sufficient to produce sufficient force (Allard and Cytrynbaum, 2009).

Resistance to bending is an inherent property of stiff filaments and opposition to bending generates a force. However, instead of the stiffness of an entire filament generating force, individual monomers within the filament may be more significant. Modeling of nucleotide-bound FtsZ filaments shows hydrolysis drives a conformation change between subunits in FtsZ-GTP filaments compared to FtsZ-GDP filaments leading to significant bending (Hsin et al., 2012). The stiffness with which monomers resist bending generates 20-30 pN of force per
polymerized monomer, enhanced throughout filaments condensed in the Z ring and sufficient to
generate constriction (Hsin et al., 2012).

A problem models utilizing nucleotide hydrolysis induced bending of filaments must
reconcile is experimental observations showing cell division in GTP-hydrolysis-deficient FtsZ
mutants. An FtsZ mutant in the “synergy loop” (D212G) is GTPase-dead but still supports cell
division (Mukherjee et al., 2001). GTP hydrolysis clearly is not necessary for constriction.
However, Hsin et al., show even the slightly bent FtsZ-GTP filaments can generate ~10 pN of
force, still sufficient to carry out division (Hsin et al., 2012). In these GTPase deficient mutants,
other cellular factors might contribute to force generation as well. A recent model also shows
curvature of filaments can drive formation of either a circular or helical Z ring, as straighter
filaments generate helices or spirals (Fischer-Friedrich et al., 2012). This model perhaps explains
structures observed in different ftsZ mutants (Addinall and Lutkenhaus, 1996; Monahan et al.,
2009).

Other models of the constrictive force utilize an ensemble of bundled and condensed FtsZ
filaments in the Z ring. The ability of FtsZ polymers to laterally associate is enthalpically
favorable but only at high enough concentrations able to overcome the entropic costs that prefer
to keep filaments uniformly distributed in space (Sun and Jiang, 2011). A condensing Z ring is
such an environment. This is achieved by decreasing the cell radius while keeping the number of
FtsZ subunits in the Z ring the same (Lan et al., 2009). The bundled FtsZ filaments anchored to
the membrane can then generate enough force (< 8 pN) for cytokinesis in E. coli (Sun and Jiang,
2011).

FtsZ alone cannot complete cytokinesis though. Complete scission seems to require FtsA
as well (Osawa and Erickson, 2013), and the force generated by FtsZ might not be enough to
constrict the stiff cell wall. Peptidoglycan remodeling proteins are directed to the division site by FtsZ and help drive invagination of the septum to complete cytokinesis (Adams and Errington, 2009). The study of L-forms, bacteria that are able to switch into a cell wall-deficient state, also presents an interesting case.

**Factors That Regulate FtsZ Assembly**

In the model organisms in which the process of bacterial cell division is most thoroughly studied, Gram-positive *B. subtilis* and Gram-negative *E. coli*, FtsZ levels remain unchanged throughout the cell cycle (Weart and Levin, 2003). Therefore control over the exact timing and location of Z ring formation (and consequently cell division and cytokinesis) occurs at the level of FtsZ assembly (Romberg and Levin, 2003). Many proteins have been identified in both *B. subtilis* and *E. coli* that affect cell division and either directly or indirectly affect FtsZ assembly.

**Inhibitors of FtsZ Assembly:**

The FtsZ concentration in the cell is 4-10 μM depending on bacterial species and strain, significantly higher than the critical concentration for assembly at ~1 μM (Erickson et al., 2010). This suggests FtsZ assembly is favored in the energy-rich environment of the cell; however, bacterial cells have evolved proteins that inhibit FtsZ assembly to prevent aberrant Z ring formation.

One such set of proteins is the Min system. Found in most rod-shaped bacteria, the Min system consists of a series of oscillating proteins that move from pole to pole to block polar FtsZ ring formation and septation (Adams and Errington, 2009). In *E. coli*, this system is comprised of the proteins MinC, MinD and MinE (de Boer et al., 1989). *B. subtilis* also has MinC and MinD,
but instead of MinE it utilizes an unrelated protein DivIVA (Edwards and Errington, 1997; Marston and Errington, 1999; Marston et al., 1998). Of these proteins MinC is the FtsZ antagonist and acts by inhibiting lateral filament interactions (Dajkovic et al., 2008a; Shen and Lutkenhaus, 2010). MinC binds to the FtsZ GHP in *E. coli* but evidence for this interaction is lacking in *B. subtilis* (Shen and Lutkenhaus, 2009; de Oliveira et al., 2010).

Another spatial regulator of FtsZ assembly is the nucleoid occlusion (NO) system. NO prevents aberrant Z ring formation by preventing FtsZ assembly over the nucleoid (the organized chromosomal mass in the cytoplasm). This ensures the Z ring will only form at midcell after DNA replication has occurred and the chromosomes begin to separate toward the cell poles (Margolin, 2005). SlmA is the *E. coli* NO effector. A direct interaction between SlmA and FtsZ has been shown, but the exact mechanism by which SlmA acts on FtsZ remains controversial. Cho et al., demonstrate SlmA prevents FtsZ assembly into individual protofilaments in a manner that requires FtsZ GTP hydrolysis and is enhanced by SlmA binding to DNA (Cho et al., 2011). Conversely, SlmA has been shown to prevent higher-order FtsZ structures in a GTP-independent manner (Tonthat et al., 2011). Likely differences stem from the techniques used to analyze the SlmA-FtsZ interaction and buffer conditions. Both studies agree that SlmA functions as a dimer. A homologous system exists in *B. subtilis* and utilizes a DNA-binding protein called Noc, though no direct interaction with FtsZ has been shown (Adams and Errington, 2009).

FtsZ assembly is also inhibited in a nutrient-dependent manner by two additional proteins: UgtP in *B. subtilis* and OpgH in *E. coli*. Remarkably, though these proteins are highly unrelated, they both are moonlighting enzymes that utilize the metabolite UDP-glucose to couple the nutritional state of the cell to a modulation FtsZ activity (Hill et al., 2013).
Our lab first identified UgtP as a component of a metabolic pathway that links nutrient availability to cell division (Weart et al., 2007). Loss of UgtP leads to smaller *B. subtilis* cells during fast growth. UgtP also localizes to the Z ring in a nutrient-dependent manner; Ugtp localizes to the division site in nutrient-rich media while growth in nutrient-poor media results in distinct puncta distributed throughout the cytoplasm. These puncta appear to be UgtP oligomers that depend on the intracellular level of UDP-glucose (Chien et al., 2012). UgtP inhibits FtsZ assembly *in vitro* as demonstrated using 90° light scattering assays, but UgtP does not affect the GTPase activity of FtsZ (Weart et al., 2007). The affinity of UgtP for FtsZ is very high ($K_D \sim 20$ nM), and it appears to inhibit assembly of an FtsZ mutant unable to laterally associate, suggesting the mechanism of activity on FtsZ filaments is either capping or severing (Chien et al., 2012).

OpgH similarly binds UDP-glucose. Because *B. subtilis* and *E. coli* both use UDP-glucose as sensor molecules for nutrient availability, *opgH* was identified looking for genes that utilized similar pathways to those in *B. subtilis*. OpgH also localizes to the Z ring and biochemical evidence suggests it interacts with FtsZ by sequestering FtsZ monomers or capping filaments (Hill et al., 2013). More information about OpgH can be found in the Appendix.

SulA is another inhibitor of FtsZ assembly found in *E. coli*. SulA behaves as a dimer in solution and binds to FtsZ at the protofilament interface (Cordell et al., 2003). SulA inhibits FtsZ’s GTPase activity and acts as a monomer sequesterer (Chen et al., 2012; Dajkovic et al., 2008b). A functional homolog of SulA, YneA, functions in a similar way *in vivo* in *B. subtilis*, though no mechanism is known (Kawai et al., 2003).

An additional negative regulator of FtsZ assembly is the conserved AAA+ ATPase ClpX, the substrate recognition complex of the ClpXP protease. ClpX was identified in a screen for
suppressors of a temperature-sensitive ftsZ-GFP mutant in *B. subtilis* (Weart et al., 2005). *In vitro*, ClpX inhibits FtsZ assembly in a concentration-dependent manner that does not affect FtsZ GTP hydrolysis, suggesting it acts on FtsZ filaments, not monomers. ClpX also does not localize to the Z ring hinting it acts on the cytoplasmic pool of FtsZ (Weart et al., 2005). ClpX interacts with FtsZ in an ATP-independent manner (Haeusser et al., 2009; Weart et al., 2005). Since ClpX requires ATP for the proteolysis activity of ClpXP, this suggests ClpX affects FtsZ in a manner independent of ClpP. Deletion of the *clpX* gene does not alter FtsZ levels *in vivo* and *in vitro*. Degradation assays using FtsZ as a substrate of ClpXP show FtsZ cannot be proteolyzed (Weart et al., 2005). In *E. coli*, ClpX has also been shown to be an negative regulator of FtsZ assembly, though whether or not it does so through degradation via ClpXP or solely owing to the action ClpX as in *B. subtilis* remains controversial (Camberg et al., 2009; Sugimoto et al., 2010).

EzrA is another negative regulator of FtsZ assembly identified in a screen for extragenic suppressors of a temperature-sensitive ftsZ-GFP mutant in *B. subtilis* (Levin et al., 1999). Loss of function mutations in *ezrA* lowers the critical concentration of FtsZ *in vivo* leading to cells that form extra Z rings in at the poles and quarter-cell positions (Levin et al., 1999). EzrA is anchored in the plasma membrane via an N-terminal transmembrane domain while its cytoplasmic domain is predicted to consist of four coiled-coil domains (Haeusser et al., 2004). Pre-division, EzrA is uniformly distributed throughout the plasma membrane but then concentrates to the Z ring via a conserved sequence called the QNR patch (Haeusser et al., 2007). *In vitro* EzrA interacts directly with FtsZ and inhibits FtsZ assembly in a concentration dependent manner and modestly increases FtsZ GTPase activity (Haeusser et al., 2004). This interaction likely requires the FtsZ GHP (Singh et al., 2007). EzrA likely acts by preventing FtsZ lateral interactions (L. Romberg,
personal communication). Because it localized to midcell during cell division, it is thought that EzrA works by helping maintain the dynamic nature of the Z ring.

**FtsZ Stabilizers:**

Just as there are cellular factors that prevent Z ring formation, there are others that promote FtsZ assembly. Many of these proteins are able to stabilize FtsZ protofilament formation and promote FtsZ bundling. These factors are believed to counteract the negative regulators of FtsZ assembly so that the Z ring is able to maintain its structure throughout the cell cycle (Romberg and Levin, 2003).

FtsA was the first protein identified that stabilizes the Z ring in *E. coli* and subsequently in *B. subtilis* (Beall and Lutkenhaus, 1992; Beall et al., 1988; Dai and Lutkenhaus, 1992; Donachie et al., 1979; Robinson et al., 1988; Tormo et al., 1985). FtsA localizes to the Z ring early in the division process and anchors FtsZ to the membrane (Erickson et al., 2010; Pichoff and Lutkenhaus, 2005). This anchoring is mediated through an interaction between FtsA and the GHP of FtsZ (Haney et al., 2001; Ma and Margolin, 1999; Yan et al., 2000). In *E. coli*, FtsA helps recruit downstream division proteins to the new division site once FtsZ is recruited to midcell (Corbin et al., 2004; Pichoff and Lutkenhaus, 2002; Rico et al., 2004). Because of this function, it is essential in *E. coli*, though Z ring formation still occurs in when it is depleted or inactivated through a temperature sensitive mutation (Addinall et al., 1996; Hale and de Boer, 1999). However, FtsA is not required for cell division in *B. subtilis* despite cells being highly filamentous (Beall and Lutkenhaus, 1992; Jensen et al., 2005). These data suggest other factors help anchor FtsZ to the membrane (see below).
FtsA is an ATPase in the actin superfamily and well-conserved throughout many bacterial species (Bork et al., 1992). As such, FtsA has been shown to polymerize into actin-like filaments using a lipid monolayer as substrate, suggesting a self-interaction is mediated by the plasma membrane (Szwedziak et al., 2012). While FtsA displays ATPase activity, it is unknown how this is related to polymerization of FtsA filaments (Feucht et al., 2001; Singh et al., 2012). However, reconstitution of the FtsZ-FtsZ complex from *E. coli* in unilamellar liposomes shows ATP is required in order to generate constriction suggesting the complex uses the energy from nucleotide hydrolysis to create force (Osawa and Erickson, 2013).

One additional membrane anchor of FtsZ is ZipA in *E. coli*, a bitopic protein containing a transmembrane helix, a flexible linker domain, and a globular cytoplasmic domain required for binding FtsZ (Mosyak et al., 2000; Moy et al., 2000). ZipA is found only in the γ-Proteobacteria and serves a role similar to FtsA with both proteins having redundant function (Hale and de Boer, 1997; Pichoff and Lutkenhaus, 2002). *In vivo*, ZipA directly interacts with FtsZ to stabilize the Z ring and localizes to midcell early in cell division in an FtsZ-dependent (Hale and de Boer, 1997; 1999; Liu et al., 1999). Purified ZipA causes the formation of extensive FtsZ bundles *in vitro* and binds to the FtsZ GHP, though the mechanism of bundling is unknown (Hale et al., 2000; Haney et al., 2001; Ma and Margolin, 1999; Mosyak et al., 2000; RayChaudhuri, 1999). Reconstitution of ZipA and FtsZ in spherical giant unilamellar vesicles shows ZipA anchors FtsZ to lipids and suggests both proteins might work in tandem to help produce constrictive force for cytokinesis (López-Montero et al., 2013).

While no homolog of ZipA exists in *B. subtilis*, another protein, SepF, shares similar functions to both FtsA and ZipA. Conserved in Gram-positive bacteria, SepF was discovered independently by two labs as a component of the division machinery (Hamoen et al., 2006;
Ishikawa et al., 2006). SepF localizes to the Z ring in an FtsZ-dependent manner and has been shown to directly interact with FtsZ in vivo (Ishikawa et al., 2006). In vitro, SepF assembles into rings ~50 nm in diameter that are able to bundle FtsZ filaments into tube-like structures, supporting genetic evidence that it stabilizes the Z ring (Gündoğdu et al., 2011; Singh et al., 2008). The FtsZ-SepF interaction is mediated through SepF binding the FtsZ GHP (Król et al., 2012).

The final group of FtsZ-stabilizing proteins in both B. subtilis and E. coli is the Zap family. ZapA was the first of these discovered in a screen of proteins that, when overexpressed, overcome lethality induced by MinD overexpression in B. subtilis (Gueiros-Filho and Losick, 2002). Conserved in many bacteria, ZapA localizes to the Z ring early in the division process and promotes lateral FtsZ interactions, but its deletion in both B. subtilis and E. coli yields little noticeable phenotype (Gueiros-Filho and Losick, 2002; Mohammadi et al., 2009). ZapA is able to dimerize and tetramerize in vitro and must interact with FtsZ as a tetramer (Low et al., 2004; Pacheco-Gómez et al., 2013; Small et al., 2007). Active ZapA oligomers crosslink FtsZ filaments into bundles through changing the conformation of GTP in the FtsZ active site, leading to a reduction of FtsZ GTP hydrolysis activity (Dajkovic et al., 2010; Small et al., 2007). ZapA is also able to compete with MinC for binding to FtsZ hinting at a dynamic equilibrium of FtsZ bound to negative modulators or stabilizers that control stability of the Z ring (Scheffers, 2008).

Other Zap proteins have also been identified in E. coli. ZapB is a small molecular weight protein that is recruited to the Z ring by ZapA and forms a complex multi-protein complex with FtsZ and ZapA in which ZapB is linked between FtsZ filaments by ZapA (Galli and Gerdes, 2010; 2012). ZapC and ZapD have also been identified as components of the division apparatus; these proteins are able to interact with FtsZ and form filament bundles (Durand-Heredia et al.,
The exact cellular functions and reason for the redundancies of the Zap proteins is unclear, but they imply that lateral FtsZ interactions are important \textit{in vivo}. The differences in modulators between bacterial species also might explain the variations in the \textit{in vitro} assembly properties of FtsZ from different species described in Chapter 2.

\textbf{Toward a Unified Model of FtsZ Assembly}

The \textit{in vitro}, \textit{in vivo}, and \textit{in silico} works on the nature of FtsZ and the division machinery have all been impressive and crucial for our understanding of bacterial cell division. However, they each have limitations and sometimes generate contradictory conclusions. Data generated using these different methodologies must be carefully scrutinized, and results interpreted while weighing the pros and cons of the techniques used. Achieving a molecular-level resolution of FtsZ and the Z ring in living cells is an ultimate goal in the bacterial cell division field. Until that is possible, if it even is, the \textit{in vivo} cell biology, genetic, and microscopic work must ultimately serve as the framework for which details elucidated \textit{in vitro} and computationally fit into a model for FtsZ assembly and function in the cell. The more recent use of synthetic biology approaches, such as reconstituting protein structures in cell-like liposomes, will also be an important tool. Each approach is critical for continuing to advance the knowledge of FtsZ and developing a more complete model of the Z ring.

Perhaps the best example of the thorough consideration that must be given to developing a working model of the Z ring is the relevance for lateral FtsZ filament interactions in the cell. This is one of the primary questions raised by this thesis. Chapter 2 discusses in detail how changing FtsZ lateral interactions \textit{in vitro} correspond to a less-stable Z ring \textit{in vivo}, suggesting they are important. However, Chapter 3 shows FtsZs that favor forming single-stranded
filaments support normal cell division. Chapter 4 proposes future areas of study on this concept and further implications for cell division.

The mechanism by which bundling affects Z ring structure (linked to the arrangement of individual filaments) remains unknown. There are hints, though, from additional in vitro and in vivo work. FtsZ readily bundles in vitro in the presence of molecular crowding agents meant to simulate the cytoplasmic environment. Careful examination of these FtsZ structures reveals they are packed loosely in a liquid-crystalline order and not held together by regular lateral contacts like those that produce the microtubule wall (Erickson et al., 2010; Popp et al., 2009). These data complement well with PALM data from E. coli cells and support a type of bundling in which filaments do not organize in a regular arrangement (Fu et al., 2010). FtsZ-GFP expressed in yeast also forms large bundles and shows subunit turnover with a half-time of 11 seconds, suggesting they are dynamic structures (Srinivasan et al., 2008). Tightly bundled filaments would be predicted to have a much slower turnover, implying the freely bundled filaments are essential for Z ring dynamics.

The dynamic nature of bundles are supported by recent 3D-SIM data showing the Z ring composed of regions of varying density moving throughout the cell cycle (Strauss et al., 2012). The denser regions of FtsZ likely contain bundled FtsZ and it seems these bundles must assemble and disassemble quickly. Bundling also has been shown to be important computationally (Lan et al., 2009). The Z ring requires quick re-arrangement of filaments in order to condense and constrict. This model is supported by evidence of a temperature-sensitive B. subtilis FtsZ mutant. Growth of this mutant at the non-permissive temperature results in helical Z rings with a long pitch; in vitro the mutant FtsZ only forms single-stranded filaments while wild-type FtsZ forms bundles (Monahan et al., 2009). FtsZ rings have also been
reconstituted on synthetic lipid tubules and shown to associate laterally; however, filaments are in very tight and regularly arranged with little space in between and might be an artifact of the liposome system (Milam et al., 2012). Regardless, lateral filament interactions seem to be a key aspect of FtsZ behavior both in and out of the cell.

Conversely, in vitro FtsZ filaments show little evidence for lateral interactions and form predominantly single-stranded filaments in buffer more closely resembling physiological (pH 7.4-7.5, 250 mM KCl) (Erickson et al., 2010). Despite being crowded, the cytoplasm may also resemble these more dilute buffer conditions due to favorable ionic and hydrophobic interactions between FtsZ subunits that favor single-stranded filaments (McGuffee and Elcock, 2010). Strong evidence for lack of bundling in vivo also comes from ECT of the Z ring from C. crescentus which showed that short FtsZ filaments do not make contact with each other (Li et al., 2007). A downside of the ECT study is that cells were only able to be imaged at a certain angle meaning other orientations of FtsZ filaments could have been overlooked.

It seems that many of the FtsZ structures likely do not exactly represent the arrangement of filaments in the cell. A significant factor likely is that many of these studies only examine the behavior of FtsZ alone. The division apparatus contains other protein factors that inhibit or stabilize FtsZ assembly. Given the number of FtsZ bundling proteins, the single-stranded nature of FtsZ filaments alone is likely to be overcome by these factors. And now given recent evidence that FtsZ and FtsA together generate enough contractile force to divide liposomes (Osawa and Erickson, 2013), future studies of the Z ring need to move toward including different division machinery components to understand their interactions both in vitro and in vivo.
Significance, Summary, and Scope of Dissertation

The goal of my thesis has been to understand the molecular details of FtsZ assembly and how small changes to protein structure have significant cellular impacts. By detailing how previously uncharacterized domains of FtsZ work synergistically to promote assembly, I have provided significant insight into in vitro and in vivo FtsZ behavior. These domains are central to FtsZ function and establishing their significance has improved our understanding FtsZ’s role in cell division. Because of the ubiquitousness of FtsZ in many prokaryotes, cell division is likely conserved at a certain level. Broadening the insight into how FtsZ functions will allow us to uncover how cytokinesis evolved and potentially exploit it as a drug targets against pathogenic microbes.

The elucidation of the FtsZ crystal structure was the first step towards understanding the molecular details of FtsZ assembly (Löwe and Amos, 1998). The mapping of residues important for nucleotide binding and forming longitudinal bonds proved FtsZ assembly is crucial for cell division in vivo (Lu et al., 2001; Redick et al., 2005). However, crystal structures only resolve the well-conserved globular core domain of FtsZ. The entire C-terminus of FtsZ including the non-conserved C-terminal linker (CTL) and the grappling hook peptide (consisting of the conserved C-terminal constant region (CTC) and the C-terminal variable region (CTV)) does not appear in the structure and until now has remained largely ignored. The CTC has been shown to be the binding site for different FtsZ modulatory proteins, and the CTL displayed having a flexible nature; however, the exact roles that these domains play in the ability of FtsZ self-assemble were unknown.

My thesis aimed to clarify the function of the previously uncharacterized C-terminus in FtsZ assembly. Chapter 2 describes the characterization of the FtsZ C-terminus as a mediator of
lateral filament interactions. I show the FtsZs from two model organisms in the field of bacterial cell division, *B. subtilis* and *E. coli*, have different inherent abilities to form lateral interactions *in vitro* using 90° light scattering, electron microscopy, and a regenerative assay for GTPase activity. Using a *B. subtilis* FtsZ mutant in which the grappling hook peptide is deleted, I demonstrate this region of FtsZ is important for lateral bond formation but not individual protofilament assembly. I then create FtsZ chimeras swapping the CTV sequences from *B. subtilis* and *E. coli* to show specific charged residues facilitate FtsZ bundling and charge in general as an important factor in determining whether bundling occurs. I find that changes to the FtsZ CTV affect Z-ring stability when expressed in *B. subtilis*, suggesting lateral FtsZ interactions are important *in vivo*. These data lead to a model in which the FtsZ CTV is positioned by the flexible linker in such a way that it interacts with the globular core to allow filament bundling to occur.

In Chapter 3, I further explore this model and determine an essential role for the *B. subtilis* FtsZ CTL in protofilament assembly and Z-ring formation. By deleting the linker, I establish that it is necessary for normal FtsZ assembly *in vitro* and cell division *in vivo*. I determine that the linker behaves as an IDP, as replacing it with an alpha-helical sequence yields an FtsZ deficient in protofilament assembly and unable to support cell division. Using different chimeric FtsZs in which the linker sequence is altered or replaced with those from other bacterial species, I show a functional linker is between 25-100 residues in length but sequence is not important as long as the structure remains intrinsically disordered. These chimeras support normal protofilament assembly *in vitro* and *B. subtilis* cells divide normally. This data support a model in which the FtsZ linker acts as a flexible tether allowing filaments to anchor to the
membrane and respond to its curvature, possibly helping generate constrictive force as filaments bend.

Finally, in Chapter 4, I describe future directions for the study of FtsZ assembly, establishing the \textit{in vivo} importance of lateral FtsZ interactions, testing how the intrinsically disordered CTL conformation depends on primary sequence, and determining the actual physical flexibility of the FtsZ linker using single-molecule approaches. I also propose the development of new tools to explore the mechanisms of interaction between FtsZ and its modulatory proteins.
References


Romberg, L. EzrA prevents FtsZ bundling. *Personal communication.*


Figure 1

Cell division in a rod-shaped bacterium.

Cell division is coordinated with DNA replication. In a non-dividing cell, FtsZ (blue circle) exits primarily unassembled in the cytoplasm as either monomers or small oligomers. Upon some unknown signal, chromosome replication begins. As the chromosomes (grey ovals) continue to replicate, they are pulled toward the cell poles. Once there is a nucleoid-free space over midcell, FtsZ assembles into a ring-like structure (blue ring). The Z ring is composed of loosely-associated FtsZ filaments. As division continues, the Z ring condenses helping constrict the leading edge of the invaginating septum. Cytokinesis completes when the two daughter cells split and the Z ring disassembles back into a cytoplasmic pool of monomers and small oligomers.
Figure 1
**Figure 2**

**FtsZ assembly dynamics *in vitro***.

FtsZ assembly is a multi-step process. Assembly is initiated when FtsZ exchanges GDP for GTP. FtsZ subunits then undergo a switch to a conformationally active state and assemble into single-stranded protofilaments. Free FtsZ-GTP subunits can exchange with other FtsZ-GTP subunits at filament ends. These FtsZ-GTP protofilaments can also associate laterally to form bundles. Filament formation also leads to GTP hydrolysis within the filament at random subunits. The FtsZ-GDP within a filament can cause destabilization and fragmentation of the filament at subunit interface. The fragmented FtsZ-GTP filament can then anneal with other FtsZ-GTP subunits or filament ends leading to growing filaments. FtsZ-GDP at filament ends can exchange nucleotide with solvent or dissociate into the free pool of FtsZ-GDP subunits. A filament that does not fragment can stay stable until all subunits hydrolyze GTP. The resulting FtsZ-GDP filament becomes highly curved and destabilized where it disassembles.
Figure 2
Chapter 2:

The extreme C-terminus of the bacterial cytoskeletal protein FtsZ plays a fundamental role in assembly independent of modulatory proteins

PJ Buske and Petra Anne Levin

Journal of Biological Chemistry (2012)
Abstract

Bacterial cell division typically requires assembly of the cytoskeletal protein FtsZ into a ring (Z-ring) at the nascent division site that serves as a foundation for assembly of the division apparatus. High-resolution imaging suggests the Z-ring consists of short, single stranded polymers held together by lateral interactions. Several proteins implicated in stabilizing the Z-ring enhance lateral interactions between FtsZ polymers in vitro. Here we report that residues at the C-terminus of Bacillus subtilis FtsZ (CTV for C-terminal variable region) are both necessary and sufficient for stimulating lateral interactions in vitro in the absence of modulatory proteins. Swapping the 6-residue CTV from B. subtilis FtsZ with the 4-residue CTV from Escherichia coli FtsZ completely abolished lateral interactions between chimeric B. subtilis FtsZ polymers. The E. coli FtsZ chimera readily formed higher order structures normally seen in only the presence of molecular crowding agents. CTV mediated lateral interactions are important for integrity of the Z-ring, as B. subtilis cells expressing the B. subtilis FtsZ chimera had a low frequency of FtsZ ring formation and a high degree of filamentation relative to wild-type cells. Site-directed mutagenesis of the B. subtilis CTV suggests electrostatic forces are an important determinant of lateral interaction potential.
Introduction

FtsZ is an essential cytoskeletal protein that plays a central role in bacterial cell division. Conserved in bacteria, archaea and chloroplasts, FtsZ assembles into a ring structure at the nascent division site (the FtsZ ring) that serves as a scaffold for assembly of the division machinery (Harry et al., 2006). The FtsZ ring is dynamic, with average subunit turnover time estimated to be between 8 and 30 seconds (Anderson et al., 2004). At the end of the cell cycle the FtsZ ring constricts at the leading edge of the invaginating septum. Approximately 30% of total FtsZ is in the FtsZ ring, while the remainder appears to be largely cytoplasmic (Anderson et al., 2004).

Like its eukaryotic homolog tubulin, FtsZ binds GTP as a monomer; however a GTPase active site is formed only after dimerization (Erickson et al., 2010). In vitro, FtsZ assembles into single-stranded polymers, or protofilaments, in the presence of GTP (Mukherjee and Lutkenhaus, 1994). Increasing the concentrations of divalent cations like Mg$^{2+}$ or Ca$^{2+}$ or adding crowding agents such as methyl cellulose or DEAE-dextran to purified *Escherichia coli* and *Bacillus subtilis* FtsZ stimulates lateral interactions between protofilaments, leading to the formation of bundles, spirals, tubes and sheets (Gueiros-Filho and Losick, 2002; Gündoğdu et al., 2011; Mukherjee and Lutkenhaus, 1999; Popp et al., 2009).

High resolution imaging techniques suggest that in vivo the FtsZ ring is composed of short, single-stranded protofilaments that are linked together via lateral interactions. Cryo-EM tomography indicates the FtsZ ring is made up of short filaments (~100 nm) concentrated at the division site (Li et al., 2007). Photo-activated localization microscopy (PALM) suggests that FtsZ protofilaments are arranged around midcell in a randomly oriented loose bundle (Fu et al., 2010). Lateral interactions are mediated in part through the actions of FtsZ modulatory proteins.
including the widely conserved ZapA protein as well as ZipA and SepF, which are limited to the Gamma group of Gram-negative bacteria and the Firmicutes, respectively (Adams and Errington, 2009).

Structural and phylogenetic analysis divides the FtsZ subunit into five domains: the unstructured and poorly conserved N-terminus, the globular highly conserved core, an unstructured C-terminal linker, a short, well conserved “C-terminal tail” (CTT), and finally a small, highly variable set of residues (Löwe and Amos, 1998; Margolin, 2005; Vaughan et al., 2004), termed here the C-terminal variable region, or CTV (Fig. 1A). For simplicity we have grouped the N-terminal peptide and core together. FtsZ’s core displays a Rossman fold typical of nucleotide binding proteins including the residues comprising the GTP binding site and the T7 synergy loop required for GTP hydrolysis (Erickson et al., 2010). The core has been extensively studied and a handful of residues essential to longitudinal subunit bonds at the dimer interface have been mapped to this region (Koppelman et al., 2004; Lu et al., 2001; Redick et al., 2005). Residues implicated lateral interactions between protofilaments have also been mapped to the core (supplemental Table 1).

Following the core is the C-terminal linker (CTL). In B. subtilis and E. coli, the region spans ~50 residues, but can span over 300 residues in some bacterial species (Vaughan et al., 2004). The CTL is irresolvable by crystal structure and we have modeled it here as an unstructured peptide as proposed by Erickson (Erickson et al., 2010) (Fig. 1A).

The next 11 residues of FtsZ constitute the conserved CTT (Fig. 1A). The CTT is critical for interactions between FtsZ and several modulatory proteins, a function analogous to tubulin’s C-terminal tail. The stabilizing proteins FtsA and ZipA require the CTT for an interaction with FtsZ in E. coli (Haney et al., 2001; Ma and Margolin, 1999; Mosyak et al., 2000). In Caulobacter
*crescentus*, deletion of the CTT disrupts the FtsZ-FtsA interaction and leads to a punctate FtsZ localization pattern (Din et al., 1998). Similarly, *E. coli* FtsZ CTT point mutants are resistant to overexpression of the division inhibitor MinC<sup>C</sup>/MinD and at least one CTT mutant is unable to bind MinC *in vitro* (Shen and Lutkenhaus, 2009). EzrA, an inhibitor of FtsZ assembly in *B. subtilis*, disrupts assembly of full length FtsZ but does not appear to interact with a truncated protein missing the last 16 residues, a region that includes the CTT (Singh et al., 2007).

Although the proteins that bind to it are not all conserved the majority of CTT residues implicated in interactions with modulatory proteins are themselves conserved (Fig. 1B, supplemental Table 1). The four CTT residues, D373, I374, F377 and L378, implicated in interactions between FtsZ and FtsA, MinC and ZipA, are conserved in FtsZs from a diverse array of species (Fig. 1B). This high degree of conservation suggests the CTT may play a role in FtsZ assembly beyond its function as a “landing pad.”

The residues between the CTT and the very C-terminus of FtsZ are highly variable both in length and content. In *B. subtilis* FtsZ, this region is 6 residues but spans up to 10 residues in *Staphylococcus aureus* FtsZ, and can be positive, negative or neutral in charge. We have termed this region the C-terminal variable, or CTV, domain.

Here we report that the *B. subtilis* CTV is both necessary and sufficient for promoting lateral interactions between FtsZ protofilaments in the absence of modulatory proteins. Comparative analysis of mutant and chimeric *B. subtilis* and *E. coli* FtsZs indicate that changes in the CTV have dramatic impacts on the bundling capacity of each protein. CTV charge appears to be the primary determinant of its potential to promote lateral interactions. Supporting a role for the CTV in mediating lateral interactions between FtsZ polymers *in vivo*, defects in the *B. subtilis* FtsZ CTV severely impair FtsZ ring formation and cell division. This work has
implications for our understanding of FtsZ assembly processes as well as the role of modulatory proteins in controlling FtsZ lateral interactions.
Results

FtsZ from both *B. subtilis* and *E. coli* display distinct assembly properties:

Despite a high degree of conservation at the sequence level, particularly in the core domain (54% identical, 76% similar), purified *B. subtilis* and *E. coli* FtsZs behave very differently in standard assays for FtsZ assembly. Transmission electron micrographs of 3 µM FtsZ assembled in our standard FtsZ polymerization buffer (50 mM MES pH 6.5, 50 mm KCl, 2.5 mM MgCl₂, 1 mM EGTA, 1 mM GTP) indicate that *B. subtilis* FtsZ (Bs FtsZ) has a strong propensity to form stable lateral interactions while *E. coli* FtsZ (Ec FtsZ) does not (Fig. 2). Bs FtsZ formed both large bundles of filament rings (Fig. 2A) and sheets of single-stranded filaments (protofilaments) (Fig. 2B), while Ec FtsZ was found primarily as unbundled single-stranded filaments (Fig. 2C). Ec FtsZ filaments were also longer than Bs FtsZ filaments, averaging 218.88 ± 56.54 nm in length versus 129.32 ± 36.33 nm for their *B. subtilis* counterparts (Fig. 2F).

Consistent with the presence of increased lateral interactions and larger structures, Bs FtsZ produces a 23-fold higher signal than Ec FtsZ when assembly is assayed by 90° angle light scattering (Fig. 2G). 90° angle light scattering is a standard method to measure FtsZ assembly in real time (Mukherjee and Lutkenhaus, 1999). The nature of the assay is such that highly bundled polymers will produce a larger signal than the same number of unbundled single stranded polymers due to increased polymer mass (Mukherjee and Lutkenhaus, 1999). In light of our EM data, we therefore interpret the large difference in light scattering signal between the Bs FtsZ and Ec FtsZ to be a reflection of the increased lateral interactions between Bs FtsZ filaments. In agreement with previous studies, both Bs FtsZ and Ec FtsZ assemble in a GTP-dependent
manner, reaching steady-state approximately one minute after the addition of GTP (Fig. 2, G and H) (Haeusser et al., 2004; Mukherjee and Lutkenhaus, 1999).

The differences in bundling appear to be electrostatically driven, as increasing the KCl concentration in the buffer lowers the Bs FtsZ light scattering signal ~40-fold while that of Ec FtsZ is largely unaffected (supplemental Fig. 1). In the same buffer used to measure FtsZ assembly, Bs FtsZ hydrolyzed GTP at a rate of 2.25 ± 0.40 GTP/FtsZ/min whereas Ec FtsZ hydrolysis was 5.40 ± 0.39 GTP/FtsZ/min. The GTPase rate of Bs FtsZ is significantly lower than that of Ec FtsZ, presumably due to reduced turnover of subunits in bundled protofilaments of Bs FtsZ (Table 2).

Note that the same polymerization buffer is used throughout this study. Ec FtsZ has been reported to form structures several protofilaments thick in the presence of higher levels of magnesium, 10 mM MgCl$_2$ (Mukherjee and Lutkenhaus, 1998; Pacheco-Gomez et al., 2011). Mg$^{2+}$ along with Ca$^{2+}$ and the molecular crowding agent DEAE-dextran have been shown to stimulate lateral interactions and the formation of higher order structures (Romberg and Levin, 2003). However, the 2.5 mM concentration of Mg$^{2+}$ in our standard polymerization buffer is more physiologically relevant (Froschauer et al., 2004). All EM was conducted with FtsZ at a concentration of 3µM to allow us to visualize single stranded filaments more easily, and light scattering and GTPase assays at a concentration of 5µM.

**Deletion of the conserved C-terminal tail eliminates B. subtilis FtsZ lateral interactions:**

In light of these findings, we next sought to determine which regions of FtsZ might account for the differences in the ability of Bs FtsZ and Ec FtsZ to bundle. Since the N-terminal core domain is relatively well conserved between *E. coli* and *B. subtilis*, we speculated that the
C-terminal region, particularly the CTT and CTV, might account for the difference in assembly properties. Although the role of the CTT in interactions with FtsZ modulatory proteins has been studied in some depth (Haney et al., 2001; Ma and Margolin, 1999; Shen and Lutkenhaus, 2009; Singh et al., 2007), a function for the C-terminus in FtsZ polymer assembly in the absence of modulatory proteins has not been established.

To determine the role of the CTT and CTV in FtsZ assembly, we engineered a *B. subtilis* FtsZ protein that is missing the last 17 residues starting at Asp 366, a region that includes both the CTT and the CTV (Bs FtsZ ΔC17). As most of the longitudinal contacts between adjacent FtsZ subunits have been mapped to the N-terminal core (Lu et al., 2001; Redick et al., 2005), we hypothesized that the Bs FtsZ ΔC17 mutant would still be able to assemble into protofilaments and maintain GTPase activity needed for oligomerization. Bs FtsZ ΔC17 and all other FtsZ constructs used in this work are depicted in Fig. 1C.

Our data suggest the C-terminus of *B. subtilis* FtsZ plays an important role in assembly (Fig. 2, D and G). EM images of Bs FtsZ ΔC17 assembled in the presence of GTP indicate it is almost exclusively in single-stranded protofilaments. Consistent with a lack of bundling Bs FtsZ ΔC17 reached maximal assembly at only 1.8% of the peak value of Bs FtsZ WT (Fig. 2, G and H). Similarly, the GTP turnover rate of Bs FtsZ ΔC17 was 71% higher than that of wild-type Bs FtsZ, although not as high as that observed for Ec FtsZ, which also displays unbundled single filaments (Table 2). Bs FtsZ ΔC17 filaments averaged 107.26 ± 37.84 nm in length, significantly shorter than Bs FtsZ WT (Fig. 2F).

In contrast to *B. subtilis* FtsZ, deleting the last 15 residues of *E. coli* FtsZ (residues 369 to 383), the region constituting its CTT and CTV, had only a small impact on assembly. Like wild-type Ec FtsZ, Ec FtsZ ΔC15 readily assembled into single protofilaments upon the addition of
GTP and no bundling was observed (Fig. 2E). The Ec FtsZ ΔC15 filaments, however, were somewhat shorter than wild-type Ec FtsZ (182.35 ± 47.67 nm in length versus 218.88 ± 56.54 nm) (Fig. 2F). The average peak light scattering value for Ec FtsZ ΔC15 was 83% of wild-type Ec FtsZ (p < 0.05), consistent with the reduction in average filament length (Fig. 2H). GTP hydrolysis rates of Ec FtsZ ΔC15 were near that of wild-type Ec FtsZ, with average values of 4.76 ± 0.29 GTP/FtsZ/min and 5.40 ± 0.39 GTP/FtsZ/min, respectively (p > 0.05) (Table 2).

The *B. subtilis* FtsZ CTV is sufficient to induce lateral interactions between FtsZ protofilaments:

Based our finding that the C-terminus of Bs FtsZ was required for lateral interactions *in vitro*, we next investigated the role of the CTV in this process. In contrast to the highly conserved CTT, the CTV is highly variable both in length and content between FtsZs from different species. To test the role of the CTV in modulating lateral interactions, we swapped the last six residues of Bs FtsZ with the last four residues of *E. coli* FtsZ. The fusion boundaries for the chimeric proteins were at the very end of the CTT, R376 in Bs FtsZ and R379 Ec FtsZ (Fig. 1C). We designated the resulting chimeras Bs FtsZ CTVE and Ec FtsZ CTVB.

Data from the chimeric proteins supports a central role for the CTV in modulating lateral interactions. Under standard polymerization conditions, Bs FtsZ CTVE was able to form single stranded polymers; however, no FtsZ filament bundles were observed (Fig. 3A). Consistent with the EM data, Bs FtsZ CTVE exhibited a peak light scattering value on par with that of Bs FtsZ ΔC17 and only 1.65% of the peak light scattering value of wild-type Bs FtsZ (Fig. 3E). Similarly, the GTPase turnover rate of Bs FtsZ CTVE was essentially equivalent to Bs FtsZ ΔC17 (3.86 ± 0.19 GTP/FtsZ/min), significantly higher than wild-type Bs FtsZ (2.25 ± 0.40 GTP/FtsZ/min).
GTP/FtsZ/min) (p < 0.005). Average Bs FtsZ CTVE filament lengths measured 95.92 ± 22.16 nm, shorter than the 129.32 ± 36.33 nm observed for Bs FtsZ (Fig. 3D).

Data from Ec FtsZ CTVB indicates that the CTV from *B. subtilis* is sufficient to promote lateral interactions between single stranded FtsZ polymers. In contrast to both wild-type *E. coli* FtsZ and Bs FtsZ CTVE, the Ec FtsZ CTVB chimera readily formed lateral interactions *in vitro* (Fig. 3B). In the presence of GTP, purified Ec FtsZ CTVB formed thick filament sheets as well as rings and toroidal structures (Fig. 3C). Such structures have previously been observed for Ec FtsZ only in the presence of higher concentrations of divalent cations or molecular crowding agents (7). The diameter of the Ec FtsZ CTVB rings was on average 370.64 ± 46.24 nm with a width of 58.27 ± 20.37 nm, somewhat larger than those observed for similar structure composed of wild-type Bs FtsZ (183.41 ± 34.27 nm diameter; 30.66 ±10.85 nm width) (supplemental Fig. 2D).

Consistent with increased lateral interactions and the presence of very large macromolecular weight complexes, Ec FtsZ CTVB had a peak light scattering value ~30-fold higher than wild-type Ec FtsZ and ~ 30% greater than Bs FtsZ (Fig. 4E). The GTPase turnover rate of the Ec FtsZ CTVB was significantly lower than wild-type Ec FtsZ at only 2.19 ± 0.29 GTP/FtsZ/min, also consistent with an increase in lateral interactions. Individual Ec FtsZ CTVB protofilaments were typically longer than wild-type Ec FtsZ (277.58 ± 49.40 nm vs.218.88 ± 56.54 nm) (Fig. 3D) and the range of lengths was significantly broader (supplemental Fig. 2).

CTV charge plays a critical role in lateral interaction potential:

At pH 6.5, the Bs FtsZ CTV (NRNKRG) is highly positive, whereas the Ec FtsZ CTV (KQAD) has a net neutral charge. We therefore speculated that the positive charge of the Bs FtsZ
CTV conferred the ability in Bs FtsZ to form lateral filament interactions. To test this possibility, we examined the assembly properties of two Bs FtsZ mutants, one in which the positive charge of the CTV was conserved but replaced with different amino acids (Bs FtsZ NKNRKG) and another in which the charges were completely reversed (Bs FtsZ NENDEG).

Our data suggest that CTV charge is the primary determinant of Bs FtsZ lateral interaction potential. While the positively charged Bs FtsZ NKNRKG mutant, readily formed filament bundles and sheets in vitro, the negatively charged Bs FtsZ NENDEG did not (Fig. 4, A and B). Similarly, in light scattering reactions Bs FtsZ NKNRKG assembled robustly, albeit 2-fold less than wild-type Bs FtsZ. However, the light scattering signal of negatively charged Bs FtsZ NENDEG was ~16-fold less than that of wild-type Bs FtsZ (Fig. 4D), a level comparable to Ec FtsZ and Bs FtsZ ΔC17.

Although the positively charged Bs FtsZ NKNRKG readily formed bundles in vitro (Fig. 4A), we did not observe any of the bundled rings structures characteristic of wild-type Bs FtsZ, suggesting a possible role for specific CTV residues in mediating lateral interactions. This finding is consistent with the somewhat reduced signal of this mutant in the light scattering reaction (Fig. 4D). Filaments averaged a length of 133.67 ± 37.42 nm (Fig. 4C).

GTPase data reflects the relative lateral interaction potential of the two FtsZ mutants. Negatively charged Bs FtsZ NENDEG had elevated GTPase activity with a rate of 5.85 ± 0.29 GTP/FtsZ/min, comparable to that of Ec FtsZ. In contrast, positively charged Bs FtsZ NKNRKG exhibited a rate of 2.32 ± 0.19 GTP/FtsZ/min, in the range of wild-type Bs FtsZ. Despite its inability to form lateral interactions, the average filament length of negatively charged Bs FtsZ NENDEG was 176.00 ± 51.64 nm, significantly longer than the average for wild-type Bs FtsZ (129.32 ± 36.33 nm).
Defects in lateral interaction potential impair cell division in *B. subtilis*:

To determine if the lateral interactions we observed for *B. subtilis* FtsZ *in vitro* (Fig. 2A), were relevant *in vivo* we engineered an otherwise wild-type *B. subtilis* strain expressing Bs FtsZ CTVE from the native FtsZ promoter (see Experimental Procedures). As a control, we engineered a congenic strain expressing wild-type *Bs ftsZ*. Cells were grown to mid-exponential phase (OD$_{600}$ ~ 0.4), fixed, stained for cell wall and FtsZ and visualized by fluorescence microscopy. Quantitative Western blot analysis demonstrated the mutant FtsZ CTVE construct is stable *in vivo* (supplemental Fig. 4)

Analysis of *Bs ftsZ CTVE* mutants suggests that the lateral interactions we observed *in vitro* are critical for FtsZ ring integrity *in vivo*, and support a role for the CTV in stabilizing FtsZ polymers within the cytokinetic ring. Although *B. subtilis* cells expressing *Bs ftsZ CTVE* grew normally and exhibited exclusively medial FtsZ rings (Fig. 5A), they were on average ~85% longer than congenic wild-type cells (Fig. 5D). ~31% (90/290) of *Bs ftsZ CTVE* mutants were filamentous (>5 cell lengths) suggesting cells expressing the chimera failed to divide at a relatively high frequency.

Comparisons of cell length (L) to number of FtsZ rings (R) indicated that the L/R ratio of the *Bs ftsZ CTVE* mutants was nearly 75% greater than wild-type cells. While the congenic wild-type strain had an L/R ratio of 7.42 ± 0.71 µm/ring, similar to that of wild-type JH642 cells (33), *Bs ftsZ CTVE* mutants had a L/R ratio of 12.97 ± 0.40 µm/ring, consistent with a reduction the frequency of FtsZ ring formation (Fig. 5C).

Not surprisingly, none of the CTV chimeras were able to support division outside of their native species, in support of previous work (Redick et al., 2005). Neither wild-type Ec FtsZ nor
Ec FtsZ CTVB supported division in *B. subtilis* cells depleted for wild-type Bs FtsZ (Fig. 5E). Both Ec FtsZ and Ec FtsZ CTVB were dispersed throughout the cytoplasm, suggesting they lacked a targeting determinant required for medial localization and/or failed to interact with critical cell division proteins. Bs FtsZ and Bs FtsZ CTVE expressed from a plasmid vector were similarly unable to rescue cell division in the heat sensitive *E. coli ftsZ84* mutant at the restrictive temperature (supplemental Fig. 3).
Discussion

Taken together our data strongly support a role for FtsZ’s C-terminus, particularly the poorly conserved residues at the very end of the protein (the CTV), in modulating lateral interactions between protofilaments in the absence of modulatory proteins. Specifically, we find that the last six residues of *B. subtilis* FtsZ are both necessary and sufficient to promote the high degree of lateral interactions characteristic of wild-type *B. subtilis* FtsZ. Swapping the six residue CTV of *B. subtilis* FtsZ with the four residue *E. coli* FtsZ CTV reverses the bundling potential of both proteins, reducing light scattering signal 60-fold for Bs FtsZ and increasing it 30-fold for Ec FtsZ (Fig. 3). Importantly, *B. subtilis* FtsZ CTV has a significant cell division defect *in vivo*, strongly supporting the physiological relevance of our biochemical data.

Although the CTT has been implicated in interactions between FtsZ and modulatory proteins (Koppelman et al., 2004; Lu et al., 2001; Monahan et al., 2009; de Oliveira et al., 2010), the role of FtsZ’s C-terminus in FtsZ assembly has not been subject to direct analysis until this work. Below we discuss the implication of these findings with regard to our understanding of the mechanisms driving FtsZ lateral interactions both alone and in the presence of modulatory proteins.

Electrostatic forces as the primary determinant of lateral interaction potential:

Our findings support a model in which CTV charge is the primary determinant of its effect on bundling. Both wild-type Bs FtsZ and one in which positively charged residues in the CTV have been replaced by residues with similar charge exhibit a high degree of lateral interaction potential and low GTPase activity (Figs. 2 and 4). Conversely, Bs FtsZ mutants in which the positively charged wild-type CTV was replaced with either the neutral CTV from *E.
coli FtsZ or a negatively charged CTV sequence, NENDEG, were more or less equivalent to wild-type E. coli FtsZ with regard to bundle formation, light scattering, and GTPase activity (Figs. 3 and 4). In other words, while a positively charged CTV appears to be an indicator of a high degree of lateral interaction potential, a negative or neutral CTV is suggestive of a low level of lateral interaction potential.

In light of these findings we propose a model in which the FtsZ filament is in essence a linear polyelectrolyte separate from the flexible linker, CTT, and CTV. The cores of both Bs FtsZ and Ec FtsZ carry a net-negative charge at physiological pH. While a negatively charged or neutral CTV would have little impact on lateral interactions between negatively charged strands, a positively charged CTV would function as a polycation, shielding adjacent, negatively charged filaments from one another and thus facilitating lateral interactions. Data indicating that increasing the salt concentration of the polymerization buffer also inhibits lateral interactions between polymers of Bs FtsZ further supports this model (supplemental Fig. 1).

High concentrations of other charged ions, for instance Ca\(^{2+}\) and Mg\(^{2+}\), also favor protofilament bundling in Ec FtsZ even in the absence of the CTT and CTV (Geissler et al., 2003). We have observed a similar phenomenon for Bs FtsZ, suggesting the cations play a similar charge-shielding role the CTV plays to promote bundling in its absence.

Modulatory proteins that promote lateral interactions between FtsZ protofilaments, such as ZapA and B. subtilis SepF may function by mimicking the positively charged CTV. Consistent with this idea, data from the crystal structure of ZapA suggests the ZapA tetramer has an exposed patch of positive residues in its more conserved globular domain (Low et al., 2004). Depending on its orientation relative to the FtsZ polymer, it is possible that ZapA’s charged patch might promote bundling by shielding negatively charged FtsZ cores on adjacent polymers,
in a manner similar to the model we propose for the Bs FtsZ CTV. More work is needed, though, to detail electrostatic potential of the SepF protein.

While charge appears to be the primary determinant of lateral interaction potential, other forces cannot be ruled out as contributors to FtsZ bundling. A conservative change in Bs FtsZ CTV (NRNKRG to NKNRKG) that altered amino acid composition but retained the positively charged nature of the Bs CTV reduced the light scattering signal two-fold, although sheets and smaller bundles were still readily visible by EM (Fig. 4, A and D). This finding suggests that other factors such as salt bridges between specific residues or van der Waals interactions may also play a role in FtsZ filament bundling.

In support of the idea that specific interactions between residues in the CTV and the core contribute to bundle formation, a handful of residues on the surface of FtsZ’s core have been implicated in modulating lateral interactions (supplemental Table 1) (Koppelman et al., 2004; Lu et al., 2001; Monahan et al., 2009; de Oliveira et al., 2010). Specifically which core residues might interact with the CTT is not clear. Based on our observation that the Bs FtsZ CTV is sufficient to enhance bundling of Ec FtsZ 30-fold, we would predict that the Bs FtsZ CTV interacts with conserved residues on the core of Ec FtsZ (Fig. 3). To date, however, core residues implicated in lateral interactions are either not well conserved between B. subtilis and E. coli FtsZs (Lu et al., 2001) or mutants in these residues are significantly deficient in GTPase activity (de Oliveira et al., 2010), suggesting longitudinal interactions between subunits are perturbed in contrast to our Bs FtsZ ΔC17 and Bs FtsZ CTVE mutants (supplemental Table 1).

**Filament length appears to be independent of lateral interaction potential:**
Somewhat surprisingly, we did not observe a strong correlation between filament length and lateral interaction potential. While the relationship between filament length and bundling is not well understood, it has been addressed through modeling (Lan et al., 2008). At steady state, lateral interactions are predicted to be more favorable as filament length increases. This was the observed case for our Ec FtsZ CTVB mutant, which bundled with high efficiency and had significantly longer filaments than wild-type Ec FtsZ in vitro (Fig. 3D). However, this notion does not apply when comparing Bs FtsZ to Ec FtsZ. Generally, the better bundling Bs FtsZ had average single filament lengths less than the single stranded Ec FtsZ as did the poorly bundling Bs FtsZ ΔC17 and Bs FtsZ CTVE variants (Fig. 2F). The possibility exists that there could very well be intrinsic length limits to FtsZ filaments from different species. This idea is supported by looking at the lengths of our Bs FtsZ ΔC17 and Ec FtsZ ΔC15 mutants (Fig. 2F). Bs FtsZ ΔC17 had significantly shorter single filament lengths than Ec FtsZ ΔC15, indicating that length is not dependent on ability of the FtsZ to bundle.

Regardless of species, deleting the C-termini (CTT and CTV) of either B. subtilis or E. coli FtsZ significantly reduced the average length of single stranded polymers (Fig. 2F), suggesting a possible role for this region in modulating interactions between subunits within individual protofilaments. For example, the C-terminus may increase nucleation rates without altering elongation rates, a situation that would lead to the formation of a higher number of shorter filaments due to an overall reduction in pool of FtsZ subunits available for assembly. Regardless of mechanism, our findings highlight the need for closer examination of the assembly dynamics of both wild-type FtsZ and C-terminal deletion mutants, to illuminate the factors that contribute to FtsZ filament length.
Changes in lateral interaction potential *in vitro* impact FtsZ ring stability *in vivo*:

Our data support a model for cell division in which lateral interactions between protofilaments are important *in vivo*. The high ionic strength and pH of the cytoplasm differ from our *in vitro* conditions, and are likely weaken the ability of the positively charged CTV to promote lateral interactions to some degree. However, *B. subtilis* cells expressing the poorly bundled Bs FtsZ CTVE chimera as the sole copy of FtsZ exhibited a mild cell division defect, suggesting the wild-type CTV plays a role in modulating lateral interactions *in vivo*. Bs FtsZ CTVE mutant cells were on average ~85% longer than congenic cells expressing wild-type FtsZ, exhibited a high frequency of filamentation, and had an elevated cell length to FtsZ ring ratio (Fig. 5, A and B), consistent with a reduction in the integrity of the FtsZ ring. Over 30% of Bs *ftsZ CTVE* mutants were filaments, suggesting that at least a subset of these cells is at the precipice between being able to divide and failure of the division machinery.

Although we do not favor the possibility, the *Bs ftsZ CTVE* cell division phenotype may be due to loss of interaction between FtsZ and a modulatory protein that interacts with the CTV. A potential candidate for such a modulatory protein is FtsA, which helps anchor FtsZ to the membrane through interactions with CTT (Erickson et al., 2010; Haney et al., 2001; Ma and Margolin, 1999; Yan et al., 2000). In support of this idea, an *ftsA* deletion in *B. subtilis* also leads to a mild to moderate cell-division defect (Jensen et al., 2005). However, the length of Bs *ftsZ CTVE* mutant cells is significantly more variable than that of *ftsA* deletion mutants. Similarly, while we observed irregular FtsZ localization in the Bs FtsZ CTVE mutant (Fig. 5), consistent with a disruption in Z-ring integrity, Z-rings are regularly positioned in the *ftsA* deletion, and though irregular in shape, Z-rings are still assembled (Jensen et al., 2005). Moreover, there is no data suggesting FtsA interacts directly with residues in the CTV of either *E. coli* FtsZ or *B.*
*subtilis* FtsZ. Finally, while it is possible the CTV is needed for an interaction with a modulatory protein other than FtsA, it is unclear what such a protein would be as the Bs *ftsZ CTVE* phenotype is unlike those of other cell division mutants, including *zapA* and *sepF* (Gueiros-Filho and Losick, 2002; Ishikawa et al., 2006).

Thus far, the native CTT is the primary location for interactions between FtsZ and its regulatory proteins, and it is unchanged in the *Bs ftsZ CTVE* mutant. We therefore favor the more straightforward explanation that our *in vivo* and *in vitro* phenotypes are related.

What is perhaps more surprising is that *Bs* FtsZ CTVE is able to support division at all given its relatively poor ability to bundle *in vitro*. In this way, the *Bs ftsZ CTVE* mutant is reminiscent of two other FtsZ mutants, *tsl* from *B. subtilis* and *ftsZ84* from *E. coli*. Purified *E. coli* FtsZ84 is unable to assemble to a significant degree *in vitro* and is moderately defective in GTP binding and hydrolysis (although it is able to bind and hydrolyze ATP as well) (RayChaudhuri and Park, 1994; Redick et al., 2005). However, *in vivo* the *ftsZ84* mutation is able to support division at the permissive temperature of 30°C and/or in the presence of NaCl (Bi and Lutkenhaus, 1992; Dai et al., 1994). Similarly, although the conditional *B. subtilis* mutant FtsZ(Ts1) is unable to form lateral interaction *in vitro*, it is capable of supporting division *in vivo* at permissive temperatures (Monahan et al., 2009). We speculate that in all three cases, the Bs FtsZ CTVE, FtsZTs1, and FtsZ84 mutant proteins retain their ability to interact with modulatory proteins, which in turn promote stabilizing lateral interactions thereby ameliorating any assembly defect *in vivo*. Consistent with this hypothesis, overexpression of the stabilizing protein ZipA represses the thermosensitivity of the *ftsZ84* mutant at 42°C (RayChaudhuri, 1999).

Our finding that neither the wild-type Ec FtsZ nor the Ec FtsZ CTVB chimera was able to support division in *B. subtilis* (Fig. 5E) is consistent with previous work indicating that *B.
*B. subtilis* FtsZ is unable to support division in wild-type *E. coli* (Beall et al., 1988; Redick et al., 2005). Moreover, the wild-type *E. coli* FtsZ and *E. coli* FtsZ CTVB we observed in *B. subtilis* cells depleted for native FtsZ (Fig. 5E) are strikingly similar to the foci generated by expression of a YFP-fusion to a chimeric FtsZ in which the first 323 residues from *B. subtilis* (the core) were fused to residues 325 to 383 of *E. coli* (the linker, CTT, and CTV) [Fig. 5A from (Osawa and Erickson, 2006)]. Intriguingly, in their study Osawa and Erickson were able to obtain second site suppressor mutations in the *E. coli* chromosome that permitted growth of *E. coli* cells expressing the *B. subtilis* chimera protein as its only source of FtsZ (Osawa and Erickson, 2006). Coupled with the localization data, this finding would support the view that an unidentified FtsZ interacting protein may titrate ectopic FtsZ away from the site of division. However, this is only speculative, as the suppressor mutations isolated in the original paper were never identified at the molecular level.

**A role for FtsZ’s C-terminus in mediating lateral interactions between FtsZ protofilaments:**

Based on our findings, we propose a model in which the C-terminus of FtsZ plays a central role in mediating interactions between FtsZ protofilaments. Specifically, we envision the FtsZ subunit as a “bucket truck” or “cherry picker” in which the linker serves a function analogous to the boom and the CTT a function analogous to the bucket. During FtsZ assembly, the flexible linker would provide the CTT access to subunits on adjacent protofilaments. Interactions between the CTV, and alternatively modulatory proteins bound to the CTV, and the core of adjacent subunits would then function to either promote or inhibit lateral interactions, stabilizing or inhibiting FtsZ assembly as appropriate.
This model is particularly appealing with regard to our understanding of the activity of various FtsZ modulatory proteins. For example, a bundling protein such as ZipA from *E. coli* would use the CTT as the equivalent of the bucket on a “cherry picker” enhancing lateral interactions either through charge shielding or through direct interactions between ZipA and monomers on adjacent protofilaments. Conversely, an inhibitor such as EzrA from *B. subtilis* could use its interactions with the CTT terminus to disrupt interaction between the CTV and the core. In the case of EzrA, such an activity would be consistent with both the high degree of bundling observed for *B. subtilis* FtsZ in vitro as well as data suggesting EzrA is unable to inhibit assembly of *B. subtilis* FtsZ missing 17 C-terminal residues (Singh et al., 2007).

In summary, we have defined a new role for FtsZ’s C-terminus that complements its previously identified function as an interaction site for modulatory proteins. Studies of the in vitro assembly properties of FtsZs from various species suggest that some (e.g. *B. subtilis*, *Mycobacterium tuberculosis*) (Chen et al., 2007; Monahan et al., 2009) bundle to a significantly greater degree than others (e.g. *E. coli*, *C. crescentus*) (Erickson et al., 2010; Goley et al., 2010). It will be exciting to determine the impact of the CTV from various species of FtsZ on assembly. For example, *S. aureus* FtsZ, with the highly positively charged CTV sequence NREERRSRRRTRRR, would be expected to display a high degree of bundling. Conversely, species of *Actinobacteria* without CTV sequences (Fig. 1B) would be expected to be poor bundlers. Interestingly, the two extreme C-terminal arginines of *M. tuberculosis* FtsZ have been shown to be required for assembly in vitro (Gupta et al., 2010). It will also be attractive to identify the conserved residues on the FtsZ core involved in stabilizing lateral interactions with the CTT. Finally, this work also highlights the need to characterize the role of the poorly conserved linker in modulating FtsZ assembly dynamics.
Experimental Procedures

General methods and strain construction:

All *B. subtilis* strains are derivatives of the strain JH642 (Perego et al., 1988). Cloning and genetic manipulation were performed using standard techniques (Harwood and Cutting, 1990; Sambrook and Russell, 2001). All cloning was done using the *E. coli* strain AG1111 (Ireton et al., 1993), with the exception that *B. subtilis ftsZ* vectors were cloned in the AG1111 derivative PL930. PL930 contains the low copy plasmid pBS58 expressing *E. coli ftsQAZ*, which facilitates cloning of *B. subtilis* FtsZ (Wang and Lutkenhaus, 1993). Vent DNA polymerase was used for PCRs (NEB).

Cells were grown in Luria-Bertani (LB) medium at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin = 100 µg ml\(^{-1}\), spectinomycin = 100 µg ml\(^{-1}\), tetracycline = 12.5 µg ml\(^{-1}\), chloramphenicol = 5 µg ml\(^{-1}\).

Strains and plasmids used in this study are listed in Table 1. A list of primers used with restriction sites can be found in supplemental Table 2. The plasmids pPJ1-12 were constructed first by PCR amplifying the inserts from genomic *B. subtilis* (strain JH642) or *E. coli* (strain MG1655) DNA. Plasmids pPJ1-8 were constructed by ligating restriction-digested insert into digested pET-21b(+). pPJ9-12 were constructed by ligating restriction-digested insert into the digested pDR67 plasmid. pPJ11-12 plasmids were constructed with forward primers containing the *B. subtilis* ribosome binding site.

For construction of the congenic *B. subtilis* strains PL3188 (*ftsZ::ftsZ*) and PL3189 (*ftsZ::ftsZ CTVE*) which express wild-type *ftsZ* and *Bs ftsZ CTVE* from the native *ftsZ* promoter, *B. subtilis ftsZ* or *ftsZ CTVE* genes with *NdeI/XhoI* (NEB) linkers were amplified from the pPJ1 or pPJ5 plasmids, digested with *PstI* and *XhoI* and ligated into pJL74 (LeDeaux and
Grossman, 1995). The resulting plasmids were transformed into JH642, selecting for single
crossover recombination events at the native \textit{ftsZ} locus by growth on spectinomycin-containing
media. Mutations were confirmed by plasmid sequencing. Integration was confirmed by
chromosomal \textit{ftsZ} locus sequencing.

\textit{B. subtilis} strains PL3176 and PL3178, which permit depletion of wild-type FtsZ and
expression of \textit{E. coli} FtsZ variants, were constructed by first transforming pPJ11 or pPJ12
plasmids into wild-type JH642 cells. This allowed the insertion of a copy of \textit{E. coli ftsZ} or \textit{E. coli}
\textit{ftsZ CTVB} with a \textit{B. subtilis} ribosome-binding site upstream of the start codon at the \textit{amyE} locus
under control of the P_{spac} promoter. Chromosomal DNA from the resulting strains was then
transformed into competent PL2084 cells (\textit{JH642 thrC::P_{xyl-ftsZ_{Bsp}} ftsZ_{Bsp}::spc, xylA::tet}), which
have their only copy of native FtsZ expressed from a xylose inducible promoter at the \textit{thrC}
locus. This strain permits depletion of wild-type FtsZ and is xylose dependent for normal growth
(Weart and Levin, 2003).

\textbf{Growth Conditions:}

\textit{(i)} \textit{B. subtilis} congenic strains PL3188 and PL3189 were grown in LB containing
spectinomycin from a single colony overnight and then back-diluted at a 1:100 ratio in fresh LB
the next day. Cells were grown to an optical density at 600 nm (OD$_{600}$) \sim 0.5, then back diluted
again at a 1:100 ratio in fresh LB and grown until an OD$_{600}$ \sim 0.4, at which point cells were
harvested for analysis via immunofluorescence.

\textit{(ii)} \textit{B. subtilis} strains PL3176 and PL3178 expressing \textit{E. coli} FtsZ variants were first
grown from single colony overnight in LB containing spectinomycin and xylose to a final
concentration of 0.5\%. The next day, cells were diluted 1:100 in fresh LB medium supplemented
with 0.5% xylose and grown to an OD$_{600}$ ~0.5 at which point 1 mL of cell culture was harvested by centrifugation. Cells were then washed 2X with a fresh 1 mL of LB medium and then diluted 1:100 into a fresh 10 mL LB medium plus IPTG to a final concentration of 1 mM to induce expression of *E. coli* FtsZs. OD$_{600}$ measures were taken every 1 hour to monitor cell growth and at an OD$_{600}$ ~0.4, cells were harvested and prepared for immunofluorescence.

**Dilution plating:**

Strain PL2452 (*MG1655 leu::Tn10-ftsZ84 (ts)*) transformed with the plasmids pPJ9-12 was used for dilution plating. Cells were grown overnight in LB medium containing ampicillin at 30°C and the following morning cultures were diluted 1:100 into a fresh of 5 mL low-salt (LB with 0.05% NaCl) medium. Cultures were grown at 30°C until OD$_{600}$ ~0.5 upon which serial dilutions from $10^{-1}$ to $10^{-8}$ were made in low-salt medium. 10 µl of each dilution was then plated in series using a multichannel pipette onto either pre-warmed LB agar or low salt agar with or without 1mM IPTG. Liquid cultures were allowed to dry on the plates at room temperature for upwards of 6 hours and then plates were incubated at either the permissive (30°C) or restrictive (42°C) temperatures overnight. The plates were then imaged and relative growth at each temperature qualitatively assessed.

**Immunofluorescence microscopy:**

Immunofluorescence was performed as previously described (Haeusser et al., 2007). An Olympus BX51 microscope with Chroma filters and a Hamamatsu OrcaERG camera were used for image capture. Images were processed using Openlab version 5.2.2 (Improvision) and Adobe
Photoshop CS version 8.0 (Adobe Systems). All cell or ring measurements for collected images were obtained with a minimum population of 200 cells/strain.

*B. subtilis* FtsZ was detected using affinity-purified polyclonal rabbit anti-FtsZ sera (Levin and Losick, 1996) in combination with goat anti-rabbit sera conjugated to Alexa488 (Invitrogen). *E. coli* FtsZ was detected using affinity-purified polyclonal rabbit anti-FtsZ, a gift kindly provided by the William Margolin lab, in combination with goat anti-rabbit sera conjugated to Alexa488 (Invitrogen). Cell walls were visualized with wheat germ agglutinin conjugated to tetramethylrhodamine (Invitrogen). Nucleoids were stained with DAPI.

**Determination of Cell Length to FtsZ ring Ratio:**

Cell length to FtsZ ring (L/R) ratio was calculated as the sum total length of a population of cells divided by the number of FtsZ rings in that population as described previously (Weart et al., 2007).

**Quantitative Immunoblotting:**

Immunoblotting was done as previously described (Weart and Levin, 2003). Briefly, *B. subtilis* cell samples were washed with TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and resuspended in lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl). Cells were lysed with lysozyme and detergent and loading was normalized to the OD$_{600}$ at sampling. Immunoblot analysis was performed by using affinity-purified polyclonal rabbit anti-FtsZ antibodies (Levin and Losick, 1996) and goat anti-rabbit antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories.). Immunoblots were developed by using ECL Western Blotting Detection Reagents (GE Healthcare) and visualized with the luminescent
image analyzer ImageQuant LAS 4000 mini (GE Healthcare). Blot density was then quantified using ImageJ and the linear range for the signal was established from serial dilutions of whole-cell lysates.

**Protein Purification:**

FtsZ variants were cloned into the pET-21b(+) expression vector through *E. coli* strain AG1111. The resulting plasmids were mini-prepped and freshly transformed into C41(DE3) cells (Miroux and Walker, 1996) and consequently used for expression of protein. No frozen stocks were used. Briefly, 1 L of LB medium was inoculated 1:100 with overnight culture from single colony. Cells were grown at 37°C until OD_	ext{600} \sim 0.6, then cells were induced with isopropyl-1-thio-β-d-galactopyranoside (IPTG) to a final concentration of 1 mM. Cells were grown for an additional 4 hours at 37°C, and then cells were harvested by centrifugation and cell pellets stored at -80°C.

Purification of FtsZs were performed as previously described (31,35) with the following modifications: After precipitation of FtsZ with ammonium sulfate, FtsZ pellets were re-suspended in 50 mL FtsZ anion-exchange buffer (low salt) (50 mM Tris, pH 8.5, 50 mM KCl, 1mM EGTA, 10% sucrose). The re-suspended FtsZ was then filtered through a 0.45 µm nylon pore membrane. The FtsZ was further purified on a UnoQ6 (Bio-Rad) with a linear gradient of 50 to 500 mM KCl in 50 mM Tris, pH 8.5, 1 mM EDTA, 10% sucrose. Peak fractions were analyzed by SDS-PAGE, pooled together and dialyzed overnight in 1 L FtsZ dialysis buffer pH 7.5 (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 50 mM KCl, 2.5 mM MgCl₂,1 mM EGTA, 10% sucrose). Protein preparations were concentrated using PEG-12,000, aliquoted, flash frozen on liquid N₂, and stored at -80°C. Bs FtsZ NENDEG, which has a
negatively charged CTV, was precipitated with a single 30% ammonium sulfate cut instead of the 30%/35% cuts used for all other *B. subtilis* FtsZ constructs.

**90° Angle Light Scattering Assay:**

Light scattering experiments were performed essentially as described earlier (Haeusser et al., 2007) using a DM-45 spectrofluorimeter (Olis). Readings were taken every 0.25 s at 30°C and a baseline was established for 1 min prior to addition of 1 mM GTP. Assembly reactions contained 5 µM FtsZ in assembly buffer (50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, 50 mM KCl, 2.5 mM MgCl₂, 1 mM EGTA). Data was collected by SpectralWorks (Olis) and exported into Microsoft Excel for processing and compiled in Kaleidagraph.

**Electron Microscopy:**

Electron microscopy was performed essentially as described (Weart et al., 2007). 3 µM FtsZ was used as it was found to be the best concentration in which to visualize single FtsZ filaments. FtsZ was assembled with GTP as for light scattering, and samples were visualized using a JEOL 1200EX transmission electron microscope. FtsZ filament lengths were measured using ImageJ and data compiled in Microsoft Excel and Kaleidagraph.

**GTPase assay:**

GTPase activity was measured using the continuous, regenerative coupled GTPase assay of Ingerman and Nunnari (Ingerman and Nunnari, 2005). Assays were conducted in buffer conditions identical to those used for light scattering. A 150 µL reaction volume included 5 µM FtsZ, 1 mM GTP, 1 mM phosphoenolpyruvate, 250 µM NADH, 80 units/ml lactose
dehydrogenase, and 80 units/ml pyruvate kinase. A linear decline of absorbance at 340 nm for NADH was observed at 30°C for 3 minutes in a quartz cuvette (1 cm pathlength) using a SPECTRAmax Plus spectrophotometer (Molecular Devices). The raw data of absorbance per minute was then converted activity using the extinction coefficient for NADH at 340 nm of 6220 M⁻¹ cm⁻¹. The raw data was then exported to Microsoft Excel for analysis. GTPase data are the average of at least 3 independent experiments.

Statistical Analyses:

Statistical significance was assessed with either unpaired t test (Graphpad Quick Calcs, http://www.graphpad.com/quickcalcs/index.cfm) or one-way ANOVA (http://faculty.vassar.edu/lowry/anova1u.html). Results were significant if the p value was less than 0.05.
References


Wang, X., and Lutkenhaus, J. (1993). The FtsZ protein of Bacillus subtilis is localized at the division site and has GTPase activity that is dependent upon FtsZ concentration. Mol. Microbiol. 9, 435-442.


Acknowledgements

The authors would like to thank Bill Margolin for the generous gift of antisera against *E. coli* FtsZ. We also thank Wandy Beatty for assistance with electron microscopy. We are indebted to Laura Romberg, Daniel Haeusser, and Corey Westfall for valuable comments on the manuscript, and to members of the Levin lab for their technical advice, lively discussions, and support. This work was supported by NIH Public Health Service grant GM64671 to PAL.
List of Abbreviations

CTL: C-terminal linker
CTT: C-terminal tail
CTV: C-terminal variable region
CTVB: C-terminal variable region from \textit{B. subtilis}
CTVE: C-terminal variable region from \textit{E. coli}
### Table 1

Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH642</td>
<td><em>B. subtilis</em> trpC2 pheA1</td>
<td>(Perego et al., 1988)</td>
</tr>
<tr>
<td>PL2084</td>
<td>JH642* thrC::*P&lt;sub&gt;xyl&lt;/sub&gt;*ftsZ&lt;sub&gt; Bs &lt;/sub&gt;, *ftsZ&lt;sub&gt; Bs &lt;/sub&gt;::*spc, <em>xylA::tet</em></td>
<td>(Weart and Levin, 2003)</td>
</tr>
<tr>
<td>PL3176</td>
<td>PL 2084* amyE::*P&lt;sub&gt;spac&lt;/sub&gt; -ftsZ&lt;sub&gt; Ec &lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PL3178</td>
<td>PL 2084* amyE::*P&lt;sub&gt;spac&lt;/sub&gt; -ftsZCTVB&lt;sub&gt; Ec &lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PL3188</td>
<td>JH642* ftsZ::ftsZ</td>
<td>This study</td>
</tr>
<tr>
<td>PL3189</td>
<td>JH642* ftsZ::ftsZCTVE&lt;sub&gt; Bs &lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td><em>F·λ·ivG· rfb·50 rph·1</em></td>
<td></td>
</tr>
<tr>
<td>AG1111</td>
<td>DZR200- MC1061* F&lt;sup&gt;lacIQ&lt;/sup&gt; lacZM15 Tn10 (tet)</td>
<td>(Ireton et al; 1993)</td>
</tr>
<tr>
<td>C41 (DE3)</td>
<td><em>F·ompT·hsdSB (rB·mB·)·gal·dcm (DE3)</em></td>
<td>(Miroux and Walker, 1996)</td>
</tr>
<tr>
<td>PL930</td>
<td>AG111 + pB5S8</td>
<td>(Weart and Levin, 2003)</td>
</tr>
<tr>
<td>PL2452</td>
<td>MGI655* leu::Tn10-ftsZ84 (ts)</td>
<td>Lab Stock</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPJ1</td>
<td>pET-21b(+) -ftsZ&lt;sub&gt; Bs &lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ2</td>
<td>pET-21b(+) -ftsZ&lt;sub&gt; Ec &lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ3</td>
<td>pET-21b(+) -ftsZΔC17&lt;sub&gt; Bs &lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ4</td>
<td>pET-21b(+) -ftsZΔC15&lt;sub&gt; Ec &lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ5</td>
<td>pET-21b(+) -ftsZCTVE&lt;sub&gt; Bs &lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ6</td>
<td>pET-21b(+)-ftsZTVBE&lt;sub&gt;E&lt;/sub&gt;&lt;sub&gt;c&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>pPJ7</td>
<td>pET-21b(+)-ftsZNKNRKG&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ8</td>
<td>pET-21b(+)-ftsZNENDEG&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ9</td>
<td>pDR67-ftsZ&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ10</td>
<td>pDR67-ftsZTVEB&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ11</td>
<td>pDR67-ftsZ&lt;sub&gt;E&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ12</td>
<td>pDR67-ftsZTVBE&lt;sub&gt;E&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ13</td>
<td>pJL74-ftsZ&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ14</td>
<td>pJL74-ftsZTVEB&lt;sub&gt;Bs&lt;/sub&gt; stop</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2

**FtsZ GTPase Turnover Rates**

GTPase assays were carried out under conditions identical to those used for light scattering and EM (50 mM MES pH 6.5, 50 mM KCl, 2.5 mM MgCl$_2$, 1 mM EGTA, 1 mM GTP, 30°C). Rates were calculated as GTP consumed per FtsZ subunit per minute. Observed FtsZ structures (by EM) are noted in the right column.

<table>
<thead>
<tr>
<th>FtsZ species</th>
<th>GTP FtsZ$^{-1}$ min$^{-1}$</th>
<th>Relative Bundling at pH 6.5, 50 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec FtsZ WT</td>
<td>5.40 ± 0.39</td>
<td>Single filaments</td>
</tr>
<tr>
<td>Ec FtsZ ΔC15</td>
<td>4.76 ± 0.29</td>
<td>Single filaments</td>
</tr>
<tr>
<td>Bs FtsZ NENDEG</td>
<td>5.85 ± 0.29</td>
<td>Single filaments</td>
</tr>
<tr>
<td>Bs FtsZ ΔC17</td>
<td>3.86 ± 0.19</td>
<td>Single filaments</td>
</tr>
<tr>
<td>Bs FtsZ CTVE</td>
<td>3.86 ± 0.19</td>
<td>Single filaments</td>
</tr>
<tr>
<td>Bs FtsZ NKNRKG</td>
<td>2.32 ± 0.19</td>
<td>Bundled filament sheets</td>
</tr>
<tr>
<td>Bs FtsZ WT</td>
<td>2.25 ± 0.40</td>
<td>Filaments sheets and bundled rings</td>
</tr>
<tr>
<td>Ec FtsZ CTVB</td>
<td>2.19 ± 0.29</td>
<td>Long filaments, bundles and rings</td>
</tr>
</tbody>
</table>
Figure 1

FtsZ domain structure, C-terminal alignments, and protein constructs.

A. Structure of *B. subtilis* FtsZ showing the N-terminal core in maroon bound to GDP (from PDB entry 2RHO (Raymond et al., 2009)). The flexible C-terminal linker (red) is depicted here as an unstructured peptide. The conserved C-terminal tail (CTT), in blue, is depicted as a short α helix based on the structure of the *E. coli* CTT in complex with ZipA (Mosyak et al., 2000). B. Alignment of the FtsZ C-termini from 15 different organisms. Conserved in all organisms, a proline (*B. subtilis* position 372) and phenylalanine (*B. subtilis* position 374) are highlighted in yellow. The C-terminal variable region (CTV) is boxed. C. Schematic of the different FtsZ constructs used in this study, drawn to scale.
Figure 2

The *B. subtilis* FtsZ C-terminus is required for lateral interactions *in vitro*.

A-E. Electron micrographs of 3µM FtsZ assembled in the presence of GTP. Bar = 100 nm. A. Bundled rings of Bs FtsZ. B. Ordered sheets of Bs FtsZ filaments on the same grid as A. C. In the presence of GTP Ec FtsZ assembles into more or less randomly positioned single stranded filaments. D. In contrast to full length Bs FtsZ, Bs FtsZ ΔC17 assembles into predominantly single stranded filaments. E. Ec FtsZ ΔC15 assembles into predominantly single filaments that are indistinguishable from full length Ec FtsZ. F. Average filament lengths. Bs FtsZ ΔC17 (black-diagonal stripes) and Ec FtsZ ΔC15 filaments (black horizontal bars), 107.26 ± 37.84 nm and 182.35 ± 47.67 nm, respectively, are significantly shorter than full length Bs FtsZ (solid black) and Ec FtsZ (solid gray), 129.32 ± 36.33 nm and 218.88 ± 56.54 nm, respectively (p < 0.001). More than 100 filaments were measured for each FtsZ. Error bars represent the standard deviation from the mean. G. Representative traces of 5 μM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. Bs FtsZ ΔC17 and Ec FtsZ show ~90 and ~23 fold decreases in light scattering signal relative to Bs FtsZ, respectively. The Ec FtsZ ΔC15 signal is reduced by 17% compared to the wild-type Ec FtsZ. H. Same plot as in G but scaled to highlight GTP-dependent increase in light scattering of Ec FtsZ, Bs FtsZ ΔC17, and Ec FtsZ ΔC15. Note: Buffer conditions for all *in vitro* work are: 50 mM MES pH 6.5, 50 mM KCl, 2.5 mM MgCl₂, 1 mM EGTA, 1 mM GTP. FtsZ was used at a concentration of 3 μM for all electron microscopy to facilitate visualization of single stranded polymers. For 90° angle light scattering and GTPase assays FtsZ concentration is always 5 μM.
Figure 3

The *B. subtilis* CTV is sufficient to promote lateral interactions *in vitro*.

A-C. Electron micrographs of Bs FtsZ CTVE and Ec FtsZ CTVB assembled in the presence of GTP. Bar = 100 nm. A. Bs FtsZ CTVE forms predominantly short single stranded filaments with little evidence of lateral interactions. B. Ec FtsZ CTVB forms large sheets consistent with extensive lateral interactions. C. Ec FtsZ CTVB filament bundle. D. Average filament lengths of Bs FtsZ CTVE (diagonal black stripes) and Ec FtsZ CTVB (horizontal black stripes), 95.92 ± 22.16 nm and 277.58 ± 49.40 nm, respectively, are significantly shorter than wild-type Bs FtsZ (solid black) and Ec FtsZ (solid gray), 129.32 ± 36.33 nm and 218.88 ± 56.54 nm, respectively (p < 0.001). More than 100 filaments were measured for each FtsZ. Error bars represent the standard deviation from the mean. E. Representative traces of 5 μM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. Note 60-fold decrease in light scattering signal of Bs FtsZ CTVE relative to wild-type Bs FtsZ and 30-fold increase in signal of Ec FtsZ CTVB relative to wild-type Ec FtsZ.
Figure 4

Bs FtsZ CTV charge appears to be the primary determinant of lateral interaction potential.

*In vitro* assembly of *B. subtilis* FtsZ NKNRKG (positively charged CTV) and *B. subtilis* FtsZ NENDEG (negatively charged CTV). A and B. Electron micrographs of 3μM Bs FtsZ NKNRKG and Bs FtsZ NENDEG. Bar = 100 nm. A. Bs FtsZ NKNRKG assembled into filament bundles running across the width of the field. Note ordered sheet visible in bottom right corner. B. Single filaments of Bs FtsZ NENDEG. C. The average filament lengths of Bs FtsZ NKNRKG (solid gray) are similar to wild-type Bs FtsZ (solid black) (133.67 ± 37.42 nm versus 129.32 ± 36.33 nm), while Bs FtsZ NENDEG (black stripes) filaments measure 176.00 ± 51.64 nm are significantly longer (one-way ANOVA analysis with α = 0.001). More than 100 filaments measured per FtsZ. Error bars represent the standard deviation from the mean. D. Representative traces of 5 μM FtsZ assembled in presence of GTP and measured by 90° angle light scattering.
Figure 5

The FtsZ CTV plays a critical role in maintenance of FtsZ ring integrity in vivo.

A. Immunofluorescence microscopy of cells expressing Bs ftsZ (top) and Bs ftsZ CTVE (bottom). Bs FtsZ CTVE assembles into rings at midcell (arrows) and supports division. B. ~31% (90/290) of Bs ftsZ CTVE mutants were filamentous (>5 cell lengths) consistent with a reduction in FtsZ ring integrity. Arrow highlights Z-ring within filament of cells still occur. C. The length to FtsZ ring (L/R) ratio of Bs FtsZ CTVE cells (gray bar) is ~75% higher than that of Bs FtsZ WT cells (black bar) (p < 0.001) Error bars represent the standard deviation from the mean. The Bs ftsZ CTVE error bar is large reflecting the high heterogeneity of cell lengths in the population. D. Bs ftsZ CTVE cells (gray bar) are ~85% longer than congenic Bs ftsZ WT cells (black bar) (p < 0.001). Error bars represent the standard deviation from the mean. E. Neither Ec FtsZ and Ec FtsZ CTVB support division in B. subtilis cells depleted for wild-type FtsZ. Note punctate localization of Ec FtsZs, indicating failure to localize to midcell. Bar = 5 µm.
## Supplemental Table 1

### FtsZ Point Mutants that Abolish Modulator or Lateral Interactions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZipA (E. coli)</td>
<td>I374A, F377A</td>
<td>C-terminal tail</td>
<td>Ma and Margolin, 1997</td>
</tr>
<tr>
<td>FtsA (S. aureus)</td>
<td>F376A</td>
<td>C-terminal tail</td>
<td>Yan et al., 2000</td>
</tr>
<tr>
<td>MinC (E. coli)</td>
<td>D373E, I374V, L378V, K380M</td>
<td>C-terminal tail</td>
<td>Shen and Lutkenhaus, 2009</td>
</tr>
<tr>
<td>MinC (E. coli)</td>
<td>N280D</td>
<td>Core</td>
<td>Shen and Lutkenhaus, 2010</td>
</tr>
<tr>
<td>MinC (B. subtilis)</td>
<td>S219L, A285T, L302P &amp; Q353R</td>
<td>Core</td>
<td>de Oliveira et al., 2010</td>
</tr>
</tbody>
</table>

### FtsZ Lateral Interaction

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutation</th>
<th>Location</th>
<th>Assembly Defect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>D86K</td>
<td>Core</td>
<td>cannot complement fisZ84 mutant cells, twined protofilaments and tubes, GTPase 49% of WT</td>
<td>Lu et al., 2001</td>
</tr>
<tr>
<td>E. coli</td>
<td>R174D</td>
<td>Core</td>
<td>Able to localize to midcell, but not support cell division; in vitro cannot bundle in presence of CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Koppelman et al., 2004</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>A240V</td>
<td>Core</td>
<td>Does not bundle at high temperature (35°C)</td>
<td>Monahan et al., 2009</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>S219L</td>
<td>Core</td>
<td>GTPase lowered by ~30%, no lateral interactions by EM and LS</td>
<td>de Oliveira et al., 2010</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>A285T</td>
<td>Core</td>
<td>No GTPase activity, no assembly by EM or LS</td>
<td>de Oliveira et al., 2010</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>L302P &amp; Q353R</td>
<td>Core</td>
<td>GTPase lowered by ~50%, no lateral interactions by EM and LS</td>
<td>de Oliveira et al., 2010</td>
</tr>
<tr>
<td>Name</td>
<td>Sequence (5’-3’)</td>
<td>Site</td>
<td>Plasmids</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------------------------------------</td>
<td>--------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZFwd</td>
<td>GATCGATCC<strong>CATATG</strong>TTGGA GTTCGAAAACAAACATAGAG</td>
<td>NdeI</td>
<td>pPJ1, pPJ3, pPJ5, pPJ13, pPJ14</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZRev</td>
<td>GATCAGATCTCGATTTTGT CTTTACATTAGCCCGG</td>
<td>BamHI</td>
<td>pPJ1</td>
<td></td>
</tr>
<tr>
<td>PJB EcFtsZFwd</td>
<td>GATCGATCC<strong>CATATG</strong>CGGAG AGAAACTATGTTGAACCA</td>
<td>NdeI</td>
<td>pPJ2, pPJ4, pPJ6</td>
<td></td>
</tr>
<tr>
<td>PJB EcFtsZRev</td>
<td>GATCAGATCTCCAAATTCC AGTCAATTCTTAATCAGC</td>
<td>BamHI</td>
<td>pPJ2</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZΔC17R</td>
<td>GTCGGGATCCATTAAGCGG GCCTGTGAAGTATGACG</td>
<td>BamHI</td>
<td>pPJ3</td>
<td></td>
</tr>
<tr>
<td>PJB EcFtsZΔC15R</td>
<td>GCATGGATCCTTACTCTTT CGCAGTTTGCAGG</td>
<td>BamHI</td>
<td>pPJ4</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZCTVERev</td>
<td>GATCCCTCGAGACATTAATC AGCTTGCTTTCTTAAGATG TCGGGA</td>
<td>XhoI</td>
<td>pPJ5, pPJ14</td>
<td></td>
</tr>
<tr>
<td>PJB EcFtsZCTVBRev</td>
<td>GATCCCTCGAG CAATTTTAAAGCCGCCTTTAT TACGTTACGAGAGATGT</td>
<td>XhoI</td>
<td>pPJ6</td>
<td></td>
</tr>
<tr>
<td>PJBBsNKNRKGRev</td>
<td>GATCCCTCGAGACATTAGCC CTTACGATTTTTGTCTTAA AGAATGTCG</td>
<td>XhoI</td>
<td>pPJ7</td>
<td></td>
</tr>
<tr>
<td>PJBBsNENDEGRev</td>
<td>CATGCTCGAGTGGCTTCTTA CATTAGCCCTCATATTCTCAGTGGATTC</td>
<td>XhoI</td>
<td>pPJ8</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZH3Fwd</td>
<td>GATCAAAGCTCTATTAAGC AGTTTTTGGGAGATAG</td>
<td>HindIII</td>
<td>pPJ9, pPJ10</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZB2Rev</td>
<td>GATCAGATCTCGATTTTGT CTTTACATTAGC</td>
<td>BglII</td>
<td>pPJ9</td>
<td></td>
</tr>
<tr>
<td>Construct</td>
<td>Sequence</td>
<td>Restriction Enzyme</td>
<td>Plasmid</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------------------</td>
<td>-------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZCTVES1R</td>
<td>GATCGAGCATGC ACATTAATCAGCTTGCTTTCTTAAGAATGTCCGGGA</td>
<td>SphI</td>
<td>pPJ10</td>
<td></td>
</tr>
<tr>
<td>PJBEcFtsZH3Fwd</td>
<td>GATCAAGCTTCAAAAAGGAGGAGAAAACATATGTTTGAAACCAATGGA</td>
<td>HindIII</td>
<td>pPJ11, pPJ12</td>
<td></td>
</tr>
<tr>
<td>PJBEcFtsZB2Rev</td>
<td>GATCAGATCTCCAAATTCACGATTTTCCTTAATCAGC</td>
<td>BglII</td>
<td>pPJ11</td>
<td></td>
</tr>
<tr>
<td>PJBEcFtsZCTVBB2R</td>
<td>GATCAGATCTCAATTCTTACCACGCTTTATTACGTTACGCAGGAATGCT</td>
<td>BglII</td>
<td>pPJ12</td>
<td></td>
</tr>
<tr>
<td>PJBBsFtsZRev2</td>
<td>GATCCCTCGAGCGATTTTGTCCCTACATTAGCCCGG</td>
<td>XhoI</td>
<td>pPJ13</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figure 1

Salt affects lateral filament interactions between FtsZ filaments.

A and B. Electron micrographs of 3µM FtsZ assembled in the presence of GTP in high salt assembly buffer (50 mM MES, pH 6.5, 250 mM KCl, 2.5 mM MgCl2, 1 mM EGTA, 1 mM GTP). Bar = 100 nm. A. Bs FtsZ forms predominantly single filaments at 250 mM KCl. B. Ec FtsZ also forms single filaments in the same high salt buffer. C. Representative traces of 5 µM FtsZ assembly as measured by 90° angle light scattering. Bundling of Bs FtsZ at 50 mM KCl (solid black) is evident by the large light scattering signal. At 250 mM KCl, Bs FtsZ (black dashed) shows a ~50 fold decrease in assembly relative to 50 mM KCl. Assembly of Ec FtsZ is not affected by KCl concentration as the light scattering signal at 50 mM KCl (gray small dashed) is nearly the same as 250 mM KCl (gray large dashed).
Supplemental Figure S2

Electron microscopy measurements.

A-C. The length distributions of filaments from electron micrographs of 3µM FtsZ assembled in the presence of assembly buffer (50 mM MES, pH 6.5, 250 mM KCl, 2.5 mM MgCl$_2$, 1 mM EGTA, 1 mM GTP). At least 100 filaments were measured for each FtsZ. A. Bs FtsZ and Bs FtsZ ΔC17 mostly measure shorter than Ec FtsZ and Ec FtsZ ΔC15 filaments. B. Bs FtsZ and Bs FtsZ CTVE filaments populate a smaller majority than Ec FtsZ. Ec FtsZ CTVB filaments are mostly longer than Ec FtsZ. C. Bs FtsZ and Bs FtsZ NKNRKG show a very similar size distribution while Bs FtsZ NENDEG filaments have a population that measures slightly longer. D. Average diameters of filament toroids and filament bundle widths and of Bs FtsZ versus Ec FtsZ CTVB.
Supplemental Figure S3

Expression Ec FtsZ and Ec FtsZ CTVB are able to rescue growth in a temperature sensitive background.

Ec FtsZ, Ec FtsZ CTVB, Bs FtsZ, and Bs FtsZ CTVE were cloned into the pDR67 plasmid and transformed into an *E. coli* strain with the temperature sensitive *ftsZ84* allele. Cells were grown to mid log at 30°C then serial dilutions made as labeled and plated on low salt agar plates. At permissive temperature (30°C, left), FtsZ84 is able to support cell growth in all cell backgrounds. At restrictive temperature (42°C, right) only cells expressing the Ec FtsZ and Ec FtsZ CTVB (upper) off the plasmid are able grow as at permissive temperature. Bs FtsZ and Bs FtsZ CTVE (lower) are not able to support growth in *E. coli*. 
Supplemental Figure S4

Quantitative Western blot of Bs \textit{ftsZ} WT (PL 3188) and Bs \textit{ftsZ} CTVE (PL 3189) \textit{in vivo} expression.

A. Lysates of cells expressing Bs FtsZ WT (PL3188) and Bs FtsZ CTVE (PL3189) were prepared and run on SDS-PAGE at different serial dilutions. Lanes 1 and 6 have dilution factor of 1. Lanes 2 and 7 have dilution factor of 2. Lanes 3 and 8 have dilution factor of 3. Lanes 4 and 9 have dilution factor of 4. Lanes 5 and 10 have dilution factor of 8. After transfer, blot was probed using α-Bs FtsZ antibody and developed. B. Plot of average band intensities after correcting for dilution factor for PL 3188 and PL 3189 cells show that FtsZ levels in each strain are not significantly changed.
Addendum

Chapter 2 was previously published in the Journal of Biological Chemistry (Buske and Levin, 2012).


The work in Chapter 2 was performed as follows:

1. All experiments designed and conceived by PJ Buske and Petra Levin.
2. PJ Buske performed all experiments and collected data.
3. PJ Buske and Petra Levin analyzed the data.
Chapter 3:

A flexible C-terminal linker is required for proper FtsZ assembly in vitro and cytokinetic ring formation in vivo

PJ Buske and Petra Anne Levin

Molecular Microbiology (2013)
Abstract

Assembly of the cytoskeletal protein FtsZ into a ring-like structure is required for bacterial cell division. Structurally, FtsZ consists of four domains: the globular N-terminal core, a flexible linker, 8-9 conserved residues implicated in interactions with modulatory proteins, and a highly variable set of 4-10 residues at its very C terminus. Largely ignored and distinguished by lack of primary sequence conservation, the linker is presumed to be intrinsically disordered. Here we employ genetics, biochemistry and cytology to dissect the role of the linker in FtsZ function. Data from chimeric FtsZs substituting the native linker with sequences from unrelated FtsZs as well as a helical sequence from human beta-catenin indicate that while variations in the primary sequence are well tolerated, an intrinsically disordered linker is essential for \textit{B. subtilis} FtsZ assembly. Linker lengths ranging from 25-100 residues supported FtsZ assembly, but replacing the \textit{B. subtilis} FtsZ linker with a 249-residue linker from \textit{A. tumefaciens} FtsZ interfered with cell division. Overall, our results support a model in which the linker acts as a flexible tether allowing FtsZ to associate with the membrane through a conserved C-terminal domain while simultaneously interacting with itself and modulatory proteins in the cytoplasm.
Introduction

The prokaryotic cytoskeletal protein FtsZ plays a central role in bacterial cell division. In rod-shaped bacteria such as *Bacillus subtilis* and *Escherichia coli*, FtsZ assembles into a ring-shaped structure (Z ring) at midcell that establishes the location of the future division site and serves as a platform for assembly of the division machinery. As cell division progresses, the Z ring constricts to begin separation of the newly forming daughter cells. The Z ring is required for recruitment of downstream cell division proteins, including those required for cross wall synthesis, to the nascent division site (for reviews see: (Adams and Errington, 2009; Kirkpatrick and Viollier, 2011; Lutkenhaus et al., 2012).

High-resolution microscopy suggests that the Z ring consists primarily of heterogeneously distributed short filaments (~100 nm in length) held together via lateral interactions (Fu et al., 2010; Strauss et al., 2012). FtsZ protofilaments are believed to associate with the membrane through interactions with FtsA and other, less widely conserved, cell division proteins. In addition, several studies have shown that FtsZ filaments targeted to the membrane in *in vitro* liposomes are able to generate visible distortions where FtsZ is bound and suggest the force being applied is solely through FtsZ (Osawa et al., 2009; 2008). Computational modeling suggests that GTP-hydrolysis-dependent bending of FtsZ filaments generates sufficient force to drive inward cell-wall growth during division (Hsin et al., 2012).

*In vitro*, FtsZ assembly into single stranded “protofilaments” is stimulated by GTP (Erickson et al., 2010; Romberg and Levin, 2003). FtsZ, a tubulin homolog, binds to GTP as a monomer. Polymerization leads to the formation of active sites for GTP hydrolysis at the interface between FtsZ subunits. FtsZ protofilaments are able to associate laterally to form filament bundles, spirals, tubes, and sheets depending on the buffer conditions. Lateral
interaction potential varies between FtsZs from different species and is dependent in part on the charge of the variable residues at the very C-terminus of the protein (Buske and Levin, 2012; Gueiros-Filho and Losick, 2002; Gündoğdu et al., 2011; Mukherjee and Lutkenhaus, 1999; Popp et al., 2009).

The FtsZ monomer is divided into 5 domains: an unstructured N-terminal peptide, a highly conserved globular core, an unstructured C-terminal linker (CTL), a conserved set of ~11 residues referred to here as the C-terminal constant region (CTC), and a small, highly variable group of residues at the extreme C-terminus of FtsZ termed the C-terminal variable region (CTV) (Buske and Levin, 2012; Erickson et al., 2010; Vaughan et al., 2004). For simplicity, we treat the N-terminal peptide and core as a single unit. The core contains residues required for GTP binding and hydrolysis as well as residues involved in longitudinal interactions between subunits (Lu et al., 2001; Redick et al., 2005). In vitro, the core has also been shown to be sufficient for filament formation and has also been implicated in protofilament bundling (Wang et al., 1997). The very C-terminus of FtsZ, a 15 residue region in E. coli FtsZ and a 17 residue region in B. subtilis FtsZ that includes both the CTC and the CTV, has been implicated in interactions between FtsZ and a host of modulatory proteins. Such proteins include FtsA, ZipA and MinC in E. coli and EzrA and SepF in B. subtilis (Haney et al., 2001; Król et al., 2012; Ma and Margolin, 1999; Mosyak et al., 2000; Shen and Lutkenhaus, 2009; Singh et al., 2007; Szwedziak et al., 2012; Yan et al., 2000). The CTV also appears to be a key determinant of lateral interaction potential. We have designated the region containing the CTC and CTV (residues 366-382 of B. subtilis FtsZ) the “grappling hook peptide” (GHP) to reflect its role in mediating interactions between modulatory proteins in both the cytoplasm and the plasma membrane.
While recent work has begun to clarify the role of the core, the CTC and the CTV in FtsZ assembly and Z-ring integrity, the function of the unstructured C-terminal linker has remained largely mysterious. The CTL spans the gap between the core and the CTC, suggesting it may be required to maintain a flexible connection between FtsZ protofilaments and modulatory proteins bound to the GHP of FtsZ subunits. Phylogenetic analysis of FtsZs across domains of life showed that there is little sequence conservation of the CTL even amongst bacteria from the same taxonomic phylum.

Intriguingly, while the length of the CTL can range 2 to 330 residues (Vaughan et al., 2004), it is ~50-100 residues long in the majority of FtsZs sequenced that contain all structural domains. An exception to this rule is FtsZs from the Alpha-Proteobacteria, in which linkers are significantly longer, between 119 amino acids in Rickettsia prowazeckii and 251 amino acids in Agrobacterium tumefaciens. Amongst the model organisms for bacterial cell division, the CTL of B. subtilis and E. coli is ~50 residues, while that of Caulobacter crescentus, a member of the Alpha-Proteobacteria, is 176. Notably, the CTL is irresolvable on crystal structures of bacterial FtsZ (Haydon et al., 2008; Leung et al., 2004; Läppchen et al., 2008; Matsui et al., 2012; Oliva et al., 2007; Raymond et al., 2009), and therefore has been presumed to be an intrinsically disordered peptide (IDP) (Erickson et al., 2010). Previous work suggests the CTL is flexible with a contour length of 17 nm, and an average end-to-end distance of 5.2 nm for the relaxed peptide (Ohashi et al., 2007).

Here we employ genetics, cell biology, and biochemistry to clarify the role of the FtsZ CTL. Our data indicate the CTL is required for protofilament assembly in vitro and formation of a curved ring in vivo, suggesting a role for the CTL in the geometry of longitudinal interactions between individual subunits. While changes in linker length were generally tolerated in vitro,
increases in length greater than 100 residues led to disruptions in the frequency and position of FtsZ assembly in vivo. Changes in the linker’s primary sequence had little impact on assembly in vivo or in vitro when retained as an IDP, whereas replacing the linker with an inflexible domain from human beta-catenin was not tolerated. Notably, our findings in B. subtilis closely mirror those from the Erickson laboratory working in E. coli (Gardner et al., 2013), who similarly determined CTL sequence to be unimportant while length being crucial. Taken together our findings have significant implications for our understanding of FtsZ assembly dynamics at the molecular level, as well as the role of various regions of FtsZ in assembly of a productive cytokinetic ring.
Results

The FtsZ linker is required for normal function in *B. subtilis*:

Deletion analysis indicated that the linker region of FtsZ was essential for efficient assembly *in vitro* and ring formation *in vivo*. As a first step towards assessing the role of the linker in FtsZ activity, we generated an FtsZ mutant in which the linker was deleted, leaving only the core followed immediately by the CTC and CTV domains (FtsZ ΔCTL50) (Fig. 1).

Transmission electron microscopy (TEM) of wild-type FtsZ assembled in our standard FtsZ polymerization buffer (50 mM MES, pH 6.5, 50 mm KCl, 2.5mM MgCl2, 1mM EGTA, 1mM GTP) indicated the present of filament bundles and sheets (Fig. 2A and B), consistent with our previous findings (Buske and Levin, 2012). In contrast, FtsZ ΔCTL50 appeared impaired for filament formation and filament bundling. By TEM we observed primarily short oligomers (<25 nm) interspersed amongst single- or double-stranded filaments of FtsZ ΔCTL50 5 minutes after initiation of polymerization by GTP (Fig. 2C). 15 minutes after addition of GTP, FtsZ ΔCTL50 filaments became more pronounced and longer, suggesting the rate of filament formation was delayed compared to wild type, though many small oligomers could still be observed also suggesting gross filament formation was diminished (Fig. 2D).

FtsZ ΔCTL50 assembly was GTP-dependent, as we observed a modest increase in 90° light scattering signal following the addition of 1 mM GTP (Fig. 2E). Here, as previously, we use light scattering to measure increase in polymer mass and FtsZ bundling (Buske and Levin, 2012; Mukherjee and Lutkenhaus, 1999), and define assembly as a global term to include head-to-tail polymerization into protofilaments and lateral interactions. Consistent with its limited assembly in TEM micrographs, the FtsZ ΔCTL50 light scattering signal was 37-fold lower than that of wild-type FtsZ in the presence of GTP. Similarly, the critical concentration (C_c) of FtsZ
ΔCTL50 was 1.08 μM compared to 0.68 μM for wild type, suggesting a reduction in cooperative assembly. We also observed that time needed to reach a peak light scattering value was on the order of minutes whereas wild-type FtsZ was on seconds. The delay in filament formation suggested that head-to-tail assembly of filaments was impaired without the FtsZ linker. The GTPase activity of FtsZ ΔCTL50 was 1.48 GTP FtsZ⁻¹ min⁻¹, significantly lower than the 3.82 GTP FtsZ⁻¹ min⁻¹ measured for wild type (Table 1). Notably, CD spectra indicated that FtsZ ΔCTL50 folded normally despite the loss of the linker, eliminating a trivial explanation for its defect in polymerization (Fig. S1).

In vivo, FtsZ ΔCTL50 was unable to support either FtsZ assembly or division. Taking advantage of a strain that allowed for depletion of wild type FtsZ, we expressed FtsZ ΔCTL50 as the sole copy of FtsZ in B. subtilis cells under the control of an isopropyl 1-thio-β-D-galactopyranoside (IPTG) - inducible promoter at the amyE locus (see “Experimental Procedures”). Expression FtsZ ΔCTL50 in the absence of wild-type FtsZ led to the rapid demise of cells: cell density as measured by absorbance at 600 nm (A₆₀₀) declined ~2-fold four hours after reaching a peak density of 0.4 following induction of FtsZ ΔCTL50 and depletion of wild-type FtsZ (Fig. 3A). It should be noted we were only able to observe growth when cells were grown in inducer at a starting A₆₀₀ twice that of all other constructs used in the study, suggesting that expression of FtsZ ΔCTL50 had an immediate and deleterious impact on cell viability.

Immunofluorescence microscopy of FtsZ ΔCTL50 cells 2.5 hours post induction in the absence of wild-type FtsZ supported the idea that the linker was required for proper FtsZ assembly and the formation of an intact cytokinetic ring. Cells were filamentous (> 80 μm, the length of our microscopy field) and FtsZ was mostly in punctate dots (88% of cells) throughout the cytoplasm in contrast to the Z rings of cells expressing wild-type FtsZ (Fig. 2F, arrow).
Strikingly, in 30% of cells FtsZ ΔCTL50 formed long and relatively straight filaments that extended longitudinally through the cell similar to what we observed *in vitro* and suggested defects in the geometry of FtsZ ΔCTL50 assembly. Co-expression of FtsZ ΔCTL50 with wild-type FtsZ resulted in a dominant lethal phenotype and loss of cell viability when assessed by dilution plating assay (Fig. S2).

Together, these findings suggested the linker played a critical role in FtsZ filament assembly counter to previous reports indicating that *E. coli* FtsZ mutants lacking the entire CTR readily assemble *in vitro* (Wang et al., 1997; Yu and Margolin, 1997). In light of this discrepancy, we examined the ability of *B. subtilis* and *E. coli* FtsZ core constructs lacking the entire C-terminal region (CTR; *B. subtilis* residues 316-382) to assemble *in vitro* under our standard conditions (Fig. S4). Somewhat surprisingly, both the *B. subtilis* and *E. coli* core domains were defective in both protofilament formation and lateral interactions, counter to the earlier studies (Singh et al., 2007; Wang et al., 1997; Yu and Margolin, 1997). A potential explanation for this discrepancy may be buffer conditions: while earlier studies employed 10 mM MgCl$_2$ or CaCl$_2$, MgCl$_2$ was at 2.5 mM in our standard conditions. Divalent cations have been shown to promote the formation of stabilizing lateral interactions between protofilaments most likely through charge shielding of the core (Mukherjee and Lutkenhaus, 1999).

**FtsZ function is independent of its linker sequence:**

We next determined if the linear sequence of the FtsZ linker was critical for function. The linker exhibited little or no conservation between even closely related bacterial species, suggesting that its presence and potentially its flexible nature, but not its precise sequence, were critical for function. To explore this possibility we generated an FtsZ mutant in which the
sequence of linker was randomly scrambled (FtsZ CTLScr), keeping the same amino acids as wild-type B. subtilis FtsZ but changing their order. In addition we swapped the 50-residue B. subtilis linker with the equivalent 50 residues from the E. coli linker to yield a linker of 54 residues (CTLE) and with the first 46 amino acids of the 251 residue long linker from Agrobacterium tumefaciens FtsZ to yield a linker of 50 residues (CTLA50) (Fig. 1).

Our data suggested that linker sequence had a small impact on FtsZ assembly in vitro. FtsZ CTLScr showed no evidence of compromised protofilament assembly and readily formed single stranded protofilaments and small bundles 3-5 filaments thick as seen by TEM (Fig. 4B). In agreement with TEM data, the 90° light scattering assays of FtsZ CTLScr indicated robust, GTP-dependent assembly to ~60% that of FtsZ WT (Fig. 4E). This level of assembly was consistent with the smaller-than-wild-type bundles we observed by EM but significantly (95-98%) higher than that of FtsZ mutants that were incapable of forming lateral interactions (Buske and Levin, 2012). Evidence of bundling was also seen by a lowered GTPase rate of 2.68 GTP FtsZ⁻¹ min⁻¹. The Cₐ of CTLScr was 0.88 μM, similar to the wild-type value of 0.68 μM (Table 1).

When FtsZ CTLScr was expressed as the sole copy in B. subtilis, cells were able to divide normally and Z rings localized to midcell as wild type (Fig. 4F). Here, and in all cell types that displayed the ability to form Z rings, we employed measurement of the length to ring (L/R) ratio as proxy for cell size. As some cell types became filamentous, using interseptal distance underestimated the average length of cells in a given population due to an inability to distinguish between partial and completed septa in these longer cells. FtsZ CTLScr-expressing cells had an average 5.48 μm/Z ring compared to 5.82 μm/Z ring for wild type (Fig. 3B).
Replacing the native linker with heterologous sequences had modest impacts on FtsZ assembly, particularly lateral interactions between single stranded protofilaments. Both FtsZ CTLE and FtsZ CTLA50 readily assembled in the presence of GTP (Fig. 4C and D); however, the structures appeared to be primarily single stranded by TEM. Similarly, the light scattering signals for CTLE and CTLA50 were ~62 fold and 37-fold lower than FtsZ WT, respectively, consistent with a reduction in lateral interactions and the formation of higher order structures. Finally, the GTPase activity of CTLE and CTLA50 was also elevated relative to wild-type FtsZ, 5.04 GTP FtsZ$^{-1}$ min$^{-1}$ and 5.51 GTP FtsZ$^{-1}$ min$^{-1}$ for CTLE and CTLA50 (Table 1), a phenotype that is also consistent with a reduction in lateral interaction potential (Buske and Levin, 2012). The apparent C_c was near wild type for both chimeras.

In vivo, FtsZ CTLE and FtsZ CTLA50 all appeared to support normal division, despite their reduction in lateral interaction potential in vitro. Expression of FtsZ CTLE and FtsZ CTLA50 in the absence of wild-type FtsZ fully supported normal division and FtsZ localization (Fig. 4F) with near wild-type L/R ratios (Fig. 3B). Cells expressing these FtsZ chimeras displayed growth rates near that of wild type (Fig. 3A). Some irregular cell wall staining seen in these mutants we attributed to lysozyme treatment for IFM, as we observed similar staining in wild-type cells (Fig. S3A). The ability of these mutants to support normal division despite reduced lateral interaction potential was counter to our previous findings that ability to form lateral FtsZ interactions in vitro affected cell division in vivo with a CTV mutant (Buske and Levin, 2012). This finding will be addressed in the discussion.

Linker length is an important determinant for normal FtsZ function:
To determine the role of linker length in FtsZ function we generated FtsZ chimeras of different length and examined the impact of these changes on FtsZ assembly in vitro and in vivo. Reducing the length of the native FtsZ linker by deleting the last 25 residues (FtsZ ΔCTL25), significantly altered FtsZ assembly in vitro. Purified FtsZ ΔCTL25 formed short, irregular protofilaments with little evidence of lateral interactions (Fig. 5B). Consistent with reduced assembly, FtsZ ΔCTL25 assembly peaked at a level ~79 fold lower than wild-type FtsZ in 90° light scattering assays (Fig. 5E and F). FtsZ ΔCTL25 hydrolyzed GTP at a rate of 4.66 GTP FtsZ⁻¹ min⁻¹, higher than wild-type FtsZ and FtsZ CTLScr, presumably due to a reduction in stabilizing lateral interactions. Interestingly, the C_c for FtsZ ΔCTL25 was 0.30 μM, about half that for wild type (Table 1).

Surprisingly, despite significantly altering FtsZ assembly dynamics in vitro, reducing the length of the linker by 50% had little effect on FtsZ ring formation or division in vivo. Expression of FtsZ ΔCTL25 in B. subtilis fully supported Z-ring assembly and cell division. Cells were able to grow at a rate near that of wild type (Fig. 3A), and FtsZ ΔCTL25 assembled into apparently normal FtsZ rings that were appropriately localized to midcell (Fig. 5G). The L/R ratio of FtsZ ΔCTL25-expressing cells was also near wild type at 5.62 µm/Z ring (Fig. 3B).

In a complementary set of experiments we generated FtsZ chimeras with longer linkers by swapping the native linker sequence with either the entire linker (FtsZ CTLA249) from A. tumefaciens (FtsZ CTLA249) or the first 96 residues (FtsZ CTLA100) to make a total linker length of 100 residues (FtsZ CTLA100) (Fig. 1). Both FtsZ CTLA249 and FtsZ CTLA100 assemble into single-stranded protofilaments in the presence of GTP as observed by TEM (Fig. 5C and D). However, there were few, if any, bundles apparent in any field of view, suggesting a reduction in lateral interactions. Consistent with this view FtsZ CTLA249 and FtsZ CTLA100
exhibited ~43 and ~33 fold decreases in signal relative to wild-type FtsZ in 90° light scattering assays (Fig. 5E and F). The apparent critical concentrations for both chimeras were near wild type; FtsZ CTLA249 had a C_c of 0.80 μM and FtsZ CTLA100 a C_c of 0.78 μM. GTPase activities were 4.40 and 5.09 GTP FtsZ^{-1} min^{-1} for FtsZ CTLA249 and FtsZ CLTA100, respectively (Table 1).

Despite the near wild-type in vitro assembly dynamics of the two chimeras, only FtsZ CTLA100 was able to support division in vivo. Cells expressing FtsZ CTLA100 were essentially wild type with regard to growth rate, FtsZ ring assembly, and localization with a wild-type L/R ratio of 5.44 µm/Z ring (Fig. 3). In contrast, cells expressing FtsZ CTLA249 as their only copy of FtsZ were unable to divide and formed extremely long filaments with what appeared to be partial invaginations of the cell wall. Cell growth was significantly reduced as A_{600} reached a peak value of ~0.5 four hours post-induction (Fig. 3A). Cells expressing FtsZ CTLA249 were also not viable in a plating assay, though co-expression with wild-type FtsZ did restore some growth (Fig. S2B). Although FtsZ rings were present in FtsZ CTLA249 cells, their localization appeared somewhat irregular, with closely spaced rings frequently followed by longer lengths of cells with no rings at all (Fig. 5G). The L/R of FtsZ CTLA249 cells was 4.66 µm/Z ring, which was not statistically significantly different from cells expressing wild-type FtsZ (Fig. 3B).

The FtsZ linker must be flexible and unstructured:

To determine if linker flexibility and disorder was essential for FtsZ function, we replaced the linker from B. subtilis FtsZ with residues 398-455 from the human beta-catenin protein (FtsZ CTLH) (Fig. 1). This region of beta-catenin was selected both for its rigid nature — it encompasses ~ 4 of the 12 alpha-helical Armadillo repeats from the protein — and for its
neutral charge, to eliminate potentially interfering effects from electrostatic interactions (Xing et al., 2008).

Our data suggested that linker flexibility was critical for proper FtsZ function both in vitro and in vivo. We observed only small oligomers of FtsZ CTLH by TEM and there was only a negligible increase in signal following the addition of GTP to a 90° light scattering assay (~136 times lower than FtsZ WT) (Fig. 6B and C). Although it was unable to assemble to a significant degree, FtsZ CTLH hydrolyzed GTP at a rate of 0.71 GTP FtsZ\(^{-1}\) min\(^{-1}\). The apparent \(K_c\) for FtsZ CTLH was 0.70 \(\mu\)M (Table 1). Together, these findings suggested that FtsZ CTLH was still able to bind GTP but was significantly impaired for hydrolysis. We could not attribute assembly defects toward any large changes in FtsZ subunit secondary structure as CD spectra of FtsZ CTLH was nearly identical to that of wild type (Fig. S1).

Consistent with its impaired assembly in vitro, FtsZ CTLH was unable to support division in the absence of wild-type FtsZ (Fig. 6D, bottom panel). Cells expressing FtsZ CTLH in the absence of wild-type FtsZ were extremely filamentous and unable to divide. Immunofluorescence microscopy indicated that instead of forming a coherent ring, FtsZ CTLH localized in small punctae that were distributed randomly throughout the filaments (Fig. 6D, bottom panel). This punctate localization pattern was consistent with the inability of FtsZ CTLH to assemble in vitro (Fig. 6B). We also observed some crosswalls in cells that we attributed to remnants of previous cell divisions before wild-type FtsZ was depleted, as we saw similar crosswalls in cells in which no FtsZ was expressed (Fig. S3B). Expression of FtsZ CTLH also had a deleterious effect on growth as the growth curve reveals cells only reached a peak \(A_{600}\) of ~0.2 3 hours after induction followed by a slow 1.7-fold decrease in cell density over 4 hours.
(Fig. 3A). Co-expression with wild-type FtsZ was also dominant lethal as indicated by dilution plating (Fig. S2B).
Discussion

Previous studies of FtsZ have largely ignored its C-terminal linker, in large part because this region is irresolvable in crystal structures. Our data suggest the presence of a flexible and disordered C-terminal linker is critical for protofilament assembly and the architecture of the cytokinetic ring. At the same time, the specific sequence composition and/or length of the CTL appear to be less critical, with FtsZ assembly and Z-ring formation being largely wild type in *B. subtilis* expressing FtsZ variants with heterologous CTLs up to 100 residues in lengths. Below we discuss the implications of these data in the context of our current understanding of FtsZ assembly and the ultrastructure of the FtsZ ring.

An intrinsically disordered linker is essential for FtsZ assembly and Z-ring formation:

Somewhat unexpectedly, our data support a role for the flexible linker not only *in vivo*, but also in longitudinal interactions between FtsZ subunits *in vitro*. FtsZ ΔCTL50 exhibits a long delay in protofilament assembly (Fig. 2D and E) and an elevated critical concentration (Table 1), suggesting the linker is needed for efficient incorporation of subunits into a filament. Its delayed assembly dynamics provide a potential explanation for the long straight filaments formed by FtsZ ΔCTL50 *in vivo* (Fig. 2F), as do aberrant interactions with modulatory proteins via its C-terminal grappling hook peptide (GHP). At the same time, we cannot discount possibility that in the absence of the linker, the GHP itself sterically interferes with subunit interactions. A role for the unstructured linker in coordinating longitudinal interactions between FtsZ subunits assembly is supported by data indicating that a chimeric FtsZ in which the linker has been replaced with a helical repeat (FtsZ CTLH) is unable to assemble *in vitro* or *in vivo* (Fig. 6).
Conservation of an intrinsically disordered peptide:

The inability of the helical repeat to functionally replace the FtsZ linker suggests that the ability of the linker to behave as an intrinsically disordered peptide (IDP) is a key determinant of linker functionality. Regardless of primary sequence, our FtsZ CTLScr, FtsZ CTLE, and FtsZ CTLA50 chimeras support cell division (Fig. 4), and all maintain disorder when run through multiple secondary structure predictors (Ishida and Kinoshita, 2008; Rost et al., 2004). Importantly, the lack of sequence conservation and the ability of highly divergent linkers to substitute for one another in vivo strongly argue against the linker as a site for interaction with modulatory proteins.

Although disorder is a key feature of the functional FtsZ linker, there appear to be some constraints on its size in the cell as a large increase in linker length has a negative impact on B. subtilis FtsZ assembly in vivo. While FtsZ CTLA249 assembles into ring-like structures in vivo (Fig. 5G), Z-ring localization was abnormal (4.66 µm length-to-ring ratio) and cells were unable to constrict. Specifically, why FtsZ CTLA249 fails to support division in B. subtilis, despite its relatively normal in vitro assembly dynamics, is not clear. One possibility is the large linker interferes with interactions between FtsZ and modulatory proteins. Alternatively, the longer linker may provide too much freedom to FtsZ subunits, disrupting the ultrastructure of the FtsZ ring. It remains to be seen why such long linkers are present in FtsZs from the Alpha-Proteobacteria, where exceptionally long (119-330 amino acids) are the norm and where different sets of FtsZ-interacting proteins exists.
FtsZ linker charge impacts lateral filament interactions:

Our results reinforce previous work implicating C-terminal charge as an important determinant of lateral interactions between FtsZ protofilaments. While CTLE and CTLA50 have little impact on protofilament assembly and Z-ring formation, both carry net positive charges well below wild type (wild-type FtsZ linker net charge +4; CTLE and CTLA50 net charge of +1.5 and -0.3, respectively) and display reduced lateral interaction potential (Fig. 4 C-E). The FtsZ CTLScr chimera, which retains the same charge composition as wild-type FtsZ, readily forms bundled structures in vitro, although its light scattering signal is only half that of wild type (Fig. 4E), suggesting that the position of charge within the linker may also contribute to lateral interactions. These findings are consistent with data indicating that CTV charge, as well as buffer pH and salt concentration, have a strong impact on FtsZ’s lateral interaction potential, most likely by shielding negatively charged core domains on different protofilaments from one another (Buske and Levin, 2012; Pacheco-Gomez et al., 2011).

At the same time, the finding that FtsZ CTLE and CTLA50 are essentially wild type for localization and division, despite the absence of lateral interactions in vitro, is somewhat surprising based on our previous work suggesting that loss of lateral interaction potential, via changes in CTV charge, significantly undermines the integrity of the FtsZ ring (Buske and Levin, 2012). One explanation for the more severe phenotype of the CTV mutant (Bs FtsZ CTVE) is that the CTV, like the CTC, is required for interaction between FtsZ and a subset of modulatory proteins in vivo, and that loss of these interactions negatively impacts the stability of the FtsZ ring.

Alternatively, in vivo, CTV charge may be a more important determinant of lateral interaction potential than linker charge, either through a better ability to access adjacent
protofilaments or its proximity to the CTC and associated modulatory proteins. In support of the former, a recent crystal structure showed that the CTV was bound to FtsA from *Thermotoga maritima* (Szwedziak et al., 2012). Similarly, it was also recently reported that mutations in CTV residues 377, 378, 380 and 381 significantly reduce interactions between FtsZ and the bundling protein SepF in *B. subtilis* (Król et al., 2012). Monitoring cell division in cells expressing the different FtsZ CTL and CTV mutants in the absence of these modulatory proteins will help clarify such questions.

**The FtsZ linker is a flexible tether:**

We propose that the primary role of the linker is to allow FtsZ to be anchored in the membrane via interactions between its GHP and modulatory proteins. At the same time, the linker permits FtsZ filaments to maintain a sufficient distance from the membrane for protofilaments to form the curved structure necessary for assembly of the Z ring and division machinery needed for cytokinesis to proceed along the short axis of the cell, similar to what has been proposed previously (Erickson et al., 2010) (Fig. 7A). Based on the near-linear localization pattern of the FtsZ ∆CTL50 construct, linkerless FtsZ filaments instead remain in close proximity to the membrane unable to respond to its curvature in a manner that allows Z ring formation (Fig. 7B). A structural role for the linker as a flexible tether is consistent with our finding that relatively large changes in linker length are tolerated both for FtsZ assembly *in vitro* and Z-ring formation *in vivo*. FtsZ with linkers ranging in lengths from 25 residues up to 100 residues appear to be wild type for both assembly and cell division. The Erickson lab has reported a similar finding for *E. coli* FtsZ (Gardner et al., 2013).
A flexible linker may also be essential to allow FtsZ to adopt a conformation that allows it to position the CTV in a way that promotes lateral filament interactions, likely with the core of adjacent filaments and similar to what we previously proposed (Buske and Levin, 2012) (Fig. 7A). In the case of native \textit{B. subtilis} FtsZ, this can be accomplished in the absence of modulatory proteins due to its proclivity toward forming FtsZ bundles. In FtsZs where filaments do not bundle, the interaction between the GHP and modulatory proteins that promote bundling can be positioned near neighboring filaments through the linker.

While the precise nature of the mechanisms responsible for initiating FtsZ assembly at the nascent division site, modulating the architecture of the FtsZ ring, and driving constriction of the ring at the beginning of cytokinesis remain to be determined, our findings together with those of the Erickson lab (Gardner et al., 2013), reinforce the need to consider the role of the linker in all aspects of FtsZ dynamics.
Experimental procedures

General methods:

All *B. subtilis* strains are derivatives of the strain JH642 (Perego et al., 1988). Cloning and genetic manipulation were performed using standard techniques (Harwood et al., 1990; Sambrook and Russell, 2001). All cloning was done using the *E. coli* strain AG1111 derivative PL930 (Wang and Lutkenhaus, 1993). PL930 contains the low copy plasmid pBS58 expressing *E. coli* *ftsQAZ*, which facilitates cloning of *B. subtilis* FtsZ. Vent DNA polymerase was used for PCRs (New England Biolabs). Cells were grown in Luria-Bertani (LB) medium at 37 °C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin = 100 μg ml⁻¹, spectinomycin = 100 μg ml⁻¹, chloramphenicol = 100 μg ml⁻¹. Strains and plasmids used in this study are listed in Table 2. Primers used are listed in Table S1.

Cloning:

The pPJ15 plasmid was made by Quick Change II site-directed mutagenesis (Agilent Technologies) of pPJ1 plasmid to delete *B. subtilis* FtsZ aa 316-365. All other FtsZ chimeras were constructed as follows. Novel restriction sites were introduced into the *B. subtilis* FtsZ sequence by site-directed mutagenesis (BamHI after aa 315 and XmaI before aa 366) to yield the pPJ19 plasmid. This resulted in the amino acid pairs GS and PG at the beginning and end of the FtsZ linker. Gene sequences were PCR amplified, restriction digested, and ligated into the pPJ19 plasmid between the BamHI and XmaI sites. FtsZ ΔCTL25 was made by PCR amplification of *B. subtilis* FtsZ amino acids 1-338 and ligating into pPJ19. Including the XmaI site, this resulted in a linker length of 25 amino acids. FtsZ CTLScr was created by assigning each linker codon a number and running it through a random number generator (www.random.org) to yield the
resulting sequence:

KQSTNTPQEKQPSIKVRRQQEVKPSLQKQENTVHIRHSDNAPTSDEEQ. A synthetic oligonucleotide encoding this sequence was made (Integrated DNA Technologies) and PCR amplified to insert into pPJ19. Including the restriction sites, the total linker length was 54 amino acids. The *E. coli* linker sequence (amino acids 318-366) was amplified from genomic DNA from strain MG1655 DNA and ligated into pPJ19. Including the restriction sites, the total linker length was 52 amino acids. FtsZ CTLH was made through the creation of a gBlocks™ Gene Fragments synthetic double-stranded oligonucleotide encoding human beta-catenin residues 398-455 (Integrated DNA Technologies). The oligonucleotide was then restriction digested and inserted into pPJ19. *A. tumefaciens* FtsZ1 linker sequences were amplified from genomic DNA from strain A136 (gift of W. Margolin). FtsZ CTLA249 contained amino acids 322-566 from *A. tumefaciens* FtsZ1 in addition to extra residues from the novel restriction sites resulting in a total linker of 249 residues. FtsZ CTLA100 contained amino acids 322-417 from *A. tumefaciens* FtsZ1 plus restriction sites yielding a linker total length of 100 amino acids. FtsZ CTLA50 contained amino acids 322-367 from *A. tumefaciens* FtsZ1 along with restriction sites producing a linker of 50 residues.

pPJ27-35 were constructed by ligating restriction-digested full-length FtsZ chimera insert into the digested pDR67 plasmid with forward primers containing the *B. subtilis* ribosome binding site. All DNA sequences were confirmed by Sanger sequencing.

**Strain Construction:**

*B. subtilis* strains PL3400-07 were constructed as previously described (Buske and Levin, 2012). Briefly, these strains allow depletion of wild-type FtsZ and expression of *B. subtilis* FtsZ...
variants. They were constructed by first transforming pPJ27-35 plasmids into wild-type JH642 cells. This allowed the insertion of a copy of FtsZ chimeras with the *B. subtilis* ribosome-binding site upstream of the start codon at the *amyE* locus under control of the isopropyl 1-thio-β-D-galactopyranoside-inducible P\textsubscript{spac} promoter. Chromosomal DNA from the resulting strains was then transformed into competent PL2084 cells (JH642 *thrC::P\textsubscript{xyl}-ftsZ\textsubscript{Bs}, ftsZ\textsubscript{Bs}::spc, xylA::tet*), which have their only copy of native FtsZ expressed from a xylose-inducible promoter at the *thrC* locus. This strain permits depletion of wild-type FtsZ and is xylose-dependent for normal growth (Weart and Levin, 2003).

**Growth Conditions:**

Growth conditions were as follows. *B. subtilis* strains PL3171 (FtsZ WT) and PL3400-3407 (FtsZ variants) were first grown from single colony overnight in LB containing spectinomycin and xylose to a final concentration of 0.5%. The next day, cells were diluted 1:100 in fresh LB medium supplemented with 0.5% xylose and grown to an *A\textsubscript{600}* of 0.4 – 0.5, at which point 1 ml of cell culture was harvested by centrifugation. Cells were then washed twice with a fresh 1 ml of LB medium and then diluted 1:100 into a fresh 20 ml of LB medium plus isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM to induce expression of FtsZs. IPTG concentration was determined by titration in PL3171 to match FtsZ expression to that of wild-type JH642 cells. *A\textsubscript{600}* measures were taken every 30 min to monitor cell growth, and after 2.5 hours post-induction with IPTG cells were harvested and prepared for immunofluorescence.

**Immunofluorescence Microscopy:**
Immunofluorescence was performed as described previously (Buske and Levin, 2012) using 2.5% paraformaldehyde and 0.04% glutaraldehyde. An Olympus BX51 microscope with Chroma filters and a Hamamatsu OrcaERG camera were used for image capture. Images were processed using Openlab version 5.2.2 (Improvision) and Adobe Photoshop CS version 8.0 (Adobe Systems). All cell or ring measurements for collected images were obtained with a minimum population of 200 cells/strain. *B. subtilis* FtsZ was detected using affinity-purified polyclonal rabbit anti-FtsZ serum (Levin and Losick, 1996) in combination with goat anti-rabbit serum conjugated to Alexa488 (Life Technologies). Cell walls were visualized with wheatgerm agglutinin conjugated to tetramethylrhodamine (Invitrogen). Nucleoids were stained with DAPI.

**Determination of Cell Length/FtsZ Ring Ratio:**

The cell length/FtsZ ring (L/R) ratio was calculated as the sum total length of a population of cells divided by the number of FtsZ rings in that population as described previously (Weart et al., 2007).

**Dilution Plating:**

Strains 3171, 3400, 3404, and 3405 were used for dilution plating. Cells were grown overnight in LB medium containing ampicillin at 30 °C, and the following morning, cultures were first grown from single colony overnight in LB containing spectinomycin and xylose to a final concentration of 0.5%. The next day, cells were diluted 1:100 in fresh LB medium supplemented with 0.5% xylose and grown to an A600 of 0.4 – 0.5, at which point 1 ml of cell culture was harvested by centrifugation. Cells were then washed twice with a fresh 1 ml of LB medium upon which serial dilutions from $10^{-1}$ to $10^{-8}$ were made in LB medium. 10 µl of each
dilution was then plated in series using a multichannel pipette onto pre-warmed LB agar containing spectinomycin and 0.1 mM isopropyl 1-thio-β-D-galactopyranoside with or without 0.5% xylose. Liquid cultures were allowed to dry on the plates at room temperature for upwards of 6 h, and then plates were incubated at 37 °C overnight. The plates were then imaged, and relative growth was qualitatively assessed.

Protein Purification:

FtsZ variants were cloned into the pET-21b(+) expression vector through E. coli strain AG1111. The resulting plasmids were miniprepped and freshly transformed into C41(DE3) cells (Miroux and Walker, 1996) and consequently used for expression of protein. No frozen stocks were used. Briefly, 1 liter of LB medium was inoculated 1:100 with overnight culture from a single colony. Cells were grown at 37 °C until A600 ~0.6, and then cells were induced with isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Cells were grown for an additional 4 h at 37 °C, and then cells were harvested by centrifugation, and cell pellets were stored at -80 °C. Purification of FtsZs were performed as described previously (Buske and Levin, 2012) with the following modifications. FtsZ CTLE, CTLH CTLA249, CTLA100, and CTLA50 were all precipitated with one ammonium sulfate cut at 30%. The FtsZ was further purified on a MT-20 column manually packed with UNOsphere™ Q beads (Bio-Rad) with a linear gradient of 50–500 mM KCl in 50 mM Tris, pH 8.5, 1 mM EDTA, 10% sucrose. Peak fractions were analyzed by SDS-PAGE, pooled together, and dialyzed overnight twice, first in 1 liter of FtsZ dialysis buffer, pH 6.5 (50 mM MES, pH 6.5, 50 mM KCl, 2.5 mM MgCl2,1mM EGTA, 10% sucrose), then in 1 liter of FtsZ dialysis buffer, pH 7.5 (50 mM HEPES, pH 7.5, 50 mM KCl, 2.5 mM MgCl2,1mM EGTA, 10% sucrose). Protein preparations were concentrated in dialysis
tubing using PEG 12,000, aliquoted, flash frozen on liquid N$_2$, and stored at -80 °C. Prior to use in an assay to remove any insoluble polymers, FtsZ aliquots were thawed on ice, then spun at 80,000 rpm in a TLA-100 rotor (Beckman, Inc.) for 10 minutes at 4 °C and the supernatant used. FtsZ was then quantified using Coomassie Plus™ Protein Assay Reagent (Fisher) with porcine tubulin (Cytokeleton, Inc.) as a standard.

90° Angle Light Scattering Assay:

Light scattering experiments were performed essentially as described earlier (Buske and Levin, 2012) using a DM-45 spectrofluorimeter (Olis). Assembly reactions contained 5 μM FtsZ in assembly buffer (50 mM MES, pH6.5, 50 mM KCl, 2.5mM MgCl$_2$, 1 mM EGTA, 1 mM GTP).

Electron Microscopy:

Electron microscopy was performed essentially as described (Buske and Levin, 2012). 3 μM FtsZ was used because it was found to be the best concentration in which to visualize single FtsZ filaments. FtsZ was assembled with GTP as for light scattering, and samples were visualized using a JEOL 1200EX transmission electron microscope. FtsZ filament lengths were measured using ImageJ, and data were compiled in Microsoft Excel and Kaleidagraph.

GTPase Assay and Critical Concentration:

GTPase activity was measured using a continuous, regenerative coupled GTPase assay (Ingerman and Nunnari, 2005). Assays were conducted in buffer conditions identical to those used for light scattering. Each reaction included 1-8 μM FtsZ, 1 mM GTP, 1 mM
phosphoenolpyruvate, 250 μM NADH, 80 units/ml lactose dehydrogenase, and 80 units/ml pyruvate kinase. A linear decline of absorbance at 340 nm for NADH was observed at 30 °C for 5 min in a 96 well plate (path length calculated as 0.425 cm) using a SPECTRAmax Plus spectrophotometer (Molecular Devices). The raw data of absorbance per minute were then converted to activity using the extinction coefficient for NADH at 340 nm of 6220 M$^{-1}$ cm$^{-1}$ and the path length. The raw data were then exported to Microsoft Excel for analysis. The critical concentration was determined as the X-intercept of a plot of the GTP consumed per minute versus FtsZ concentration as has been done previously (Chen et al., 2012).
References


Wang, X., and Lutkenhaus, J. (1993). The FtsZ protein of *Bacillus subtilis* is localized at the division site and has GTPase activity that is dependent upon FtsZ concentration. Mol. Microbiol. 9, 435-442.


Acknowledgments

We thank Harold Erickson for sharing data and helpful discussions on the manuscript. We thank Bill Margolin for the generous gift of *A. tumefaciens* strain A136. We also thank Wandy Beatty for assistance with electron microscopy and all members of the Levin lab for their helpful discussions and feedback. This work was supported by a National Institutes of Health Public Health Service Grant GM64671 to PAL.
List of Abbreviations

aa: amino acid

CTC: C-terminal tail

CTL: C-terminal linker

CTR: C-terminal region

CTV: C-terminal variable region

EDTA: ethylenediaminetetraacetic acid

GHP: grappling hook peptide

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IDP: intrinsically disordered peptide

IFM: immunofluorescence microscopy

IPTG: isopropyl 1-thio-β-D-galactopyranoside

MES: 2-(N-morpholino)ethanesulfonic acid

SDS-PAGE: sodium dodecyl sulfate – polyacrylamide gel electrophoresis

TEM: transmission electron microscopy

WT: wild type
Table 1

<table>
<thead>
<tr>
<th>FtsZ species</th>
<th>Cc (μM)</th>
<th>GTP FtsZ⁻¹ min⁻¹</th>
<th>FtsZ Polymers at pH 6.5, 50 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.68</td>
<td>3.82</td>
<td>Filament bundles and rings</td>
</tr>
<tr>
<td>ΔCTL50</td>
<td>1.08</td>
<td>1.48</td>
<td>Single filaments</td>
</tr>
<tr>
<td>ΔCTL25</td>
<td>0.30</td>
<td>4.66</td>
<td>Single filaments</td>
</tr>
<tr>
<td>CTLScr</td>
<td>0.88</td>
<td>2.68</td>
<td>Filament bundles</td>
</tr>
<tr>
<td>CTLH</td>
<td>0.70</td>
<td>0.71</td>
<td>Small oligomers</td>
</tr>
<tr>
<td>CTLE</td>
<td>0.64</td>
<td>5.04</td>
<td>Single filaments</td>
</tr>
<tr>
<td>CTLA249</td>
<td>0.80</td>
<td>4.40</td>
<td>Single filaments</td>
</tr>
<tr>
<td>CTLA100</td>
<td>0.78</td>
<td>5.09</td>
<td>Single filaments</td>
</tr>
<tr>
<td>CTLA50</td>
<td>0.64</td>
<td>5.51</td>
<td>Single filaments</td>
</tr>
</tbody>
</table>
Table 2

Bacterial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL 2084</td>
<td>JH642 thrC::P&lt;sub&gt;xyl&lt;/sub&gt;/ftsZ, ftsZ::spc, xylA::tet</td>
<td>(Weart and Levin, 2003)</td>
</tr>
<tr>
<td>PL 3171</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZ</td>
<td>(Buske and Levin, 2012)</td>
</tr>
<tr>
<td>PL 3400</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZACTL50</td>
<td>This study</td>
</tr>
<tr>
<td>PL 3401</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZ ΔCTL25</td>
<td>This study</td>
</tr>
<tr>
<td>PL 3402</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZCTLE</td>
<td>This study</td>
</tr>
<tr>
<td>PL 3403</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZCTLA249</td>
<td>This study</td>
</tr>
<tr>
<td>PL 3404</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZCTLA100</td>
<td>This study</td>
</tr>
<tr>
<td>PL 3405</td>
<td>PL 2084 amyE::P&lt;sub&gt;spac&lt;/sub&gt;-ftsZCTLA50</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td><em>F' ilvG</em> rfb-50 rph-1</td>
<td></td>
</tr>
<tr>
<td>AG1111</td>
<td>DZR200- MC1061 F' lacIQ lacZM15 Tn10 (tet)</td>
<td>(Ireton et al., 1993)</td>
</tr>
<tr>
<td>C41 (DE3)</td>
<td>*F' ompT hsdSB (rB&lt;sup&gt;R&lt;/sup&gt; mB&lt;sup&gt;R&lt;/sup&gt;) gal dcm (DE3)</td>
<td>(Miroux and Walker, 1996)</td>
</tr>
<tr>
<td>PL930</td>
<td>AG111 + pB5S8</td>
<td>(Weart and Levin, 2003)</td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype and/or features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPJ1</td>
<td>pET21b(+)-ftsZ stop</td>
<td>(Buske and Levin, 2012)</td>
</tr>
<tr>
<td>pPJ15</td>
<td>pET21b(+)-ftsZACTL50 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ19</td>
<td>pET21b(+)-ftsZ946-51 BamHI 1090-95 XmaI stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ21</td>
<td>pET21b(+)-ftsZACTL25 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ22</td>
<td>pET21b(+)-ftsZCTLE stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ23</td>
<td>pET21b(+)-ftsZCTLA249 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ24</td>
<td>pET21b(+)-ftsZCTLA100 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ25</td>
<td>pET21b(+)-ftsZCTLA50 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ26</td>
<td>pDR67-ftsZACTL50 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ29</td>
<td>pDR67-ftsZ ΔCTL25 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ30</td>
<td>pDR67-ftsZCTLE stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ31</td>
<td>pDR67-ftsZCTLA249 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ32</td>
<td>pDR67-ftsZCTLA100 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ33</td>
<td>pDR67-ftsZCTLA50 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ34</td>
<td>pDR67-ftsZCTLA249 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ35</td>
<td>pDR67-ftsZCTLA100 stop</td>
<td>This study</td>
</tr>
<tr>
<td>pPJ36</td>
<td>pDR67-ftsZCTLA50 stop</td>
<td>This study</td>
</tr>
</tbody>
</table>
Figure 1

FtsZ Constructs.

Schematic of the different *B. subtilis* FtsZ constructs used in this study, drawn to scale. FtsZ ΔCTL50 is a complete deletion of the linker, leaving only the core and conserved CTC and CTV. FtsZ ΔCTL25 is a deletion of the last 25 linker residues. FtsZ CTLScr is a randomly generated scramble of the native linker sequence. FtsZ CTLH has the linker replaced with helical repeats (residues 398-455) from human beta-catenin. FtsZ CTLE has the native *B. subtilis* linker replaced with residues 318-366 from *E. coli* FtsZ. FtsZ CTLA249 is a replacement of the native linker with residues 322-566 from *A. tumefaciens* FtsZ, while FtsZ CTLA100 and CTL50 are replacements with residues 322-417 and 322-367, respectively.
Figure 2

The FtsZ linker is required for cell division in *B. subtilis*.

Note: Buffer conditions for all *in vitro* work are: 50 mM MES pH 6.5, 50 mM KCl, 2.5 mM MgCl$_2$, 1 mM EGTA, 1 mM GTP. FtsZ is used at a concentration of 3 µM for all electron microscopy to facilitate visualization of single stranded polymers. For 90° angle light scattering and GTPase assays FtsZ concentration is always 5 µM. A-C. Electron micrographs of 3µM FtsZ assembled in the presence of GTP. Bar = 100 nm. A. Bundled rings of FtsZ. B. Ordered sheets of FtsZ filaments on the same grid as A. C. FtsZ ΔCTL50 assembles mostly into small oligomers with occasional short, single or double-stranded filaments positioned randomly 5 minutes after addition of GTP. D. FtsZ ΔCTL50 filaments 15 minutes after addition of GTP. Note increase in filament length compared to 5 minutes. E. Representative traces of 5 µM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. Time course is over 60 minutes to denote delay in peak light scattering signal displayed by FtsZ ΔCTL50 compared to FtsZ WT. FtsZ ΔCTL50 shows a ~37 fold decreases in light scattering signal relative to FtsZ. F. Immunofluorescence microscopy of *B. subtilis* cells expressing *ftsZ* or *ftsZ ΔCTL50* as the sole copy of FtsZ. FtsZ ΔCTL50 fails to form Z rings and support cell division instead showing punctate localization or forming strands throughout the cytoplasm. Bar = 5 µm.
Figure 3

FtsZ chimeras and their effect on cell growth.

A. Growth curves monitoring optical density of *B. subtilis* cells post-induction of chimeric FtsZs with IPTG at 37°C. B. Chimeric FtsZs that form Z rings exhibit regular Z-ring localization. Length-to-ring ratios were measured for all *B. subtilis* cell types that showed the formation of Z rings. There is no significant difference between groups as determined by one way ANOVA analysis with α = 0.001.
Figure 4

The sequence of the FtsZ linker does not affect protofilament assembly or cell division in *B. subtilis*.

A-D. Electron micrographs of 3μM FtsZ assembled in the presence of GTP. Arrows indicate individual filaments. Bar = 100 nm. A. Bundled rings of FtsZ. Same as Fig. 2A to allow direct comparison of wild type with FtsZ chimeras. B. FtsZ CTLScr assembles into filaments able to associate laterally and form ordered sheets and bundles 3-5 protofilaments thick. C. FtsZ CTLE forms predominantly single stranded filaments with little evidence of lateral interactions. D. FtsZ CTLA50 also forms predominantly single- or double-stranded filaments. E. Representative traces of 5 μM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. FtsZ CTLScr displays a high signal, 60% that of wild-type FtsZ, indicating it is able to form lateral interactions. FtsZ CTLE and CTLA50 show ~62 and ~37 fold decreases in light scattering signal relative to FtsZ, respectively. The low light scattering signals, coupled with the electron micrographs, indicate FtsZ CTLE and CTLA50 primarily form single-stranded protofilaments. F. Immunofluorescence microscopy of *B. subtilis* cells expressing *ftsZ*, *ftsZ CTLScr*, *ftsZ CTLE*, or *ftsZ CTLA50* as the sole copy of FtsZ. All FtsZ chimeras are able to support cell division normally and display properly positioned Z rings. Bar = 5 μm.
**Figure 5**

**FtsZ linker length does not affect protofilament formation but is important for cell division.**

A-D. Electron micrographs of 3µM FtsZ assembled in the presence of GTP. Bar = 100 nm. A. Bundled rings of FtsZ. Same as Fig. 2A to allow direct comparison of wild type with FtsZ chimeras. B. FtsZ ΔCTL25 assembles into short single-stranded filaments unable to associate laterally. C. FtsZ CTL249 forms predominantly single stranded filaments with little evidence of lateral interactions. D. FtsZ CTLA100 also forms predominantly single-stranded filaments with little evidence of lateral interactions. E. Representative traces of 5 µM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. FtsZ ΔCTL25 displays a signal ~79 fold lower than that of wild-type FtsZ. FtsZ CTLA249 and CTLA100 show ~43 and ~33 fold decreases in light scattering signal relative to FtsZ, respectively, indicative of single-stranded filaments. F. Same plot as in E but scaled to highlight GTP-dependent increase in light scattering of FtsZ ΔCTL25, FtsZ CTLA249, and FtsZ CTLA100. G. Immunofluorescence microscopy of *B. subtilis* cells expressing *ftsZ*, *ftsZ ΔCTL25*, *ftsZ CTLA249*, or *ftsZ CTLA100* as the sole copy of FtsZ. The short linker in FtsZ ΔCTL25 supports normal cell division and Z-ring localization. Cells expressing *ftsZ CTLA249* are able to form Z rings, but cells cannot divide and instead are filamentous. A linker of intermediate length does not affect cell division, as cells expressing *ftsZ CTLA100* are able to divide normally. Bar = 5 µm.
Figure 6

A flexible and disordered linker is essential for FtsZ assembly and cell division.

A-B. Electron micrographs of 3μM FtsZ assembled in the presence of GTP. Bar = 100 nm. A. Bundled rings of FtsZ. Same as Fig. 2A to allow direct comparison of wild type with FtsZ chimeras. B. FtsZ CTLH is unable to assemble into protofilaments, instead seemingly forming small oligomers. C. Representative traces of 5 μM FtsZ assembled in presence of GTP and measured by 90° angle light scattering. FtsZ CTLH displays a signal ~136 fold lower than wild-type FtsZ, indicating it is very poor at assembling into protofilaments. D. Immunofluorescence microscopy of B. subtilis cells expressing ftsZ or ftsZ CTLH as the sole copy of FtsZ show the cells are unable to divide and FtsZ is punctate throughout the cell.
**Figure 7**

**The FtsZ linker is a flexible tether.**

A. FtsZ filaments (tan) are anchored to the membrane through the interaction between its grappling hook peptide (GHP; green) and FtsA (light blue). As the filament ends move further away from the membrane due to the intrinsic bend of the filament, the FtsZ linker (dark blue) extends and takes on a conformation which allows the GHP to remain tethered to FtsA. Lateral FtsZ filament interactions area also capable of forming through the positioning of the GHP near the N-terminal core of adjacent subunits by the linker. B. A FtsZ ΔCTL50 filament without the linker is able to anchor to the membrane through its GHP. However, in the absence of the linker, the filament remains near the membrane in a straighter positioning that cannot respond to the membrane curvature and form a proper Z ring. The FtsZ ΔCTL50 filament is also unable to form bundles.
Supplemental Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Site</th>
<th>Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJBBsFtsZFwd</td>
<td>GATCGATCCCATATGTGGAGTTGCAGAAACATAGACG</td>
<td>NdeI</td>
<td>pPJ1, pPJ19, pPJ21</td>
</tr>
<tr>
<td>PJBBsFtsZRev</td>
<td>GATCGATCTCGATTTTGTCTTTACATTAGCCGC</td>
<td>BamHI</td>
<td>pPJ1</td>
</tr>
<tr>
<td>PJBBsFtsZRev2</td>
<td>GATCGCTGAGCGATTTTGTCTTTACATTAGCCGC</td>
<td>XhoI</td>
<td>pPJ19</td>
</tr>
<tr>
<td>PJBBsFtsZΔCTL50Fwd</td>
<td>AGTGTTGCACGCTTTTTGATCATACGCGATTTAGGGTTGACATC</td>
<td>none</td>
<td>pPJ15</td>
</tr>
<tr>
<td>PJBBsFtsZΔCTL50Rev</td>
<td>GATGTCAAGCTATCATCAAAGCCGGTTGCAATCATC</td>
<td>none</td>
<td>pPJ15</td>
</tr>
<tr>
<td>PJBsFtsZSDM946-5Fwd</td>
<td>TGGTGACAGTGATTGCAGCCGGCTTTGGATCCCAAGAGAAGGACGATGACGAAGCCTTCAGCGGAAGGACTCAGCG</td>
<td>BamHI</td>
<td>pPJ19</td>
</tr>
<tr>
<td>PJBBsFtsZSDM946-5Rev</td>
<td>GAGGCTTCGTACGCTCTCTCTCTGGATCCCAAGAGAAGGACGATGACGAAGCCTTCAGCGGAAGGACTCAGCGGAAG</td>
<td>BamHI</td>
<td>pPJ19</td>
</tr>
<tr>
<td>PJBBsFtsZ1090-109SDMFwd</td>
<td>CCGTCATACTTCAAGCGCCTTTTTGGATCAGCTTGACATC</td>
<td>XmaI</td>
<td>pPJ19</td>
</tr>
<tr>
<td>PJBBsFtsZ1090-109SDMRev</td>
<td>GATGTCAGCCGTATCATCCCAAGAGAAGGACGATGACGAAGCCTTCAGCGGAAGGACTCAGCGGAAGGACTCAGCGG</td>
<td>XmaI</td>
<td>pPJ19</td>
</tr>
<tr>
<td>PJBBsFtsZΔCTL25Rev</td>
<td>CATGCGCGATTTGTGTGTGTGATGCTTGATTTAAGC</td>
<td>XmaI</td>
<td>pPJ21</td>
</tr>
<tr>
<td>PJBBsFtsZCTLScrOli</td>
<td>GATCGATCAGATCCAAACAGCATACGAAAGCTTCATGAAGGACGATGACGAAGCCTTCAGCGGAAGGACTCAGCGGAAGG</td>
<td>BamHI, XmaI</td>
<td>pPJ22</td>
</tr>
<tr>
<td>Sequence Name</td>
<td>Sequence</td>
<td>Restriction Enzyme</td>
<td>Plasmid</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------</td>
<td>--------------------</td>
<td>---------</td>
</tr>
<tr>
<td>PJBBsFtsZCTLScrFwd</td>
<td>GATCGGATCCAAACAGTCAACGAATACTC</td>
<td>BamHI</td>
<td>pPJ22</td>
</tr>
<tr>
<td>PJBBsFtsZCTLScrRev</td>
<td>GATCCCGGGCTGGCTCTTTC</td>
<td>XmaI</td>
<td>pPJ22</td>
</tr>
<tr>
<td>PJBBsFtsZCTLEFwd</td>
<td>GATCGGATCCAACGTCTGAAAATCACTCTGGTG</td>
<td>BamHI</td>
<td>pPJ23</td>
</tr>
<tr>
<td>PJBBsFtsZCTLEFwd</td>
<td>GATCCCGGGGCGCAGTTTGGCGGCCGCATTTG</td>
<td>XmaI</td>
<td>pPJ23</td>
</tr>
<tr>
<td>PJBBsFtsZCTLHOLi</td>
<td>GATCGGATCCATGGAAGGACTTTCTTGGGAACTTTGGCTCACTGATGA</td>
<td>BamHI, XmaI</td>
<td>pPJ24</td>
</tr>
<tr>
<td>PJBBsFtsZCTLAfwd</td>
<td>GATCGGATCCGGTTCGCGGGCATCGGGCGAAGCAG</td>
<td>BamHI</td>
<td>pPJ25, pPJ26, pPJ27</td>
</tr>
<tr>
<td>PJBBsFtsZCLTA249Rev</td>
<td>GATCCCGGGGATGGCTGGACGGAGGAAGGCCGTC</td>
<td>XmaI</td>
<td>pPJ25</td>
</tr>
<tr>
<td>PJBBsFtsZCLTA100Rev</td>
<td>GATCCCGGGGACGAGAGGCCGCGGCGCTTT</td>
<td>XmaI</td>
<td>pPJ26</td>
</tr>
<tr>
<td>PJBBsFtsZCLTA50Rev</td>
<td>GATCCCGGGGGTGCTTTGGTGCGTGCAGTAATTGGTGACGGTTAG</td>
<td>XmaI</td>
<td>pPJ27</td>
</tr>
<tr>
<td>PJBBsFtsZpDR67Fwd</td>
<td>GATCAAGCTTTTTAAGAGGAGGAGGTTTAAGCATGTTGGAGTTCGAAACAAACATAG</td>
<td>HindIII</td>
<td>pPJ28, pPJ30-35</td>
</tr>
<tr>
<td>PJBBsFtsZpDR67Fwd2</td>
<td>GATCTTCTAGTTTTAAGAGGAGGAGGTTTAAGCATGTTGGAGTTCGAAACAAACATAG</td>
<td>XbaI</td>
<td>pPJ29</td>
</tr>
<tr>
<td>PJBBsFtsZpDR67Rev</td>
<td>ATGCGCATGCACATTAGCCGCGTCTTATTACGGTT</td>
<td>SphI</td>
<td>pPJ28-35</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

Circular dichroism spectra of FtsZ, FtsZ ΔCTL50, and FtsZ CTLH.

Spectra for 8 μM FtsZ was measured. FtsZ (black circles), FtsZ ΔCTL50 (blue squares) and FtsZ CTLH (purple open circles) showed nearly identical spectra when scanned from 200-260 nm.
Supplemental Figure 2

_B. subtilis_ cells that express FtsZ ΔCTL50, FtsZ CTLH, or FtsZ CTLA249 display significant growth deficiencies.

A-B. PL strains 3171, 3400, 3404, and 3405 were grown to mid-log in presence of xylose to promote induction of wild-type FtsZ. Cells were harvested and washed, serial dilutions were made as labeled, and cells plated on LB agar plates with various inducers. A. Cells were plated in the presence of 0.1 mM IPTG to induce expression of only the chimeric FtsZ. Expression of FtsZ ΔCTL50, FtsZ CTLH, or FtsZ CTLA249 alone failed to support cell growth. B. Cells were plated in the presence of 0.1 mM IPTG and 0.5% xylose to induce expression of both wild-type FtsZ and the FtsZ chimera. Expression of FtsZ ΔCTL50 and FtsZ CTLH was dominant lethal; however, cells expressing FtsZ CTLA249 and wild-type FtsZ still were viable, but to a lesser degree compared to wild type alone.
Supplemental Figure 3

Cell wall staining of FtsZs.

A. Immunofluorescence microscopy of PAL 522 (wild type) and PAL 3171 cells (wild-type FtsZ induced with IPTG) showed similar cell wall-staining to FtsZ CTLScr, FtsZ CTLE, and FtsZ CTLA50 when treated with lysozyme. B. Crosswalls observed in PAL 3404 (FtsZ CTLH) cells in which no FtsZ was induced were similar to crosswalls observed in which FtsZ CTLH was expressed (see Fig. 6D).
**Supplemental Figure 4**

**Deletion of the entire FtsZ C-terminus abolishes ability to form protofilaments.**

FtsZs from *B. subtilis* and *E. coli* in which the entire C-terminus (CTR) were cloned, expressed, and purified. A-B. Electron micrographs of 3μM FtsZ assembled in FtsZ assembly buffer (50 mM MES, pH 6.5, 50 mM KCl, 2.5 mM MgCl$_2$, 1 mM EGTA, 1 mM GTP). Bar = 100 nm. A. Bs FtsZ ΔCTR is only able to form small oligomers. B. Ec FtsZ ΔCTR is also only able to form small oligomers.
Addendum

Chapter 3 was previously published in *Molecular Microbiology* (Buske and Levin, 2013).


Work in Chapter 3 was as follows:

1. PJ Buske and Petra Levin designed and devised all experiments.
2. All experiments performed/data collected by PJ Buske.
3. PJ Buske and Petra Levin wrote the manuscript.
Chapter 4:
Summary, discussion, and future directions
Summary

This dissertation establishes a new structural domain of FtsZ, the C-terminal variable (CTV) region and describes an essential role for FtsZ C-terminal linker (CTL) in FtsZ assembly and cell division in *B. subtilis*. Because these domains are irresolvable by X-ray crystallography, little was known about their functions prior to this work.

Here, I confirm these FtsZ domains play an important role in FtsZ assembly independent of modulatory proteins or other outside factors. In Chapter 2, I show that the CTV region of FtsZ facilitates lateral filament interaction potential and that the charge of the CTV is a main determinant in modulating bundling. I also show that *in vivo*, changing the *B. subtilis* CTV sequence to that of *E. coli* has significant adverse effects on cell division. This leads to a model in which the CTV is positioned by the flexible CTL to interact with adjacent filaments and allow filament bundling.

In Chapter 3, I establish that the CTL is indeed a flexible domain and that is essential for FtsZ function both *in vitro* and *in vivo*. Deletion of the CTL results in deformed FtsZ filaments and mislocalized FtsZ in *B. subtilis* cells unable to support cytokinesis. In addition, I show the CTL behaves as an intrinsically disordered peptide (IDP). Together, these works result in a model in which the CTL functions as a flexible tether coupling FtsZ filaments to the membrane and allowing filaments to respond to the curvature of the cell in a way which helps generate constrictive force. In addition, this dissertation provides significant insight into the requirements for a conformationally active FtsZ subunit leading to filament formation.
Discussion

The FtsZ CTV Mediates Lateral Filament Interactions:

*B. subtilis* and *E. coli* have long been the standard model organisms for understanding cell division in rod-shaped bacteria. They are easily genetically tractable and represent the two classic taxonomies of microbiology, Gram-positive and Gram-negative bacteria. As such, understanding cell division in each organism has led to the extensive study of the proteins involved in this process and how they interact, including FtsZ and its modulatory proteins. Originally I had intended to explore the interactions between FtsZ and certain modulatory proteins discovered in *B. subtilis* by our lab such as EzrA and ClpX (Levin et al., 1999; Weart et al., 2005). I also wanted to compare the interactions between FtsZ and modulatory proteins, such as MinC and FtsA, shared between *B. subtilis* and *E. coli*. However, in preparing for these types of experiments, I discovered that the FtsZs from these two species behave quite different *in vitro*.

In Chapter 2, I establish that *B. subtilis* and *E. coli* have different inherent abilities to form lateral filament interactions. While *B. subtilis* FtsZ (Bs FtsZ) forms bundled filament rings, *E. coli* FtsZ (Ec FtsZ) forms primarily single-stranded filaments under the same buffer conditions and protein concentrations. Deleting the very last C-terminal 17 amino acids from Bs FtsZ (Bs FtsZ ΔC17) yields an FtsZ that becomes bundling-deficient but is still able to assemble into single filaments. A homologous deletion of the last 15 residues from Ec FtsZ (Ec FtsZ ΔC15) does not affect filament assembly or lateral interactions. The last 17 Bs FtsZ and 15 Ec FtsZ residues that were deleted correspond to what has been termed the C-terminal tail and determined to be a binding site for modulatory proteins to FtsZ (Adams and Errington, 2009). In analyzing these tail sequences across bacterial species, this region of FtsZ is re-classified into the conserved C-terminal tail (CTT) encompassing the first ~10 residues and highly irregular C-
terminal variable (CTV) domain. In Chapter 3, we change the nomenclature of the CTT to CTC for C-terminal constant region. We also term the combined CTC and CTV domains the grappling hook peptide (GHP) to portray their roles as binding sites for modulatory proteins.

Because of its non-conserved nature, I show that the CTV is the domain responsible for the different bundling capabilities observed between Bs FtsZ and Ec FtsZ. Swapping the Bs FtsZ CTV with that from *E. coli* (Bs FtsZ CTVE) yields in protein that still assembles into filaments but cannot bundle. Conversely, the corresponding swap in Ec FtsZ (Ec FtsZ CTVB) results in an FtsZ that forms large bundled rings and sheets of FtsZ. The CTVs from *B. subtilis* and *E. coli* differ significantly in charge composition. The Bs FtsZ CTV is highly positive, while the Ec FtsZ CTV is neutral in charge. I show charge composition to be an important determinant for mediating lateral FtsZ filament interactions. Keeping the Bs FtsZ CTV positively charged (but changing the sequence composition) still allows filaments to bundle; reversing the charge results in primarily single-stranded filaments.

These data suggest electrostatic interactions mediate FtsZ lateral interactions. I put forth a model depicting the FtsZ filament as a linear rod of charge consisting of the negative core with the CTV positioned outside the rod by the CTL. A positive CTV shields the negative core of the filament from neighboring filaments to promote bundling. The sensitivity of FtsZ bundling to salt supports the importance of charge. Increasing the concentration of KCl or NaCl in buffer result in single-stranded Bs FtsZ filaments, suggesting the binding energies between filaments depends on counterion concentration. Likely, the ions inhabit space around filaments to promote or suppress bundling as has been proposed for actin filaments (Tang and Jamney, 1996). The polyelectrolytic nature of FtsZ filaments should be an important characteristic to consider when determining how modulatory proteins affect FtsZ assembly. Instead of specific residue contacts
being important for interactions, instead inhibition or stabilization of FtsZ assembly by other proteins may occur through general charge effects. Furthermore, while an exact mechanism of bundling observed between FtsZ filaments in Chapter 2 needs to be determined, it likely differs from bundling induced by divalent cations like Ca$^{2+}$ which involves a change to way GTP binds FtsZ (Marrington et al., 2004).

*In vivo* the Bs FtsZ CTV also is important. When *B. subtilis* cells express Bs FtsZ CTVE as the sole copy of FtsZ, 30% of cells become filamentous and display sparse Z rings. This suggests the loss of bundling caused by the CTVE mutation results in a less-stable Z ring. This might be solely due to the inherent loss of FtsZ bundling. However, new data suggest that it may be due to the loss of an interaction between FtsZ and SepF. SepF binds to the FtsZ C terminus, and CTV residues N377, K380, and R381 may be important for this interaction (Król et al., 2012). Because SepF mediates FtsZ lateral interactions as well, it still suggests the CTV plays an important role in forming FtsZ bundles.

**The FtsZ CTL Plays an Important Role in Protofilament Assembly and Z ring Stability:**

In Chapter 2, I propose the “bucket truck” model for FtsZ in which the globular core represents the body of the truck while the CTL is the flexible boom that positions the CTV (bucket) in a way to interact with other FtsZ filaments or modulatory proteins. Chapter 3 further tests this model and establishes that the FtsZ CTL is required for normal protofilament assembly *in vitro* and function *in vivo* by acting as a flexible tether to the membrane.

Prior to the work in Chapter 3, little was known about the function of the FtsZ linker. A truncated *E. coli* FtsZ (residues 1-320; lacking the CTL, CTC, and CTV) shows a greater propensity to bundle, suggesting it is not necessary for assembly and perhaps even hinders it;
however, the buffer conditions used in the study contain a high concentration of magnesium known to promote lateral interactions (Wang et al., 1997). Crystal structures of FtsZ across prokaryotic species cannot resolve the CTL (Haydon et al., 2008; Leung et al., 2004; Läppchen et al., 2008; Löwe and Amos, 1998; Matsui et al., 2012; Oliva et al., 2007; Raymond et al., 2009). Thus, it has been presumed unstructured. Analysis of FtsZs across domains of life show the CTL has very little conservation in both primary sequence and length, suggesting it serves merely as a placeholder between the globular core and the very C terminus (CTC and CTV) (Vaughan et al., 2004). While the CTL from *E. coli* has been shown to be flexible with a contour length of 17 nm, it was used as a linker between GFP FRET pairs and not part of a continuously folded protein as it would be in an FtsZ subunit (Ohashi et al., 2007). My work establishes a role for the CTL in the context of a complete FtsZ subunit and functional filament in *B. subtilis*. Recent work from Harold Erickson’s lab at Duke University Medical Center finds a similar role for the linker in *E. coli* (Gardner et al., 2013). Together, these works show the CTL serves a distinct structural role in FtsZ function and that what may actually be conserved is nature of the CTL to be an IDP.

However, there still might be some key differences between behavior of the CTL in *B. subtilis* and *E. coli*, namely the role of the linker in head-to-tail protofilament assembly. While *E. coli* FtsZ missing the CTL shows little problem assembling, I show the linker is critical for *B. subtilis* FtsZ assembly. My data suggest the linker plays an important structural role in maintaining a proper FtsZ subunit conformation needed for assembly. Conformational flexibility within the FtsZ subunit has been shown to play an important role in FtsZ assembly and ability to switch from GTP-bound to GDP-bound (Chen and Erickson, 2011; Díaz-Espinoza et al., 2007; Martín-Galiano et al., 2010). Replacing the linker with a helical structure (FtsZ CTLH) shows
that adding rigidity to the FtsZ subunit may trap it in a conformation insufficient to form filaments by blocking subunit addition onto an elongating polymer. Deleting the linker altogether (FtsZ ΔCTL50) results in delayed kinetics of filament formation. The FtsZ ΔCTL50 mutant also has a higher critical concentration that wild-type FtsZ indicating a lower affinity between subunits. This decrease in affinity also might be an indication that the conformation of FtsZ ΔCTL50 has changed in a way that slows elongation. The decreased GTPase activity observed for the ΔCTL50 and CTLH mutants also suggests a conformational deficiency in the ability to switch from GTP-bound to GDP-bound state.

*In vivo* results also associate well with the *in vitro* data on FtsZ ΔCTL50 and CTLH assembly. FtsZ CTLH displays a punctate localization throughout *B. subtilis* cells, which is expected given the short oligomers observed *in vitro*. FtsZ ΔCTL50 also forms similar punctae reflective of the small oligomers *in vitro*, but FtsZ ΔCTL50 also formed putative filaments along the longitudinal axis of the cell reflective of longer filaments observed *in vitro*. Perhaps the FtsZ core sits too close to the membrane to allow sufficient space for the filament to orient itself along the circumferential axis when anchored through the GHP. It seems a flexible linker helps the entire anchored filament to align itself and other filaments along with other divisome proteins to make a Z ring, perhaps through an additional unidentified structural feature as has been proposed (Erickson et al., 2010). These data support our model of the CTL behaving as a flexible tether linking FtsZ filaments to the membrane.

It is also interesting to note that filament formation is not prerequisite for a Z ring. FtsZs that do not form protofilaments still can form a Z ring (Redick et al., 2005). However, the FtsZ ΔCTL50 mutant cannot construct Z rings despite forming filaments *in vitro*. It seems that the linker is an essential component in allowing FtsZ to organize at the membrane.
Another question the work in Chapter 3 raises is the role of linker length. I show that the linker is functional with a length between 25-100 residues and sequence is not important as long as it retains itself as an IDP. However, replacing the wild-type CTL with that from A. tumifaciens (FtsZ CTLA249), comprising a length ~5 times that of wild type, does not support cell division even though Z rings develop. Interestingly, though, this mutant shows no deficiency in ability to form protofilaments \textit{in vitro}. Cells expressing FtsZ CTLA249 clearly have a defect in generating the constrictive force needed for cytokinesis. How does the linker length contribute to this phenotype? Conceivably the longer linker, due to its size, physically interferes with other proteins in the division machinery from working properly.

The other possibility is that the CTL plays a direct role in generating constrictive force. Deformation of the membrane needed for cytokinesis requires ~8 pN of force (Lan et al., 2007). An FtsZ filament has been modeled to generate 10-30 pN of force through bending due to GTP hydrolysis (Hsin et al., 2012). How does the bending force act upon the membrane? Because a filament will be anchored to the membrane through another protein like FtsA, it will not interact directly with the membrane. Therefore, the force must be translated through another mechanism. Presumably, due to the intrinsic bend of FtsZ filaments, subunits at the end of a filament bend inward away from the membrane. In response to this bending, the linker, because it is flexible, extends to keep the filament anchored to the membrane. In effect, the linker pulls the membrane inward as the filament continues to bend and the linker approaches its contour length. Filament ends have been estimated to be approximately 20 nm from the membrane when bent in an intermediate conformation and their center anchored at the membrane (Erickson et al., 2010). With a contour length of ~17 nm, the FtsZ linker would just about be fully extended in this conformation and further bending would likely then cause distortion of the membrane. In the
case of the FtsZ CTLA249 mutant, a very long linker might not ever take a taut conformation that can generate enough bending force.

That the linker is important for generating constrictive force may also be reflected in its ability to modulate later filament interactions through positioning the FtsZ CTV. As the Z ring constricts, filaments become condensed and bundling becomes more favorable. The constrictive force generated has been modeled to increase as the bundling increases, possibly up to 50 pN (Sun and Jiang, 2011). In the case of B. subtilis, FtsZ shows a greater propensity to bundle. Because of the thicker and stiffer cell wall in the Gram-positive organism, it is reasonable to presume that a greater constrictive force must be generated for cytokinesis. The difference in the inherent bundling abilities between B. subtilis and E. coli could very well be due to the force requirements for cytokinesis in Gram-positive versus Gram-negative organisms.
Future Directions

The data presented in this dissertation establish that the FtsZ C terminus plays important roles in modulating the assembly of FtsZ. However, this work also raises many important questions regarding FtsZ function both in vitro and in vivo. Some future studies to help better understand FtsZ dynamics and the composition of the Z ring are discussed below.

FtsZ Bundling In Vivo: Is It Important?

FtsZ forms a variety of different structures in vitro depending on buffer conditions and ion concentration (Buske and Levin, 2012; Erickson et al., 2010; Popp et al., 2009), but an issue regarding these structures is whether or not they are physiologically relevant. Work in Chapter 2 shows that changing the lateral interaction potential of B. subtilis FtsZ through mutations to the CTV result in cells with a fewer Z rings and suggests FtsZ bundling is important for Z ring stability. However, some of the FtsZ chimeras studied in Chapter 3 question this interpretation. Namely, Ftzs that form predominantly single-stranded filaments (FtsZ CTLE, FtsZ CTLA50, and FtsZ CTLA100) are able to support cell division in B. subtilis. As noted above, perhaps the filamentous cell phenotype observed in cells expressing Bs FtsZ CTVE is due to disrupting the FtsZ-SepF interaction (Król et al., 2012). The FtsZ CTLE, FtsZ CTLA50, and FtsZ CTLA100 proteins all contain the wild-type CTV sequence and should maintain a normal interaction with SepF. The lack of lateral filament interactions observed in vitro could be compensated for by SepF and other FtsZ-bundling proteins.

To test this idea, I am currently constructing B. subtilis strains in which the genes for the FtsZ stabilizers sepF and zapA are deleted individually. These gene deletions will then be introduced into the strains that can express different FtsZ CTL and CTV mutants. Using
immunofluorescence microscopy and other cell biology techniques to evaluate Z ring composition, cell size and cell growth, the effect of FtsZ lateral interactions on cell division can be assessed independent of stabilizing proteins.

Understanding the effects these stabilizing proteins have on the Z ring requires a high-resolution examination of the Z ring. Our lab is currently collaborating with the lab of Dr. Jie Xiao at Johns Hopkins School of Medicine to obtain a high-resolution image of the *B. subtilis* Z ring using photoactivated localization microscopy (PALM). The Xiao lab has already established the use of PALM to image the Z ring in *E. coli* (Fu et al., 2010). Of particular interest will be to compare the Z ring composition between *B. subtilis* and *E. coli* given the differences in bundling we observe *in vitro*. Determining the organization of FtsZ in FtsZ CTL and CTV mutants will also be of interest, particularly the composition of the filamentous structures observed in cells expressing FtsZ ΔCTL50. Resolving the orientation of filaments in these arrangements could provide insight whether the linker helps align FtsZ filaments in a certain direction in the ring. Imaging the Z ring in *sepF* and *zapA* null cells will also shed light on the roles of these proteins during cell division. One would expect a “looser”, less organized Z ring in their absence.

It will also be important to extend similar studies to bacteria besides *B. subtilis* and *E. coli*. Though these are the two model organisms for studying prokaryotic cell division, they could be the exception instead of the rule. The study of cell division, and particularly FtsZ, is relatively unexplored in many bacteria. Will predictions about lateral interaction potential correlating to the charge composition of the CTV hold true? Does cell division require different sets of protein for organisms with particularly long CTLs, such as *A. tumifaciens*? How can a linker of 249 residues function in this bacterial species but not *B. subtilis*? While the overall
process and the central role of FtsZ in cell division are likely conserved, the individual component might vary from species to species and open an entirely new avenue of study.

Role of the Linker in FtsZ Protofilament Assembly:

The work in Chapter 3 shows that deleting the FtsZ linker impacts head-to-tail assembly of FtsZ filaments in B. subtilis. The kinetics of assembly seem to be slower for the FtsZ ΔCTL50 mutant compared to wild-type FtsZ as assessed by 90° light scattering. The peak light scattering value for FtsZ ΔCTL50 is reached in ~15 min while FtsZ WT only takes on the order of seconds to reach a peak value that then reaches steady-state. While 90° light scattering allows detection of FtsZ assembly in real time, it is only a semi-quantitative method that cannot distinguish single-stranded filaments from bundles. It also underreports filaments that are smaller than the wavelength of light used to illuminate the sample and therefore cannot differentiate the initial steps of FtsZ assembly including nucleation and elongation.

To better quantify the assembly kinetics of B. subtilis FtsZ and the FtsZ ΔCTL50 mutant, I propose using fluorescent reporters of FtsZ assembly as has been established for E. coli FtsZ. The discovery that the E. coli FtsZ L68W mutant showed an increase in fluorescence upon incorporation of subunits into filaments allowed the first pre-steady-state kinetic examination of FtsZ assembly (Chen et al., 2005). One caveat of this mutant is that it seems to decrease the FtsZ critical concentration and assembly might differ from wild type. To circumvent that problem, I have created a similar B. subtilis FtsZ mutant, L161W, equivalent to an E. coli L160W mutant that displays a near wild-type critical concentration and an increase in fluorescence upon assembly as well (Chen et al., 2011). Such a mutant should display assembly much closer to what would be expected of wild-type FtsZ. I have also introduced this mutation into the Bs FtsZ
ΔCTL50 mutant. It would be expected to see that the kinetic rate constants for nucleation and elongation phases of Bs FtsZ ΔCTL50 assembly would be slower than wild-type when using a simple dimer nucleus model similar to that of *E. coli* FtsZ (Chen et al., 2005).

The use of tryptophan mutants has only been examined using *E. coli* FtsZ. In the case equivalent tryptophan mutants prove non-functional for *B. subtilis* FtsZ, I also propose using a fluorescence resonance energy transfer (FRET)-based assay to measure FtsZ assembly in real time. Using differentially labeled subunits, FtsZ assembly has been measured using FRET for both *E. coli* FtsZ and *M. tuberculosis* FtsZ (Chen and Erickson, 2005; 2009; Chen et al., 2007). An advantage of the FRET assay is that it allows a direct measurement of subunit exchange from filaments whereas the tryptophan fluorescence assay only measures kinetics of filament formation. I have already cloned a *B. subtilis* FtsZ F268C mutant necessary to created labeled subunits for future fluorescence applications. These biochemical experiments can provide insight into the assembly mechanism of *B. subtilis* FtsZ and allow a comparison to that established for *E. coli* FtsZ to determine whether FtsZ assembly at the subunit level remains conserved.

**Conformational Flexibility of the FtsZ Linker:**

In Chapter 3, our model predicts that the *B. subtilis* FtsZ CTL is a flexible IDP able to undertake different conformations depending on whether FtsZ is anchored to the membrane, involved in lateral filament interactions, or interacting with modulatory proteins. The Erickson lab reaches similar conclusions that the CTL of *E. coli* FtsZ is in fact an IDP with little sequence specificity but a length requirement for function (Gardner et al., 2013). However, there is disagreement on how flexible the CTL really is. Assuming the CTL behaves as a worm-like chain, Gardner et al., predict the linker to be an entropic spring and estimate the force required to
stretch it to half its contour length (~8.5 nm) would be 9.3 pN (Gardner et al., 2013). They conclude that this force is likely too much to be generated by a constricting Z ring and that the CTL must therefore be relatively stiff.

Our basis for the conformational flexibility of the linker is derived from its functioning as an IDP. While traditional polymer physics theory, like the worm-like chain, has been used to model IDPs, it seems now that those models are limiting and inadequate (Mao et al., 2013). To really understand the behavior of the CTL as an IDP, we are collaborating with the lab of Dr. Rohit Pappu in the Department of Biomedical Engineering here at Washington University in St. Louis. The Pappu lab has pioneered work establishing the relationship between the primary sequence of IDPs and the different conformations they undertake. One key insight they have revealed is that IDPs can accomplish a continuum of conformations depending on the charge content and distribution of charged residues throughout a primary sequence (Mao et al., 2013). In the context of a cell, this proposes that IDPs can take on different conformations depending on the cellular environment, either through changes to the solvent environment (i.e. pH and ion content of the cytoplasm) or through interactions with other nearby proteins. For FtsZ, these could be neighboring filaments or modulatory proteins.

Using computational predictions, the Pappu lab has generated sequence variants of the B. subtilis FtsZ CTL with different inherent properties. By changing the order of charged residues in a given IDP sequences, linkers that are either more extended or undertake non-globular conformations with hairpin-like structures have been generated. I have successfully cloned these CTL variants into B. subtilis using an approach identical to the way I created CTL chimeras in Chapter 3. I am currently investigating the in vitro assembly properties of purified protein and in vivo phenotypes of cells expressing these FtsZ linker variants. These experiments will allow us
to test the computational predictions and determine whether they have biological significance. Another goal is to continue to explore how the domains of FtsZ, specifically the core and CTL, work together to yield self-assembling subunits and maintain cytokinetic ring integrity during cell division.

While determining whether the CTL conformation affects FtsZ function is critical, it is also important to experimentally determine how flexible the native CTL and its variants really are. To accomplish this, single-molecule force spectroscopy can be used. Using a dual-beam trap, one can envision an FtsZ subunit adhering to a polystyrene bead trapped by one laser that also bound to FtsA (or another modulatory protein known to bind the GHP) on a bead trapped by the other laser. The FtsZ-FtsA interaction links the two beads through the CTL. Force is then applied by moving one of the traps to measure how much force it takes to extend the CTL. Information from these types of experiments would help determine whether a force like that modeled to be created by FtsZ filament bending (~10-30 pN) would be enough to extend the CTL. This would have implications for the force generation capabilities of FtsZ and whether or not FtsZ actually does generate most of the constrictive force.

**FtsZ CTL and CTV Chimeras as Tools for Understanding FtsZ-Modulatory Protein Interactions:**

The different FtsZ chimeras described in Chapters 2 and 3 can be used to study FtsZ behavior beyond how FtsZ interacts with itself. These modified FtsZs display distinctive assembly properties, such as ability to form lateral interactions or single-stranded filaments under different buffer conditions, which can be exploited to reveal how different modulatory proteins interact with FtsZ.
Of the many FtsZ modulatory proteins identified so far, a clear mechanism for how they affect FtsZ assembly has only been established in a few. SulA has been identified as a monomer sequester (Chen et al., 2012), while ZapA bundles FtsZ filaments through crosslinking (Dajkovic et al., 2010). For others, a definitive mechanism has not been determined but a combination of in vitro and in vivo work has developed models for filament severers, capping proteins, and bundling inhibitors (Chien et al., 2012; Haeusser et al., 2004; Shen and Lutkenhaus, 2010).

Testing the interaction between different FtsZ modulators and FtsZ CTL and CTV variants will allow better clarification of these mechanisms. Our lab has already provided such an example. To better establish how the inhibitor UgtP modulates FtsZ, assembly of the B. subtilis CTV variant Bs FtsZ NENDEG was tested in the presence of UgtP. By measuring a 60% reduction in assembly of Bs FtsZ NENDEG in the presence of UgtP, it was concluded that UgtP either acts as a severer of filament or a filament cap (Chien et al., 2012). If UgtP modulated FtsZ assembly by preventing bundling, Bs FtsZ NENDEG should show no reduction in assembly in the presence of UgtP. Though additional experiments would be needed to firmly differentiate severing from capping, the use of the FtsZ NENDEG mutant proved useful in eliminating one mechanism of inhibition.

Other FtsZ chimeras can be used in a similar approach. Some CTL variants described in Chapter 3 (FtsZ CTLE, FtsZ CTLA50) do no retain the ability to form lateral filament interactions. These chimeras provide an advantage over CTV variants in that they maintain the native CTV sequence. Since many FtsZ modulatory protein bind the FtsZ GHP (which includes the CTV), alterations to this region may impact the binding of FtsZ to those modulatory proteins. The CTL variants overcome this potential problem, especially if comparing to wild-type FtsZ to determine whether FtsZ bundling is affected or not.
Concluding Remarks

This dissertation establishes previously unidentified roles for the C-terminal domains of the FtsZ in protofilament assembly and lateral interaction formation in vitro and Z-ring stability in vivo. Through elucidating the mechanism by which FtsZs from two different bacterial species, *B. subtilis* and *E. coli*, differ in ability to form filament bundles, this work defines a new region of the FtsZ subunit, the CTV. Charge composition of the CTV modulates FtsZ lateral interaction potential and changing the sequence to a form that promotes single-stranded filament formation causes a destabilization of the Z-ring in *B. subtilis* cells. Moreover, the FtsZ CTL, irresolvable by crystallography and with a role previously unclear, is shown to function as an IDP and is crucial for FtsZ head-to-tail assembly of filaments and Z-ring formation. While the sequence of the CTL is unimportant as long as it remains an IDP, the length of the linker must be between 25 to 100 residues. Longer CTLs do not support division in *B. subtilis*. Together, these data support a model in which the CTL behaves as a flexible tether that allows FtsZ filaments to anchor to the membrane and help generate a constriction force while also undertaking other conformations that position the CTV next to adjacent FtsZ filaments to promote filament bundling necessary for maintaining a Z ring throughout cell division.

This dissertation has furthered our understanding of FtsZ self-assembly and how a Z ring functions during cell division. New opportunities for exploring FtsZ assembly have been uncovered. It will be particularly exciting to see how FtsZs from other bacterial species behave and whether assembly mechanisms are conserved. Most importantly, this work shows that studying proteins requires an understanding beyond conventional structural determinations. Proteins are dynamic, flexible molecules that require multiple parts working together to result in a particular function and all domains must be considered in their analysis.
References


Appendix:

A moonlighting enzyme links *Escherichia coli* cell size with central metabolism

Norbert S. Hill, Paul J. Buske, Yue Shi, and Petra Anne Levin

Abstract

Growth rate and nutrient availability are the primary determinants of size in single-celled organisms: rapidly growing *Escherichia coli* cells are more than twice as large as their slow growing counterparts. Here we report the identification of the glucosyltransferase OpgH as a nutrient-dependent regulator of *E. coli* cell size. During growth under nutrient-rich conditions, OpgH localizes to the nascent septal site, where it antagonizes assembly of the tubulin-like cell division protein FtsZ, delaying division and increasing cell size. Biochemical analysis is consistent with OpgH sequestering FtsZ from growing polymers. OpgH is functionally analogous to UgtP, a glucosyltransferase that is a growth rate-dependent inhibitor of cell division in *Bacillus subtilis*. In a striking example of convergent evolution, OpgH and UgtP share no homology, have distinct enzymatic activities, and appear to inhibit FtsZ assembly through different mechanisms. Comparative analysis of *E. coli* and *B. subtilis* reveals conserved aspects of growth rate regulation and cell size control that are likely to be broadly applicable. These include the conservation of uridine diphosphate glucose as a proxy for nutrient status and the use of moonlighting enzymes to couple growth rate-dependent phenomena to central metabolism.
Author Summary

The observation that growth rate and nutrient availability strongly influence bacterial cell size was made over forty years ago. Yet, the molecular mechanisms responsible for this phenomenon have remained elusive. Using a genetic approach, we identified proteins responsible for increasing *Escherichia coli* cell size under nutrient-rich conditions. Our data indicate that OpgH, a glucosyltransferase involved in cell envelope biogenesis, interacts with FtsZ, a key component of the bacterial cell division machinery. In the presence of a modified sugar, UDP-glucose, OpgH interacts with FtsZ to delay the timing of division machinery assembly. Comparison of the *E. coli* pathway with the parallel *Bacillus subtilis* pathway illuminates a striking example of convergent evolution in which two highly divergent bacteria employ unrelated glucosyltransferases for an essential part of cell cycle regulation and reveals aspects of metabolic and physiological control that are potentially applicable to all forms of life.
Introduction

Cell size control is a fundamental aspect of the cell cycle. Coordinating cell growth with division is essential to ensure that daughter cells have sufficient room for cytoplasmic and genetic material and are the correct size for a given condition or developmental fate. Despite the universal requirement for size control, how cells are able to detect the achievement of a particular size and communicate this information to the division apparatus remains an unresolved question in cell biology [1].

Nutrient availability is a primary determinant of cell size for single-celled organisms. In their seminal 1958 studies Schaechter, Maaløe, and Kjeldgaard determined that Salmonella cell size is coupled to growth rate, which is itself a function of nutrient availability [2]. Later work established that growth rate and nutrient availability are conserved determinants of cell size. Escherichia coli and Bacillus subtilis both coordinate cell size with nutrient availability, as do single-celled eukaryotes including the classic cell cycle model organism Schizosaccharomyces pombe [3,4,5].

To coordinate growth rate and nutrient availability with size, cells must have a mechanism to transmit information about growth rate and metabolic status to the division machinery. In B. subtilis, cell size is coordinated with central metabolism in part through uridine diphosphate glucose (UDP-glucose)-dependent changes in the oligomerization potential of the glucosyltransferase, UgtP [6,7]. During growth in nutrient-rich medium UDP-glucose, synthesized in a two-step pathway from glucose-6-phosphate, stimulates interaction between UgtP and the highly conserved tubulin-like cell division protein FtsZ, delaying assembly of the division machinery and increasing cell size. Conversely, during growth in nutrient-poor medium and/or in the absence of UDP-glucose, UgtP favors self-interaction. This permits division to
proceed unimpeded yielding a smaller size. UgtP is a moonlighting enzyme also required for synthesis of the diglucosyl-diacylglycerol (Di-glc-DAG) anchor for lipoteichoic acid (LTA), an anionic polymer that is a major component of the Gram-positive cell wall. Loss-of-function mutations in \textit{ugtP} disrupt synthesis of the Di-glc-DAG moiety, but does not impact LTA synthesis [8].

Previous reports have indicated that inactivating UDP-glucose synthesis by inactivating the phosphoglucomutase, \textit{pgm}, results in a \~25\% reduction in \textit{E. coli} cell size under nutrient-rich conditions [9,10]. This phenotype suggests that \textit{pgm} mutant cells are unable to properly coordinate cell division with nutritional conditions and, furthermore, implicate UDP-glucose as a widely conserved intracellular proxy for nutrient-dependent size control. Interestingly, despite the apparent conservation of UDP-glucose as a signaling molecule, the identity of the effector is less obvious. As a Gram-negative bacterium, \textit{E. coli} does not synthesize LTA and computational analysis does not reveal a \textit{ugtP} homolog within its \~4.6 MB genome.

Here we report the identification and characterization of the integral inner-membrane protein OpgH as a UDP-glucose-activated inhibitor of FtsZ ring formation in \textit{E. coli}. Genetic and biochemical data indicate that OpgH interacts directly with FtsZ via its N-terminal domain to inhibit division in a UDP-glucose-dependent manner. Although OpgH and UgtP are both glucosyltransferases they share no homology, have distinct enzymatic activities, and inhibit FtsZ assembly through different mechanisms. In all, this work is a significant advance in our understanding of cell size control in \textit{E. coli}. However, taken with our previous work in \textit{B. subtilis} [6], this work exposes a remarkable instance of convergent evolution revealing themes in growth rate regulation that are potentially applicable to all domains of life.
Results

*E. coli* utilizes UDP-glucose to couple cell size with nutrient availability:

Previous work from our lab and others suggests that *E. coli* may employ UDP-glucose as an intracellular proxy for nutrient availability in the regulatory circuit responsible for coupling cell size with growth rate [10,11]. To test this we examined the size of wild type cells and pgm null mutants that are defective in the first step of UDP-glucose biosynthesis (Figure 1A). If UDP-glucose is central to the nutrient-dependent control of *E. coli* cell size, the size differential between wild type and pgm mutant cells should be greatest under nutrient-rich conditions and least under nutrient-poor conditions.

Measurements of the cross-sectional area of cells cultured under a range of nutrient conditions indicate that pgm mutants are indeed defective in the growth-rate-dependent control of cell size (Figure 1B). In LB + 0.2% glucose (LB-glucose), the average cross-sectional area of wild-type *E. coli* was 5.66μm² while pgm mutants were 4.24μm², a difference of over 25%. However, the size inequity dissipated under nutrient-poor conditions. pgm mutants were only 4% smaller than wild type when cultured at a growth rate 4-times slower in a minimal growth medium supplemented with 0.4% succinate (AB-succinate).

Defects in UDP-glucose biosynthesis increase the frequency of FtsZ rings over incompletely segregated nucleoids:

Rapidly growing cells are not only longer, but also have more total DNA, a consequence of multifork replication. Multifork replication is a phenomenon that allows *E. coli* and other bacteria to sustain mass doubling times shorter than the period required to complete chromosome replication and division. In previous work we determined that growth rate-dependent increases in
cell size help prevent aberrant assembly of the division machinery over unsegregated bacterial chromosomes (nucleoids) in \textit{B. subtilis} [6]. To determine the impact of a reduction in \textit{E. coli} cell size on the frequency of division rings formed over nucleoids, we visualized FtsZ ring formation in rapidly growing \textit{pgm} mutant cells with an inducible copy of \textit{ftsZ} fused to \textit{gfp} [12].

Consistent with growth rate-dependent increases in cell size ensuring that \textit{E. coli} cells have sufficient room for DNA segregation during multifork replication, we observed a near two-fold increase in the frequency of division rings positioned over nucleoids in \textit{pgm} mutants relative to wild type. In accordance with previous studies [13], approximately 20\% (130/654) of wild type cells had an FtsZ ring over chromosomal material. The frequency increased to 35\% (246/662) in diminutive \textit{pgm::kan} cells (Figure 1C; Table S1). This suggests that the growth rate-dependent size increase is, in part, a mechanism to spatially coordinate the excess DNA generated by multifork replication.

**Defects in UDP-glucose biosynthesis stabilize FtsZ assembly at midcell:**

To coordinate size with growth rate, cells must evaluate nutrient availability and subsequently transmit that information to the division machinery. To assess the impact of UDP-glucose on FtsZ assembly in \textit{E. coli}, we determined if defects in UDP-glucose biosynthesis were sufficient to suppress the conditional lethality of the heat-sensitive \textit{ftsZ84} allele. FtsZ84 (G105S) supports assembly of the division ring at 30\degree C, but is unable at 42\degree C, leading to extensive filamentation and cell death [14,15].

Consistent with increases in UDP-glucose activating an inhibitor of FtsZ assembly, the viability of \textit{pgm::kan ftsZ84} double mutants was ~4.5-fold higher than \textit{ftsZ84} alone under restrictive conditions (Figure 1D). The plating efficiency (CFU restrictive/CFU permissive) of
ftsZ84 cells grown at 30°C versus 42°C was just 0.013% (± 0.001). In contrast, pgm::kan ftsZ84 double mutants exhibited essentially no reduction in viability under identical conditions 99.2% (± 0.3). Notably, this is the first example of a loss-of-function mutation that suppresses the heat sensitivity of ftsZ84 without increasing the intracellular concentration of FtsZ84 (Figure S1). Together, the data presented in Figure 1 provides evidence for a UDP-glucose-activated factor coupling cell division directly to carbon metabolism.

Defects in the glucosyltransferase OpgH reduce E. coli cell size:

To identify the UDP-glucose-dependent regulator of E. coli cell size, we systematically screened kanamycin resistance cassette insertions in genes predicted to be associated with UDP-glucose synthesis or utilization for defects in cell size, taking advantage of the Keio mutant collection (Figure 1A) [16]. Importantly, mass doubling rates in these mutants were indistinguishable from wild type in both nutrient-rich and nutrient-poor conditions (Table S2).

Of the 16 mutants we screened, knockouts in only three genes pgm, galU encoding a pyrophosphorylase required for synthesis of UDP-glucose from glucose-1-phosphate, and opgH (mdoH, b1049, EcoCyc:EG11886, UniProt:P62517) encoding a Family II glucosyltransferase, resulted in a statistically significant reduction in cell size (Figure 2A, 2B; Figure S3). Wild-type MG1655 cells had an average area of 5.66μm². The galU::kan and opgH::kan mutants were 18% (4.66μm²) and 12% (5.01μm²) smaller than wild type, respectively. As previously shown in Figure 1B, pgm null cells were 25% smaller at 4.24μm². Importantly, of the six known E. coli enzymes that utilize UDP-glucose (GalE, GalT, Ugd, OpgG, OpgH, and OtsA) mutations in only one, opgH::kan, led to a statistically significant size reduction (p > 0.05). (Length and width data of the various mutants is presented in Table S2.) Why mutations in galU and opgH do not reduce
cell size to the same extent as the *pgm::kan* mutation is not readily apparent, however it is reminiscent of what we have observed for mutations in the analogous genes in *B. subtilis* [6].

**OpgH inhibits division in a UDP-glucose-dependent fashion:**

Based on our study of the parallel pathway in *B. subtilis*, we speculated that OpgH, rather than Pgm or GalU, was the UDP-glucose-dependent regulator of cell division in *E. coli*. To test this hypothesis, we measured the average size of cells expressing either *pgm*, *galU*, and *opgH* (fused with an N-terminal thioredoxin and a C-terminal polyhistidine) from a high-copy plasmid cultured in nutrient-rich media.

Consistent with our prediction, induction of *thio-opgH-his*, though not *thio-pgm-his* nor *thio-galU-his*, led to severe filamentation indicative of a block in cell division (Figure 2C, 2D). Cells expressing *thio-opgH-his* increased cell length over five-fold, from an average of ~2.5μm to ~13.2μm after 4h of induction. Expression of either the *thio-pgm-his* or *thio-galU-his* fusion had a negligible impact on cell size.

UDP-glucose was absolutely required for OpgH-mediated division inhibition. Expression of wild-type *thio-opgH-his* in a *pgm::kan* background had no significant impact on cell size (Figure 2C, 2D; Figure S4). Cell size was similarly wild-type in cells expressing a mutant allele of *opgH* (*PIC249AIA*) defective in residues of the putative UDP-glucose binding site [17]. Strains with this mutation were also unable to complement normal glucosyltransferase function (Figure S5). Together, the data presented in Figure 2 suggests OpgH functions as a UDP-glucose-dependent antagonist of cell division.

**OpgH regulates cell size independent from its role in OPG synthesis:**
OpgH is an inner-membrane glucosyltransferase that synthesizes osmoregulated periplasmic glucans (OPGs); branched glucans consisting of 5 to 13 glucose residues joined by $\beta$-1-2 linkages and branched by $\beta$-1-6 linkages [18,19]. OpgH is responsible for the synthesis and subsequent periplasmic delivery of $\beta$-1-2 poly-glucose chains from UDP-glucose. $opgH$ is co-transcribed with $opgG$, which encodes a periplasmic protein required for generating the $\beta$-1-6 branches [20].

The loss of OPG synthesis has been implicated in a range of abnormal phenotypes including deficiencies in: envelope stability, flagellar synthesis and motility, phage infectivity, biofilm formation, and pathogenicity [21,22,23,24,25,26,27,28,29]. While some of these phenotypes can be attributed to an altered cell envelope, the majority are tied to aberrant activation of the Rcs phosphorelay [22,30,31]. To eliminate the possibility that the cell size defect was a secondary consequence of either lacking OPGs and/or Rcs-mediated gene expression, we examined the size of cells defective for OPG synthesis or Rcs activity.

Our data shows that OpgH’s role in size modulation is independent of OPG synthesis or activation of the Rcs system. Inactivating $opgG$, thereby eliminating OPG production either by a non-polar mutation in $opgG$ or by complementing $opgH$ in trans in an $opgGH$ double mutant, had no impact on cell size (Figure 2A; Figure S6A).

Based on previous reports indicating that one of the six promoters driving $ftsZ$ is positively regulated by Rcs [32,33], we were particularly concerned that induction of Rcs in the absence of UDP-glucose or OPGs might lead to overexpression of $ftsZ$, which in turn would reduce cell size. However, FtsZ levels are wild-type in $\Delta pgm$, $\Delta galU$, and $\Delta opgH$ mutant cells (Figure S6B). (FtsZ would have to be overexpressed by ~50% to translate into a size reduction of 15-20% [10,34].) Furthermore, a $\Delta opgH/\Delta rcsB$ double mutant was ~10% smaller than an $\Delta rcsB$
single mutant (Figure S6B). Deletion of rpoS, a transcriptional target of the Rcs system encoding the stationary phase transcription factor Sigma S [35,36], had no impact on ΔopgH or Δpgm mutant size (Figure S6C). This data is not the first example of wild-type FtsZ levels in circumstances of Rcs induction [37,38].

OpgH localizes to the division ring only in nutrient-rich conditions, yet independent of UDP-glucose:

To determine OpgH’s subcellular localization pattern, we used antiserum raised against OpgH’s N-terminal cytoplasmic domain. During growth in LB-glucose (□ = 21), OpgH exhibited both peripheral and midcell localization. Of the 203/227 (89%) cells with an FtsZ ring, OpgH colocalized with FtsZ at midcell in 96% (195/203) of cells (Figure 3A). OpgH midcell localization was contingent on nutrient-rich conditions. At a slower growth rate (□ = 38), the covariance of OpgH and FtsZ medial localization was significantly reduced [FtsZ at midcell 69% (170/248); covariance 36% (61/170)]. When growth rates were further reduced by culturing cells in AB-glucose (□ = 60), OpgH was relegated to the cell periphery, rarely colocalizing with an FtsZ ring (3% 3/112) [55% (112/207) of cells had an FtsZ ring]. OpgH localization to midcell was dependent on FtsZ. We were unable to detect an instance in which OpgH localized to midcell in the absence of an FtsZ ring. Further, medial localization of OpgH was abolished when ftsZ was depleted (Figure 3B).

Based on our work the B. subtilis functional homolog UgtP, we speculated that changes in the intracellular levels of OpgH’s substrate, UDP-glucose, might be responsible for its nutrient-dependent localization [6]. However, OpgH localization was unperturbed in either a background unable to synthesize UDP-glucose (pgm::kan) or a mutation in the putative UDP-
glucose binding region (Figure 3C). Midcell covariance of OpgH and FtsZ localization at midcell in the pgm::kan cells was 91% (178/196) and 93% (212/227) in the nucleotide sugar-binding mutant when cultured in LB-glucose. Together, this data supports a model in which OpgH dynamically localizes to the division machinery in a growth rate-dependent, but UDP-glucose-independent manner.

The N-terminus of OpgH is both necessary and sufficient for division inhibition:

OpgH is an 848 amino acid (97 kDa) integral inner-membrane protein. Reporter fusion analysis indicates that OpgH is composed of eight transmembrane domains with both N- and C-termini residing in the cytoplasm (Figure 4A) [39]. OpgH has three significant cytoplasmic domains referred to here as OpgH<sup>N</sup> (1-138), OpgH<sup>M</sup> (211-514), and OpgH<sup>C</sup> (702-848). OpgH<sup>M</sup>, the largest domain, contains the protein’s putative UDP-glucose binding domain based upon sequence homology.

To identify determinants within OpgH required for division inhibition, we expressed thioredoxin/polyhistidine fusions to opgH<sup>N</sup>, opgH<sup>M</sup>, or opgH<sup>C</sup> from an arabinose inducible promoter on multicopy vectors in an opgH::kan mutant background. Induction of thio-opgH<sup>N</sup>-his, but not thio-opgH<sup>M</sup>-his or thio-opgH<sup>C</sup>-his, resulted in a substantial increase in cell size relative to an uninduced control (Figure 4B, 4C). The area of cells expressing Thio-OpgH<sup>N</sup>-His increased more than two-fold ~3h post-induction (from 2.9μm to 5.9μm), indicative of a partial block in division. The differential in division inhibition efficacy between OpgH and OpgH<sup>N</sup> may be the consequence of the former being at the membrane where it can more readily access the division apparatus. Expression of an OpgH<sup>N</sup> deletion mutant (thio-opgH(Δ1-210)-his) had no impact on cell size (Figure 4C). These data are consistent with a model in which UDP-glucose
binding to OpgH^M leads to a conformational change that promotes OpgH^N mediated division inhibition.

Expression of thioredoxin fusions to a set of nested deletions within the OpgH^N domain identified an 18 amino acid peptide (residues 83-101) that was sufficient for division inhibition (Figure 4C; Figure S7). However, systematic alanine substitutions to this region or deletion of all 18 residues had no significant impact on the ability of OpgH^N to mediate division inhibition (Figure 4C; data not shown). This finding suggests the presence of at least one additional determinant within OpgH^N that is sufficient for its role in division inhibition.

Consistent with a role in cell division, OpgH^N localized to midcell independent of the rest of the protein. Immunofluorescence microscopy using antibodies against the His tag, indicated that of the three cytoplasmic domains, only Thio-OpgH^N-His exhibited medial localization on its own. During growth in LB-glucose, 84% (379/452) of cells expressing thio-opgH^N-his displayed colocalization with FtsZ at midcell (Figure 4D). Strikingly, a Thioredoxin fusion to the 18 residue fragment of OpgH^N (residues 83-101) that was sufficient for division inhibition, also colocalized with FtsZ in 82% (169/205) of cells (Figure S7B).

Although OpgH^N was sufficient for medial localization, it was not necessary. A thioredoxin fusion to the OpgH(Δ1-210), the N-terminal deletion mutant, colocalized with FtsZ 67% of the time (148/221) during growth in LB-glucose, implying the N-terminal domain is not necessary for medial localization of OpgH (Figure S7B). Given the inability of the OpgH^M or OpgH^C domains to localize on their own, we hypothesize that the second localization determinant is situated in a transmembrane and/or periplasmic region of the protein (see Discussion).
OpgH inhibits division by blocking FtsZ ring formation:

In light of our finding that cytoplasmic OpgH\(^N\) was capable of blocking division in vivo (Figure 4B, 4C), as well as genetic data suggesting that the loss of UDP-glucose positively impacts FtsZ assembly at midcell (Figure 1D), we speculated that OpgH might modulate cell division through direct interactions with FtsZ. To test this possibility, we measured the proportion of cells with FtsZ rings following induction of either thio-his, thio-opgH-his or thio-opgH\(^N\)-his. If OpgH inhibits division by antagonizing FtsZ polymerization dynamics, then induction of thio-opgH-his or thio-opgH\(^N\)-his should reduce the number of FtsZ rings.

For this experiment, dilutions were calibrated to ensure cells would be in early exponential growth at each time point. Samples were taken at 25-minute intervals for ~3h, fixed, and the percentage of cells with an FtsZ ring were scored using immunofluorescence microscopy. Induction of both thio-opgH-his as well as thio-opgH\(^N\)-his nearly abolished cells with FtsZ rings by ~2.5h post-induction (Figure 5A).

As an additional test of the impact of UDP-glucose and OpgH on FtsZ assembly dynamics in vivo, we evaluated the effects of overexpressing the spatial cell division regulator MinD. The precise temporal and spatial regulation of cell division is achieved through a concert of factors that modulate FtsZ assembly. Overexpression of an FtsZ inhibitor, and the associated lethality, can be balanced by deletion of another inhibitor [6,40].

Consistent with defects in UDP-glucose synthesis or deletion of opgH enhancing FtsZ assembly, inactivating genes in the metabolic pathway suppressed lethality associated with minD overproduction (Figure 5B). Two-fold overexpression of MinD reduced the plating efficiency of wild-type E. coli to 0.03%. However, overexpression of MinD to identical levels in pgm null
cells resulted in a plating efficiency of 3.9%, >100-fold higher than in the wild-type background (Figure 5B; Figure S2).

Consistent with OpgH interacting directly with FtsZ to delay division, an opgH::kan mutation increased the viability of cells overexpressing minD by ~1.5 fold (Figure 5B; Figure S2). Cells expressing opgH(PIC249AIA) phenocopied the opgH null results, further corroborating that UDP-glucose binding is essential for OpgH mediated inhibition of FtsZ assembly. In contrast to a loss-of-function mutation in pgm, opgH::kan was unable to suppress the heat-sensitivity of ftsZ84 allele.

Genetic data suggests that OpgH reduces the pool of FtsZ available for assembly into the cytokinetic ring:

To position OpgH within the regulatory hierarchy responsible for the temporal and spatial control of cell division, we examined the phenotypes of cells defective in either UDP-glucose synthesis (Δpgm) or opgH with defects in one of three previously characterized FtsZ antagonists: minCDE, slmA, or clpX. We reasoned that if OpgH shared a role in nucleoid occlusion, ring constriction, or preventing immediate division ring reassembly, then the double knockouts might exacerbate defects in division rings bisecting nucleoids, augmented cell size, or increased rate of minicells.

None of the double mutants had defects in growth rate, increased rates of division rings over unsegregated nucleoids, or a higher rate of inaccurately placed FtsZ rings during growth in LB-glucose (Table S1, data not shown). However, supporting a model in which OpgH reduces the effective concentration of FtsZ, a loss-of-function mutation in either opgH or pgm reduced the length of the abnormally long minCDE null mutants by approximately half (Figure S8; Table
S1). This finding suggests that the pools of FtsZ available for assembly into the cytokinetic ring are elevated in the absence of either opgH or pgm.

**OpgH\(^N\) interacts directly with FtsZ to inhibit assembly:**

Based on the genetic evidence suggesting that OpgH antagonizes division through direct interactions with FtsZ, we next determined if purified OpgH\(^N\) was sufficient to inhibit FtsZ assembly in vitro. For this experiment we employed 90° angle light scattering, a functional assay for FtsZ assembly [41,42]. All experiments were performed with OpgH\(^N\) (residues 1-138) and *E. coli* FtsZ in their native (untagged) form.

Consistent with our genetic data, OpgH\(^N\) inhibited FtsZ assembly in a dose-dependent fashion (Figure 5C, 5D). At a 1:1 ratio of FtsZ to OpgH\(^N\), FtsZ assembly was reduced by more than 30%. At higher ratios of FtsZ:OpgH\(^N\), 1:1.5 and 1:2, FtsZ assembly was reduced further by ~60% and ~80%, respectively. Heat-inactivating OpgH abolished its ability to inhibit FtsZ assembly (Figure S9A, S9B). This level of inhibition is slightly less potent than other *E. coli* FtsZ inhibitors [43,44,45,46], however it is consistent with a model in which OpgH functions to delay division, not prevent it.

**OpgH raises the apparent critical concentration of FtsZ required for GTP hydrolysis:**

Although 90° angle light scattering is valuable for measuring gross FtsZ assembly, it does not provide insight into the particular mechanism by which FtsZ assembly is obstructed. To elucidate the mechanism by which OpgH inhibits FtsZ assembly, we next determined the impact of OpgH\(^N\) on FtsZ’s intrinsic GTPase activity. FtsZ binds to GTP as a monomer, however dimerization is required for the formation of the GTPase active site and GTP hydrolysis (for
reviews of FtsZ assembly dynamics see [7,47,48,49,50]). Factors that bind to FtsZ monomers and prevent them from being added to growing polymers (so-called sequesters) reduce GTP hydrolysis activity by interfering with dimerization and the formation of the GTPase active site [7,51,52,53]. Conversely, factors that inhibit FtsZ assembly through other means (severing preformed polymers or interfering with lateral interactions between polymers) have little impact on GTP hydrolysis [43,54,55,56].

Consistent with a mechanism in which OpgH sequesters FtsZ monomers and prevents them from assembling into single-stranded polymers, OpgH\textsuperscript{N} reduced FtsZ’s intrinsic GTP hydrolysis activity in a dose-dependent manner. Under our conditions, FtsZ hydrolyzed GTP at a rate of 5.1 GTP/minute/FtsZ (Figure 6A). GTP hydrolysis was reduced by 25% at a 1:1 ratio (FtsZ:OpgH\textsuperscript{N}), 55% at 1:1.5, and 84% at 1:2. OpgH\textsuperscript{N} did not exhibit any significant GTPase activity on its own. Heat-treated OpgH\textsuperscript{N} had no effect on FtsZ’s GTPase activity (Figure S9C). Further, control experiments using a different GTPase, demonstrate that OpgH\textsuperscript{N} does not interfere with the regenerative, NADH coupled GTPase assay (Figure S9D).

Monomer binding reduces the pool of FtsZ subunits available for assembly. A hallmark of a sequestration-like mechanism is an increase in the apparent critical concentration (CcApp) of FtsZ required for GTP hydrolysis. To determine if OpgH\textsuperscript{N} impacts FtsZ’s CcApp, we measured GTP hydrolysis rates in reactions that kept levels of OpgH\textsuperscript{N} constant, but increased the FtsZ concentration. Plotting GTP hydrolysis per minute versus FtsZ concentration identifies the X intercept for each OpgH\textsuperscript{N} concentration. This intercept is equivalent to FtsZ’s critical concentration under that condition [53].

In support of a model in which OpgH is a monomer binding protein, the addition of OpgH\textsuperscript{N} increased FtsZ’s CcApp for GTP hydrolysis. In the absence of OpgH\textsuperscript{N}, \textit{E. coli} FtsZ
exhibited a CcApp of 0.69μM. The CcApp was increased to 1.31μM at a 2.5μM OpgH^N concentration, to 1.85μM at 5μM OpgH^N, and finally to 4.08μM in 10μM OpgH^N (Figure 6B).
Discussion

Classic work conducted over 50 years ago first identified nutritional content and growth rate as primary determinants of bacterial cell size [2]. Here we report that two factors, UDP-glucose and the glucosyltransferase OpgH, govern the regulatory circuit responsible for coordinating *E. coli* cell size with nutrient availability. Our genetic and biochemical data support a model in which binding of UDP-glucose by OpgH promotes a conformational change revealing an FtsZ interaction site on the OpgH$^N$ domain. Interactions between OpgH$^N$ and FtsZ obstruct assembly and/or maturation of the cytokinetic ring, delaying division and increasing cell size (Figure 7A). During slow growth in nutrient-poor conditions or defective for UDP-glucose production, OpgH assumes a conformation that obscures the interaction between OpgH$^N$ and FtsZ. FtsZ assembly is able to proceed unimpeded, reducing cell size (Figure 7B). OpgH, specifically OpgH$^N$, is well conserved in the order Enterobacteriales, suggesting functional conservation in close relatives of *E. coli* (Figure S10).

Based on our genetic and biochemical data, we propose that nutrient-dependent activation of OpgH leads to a reduction of FtsZ available for assembly into the cytokinetic ring. FtsZ levels are constant regardless of growth rate [57]. Nutrient-dependent increases in OpgH activity should translate into proportional reductions in the pool of available FtsZ subunits. Cells increase in size until they have accumulated sufficient FtsZ to overcome OpgH-mediated inhibition and support assembly of a mature cytokinetic ring [1]. This model is consistent with data from the Vicente lab demonstrating that modest reductions in FtsZ pools lead to a transient increases in cell size, but do not impact the timing of division under steady-state conditions [58].

Multiple determinants ensure OpgH localization to the division machinery:
OpgH’s localization to the division apparatus only occurs in nutrient-rich conditions (Figure 3). Intriguingly, this growth rate-dependent localization is not controlled by interaction with its substrate, UDP-glucose, unlike its functional *B. subtilis* homolog UgtP [6]. Thus, the mechanism behind OpgH’s dynamic nutrient-dependent localization remains to be elucidated.

Analysis of OpgH deletion constructs suggests the presence of at least two determinants within the OpgH polypeptide are required for localization to the cytokinetic ring (Figure 4D; Figure S7B). One such determinant is within the soluble OpgH subdomain. The other determinant resides somewhere in residues 211-848. Intriguingly, residues 651-713, encoding OpgH’s 7th and 8th transmembrane domains, share homology to the SEDS (shape, elongation, division, and sporulation) family of proteins. The essential division protein FtsW and the shape determining protein RodA, two well-studied *E. coli* SEDS proteins, localize to the division apparatus through FtsZ-independent interactions [59,60]. We hypothesize that OpgH employs its SEDS-like domain to bolster its medial localization by interacting with other components of the division machinery.

Specifically why OpgH employs more than one mechanism for localization to the cytokinetic ring is not immediately apparent. However, we speculate that concentrating OpgH at midcell may be essential to ensure that its effective concentration is high enough to meaningfully impact cell division. Although we were unable to obtain quantitative measurements of native OpgH levels, similar to other groups [39], qPCR data suggests *opgH* transcripts are ~1000-fold less abundant than *ftsZ* transcripts (data not shown). A localization mechanism that depends on factors that localize to the division site downstream of FtsZ, would ensure that OpgH remains at midcell during the duration of the Z-period and is not displaced by competition with other FtsZ binding proteins.
A remarkable example of convergent evolution:

When viewed in the context of the parallel pathway in *B. subtilis*, this study highlights an exceptional example of convergent evolution. *E. coli* and *B. subtilis*, organisms separated by a greater evolutionary distance than *Homo sapiens* and *Saccharomyces cerevisiae*, both utilize UDP-glucose and unrelated glucosyltransferases in the regulatory circuit coupling cell size with growth rate and nutrient availability (Figure 7C).

Why UDP-glucose-utilizing glucosyltransferase enzymes were chosen to coordinate this phenomenon is an intriguing question. In general, moonlighting enzymes tend to be more valuable to an organism if the multiple functions are both beneficial at the same time [61]. Thus, the question arises are there circumstances in which an increased need for their sugar transferase activity coincides with a need to block division.

Both effectors have previously described roles in envelope biogenesis: OpgH produces periplasmic glucans and UgtP synthesizes the Di-glc-DAG anchor for LTA. These products are particularly important during conditions that prompt the osmotic stress response [8,62,63]. Curiously, shifting the osmotic or turgor pressure has been reported to cause a temporary arrest of cell division that is alleviated only after cells equilibrate to the new conditions [64,65]. Thus, while our work describes a role for OpgH and UgtP in nutrient-dependent size control, we speculate that both proteins may also function to inhibit division during osmotic stress or other cell envelope perturbations.

UDP-glucose is an ideal signaling molecule for transmitting information to the division apparatus under both conditions of rapid growth and cell envelope stress. Its accumulation is directly coupled to central carbon metabolism and is likely to accumulate primarily under
nutrient-rich conditions, the same conditions that support multifork replication and necessitate an increase in cell size. Likewise, consistent with a role in the osmotic stress response pathway, genes involved in UDP-glucose synthesis are up-regulated during cell envelope stress [66,67]. UDP-glucose is directly incorporated into the cell envelope in both Gram-positive and Gram-negative organisms.

Intriguingly, synthesis of both OPG and LTA is the primary source of diacylglycerol in *E. coli* and *B. subtilis* [68], raising the possibility that diacylglycerol may serve as a secondary messenger in the regulatory circuit governing bacterial cell size. (FabH, an enzyme involved in key steps of fatty acid biosynthesis, has recently been implicated in the nutrient-dependent control of cell size, suggesting another potential role for lipids in cell size homeostasis [69].)

In closing, our findings represent a major advance in our understanding of cell size control in *E. coli*, the predominant model system for the study of bacterial physiology. Moreover, through comparison with a parallel pathway in *B. subtilis*, our work reveals conserved aspects of growth rate regulation and cell size control -- including conservation of UDP-glucose, a molecule common to all domains of life, as a proxy for nutrient availability and the use of moonlighting enzymes to couple growth rate-dependent phenomena to central metabolism -- that are likely to be broadly applicable.
Experimental Procedures

Strains and Media:

*E. coli* strains and plasmids and their construction are described in Supporting Information (Text S1, Table S3, and Table S4). Cells were cultured in Luria-Bertani (LB) ± 0.2% glucose or AB defined media [70] supplemented with 10μg/ml thymine, ± 0.5% casamino acids (CAA), and either 0.2% glucose or 0.4% succinate as a carbon source. Cells were grown at 37°C and used for experimentation at early exponential growth phase (an OD of 0.15-0.3) unless otherwise stated. Standard techniques were employed for cloning, P1vir transductions, and other genetic manipulations.

Quantitative Immunoblotting:

Cells were lysed at early to mid-log phase (an OD<sub>600</sub> of 0.2-0.5) using physical or chemical techniques. Lysates were then normalized to either OD or total protein using a bicinchoninic acid (BCA) assay and subjected to SDS-PAGE. Immunoblots were performed using either rabbit α-MinD antibody (the gift of William Margolin), chicken α-His antibody (Millipore), rabbit α-FtsZ (the gift of David Weiss), or rabbit α-DnaA (the gift of Jon Kaguni) with cognate goat α-rabbit or donkey α-chicken secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Band intensity was determined using ImageJ software and processed in Microsoft Excel [71].

Immunofluorescence Microscopy:
Microscopy performed was essentially similar to [10]. Cells were fixed in paraformaldehyde similar to as described in [72]. His-tagged proteins were detected using a chicken α-polyhistidine tag antibody (Millipore) with cognate goat α-chicken serum conjugated to Alexa488 (Invitrogen). FtsZ was detected using affinity-purified polyclonal rabbit α-FtsZ serum (the gift of William Margolin) in combination with goat α-rabbit serum conjugated to Alexa546 (Invitrogen). OpgH was detected using mouse △-OpgH^N polyclonal serum with goat anti-mouse Alexa488 (Invitrogen). Genetic content was stained with DAPI.

Cell Size Measurements:

Cells in early to mid-log phase were stained with FM-464 and adhered to 15-well glass slides using poly-L-lysine. The length and width of cells were calculated using the Openlab software. Only length was measured in cases of severe cell filamentation when width was irrelevant to the interpretation.

*ftsZ84* and *minD* Plating Efficiency:

Plating efficiency was done as previously described [73]. Briefly, strains encoding *ftsZ84* or an inducible copy of *minD* were cultured in permissive conditions to early/mid-log phase (OD_{600} 0.1-0.4), back-diluted to an OD_{600} of 0.01 and grown to an OD_{600} of 0.15-0.3. Cultures were normalized to optical density and then serially diluted from 10^{-1} to 10^{-8}. Equal volumes were plated at permissive conditions (LB with 0.05% NaCl at 30°C for *ftsZ84*; LB-glucose + 0mM IPTG for *minD*) and restrictive conditions (LB with 0.05% NaCl at 42°C for *ftsZ84*; LB-
glucose + 0.15mM IPTG for minD). Plating efficiency was calculated as the ratio of colony forming units at restrictive versus permissive conditions.

**Protein Purification:**

Untagged *E. coli* FtsZ was purified as described previously [42]. Native OpgH\textsuperscript{N} was purified using the IMPACT Protein Purification System (New England Biolabs) as follows. BL21(DE3) cells harboring pBH616 (pTYB4 + opgH\textsuperscript{N}) was grown in one-liter cultures with 100\(\mu\)g/ml ampicillin at 37\(^\circ\)C to mid-log, then induced with 1mM IPTG for 4-6h. (Inducting at temperatures under 37\(^\circ\)C did not yield functional OpgH\textsuperscript{N}.) Cells were pelleted, washed in 1XPBS (pH 7.4), repelleted, and frozen at \(-80^\circ\)C for later use. On the day of purification, cell pellets were thawed and resuspended in 30ml of ice-cold IMPACT lysis buffer (20mM Tris-HCl, 500mM NaCl, 1mM EDTA, pH 8) containing 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma). Multiple passes in a prechilled French press cell at 1,000 lb/in\(^2\) lysed cells. All subsequent steps were performed at 4\(^\circ\)C. Lysates were cleared by centrifugation, then loaded on to pre-equilibrated columns with 10-15ml of chitin beads (New England Biolabs). The column was then washed with \(~10\) column volumes of IMPACT lysis buffer. Subsequently, IMPACT lysis buffer containing 0.05M dithiothreitol (DTT) was loaded to stimulate Intein autocleavage. The column was left incubating with DTT at 4\(^\circ\)C overnight (16-20h). The eluate yielded both the OpgH\textsuperscript{N}-Intein fusion (71.9 kDa) and OpgH\textsuperscript{N} (15.9kDa). The fractions were pooled and transferred into dialysis tubing (7,000 MWCO) and concentrated from \(~25\)ml to \(~1\)ml using polyethylene glycol (PEG) 12,000. The 1mL was immediately loaded onto a S-300 size exclusion column and equilibrated in OpgH\textsuperscript{N} buffer (20mM Tris-HCl, 100mM NaCl, 10%
glycerol, pH 8). OpgH^N fractions were pooled and transferred to dialysis tubing (7,000 MWCO) and concentrated with PEG. Aliquots were flash frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined by a Coomassie Plus (Pierce) assay using a SPECTRAmax Plus spectrophotometer (Molecular Devices) using a BSA standard.

90° Angle Light Scattering Assay:

Light scattering assays were performed as described previously using a DM-45 spectrofluorimeter (Olis) [40,73]. Readings were taken every 0.5s at 30°C. A baseline was established ~60s previous to the addition of 1mM GTP. The reaction mixtures contained a final concentration of 5μM FtsZ diluted in polymerization buffer (50 mM morpholineethanesulfonic acid (MES), 2.5 mM MgCl₂, 1 mM EGTA, 50 mM KCl, pH 6.5) ± OpgH^N or OpgH^N buffer (20mM Tris-HCl, 100mM NaCl, 10% glycerol, pH 8). Data was collected by SpectralWorks (Olis) and exported into Microsoft Excel for processing. Baseline corrections were applied in Microsoft Excel to remove the background signal from unassembled FtsZ.

Regenerative Coupled GTPase Assay:

FtsZ’s GTPase activity was evaluated as previously described [42] using a continuous, regenerative coupled GTPase assay [74]. Briefly, experiments were done in the same buffer conditions as used for light scattering. A 150μl reaction volume included: 1mM phosphoenolpyruvate (PEP), 80 units/ml lactose dehydrogenase, 80 units/ml pyruvate kinase, 250μM NADH, 1mM GTP then varying amounts of FtsZ and OpgH^N or equivalent volume of
respective buffer. Absorbance at 340nm was measured at 30°C for 3 min in a quartz cuvette (1cm path length) using a SPECTRAmax Plus spectrophotometer (Molecular Devices). Raw data from 60-120s was translated into activity with the extinction coefficient for NADH at 340nm of 6220 M$^{-1}$ cm$^{-1}$. 
References


**Figure 1**

*E. coli* utilizes UDP-glucose to coordinate nutrient availability with cell size.

(A) A simplified schematic of UDP-glucose production and utilization in *E. coli*. Substrates are in black font, enzymes are in red font. Enzymes crucial to coordination of size with growth rate are in dark red and bold. Metabolic pathway information was derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website [75,76]. (B) Cell area measurements of wild type (black squares) versus pgm::kan (red circles) in various growth media (from left to right: AB-succinate, AB-glucose, AB-succinate + casamino acids (CAA), AB-glucose + CAA, LB, and LB-glucose). (C) Occurrence of FtsZ rings over incompletely segregated nucleoids in wild type or smaller pgm::kan cells. Both strains encode a chromosomal copy of $P_{lac}$::gfp-ftsZ and were cultured in nutrient-rich conditions (LB-glucose with 1mM IPTG). GFP-FtsZ (top), DAPI stained DNA (middle), and the overlay with FtsZ in red and DNA in blue (bottom). White arrows indicate cells with a division ring over partially segregated chromosomes. Bar = 2.5μm. (See Table S1 for expanded analysis.) (D) Disrupting UDP-glucose production by disrupting pgm suppresses lethality of FtsZ84 in non-permissive conditions (42°C). Serial dilution plating of strains with ftsZ84 ± pgm::kan grown on nutrient-rich, low-salt media at 30°C (permissive) or 42°C (restrictive). See also Figure S1 (expression levels of FtsZ84).
Figure 2

OpgH acts as a nutrient-dependent division antagonist.

(A) Cell area measurements of mutants in UDP-glucose synthesis or utilization cultured in LB-glucose. WT is set to 100%. >250 cells were measured per sample, error bars are standard deviation (n > 3). The opgH null is complemented with a plasmid encoding $P_{lac}$::$opgH$-gfp and was cultured with 0.08mM IPTG. This fusion is shown to be functional for glucosyltransferase ability (Figure S5). * denotes p > 0.001, ** signifies p > 0.05 as judged by chi$^{2}$ analysis. (B) Micrographs of WT and knockouts of genes involved in UDP-glucose pathway grown in LB-glucose and stained with the membrane dye FM4-64. Bar = 5μm. (C) Representative micrographs and (D) cell length measurements of strains cultured in LB overexpressing genes in the UDP-glucose pathway from an arabinose inducible promoter. Uninduced cells are on the upper panel. Induced constructs are on the bottom panel. Cells are stained with FM4-64. Bar = 5μm. Error bar denotes standard deviation (n = 3). See also Table S2 (information of length, width, and growth rate), Figure S3 (measurements of cells defective in factors adjacent to the UDP-glucose biosynthesis pathway), and Figure S4 (Thio-OpgH-His overexpression levels).
Figure 3

OpgH localizes to midcell in a growth rate- and FtsZ-dependent manner.

Immunofluorescence localization of FtsZ and OpgH in various growth conditions or genetic backgrounds. (A) OpgH localizes at midcell with FtsZ only at fast growth rates. Wild type cells were grown in either LB-glucose (τ = 21’), AB-glucose + casamino acids (τ = 38’), or AB-glucose (τ = 60’). (B) OpgH is unable to localize to midcell in the absence of FtsZ. A strain encoding a sodium salicylate inducible copy of ftsZ (PL3180) was grown to mid-log phase and back-diluted into LB broth ± inducer (2.5µM sodium salicylate) for 2.5h. (C) The frequency of OpgH at midcell is independent of UDP-glucose. Congenic strains either encoding a deletion of a key gene in UDP-glucose biosynthesis (pgm::kan) or a mutation in OpgH’s putative UDP-glucose binding site. (A-C) DNA is stained by DAPI. Bar = 5μm. White arrowheads indicate OpgH midcell localization. OpgH is in green and FtsZ is in red in the overlays. The percent covariance of FtsZ and OpgH at midcell is indicated below the micrographs.
Figure 4

The N-terminal cytoplasmic region of OpgH is necessary and sufficient to inhibit cell division.

(A) A schematic representation of the inner-membrane glucosyltransferase OpgH [39]. (B) Overexpression of OpgH\textsuperscript{N} increases cell size in the opgH::kan strain. Micrographs of cells encoding arabinose inducible N-terminal thioredoxin fusions to each of OpgH’s cytoplasmic domains cultured in LB ± 0.5% arabinose. Cells are stained with FM4-64. Bar = 5μm. (C) Cell area measurements of cells with the various thio-opgH-his constructs. Cells were cultured in LB with either 0% arabinose (dark red bars) or 0.5% arabinose (light red bars). Error bars equal standard deviation (n = 3). (D) Immunofluorescence micrographs of various P\textsubscript{ara}:thio-opgH-his constructs following 2h of induction in LB + 0.5% arabinose. Overlays of OpgH (green) and FtsZ (red) localization are on the bottom row. Bar = 3μm. See also Figure S7 (localization data on additional deletion constructions).
Figure 5

OpgH^N is an inhibitor of FtsZ assembly.

(A) Induction of OpgH inhibits FtsZ assembly in vivo. Cells were sampled and imaged for immunofluorescence microscopy at 25 minute intervals following the induction of either thio-his (black squares), thio-opgH-his (dark grey diamonds), or thio-opgH^N-his (light grey circles). Cells were cultured in LB. >200 cells were evaluated per sample. Representative α-FtsZ immunofluorescence micrographs from time points 0’ and 150’ are shown in the lower left. (B) Mutations that disrupt synthesis of UDP-glucose or OpgH itself suppress the lethality of MinD overexpression. MinD is overexpressed by ~2-fold, which is at the threshold of lethality in WT. Error bars equals standard deviation (n = 3). (See Figure S2 for relative MinD expression levels.) (C) A representative 90º angle light scattering plot of FtsZ assembly ± OpgH^N. FtsZ is at 5μM, OpgH^N is at 10μM. Arrow indicates addition of 1mM GTP. (D) Concentration-dependent inhibition of FtsZ polymerization by OpgH^N. The ratio of FtsZ to OpgH^N is listed below. FtsZ is at 5μM in all cases. (See Figure S9A, S9B for additional controls.)
**Figure 6**

OpgH$^N$ appears to function as an FtsZ monomer sequestering protein.

(A) Concentration-dependent inhibition of FtsZ’s GTPase activity by OpgH$^N$. The GTP hydrolysis rate of 5μM FtsZ is shown at differing ratios of OpgH$^N$. OpgH$^N$ alone is at 5μM. Error bars equal standard deviation (n = 3). (B) FtsZ GTPase rates at increasing concentrations of OpgH$^N$. The critical concentration for assembly of FtsZ was determined at OpgH$^N$ concentrations of 0μM, 2.5μM, 5μM, or 10μM. (See Figure S9C, S9D for additional controls.)
Figure 7

Glucosyltransferase OpgH couples cell size to nutritional availability and growth rate in *E. coli*.

(A) The nucleotide sugar UDP-glucose acts as a proxy for nutritional status to ensure cells maintain an optimal size for a given growth rate. OpgH blocks division by inhibiting assembly of the essential bacterial cytoskeleton protein FtsZ. OpgH bound to UDP-glucose assumes a conformation where the N-terminus is able to interact with FtsZ. This interaction effectively reduces the pool of FtsZ able to participate in the formation and maturation of the FtsZ ring. (B) In nutrient-poor conditions, UDP-glucose is less available and no longer serves to promote interaction between OpgH and FtsZ. Consequently, division is unobstructed and cell size does not increase. (C) Evolutionarily divergent organisms *E. coli* and *B. subtilis* both utilize UDP-glucose and unrelated glucosyltransferases to coordinate growth rate-dependent size homeostasis. Both organisms have co-opted sugar transferases activated by UDP-glucose to antagonize assembly of FtsZ by different mechanisms.
Supplemental Text

Media, plasmid and strain construction:

Antibiotics were used at 100µg/ml ampicillin (Amp), 25µg/ml chloramphenicol (Cm), 12.5µg/ml tetracycline (Tet), and 25 or 50µg/ml kanamycin (Kan). Strains and plasmids employed are listed in Tables S3 and S4. Plasmid DNA and PCR fragments were purified using the Wizard miniprep or gel and PCR clean-up kits (Promega). PCR reactions used MG1655 genomic DNA as template and the high-fidelity VENT polymerase (NEB). A brief description of plasmid and strain construction is given below. Oligonucleotides used in PCR reactions are highlighted in bold, italics, or underlined to indicate initial annealing regions, restriction enzyme sites, and/or sites of mutagenesis, respectively. Recombineering strategies used plasmids (pKD3, pKD4, pKD46, & pCP20) from [1]. Many of the constructs were cloned into a pBAD/TOPO Thio-Fusion expression vector (Invitrogen) with a 5’ NcoI site.

pBH494:

\[ P_{lac}::opgH(PIC249AIA)-gfp \] was constructed by site directed mutagenesis of pBH425 \([P_{lac}::opgH-gfp]\) using the Quikchange method (Stratagene), the oligo

CGCACGGCGTTGATCATGGCTATCGCTAACGAAGACGTGAAC, and the complementing oligo.

pBH536:
pgm was amplified using oligos CCATGGCAATCCACAATCGTGCA
and TTACGCGTTTTTCAGAATTCGC. The resulting 1638bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH537:

galU was amplified using oligos CCATGGATGGCTGCCATTAATACGAAAGTC and TTACTTCTTAATGCCCATCTCTTC. The resulting 906bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH538:

The N-terminus of opgH was amplified using oligos

CCATGGATGAATAAGACAACCTGAGTACATT and ACGCAGGATGGTACCGACG.
The resulting 414bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH539:

The sugar-binding domain (middle cytoplasmic domain) of opgH was amplified using oligos CCATGGACGGCGTTAATGGGCTTCCTGCAA and CATACCCCTCACCAGGAACAGACG. The resulting 909bp amplicon was TOPO cloned into the ThioFusion expression vector.
pBH540:

The C-terminus of *opgH* was amplified using oligos

\[ \text{CCATGGCGTGCCACCGTTGGTCTGCGCACC and CGCATCCGGTTTACGCAATGC.} \]

The resulting 438bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH541:

*opgH* was amplified using oligos 5’ *CCATGGATGAATAAGACAACCTGAGTACATT* and 5’ *CGCATCCGGTTTACGCAATGC*. The resulting 2455bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH580:

The first half of the N-terminus (residues 1-67) of *opgH* was amplified using oligos

\[ \text{CCATGGATGAATAAGACAACCTGAGTACATT and TCCATCAGCAATCTGGCCA.} \]

The resulting 201bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH581:

The second half of the N-terminus (residues 68-138) of *opgH* was amplified using oligos
CCATGGCAGTTAATTAAGACGACGAAGGG and ACGCGCGATGGTACCGACG. The resulting 201bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH582:

opgH was amplified using oligos CCATGGGAAGCAAAACGCTCCTCGATG and ACGCGCGATGGTACCGACG. The resulting 165bp amplicon (coding for residues 83-138) was TOPO cloned into the ThioFusion expression vector.

pBH583:

opgH was amplified using oligos CCATGGGGCGCGTCTCTGGGGATCGC and ACGCGCGATGGTACCGACG. The resulting 120bp amplicon (coding for residues 98-138) was TOPO cloned into the ThioFusion expression vector.

pBH584:

opgH was amplified using oligos CCATGGCCGCGCTATCTGGGCTCGTTTG and ACGCGCGATGGTACCGACG. The resulting 75bp amplicon (coding for residues 113-138) was TOPO cloned into the ThioFusion expression vector.

pBH585:
opgH was amplified using oligos CCATGGCAGTTAATTAAGACGACGAAGGG and CTCTTCTTTGGTCAAACGAGCCAG. The resulting 165bp amplicon (coding for residues 68-123) was TOPO cloned into the ThioFusion expression vector.

pBH586:

opgH was amplified using oligos CCATGGCAGTTAATTAAGACGACGAAGGG and CGTGCATCGCGTCCACG. The resulting 165bp amplicon (coding for residues 68-112) was TOPO cloned into the ThioFusion expression vector.

pBH587:

opgH was amplified using oligos CCATGGCAGTTAATTAAGACGACGAAGGG and AAACATCGAGGAGCGTTTTGC. The resulting 165bp amplicon (coding for residues 68-90) was TOPO cloned into the ThioFusion expression vector.

pBH588:

opgH was amplified using oligos CCATGGGAAGCAAAACGCTCCTCGATG and CGTGCATCGCGTCCACG. The resulting 87bp amplicon (coding for residues 83-112) was TOPO cloned into the ThioFusion expression vector.
pBH589:

*opgH* was amplified using oligos **CCATGGCAGTTAATTAAAGACGACGAAGGG** and **CGTGACATCGCGTCCACG**. The resulting 165bp amplicon (coding for residues 83-101) was TOPO cloned into the ThioFusion expression vector.

pBH608:

*opgH* was amplified using oligos **CCATGGATGAATAAGACAACTGAGTACATT** and **ATAGGATCC TTCTGGCATCGCCTTCAGCTG** as well as **ATAGGATCCGTTAGGCCGTTTCCTGGGATC** and **ACGGCGGATGGTACCGACG** to create amplicons bp1-249 and bp303-414, respectively. The amplicons were then digested with BamHI, ligated, amplified using oligos **CCATGGATGAATAAGACAACTGAGTACATT** and **ACGGCGGATGGTACCGACG**. The resulting 375bp amplicon was TOPO cloned into the ThioFusion expression vector.

pBH664:

A *opgH* construct excluding the N-terminus and first two transmembrane domain was constructed by using oligos **CCATGGACGGCGTTAATGGGCTTCTGCAA** and **CGCATCCCGTTTACGCAATGC**. The resulting 1971bp amplicon was TOPO cloned into the ThioFusion expression vector (Invitrogen).
BH671:

opgH was amplified from pBH494 using oligos

CCATGGATGAATAAGACAACTGAGTACATT and CGCATCCGGTTTACGCAATGC.
The resulting 2455bp amplicon was TOPO cloned into the ThioFusion expression vector.

BH616:

The N-terminus of opgH was amplified using oligos

ATTCCATGGTTATGAATAAGACAACTGAGTACATT and

ATTCCCGGGACGGCGGATGGTACCGACG. The resulting fragment was digested with
NcoI and SmaI and ligated into pTYB4 (Invitrogen) yielding an in-frame opgHN-intein fusion.

BH643:

An insertion into opgG was achieved using lambda red recombination [1]. Amplifying
using oligo

CGTTGGTTGAGTGCTGCAGTAATGTTAACCCTGTATACAGTGTTAGGCTGGAGCTGC

TTC with

GCTGGTTTGCCTGACGCGGATTCCTCTGCGAGTTATGGGAATTAGCCATGGT

CC using pKD3 as template created a PCR product containing a chloramphenicol resistance
cassette flanked with homologous regions to opgG. The resulting amplicon was transformed into
BH249 (MG1655 + pKD46) to knockout opgG. The cmR cassette was removed using pCP20.
BH663:

opgH was amplified using pBH494 (\(P_{\text{lac}}::\text{opgH(PIC249AIA)-gfp}\)) as template with oligos CATCCGGTTTACGCAATGC and AAGCAGCTCCAGCCTACATTATTGGCAAGCCGCATCCG. Separately, a kanamycin resistance cassette was amplified with a homologous region 36 bp distal to the 3’ end of opgH from pKD4 [1] using oligos TGTAGGCTGGAGCTT and TGCAAAATCAATAAATTGCAGGAACGATGTA TGACATGGGAATTAGCCATGGT.

The two PCR products were then SOE’ed together by PCR using CATCCGGTTTACGCAATGC and TGCAAAATCAATAAATTGCAGGAACGATGTA TGACATGGGAATTAGCCATGGTC, which created a PCR product of ~4.0kb. This was then electroporated into WT MG1655 cells harboring the lambda red plasmid pKD46 to facilitate chromosomal integration. Kan\(^R\) colonies were then screened for the mutation by sequencing.

Chemotaxis assay:

For our purposes chemotaxis was used as an indirect assessment of OPG production. OPGs are monitored by the Rcs phosphorelay system. An absence of OPGs in the periplasm causes a down-regulation of flagellar synthesis and chemotaxis [2]. Early-log cultures (\(OD_{600} 0.15-0.4\)) were normalized to optical density. Subsequently, 1\(\mu\)l of culture was stabbed into a swarm plate (1% tryptone, 0.5% NaCl, and 0.3% agar) ± inducer. Plates were incubated at 30°C
for 20 hours and imaged. The diameter was calculated by the average of three measurements.

Quantitative Real-Time PCR:

_E. coli_ cells were harvested for RNA in early-log (an OD<sub>600</sub> of ~0.25) with the RiboPure™ Kit (Ambion), treated with the Turbo DNA-Free Kit™ (Ambion), and reverse transcribed for 1 hour at 42˚C using the RETROscript® Kit (Ambion). Template was diluted 10-fold and added to iTaq SYBR Green Supermix (Bio-Rad) and amplified with primers to either opgH (GGTGCTGTGTTTCCCTGCGAAGTTATTG and CAGCAACGATAATGTAACGCAGCAGAAG) or ftsZ (ATTTGGGTATCCTGACCGTTGCTG and ACTTTCAGCAGTTTTGCCTGTTCGGG) using an Applied Biosystems model 7500 thermocycler. Results were analyzed using the comparative Pfaffl method [3].

Chromosomal origins per cell:

Determination of _oriC_ per cell was determined by evaluating the flow cytometry profiles after replication run out identically to described in [4].
Supplemental Text References


Supplemental Table 1

Phenotypes of combining defects in UDP-glucose synthesis (Δpgm) or ΔopgH with inactivating characterized *E. coli* division inhibitors.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth rate (min)</th>
<th>Cell area (µm²)</th>
<th>Rings over nucleoids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wt</em></td>
<td>21.2</td>
<td>5.66</td>
<td>19.9% (130/654)</td>
</tr>
<tr>
<td>Δpgm</td>
<td>22.7</td>
<td>4.28</td>
<td>37.2% (246/662)</td>
</tr>
<tr>
<td>ΔopgH</td>
<td>21.2</td>
<td>5.03</td>
<td>32.1% (212/660)</td>
</tr>
<tr>
<td>ΔminCDE</td>
<td>24.0</td>
<td>10.2</td>
<td>23.5% (50/213)</td>
</tr>
<tr>
<td>ΔminCDE Δpgm</td>
<td>22.7</td>
<td>5.50</td>
<td>21.1% (114/540)</td>
</tr>
<tr>
<td>ΔminCDE ΔopgH</td>
<td>22.7</td>
<td>5.72</td>
<td>22.9% (120/524)</td>
</tr>
<tr>
<td>ΔslmA</td>
<td>21.9</td>
<td>5.12</td>
<td>25.6% (160/632)</td>
</tr>
<tr>
<td>ΔslmA Δpgm</td>
<td>23.0</td>
<td>4.00</td>
<td>37.3% (208/558)</td>
</tr>
<tr>
<td>ΔslmA ΔopgH</td>
<td>21.8</td>
<td>4.64</td>
<td>31.4% (244/776)</td>
</tr>
<tr>
<td>ΔclpX</td>
<td>21.4</td>
<td>5.44</td>
<td>18.1% (104/576)</td>
</tr>
<tr>
<td>ΔclpX Δpgm</td>
<td>22.6</td>
<td>4.39</td>
<td>35.4% (216/710)</td>
</tr>
<tr>
<td>ΔclpX ΔopgH</td>
<td>21.8</td>
<td>4.72</td>
<td>30.0% (212/706)</td>
</tr>
</tbody>
</table>
### Supplemental Table 2

Detailed cell size measurements of mutants associated with UDP-glucose synthesis or utilization cultured in LB-glucose.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth ratea</th>
<th>Area (μm²)</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>oriC/cellb</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>21.2/78.3</td>
<td>5.66 ± 0.13</td>
<td>4.55 ± 0.10</td>
<td>1.22 ± 0.03</td>
<td>10.2 ± 0.38</td>
</tr>
<tr>
<td>Δpgm</td>
<td>22.7/82.1</td>
<td>4.24 ± 0.27</td>
<td>3.15 ± 0.28</td>
<td>1.40 ± 0.01</td>
<td>4.33 ± 0.99</td>
</tr>
<tr>
<td>ΔgalU</td>
<td>22.2/81.0</td>
<td>4.66 ± 0.19</td>
<td>3.94 ± 0.07</td>
<td>1.13 ± 0.03</td>
<td>4.76 ± 0.10</td>
</tr>
<tr>
<td>ΔgalE</td>
<td>21.8/78.9</td>
<td>5.53 ± 0.25</td>
<td>4.41 ± 0.16</td>
<td>1.24 ± 0.01</td>
<td>10.2 ± 0.33</td>
</tr>
<tr>
<td>ΔgalT</td>
<td>21.1/79.3</td>
<td>5.70 ± 0.27</td>
<td>4.43 ± 0.23</td>
<td>1.27 ± 0.11</td>
<td>10.2 ± 0.42</td>
</tr>
<tr>
<td>Δugd</td>
<td>21.5/79.0</td>
<td>5.72 ± 0.27</td>
<td>4.53 ± 0.21</td>
<td>1.25 ± 0.03</td>
<td>9.54 ± 0.35</td>
</tr>
<tr>
<td>ΔotsA</td>
<td>21.5/78.8</td>
<td>5.44 ± 0.27</td>
<td>4.33 ± 0.20</td>
<td>1.23 ± 0.04</td>
<td>10.1 ± 0.42</td>
</tr>
<tr>
<td>ΔopgG</td>
<td>21.7/78.2</td>
<td>5.61 ± 0.30</td>
<td>4.50 ± 0.17</td>
<td>1.24 ± 0.01</td>
<td>10.4 ± 0.35</td>
</tr>
<tr>
<td>ΔopgH</td>
<td>21.2/78.6</td>
<td>5.01 ± 0.06</td>
<td>3.7 ± 0.03</td>
<td>1.36 ± 0.03</td>
<td>6.14 ± 0.27</td>
</tr>
<tr>
<td>opgH (PIC249AIA)</td>
<td>21.4/78.9</td>
<td>5.02 ± 0.06</td>
<td>3.75 ± 0.05</td>
<td>1.34 ± 0.02</td>
<td>n/a</td>
</tr>
<tr>
<td>ΔpgmΔopgH</td>
<td>22.9/ n/a</td>
<td>4.34 ± 0.25</td>
<td>3.07 ± 0.13</td>
<td>1.42 ± 0.03</td>
<td>n/a</td>
</tr>
<tr>
<td>Δpgi</td>
<td>21.5/n/a</td>
<td>5.64 ± 0.04</td>
<td>4.54 ± 0.01</td>
<td>1.24 ± 0.01</td>
<td>n/a</td>
</tr>
<tr>
<td>Δzwf</td>
<td>21.6/n/a</td>
<td>5.90 ± 0.15</td>
<td>4.56 ± 0.11</td>
<td>1.29 ± 0.01</td>
<td>n/a</td>
</tr>
<tr>
<td>ΔrfbA</td>
<td>n/a</td>
<td>6.03 ± 0.08</td>
<td>4.89 ± 0.13</td>
<td>1.23 ± 0.02</td>
<td>n/a</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----</td>
</tr>
<tr>
<td>ΔrfbB</td>
<td>n/a</td>
<td>5.75 ± 0.12</td>
<td>4.65 ± 0.13</td>
<td>1.24 ± 0.02</td>
<td>n/a</td>
</tr>
<tr>
<td>ΔglgB</td>
<td>n/a</td>
<td>5.54 ± 0.08</td>
<td>4.49 ± 0.04</td>
<td>1.23 ± 0.02</td>
<td>n/a</td>
</tr>
<tr>
<td>ΔglgC</td>
<td>n/a</td>
<td>5.43 ± 0.31</td>
<td>4.43 ± 0.16</td>
<td>1.23 ± 0.03</td>
<td>n/a</td>
</tr>
<tr>
<td>ΔgalF</td>
<td>21.8/n/a</td>
<td>5.58 ± 0.44</td>
<td>4.53 ± 0.25</td>
<td>1.23 ± 0.04</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The average is presented ± SD (n = 3).

a Growth rate for the strains cultured in LB-glucose/AB-succinate. The rate is listed in minutes during the exponential period of growth.

b Cell size is coupled to chromosomal replication in E. coli. A reduction in chromosomal origins (oriC) per cell indicates a reduction in cell mass. It is shown here to demonstrate the pgm, galU, and opgH deletions have significant oriC/cell reductions in agreement with the size measurements.
### Supplemental Table 3

Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Relevant genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH140 (MG1655)</td>
<td>$F\lambda:\text{ilvG-}rfb-50\text{rph-1}$</td>
<td>[1]</td>
</tr>
<tr>
<td>BH121 (W3110)</td>
<td>$F\lambda:\text{rph-1}\ INV(\text{rrnD, rrnE})$</td>
<td>[2]</td>
</tr>
<tr>
<td>PL1679 (BL21(DE3))</td>
<td>$F\lambda:\text{ompT gal dcm lon hsdS}\lambda(\text{DE3})$</td>
<td>[3]</td>
</tr>
<tr>
<td>PL2432 (BW25113)</td>
<td>$F\lambda:\text{rph-1}\ \Delta(\text{araD-araB})\ \Delta\text{lacZ} \ \Delta\text{hsdR} \ \Delta(\text{rhaD-rhaB})$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH141</td>
<td>MG1655 $\Delta\text{pgm::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2449</td>
<td>MG1655 $\Delta\text{galU::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH167</td>
<td>MG1655 $\Delta\text{galT::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH169</td>
<td>MG1655 $\Delta\text{otsA::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH171</td>
<td>MG1655 $\Delta\text{galE::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH177</td>
<td>MG1655 $\Delta\text{ugd::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>BH643</td>
<td>MG1655 $\Delta\text{opgG::frt}$</td>
<td>Recombineering</td>
</tr>
<tr>
<td>PL2450</td>
<td>MG1655 $\Delta\text{opgG::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2455</td>
<td>MG1655 $\Delta\text{opgH::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2325</td>
<td>W3110 $\Delta\text{pgi::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2317</td>
<td>W3110 $\Delta\text{zwf::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2342</td>
<td>W3110 $\Delta\text{glgB::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2343</td>
<td>W3110 $\Delta\text{glgC::kan}$</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PL2340</td>
<td>W3110 ΔrfbA::kan</td>
<td>[4]</td>
</tr>
<tr>
<td>PL2341</td>
<td>W3110 ΔrfbB::kan</td>
<td>[4]</td>
</tr>
<tr>
<td>PL3236</td>
<td>BW25113 ΔgalF::kan</td>
<td>[4]</td>
</tr>
<tr>
<td>BH317</td>
<td>BH141 Δpgm::frt</td>
<td>BH141/pCP20</td>
</tr>
<tr>
<td>BH265</td>
<td>PL2455 ΔopgH::frt</td>
<td>PL2455/pCP20</td>
</tr>
<tr>
<td>BH666</td>
<td>MG1655 ΔrcsB::kan</td>
<td>[4]</td>
</tr>
<tr>
<td>BH183</td>
<td>MG1655 ΔrpoS::tet</td>
<td>[5]</td>
</tr>
<tr>
<td>PL3035</td>
<td>MG1655 ΔminCDE::kan</td>
<td>[6]</td>
</tr>
<tr>
<td>PL3036</td>
<td>MG1655 ΔslmA::kan</td>
<td>[7]</td>
</tr>
<tr>
<td>PL2974</td>
<td>MG1655 ΔclpX::kan</td>
<td>[4]</td>
</tr>
<tr>
<td>BH657</td>
<td>MG1655 opgH::opgH-6xhis</td>
<td>Recombineering</td>
</tr>
<tr>
<td>BH663</td>
<td>MG1655 opgH (PIC249AIA)</td>
<td>Recombineering</td>
</tr>
<tr>
<td>BH270</td>
<td>BH265 ΔgalU::kan</td>
<td>P1(PL2449) x BH265</td>
</tr>
<tr>
<td>BH271</td>
<td>BH265 ΔopgG::kan</td>
<td>P1(PL2450) x BH265</td>
</tr>
<tr>
<td>BH485</td>
<td>BH265 Δpgm::kan</td>
<td>P1(BH141) x BH265</td>
</tr>
<tr>
<td>BH667</td>
<td>BH317 ΔrcsB::kan</td>
<td>P1(BH666) x BH141</td>
</tr>
<tr>
<td>BH668</td>
<td>BH265 ΔrcsB::kan</td>
<td>P1(BH666) x BH265</td>
</tr>
<tr>
<td>BH192</td>
<td>BH183 Δpgm::kan</td>
<td>P1(BH141) x BH183</td>
</tr>
<tr>
<td>BH194</td>
<td>BH183 ΔgalU::kan</td>
<td>P1(PL2449) x BH183</td>
</tr>
<tr>
<td>BH206</td>
<td>BH183 ΔopgH::kan</td>
<td>P1(PL2455) x BH183</td>
</tr>
<tr>
<td>BH652</td>
<td>BH317 ΔminCDE::kan</td>
<td>P1(PL3035) x BH317</td>
</tr>
<tr>
<td>BH653</td>
<td>BH265 ΔminCDE::kan</td>
<td>P1(PL3035) x BH265</td>
</tr>
<tr>
<td>BH639</td>
<td>BH317 ΔslmA::kan</td>
<td>P1(PL3036) x BH317</td>
</tr>
<tr>
<td>BH640</td>
<td>BH265 ΔslmA::kan</td>
<td>P1(PL3036) x BH265</td>
</tr>
<tr>
<td>BH641</td>
<td>BH317 ΔclpX::kan</td>
<td>P1(PL2974) x BH317</td>
</tr>
<tr>
<td>BH642</td>
<td>BH265 ΔclpX::kan</td>
<td>P1(PL2974) x BH265</td>
</tr>
<tr>
<td>PL2452</td>
<td>MG1655 ftsZ84 (tet)  [8]</td>
<td></td>
</tr>
<tr>
<td>BH173</td>
<td>PL2452 Δpgm::kan</td>
<td>P1(BH141) x PL2452</td>
</tr>
<tr>
<td>BH175</td>
<td>PL2452 ΔgalU::kan</td>
<td>P1(PL2449) x PL2452</td>
</tr>
<tr>
<td>BH352</td>
<td>PL2452 ΔopgH::kan</td>
<td>P1(PL2455) x PL2452</td>
</tr>
<tr>
<td>BH330</td>
<td>MG1655 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>[9]</td>
</tr>
<tr>
<td>BH331</td>
<td>BH141 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH141</td>
</tr>
<tr>
<td>BH647</td>
<td>PL2455 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x PL2455</td>
</tr>
<tr>
<td>PL644</td>
<td>PL3035 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x PL3035</td>
</tr>
<tr>
<td>BH654</td>
<td>BH652 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH652</td>
</tr>
<tr>
<td>BH669</td>
<td>BH653 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH653</td>
</tr>
<tr>
<td>BH645</td>
<td>PL3036 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x PL3036</td>
</tr>
<tr>
<td>BH648</td>
<td>BH639 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH639</td>
</tr>
<tr>
<td>BH649</td>
<td>BH640 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH640</td>
</tr>
<tr>
<td>BH647</td>
<td>PL2974 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH647</td>
</tr>
<tr>
<td>BH650</td>
<td>BH641 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH650</td>
</tr>
<tr>
<td>BH651</td>
<td>BH642 P₇₇₄lac::gfp-ftsZ (bla)</td>
<td>P1(BH330) x BH651</td>
</tr>
<tr>
<td>PL3180^c</td>
<td>W3110 ftsZ::kan</td>
<td></td>
</tr>
</tbody>
</table>

---

^a P₇₇₄lac indicates the lactose promoter. bla, tet and kan indicate ampicillin, tetracycline and kanamycin resistance cassettes. The kan resistance cassettes are flanked by frt sites for excision by the FLP recombinase leaving a frt scar sequence.

^b P1 transduction is succinctly described as: P1(donor strain) x recipient strain.

^c Supplemented with pKG110-ftsZ.
### Supplemental Table 3 References


### Supplemental Table 4

Bacterial plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype(^a)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDR155</td>
<td>$P_{lac}::\text{minD (bla)}$</td>
<td>[1]</td>
</tr>
<tr>
<td>pBH228</td>
<td>$P_{ara}::\text{thio-6xhis (bla)}$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBH421</td>
<td>$P_{lac}::\text{pgm-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH422</td>
<td>$P_{lac}::\text{galT-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH423</td>
<td>$P_{lac}::\text{galE-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH424</td>
<td>$P_{lac}::\text{opgG-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH425</td>
<td>$P_{lac}::\text{opgH-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH426</td>
<td>$P_{lac}::\text{ugd-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH427</td>
<td>$P_{lac}::\text{galU-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH429</td>
<td>$P_{lac}::\text{otsA-gfp (cat)}$</td>
<td>[2]</td>
</tr>
<tr>
<td>pBH494</td>
<td>$P_{lac}::\text{opgH (PIC249AIA)-gfp (cat)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH536</td>
<td>$P_{ara}::\text{thio-pgm-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH537</td>
<td>$P_{ara}::\text{thio-galU-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH538</td>
<td>$P_{ara}::\text{thio-opgH(1-414)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH539</td>
<td>$P_{ara}::\text{thio-opgH(573-1542)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH540</td>
<td>$P_{ara}::\text{thio-opgH(2106-2455)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH541</td>
<td>$P_{ara}::\text{thio-opgH-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH580</td>
<td>$P_{ara}::\text{thio-opgH(1-201)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH581</td>
<td>$P_{ara}::\text{thio-opgH(204-414)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH582</td>
<td>$P_{ara}::\text{thio-opgH(249-414)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH583</td>
<td>$P_{ara}::\text{thio-opgH(294-414)-6xhis (bla)}$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH584</td>
<td>$P_{ara}::thio-opgH(339-414)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>pBH585</td>
<td>$P_{ara}::thio-opgH(204-369)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH586</td>
<td>$P_{ara}::thio-opgH(204-336)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH587</td>
<td>$P_{ara}::thio-opgH(204-270)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH588</td>
<td>$P_{ara}::thio-opgH(249-336)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH589</td>
<td>$P_{ara}::thio-opgH(249-303)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH608</td>
<td>$P_{ara}::thio-opgH(249-303)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH616</td>
<td>$P_{T7}::opgH^N$-intein (bla)</td>
<td>This study</td>
</tr>
<tr>
<td>pBH664</td>
<td>$P_{ara}::thio-opgH(573-2544)-6xhis (bla)$</td>
<td>This study</td>
</tr>
<tr>
<td>pBH671</td>
<td>$P_{ara}::thio-opgH(PIC249AIA)-6xhis (bla)$</td>
<td>This study</td>
</tr>
</tbody>
</table>

$a$ $P_{lac}$, $P_{ara}$, or $P_{T7}$ indicate the lactose, arabinose, or the phage T7 promoters. $bla$ and $cat$ indicate ampicillin and chloramphenicol resistance cassettes.

**Supplemental Table 4 References**


**Supplemental Figure 1**

FtsZ84 levels are not elevated in *pgm* null strain.

A representative quantitative immunoblot of FtsZ84 levels for WT and *pgm::kan* strains encoding the *ftsZ84* allele at both permissive (30°C) and restrictive (42°C) growth conditions (See Figure 1D). The replication protein DnaA is shown as a loading control (LC). Relative expression (below) was calculated using WT grown at 30°C as the reference (n = 3).
<table>
<thead>
<tr>
<th></th>
<th>30°C</th>
<th></th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pgm::kan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FtsZ84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>100%</td>
<td>103% (±1)</td>
<td>106% (±3)</td>
</tr>
</tbody>
</table>
Supplemental Figure 2

MinD overexpression and FtsZ levels are congruent between in wild type and UDP-glucose pathway mutants.

A representative quantitative immunoblot of MinD overexpression and FtsZ levels in WT and the UDP-glucose pathway mutants encoding a plasmid with \( P_{\text{lac}}::\text{minD} \). Cells were cultured in LB ± 0.15mM IPTG for ~3h. Average fold overexpression level displayed below (n = 3).
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>pgm::kan</th>
<th>galU::kan</th>
<th>opgH::kan</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG (mM)</td>
<td>0</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>MinD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FtsZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MinD Fold Overexpression</td>
<td>1.8</td>
<td>1.9</td>
<td>2.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Diagram: Western blot showing protein expression with $P_{lac::minD}$.
Supplemental Figure 3

The cell size defect is confined to the UDP-glucose biosynthesis pathway.

Cell area measurements of loss-of-functions mutations in genes adjacent to the UDP-glucose (see Figure 1A). Cells were grown in LB-glucose. >250 cells per strain were evaluated per replicate. Error bar equals standard deviation (n = 3).
Supplemental Figure 4

Thio-OpgH-His expressed to similar levels.

Expression levels for wild-type Thio-OpgH-His, a putative UDP-glucose binding mutant (PIC249AIA), or wild-type OpgH in a pgm::kan background. Cells were grown in LB ± 0.5% arabinose.
<table>
<thead>
<tr>
<th>Arabinose</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-FtsZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PIC249AlA</th>
<th>pgm::kan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thio-OpgH-His</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figure 5

opgH-gfp and thio-opgH-his fusion constructs complement for size and glucosyltransferase activity.

(A) Cell area distribution of WT (black), opgH::kan (white), opgH::kan + P_{lac}::opgH-gfp (green), and opgH::kan + P_{ara}::thio-opgH-his (red) grown in LB-glucose. The P_{lac}::opgH-gfp is induced with 0.08mM IPTG, while P_{ara}::thio-opgH is induced with 0.25% arabinose. >250 cells were evaluated. Averages shown in the inset. (B) Swarm phenotypes are a proxy for glucosyltransferase activity (see Text S1). Expression of either P_{lac}::opgH-gfp (in 0.08mM IPTG) or P_{ara}::thio-opgH-his (0.25% arabinose) in an opgH null led to wild-type swarming. Similar expression using a opgH sugar-binding mutant (*) or inactivating UDP-glucose biosynthesis (pgm::kan) was unable to complement normal activity. Average diameter of swarm distance shown below (n = 3).
Supplemental Figure 6

The ΔopgH cell size defect is independent of osmoregulated periplasmic glucans and Rcs activation.

(A) Cell area measurements of an opgGH null complemented with either $P_{lac}::opgG$-gfp or $P_{lac}::opgH$-gfp. Cells grown in LB-glucose with 0.1mM IPTG. (B) A representative quantitative immunoblot of FtsZ levels for WT and the UDP-glucose null strains. Lysates were normalized to total protein using a BSA assay. Relative expression (below) was calculated using WT as the reference (n = 3). (C) Loss-of-function mutations in the UDP-glucose pathway genes combined with null mutations in either the Rcs response regulator $rcsB$ (left) or the alternative sigma factor $rpoS$ (right). >250 cells per strain were evaluated three times. Error bars equals standard deviation (n = 3).
Supplemental Figure 7

An 18-amino acid peptide of OpgH is sufficient, though unnecessary, to inhibit division and localize to the division ring.

(A) Cell area measurements of $P_{ara}$::thio-opgH$^N$-his deletion constructs in a opgH::kan background cultured in LB ± 0.5% arabinose. Cells were cultured with inducer for ~3h. >250 cells were assessed for cell area in each replicate. (B) Immunofluorescence of Thio-OpgH(83-101)-His and Thio-OpgH(211-848)-His after being cultured in LB + 0.5% arabinose for 2h. OpgH localization using an α-His antibody is shown in the top panel, α-FtsZ in the middle panel, with the colocalization displayed on the bottom panel (OpgH in green, FtsZ in red). The covariance of OpgH/FtsZ midcell localization is denoted below. Bar = 3μm. (C) Quantitative immunoblot of the various Thio-OpgH-His constructs in the opgH null cultured in LB ± 0.5% arabinose. FtsZ is used as a loading control (below).
Supplemental Figure 8

UDP-glucose pathway mutants reduce filamentation of ΔminCDE.

minCDE::kan, Δpgm minCDE::kan, ΔopgH minCDE::kan strains grown in LB-glucose, stained with FM4-64. The average area is listed below (>150 cells counted, n = 3). Bar = 5μm. A representative immunoblot of FtsZ levels of the mutants is shown below.
Supplemental Figure 9

Heat-treated OpgH\textsuperscript{N} loses ability to inhibit FtsZ.

(A) A representative 90° angle light scattering plot and (B) percent assembly of FtsZ assembly ± heat-treated OpgH\textsuperscript{N}. Arrow indicates addition of 1mM GTP. (C) The rate of GTP hydrolysis of FtsZ ± heat-treated OpgH. (A-C) FtsZ is at 5µM, heat-treated OpgH\textsuperscript{N} is at 10µM. OpgH\textsuperscript{N} was subjected to 30 minutes at 90°C to act as the heat-treated control. (D) The GTPase activity of calf intestinal phosphatase (CIP) was measured with either OpgH\textsuperscript{N} buffer (black) or 10µM OpgH\textsuperscript{N} (blue). CIP’s ability is to hydrolyze GTP is unaltered with the addition of OpgH\textsuperscript{N}. This demonstrates that OpgH\textsuperscript{N}’s activity is specific to FtsZ and is not inhibiting a different reaction in the regenerative NADH-coupled assay. Error bars equal standard deviation (n = 3).
Supplemental Figure 10

Phylogenetic tree and alignment of the FtsZ inhibiting domain of OpgH.

(A) A phylogenetic tree comparing the C-terminus of OpgH\textsuperscript{N} (66-138) with other gammaproteobacteria. The percent identity and percent similarity are listed in parentheses. (B) The protein alignment of OpgH\textsuperscript{N} (66-138) with various other gammaproteobacteria that encode an OpgH homolog. The black box indicates the 18 amino acid region that is sufficient to block division \textit{in vivo}.
Addendum

This appendix was previously published in PLoS Genetics (Hill et al., 2013).


The work in this appendix performed by PJ Buske is as follows:

1. Purification of E. coli FtsZ.
2. GTPase assays (with Norbert S. Hill).

All other experiments were performed by Norbert S. Hill with the exception of the chemotaxis assay (Supplemental Figure 5), which was done by Yue Shi. The manuscript was written by Norbert S. Hill and Petra Anne Levin with input and editing by PJ Buske.