A Genomic and Structural Study of FtsZ Function for Bacterial Cell Division

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

The tubulin homolog FtsZ provides the cytoskeletal framework for bacterial cell division. FtsZ is an essential protein for bacterial cell division, and is the only protein necessary for Z-ring assembly and constriction force generation in liposomes *in vitro*. The work presented here utilizes structural and genomic analysis methods to investigate FtsZ function for cell division with three separate questions: (1) What is the function of the C-terminal linker peptide in FtsZ? (2) Are there interacting proteins other than those of the divisome that facilitate FtsZ function? (3) Do lateral contact sites exist between protofilaments in the Z ring, resulting in an organized Z-ring substructure?

The FtsZ protein has an ~50 aa linker between the protofilament-forming globular domain and the C-terminal (Ct) membrane-tethering peptide. This Ct linker is widely divergent across bacterial species, and has been thought to be an intrinsically disordered peptide (IDP). We have made chimeras where we have swapped the *Escherichia coli* IDP for Ct linkers from other bacteria, and even for an unrelated IDP from human a-adducin. Most of these substitutions allowed for normal cell division, suggesting that sequence of the IDP did not matter –any IDP appears to work (with some exceptions). Length, however, was important: IDPs shorter than 39 or longer than 89 aa’s had compromised function. We conclude that the Ct linker of FtsZ functions as a flexible tether between the globular domain of FtsZ in the protofilament, and its attachment to FtsA and ZipA at the membrane. As a worm-like-chain, the Ct linker will function as a stiff entropic spring linking the constricting protofilaments to the membrane.
Previous work from our laboratory found that mutant and foreign FtsZ that do not normally function for cell division can function upon acquisition of a second site suppressor mutation, somewhere in the *E. coli* genome. We expect that some mutant or foreign FtsZ are partially functional for division in *E. coli*. As such, these FtsZ require another mutation that further enables their function. These suppressing mutations may reveal proteins interacting with FtsZ and the divisome, that have previously been unknown. In the present study, we have identified, via whole genome re-sequencing, single nucleotide polymorphisms that allow 11 different foreign and mutant FtsZ proteins to function for cell division. While we see a trend toward mutations in genes related to general metabolism functions in the cell, we have also identified mutations in two genes, *ispA* and *nlpI*, that may be interacting more directly with the cell division mechanism.

Finally, we have devised a screen to identify mutations in FtsZ that may be involved in lateral bonding between protofilaments. There are presently two proposed models of FtsZ substructure: the scattered or the ribbon model. A major difference between these models is that the scattered model proposed no interaction between adjacent protofilaments in the Z ring, while the ribbon model suggests that adjacent protofilaments are bonded laterally to create an organized substructure of aligned protofilaments. Our screen was designed to identify complementary surface-exposed residues that may be involved in lateral bonding. We initially identified two lateral contact candidate residues: R174, and E250 and mutated them to abrogate FtsZ function. We also mutated L272, which is known to make contacts across the protofilament
interface, to look for compensating mutations in these contact residues. Using the screen, we identified a number of secondary mutations in FtsZ that can complement these initial loss-of-function mutations. While this screen has not yielded strong candidates for lateral bonding partners, it has emerged as a high-throughput method for screening large libraries of mutant FtsZ proteins in order to identify compensating mutation pairs.
For their unwavering love, encouragement, and support,

this work is dedicated to

Mom, Dad, and Matt.
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List of Abbreviations

aa: amino acid(s)
amp: ampicillin
bp: base pair(s)
cfu: colony forming units
cm: chloramphenicol
Ct: C-terminal

$E$: Young’s modulus
EDTA: ethylenediaminetetraacetic acid
EtOH: ethyl alcohol
EM: electron microscopy

FRAP: fluorescence recovery after photobleaching
FRET: fluorescence resonance energy transfer
FtsZ: filamentous temperature sensitive Z

GAP: GTPase activating protein
GDP: guanidine 5’-diphosphate
GDP-AlF: guanidine 5’-diphosphate-aluminum fluoride
GFP: green fluorescent protein

GMPCPP: guanosine 5’-[(α,β)-methylene]triphosphate, sodium salt
GTP: guanidine 5’-triphosphate
$I$: second moment of inertia

IDP: intrinsically disordered peptide

kan: kanamycin

KOAc: potassium acetate

$L$: contour length

LB: Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl)

mts: membrane-targeting sequence

Nt: N-terminal

nt: nucleotide(s)

$P$: persistence length

PCR: polymerase chain reaction

pf: protofilament(s)

SDS: sodium dodecyl sulfate

SNP: single nucleotide polymorphism

TE: tris hydroxymethylaminoethane, ethylenediaminetetraacetic acid

wlc: worm-like chain

wt: wild-type

YFP: yellow fluorescent protein
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Chapter 1: Introduction

The division of a mother cell to produce two daughter cells is a universal characteristic of life. This process, known as cytokinesis, is highly regulated temporally and spatially, and allows for reliable and equal segregation of the cell contents and genetic material into the daughter cells. The cytoskeleton is an important regulator of cytokinesis in all cells. In animal cells, actin and myosin produce the contractile force to divide the cells. In plants, the cell plate, aided by actin and tubulin, generates a new cell wall at the division site to divide the cell in two. In prokaryotes, division is achieved by homologs of eukaryotic cytoskeletal proteins, which usually divide the cell symmetrically between the segregated nucleoids. Most notable of these cytoskeletal proteins is FtsZ, a prokaryotic homolog of tubulin (1). In Escherichia coli, division occurs by an invagination of the inner membrane. The outer membrane and cell wall follow the invagination to form a septum that will completely divide the bacteria. FtsZ is at the leading edge of this septation (2).

FtsZ is a highly conserved protein, found in most of the major groups of bacteria and in the Euryarchaeal branch of Archaea (3). Originally identified as a temperature sensitive mutant in E. coli, the fis genes (filamentous temperature sensitive) are named for the distinct filamentous phenotype of mutant cells at the non-permissive temperature, indicating septation and division defects. Several fis genes were initially identified (4) as contributing to this division defect, with the fisZ gene product emerging as a major part of the division machine.
Figure 1: Time-lapse observation of Z rings in *E. coli*, using FtsZ-GFP as a dilute label, expressed at about one-third the level of genomic FtsZ. Three cells are shown, and the cell indicated with an arrow in the first frame is undergoing division. The constriction of the Z ring, and its concurrent disassembly, as well as the assembly of new Z rings in the two daughter cells can be seen. Figure reproduced from (5) with permission of the author.
FtsZ is the first protein to localize to the site of future division. Individual FtsZ molecules assemble into protofilaments which further assemble into the Z ring at midcell. This Z-ring assembly at the future division site is the initial step of cytokinesis (2). A continuous circle of protofilaments, the Z ring is capable of generating a constriction force that can deform a membrane to start cell division (6). FtsZ generates the constriction force by itself, without any motor molecules. After division, new Z rings assemble rapidly in the newly formed daughter cells, and these Z rings are present for the majority of the cell cycle, as 85 to 95% of rapidly dividing Bacillus subtilis and E. coli cells have detectable Z rings (7). The Z ring was first visualized by immunoelectron microscopy (2) and later by immunofluorescence microscopy (7). The ability to tag FtsZ with various fluorescent proteins, such as green fluorescent protein (GFP) (8), has enabled visualization of the Z ring in living cells (Figure 1).

In E. coli, upon complete formation of the Z ring, at least nine other essential proteins are recruited to the septation site to form the division machinery known as the divisome (Figure 2)(9). FtsA appears to play a key role in the addition of these proteins to the Z ring(9). These proteins appear to be recruited in a linear fashion (10) and have various functions that work together to remodel the cell wall behind the invagination of the membrane caused by Z-ring constriction and to ensure proper partitioning of cellular contents into each of the resulting daughter cells (9). Z-ring formation is also negatively regulated by the proteins MinC and SulA, which serve to ensure that the divisome forms only at mid-cell and only when the DNA is copied and intact (11).
The FtsZ protein is comprised of three domains: a conserved globular domain which is the tubulin-homologous core, a divergent and flexible peptide termed the Ct linker, and a conserved extreme Ct peptide referred to as the FtsA-binding peptide (Figure 3). The globular domain can be further divided into two subdomains, the N- and C-terminal subdomains, which can be expressed separately and fold independently (12). The N-terminal subdomain has the structure of a Rossman fold and contains the entirety of the GTP binding site as well as the lower half of the interface between adjacent FtsZ subunits arranged in a protofilament. The C-terminal subdomain contains the upper half of the subunit interface and the T7 “synergy” loop (5).

Following the C-terminal subdomain of the globular domain is a highly divergent peptide termed the Ct linker. This peptide is 50 aa long on average, including in E. coli, but it can vary widely in length and sequence across bacterial species. The Ct linker is invisible in crystal structures of the complete FtsZ protein, where electron density is only seen for the globular domain. This would indicate that the Ct linker is a disordered peptide. That conclusion is further supported by the high variability between Ct linkers of different bacterial species, which contrasts sharply with the high conservation in the globular domain and FtsA-binding peptides of divergent FtsZ.
Figure 2: Schematic of the assembly of the Z ring and the entire divisome in *E. coli*
Figure 3: (A-B) Structure of the FtsZ subunit. The globular domain is comprised of two subdomains, colored blue (N-terminal) and cyan (C-terminal). The structure is from *P. aeruginosa*, PDB 1OFU (13). The GDP is shown in orange, and the D212 residue of the synergy loop in red. The first 10 amino acids on the N-terminal and the 50 aa C-terminal linker are shown as unstructured peptides in magenta. The 17-aa FtsA binding peptide is shown as an extended beta strand and alpha helix in dark purple (from PDB 1F47 (14)). (C) A protofilament is assembled by stacking subunits on top of each other so that the D212 of the upper subunit is just above the GDP of the one below. Figure reproduced from (5) with the permission of the author.
The FtsA-binding peptide is the last domain of FtsZ. Composed of the final ~17 aa, this highly conserved peptide can bind to FtsA or ZipA to tether FtsZ, and any protofilaments or the Z ring, to the cell membrane. FtsA is a membrane associated protein with a C-terminal amphipathic helix that can insert into the lipid bilayer and anchor FtsA to the membrane (15). ZipA is a transmembrane protein. Thus, the Z ring can tether itself to the membrane by binding either FtsA or ZipA (16). In fact, cells require either FtsA or ZipA to be present in order to form a Z ring (17). Though FtsA or ZipA are required in vivo, it appears that Z-ring formation requires only FtsZ and can be reconstituted in vitro in tubular liposomes (6) if FtsZ is provided with a membrane targeting sequence (MTS) that can act in place of FtsA or ZipA to attach the Z ring to the membrane.

Though FtsZ has only a 10% amino acid identity to eukaryotic tubulin (1), the two are considered homologs because of the identical structure at the level of C-alpha chain folding. Both FtsZ and tubulin bind GTP at one end of the molecule (often called the “+ end” or “top”). The FtsZ filaments formed in the presence of GTP are structurally similar to microtubule protofilaments Erickson et al 1996, (18).

FtsZ is both a GTPase and a GTPase-activating protein (GAP). One molecule of FtsZ binds GTP on its + end, but hydrolysis requires contact with another FtsZ molecule which will act as a GAP and provide catalytic side chains from its synergy loop to the hydrolysis site of the GTPase FtsZ. These catalytic side chains, sequence 207NxDFAD212 in _E. coli_, are highly conserved in all FtsZ and have high homology to the NxDxxE GAP sequence of all α-tubulins (1). The GTPase activity of FtsZ is severely
decreased by mutations of the synergy loop, further indicating the GAP role of adjacent FtsZ subunits in a protofilament (19).

In the presence of GTP, FtsZ polymerizes to form dynamic filaments in vitro. These protofilaments have a basic structure that is directional and one subunit thick, though these protofilaments are capable of lateral association or bundling in certain buffer conditions (20). Studies of this FtsZ assembly in vitro revealed that protofilament assembly occurs in a cooperative manner, elongating rapidly after an initial nucleating lag time corresponding to the formation of an FtsZ dimer. Once formed, these protofilaments are dynamic; able to rapidly exchange subunits within the protofilaments with cytoplasmic FtsZ molecules with a half-time of 7-9 seconds (21). This is similar to the subunit turnover in vivo demonstrated by FRAP (22).

While subunits appear to be able to exchange between protofilaments, nucleotide exchange within a protofilament does not occur. FtsZ subunits bind GTP before assembly, and that GTP is trapped between adjacent FtsZ subunits. As such, nucleotide exchange can only occur for free monomers or at the plus end of a protofilament, where the GTP binding pocket is solvent-exposed (23). Early studies indicated that GTP binding and hydrolysis was necessary for protofilament assembly, as FtsZ assembly seemed to require GTP concentration in the range of 50 to 100 µM (24). However, other studies showed that FtsZ assembly can occur when GTP hydrolysis is blocked with EDTA (25), when GDP is bound to the monomers, rather than GTP (26), or when slow-hydrolyzing or non-hydrolyzable analogs of GTP (GMPCPP or GDP-AlF) were present in place of GTP (27). Though the presence of nucleotides other than GTP affected assembly...
dynamics, this suggests that GTP is not required for assembly. This was further shown by the ability of apo-FtsZ to assemble with a similar critical concentration as FtsZ-GTP (28). GDP was able to destabilize these apo-FtsZ protofilaments, suggesting that GTP binding and subsequent hydrolysis is important for protofilament destabilization and subsequent subunit exchange.

The protofilaments formed by FtsZ polymerization, as observed by electron microscopy and atomic force microscopy, seemed to exist in one of three distinct curvatures: straight, high curvature and intermediate curvature (Figure 4). The existence of these different curved conformations was the initial indication that FtsZ may be able to generate a constriction force on its own. Highly curved protofilaments formed mini-rings of 16 FtsZ subunits with a 23-degree bend at each interface, resulting in a ring of 24 nm diameter (29, 30). Protofilaments with the intermediate curvature exhibit a 2.5-degree bend between subunits, which would correspond to a closed circle of ~200 nm diameter (27). It was initially postulated that protofilaments with bound GTP favored a straight conformation, while protofilaments with bound GDP favored a curved conformation, thus indicating that GTP hydrolysis could generate a constriction force by transitioning protofilaments from the straight to the curved conformation (30). However, the intermediate curvature does not seem to require GTP hydrolysis (5). Further, FtsZ with a D212G mutation is essentially GTPase dead (31), but it can function for cell division upon the generation of a genomic suppressor mutation (32). Taken together, this indicates that while the conformational change of FtsZ protofilaments may produce the
constriction force necessary for cell division, GTP hydrolysis may not be the only determinant in conformational change.

That FtsZ alone is sufficient to produce a constriction force was demonstrated by reconstituting Z rings in tubular liposomes in vitro (Figure 5). These Z rings were made of FtsZ-YFP-mts, which contained yellow fluorescent protein as a tracker and a C-terminal amphipathic helix enabling the Z ring to assemble and tether directly to the liposomes without FtsA or ZipA. These reconstituted Z rings were able to produce a membrane-deforming force in the presence of GTP. This result showed that FtsZ and GTP alone were able to produce a bending that was capable of deforming a lipid membrane.
Figure 4: Protofilaments, small sheets, and minirings formed by FtsZ in GDP and adsorbed to the polycationic lipid monolayer. The arrows indicate protofilaments that have separated from a sheet, remaining straight for some length and then changing abruptly to the curved conformation. Scale bar = 100 µm. Figure reproduced from (33) with the permission of the author.
Figure 5: Z rings assembled in tubular liposomes from purified mts-FtsZ-YFP. The upper frames at the two timepoints uses Nomarski optics to show the profile of the liposome. The lower panel shows FtsZ localization by yellow fluorescent protein (YFP) fluorescence. At time zero, there are many dim Z rings. After 350s, the dim Z rings have coalesced into fewer bright ones, and these are generating constrictions in the liposomes, as seen by indentations in the contour of the liposome. This figure is reproduced from (6) with permission of the author.
The force required to deform a bacterial membrane has been estimated to be on the order of several pN, which is quite high (34). However, we can model a single protofilament as a cantilevered beam, where the protofilament is a rod fixed at one point (to the membrane by FtsA or ZipA) (5). With this model, the small-angle approximation of the beam equation can estimate the force produced by bending a protofilament (Figure 6). Using this approximation, the force \( F \) needed to bend a 130-nm protofilament that is fixed at its center \( L = 65 \text{ nm} \) by 20 nm at each end \( (y) \) into the \( \sim 200\text{-nm-diameter} \) intermediate curved conformation can be calculated as \( F = \left( \frac{3EI}{L^3} \right) \cdot y \). Using 1.4 GPa for the Young’s modulus \( (E) \) (35) and \( 8.4 \times 10^{-34} \text{ m}^4 \) for the second moment of inertia \( (I) \) (36), we calculate that such bending requires 2.6 pN of force at each end of the protofilament. This force is in keeping with the estimates of required membrane deforming force, indicating that the bending of protofilaments alone can produce enough force to constrict the membrane, and the transition of the protofilaments from straight to highly curved could create a constriction that would account for the majority of the cell division process (Figure 7).

FtsZ is a major component of the bacterial divisome, forming the Z ring and producing the constriction force that divides the cell. Because it is highly conserved between species, a thorough understanding of \textit{E. coli} FtsZ structure and function can lend great insight into the replication process for most bacteria. This conservation of Z-ring structure and function across species also makes FtsZ an excellent target for antibiotic drug design.
Figure 6: Scale model of protofilament bending. A 130-nm-long protofilament (solid line) is shown fixed at the center (dot) in the straight and the intermediate curved conformations. The two 65-nm halves behave as individual cantilevered beams as the protofilament bends to the radius of the intermediate curved conformation. Arrows show the direction of force (lighter central arrows represent the opposite force that would necessarily push back against the membrane if the protofilaments were free). Figure reproduced from (5) with permission of the author.
Figure 7: The conformational change of protofilaments could constrict a cell membrane. (A) An FtsZ protofilament is shown tethered to the membrane of an undivided cell. The blue FtsZ subunits are connected into a protofilament that has a preferred bend. Approximately every fifth subunit is tethered to the membrane via FtsA, indicated by a brown circle. (B) When in the highly curved conformation, the 24-nm miniring plus the C-terminal linker tether plus FtsA could constrict the membrane to about 57 nm on the outer diameter. (C) The diameters of the undivided cell, the intermediate curved conformation, and the miniring are compared. Figure reproduced from (5) with permission of the author.
Chapter 2: The C-terminal Linker of *Escherichia coli* FtsZ Acts as an Intrinsically Disordered Peptide

**Introduction**

FtsZ (filamentous temperature sensitive Z), an essential cell division protein that is highly conserved across bacterial species, is a bacterial tubulin homologue required for prokaryotic cell division. Individual FtsZ molecules assemble into protofilaments, which further associate to make the Z ring, probably a ribbon of 3-9 protofilaments (Milam EM), at the site of cytokinesis. In addition to providing the cytoskeletal framework for the division apparatus, FtsZ appears to generate the constriction force necessary for cell division (6). The process of cell division, and details of FtsZ itself, have been reviewed by Adams and Errington (37) and by Erickson et al (5).

The structure of an FtsZ subunit is shown in cartoon form in Figure 8. The globular domain, which has the tubulin homology fold, is shown as two subdomains colored blue and cyan. The 67 C-terminal aa, which we refer to as the Ct tail, comprises two parts. The extreme C-terminal 15-17 aa segment, which is highly conserved across bacterial species, binds to FtsA (and in *E. coli*, ZipA); we call this the FtsA-binding peptide. These are membrane proteins, and thereby tether FtsZ to the membrane. That segment is shown in Figure 8 as a beta strand and alpha helix, which is the structure the peptide assumes in a co-crystal with ZipA (14). The ~50 aa between the globular domain and the FtsA-binding peptide are termed the Ct linker.
Figure 8: FtsZ Structure. (A) Front view of the FtsZ subunit from *P. aeruginosa*, PDB 1OFU (13) (modified from (5)). This view is equivalent to that of tubulin from the outside of a microtubule. The two subdomains of the globular domain are colored blue (Nt) and cyan (Ct). GDP and the synergy loop aa D212 (*E. coli* numbering) are in red. A 10 aa segment on the N terminus and the 50 aa C-terminal linker are modeled as flexible peptides in magenta. The C-terminal FtsA-binding peptide is shown in dark purple. (B.) Model of the Ct-linker stretched to 8.5 nm, or half its contour length, which would require a force of 9 pN. In this case the molecule is rotated so that the front surface, to which the Ct linker is attached, is facing left.
Several lines of evidence suggest that the Ct linker is unstructured, an intrinsically disordered protein (IDP). (a) A number of crystal structures are available where the complete protein was crystallized, but these generally show electron density only for aa 10-316 of the *E. coli* FtsZ (EcFtsZ) sequence. This suggests that the first nine aa’s, and the C-terminal aa 317-383 are disordered. (b) The sequence of the Ct FtsA-binding peptide is highly conserved across bacterial species, as is that of the globular domain. In contrast, the sequence of the Ct linker shows almost no sequence conservation. (c) Sequences of FtsZ were analyzed by the PONDR program (http://www.pondr.com/(38)) and the Ct-linker region in most species was indicated as disordered.

In the present study we have addressed the question of whether the Ct linker behaves in vitro as an IDP, and we tested in vivo whether it could be swapped with Ct linkers from other bacterial species, and with IDPs from unrelated proteins.

**Methods**

**Cloning**

All variant forms of FtsZ presented in this paper were derived from *E. coli* FtsZ in a pJSB2 plasmid, which is derived from pBAD and has an arabinose-inducible promoter. Novel restriction sites were inserted into the FtsZ sequence at the start and end of the Ct linker (NotI before aa317 and XhoI after aa366) to produce the EcFtsZ construct presented here. Alternative linker sequences to splice into FtsZ were amplified via PCR to contain a 5’ GCGGCCGC (NotI, where the GC indicates nucleotides already present in
the FtsZ sequence, thus requiring the insertion of only 6 nt) and a CTGAG 3 (XhoI). This results in aa pairs GR and LE inserted before and after the linker. *Bacillus subtilis* FtsZ Ct-linker sequence (EQEK… RHTSQ) was amplified from the pBS58 plasmid (39). *Rhizobium mellioti* FtsZ Ct-linker sequence (RTAA…QMRS) was amplified from pJC06 (provided by W. Margolin, (40)). *Azotobacter vinelandii* FtsZ Ct-linker sequence (TRAD…INPQ) was taken from pET11b-AzbFtsZ (H. Erickson). *Caulobacter crescentus* FtsZ Ct-linker sequence (GASI…DDAE ) was amplified from a pET15b plasmid cocon optimized for *E. coli*, provided Dr. M. Schumacher, Duke). The ZipA PQ domain sequences were taken from the *E. coli* ZipA PQ domain previously characterized as unstructured (41). The various adducin Ct-linker sequences were put together from a 66 aa peptide of human α-adducin (residues 440-505) previously characterized as unstructured (42). Sequences were repeated according to the color code in Figure 9. Each is labelled as Add#, where the # indicates the length of the adducin peptide included the in the FtsZ fusion protein. Amplified PCR products were restricted with NotI and XhoI and ligated into the Ec FtsZ construct at the complementary cut sites. Resultant plasmids were verified by Sanger sequencing across the ligation sites. The ΔLink FtsZ construct was made by PCR amplification of the Ec FtsZ plasmid with primers

5’gtctcgagagacgcggattatctgatccacg3’ and

5’ggagcggccggtatactgtgcaaaacggtaacg3’, followed by the blunt end ligation of the PCR product to produce an FtsZ construct in which the Ct linker was replaced with a serine-glycine dipeptide. Charge mutants of the EcFtsZ linker were made by the directed mutagenesis of lysines (K319, K329, K356) to glutamate. Ec-1KE refers to the K319E,

**Growth of JKD7-1 Cells**

The JKD7-1/pKD3 *E. coli* strain was developed by Dai and Lutkenhaus (43), and has been useful to test for complementation of *ftsZ* mutants (19). JKD7-1 is *ftsZ*-null due to a kanamycin cassette insertion into the genomic *ftsZ*. FtsZ for cell division is provided by the pKD3 plasmid, which expresses wild-type *E. coli* FtsZ and is temperature sensitive for replication. The cell strain was maintained at 30°C in Repression Media: Luria-Bertani media containing 100 µg mL⁻¹ of ampicillin (selecting for pKD3 plasmid), 100 µg mL⁻¹ of kanamycin (selecting for the kanamycin inserted at genomic *ftsZ*), 34 µg mL⁻¹ of chloramphenicol (selecting for the pJSB plasmid containing the variant *ftsZ*), and 0.2% (w/v) glucose (suppressing expression of the pJSB-FtsZ protein).

**Complementation**

The ability of variant Ct-linker FtsZ proteins to complement the loss of genomic FtsZ was assayed *in vivo* using a plate complementation assay. JKD7-1/pKD3 cells containing FtsZ with Ct-linker variants on pJSB were grown in Repression Media at 30°C overnight. The cultures were then diluted 1:1000, and 10³ cells were plated on dishes containing Induction Media: Luria-Bertani-agar media containing 100 µg mL⁻¹ kanamycin, 34 µg mL⁻¹ chloramphenicol and 0.2% (w/v) arabinose (to induce expression of the FtsZ protein from pJSB). Plates were grown at 42°C to eliminate the temperature sensitive pKD3 rescue plasmid. In Induction Media at 42°C, the pJSB plasmid is the
only source of FtsZ for cell division. An equal volume of cells was also plated on Repression Media plates and grown at 30°C, to assay the total number of cells plated.

**Growth Curves and Cell Morphology**

JKD7-1 cells with pJSB FtsZ were grown in liquid Repression Media at 30°C with shaking overnight. To track the ability of various FtsZ proteins to function for cell division and normal cell morphology, an aliquot of these cells were transferred to liquid Induction Media at 42°C with shaking. Cell growth was assayed as the optical density at 600 nm at various timepoints following the transfer. 222 minutes after switching to Induction Media and 42°C, cell samples were fixed in cold methanol for DIC microscopy. Some mutants of FtsZ that did not complement were assessed for dominant-negative effects. Non-complementing protein was expressed in Induction Media containing 100 µg mL⁻¹ ampicillin at 30°C, to maintain the pKD3 rescue plasmid. The expression of wt FtsZ from pKD3 should allow for cell division to occur, while testing whether the non-complementing FtsZ would interfere with the wt FtsZ. These cells were also fixed in cold methanol and observed for cell morphology 190 minutes after induction of mutant FtsZ with 0.2% arabinose.

**Results**

To examine the specific role that the Ct linker plays in FtsZ function for cell division, we produced a number of FtsZ variants and tested them for ability to function for cell division. In this complementation assay, JKD7-1 cells, which are genomic ftsZ null, are supported by the expression of wild-type FtsZ from a pKD3 rescue plasmid.
pKD3 is temperature sensitive for replication, and after several generations at 42°C the wild-type FtsZ is depleted. The mutant FtsZ to be tested is introduced to the cell on the arabinose inducible pJSB plasmid. In the presence of arabinose (Induction Media) and at the restrictive temperature of 42°C, the pJSB plasmid is the only source of FtsZ available for cell division. The ability of the mutant FtsZ to function for cell division is demonstrated by the ability of a single plated cell expressing that Ct-linker variant to form a colony. A variant form of FtsZ was considered to complement ftsZ-null when the number of cfu on the Induction plate at the restrictive temperature (cells expressing FtsZ solely from pJSB) was at least 80% that on the Repression plate at the permissive temperature (cells expressing FtsZ solely from pKD3). In general, plating 1000 initial cells resulted in 1000 colonies. This indicates that that FtsZ protein is fully functional for cell division. FtsZ variants that are unable to complement the ftsZ null phenotype do not produce any colonies in this assay, and are thus considered non-functional for cell division. In a previous study, Osawa and Erickson (32) found that some FtsZ mutants produced no colonies when 1000 cells were plated, but when 1,000,000 cells were plated 100-500 colonies grew. These were attributed to suppressor mutations that arose in the E. coli genome. The FtsZ mutant is functional for cell division in the suppressor strain. We tested all FtsZ linker substitutions for complementation, and for those that did not complement we tested for the generation of suppressor strains.

We constructed 32 different Ct-linker constructs from the original pJSB-EcFtsZ plasmid. All new constructs contain a glycine-arginine at the start of the Ct linker (corresponding to the inserted NotI restriction site) and a leucine-glutamate at the end of
the Ct linker (corresponding to the inserted XhoI restriction site). The presence of these four residues in the *E. coli* FtsZ Ct linker did not affect the ability of the FtsZ protein to complement *ftsZ*-null (Table 1), and had no effect on growth (data not shown). For the purposes of this paper we refer to this as Ec (Table 1, Figures 10-12).
Table 1: Complementation Results for All Linker Constructs Tested. The first column lists all C-terminal linker constructs tested. The length reported includes the GR and LE residues from the NotI and XhoI restriction sites. Complementation is reported as ++ for constructs that fully complement the ftsZ null; (+) for constructs that can complement after generation of a second site suppressor mutation; – for constructs that can neither complement nor generate suppressor strains.

<table>
<thead>
<tr>
<th>Ct-Linker Peptide</th>
<th>Length (aa)</th>
<th>Ct-Linker Charge</th>
<th>Complementation</th>
</tr>
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<tbody>
<tr>
<td>Ec</td>
<td>54</td>
<td>1.1</td>
<td>++</td>
</tr>
<tr>
<td>Bs</td>
<td>51</td>
<td>3.2</td>
<td>++</td>
</tr>
<tr>
<td>Av</td>
<td>54</td>
<td>3.1</td>
<td>(+)</td>
</tr>
<tr>
<td>Cc</td>
<td>179</td>
<td>-11</td>
<td>-</td>
</tr>
<tr>
<td>Rm</td>
<td>257</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>CeB</td>
<td>54</td>
<td>-7</td>
<td>++</td>
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<td>RmA</td>
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<td>RmB</td>
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<tr>
<td>RmC</td>
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<td>++</td>
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<tr>
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<td>++</td>
</tr>
<tr>
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<tr>
<td>PQb</td>
<td>54</td>
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<td>++</td>
</tr>
<tr>
<td>PQc</td>
<td>54</td>
<td>-2.9</td>
<td>++</td>
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<td>6</td>
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<tr>
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</tr>
<tr>
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<td>++</td>
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</tr>
<tr>
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<td>-0.8</td>
<td>(+)</td>
</tr>
<tr>
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<td>1.2</td>
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<tr>
<td>Add126blk</td>
<td>126</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Add168blk</td>
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<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>FNII/β-8</td>
<td>188</td>
<td>-12.6</td>
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Initially, we tested the Ct linkers from the FtsZ of other bacterial species for function in *E. coli* FtsZ. The Bs construct, containing the 47 aa Ct linker from *Bacillus subtilis*, fully complemented the *ftsZ* null. The Av construct, containing the 50 aa Ct linker from *Azotobacter vinelandii*, was also functional for cell division, but required the generation of a suppressor strain. However, two other foreign Ct-linker constructs, Cc from *Caulobacter crescentus* and Rm from *Rhizobium meliloti*, did not produce any colonies in the complementation assay, and they did not generate suppressor strains. Since these Ct linkers were much longer than the native *E. coli* Ct linker, 175 and 253 aa respectively, we tested a 50 aa segment from the Cc linker, which is the same length as the Ec Ct linker. This shorter linker, termed CcB, was fully functional for cell division. Five other Ct-linker constructs were made by dividing the 253 aa Rm Ct-linker into 50 aa segments (constructs RmA, RmB, RmC, RmD, and RmE). All five of these chimeric proteins were fully functional for cell division. The sequence of the linkers from the different bacteria, and the other IDP constructs tested, showed only about 15-20% identity to that of *E. coli*, which is essentially random. These initial results suggested that length was the major determining factor for Ct-linker function, rather than the sequence or structure of the peptide.

To further test whether the Ct linker had no structural requirementes, we turned to peptides from the bacterial division protein ZipA, which has a 150 aa “charged plus PQ domain” that forms a separate link between FtsZ and the membrane. This domain, called PQ in Table 1, has been considered to be an IDP (16, 41). The full length PQ domain did not function as the Ct linker, which is consistent with the non-functionality of the longer
Cc and Rm linkers. We then divided the full length PQ evenly into three 50 aa peptides, PQa, PQb, PQc, and tested those for function. PQb and PQc were both fully functional for cell division, consistent with lack of a structural requirement for the FtsZ Ct linker.

We made the construct ΔLink, in which the entire Ct linker was deleted. This protein was non-functional. We also examined the role of the total charge of the Ct linker on FtsZ function, since the native Ec Ct linker and the Bs constructs have a total charge of +1 and +3 respectively. Point mutants changing one, two or three lysine to glutamate in the *E. coli* Ct linker were created from the pJSB-EcFtsZ plasmid, without the addition of the NotI and XhoI cloning sites, to create an increasing negative charge in the Ct linker. These mutants, Ec1KE, Ec-2KE, and Ec-3KE, were all completely functional for cell division. This data, in association with the functionality of the CcB construct, which carries a -7 charge, suggests that the total charge of the Ct linker is not important for FtsZ function.

Finally, we turned to an IDP from the mammalian protein α-adducin, a mammalian protein completely unrelated to bacterial cell division. The initial adducin IDP was 66 aa long, so Ct linkers of different length were either truncated versions of that peptide, or were made by piecing together repeated portions of that IDP at the inserted XhoI restriction site (Figure 9). The adducin Ct linkers were made to vary in length from 10 aa to 160 aa. We found that only Ct linkers between 39 aa and 89 aa were functional. Any Add construct outside of that length range was not able to complement. However, the Add100 construct was able to generate suppressor strains, suggesting that
the Add100 Ct linker is partially functional, but requires a suppressor mutation somewhere in the *E. coli* genome to function for cell division.

Within the acceptable 39-89 aa length range of adducin peptides for the Ct linker, the Add60blk and Add66blk constructs did not function for complementation, and did not generate suppressor strains. Comparing the sequence of these constructs to those that were functional showed that they had a unique 11 aa sequence (QQREKTRWLNS), dubbed the “black sequence”. The presence of this peptide is noted in the constructs by “blk”) (Figure 9). Replacing this black sequence from the Add60blk, with another 11 aa sequence, gave Add60, which was fully functional. Removing the black sequence from Add110blk gave Add100, which was functional in a suppressor strain.
Figure 9: Adducin sequence and constructs tested as linkers. The adducin constructs tested are indicated as bars proportional to their length. Coloring indicates which segments of the adducin peptide are included (and repeated) in each construct. The sequence of each peptide is noted with the numbering from human α-adducin. The inserted NotI and XhoI restriction sites are included as GR or LE aa insertions, respectively.
One possibility for the lethality of the black sequence is that it might provide a novel protease site within the linker. This was examined by Western blot analysis. No mobility shifts were seen in cells expressing various Ct-linker constructs, which indicates that the resultant proteins were not being cleaved cytoplasmically (Figure 10).

Cell proliferation was assessed via optical density at 600 nm, and cell morphology was examined via DIC light microscopy. Any Ct linker that showed complementation of the ftsZ-null phenotype on the plate based complementation assay, also gave normal cell growth and morphology in liquid culture (Figure 11). For those Ct linkers that did not complement in the plate based assay, growth in the liquid culture was slow and plateaued at a low optical density. This was the case for adducin constructs with the black sequence and for ΔLink (Figure 11) as well as for the other non-complementing constructs (not shown). The cell morphology showed filamentation for Add100blk and ΔLink, and Add60blk showed cell lysis. Add100 and Av Ct linkers functioned for cell division only after generation of genomic suppressor mutations. These suppressor strains grew at a rate comparable to that of the Ec Ct linker, and cells showed a normal morphology.
Figure 10: Western Blot of FtsZ expression in JKD7-1 cells at 30°C. FtsZ from the pKD3 rescue plasmid was continuously produced while mutant FtsZ from pJSB was either repressed with glucose (-) or expressed with arabinose (+). All samples were standardized for cell lysate total protein content, and 2µg total protein was loaded.
Figure 11: Growth and morphology for cells using the indicated linkers. Growth curves show turbidity at times following the switch to Induction Media (0.2% arabinose) and 42°C. Images shown are of cells fixed 222 minutes after the switch to Induction Media.
Ct-linker constructs that did not complement were tested for dominant-negative effects on FtsZ function, and thus cell morphology, by expressing the mutant Ct-linker constructs in the presence of wild-type FtsZ from the pKD3 rescue plasmid. At the 0.2% arabinose used for expression, the mutant protein is expressed at about 3-5 times the level of the wild type FtsZ (19). This over-expression of wt FtsZ had little effect on cell morphology, but all non-complementing linker constructs showed dominant-negative effects (Figure 12). The Add60blk Ct linker produced cells that tended to bulge and lyse rather than filament. The other non-complementing constructs caused moderate filamentation. The unusual bulging and cell lysis caused by Add60blk again suggests that the black peptide is disrupting the cell beyond simply preventing FtsZ to function for cell division.
Figure 12: Cell morphology under dominant negative conditions. *E. coli* cells expressing both wt FtsZ from the pKD3 rescue plasmid (at 30°C) and mutant FtsZ from pJSB (0.2% arabinose). Images shown are of cells fixed 190 minutes after induction of mutant FtsZ by addition of 0.2% arabinose.
Discussion

The Ct linker of FtsZ has long been thought to be an IDP, and to function as a flexible tether, connecting the Z ring to the cell membrane. In the most extreme interpretation any IDP would be able to substitute for the Ct linker in EcFtsZ, although its length and persistence length would probably be important. In the present study we have confirmed that almost any IDP between 39 and 89 aa can function in EcFtsZ. Of 20 IDPs tested in this length range, only PQa and adducin peptides containing the black sequence failed to function (and the Av sequence required a genomic suppressor mutation to function).

The mechanical properties of an IDP are often fit by a model of a worm-like chain (wlc), with two parameters, contour length L and persistence length P. L is fixed at 0.34 nm per aa (44), or 17 nm for 50 aa. P varies somewhat for different protein sequences, but for the 72 aa Ct tail of EcFtsZ was fit to 0.55 nm (42), which is a typical value. The average end-to-end distance for this Ct tail, in the absence of any force, was measured by FRET to be 5.2 nm (42), which corresponds well to the 4.4 nm end-to-end distance calculated for a 50 aa wlc with P = 0.55 nm (45).

The constriction force will cause the wlc to behave as an entropic spring, and the end-to-end distance will increase with force. An approximate formula is used in the fit the force to distance (46). To stretch the Ct linker to half its contour length (8.5 nm) requires a force of 9.3 pN. This is nearly twice the ~5 pN force generated by single motor molecules. The entropic spring of the Ct linker is therefore relatively stiff.
The FtsA-binding peptide, which can also bind ZipA, is at the end of the Ct linker, probably in a narrow range of 5-8 nm from the globular domain. The binding site on FtsA is in subdomain 2B, which is about 3-4 nm from the C-terminal amphipathic helix that inserts into the bilayer (47). This would place the C-terminal side of the FtsZ protifilament about 9-12 nm from the inner surface of the membrane. A modest conundrum is posed by ZipA, since its FtsZ-binding site is on the end of a 150 aa IDP, which would provide a much longer entropic spring, with an average relaxed end-to-end distance of 7.7 nm (42). This is twice the length of the FtsA link, and it is not clear how these membrane tethers could work together.

Because the Ct linker is poorly conserved across bacterial species, supplying *E. coli* FtsZ with the Ct linker from *B. subtilis*, *A. vinelandii*, *C. crescentus*, and *R. meliloti* created FtsZ mutants that varied in Ct-linker sequence and length. Any specific sequence requirement of the *E. coli* FtsZ Ct linker was unlikely to also be present in these foreign linkers, so any function seen could not be attributed to sequence identity across species. Indeed, the the Bs Ct linker was functional in *E. coli* FtsZ, suggesting that Ct-linker length was important, rather than sequence. The ability of fragments of the long Cc and Rm Ct linkers to complement the *ftsZ*-null phenotype while the full-length sequences could not further emphasizes the role of Ct-linker length in FtsZ function.

The charge of the Ct linker was manipulated by using different peptides of various charge as the Ct linker and by mutating positively charged lysine residues of the wild-type *E. coli* FtsZ Ct linker to negatively charged glutamate residues. The Ct-linker library tested peptides within the charge range of -11 to +3.2, and in every case, we did
not see an effect of Ct-linker charge on FtsZ function. We propose that FtsZ Ct-linker function is independent of charge.

The requirement of length was tested using an irrelevant and unstructured adducin peptide. We identified the range from 43 aa to 95 aa as the functional range for Ct-linker length. Ct linkers outside of that length range were unable to function for cell division. These unstructured peptides also indicate that there is no structural requirement for a functional Ct linker.

In most cases, non-functional Ct-linker peptides appeared to simply inhibit FtsZ function for cell division, resulting in filamentous cells that are unable to divide (Figure 11), which is typical of non-functional FtsZ mutants. Ct linkers that complemented the \textit{ftsZ}-null phenotype maintained normal cell morphology, indicating that the Ct linker does not have a role in the health of the cell outside of FtsZ function for cell division. Cells expressing Add60blk as the sole FtsZ did not filament, but instead tended to bulge and burst (Figure 12). When expressed with wild-type FtsZ, the tendency for Add60blk to cause cell bulging was more pronounced (Figure 12). This suggests that though a functional Ct linker only acts in the process of cell division to tether the Z ring to the cell membrane, an inhibiting sequence, such as the black sequence, can disrupt the cell beyond preventing cell division and cause global disturbances resulting in cell death.

While the mechanism by which the black peptide prevents FtsZ function is still unclear, Western blot results do not show any cleavage of the Add peptides expressing the black sequence \textit{in vivo} (Figure 10). The loss of FtsZ function cannot be attributed to
the release of the Z ring from the membrane caused by a protease site within the black peptide.

The Av and Add100 Ct linkers were unique in that they did not complement the \( ftsZ \)-null phenotype, but were able to function for cell division after the generation of a suppressor strain. These suppressor strains arose fairly frequently (10\(^{-4}\) to 10\(^{-6}\)), suggesting they are likely loss-of-function mutations in the genome. The requirement of a suppressing mutation suggests the identity of the Ct linker does not abolish FtsZ protofilament formation or force generation, since cell division can be restored in some cases of the non-functional mutant Ct linker. Rather, the Ct linker appears to be important for the attachment of the Z ring and the transmission of the generated constriction force to the membrane.

We suggest that given these results, we can treat the Ct-linker as a worm-like chain to model the function of the FtsZ Ct-linker as an entropic spring. With \textit{E. coli} FtsZ, the native Ct-linker is 54 aa long, giving a contour length of 18 nm (0.34 nm aa\(^{-1}\)). Using a persistence length of 0.55 nm, a relaxed worm-like chain of contour length 18 nm will have an end-to-end distance of 4.5 nm. This would suggest that before binding the membrane, the FtsA binding site is about 4.5 nm from the globular domain of the FtsZ, giving it considerable space and flexibility to move and seek out the membrane-bound FtsA for binding.

Upon FtsZ binding to FtsZ, the Ct-linker would act to transmit the force produced by the Z ring to the membrane. Current models for determining the force required to stretch an entropic spring estimate that the Ct linker would require 8 pN of force to
stretch it from its relaxed length (4.5 nm) to 40% of its contour length (7 nm). Compared to the ~5 pN produced by a single myosin or kinesis head, this is a considerable amount of force, indicating that the Ct-linker of FtsZ is actually a stiff spring once bound to the membrane.
Chapter 3: Identifying Suppressor Mutations of Mutant and Foreign *Escherichia coli* FtsZ by Whole Genome Re-Sequencing

Introduction

FtsZ is an essential bacterial protein that assembles into the Z ring at midcell. This Z ring produces the constriction force for cell division. FtsZ provides the cytoskeletal framework for the cell division machinery, which includes a number of downstream and interacting proteins required for division. FtsA and ZipA are membrane associated proteins that bind the C-terminal 15- to 17-amino acids of FtsZ (termed here the FtsA-binding peptide), thereby tethering FtsZ to the membrane (15, 17, 48, 49). In *Escherichia coli*, upon Z-ring assembly and binding to ZipA or FtsA, FtsK is the first downstream protein to be recruited, and the others follow in a mostly linear fashion at a later time (50-52). The constriction force for the initiation of division is believed to be produced solely by FtsZ (6). The downstream proteins are mostly involved with remodeling the peptidoglycan wall in response to the initial constriction. The overall process of cell division has been reviewed by Adams and Errington (37) and Lutkenhaus et al (53); more focused reviews of FtsZ are by Erickson et al (5) and Mingorance et al (54).

Previous work (32) has shown that FtsZ from divergent foreign bacteria (*Bacillus subtilis* and *Mycoplasma pulmonis*) can function for *E. coli* division given two conditions. (a) The foreign FtsZ must be supplied with the FtsA-binding peptide from *E. coli*. (b) A suppressor mutation in the *E. coli* genome, which facilitates the functioning of the foreign FtsZ, must be obtained. A number of suppressor strains were obtained in that study, each presumed to contain a mutation somewhere in the *E. coli* genome that
allowed the foreign FtsZ to achieve cell division. Suppressor strains were also generated that facilitated function of some point mutations in FtsZ, and of a C-terminal fusion of YFP.

The requirement for a suppressor mutation raises an interesting question: If FtsZ alone produces the constriction force, and the *E. coli* FtsA-binding peptide remains intact, how does a genomic mutation further facilitate cell division by the foreign FtsZ? One possibility is that these suppressor mutations were loss-of-function mutations in negative regulators of FtsZ, or in pathways affecting the integrity of the cell wall, thus allowing for function of partially defective FtsZ. Another possibility is that these mutations may be in genes involved in unknown pathways directly affecting division. Attempts to identify these mutations by traditional bacterial genetics methods were unsuccessful, so we turned to whole genome resequencing by high-throughput sequencing technologies (55, 56). We expected each suppressor strain to have one unique mutation relative to the parent strain.

Hayashi et al (57) sequenced the entire genomes of two K-12 *E. coli* strains (W3110 and MG1655) and showed that the mutation rate between these strains was very modest, with only 8 true differences. The strain we used to generate the suppressors, designated JKD7-1, is a W3110 strain (43), but was separated at an unknown time from the stock strain sequenced by Hayashi. In addition to identifying the suppressor mutation, our resequencing project should discover the number and type of mutations between our lab strain and the published W3110 sequence.

In the present study, we have sequenced the genomes of some of the previously reported strains (32), as well as some newly generated ones. In most cases we have
successfully identified the mutations responsible for the suppression of the *ftsZ*-null phenotype in cells expressing foreign and mutant FtsZs.

**Materials and Methods**

*Bacterial Strains and Growth Conditions*

*E. coli* strain JKD7-1/pKD3 (43) was used for complementation assays and for generating suppressor strains. JKD7-1 has a chromosomal *ftsZ* null mutation (caused by an *ftsZ* disrupting kanamycin cassette) and is supported by a pKD3 rescue plasmid, which expresses wt FtsZ and is temperature sensitive for replication. At 42°C, pKD3 replication is restricted and wt FtsZ is eliminated. The cell strain is maintained at 30°C in Repression Media: Luria-Bertani media containing 100 µg mL⁻¹ ampicillin (selecting for the pKD3 plasmid), 100 µg mL⁻¹ of kanamycin (selecting for the inserted kanamycin cassette), 34 µg mL⁻¹ of chloramphenicol (selecting for the mutant or foreign *ftsZ* on the pJSB plasmid), and 0.2% (w/v) glucose (suppressing expression of the pJSB-FtsZ protein).

*Plasmid Construction and Suppressor Strain Generation*

pJSB2 was used to express the mutant or foreign FtsZ as previously described (32, 58). pJSB2 was derived from the pBAD plasmid, which has an arabinose-inducible promoter. Foreign and mutant *ftsZ* was cloned into the pJSB2. The yellow fluorescent protein (YFP) was the Venus variant (59), which we have found to be superior to other green fluorescent proteins for fusion to FtsZ (12). The foreign *ftsZ* sequences used were those of *B. subtilis*, *A. vinelandii*, and *M. pulmonis*. Chimeric molecules with the *E. coli* FtsZ C-terminal tail (aa 325-383) fused to the N-terminal globular domain of FtsZ from
foreign bacteria were made with a silent SpeI site introduced at aa 326 in EcFtsZ in pJSB2 (32).

JKD7-1/pKD3 cells containing pJSB2 mutant/foreign ftsZ were grown in Repression Media at 30°C overnight. The cultures were then diluted 1:1000, and 10⁶ cells were plated on dishes containing Induction Media: Luria-Bertani-agar media containing 100 µg mL⁻¹ kanamycin, 34 µg mL⁻¹ chloramphenicol and 0.2% (w/v) arabinose (to induce expression of the mutant FtsZ protein from pJSB). Plates were grown at 42°C to eliminate the temperature sensitive pKD3 rescue plasmid. Suppressor mutations arose that permitted the mutant ftsZ to function for division at a frequency of 10⁻⁴ to 10⁻⁶. These were verified to be true genomic suppressors as previously described (32).

*Genome Sequencing and Analysis*

Genomic DNA was isolated from stationary phase liquid cultures of suppressor strains by a standard alkaline lysis DNA extraction protocol. Genomic DNA samples were prepared for sequencing using the Illumina Genomic DNA Sample Prep Kit (San Diego, CA). Briefly, genomic DNA was sheared to 200-800 bp using a nebulizer. 3’ A-overhangs were added to the blunt-ended fragments, and the library was amplified with 18 rounds of PCR. The resultant libraries were sequenced using the Illumina Genome Analyzer II platform, with data outputs of 36 nt single-end reads. Genome sequencing was performed by the Duke University Institute for Genome Sciences and Policy Genome Sequencing and Analysis Core, and by the Georgia Health Sciences University Integrated Genomics Core. Resultant data sets were aligned to the most recent published annotated *E. coli* W3110 genome ((57) GenBank accession number AP009048) using the Bowtie
sequence alignment package (http://bowtie-bio.sourceforge.net) and samtools utilities (http://samtools.sourceforge.net). Single nucleotide mutations were identified in the Integrated Genome Viewer (http://www.broadinstitute.org/software/igv) and later confirmed by PCR and Sanger sequencing across the mutation site.

Complementation in Slow Growth Conditions

JKD7-1/pKD3 cells containing pJSB-mutant/foreign *ftsZ* were grown in Repression Media at 30°C overnight. The cultures were then diluted 1:1000, and $10^3$ cells were plated on dishes containing Induction Minimal Media: M9 Minimal Media-agar containing 100 $\mu$g mL$^{-1}$ kanamycin, 34 $\mu$g mL$^{-1}$ chloramphenicol and 0.2% (w/v) arabinose. An equal volume of cells was also plated on Repression Minimal Media plates and grown at 30°C. A variant form of FtsZ that normally requires a suppressor mutation was considered to complement *ftsZ*-null cells in slow growth conditions when the number of cfu on the Induction plate at the restrictive temperature was at least 80% that of the Repression plate at the permissive temperature.

Results

Generation of Suppressor Strains

Suppressor strains were generated using a modification of the complementation assay originally developed by Dai and Lutkenhaus (43) (Figure 13). JKD7-1 cells, which have a chromosomal *ftsZ* null mutation (*ftsZ::kan*), are supported by a pKD3 rescue plasmid that expresses wild type FtsZ and is temperature sensitive for replication. The foreign or mutant FtsZ to be tested is produced by the arabinose-inducible plasmid, pJSB2 (19, 58). In the presence of arabinose (Induction Media) and after several
generations at the restrictive temperature of 42°C, wt FtsZ is depleted and the pJSB plasmid becomes the only source of FtsZ available for cell division. The ability of the mutant FtsZ to function for cell division is demonstrated by the ability of a single plated cell to form a colony. If the mutant FtsZ protein is fully functional for cell division, the number of colony forming units on the Induction plate at the restrictive temperature (cells expressing FtsZ solely from pJSB) was at least 80% total cells plated (the total was measured on the Repression plate at the permissive temperature). If the FtsZ could not complement the $ftsZ$-null phenotype, we plated a larger number of cells to identify any suppressor colonies. When $10^6$ cells were plated on Induction Media at 42°C, some smaller colonies were observed at a frequency usually between $10^{-4}$ and $10^{-5}$, and as high as $10^{-6}$ in the case of D212G FtsZ. These colonies were ampicillin sensitive and dependent on arabinose for growth, indicating that the rescue plasmid was eliminated and the cells were dependent on the pJSB2-FtsZ. These colonies were identified as suppressor strains, in which a randomly arising genomic mutation allowed a non-complementing FtsZ protein to function for cell division. Further testing confirmed that the ability of the mutant or foreign FtsZ to complement the $ftsZ$ null in these cells was due to a genomic, rather than a plasmid mutation.

The FtsZ that generated each suppressor strain is noted in the strain names. For example, we will use the abbreviation SUP[Av] to refer to suppressor strains generated using the pJSB-Av plasmid expressing FtsZ from *Azotobacter vinelandii*. In the instances where two or more suppressor strains were generated from a single pJSB construct, we
have designated these by appending a number, e.g., SUP[Av3] and SUP[Av4]. All abbreviations are explained in Table 2.
Figure 13: A schematic of the assay used to test a foreign or mutant FtsZ for complementation of the ftsZ-null phenotype or the generation of a suppressor strain.
In three cases reported, we generated a double-suppressor strain. These strains were first isolated and characterized as suppressor strains for the CtYFP, an FtsZ with a C-terminal YFP fusion. That strain was then transformed again with an FtsZ point mutant, which required selection of a second suppressing genomic mutation. These strains are indicated by the notation SUP[CtYFP1//Q47K], where the // separates the two pJSB constructs successively used to generate that strain.

Suppressor strains were apparently generated from random genomic mutations in *E. coli* cells expressing mutant or foreign FtsZ as their sole source of FtsZ; we did not use any mutagen. The relatively high frequency of the appearance of these suppressor strains (10^{-4} to 10^{-6}) suggests that they were the result of a single nucleotide change in the genome, likely resulting in the loss-of-function of a gene product. Because *E. coli* has a spontaneous genome mutation frequency of 0.0025 per generation (60), we expected to find only a single, unique, nucleotide change in each of the 10 sequenced suppressor strains that were derived from a single FtsZ mutant. In the three cases of double suppressors, we expected to find two independent nucleotide changes in the genome, with each one accounting for the suppression of one of the two mutant FtsZ phenotypes.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP[Av3]</td>
<td>Third strain generated against <em>A. vinelandii</em> FtsZ</td>
<td>JKD7-1/pJSB2-Av (mut?)</td>
</tr>
<tr>
<td>SUP[Av4]</td>
<td>Fourth strain generated against <em>A. vinelandii</em> FtsZ</td>
<td>JKD7-1/pJSB2-Av (mut?)</td>
</tr>
<tr>
<td>SUP[Bs/EcCt1]</td>
<td>First strain generated against <em>B. subtilis</em> FtsZ with the <em>E. coli</em> FtsA-binding peptide</td>
<td>JKD7-1/pJSB2-Bs/EcCt (mut?)</td>
</tr>
<tr>
<td>SUP[Bs/EcCt2]</td>
<td>Second strain generated against <em>B. subtilis</em> FtsZ with the <em>E. coli</em> FtsA-binding peptide</td>
<td>JKD7-1/pJSB2-Bs/EcCt (mut?)</td>
</tr>
<tr>
<td>SUP[D212G3]</td>
<td>Third strain generated against D212G FtsZ</td>
<td>JKD7-1/pJSB2-D212G (mut?)</td>
</tr>
<tr>
<td>SUP[D212G9]</td>
<td>Ninth strain generated against D212G FtsZ</td>
<td>JKD7-1/pJSB2-D212G (mut?)</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt13]</td>
<td>13th strain generated against the <em>M. pulmonis</em> FtsZ with the <em>E. coli</em> FtsA-binding peptide</td>
<td>JKD7-1/pJSB2-Mpu/EcCt (mut?)</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt28]</td>
<td>28th strain generated against the <em>M. pulmonis</em> FtsZ with the <em>E. coli</em> FtsA-binding peptide</td>
<td>JKD7-1/pJSB2-Mpu/EcCt (mut?)</td>
</tr>
<tr>
<td>SUP[Q47K2]</td>
<td>Second strain generated against Q47K FtsZ</td>
<td>JKD7-1/pJSB2-Q47K (mut?)</td>
</tr>
<tr>
<td>SUP[CtYFP1//Q47K]</td>
<td>Double suppressor, generated first against CtYFP FtsZ, then against Q47K FtsZ</td>
<td>JKD7-1/pJSB2-Q47K (mut?)</td>
</tr>
<tr>
<td>SUP[CtYFP1//D96A]</td>
<td>Double suppressor, generated first against CtYFP FtsZ, then against D96A FtsZ</td>
<td>JKD7-1/pJSB2-D96A (mut?)</td>
</tr>
<tr>
<td>SUP[CtYFP1//D212G-YFP]</td>
<td>Double suppressor, generated first against CtYFP FtsZ, then against D212G-YFP FtsZ</td>
<td>JKD7-1/pJSB2-D212G-YFP (mut? mut?)</td>
</tr>
<tr>
<td>SUP[CtYFP2]</td>
<td>Second strain generated against CtYFP FtsZ</td>
<td>JKD7-1/pJSB2-CtYFP (mut?)</td>
</tr>
</tbody>
</table>
Genome Alignment and Detection of Point Mutations

We sequenced genomic DNA from 13 unique suppressor strains, generated from the expression of 7 different mutant or foreign FtsZ (Table 2). We obtained 23 to 42 million reads per strain, each with a length of 36 nt, to cover the 4.62-Mb genome. We aligned these sequences to the published *E. coli* W3110 genome AP009048 (57) using the Bowtie sequence aligner package. Reads were aligned to the reference genome only if they contained two or fewer consecutive mismatched nucleotides, eliminating the necessity to screen mapped reads for poor quality or to trim reads of low-quality ends.

This removed between 12% and 51% of reads from the final alignment. Further, reads were suppressed if they mapped to more than one location in the genome, which was between 3% and 5% of all reads in each data set. The resultant final alignments contained the majority of the initial reads and provided genomes with an average coverage depth between 107X and 254X at each mapped nucleotide position (Table 3). Samtools was used to compile a list of SNPs between each strain and the reference genome, but for unknown reasons Samtools failed to reliably identify true differences between the sequences. We therefore used the Integrated Genome Viewer (IGV) program to visually scan each of the strain alignments. IGV provided visual identification of the mutation and a local alignment of all short reads contributing to the mutation call. All mutations identified were present in at least 99% of the reads aligned across the given nucleotide position, with the majority of the mutations occurring in 100% of the aligned short reads.
Table 3: Read Statistics for Sequence Data and Assembly of Suppressor Strain Genomes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total Reads</th>
<th>Aligned Reads</th>
<th>Suppressed Reads</th>
<th>Orphan Reads</th>
<th>Approximate Genome-Wide Depth of Aligned Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP[Av3]</td>
<td>28891402</td>
<td>16404025</td>
<td>1135072</td>
<td>3.93%</td>
<td>11352305</td>
</tr>
<tr>
<td>SUP[Av4]</td>
<td>25575027</td>
<td>13412300</td>
<td>1101800</td>
<td>4.31%</td>
<td>11060927</td>
</tr>
<tr>
<td>SUP[Bs/EcCt1]</td>
<td>40737428</td>
<td>25464712</td>
<td>1651984</td>
<td>4.06%</td>
<td>13620732</td>
</tr>
<tr>
<td>SUP[Bs/EcCt2]</td>
<td>37912312</td>
<td>16681192</td>
<td>1848748</td>
<td>4.88%</td>
<td>19382372</td>
</tr>
<tr>
<td>SUP[D212G3]</td>
<td>23989718</td>
<td>20018951</td>
<td>941445</td>
<td>3.92%</td>
<td>3029322</td>
</tr>
<tr>
<td>SUP[D212G9]</td>
<td>39865602</td>
<td>23804734</td>
<td>1976229</td>
<td>4.96%</td>
<td>14084639</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt13]</td>
<td>41766645</td>
<td>29344412</td>
<td>1525937</td>
<td>3.65%</td>
<td>10896296</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt28]</td>
<td>30542278</td>
<td>14687287</td>
<td>1180475</td>
<td>3.87%</td>
<td>14674516</td>
</tr>
<tr>
<td>SUP[Q47K2]</td>
<td>24995916</td>
<td>18275930</td>
<td>1000579</td>
<td>4.00%</td>
<td>5719407</td>
</tr>
<tr>
<td>SUP[CtYFP1//Q47K]</td>
<td>38136702</td>
<td>28493607</td>
<td>1300019</td>
<td>3.41%</td>
<td>8343076</td>
</tr>
<tr>
<td>SUP[CtYFP1//D96A]</td>
<td>39987284</td>
<td>31740360</td>
<td>1227588</td>
<td>3.07%</td>
<td>7019336</td>
</tr>
<tr>
<td>SUP[CtYFP1//D212G-YFP]</td>
<td>41485020</td>
<td>33118889</td>
<td>1590292</td>
<td>3.83%</td>
<td>6775839</td>
</tr>
<tr>
<td>SUP[CtYFP2]</td>
<td>29613232</td>
<td>16716975</td>
<td>1062132</td>
<td>3.59%</td>
<td>11834125</td>
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</table>
Table 4: Genomic Mutations Common to All Sequenced Suppressor Strains. Base
mutation locations indicate mutated nucleotide numbering in the whole AP009048 reference
genome. These mutations are identified as those where our JKD7-1 strain differs from the
reference W3110 strain.

<table>
<thead>
<tr>
<th>Base Mutations</th>
<th>Gene</th>
<th>Protein Function</th>
<th>Protein Mutation</th>
<th>b-num(^a)</th>
<th>JW id(^a)</th>
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<tbody>
<tr>
<td>A 547694 G</td>
<td>ylbE</td>
<td>hypothetical protein</td>
<td>E38E</td>
<td>b4572</td>
<td>JW0507</td>
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<tr>
<td>A 556858 T</td>
<td>folD</td>
<td>bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate cyclohydrolase</td>
<td>L36Q</td>
<td>b0529</td>
<td>JW0518</td>
</tr>
<tr>
<td>C 663526 T</td>
<td>dacA</td>
<td>penicillin-binding protein 5</td>
<td>W286Stop</td>
<td>b0632</td>
<td>JW0627</td>
</tr>
<tr>
<td>T 825317 A</td>
<td>ybhQ</td>
<td>predicted inner membrane protein</td>
<td>L89Q</td>
<td>b0791</td>
<td>JW0774</td>
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<tr>
<td>G 987574 T</td>
<td>intergenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T 1093686 C</td>
<td>ycdT</td>
<td>predicted diguanylate cyclase</td>
<td>V130A</td>
<td>b1025</td>
<td>JW5143</td>
</tr>
<tr>
<td>A 1103592 G</td>
<td>intergenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 1121926 T</td>
<td>solA</td>
<td>N-methyltryptophan oxidase, FAD-binding</td>
<td>E80K</td>
<td>b1059</td>
<td>JW1046</td>
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<tr>
<td>C 1303982 T</td>
<td>oppA</td>
<td>periplasmic-binding component of the ABC superfamily</td>
<td>Q363Stop</td>
<td>b1234</td>
<td>JW1235</td>
</tr>
<tr>
<td>A 1373590 T</td>
<td>ycjM</td>
<td>predicted glucosyltransferase</td>
<td>Q554L</td>
<td>b1309</td>
<td>JW1302</td>
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<tr>
<td>C 1966148 G</td>
<td>flhA</td>
<td>predicted flagellar export pore protein</td>
<td>R206P</td>
<td>b1879</td>
<td>JW1868</td>
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<tr>
<td>C 2005401 T</td>
<td>fiC</td>
<td>flagellar filament structural protein</td>
<td>E115K</td>
<td>b1923</td>
<td>JW1908</td>
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<tr>
<td>C 2045659 A</td>
<td>serU</td>
<td>tRNA-Ser</td>
<td></td>
<td>b1976</td>
<td>JW1958</td>
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<tr>
<td>C 2778279 T</td>
<td>ypJA</td>
<td>adhesin-like autotransporter</td>
<td>S1035N</td>
<td>b2647</td>
<td>JW5422</td>
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<tr>
<td>A 2822193 G</td>
<td>recA</td>
<td>DNA strand exchange and recombination protein with protease and nuclease activity</td>
<td>L78P</td>
<td>b2699</td>
<td>JW2669</td>
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<tr>
<td>A 3266965 C</td>
<td>intergenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 3697802 A</td>
<td>rbsR</td>
<td>DNA-binding transcriptional repressor</td>
<td>G218V</td>
<td>b3753</td>
<td>JW3732</td>
</tr>
</tbody>
</table>

\(^a\)The b number (b-num) and JW identifier (JW id) are the locus tags in the published
W3110 genome (AP009048).
Table 5: Unique Genomic Mutations Identified in Sequenced Suppressor Strains. Base mutation locations indicate mutated nucleotide numbering in the whole AP009048 reference genome.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Base Mutations</th>
<th>Gene</th>
<th>Protein Function</th>
<th>Protein Mutation</th>
<th>b-num(^a)</th>
<th>JW id(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP[Av3]</td>
<td>C 3899442 T</td>
<td>intergenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUP[Av4]</td>
<td>G 989377 A</td>
<td>asnS</td>
<td>asparaginyl-tRNA synthetase</td>
<td>Q11Amber(^b)</td>
<td>b0930</td>
<td>JW0913</td>
</tr>
<tr>
<td>SUP[Bs/EcCt1]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUP[Bs/EcCt2]</td>
<td>TA 4608134-5 AT</td>
<td>intergenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUP[D212G3]</td>
<td>G 2525323 A</td>
<td>gltX</td>
<td>glutamyl-tRNA synthetase</td>
<td>R265C</td>
<td>b2400</td>
<td>JW2395</td>
</tr>
<tr>
<td>SUP[D212G9]</td>
<td>T 439585 G</td>
<td>ispA</td>
<td>geranyltransfase</td>
<td>K247N</td>
<td>b0421</td>
<td>JW0411</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt13]</td>
<td>T 2767761 A</td>
<td>yfQ</td>
<td>hypothetical protein</td>
<td>S147R</td>
<td>b2633</td>
<td>JW2614</td>
</tr>
<tr>
<td>SUP[Mpu/EcCt28]</td>
<td>A 433159 C</td>
<td>ribD</td>
<td>fused diaminohydroxyphosphorosylaminopyrimidine deaminase and 5-amino-6-(5-phosphoribosylamino uracil reductase)</td>
<td>T161P</td>
<td>b0414</td>
<td>JW0404</td>
</tr>
<tr>
<td>SUP[Q47K2]</td>
<td>G 3308772 A</td>
<td>nplI</td>
<td>conserved hypothetical protein</td>
<td>P3V</td>
<td>b3163</td>
<td>JW3132</td>
</tr>
<tr>
<td>SUP[CtYFP1//Q47K]</td>
<td>C 1738186 A</td>
<td>ydhP</td>
<td>predicted transporter</td>
<td>L273P</td>
<td>b1657</td>
<td>JW1649</td>
</tr>
<tr>
<td>SUP[CtYFP1//D96A]</td>
<td>G 2438846 A</td>
<td>accD</td>
<td>acetylCoA carboxylase, beta carboxyltransferase subunit</td>
<td>Q176Stop</td>
<td>b2316</td>
<td>JW2313</td>
</tr>
<tr>
<td>SUP[CtYFP1//D212G-YFP]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUP[CtYFP2]</td>
<td>G 3746911 A</td>
<td>tnaB</td>
<td>low-affinity tryptophan transporter</td>
<td>P24S</td>
<td>b3709</td>
<td>JW5619</td>
</tr>
</tbody>
</table>

\(^a\)The b number (b-num) and JW identifier (JW id) are the locus tags in the published W3110 genome (AP009048).

\(^b\)Amber refers to the Amber stop codon (TAG)
Since we did not sequence our JKD7-1 lab strain from which these suppressor strains were derived, these common mutations were classified as parental mutations, indicating base differences between the genome of JKD7-1 and the published W3110 reference genome. These mutations are all in non-essential genes or non-coding regions of the DNA and are not expected to have an effect on the health of the cells. This number of differences between the lab strain and published genome is consistent with previously reported resequencing projects of laboratory bacteria strains (61, 62). One parental mutation was identified as a key difference between the JKD7-1 lab strain and the published W3110 genome: the C to A transition at genome position 2045659 occurs in the anti-codon of a Ser-tRNA. This mutation alters the anti-codon of the Ser-tRNA from CGA to CTA, which allows that Ser-tRNA to recognize and bind the TAG amber stop codon. With this mutation, our lab strain is an amber suppressor, occasionally misincorporating serine into growing peptides at the amber stop codon during translation.

A single, unique nucleotide mutation was identified in eleven of the sequenced suppressor strains. The mutations found were different in each strain, even when multiple strains were derived from the same initial non-functional FtsZ, as was the case with SUP[Av3] and SUP[Av4]. While all mutations were expected to cause protein mutations, two of the mapped SNPs were in intergenic regions that do not correspond to a coded protein. The remaining nine mutations all cause protein mutations. The mapped mutations occurred in five essential and four non-essential \textit{E. coli} genes, and corresponded to an amino acid change or a premature stop codon in the identified genes.
None of the identified mutated proteins are known to play a role in bacterial cell division. Most genes appear to be involved in secondary metabolism, important for synthesis of biological macromolecules, and thus cell growth and general health. We hypothesized that the ability of some of these mutations to suppress the non-functional FtsZ phenotype might be based on a general decrease in cell health, perhaps weakening the cell wall, or slowing the cell cycle, to facilitate division by the weakened FtsZ.

*Complementation in Slow Growth Conditions*

To explore the effect of cell cycle time on mutant and foreign FtsZ function, we examined the ability of the mutant or foreign FtsZ to complement an *ftsZ*-null phenotype when cell growth was artificially slowed. We reasoned that in some cases, the mutant or foreign FtsZ may retain some function, but at a level too low to allow for rapid cell growth and division. In that case, forcing the cell division rate to slow may allow the FtsZ enough time to produce a constriction force and divide a cell. The seven pJSB constructs expressing the mutant and foreign FtsZ that generated the suppressor strains examined here were freshly transformed into JKD7-1/pKD3 cells. Additionally, we tested the pJSB-Pa construct, which expresses the *Pseudomonas aeruginosa* FtsZ and generates a suppressor strain that was not sequenced in this study. The cells were grown to stationary phase in LB-Repression Media, then diluted to plate ~10^6 cells on Induction Minimal Media.

We found that the constructs expressing foreign FtsZ from *A. vinelandii* or *P. aeruginosa* were able to complement when grown in minimal medium (Table 6), whereas in rich medium they needed to generate suppressor strains (32). For the other six mutant
FtsZ’s, artificially slowing the cell division rate by the use of minimal media was not sufficient to restore FtsZ function. Though we plated a number of cells high enough to allow for the generation of a suppressor strain, we did not observe any colony growth indicating the accumulation of suppressing mutations. Slower growth is therefore not a general cure for weakened FtsZ mutants, and in most cases seems to inhibit the generation of suppressor strains.
Table 6: Complementation Results in Slow Growth Conditions

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Resultant Suppressor Strain(s)</th>
<th>Complementation in Minimal Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJSB2-Av</td>
<td>SUP[Ay3], SUP[Az4]</td>
<td>++</td>
</tr>
<tr>
<td>pJSB2-Bs/EcCt</td>
<td>SUP[Bs/EcCt1], SUP[Bs/EcCt2]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-D212G</td>
<td>SUP[D212G3], SUP[D212G9]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-D212G/CtYFP</td>
<td>SUP[CtYFP1//D212G-YFP]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-Mpu/EcCt</td>
<td>SUP[Mpu/EcCt13], SUP[Mpu/EcCt28]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-Q47K</td>
<td>SUP[Q47K2], SUP[CtYFP1//Q47K2]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-D96A</td>
<td>SUP[CtYFP1//D96A]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB2-CtYFP</td>
<td>SUP[CtYFP1//Q47K], SUP[CtYFP1//D96A], SUP[CtYFP1//D212G-YFP], SUP[CtYFP2]</td>
<td>-</td>
</tr>
<tr>
<td>pJSB-Pa</td>
<td>None reported here</td>
<td>++</td>
</tr>
</tbody>
</table>
Discussion

Whole genome resequencing has been shown to be a reliable method for identifying single bp mutations in the genome of bacteria (55-57, 61, 62). Here, we have shown that the majority of our suppressor strains differ from the most recently published genome at 18 locations, with 17 of those mutations being changes in our parental lab strain. Other studies that have compared newly sequenced E. coli genomes to the published reference sequence have found a conservative number of changes. While we found 17 parental mutations, Skovgaard et al (62) report finding seven parental mutations between their suppressor strains and the published MG1665 sequence (57). One of those mutations (A 547694 G) also occurred in our lab strain.

We identified the suppressor mutations in 11 of the 13 sequenced strains, but no unique mutations were found in SUP[Bs/EcCt1] and SUP[CtYFP1//D212G-YFP]. We also were only able to find one mutation in SUP[CtYFP1//Q47K] and SUP[CtYFP1//D96A]. Those strains and SUP[CtYFP1//D212G-YFP] were double suppressors, and should therefore have two independent mutations each. This indicates that there were five mutations that we did not find in our sequenced genomes. Because the double suppressors were all initially generated as a single suppressor strain against the CtYFP FtsZ, we expect them to share the CtYFP suppressing mutation. Thus, the two mutations that we did find likely correspond to the second mutation (generated by Q47K or D96A). Given the nature of Illumina whole genome sequencing, which produces millions of short reads (36 nt each) that can be aligned to a total genome, it is often impossible to identify large insertions, deletions or chromosomal rearrangements between
a sequenced genome and the reference. Skovgaard et al (62) showed that copy-number analysis could detect larger chromosomal rearrangements, but we did not attempt this extra step.

While none of the identified suppressor mutations are immediately obvious for playing a role in bacterial cell division, four were in essential genes (*asnS*, *gltX*, *ribD*, and *accD*) with roles in whole cell metabolism, such as tRNA synthesis and acetyl-CoA production. This suggests that alterations in general cell metabolism, probably mildly deleterious, can feed back into cell division and permit division by a partially defective FtsZ. This is consistent with the ability of artificially slowed cell growth, using M9 minimal media, to allow for certain foreign FtsZ to function. We did, however, find mutation in two genes (*ispA* and *nlpl*) that may offer interesting new lines of inquiry.

*ispA* is an essential gene encoding a farnesyl diphosphate synthase (63), an enzyme involved in farnesyl pyrophosphate production and belonging to the pathway producing bactoprenol for peptidoglycan synthesis. The SUP[D212G9] mutation (T2767761 A) encodes a lysine to asparagine substitution at position 247 of IspA. This removes a positive charge very near one of the two “aspartate-rich domains” believed to be important for catalysis (64, 65). This mutation may compromise isoprenoid synthesis in the suppressor strain, leading to a loss of integrity of the peptidoglycan layer, which could allow for a mutant FtsZ producing a lower constriction force to invaginate the membrane and initiate cell division. Leaver et al (61) found that a mutation in the *B. subtilis yqiD* gene, which is a homolog of *E. coli ispA*, was essential to facilitate isolation of L forms, which can grow and divide without a cell wall.
*nlpl* is a membrane-associated lipoprotein with an unclear function. However, perturbation in NlpI levels in *E. coli* causes dramatic changes to cell morphology, including filamentation in *nlpl*-null cells and the formation of single prolate ellipsoids in cell overexpressing NlpI (66). The SUP[Q47K2] mutation (G 3308772 A) encodes a proline to valine substitution at position 3 of NlpI. This is at the start of the “lipobox” signal sequence, which marks the cysteine residue targeted for lipid modification. Altering this sequence may disrupt lipid modification by the protein and thus inhibit or alter NlpI function. The ability of a P3V mutation in NlpI to allow Q47K FtsZ to function for cell division, taken together with the previously demonstrated septation defects of *nlpl*-null cells suggest that *nlpl* may be a previously unknown protein involved in *E. coli* division, and could present interesting new opportunities to study the process of cell division in bacteria.

Apart from *ispA* and *nlpl*, the suppressor mutations we found for mutant *ftsZ* were mostly in metabolic pathways with no obvious or direct link to cell division. This contrasts with a recent study of suppressors of the cell shape gene *rodZ* (67). There, suppressors were found in *rodA* and *mreB*, whose proteins interact directly with RodZ. A difference between the two cases is probably in the frequency at which the suppressor mutations arose. In the RodZ study the frequency is not known, but cells were selected over five to seven days of growth in liquid culture, suggesting that they are quite rare. This would be expected if suppressors could only be obtained in two specific genes. In our case mutations arose at relatively high frequency, $10^{-4}$ to $10^{-6}$, upon overnight culture.
on a plate. This may suggest that suppressors of \textit{ftsZ} can be obtained by mutations in multiple genes in multiple pathways. That is supported by the results of our study.
Chapter 4: A Screen to Identify FtsZ Residues Involved in Intermolecular Bonding Interactions Between Protofilaments in the Z Ring

Introduction

FtsZ, the essential cell division protein found in nearly all prokaryotes (5, 37), is a tubulin homolog that consists of three major domains: a globular domain (which can be subdivided into independently folding N- and C-terminal subdomains), the flexible C-terminal linker (~50 aa), and a highly conserved C-terminal FtsA-binding-peptide (~17 aa). The globular domain is an area of high conservation across bacterial species and is the tubulin homologous portion of the protein, responsible for the protofilament structure and the GTPase activity of the protein. Two individual FtsZ subunits can assemble to create an interface that binds GTP from the top of one subunit and adds a synergy loop from the bottom of the adjoining subunit, creating the active GTPase site. This assembly can continue across multiple subunits to create a protofilament, as seen in Figure 2.

In vivo, FtsZ assembles into a ring structure, known as the Z ring, at midcell. This Z ring can be observed by light microscopy, where it appears to made of a long, continuous filament of nearly uniform density. However, light microscopy is limited by its 250 nm resolution, so the organization of the Z ring cannot be resolved. In vitro, FtsZ assembles into short protofilaments, which are 120-250 nm in length on average (68, 69). These protofilaments further associate into the Z ring, which spans the entire inner circumference of a dividing cell (~3,000 nm for *E. coli*). Using the amount of FtsZ in cells, as determined by quantitative Western blotting, the average volume of an *E. coli* cell (2.5 μm$^3$), and the ~1 μM critical concentration of FtsZ assembly, we expect that the
Z ring is 2 to 6 protofilaments thick (5). Because the average length of protofilaments is much less than the circumference of the cell, it is believed that the Z ring is composed of many short overlapping filaments, rather than a few long protofilaments. This raises the question of how short protofilaments are arranged into the Z ring.

In response to this question, two major opposing models of Z-ring substructure organization have been proposed: the scattered model and the ribbon model (70). The scattered model suggests that multiple protofilaments arrange themselves in a scattered band at midcell, with little or no direct contact between protofilaments. The ribbon model proposes that lateral bonds connect adjacent and parallel protofilaments into an orderly band or ribbon.

Experiments in fluorescence recovery after photobleaching (FRAP) show a rapid turnover of FtsZ subunits within a Z ring (22), suggesting that subunits can be easily exchanged between the Z ring and the cytoplasm, which may not be the case if lateral bonds existed. This evidence, coupled with the tendency for *E. coli* FtsZ to polymerize into single-stranded protofilaments in vitro (68), favors the scattered model of Z-ring substructure.

However, FtsZ from other species of bacteria, including *Thermatoga maritima*, *Methanococcus jannashii*, *Mycobacterium tuberculosis*, and *Bacillus subtilis*, forms multi-stranded protofilaments in vitro (71-74), suggesting that lateral bonds between protofilaments do exist. Further, in the presence of the polycation agent DEAE dextran, or with the crowding agents Ficoll or polyvinyl alcohol, *E. coli* FtsZ can be induced to form ribbons or large bundles of protofilaments, which must involve some sort of lateral
association (33, 75, 76). Recently, “inside-out FtsZ rings” were made, in which FtsZ rings assembled and bound to the outside of a lipid tubule (77). These inside-out FtsZ rings were able to move along the tubule and exchange subunits with the environment, as we have seen with Z rings. Negative-stain electron microscopy showed that these FtsZ rings were ribbons of protofilaments, with a ~5 nm spacing. These images favor the ribbon model of Z-ring assembly.

Previous work has also identified a number of mutations in conserved, solvent-exposed residues of the FtsZ subunit that can completely abrogate FtsZ function (58, 78). Residues of interest for the study described here are R174, L272, and E250.

When looking at the FtsZ subunit from the front face, equivalent to looking at tubulin from the outside of a microtubule (Figure 3A), R174 is the most protruding residue on the right side of the N-terminal subdomain, making it an interesting possibility for lateral bond formation. We might expect that a lateral bond could be formed between R174 and a conserved residue on the left side of a subunit in the adjacent protofilament.

L272 is almost certainly involved in longitudinal contact between FtsZ subunits of a protofilament. It is found on the bottom surface of FtsZ, and appears on crystal structure models of the FtsZ protofilament to contact a number of residues on the top surface of the subunit below (79). The L272A mutant was previously determined to be non-functional for cell division (Masaki Osawa unpublished), suggesting a role for L272 in protofilament assembly and Z-ring formation.
E250 and D253 are also on the right side of the molecule, in the C-terminal subdomain. They are well conserved, and negatively charged, but tucked into a cleft in the subunit. Previous work found that the E250A mutant was functional but the E250K/D253K double mutant did not complement the *ftsZ*-null phenotype or produce suppressor strains. These residues may be interesting candidates for mutation in searching for inter-protofilament interactions.

Though it seems that lateral contacts may exist between protofilaments of the Z ring, the residues involved in these lateral bonds have not been identified. In the present study, we have chosen three solvent exposed residues that may be interesting as candidates for any type of intermolecular bonds, either lateral or longitudinal. We have mutated them to residues which abrogate function for cell division, possibly by destroying lateral bond formation. Then, we have screened a library of random FtsZ point mutant to identify second site mutations within the FtsZ gene that can compensate for the initial loss-of-function mutation, and hopefully identify sites of inter-protofilament interaction. If the original mutation were on the right hand side of the subunit (e.g. R174, E250), a compensating mutation on the left hand side would be a good candidate for a lateral contact.

**Methods**

*Cloning*

All variant forms of FtsZ presented in this paper were constructed in *E. coli* FtsZ in a pJSB2 plasmid, which is derived from pBAD and has an arabinose-inducible
promoter (19, 58). Initial point mutations were identified and introduced into pJSB by site-directed mutagenesis, changing at least two nucleotides in the codon to reduce the likelihood of a random single nucleotide mutation reverting the codon to the wild type sequence.

Construction of Mutant Libraries

Libraries of double mutants were made with the GeneMorph II Random Mutagenesis Kit from Agilent Technologies (Santa Clara, CA). Using this protocol and supplied reagents, the mutant *ftsZ* gene of interest in pJSB2 was amplified by PCR using the primers 5’ccgccattcagagaagaaaccaattgtcca3’ and 5’ttgatgcctgacgctccactctegcat3’, which amplify the entire *ftsZ* with an extra 200 bp of pJSB2 sequence flanking each end. The PCR used the Mutazyme II DNA polymerase, which randomly misincorporates nucleotides during DNA synthesis. Protocol instructions were followed to achieve a predicted mutational frequency of 1-2 nt changes in each produced gene. Mutant library PCR products were cloned into pJSB2 using BamHI and HindIII restriction sites flanking the *ftsZ* gene to produce a library of pJSB-mutFtsZ*.

Growth of JKD7-1 Cells

The JKD7-1/pKD3 *E. coli* strain was developed by Dai and Lutkenhaus (43), and has been useful to test for complementation of *ftsZ* mutants (19). JKD7-1 is an *ftsZ* null due to a kanamycin cassette insertion into the genomic *ftsZ*. Wt FtsZ for cell division is provided by the pKD3 plasmid, which is temperature sensitive for replication. The cell strain was maintained at 30°C in Repression Media: Luria-Bertani media containing 100
µg mL\(^{-1}\) of ampicillin (selecting for pKD3 plasmid), 100 µg mL\(^{-1}\) of kanamycin (selecting for the kanamycin inserted at genomic \(ftsZ\)), 34 µg mL\(^{-1}\) of chloramphenicol (selecting for the pJSB2 plasmid containing the variant \(ftsZ\)), and 0.2% (w/v) glucose (suppressing expression of the pJSB2-FtsZ protein).

**Complementation**

JKD7-1/pKD3 cells containing pJSB2-FtsZ with Ct-linker variants were grown in Repression Media at 30°C overnight. The cultures were then diluted 1:1000, and \(10^3\) cells were plated on dishes containing Induction Media: Luria-Bertani-agar media containing 100 µg mL\(^{-1}\) kanamycin, 34 µg mL\(^{-1}\) chloramphenicol and 0.2% (w/v) arabinose (to induce expression of the FtsZ protein from pJSB2). Plates were grown at 42°C to eliminate the temperature sensitive pKD3 rescue plasmid. In Induction Media at 42°C, the pJSB plasmid is the only source of FtsZ for cell division. An equal volume of cells was also plated on Repression Media plates and grown at 30°C, to assess the total number of cells plated. A mutant was considered to complement if it produced 80% as many cells on Induction Media plates as on Repression Media. \(10^6\) cells were also plated on Induction Media dishes at the restrictive temperature. On occasion, a mutant FtsZ was unable to complement the \(ftsZ\)-null phenotype in the complementation conditions (plating \(10^3\) cells), but was able to produce a few colonies when cells were plated at this higher density. These colonies, which tended to arise with a frequency of \(10^{-4}\) to \(10^{-6}\) were considered suppressor strains had generated a random genomic mutation that allowed the non-complementing FtsZ to function for cell division.

**Sequencing of \(ftsZ\)**
Plasmids were isolated from JKD7-1 cells that grew under complementation conditions using a standard miniprep procedure. Fresh DH5α cells were transformed to ensure that DNA sent for sequencing did not contain any residual pKD3 rescue plasmid. DH5α cells were miniprepped and the resulting plasmid DNA was sequenced via traditional Sanger sequencing across the entire \textit{ftsZ} gene. Sequencing reads were aligned to the wt \textit{ftsZ} sequence to identify any changes.

\textbf{Results}

We have proposed a screening method to identify interacting residues between FtsZ protofilaments. Starting with an initial intermolecular bond candidate residue, we will mutate that residue to abolish FtsZ function. Since any type of intermolecular bond will require contact between two residues, it is possible that a second mutation at the contact site could compensate for the original residue change and restore lateral bond formation. The screen described here provides a method for building and screening a library of second site mutations to identify compensating mutations in FtsZ (Figure 14).

Candidate initial mutations for the screen were selected from the crystal structure of the \textit{Pseudomonas aeruginosa} FtsZ subunit (PDB 1OFU (13)). Because we expect any lateral contacts that exist to be essential to Z-ring formation and function, residues of interest must be surface exposed on any of the side surfaces of FtsZ and to be well-conserved. We must also be able to mutate residues to produce a non-functional FtsZ that does not complement the \textit{ftsZ}-null phenotype and will not give rise to suppressor strains. Ideally, this non-functional mutant FtsZ would be the result of a conservative mutation.
Figure 14: Schematic of Library Generation and Screen. (1) Initial selection of an FtsZ mutant to screen. This mutant must have an amino acid change that renders the protein non-functional and unable to be rescued by a genomic suppressor mutation. (2) Error-Prone PCR of the mutant ftsZ produces a random collection of Mut-FtsZ* genes, in which the original mutant ftsZ has additional random mutations. (3) Production of the pJSB-MutFtsZ* library by ligation of the genes produced in step 2 into a new pJSB vector. (4) Amplification of pJSB-MutFtsZ* library by transformation of DH5α cells. (5) Screening of pJSB-MutFtsZ* library by transformation of JKD7-1 cells, and growth at 42°C in the presence of arabinose. Any colonies observed indicate FtsZ function, and thus a secondary mutation that complements the initial non-functional FtsZ.
We identified three residues (R174, L272, and E250/D253) as good candidates for our screen (Figure 15). All three of these residues are well-conserved across bacterial species (though residue 174 is usually K or R, it is always a charged residue), indicating that changes at these residues may be deleterious, as we would expect for intermolecular contact sites. These residues are also solvent-exposed in individual FtsZ subunits, and R174 and E250/D253 are exposed in the assembled protofilament. E250 was initially examined as a deleterious double mutant: E250K/D253K, which is a severe mutation, changing the charge at two neighboring residues. Individually, we found that E250K was the most severe of these two mutations, abrogating FtsZ function in vivo on its own. Using site-directed mutagenesis, we mutated each of these residues individually to produce three FtsZ mutants that were unable to function for cell division by complementation or with the generation of second site genomic suppressor mutations: R174A, L272V, and E250K. Then for each mutant we generated a library of FtsZ with additional random mutations, aiming for less than one additional mutation per FtsZ.

For each mutant library, any colony that grew on Induction Media at the restrictive temperature must have a functional FtsZ. The pJSB-mutFtsZ* from these colonies were isolated and sequenced to verify the presence of the initial mutation and to identify any new mutation that could account for the rescued phenotype. Any identified second-site mutations were generated and tested as a single mutation in pJSB-FtsZ. The double mutant with the original mutation was made to test for complementation in the
genomic $ftsZ$-null cell. The complete list of compensating mutation candidates and complementation results are listed in Table 7.
Table 7: Results of the Screen for Compensating Mutations Against Non-Functional Mutations of Solvent-Exposed Residues of FtsZ

<table>
<thead>
<tr>
<th>Initial Mutation for Screen</th>
<th>Identified Second Site Mutation</th>
<th>Complementation of Single Mutant</th>
<th>Complementation of Double Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>R174A</td>
<td>L169V</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>L169P</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Q194P</td>
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<td>(+)</td>
</tr>
<tr>
<td></td>
<td>F99S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N165K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E250K</td>
<td>D253E</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>L272V</td>
<td>E305V</td>
<td>++</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>V311A</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>R202C</td>
<td>++</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>E305V / V311A</td>
<td>++</td>
<td>(+)</td>
</tr>
</tbody>
</table>
Figure 15: Initial Candidate Residues for Intermolecular Bonds: Front view of the FtsZ subunit from *P. aeruginosa*, PDB 1OFU (13) (modified from (5)). This view is equivalent to that of tubulin from the outside of a microtubule. The two subdomains of the globular domain are colored blue (Nt) and cyan (Ct). GDP and the synergy loop aa D212 (*E. coli* numbering) are in yellow. Candidate residues for intermolecular bonding are R174 (colored in red), E250 (colored in green), and L272 (colored in pink).
The screen of R174A produced five second site mutations that appeared to suppress the non-functional R174A phenotype: L169V, L169A, Q194P, F99A, and N165K. After reintroducing each of these mutations into the pJSB-R174A-FtsZ, only L169V and L169A were verified as able to complement R174A. These two mutations were also able to complement when introduced as single mutations into FtsZ. L169 is a well conserved residue, and in the FtsZ structure, is solvent exposed on the upper right face of the subunit, very near R174 (Figure 16). While L169V/A is able to complement R174A, it seems unlikely that this is due to a restoration of a lost lateral contact as these residues are on the same side of the assembled protofilament. The other three mutations that came out of the screen (Q194P, F99A, and N165K) were unable to complement the R174A mutation when directly introduced into the pJSB-R17A-FtsZ. They were also deleterious on their own, with F99S and N165K completely abolishing FtsZ function, and Q194P requiring the generation of a suppressor mutation to function for cell division. F99 and N165 are buried in the N-terminal subdomain, and Q194 is surface-exposed on the helix H7 connecting the N- and C-terminal subdomains. The reason for these false positives in the original screen is unclear. They are reported here for potential interest as new mutants of FtsZ.

The screen of L272V produced three second site mutations that appeared to suppress the non-functional L272V phenotype: E305V, V311A, and R202C (Figure 17). All three of these mutations were able to function for cell division, either alone or as a double mutant with L272V, E305V and R202C required a genomic suppressor mutation.
E305V and V311A were isolated together in the screen, as an L272V/E305V/V311A triple mutant, and both the E305V/V311A double mutant and the triple mutant were functional. We have not tested E305V and V311A as single mutants. None of these residues are candidates for a longitudinal contact because they are all in the C-terminal subdomain, along with L272.

The screen of E250K produced one second site mutation that appeared to suppress the non-functional E250K phenotype: D253E (Figure 18). Both of these residues are surface-exposed and close to one another. While the glutamic acid to lysine mutation in the earlier construct E250K/D253K replaced an acidic residue with a basic one, the aspartic acid to glutamic acid maintains the negative charge, and simply elongates the side chain by one CH₂. The conservative nature of the second-site mutation as well at the close proximity between E250 and D253 (8 Å apart) suggests that the mechanism by which D253E complements E250K is unlikely to be related to lateral bond formation between protofilaments. The extra length of 253E may allow this negatively charged side chain to form a salt bridge with the 250K, neutralizing the introduced positive charge that rendered the original mutant non-functional.
Figure 16: Mutations of L169 Can Compensate for a Mutation in R174. The front view of an FtsZ subunit (at left) and the right view of the FtsZ subunit (at right). The original R174 mutated residue is colored in red and the compensating L169 residue is colored in green.
Figure 17: Mutations of E305 and V311 Can Compensate for a Mutation in L272. The front view of an FtsZ subunit (at left) and the right view of the FtsZ subunit (at right). The original L272 mutated residue is colored in red and the compensating E305 and V311 residues are colored in green.
Figure 18: Mutations of D253 Can Compensate for a Mutation in E250. The front view of an FtsZ subunit (at left) and the right view of the FtsZ subunit (at right). The original E250 mutated residue is colored in red and the compensating D253 residue is colored in green.
Discussion

If lateral contact sites between protofilaments of the Z ring are required for Z-ring formation and function, we would expect that contact sites would be surface-exposed on the right and left faces of the FtsZ subunit (rather than the top or bottom, which are known longitudinal contacts involved in subunit assembly and GTPase/GAP activity). Further, if we were to mutate one contact site, cell division should be halted. This defect might be rescued by a complementary mutation at the other side of the lateral contact.

The screen presented here provides a method for generating and screening for random second site mutations that may complement a pre-existing intermolecular contact candidate mutation. While this screen is successful at identifying compensating mutations, its utility in finding true intermolecular contact sites is limited by the small number of candidate starting aa’s and by the spectrum of mutations generated in the library.

While this screen has not identified strong candidates for contact pairs in FtsZ, it has yielded a number of compensating mutation pairs, as well as three new point mutations that have severely deleterious effects on FtsZ function. That we have found loss-of-function mutations in this screen indicates that its stringency is such as to allow false-positive results, and thus is unlikely to cause false-negatives. A low false-negative rate is highly beneficial for such a screen.

R174 was initially identified as a residue of interest for lateral bonding because it is the most protruding residue on the right hand side of the FtsZ subunit, residing in a loop between helix 6 and helix 7. Previous studies proposed that R174 mediated the
binding of FtsZ to the membrane, and that the mutants R174D/A were defective in formation of protofilament bundles in Ca\(^{++}\) (80). It was suggested that R174 is involved either in lateral bonding or in interactions with other divisome proteins, as the protruding loop containing R174 is protected from trypsin digestion in the Z ring, indicating some sort of shielding. However experiments from our lab have shown that R174A has no defect in assembly of bundles in Ca\(^{++}\) or polyvinyl alcohol, and it assembles ribbons of protofilaments in DEAE dextran similar to wild type FtsZ. R174 remained a favored candidate for lateral bonding because of its protruding position. However, the failure to find any compensating second mutation on the other side, dampens our enthusiasm.

L272 was not a candidate for lateral contacts, but was a a known longitudinal interface contact. If the rationale of our study were valid, we would expect that the non-functional L272V might generate compensating mutants of contacting aa’s across the interface, in the N-terminal subdomain. However, the only compensating mutations we found were in the C-terminal subdomain, perhaps changing its conformation to position the 272V for a better contact. This result questions the validity of this search to identify lateral contact sites.
Appendix A: Modeling the Forces Required to Bend an FtsZ Protofilament

In order to understand the ability of the Z ring to divide a cell, multiple attempts have been made to model the force required to bend a protofilament. By extension, the force required to bend a protofilament can give us an idea of the force generated by Z-ring constriction. Thus, we can model membrane deformation caused solely by the transition of protofilaments within a Z ring from the straight to curved conformation.

Determining the force produced by bending a protofilament requires first an estimate of protofilament rigidity. Previous work by Gittes and Howard (81) determined the Young’s modulus for both actin and microtubules as 1.4 GPa, which would give these filaments a stiffness equivalent to that of Plexiglas. Because FtsZ is a homolog of tubulin, and forms protofilaments analogous to those in the microtubule wall, we can extend this same Young’s modulus to FtsZ protofilaments.

From that, we can divide an FtsZ protofilament into two halves that act as cantilevered beams that are fixed at one end, presumably to the membrane via FtsA. The small-angle approximation of the beam equation can estimate the amount of force \( F \) needed to bend the free end of a cantilevered beam by a distance \( y \) by the formula:

\[
F = \left( \frac{3EI}{L^3} \right) \cdot y
\]

In the formula above, \( E \) is the Young’s modulus (previously determined at 1.2 GPa), \( L \) is the length of the rod, and \( I \) is the second moment of inertia. For an average FtsZ protofilament of length 130 nm, each cantilevered beam will have a length \( L \) of 65 nm \((130 \div 2)\). We will use \(8.4 \times 10^{-36} \text{ m}^4\) for the second moment of inertia \( I \), determined
by Allard and Cyantrynbaum (36). With these parameters, we can estimate the force required to bend a straight protofilament into the 200-nm diameter intermediate curvature, which would give us a deflection of 20 nm at each end. This calculated force is 2.6 pN on each end of the protofilament, or 5.2 pN across the entire protofilament.

This force calculation reinforces two ideas. Firstly, that protofilaments are very stiff structures, requiring a great deal of force to cause conformation changes. Secondly, that the Z ring itself is able to produce force on the order of several piconewtons, which is the estimated requirement to deform a bacterial membrane (34).
Appendix B: Modeling the C-terminal Linker of FtsZ as a Worm-Like Chain

The Ct linker of *E. coli* is 54 aa long (aa 316-370). If we treat the Ct linker as a worm-like chain, we can calculate the contour length \( L \) using a link length of 0.34 nm for each amino acid. From that, \( L = 0.34 \times 54 = 18.3 \) nm. We will use a persistence length \( P \) of 0.55 nm, which is typical for a wlc of this length (42). This value of persistence length states that fluctuations in the chain between two points 0.55 nm apart on the length of the chain are unrelated. This makes intuitive sense, as there are 0.34 nm between peptide bonds, so two amino acids span 0.68 nm, which is larger than the expected 0.55 nm persistence length. Thus, residues are free to move without relation to any residue except for its immediate neighbor. For \( L >> P \), \( P \) and \( L \) are related to the relaxed end-to-end distance of a wlc by the equation \( \sqrt{2PL} \), so

\[
\sqrt{2PL} = \sqrt{(2)(0.55)(18.3)} = 4.5 \text{ nm.}
\]

This is comparable to the experimentally-determined end-to-end distance of 5.2 nm (42).

After determining the contour length, persistence length, and relaxed end-to-end distance, we can estimate the force required to stretch the wlc by some fraction of its contour length with the following equation (46):

\[
F(x) = \frac{kT}{P} \left[ \frac{1}{4} \left( 1 - \frac{\Delta x}{L} \right)^2 - \frac{1}{4} + \frac{\Delta x}{L} \right]
\]

From this, we estimate that a force of 9.3 pN is required to stretch the wlc to 50% of its contour length, or 9.15 nm.
A Note on Persistence Length

Persistence length is a parameter of a filament that describes resistance to thermal forces. For a given length of a filament, we can measure thermal bending at various points along that length. The persistence length describes the distance between two points on the chain at which the bending of the filament at each point caused by thermal forces is unrelated. In short, it is a measure of a filament’s rigidity.

In our model of the Ct linker as a wlc, we expect a contour length of 18.3 nm and a persistence length of 0.55 nm, which is much shorter than the contour length. This indicates that the wlc has a great deal of freedom in motion, with amino acids free to move without regard for amino acids that are two or more residues away.

As a protofilament, FtsZ forms a filament with a persistence length of its own. Experimental measurement by cryoEM gave a persistence length of 1.2 µm for GTP-bound FtsZ protofilaments (69). This is a very long persistence length compared to the average protofilament length of 120-250 nm, indicating that the protofilament is very resistant to thermal forces and any motion along the length of the protofilament is experienced by the entire protofilament. Thus, FtsZ protofilaments are quite stiff. This persistence length also agrees well with the Young’s modulus and moment of inertia detailed in Appendix 1.
Appendix C: Extracting Genomic DNA for Next-Generation Sequence Analysis

Most genomic DNA extraction protocols utilize a phenol-chloroform extraction step. This step can leave trace amounts of phenol or chloroform in the final genomic DNA. The Illumina GAII sequencing protocol requires addition of fluorescently-labelled ddNTPs to each DNA cluster for imaging, and subsequent removal of the labelled ddNTP. The proprietary enzymes that complete this step seem to be very sensitive to phenol and/or chloroform, so the presence of these chemicals in the genomic DNA results is very low quality reads. Initial sequencing was done with DNA extracted by phenol-chloroform, and these reads gave whole genome alignments with many gaps and very low coverage (4-9x). The genomic DNA protocol below is recommended for further genome sequencing projects.

*E. coli* Genomic DNA Extraction Protocol

Start with a 50 mL culture of cells to be prepped.

1. Spin down cells. Resuspend pellet in 5.5 mL TE (50 mM Tris, pH 8.0, 50 mM EDTA). Add 1 mg lysozyme to suspension. Incubate at 37°C for one hour.
2. Add 700 µL 10% SDS and 30 µL 10mg mL⁻¹ Proteinase K. Incubate at 65°C for 10 minutes.
3. Add 1 mL 5 M KOAc, mix, incubate on ice for 60 minutes or longer. The white precipitate that forms consists mainly of insoluble potassium dodecyl sulfate and denatured proteins.
4. Transfer to a 50 mL centrifuge tube and spin at 10,000 rpm for 45 minutes.
5. Transfer supernatant for a 12 mL centrifuge tube. Add 4 mL ice-cold absolute EtOH. On mixing, the nucleic acids (2% DNA, 98% RNA) and some residual proteins will immediately precipitate.

6. Spin at 10,000 rpm for 10 minutes and discard the supernatant. Wash the pellet with 4 mL 70% EtOH. Spin at 10,000 rpm for 10 minutes and discard the supernatant again.

7. Air dry pellet. Resuspend in 1.2 mL TE (Incubate at 42°C for 10 minutes or 4°C O/N if pellet doesn’t immediately resuspend).

8. Add 60 µL 10 mg mL⁻¹ Rnase and incubate at 37°C for 30 minutes.

9. Add 60 µL 3M NaOAc and 3.6 mL isopropanol. Precipitate should be immediately visible. If not, put on dry ice for 5 minutes. Spin for 5-10 minutes. Wash with 80% EtOH. Air dry pellet.

10. Resuspend in 100-300 µL TE.
Appendix D: Utilization of Next-Generation Sequencing Analysis Software

Sites for Required Open-Source Software and Related Files

http://sourceforge.net/projects/bowtie-bio/files/bowtie/0.12.7/bowtie-0.12.7-linux-x86_64.zip/download

http://sourceforge.net/projects/samtools/files/samtools/0.1.12/samtools-0.1.12a.tar.bz2/download

http://sourceforge.net/projects/maq/files/maq/0.7.1/maq-0.7.1.tar.bz2/download

http://sourceforge.net/projects/bio-bwa/files/bwa-0.5.9rc1.tar.bz2/download

http://sourceforge.net/projects/samstat/files/samstat.tgz/download

http://bedtools.googlecode.com/files/BEDTools.v2.10.1.tar.gz

http://www.broadinstitute.org/software/igv/

http://bamview.sourceforge.net/

http://microbes.ucsc.edu/cgi-bin/hgTables?org=Escherichia+coli+K+12+substr++W3110&db=eschColi_K_12_SUBSTR_W3110&hgsid=555432&hgta_doMainPage=1

http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-40

Alignment of Next-Generation Sequencing Reads to a Reference Genome

Using the Bowtie Aligner

All commands are run from the command line in the Mac Terminal. In this example, the original fasta reference genome is “W3110.fasta” and the sequencing dataset text file is “Q47K2.txt”.

Prepare reference genome for bowtie:

bowtie-build W3110.fasta W3110
Runs bowtie with the same parameters as MAQ (default parameters):

```bash
bowtie -t -S -p 23 genomes/W3110 reads/Q47K2.txt output/defaults.sam
```

Runs bowtie with more stringent parameters (m 1 = unique matches only; v 2 = maximum of 2 mismatches per aligned read):

```bash
bowtie -t -m 1 -v 2 -S -p 23 genomes/W3110 reads/Q47K2.txt output/m1v2.sam
```

Export SAM format to BAM format

```bash
samtools view -bS defaults.sam > defaults.bam
samtools view -bS m1v2.sam > m1v2.bam
```

Sort and Index BAM files

```bash
samtools sort defaults.bam defaults_sorted.bam
samtools sort m1v2.bam m1v2_sorted.bam
samtools index defaults_sorted.bam.bam
samtools index m1v2_sorted.bam.bam
```

Visualize with IGV and/or BamView

Export Bam to Bed

```bash
bamToBed -i output/defaults_sorted.bam.bam > reads_default.bed
bamToBed -i output/m1v2_sorted.bam.bam > reads_m1v2.bed
```

Calculate coverage

```bash
genomeCoverageBed -i reads_default.bed -g genomes/my.genome > results_defaults
genomeCoverageBed -i reads_m1v2.bed -g genomes/my.genome > results_m1v2
```

Pileup SNPs from alignment

```bash
samtools pipeup --cvf W3110.fasta defaults.bam>defaults.snp
samtools pipeup --cvf W3110.fasta m1v2.bam>m1v2.snp
```
References


involved in membrane interaction and protofilament bundling, and is essential for cell division. Mol Microbiol 51:645-657.

Biography

Kiani Anela Jeniah Arkus Gardner was born to Robert and Melissa Arkus in Honolulu, HI on November 17, 1985. Kiani was able to pursue an interest in science from an early age, due to the outstanding resources and advising provided by the Kamehameha Schools, Bernice Pauahi Bishop Estate. She continued her development as a scientist when she matriculated at Washington University (St Louis, MO) in 2003. By the time she earned her AB in Biology in 2007, she had earned two federally funded fellowships, presented her work at six professional meetings, and published two research articles as a first author. She began work on her Ph.D. at Duke University (Durham, NC) in 2007. While at Duke, she has earned a Certificate of Cell and Molecular Biology, a Certificate of College Teaching, and an NIH National Research Service Award Predoctoral Fellowship.

List of Publications


