FtsZ Protofilament Curvature is the Opposite of Tubulin Rings

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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

2016
ABSTRACT
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Abstract

Bacterial tubulin homolog FtsZ assembles straight protofilaments (pfs) that form the scaffold of the cytokinetic Z ring. These pfs can adopt a curved conformation forming a miniring or spiral tube 24 nm in diameter. Tubulin pfs also have a curved conformation, forming 42 nm tubulin rings. We have previously provided evidence that FtsZ generates a constriction force by switching from straight pfs to the curved conformation, generating a bending force on the membrane. In the simplest model the membrane tether, which exits from the C terminus of the globular FtsZ, would have to be on the outside of the curved pf. However, it is well established that tubulin rings have the C terminus on the inside of the ring. Could FtsZ and tubulin rings have the opposite curvature? In the present study we explored the direction of curvature of FtsZ rings by fusing large protein tags to the N or C terminus of the FtsZ globular domain. FtsZ with a protein tag on the N terminus did not assemble tubes. This was expected if the N terminus is on the inside, because the protein tags are too big to fit in the interior of the tube. FtsZ with C-terminal tags assembled normal tubes, consistent with the C terminus on the outside. The FN extension was not visible in negative stain, but thin section EM gave definitive evidence that the C-terminal tag was on the outside of the tubes. This has interesting implications for the evolution of tubulin. It seems likely that tubulin began with the curvature of FtsZ, which would have resulted in pfs curving toward the interior of a disassembling MT. Evolution not only eliminated this undesirable curvature, but managed to reverse direction to produce the outward curving rings, which is useful for pulling chromosomes.
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1. Historical Background of FtsZ

FtsZ is the most important bacterial cytoskeletal element in cytokinesis. Forming the Z ring at the center of dividing bacteria, FtsZ exerts a bending force to constrict the membrane, leading to the eventual splitting into two bacteria. The components of the eukaryotic cytoskeleton, such as actin filaments and microtubules, have been studied for decades, and are well established fields with a large body of knowledge. In contrast, FtsZ, the first prokaryotic cytoskeletal element to be characterized, was not identified as such until the 1990’s, making the study of the bacterial cytoskeleton a much newer endeavor. Many discoveries have been made since that time, showing FtsZ to be a tubulin homologue capable of polymerization and force generation to aid in bacterial cytokinesis. Much still remains to be learned about how FtsZ and the Z ring are able to constrict the bacterial membrane during division.

1.1 Discovery

FtsZ stands for Filamenting Temperature Sensitive Z, a name which stems from the phenotype used to identify it. Random mutagenesis studies conducted in bacteria were used to characterize the effects of interrupting the functions of genes. One studied phenotype was filamenting. Bacteria with a certain mutations became unable to divide at restrictive temperatures. Some of these defects would allow all steps of division to be carried out except for cytokinesis. Those cells deficient in cytokinesis became long and filamentous. The $ftsA$ locus was one such locus that resulted in filamenting cells. This locus was discovered in the 1950’s by Hirota, but it was not studied further until 1980.
There were numerous characterized \textit{ftsA} strains, all initially assumed to be different alleles of the \textit{ftsA} gene. However, in 1980, Lutkenhaus and Donachie showed through complementation studies that there were other genes present in the \textit{ftsA} locus, one of which they named named \textit{ftsZ} (1). At this time there were multiple groups studying \textit{fts} mutations and the identifier “Z” was chosen to prevent any overlap and confusion in naming conventions. \textit{ftsZ}, however, turned out to be arguably the most important of the \textit{fts} genes. One set of observations made in this paper would turn out to be highly predictive of the function of the FtsZ protein, which was not discovered until more than a decade had passed. Lutkenhaus and Donachie noted that while both the \textit{ftsA} and \textit{ftsZ} mutants showed multinucleate filaments, there was a slightly different phenotype. \textit{ftsA} mutants showed indentations all along the filament, which were presumed to be “partially completed septa”. The \textit{ftsZ84} mutant showed no signs of constriction or septation, leading them to propose that FtsA and FtsZ were both involved in septation, but at different times. Little did they realize that FtsZ would turn out to actually comprise the machinery required for formation of the septum.

After this paper distinguishing \textit{ftsZ} and \textit{ftsA} as separate genes, there was little progress as to the function of FtsZ until 1991. The early 1990’s led to an explosion of papers as many of the functions and interactions of FtsZ were discovered.

\textbf{1.2 FtsZ and FtsA}

While it had been shown that defects in FtsZ led to an inability to form septa and undergo cytokinesis, it was still not known whether or not \textit{ftsZ} was an essential gene. It had only been shown that the temperature sensitive mutation \textit{ftsZ84} mapped to the \textit{ftsZ}
locus. In 1991, Dai and Lutkenhaus proved that \textit{ftsZ} was an essential gene, and also showed a correlation between filamentation and overall FtsZ levels (2, 3).

The most important result from this paper was to show that \textit{ftsZ} is an essential gene. They accomplished this by creating the JKD7-pKD3 cell line, which is temperature sensitive for FtsZ production. When the genomic copy of \textit{ftsZ} was disrupted by the kanamycin resistance cassette, the cells were unable to divide and would die. This was rescued by transforming in the temperature sensitive plasmid pKD3, which contained a wild type copy of \textit{ftsZ}. At 30\textdegree C cells grow normally, but at 42\textdegree C the plasmid is no longer able to replicate. This means that successive generations of bacteria will not receive the plasmid containing \textit{wtftsZ}. As the cells grow, the remaining FtsZ is diluted and degraded. Thus, after incubation at 42\textdegree C for a few hours, the cells become unable to divide properly. It is important to note that the temperature sensitivity is related to plasmid replication, not protein function, necessitating the lengthier incubation at the restrictive temperature. This strain is well suited to complementation assays to test whether various FtsZ constructs are functional as the sole source of FtsZ. By transforming the construct of interest, on a repressible/inducible plasmid, into the JKD7/pKD3 strain one can shift to the restrictive temperature while inducing the experimental plasmid, thus replacing all FtsZ in the cells with the experimental construct. This method is extremely powerful and is still widely used today.

Dai and Lutkenhaus initially tried to complement an FtsZ null phenotype with lambda phage carrying \textit{ftsZ}, but this was unsuccessful. The phage complementation most likely didn’t work because the levels of FtsZ produced were too low. This is a very important negative result to note, as it shows that wild type FtsZ function is highly
dependent on the amount of FtsZ present. This was confirmed by observing at what level of FtsZ expression filamentation started when they created the JKD7 cells.

The next year, in 1992, Dai and Lutkenhaus showed that cell division wasn’t only dependent on the overall FtsZ levels, but also on the levels of FtsA (4). The correct ratio between FtsA and FtsZ is essential for cell division in *E. coli*. In the same issue of *Journal of Bacteriology*, Dewar et al published the same findings about FtsA and FtsZ ratios (5). Raising the levels of either FtsZ or FtsA by 5 to 10 fold will prevent cellular division. However, division can be rescued by raising the levels of the other 5 to 10 fold to compensate. While a certain baseline level of FtsZ and FtsA is required to maintain cell division, it is the ratio between the two that is important when levels are elevated.

### 1.3 Z Rings and the Cytoskeleton

At the end of 1991, Bi and Lutkenhaus published a paper showing the localization of FtsZ to the septum of dividing cells (6). This is the first time that anybody had shown specific localization of FtsZ in cells, as well as the first time that electron micrographs were taken, showing this localization. This study was groundbreaking in the FtsZ field for identifying where the protein localized in cells. Additionally, this was the first paper in which it was postulated that FtsZ “may function as a cytoskeletal element.” This bold statement was made at a time when cytoskeleton was thought to exist only in eukaryotes.

They used immunogold labeling of FtsZ to observe sections of *E. coli* by electron microscopy and saw that as the cell progressed through the division cycle, FtsZ localized to the septum. Before the cell started to form a septum there was no distinct localization of FtsZ, just diffuse spots throughout the cell. Once the septum started to
form, the FtsZ localized to the membrane at the septum. The FtsZ stayed associated as the septum constricted all the way until the cell had divided. See Figure 1 for these electron micrographs. In some sections, they were able to observe a cross section through the septum of the dividing bacterium. In these images, the FtsZ appeared to form a ring all around the membrane. From the images, they proposed that FtsZ forms a ring structure in dividing bacteria that constricts as the septum tightens. After cytokinesis is completed, the ring disappears until division recommences. This model can be seen in Figure 2. This was the first model to propose the involvement of FtsZ in a ring structure during cytokinesis. However, it was still not known at this point how the FtsZ is able to localize to the septum or the causality of localization: does the septum form first, causing FtsZ localization; or does the FtsZ form a ring that becomes the septum after some unknown constriction force is applied.

Figure 1: Immunogold electron micrographs of FtsZ in dividing cells. The top two images are longitudinal sections. Note the clusters of densities at the invaginating septum in (g) and almost closed septum in (h). This two dot structure indicates a ring all around the septum. Adapted from Bi and Lutkenhaus (1991)

However, despite the novel electron micrographs collected in this study, there was a large error regarding the timing of FtsZ localization to the septum. Bi and
Lutkenhaus stated that FtsZ only localized to the septum as it started to form and delocalized after division was completed. This meant that FtsZ was only present in the ring structure for about 5 minutes of the 30-40 minute *E. coli* life cycle. This was shown to be false in series of three papers published in 1996 by Levin and Losick; Addinall, Bi, and Lutkenhaus; and Ma and Margolin (7-9). All three of these papers showed the presence of Z rings in bacteria by use of fluorescence microscopy, a technique rarely used in bacteria at the time. Levin and Losick observed Z rings in *Bacillus subtilis* by immunofluorescence, marking the first time that FtsZ had ever been observed by fluorescence microscopy. The Addinall, Bi, and Lutkenhaus paper followed by performing similar immunofluorescence microscopy, but in *E. coli*, the most common bacterium used in FtsZ studies. However, both of these experiments were conducted in fixed cells. The Ma and Margolin paper became the first to observe Z rings in live cells, using FtsZ tagged with GFP. The results of all three papers were in agreement, showing that fluorescently tagged FtsZ was localized to internucleoid rings in about 90% of an asynchronous population. They showed that FtsZ was localized in the ring structure for the vast majority of the bacterial life cycle, not just during septation and cytokinesis.
The observations that FtsZ forms a ring in dividing bacteria and the implication that FtsZ acts as a multimer an were excellent setup for studies in 1992 showing that FtsZ is a GTP binding protein with homology to the important eukaryotic cytoskeletal protein, tubulin. Consecutive articles in Nature by RayChaudhri and Park; and De Boer, Crossley, and Rothfield identified FtsZ as a GTP binding protein with GTPase activity (10, 11). After finding that FtsZ could bind and hydrolyse GTP, these groups looked for a Walker motif in FtsZ. The Walker motif is a common ATP and GTP binding motif found in a large number of proteins. As the most common motif, it was thought that it must be contained in FtsZ. However, the Walker motif was nowhere to be found. It was then that a different GTP binding motif was found. The sequence noted in FtsZ was AGMGGGTGTGAAP, found to be conserved in all three bacterial species for which FtsZ sequences were known at the time. This sequence contains the residues GGGTGTG,
also known as the tubulin signature sequence. The tubulin signature sequence, GGGTGSG, is found in alpha, beta, and gamma tubulins and is highly conserved as the GTP binding domain. Providing further evidence of this connection was the fact that the ftsZ84 mutant had an amino acid change in this signature sequence, SGGTGTG, instead of GGGTGTG.

While the discovery that FtsZ has significant homology to tubulins is arguably the most important point in the paper, it was downplayed for exactly that reason. In 1992, there had been no proven bacterial cytoskeletal proteins; FtsZ would be the first. It would be safer to merely mention the motif similarity and focus on the nucleotide aspect than to be the first one to say that a prokaryotic cytoskeletal protein had been discovered without further, concrete evidence. For such a monumental claim, it would be much easier to gain acceptance if there was more solid proof. The word cytoskeleton was not even used in either paper. It was not until the next year, 1993, that Mukherjee, Dai, and Lutkenhaus, in an independent discovery of the connection to tubulin, wrote that “Cell division in E. coli involves the localization of FtsZ to the division site in a ring structure that may be a cytoskeletal element.”(12)

1.4 Homology to Tubulin

Fortunately it was not long until more proof that FtsZ was a bacterial cytoskeletal homologue to tubulin arose. In 1994, Mukherjee and Lutkenhaus acquired the first electron micrographs of FtsZ assembly into filaments in a GTP dependent manner, as seen in Figure 3 (13, 14). FtsZ was shown to form filaments upon addition of GTP at 37C. Filaments did not form when GMP or ATP was added.
It was also shown in this paper that FtsZ filaments had the ability to associate. When incubated with DEAE-dextran, filamentous structures larger than a single pf were seen. These structures appeared to be hollow tubules, similar to a microtubule structure (15). At this time it was not known whether or not these assemblies had in vivo implications.

![Electron Micrograph](image)

**Figure 3: The first electron micrographs of in vitro FtsZ assembly into pf. Filamentation occurs only in the presence of GTP. Taken from Mukherjee and Lutkenhaus (1994)**

In addition to the groundbreaking electron microscopy, this paper contained stronger proof that FtsZ was a tubulin homolog and therefore a bacterial cytoskeletal element. 12 different amino acid sequences were aligned: alpha tubulin, beta tubulin, and gamma tubulin from *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Homo sapiens* were aligned with FtsZ from *E. coli*, *Bacillus subtilis*, and *Rhizobium meliloti*. Previous alignments had shown very low similarity except for the tubulin signature motif. However, Mukherjee and Lutkenhaus lined all 12 sequences up at the tubulin signature motif and worked outwards to find similarities. By adding gaps, they were able to discover 16 residues that were identical in all 12 sequences, as well as numerous others that were identical in most of the sequences or contained conservative mutations. It is important to note that many of the locations where gaps were added to the FtsZ
sequences already contained gaps opened to align the tubulin sequences with each other. This figure really cemented the homology of FtsZ and tubulin, a fact that was danced around only a couple years previously.

In 1998, crystal structures of both FtsZ and tubulin showed identical complex folds, providing even more proof of the homology between the two. Tubulin is a notoriously difficult protein to crystallize due to the presence of multiple isoforms in tissue and the requirement for chaperones to assist in folding. Nogales, Wolf, and Downing finally solved the structure of tubulin by electron crystallography (16). Mixing tubulin with zinc created flat, antiparallel sheets that were used to create a three dimensional reconstruction by electron microscopy. The structure was resolved to 3.7 Angstroms, showing a Rossmann fold of alternating beta strands and alpha helices, often found in nucleotide binding proteins. The very next article in that issue of Nature was Löwe’s crystal structure of FtsZ (17). The structure, from *Methanococcus jannaschii* FtsZ, was resolved to 2.8 Angstroms, and was almost identical to the tubulin structure. Both structures contained the Rossmann fold and superimpose almost perfectly, within a couple of Angstroms. The chances of two such proteins converging on identical complex folding structures are essentially zero, again confirming the homology between FtsZ and tubulin.

### 1.5 Assembly Dynamics

One of the most important aspects of FtsZ assembly into pfs was not well studied until after the turn of the millennium: the kinetics and dynamics of assembly. A 2005 paper published by Chen, Bjornson, Redick, and Erickson showed that FtsZ assembles rapidly in a cooperative manner after the formation of a dimer nucleus (18). A common
method used in the past for measuring protein assembly is light scattering. In this technique, the more pfs formed, the greater the signal. However, any association between pfs, or assembly into other structures would completely throw off light scattering measurements. For these reasons, the Erickson group developed the \textit{L68W} mutant of \textit{ftsZ}, which shows large increases in tryptophan fluorescence signal upon assembly. This provides an advantage over light scattering, as fluorescence is purely a readout of subunit assembly into filaments, independent of interactions between filaments or other structures. They found that FtsZ assembly “showed a lag, followed by nucleation, elongation, and a plateau.” The curve was shown to fit to a model containing a weak dimer nucleus, after the formation of which polymerization occurred in a simple elongation.

\textbf{Figure 4:} Electron micrograph of FtsZ tubes formed in DEAE-dextran. Inset is the diffraction pattern, which indicates a helical structure. Adapted from Lu, Reedy, and Erickson (2000)

A 2002 paper by Stricker, Maddox, Salmon, and Erickson used FRAP (Fluorescence Recovery After Photobleaching) to study the half time of subunits in the Z ring in live cells (19). They expressed FtsZ-GFP as a dilute label to visualize Z rings \textit{in vivo}. By measuring the overall levels of fluorescence in the cells, it was determined that
only about 30% of the total FtsZ was present in the Z ring. This meant that the majority of the FtsZ existed as a cytoplasmic pool, which was presumably constantly exchanging with FtsZ in the assembled pfs. The exchange of subunits was measured by FRAP. In these experiments, about half of the Z ring was bleached and the fluorescence recovery time was measured. This was the smallest spot that could be bleached based on the resolution limit of the microscope. The half time of an FtsZ subunit in the Z ring was measured in this study to be about 30 seconds. However, this figure was narrowed down to 7-9 seconds by Anderson, Filho, and Erickson in 2004 (20). The 2004 paper repeated the FRAP experiments using different methods of bacterial preparation in both \textit{E. coli} and \textit{B. subtilis}. The half time was found to be the same for both species, suggesting that the extremely rapid exchange dynamics of FtsZ are universal across many bacterial species.

In addition to the fluorescence assay of L68W, which established the kinetics and nucleation of the initial assembly, Chen and Erickson (21) developed a FRET assay to measure the exchange of FtsZ subunits between pfs of purified FtsZ. They assembled two separate pools of FtsZ, one tagged with green fluorescent dye, the other with red fluorescent dye. When the pools of assembled pfs were mixed, subunit exchange mixed the two populations, leading to an increase in FRET signal. They found a half time similar to that measured in the FRAP studies in live cells.

1.6 \textit{Functional Structures – straight and curved protofilaments}

In 1996, Erickson’s EM of FtsZ polymers showed two very different conformations for FtsZ pfs (22). This led to the question of which type of FtsZ assembly was actually the functional assembly. The two types of structures seen up to this point
were pfs and tubules. The pfs had the ability to bundle into large sheets of pfs. Lutkenhaus first observed the tubules and thought that they, being visually the most similar to microtubules, were the functional FtsZ structure. However, Erickson noted that the FtsZ tubule structure may be an artifact of DEAE-dextran addition to the assembly solution. DEAE-dextran is a polycation that was used in the tubulin field to aid in assembly. In the earlier FtsZ papers, it was thought that the FtsZ tubules formed upon DEAE-dextran addition were comparable to the microtubule structures. Erickson, however, noted that the structure of the tubules appeared to be different.

Figure 5: Electron micrograph of FtsZ minirings and pfs. Note the presence of closed minirings as well as minirings forming from the end of pfs (arrowhead). Adapted from Erickson and Stoffler (1996)

Erickson’s 1996 paper showed electron micrographs of sheets of pfs, as well as the never before seen structure of FtsZ minirings. It is important to note that these sheets are simply flat arrays of pfs next to each other, not collapsed tubules. The sheets
are also only one subunit thick, not large three dimensional arrays. All of the pfs in these arrays are straight, analogous to a microtubule wall. The minirings described in this paper were portions of FtsZ pfs that had a high degree of curvature. They were seen both freely and splitting off from the end of a sheet of pfs. The free, complete minirings were measured to have a diameter of roughly 24 nm. See figure 5 for an electron micrograph of pfs and minirings. A similar miniring structure is formed by tubulin, although with a larger diameter of 38-43 nm. The existence of the FtsZ minirings suggests that the curvature of FtsZ pfs may be involved in constriction.

Microtubules are typically a lateral assembly of 13 pfs made up of alpha and beta tubulin dimers. As seen in the top image of Figure 6 tubulin rings curl out from the end of pfs that make up the microtubule. The tubulin rings were originally thought to be helical templates corresponding to the three-start helix running around the microtubule (23). However Erickson (24) and Kirschner (25), independently showed that tubulin rings were equivalent to protofilaments, but with a strong curvature. When Erickson discovered FtsZ minirings, they were proposed to be homologs of the tubulin rings, with a slightly smaller diameter.

The Erickson lab subsequently made the connection between FtsZ minirings and the tubes assembled in DEAE dextran (26). The tubes had the same diameter as the minirings. EM and diffraction analysis showed that they were curved protofilaments similar to minirings, but instead of forming a flat circle, the protofilament had a slight pitch. Three or four protofilaments could assemble into a three- or four-start helix, making the tube. It was suggested that the natural conformation of the curved protofilament was a helix with a small pitch. The flat, closed circles of minirings were imposed when the curved protofilaments were adsorbed to the cationic lipid monolayer.
These are compared in Fig. 6. For the purposes of Chapter two it is important that the DEAE dextran tubes are structurally equivalent to the minirings.

The study of Lu, Reedy, and Erickson also explored the GTP dependence of conformation (26). It was shown that GTP favors the straight pfs, while GDP favors the tube/miniring structure. The fact that each of the conformations is related to GTP hydrolysis led to the current model of how Z rings are able to exert constriction force in cells, as detailed below. However, in order for FtsZ to apply any force to the membrane, it has to be attached to the membrane. The mechanism for this attachment was unknown at this time.

It wasn’t until 1998 that Brun provided the first evidence that the interaction of FtsZ with FtsA may be involved in membrane attachment (27). The C terminal tail of FtsZ contains an 8-15 amino acid sequence that is conserved among most bacterial species. This tail is essential, as truncation is lethal. It is not involved in GTP binding, GTPase activity, or polymerization yet is still essential and the truncation mutant cannot complement an FtsZ knockout. This leaves a potential role as a domain to interact with other proteins.
Yeast two-hybrid experiments showed that the C terminal region was essential for interaction with FtsA, the original filamenting temperature sensitive gene. This led to a 2002 paper by Pichoff and Lutkenhaus, in which it was shown that FtsA, as well as the protein ZipA, were involved in Z ring assembly (28). It was found that the C terminal tail of FtsZ is capable of interacting with both FtsA and ZipA, and that at least one is necessary for Z ring assembly. In the absence of either FtsA or ZipA, the Z ring will form, but the cell will not be able to divide. In the absence of both FtsA and ZipA, neither Z ring formation nor division will occur. It was found that both FtsA and ZipA are involved in recruitment of downstream division proteins. These proteins are not involved in Z ring formation, but are essential for division, which is why the filamenting phenotype is seen in FtsA mutants even though Z ring formation is still possible because of ZipA.

Finally, a 2005 paper by Pichoff and Lutkenhaus solved the mystery of FtsZ attachment to the membrane, tying these previous studies together (29). The C
terminus of FtsA contains a conserved helical motif that is amphipathic and thus able to insert into lipid membranes. This discovery finally solidified a model for Z ring assembly: FtsZ assembles into pfs, which are attached to the membrane by primarily FtsA, but also ZipA. At this point, constriction forces are generated and downstream division proteins are recruited to remodel the peptidoglycan and allow cytokinesis. This also explains the initial observations of the 1980 paper that saw constriction in FtsA mutants, but not in FtsZ mutants. The presence of ZipA alone was able to allow Z ring formation, but downstream recruitment was halted. Interestingly, these Z rings were able to generate partial constrictions, but not to the point where division could occur.

There are currently two main theories to explain how Z ring constriction occurs. One theory is that there are lateral interactions between pfs that allow them to slide past each other, tightening the Z ring. This theory relies primarily on the existence of lateral interactions between pfs, and secondarily on the ability of FtsZ to continually form and break these bonds. Unfortunately this theory does not seem to be energetically feasible; it requires the creation and breakage of greater numbers of bonds as constriction occurs (30). Each time more bonds are formed, it becomes more difficult to break them all simultaneously. Additionally, this theory does not explain how the Z ring would continually tighten, as opposed to loosening as the bonds are broken. For these reasons the second theory, that constriction is based upon the curvature of FtsZ pfs, is a more likely theory.

1.6.1 Curved pfs can bend membranes and generate a constriction force

It was suggested in 1997 that FtsZ might generate the constriction force for cytokinesis by itself, without any motor protein, by using the conformational change from
the straight to curved pf (31). For the next ten years there was no way to test this hypothesis. In 2008 the reconstitution of Z rings in vitro provided the needed breakthrough (32). This reconstitution was achieved by fusing an amphipathic helix (membrane targeting sequence, or mts) to the C terminus of FtsZ, so it could tether itself to the membrane. When this FtsZ-mts was incorporated inside tubular liposomes, it spontaneously assembled Z rings (Fig. 7a,c), and these Z rings constricted the liposome (constrictions are not visible in 7C, but were prominent in other liposomes). Both Z-ring assembly and constriction were achieved with purified FtsZ-mts; no other protein was required. The constriction force generated by FtsZ was strong enough to invaginate multilamellar lipid vesicles that were up to 1 µm thick.

The constriction observed in this reconstituted system was consistent with the curvature hypothesis but did not prove it. Evidence in favor of pf curvature as the mechanism of constriction force was obtained by applying FtsZ-mts to the outside of large, unilamellar liposomes. There it formed patches that bent the membrane into concave depressions (33). This direction of the bending was the same as inside liposomes, where it binds to the concave inner surface and constricts it to be more concave. These concave depressions on the outside of large liposomes were also observed by Arumugam et al (34).

The most definitive evidence that pf curvature generates the constriction force was obtained by switching the membrane tether to the opposite side of the pf. This was possible for FtsZ, because the N and C termini are flexible peptides emerging from the globular domain about 180 degrees apart. The normal C-terminal attachment in FtsZ-mts was predicted to be on the outside of the curved pf (Fig. 7a). Switching the attachment to the N terminus gave mts-FtsZ, which would place the mts on the inside of
the curved pf (Fig. 7b). As predicted by the bending hypothesis, mts-FtsZ assembled Z rings on the outside of tubular liposomes (Fig. 7d) (35). Remarkably, these “inside-out Z rings” also generated a constriction force, squeezing the liposomes from the outside. These experiments provided strong support for the pf curvature hypothesis.

Figure 7: (a) The normal tether on the C terminus is on the outside of the curved pf, and attaches to the concave membrane on the inside of a liposome or cell. (b) Switching the tether to the N terminus places it on the inside of the curved pf, for attachment to the convex surface on the outside of a liposome. (c) Z rings inside a tubular liposome reconstituted from FtsZ-mts. (d) Inside-out Z rings assembled on the outside of a liposome by mts-FtsZ. In c and d the upper panel shows FtsZ imaged by fluorescence, and the lower panels show the liposome imaged by DIC. (Reprinted from (32, 36) with permission of the authors.)
1.7 Z Ring Substructure

While understanding the mechanism of FtsZ constriction is essential to understanding bacterial division, the substructure of the Z ring itself is equally important. The arrangement and spacing of the pf's that form the Z ring is a matter of some controversy. There are two primary models for the substructural arrangement of the Z ring: the scattered model, and the ribbon model. The scattered model posits that the Z ring is greater than 100 nm wide, with pf's 40-50 nm apart, unable to make direct contact with each other (37). The ribbon model states that the Z ring is less than 25 nm wide with 2-5 parallel pf's in direct contact with each other, connected by lateral bonds. The difficulty in determining the substructure of the Z ring lies in the difficulty of its direct observation.

Epifluorescence microscopy has been the most common and powerful tool for observing the Z ring in living bacteria, but is severely limited when its resolution is compared to the molecular scale. Fluorescence microscopy is diffraction limited to a resolution of 250 nm, far larger than the 5 nm width of a single pf. Additionally, there are only an estimated 5,000 FtsZ subunits in an E. coli cell, with only ~2,000 subunits forming the Z ring (38-40). This limited amount of FtsZ means that if all of the FtsZ formed into a single, extended superfilament, it would only encircle the dividing bacterium 2.5 times. This limited amount of FtsZ in the Z ring coupled with the resolution limits of fluorescence microscopy have made it extremely difficult to observe the Z ring in high detail. Fortunately, there are microscopy methods available to bypass this resolution limit and allow greater detail information to be gleaned about the Z ring substructure.
An ideal microscopy technique for investigating Z ring substructure and assembly dynamics in vivo is PALM (PhotoActivated Localization Microscopy) (41). PALM is able to achieve 30 nm resolution, roughly 10 times better resolution than is possible with conventional imaging. PALM requires the use of a photoactivatable fluorophore, which is activated by a wavelength of light different than the wavelength used to visualize it. A short pulse of light activates a small number of fluorophores, each of which are likely to be farther apart than 300 nm, and thus can be resolved as separate points. The fluorophores are then excited with the excitation wavelength until they bleach and become nonfunctional. This creates an image of dots, each dot representing a single fluorophore. The process is repeated and eventually an overlaid image of all of the individual fluorophores is created. Each fluorophore in the image is actually a diffuse spread of photons that approximates a Gaussian distribution with a width at half maximum of 250 nm, the resolution of the light microscope. However, the centroid of each point can be calculated, using the Gaussian distribution, to a resolution of ~30 nm. The composite image is crowded with fluorophores closer than 250 nm apart, but because each fluorophore was activated separately, the centroids can be resolved. This is how PALM can achieve such an increased resolution over conventional microscopy. This technique would seem to be ideal for reconstructing images of the individual pfs in a Z ring, as well as for tracking single FtsZ molecules to study assembly and exchange dynamics in vivo.

The scattered model was first suggested by a cryoEM study of C. crescentus, which showed short pfs scattered around the Z ring and only rarely in contact with each other (37). Several studies by super-resolution light microscopy have indirectly supported this model by reporting widths of the Z ring ~110 nm (42-44); a ribbon of 2-5
pfs should be only 10-25 nm wide. A recent PALM study of the Z ring in \textit{Streptococcus pneumoniae} measured the axial width of the Z ring to be 95 nm early in the cell cycle, increasing to 127 nm as the Z ring constricted (45). It should be noted, however, that the most recent and comprehensive PALM studies (42, 45, 46) also measured the thickness of the Z ring in the radial direction. The radial thickness was very close in magnitude to the axial width (71 nm axial width vs 64 nm radial thickness (42); Fig. 7 in (45); 99 nm axial vs 59 nm radial (46)). This large radial width contradicts our expectation that all pfs are tethered to the membrane by FtsA and ZipA, necessitating a Z ring that is radially one subunit thick. It is important to recall that in \textit{E. coli} the Z ring averages only 2-5 pfs thick (both axial and radial), so there is not enough material to build in the radial direction. This suggests that the actual radial thickness should be ~5 nm. The large radial thickness measured in the PALM images may therefore reflect the achieved resolution (which is worse than the resolution predicted theoretically in the studies). Since the measured axial width was only 7-40 nm more than the radial, the actual axial width of the Z ring might well be as small as 10-25 nm. Overall, the super-resolution light microscopy does not provide convincing support for the larger width of the scattered model.

A recent cryoEM study by Szwedziak et al (47) provides strong support for the ribbon model in both \textit{C. crescentus} and \textit{E. coli}. Their tomograms showed parallel arrays of pfs encircling the cells at the constriction site; these were clearly ribbons, one pf thick radially, and with variable axial widths. Szwedziak et al (47) also presented clear images of FtsZ rings reconstituted in liposomes with FtsA (Fig. 5). A typical Z ring comprised a ribbon of 2, 3 or 4 pfs that formed a continuous helical loop around the constriction, with a zone of overlap. In all cases the ribbons were one pf thick, with all pfs
apparently linked to the membrane by FtsA. This supports the more important observation of Z rings in bacteria, which are ribbons, one pf thick.

The Erickson lab has recently made advances that allow for a fully functional FtsZ labeled with a fluorophore. An mVenus fluorophore was able to be inserted internally to a loop region of FtsZ, creating a fully functional, fluorescently labeled FtsZ. This development will allow for future superresolution studies to achieve both greater resolution and have vastly increased coverage of the Z ring.

1.8 Protofilament Curvature

The generation of directional constriction by FtsZ is highly dependent on the orientation of FtsZ monomers within the pf, leading to an orientation of the pf relative to the plasma membrane. While pfs are predicted to be thermodynamically capable of bending in any direction, experiments with both tubulin and FtsZ suggest that each possesses a preferred direction of curvature that is consistent and uniform throughout force generation (30, 48-50). That is to say that all FtsZ pfs consist of subunits that are aligned in a top-bottom manner with one terminus facing the exterior of the curvature, and the other terminus facing the interior. Because bending forces are likely responsible for FtsZ force generation, knowing the direction of curvature of FtsZ is essential to understanding both its function and evolutionary path towards tubulin.

The curvature and structure of tubulin has been well documented and understood in many experiments. The C-terminus faces the exterior of the microtubule wall, with the N-terminus facing the interior. The alpha-beta tubulin dimers running vertically along the microtubule wall are analogous to the FtsZ pf (22). Tubulin curling off the end of the microtubule is what forms the miniring structures mentioned previously.
This can be seen in the left image of Figure 8. These tubulin minirings have the N-terminus facing the outside and the C-terminus facing the inside; the outside of the microtubule wall curls around to become the inside of the miniring. These tubulin minirings are analogous to the FtsZ minirings and tubes formed in DEAE-dextran. The tubes are helical open rings. It is important to note that the minirings are analogous structures and that the FtsZ miniring and tube are NOT analogous to the circumference of the microtubule. The microtubule circumference is formed of lateral bonds between neighboring tubulin filaments. These FtsZ minirings can be seen curling off the end of individual pfs in the right image of Figure 8.

Figure 8: The left image is an electron micrograph of a microtubule. The composite pfs run vertically, with minirings curling off the end of the microtubule. This is analogous to the FtsZ in the right image. This shows individual FtsZ pfs with minirings forming by curling off the end. The arrow points to an incomplete miniring actively curling off of a pf; note the complete and sealed miniring directly to its left.
It has also been well established in FtsZ that the C-terminal tail is used for attachment to FtsA and thus the plasma membrane (27-29). For this reason, it would make sense that the C-terminus faces the exterior of the curved pf. However, this is the exact opposite curvature established in tubulin, with the N-terminus facing the exterior of the highly curved miniring. Solving this controversy and determining the direction of curvature of FtsZ is the primary focus of this dissertation.
2. FtsZ Protofilament Curvature is the Opposite of Tubulin Rings

2.1 Introduction

FtsZ is the primary cytoskeletal element in bacterial cytokinesis, and is essential for cell division. FtsZ subunits assemble into protofilaments (pfs) that then assemble further to form the Z ring at the center of dividing bacteria. This Z ring acts as a scaffold for additional division proteins (51, 52), and also exerts a constriction force on the plasma membrane to drive the invaginating septum (32, 36, 53). Recent work suggesting that proteoglycan remodeling may provide the major constriction force (46) is addressed in Discussion.

FtsZ pfs have two extreme conformations – straight and highly curved (22). We have proposed that the conformational change from straight to curved pfs generates a bending force on the membrane that leads to constriction (22). The first experimental support for this model came from reconstitution of Z rings in liposomes by purified FtsZ-mts. Normally FtsZ is tethered to the membrane by FtsA, which has an amphipathic helix that functions as a membrane targeting sequence (mts). Previous experiments in our lab (32) transferred the mts to the C terminus of FtsZ, producing FtsZ-mts, which could directly bind the membrane. When incorporated inside tubular liposomes, FtsZ-mts assembled into Z rings, and the Z rings generated force that constricted the liposomes. Both Z-ring assembly and constriction force were generated by FtsZ-mts alone, and did not require any other protein. This was consistent with the bending hypothesis but did not prove it. Additional evidence came from switching the mts from the C terminus to the N terminus, which is on the opposite side of the FtsZ pf (36). These switched pfs no longer assembled Z rings inside liposomes, but assembled
“inside-out” Z rings on the outside of liposomes. The inside-out Z rings also constricted the liposomes, squeezing from the outside. This supports the pf bending model, since the pf curvature is the same, and the preference for binding to the concave or convex lipid surface depends on which side of the pf the membrane tether was attached to.

The eukaryotic cytoskeletal protein tubulin is a homologue of FtsZ (17, 54). Tubulin also assembles pfs, and they have straight and curved conformations (24, 25). The curved conformation of tubulin is seen at the end of disassembling microtubules, where the pfs peel outward from the microtubule wall to form tight spirals and rings (55). The tubulin rings can do mechanical work, since the bulge of tubulin rings provides a mechanism by which a disassembling microtubule can drag an attached chromosome (56). The discovery of FtsZ minirings (22) suggested that that the curved conformation was conserved from FtsZ to eukaryotic α-β tubulin, with only a small change in diameter (24 nm for FtsZ, 42 nm for α-β tubulin).

However, the FtsZ bending-pf mechanism poses an apparent contradiction. In the simplest model the tether needs to be on the outside of the curved pf, to face the concave membrane surface inside the bacterium or liposome. This places the the C terminus of FtsZ, from which the tether exits, on the outside of the curved pf. In contrast, it is well established for tubulin rings that the C terminus, corresponding to the outside of a microtubule, is on the inside of the ring (57-60). If our model is correct for FtsZ, the rings of tubulin and FtsZ would have to curve in opposite directions. To test this orientation we prepared FtsZ with a protein tag fused to the N or C terminus, and used electron microscopy (EM) to determine whether the tag was on the inside or outside of the curved pfs.
2.2 EXPERIMENTAL PROCEDURES

2.2.1 Protein Purification and Assembly

FtsZ constructs were expressed in *E. coli* and purified by ammonium sulfate precipitation and Resource Q anion exchange column. 20% saturated ammonium sulfate was used to precipitate the majority of bacterial proteins. The ammonium sulfate concentration was increased to 30% saturation to precipitate the FtsZ. The protein was then dialyzed into Ltk50 buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 10% glycerol, pH 7.9) and purified on a Resource Q anion exchange column. Peak fractions were dialyzed into HMK100 buffer (50 mM HEPES, 100 mM KAc, 5 mM MgAc, pH 7.6), with 10% glycerol added for storage at -80°C. Concentration was measured by tryptophan fluorescence where available, or BCA assay using a BSA standard and multiplying by 0.75 to correct for the reduced color of FtsZ (61). The purification was essentially the same for wild type FtsZ (wtFtsZ); FN-FtsZ and FtsZ-FN (FtsZ with fibronectin (FN) fragment FN7-10 fused to the N or C terminus; and FtsZ-mVenus, with the YFP variant mVenus fused to the C terminus).

All reactions were carried out in HMK100 buffer, with 1 mg/ml protein, and 2 mM nucleotide. Filaments were assembled by adding GTP and incubating for 10 minutes at room temperature. Tubes were assembled by adding GDP, waiting 1 minute, adding DEAE-Dextran, and incubating for 10 minutes at room temperature. The amount of DEAE-Dextran added was .25 mg/ml for wtFtsZ, .5 mg/ml for FtsZ-mVenus, and 1.5 mg/ml for FtsZ-FN.
2.2.2 Assembly of Z Rings with Truncated Linkers.

To determine whether FtsZ with truncated linkers can still assemble Z rings, we used the construct FtsZ-G55-mVenus-Q56-366-mts, where the YFP variant mVenus is inserted in a loop between FtsZ aa’s G55-Q56 (D. Moore et al, submitted for publication), and the amphipathic helix from MinD is attached at aa 366, replacing the conserved FtsA-binding peptide (32). This construct has the full length linker. We prepared additional constructs 341-mts, 321-mts and 316-mts, where the mts was attached at the indicated aa. In 316-mts the mts is attached to the last aa of the globular domain, completely deleting the linker. These constructs were expressed from plasmid pJSB2 in E. coli strain JKD7-pKD3, in which the wtFtsZ can be depleted by raising the temperature to 42° C (62). JKD7 cells were diluted 1:100 in LB from overnight culture at 30° C in repression conditions, then grown at 42° C for 5 hrs. Samples were viewed with fluorescence microscopy on a Zeiss Axiophot microscope with a plan-Neofluar 100X NA 1.3 oil immersion lens. Filter cubes optimized for YFP were used for fluorescence microscopy. Images were acquired with a Coolsnap HQ CCD camera (Roper Scientific) and processed with Adobe Photoshop.

2.2.3 Electron Microscopy and Staining

Negative stain samples were prepared by adding 10 µl of assembled protein to a carbon coated grid. The sample was removed and the grid stained with 5 drops of 2% uranyl acetate. Samples for embedding and sectioning were prepared as described previously (26). Assembled tubes were fixed in 1% glutaraldehyde and centrifuged, and pellets were further fixed in 3% glutaraldehyde and 0.2% tannic acid. They were then stained with 1% Osmium Tetroxide for 30-60 minutes, followed by block staining with 2%
Uranyl Acetate. The samples were then dehydrated and embedded in Araldite resin. Silver and gray sections (50-70 nm) were collected for observation by EM. Electron microscopy was performed using a Philips 420 microscope and images were collected at 49,000x magnification unless otherwise stated.

2.2.4 Image and Statistical Analysis

Images were analyzed and measured in FIJI (Is Just ImageJ). Statistical analysis was performed in Microsoft Excel. ANOVA tests were run on all sets with a p-value result of 0 (below the calculation threshold of Excel). Brightness and contrast were adjusted in Photoshop for optimal presentation of images.

2.3 RESULTS

2.3.1 FtsZ Tubes as the Model for the Highly Curved Conformation

The highly curved conformation of Escherichia coli FtsZ was first seen for polymers assembled in vitro and adsorbed to cationic lipid monolayers, where it formed “minirings” 24 nm in diameter (22). However, minirings do not assemble in solution; the closed circular form is apparently imposed by adsorption to the flat lipid monolayer. An alternative form of the highly curved conformation is FtsZ tubes, which are assembled by FtsZ with the addition of GDP and DEAE dextran (26). The tube is formed by three or four pfs of FtsZ spiraling in a helical assembly. The tube has the same 24 nm diameter as the miniring, but the pfs also have a pitch of 22 nm. The tubes are large, stable and much easier to manipulate and image than the minirings. For these reasons, we performed our experiments with DEAE-dextran tubes instead of minirings. Negative stain EM images of tubes are shown in Figure 8.
Figure 9: Negative stain electron micrographs of tubes of FtsZ assembled with GDP and DEAE-Dextran. Tubes with FN7-10 (B) or mVenus fusions (C) have the same measurements as native FtsZ (A), and the peptide additions are not visible. Scale bar 50 nm.

2.3.2 Protein Fusions to Tag the C and N Termini of FtsZ

The C terminus of FtsZ comprises a 50 amino acid (aa) flexible linker and a conserved 17 aa peptide that binds to FtsA and hence the membrane. For the C-terminal fusion we removed this tether and fused the protein tag after aa 316, the last aa in the globular domain. The N terminus of FtsZ is a 9 aa flexible peptide on the opposite side of the pf. For the N-terminal fusion we fused the tag in front of aa 1.

The first protein tag is FN7-10, a segment of fibronectin (FN) comprising 4 FNIII domains. FN7-10 is 40 kDa, about the same mass as FtsZ, but forms an extended rod 14 nm long and 2.5 x 3 nm in width (63). Figure 9A shows a model of the structure of FtsZ-FN. If the bending pf model is correct, in the N-terminal fusion (FN-FtsZ) the FN7-10 rod should face the inside of the tube. However, this construct should be incapable of making tubes, because the FN rods would not fit in the interior (Figure 9B). FtsZ-FN, with the FN on the C terminus, should assemble tubes with the FN rods extending outward (Figure 9C).

The second tag is the yellow fluorescent protein mVenus. This is a smaller protein, 27 kDa, and globular, but still large enough that it should sterically inhibit tube
assembly if it is projecting to the inside. For FtsZ-mVenus the C-terminal tether was left intact and mVenus was fused after aa 383.

![Figure 10: Model of FtsZ with FN7-10 domains attached to C-terminus. A. FtsZ is blue globular domain on the left side. The four smaller teal through red domains are the Fibronectin type III domains. B. Model of tube if the C-terminus faced the interior of the tube. Note that there is not enough space to fit all of the FN. This tube should not be able to form. C. Model of tube if the N-terminus faced the exterior. Note the spread out FN domains that are not conflicting with each other in any way.]

### 2.3.3 Assembly of FtsZ Fusion Constructs

We first tested each of the fusion constructs for assembly without DEAE dextran. All of the constructs (FN-FtsZ, FtsZ-FN, mVenus-FtsZ and FtsZ-mVenus) were capable of forming pfs in HMK100 buffer with GTP, as imaged in negative stain EM. We had hoped that the fusions might provide a visible distinction, namely that the pfs would appear thicker or fuzzy, but there was no visible difference of pfs with or without an FN or mVenus tag.
The FN-FtsZ and mVenus-FtsZ constructs (N-terminal fusions) were unable to form tubes. These results fit with our hypothesis that the N-terminus of FtsZ is facing the interior of the tube. There should not be sufficient room in the interior of the tube to fit the FN domains or mVenus, posing a steric block for tube formation by the N-terminal fusions.

The C-terminal fusions, FtsZ-FN, and FtsZ-mVenus, were both capable of forming tubes under these conditions. However, when observed by negative stain EM the tubes of different constructs could not be distinguished (Figure 8B,C). They all had a uniform width of ~25 nm. We conclude that uranyl acetate negative stain was not sensitive enough to visualize the FN or mVenus extensions to the FtsZ.

We therefore transitioned to embedding and thin sectioning, with alternative positive staining, where the FtsZ shows up as a dark ring of stain. The embedded tubes were in random orientations. The most informative orientation was the cross section, and these were selected for measurement. The tubes appeared to cluster into parallel arrays, perhaps a result of the fixation and centrifugation process. This allowed us to measure the center to center distance of neighboring tubes. This measurement is more precise than the outside diameter of a tube, especially for the FN and mVenus constructs, where it can be difficult to distinguish boundaries between neighboring tubes.

In addition to the different measurements of the tubes discussed below, the different constructs were distinguishable by their appearance in positive stain thin section (Figure 11). The FtsZ-FN tubes have a dark ring of stain that is the same width and diameter as the wild type construct, and is identified as the FtsZ. However, they are surrounded by a large pale halo. This is presumably the FN7-10 tag, which apparently does not absorb the stain as well as the FtsZ. The mVenus construct, in contrast,
produces a uniform dark stain that cannot be distinguished from the FtsZ. The FtsZ-
mVenus tubes also have a much more regular packing, producing large fields of regular,
hexagonal lattices.

Several measurements support the conclusion that the C-terminal FN and
mVenus tags project on the outside of the tubes (Figure 11). The width of the lumen of
the wild type tubes was ~9.9 nm. The lumens of the FtsZ-FN and FtsZ-mVenus tubes
were ~9.2 nm and ~9.4 nm respectively. While this is a slight decrease in lumen width, it
is much too small of a difference to suggest that the protein addition is on the interior of
the tube.

The most informative measure of the C-terminal tags was the center to center
spacing of the tubes in cross-section clusters. This was 21.4 nm for wtFtsZ, 30.7 nm for
FtsZ-FN and 28.6 nm for FtsZ-mVenus. The FN domains are 14 nm long fully extended,
which is longer than the ~9 nm increase in tube separation. This suggests that the FN
extensions from adjacent tubes interdigitate, which is compatible with the thin
dimensions of the FN7-10 rod (2.5 x 3.5 x 14 nm) (Figure 9A). However, the ~9 nm
increased separation is less than the 14 nm length of the FN rod, which suggests that
the FN rods are not extended perpendicular to the tube axis, but are bent at the flexible
point of attachment to FtsZ aa 316. The 7 nm increased spacing for the FtsZ-mVenus
tubes is compatible with a fairly close packing of mVenus tags, which is a barrel 3 x 3 x
4.5 nm.
Figure 11: 50 nm thin sections of FtsZ tubes. 50 nm scale bar. Column A, wtFtsZ tubes. Note the distinct ring of dark stain around the open lumen. Tubes have limited clustering and are closely packed. Column B, FtsZ-FN tubes. Note the increased clustering in random orientations. The tubes cluster in a parallel manner only in small patches. The same dark ring of FtsZ can be seen as in the wtFtsZ. Note the large white halo surrounding the FtsZ rings, and the greatly increased separation. This is attributed to the FN, which does not stain as darkly as the FtsZ. Column C, FtsZ-mVenus tubes. Note the large patches of clustered, parallel tubes. The protein staining is uniform, with the mVenus staining not differentiated from the FtsZ. Section D. Model showing interdigitation of FN domains in clustered tubes. D1. Close-packed clustering of wtFtsZ tubes. D2. Single FtsZ-FN tube. Note the large, uniform halo of FN. D3. Clustering of FtsZ-FN tubes. Note that the width of the halo of a single tube is the same as the halo between neighboring tubes in thin section. This suggests interdigitation, as is seen in the model.

Figure 12: Graph of FtsZ tube measurements. "FtsZ" indicates the width of the FtsZ wall (not available for FtsZ-mVenus). "Lumen" is the inside diameter of the tubes. "Single Tube" is the diameter, including the halo for FtsZ-FN. "Center-Center" is the spacing of tubes measured from the center of the lumen. Note that the single tube width of the FtsZ-FN construct is greater than the center-center width of neighboring tubes. This is due to interdigitation of the FN domains. Statistical analysis: ANOVA was performed, error bars are S.E.M.
Figure 13: A. Models showing two possibilities for the curvature of the FtsZ pf relative to the membrane. The FtsZ globular domain is green, the 55 aa linker is black and the mts is yellow. A1. The C terminus faces the outside of curved pf, as determined in the present study. The C-terminal tail could be shortened or eliminated and the mts could still attach to the membrane. A2. The C terminus faces the inside of the curved pf, as established for tubulin. In this case the C-terminal tail would have to wrap completely around the FtsZ to attach to the membrane, and the linker could not be eliminated. B. FtsZ-YFP-mts can form Z rings in bacteria with truncated linkers. B1. The mts is directly attached to the globular domain at aa 316, these cells still form Z rings, excluding model A2. B2-B4. Various truncations of the FtsZ linker are still able to attach to the membrane to form Z rings. These images further support the model in A1. The cells form numerous Z rings all along the length of the bacterium, appearing as fluorescent bands.

2.3.4 Z Rings Assembled in *E. coli* from FtsZ-YFP-mts with Shortened and Deleted Linkers

While the simplest model of the pf bending mechanism places the C terminus and the linker on the outside of the curved pf, directly facing the membrane (Figure 12A1), an alternative model has been proposed to explain the curvature seen in a crystal structure of FtsZ from *Mycobacterium tuberculosis* (64). In this model the C terminus is on the inside of the curved pf, and the linker, a disordered peptide of 50 aa’s, loops around the pf to emerge on the outside and eventually attach to the membrane (Figure 12A2). This might be possible with the natural linker of 50 aa’s, but shortening the linker would compromise the assembly of Z rings. We tested this using a construct FtsZ-YFP-mts, where mts is an amphipathic helix that attaches FtsZ directly to the membrane. We previously showed that FtsZ-YFP-mts assembled extensive Z rings and spirals when expressed in *E. coli* strain JKD7-pKD3, in which wtFtsZ can be depleted by raising the temperature to 42°C (32). For the present study we prepared FtsZ-YFP-mts with the linker shortened or deleted entirely. Figure 12B shows that all of these constructs assembled extensive Z rings and helices when expressed in JKD7-pKD3 depleted of wtFtsZ, including the construct mts-316, where the linker is deleted completely. This is
Only possible with model Figure 12A1, adding further support to our interpretation of curvature.

2.4 DISCUSSION

2.4.1 The C-terminal Membrane Tether is on the Outside of Curved FtsZ Pfs

We previously proposed that the Z-ring constriction force is generated by FtsZ pfs switching from a straight to a curved conformation. The simplest model required that the C-terminal membrane tether exit from the outside of the curved pf (36, 65). This is opposite to the curvature of tubulin rings, which have the C terminus on the inside, presenting an apparent contradiction. In the present study we demonstrated that a large protein tag fused to the C terminus of FtsZ is indeed on the outside of the curved pfs, as predicted by our model.

A recent crystal structure of FtsZ from M. tuberculosis showed antiparallel pf pairs in a tight spiral, with the C terminus facing inward (64). A more complex model of pf bending was presented, in which the flexible linker originated on the inside of the curved pf and looped around the pf to exit on the outside of the curve and toward the membrane (Figure 12A2). However we show here that FtsZ-YFP-mts can assemble Z rings and helices in E. coli cells with the linker shortened or even deleted (Figure 12B). In the case of FtsZ-YFP-mts-316, the amphipathic helix is attached directly to the C terminus of the globular FtsZ, which would place it in direct contact with the membrane. In a separate study, C. crescentus FtsZ could function for cell division with its linker reduced to 14 aa’s (66). This also argues against the model in Figure 12A2.

The pf curvature in the M. tuberculosis crystals is the opposite of what we show for E. coli pfs, and it is not yet clear if it is physiologically important. The helix in the
crystal structure was much more highly curved, with an outside diameter of 12-13 nm, vs the 21-24 nm diameter of the tubes. Also the surface area buried in the interface of the crystal helical pfs was 1,040 Å², less than half that in the straight pf of *Staphylococcus aureus* FtsZ (2,360 Å² (67)). Modeling studies have predicted that FtsZ and tubulin pfs can bend in both directions (49, 68-70). The helix in the *M. tuberculosis* crystals suggest that the opposite curvature can actually be realized for FtsZ. However we conclude that this curvature is not the one generating a constriction force.

In addition to the highly curved miniring/tube conformation, FtsZ pfs have an intermediate curved conformation, with a diameter of ~200 nm. This curved conformation gives rise to toroids and helical bundles under various crowding conditions (71), and it may be the major source of constriction force (53). We have not been able to image protein tags on the intermediate curved conformation, so the direction of curvature has also not been determined. We can only suggest that the intermediate curved conformation is indeed intermediate to the highly curved pf, and is bending in the same direction. The Z rings reconstituted with FtsZ-YFP-mts-316 provide evidence that whatever curved conformation is relevant in vivo, its C terminus faces the membrane.

### 2.4.2 Is the Constriction Force Generated by FtsZ or by Peptidoglycan Remodeling?

The “Z-centric hypothesis,” that bending pfs generate the constriction force, has been questioned by Coltharp et al (46). They examined how mutations in either FtsZ or proteoglycan synthesis affected the timing of constriction onset and the rate of septum invagination. They found that the time of constriction onset and the rate of septum invagination were not affected by the FtsZ84 mutant, which has reduced GTPase and exchange dynamics. In contrast, alterations in proteoglycan synthesis affected both
constriction onset and the rate of invagination. They concluded that “septum closure is likely driven by septum synthesis rather than Z-ring contraction.”

The idea that peptidoglycan remodeling might provide the primary driving force for septation has a long history. In the extreme scenario, the ring of FtsZ is proposed to serve primarily as a docking site for the remodeling enzymes, and the constriction force is generated entirely by the inward remodeling of the peptidoglycan. This was largely discounted when FtsZ was discovered in mycoplasma and archaea, which have no peptidoglycan cell wall (72-74). Since FtsZ, but none of the other Fts proteins, are found in mycoplasma and archaea, FtsZ was boosted as the prime candidate for generating the constriction.

The observations of Coltharp et al (46) are actually consistent with our model, where FtsZ is the primary source of constriction force. We suggest specifically that FtsZ84, although having reduced GTPase and dynamics, can still generate a constriction force that is sufficient to drive septum invagination. However, regardless of the magnitude of the force generated by FtsZ, constriction cannot begin until peptidoglycan remodeling permits the cell wall to follow. Likewise, the rate of septum invagination is probably limited, not by the force generated by FtsZ, but by the rate at which remodeling permits the peptidoglycan wall to follow the inner membrane. Peptidoglycan remodeling is a chemical process likely independent of the force generated on the other side of the membrane. In the scenario we propose, septum invagination is driven by the constriction force of FtsZ, but its rate is limited by the peptidoglycan remodeling, which allows the wall to passively follow.

In bacteria with a peptidoglycan wall, peptidoglycan remodeling is still a candidate for contributing to the constriction force (46, 75), perhaps especially in the
later stage. However, there is no compelling evidence that proteoglycan remodeling actually does generate force. In contrast, in vitro reconstitution has shown that FtsZ alone can constrict even thick-walled multilamellar lipid tubes (32, 36, 53).

2.4.3 Evolution of the Opposite Curvature in Tubulin Pfs

A second important conclusion from our study is that pfs in tubulin rings are apparently bending in the opposite direction to FtsZ pfs. The curvature of microtubule pfs seems essential for two microtubule functions. First, the tuft of curved pfs at the end of a disassembling microtubule provides a steric wedge that permits the disassembling microtubule to drag cargo like chromosomes (56). Second, the strained bonds introduced by curvature of the terminal subunits may be key to the important mechanism of microtubule dynamic instability (76, 77). We can speculate on how tubulin pfs evolved a curvature opposite to that of FtsZ. We would propose that the precursor of tubulin in early eukaryotes had the same direction of curvature as FtsZ. However, it was the straight conformation that was important in assembling sheets of pfs, which eventually formed the microtubule wall. These pfs would have the ability to curve when they separated from neighboring pfs at the ends of the microtubules. pfs with the FtsZ curvature would bend toward the inside of the microtubule, where they might interfere with each other. This curvature may have been lost in the early stages of microtubule evolution. Subsequent evolution generated an outward curvature, which became advantageous for the mechanical work of dragging chromosomes.
3. Assessment of Conformation of HIV-1 Envelope Glycoprotein Trimers

3.1 Introduction

HIV is a disease with worldwide health implications and no current cure. The development of treatments and vaccines is a high priority in the healthcare field. The trimeric envelope glycoprotein spikes (Env gp) present on the surface of HIV-1 are essential for the virus's ability to infect cells (78, 79). Contact between the spikes and the membrane are able to trigger conformational changes in the Env gp that allow the virus to fuse with the target cell membrane, initiating an infection of that cell. The Env gps are also targets for neutralizing antibodies. If antibodies are able to bind to the Env gp, infection can be prevented (80). Because the Env gps perform such an important step in the HIV infection cycle and have a known mechanism of suppression, they present an ideal target for development of vaccines and antibody based therapies.

A vaccine would require injection of purified Env gp to initiate a host immune response. This would require expression and purification of recombinant Env gp in high amounts. Current efforts in this process aim to purify only the trimeric envelope glycoproteins, without other cytoplasmic and membrane domains that are not antigenic. These purified Env gps without the other associated domains are called gp140s and are unfortunately unstable. They quickly disassociate into their component domains, gp120 and gp41-ectodomain (gp41-ecto) (80, 81). Antibodies that respond to the native-type conformation of the Env gp are unable to recognize the dissociated gp120 and gp41-ecto fragments, making them useless for vaccine production. A modification solution is required to stabilize the gp140 trimer in order to restore antigenicity.
Multiple strategies have been applied in the past to stabilize the gp140 trimer: elimination of the internal cleavage site, addition of stabilizing motifs to the trimer, and addition of disulfide bond sites to maintain covalent linkage between the trimer components (82-84). While each of these strategies are able to stabilize the gp140 trimer, they may not preserve the native structure or conformation necessary for antibody interaction. This would destroy the viability of such constructs as vaccine candidates. Current models propose that cleavage of the gp140 trimer into the gp120 and gp41-ecto domains is actually required for native HIV infection, which would make the cleavage a requirement for vaccine development (85-87). If the trimer is prepared in any conformation that does not closely mimic the native Env gp conformation, it would be useless in vaccines. Therefore the first stabilization method, eliminating the internal cleavage site, would be an ineffective strategy. The same would apply to the second method, addition of stabilizing motifs. These motifs would prevent cleavage as well as alter the physical structure of the gp140, likely destroying any antibody binding motifs. The best method for gp140 trimer stabilization would then be addition of disulfide bond sites. This modification would maintain the ability for cleavage to occur, but would prevent complete dissociation of the gp120 and gp41-ecto domains without changing the overall trimer structure to the same degree as the addition of stabilizing motifs would.

These constructs are known as SOSIP, with the “SOS” referring to the covalent disulfide linkage of gp120 and gp41-ecto, as well as a substitution of Ile for Pro (IP) at residue 559. This mutation strengthens the interactions between neighboring gp41-ecto domains. The end goal of experimentally modifying gp140 trimers is to create protein that is easy to purify and mimics the conformation of native HIV-1 Env gp, allowing usage in vaccine research and production.
One of the strongest methods for evaluating the conformation and antigenicity of recombinant gp140 trimers is electron microscopy. One can visualize individual trimers under the electron microscope and visually compare the two dimensional and three dimensional conformations of recombinant constructs to the wild type HIV-1 Env gp. Our experiments use negative stain electron microscopy to collect images that are analysed by the class averaging program EMAN2 to determine whether or not various constructs exhibit native like conformations and behavior.

### 3.2 BG505 and CH505 Constructs

The Env gp used in the initial SOSIP studies is known as BG505 and was isolated from a patient sample. This strain was extremely valuable in that the Env gp and SOSIP displayed antigenic properties. This provided an excellent baseline and starting point to create other stabilized trimers that were able to bind antibodies (88).

Later, another strain, CH505 was isolated from a different patient. CH505 was able to bind the CH106 antibody through a CD4 binding site, as with BG505, but it also possessed a unique trait. The patient from which CH505 was isolated was also producing CH106 antibodies (89). This had never before been observed. All previous antibody interactions occurred *in vitro*. The fact that the patient was producing antibodies made CH505 extremely important to study and understand the specific conformation of its Env gp and whether or not SOSIPs produced from it would be still match the native conformation and retain antigenicity. The constructs used in our experiments are based on the CH505 SOSIP.

We are testing multiple constructs derived from BG505 SOSIP and CH505 SOSIP. We are using CH505 SOSIPs that were produced from viral samples taken from
the patient at various time points. The transmitted founder (TF) construct is based on
the initial infection strain, while other constructs are based on samples taken at 53, 78,
and 100 weeks after infection. We are also using various chimera constructs. These
consist of the BG505 backbone fused with the CD4 epitope of the CH505. The goal of
this construct is to maintain the established and studied structure of the BG505 SOSIP
while adding the antigenicity of CH505. All of these constructs consist of on the soluble,
external gp140 trimer.

We also observed constructs that are made of the gp150 trimer. The gp150
contains all of the same components of gp140, but also possesses the membrane
domain. For this reason, gp150 constructs are purified in detergent and appear in
different orientations under the electron microscope than the gp140 constructs (90).

3.3 EXPERIMENTAL PROCEDURES
3.3.1 Extraction and Purification of Membrane Expressed Env Trimers

CHO cells expressing membrane-bound CH505 gp150 were lysed in TNE
(25mM Tris, 50mM NaCl, 5mM EDTA, pH 7.5) buffer with protease inhibitor cocktail
(Roche) and 1% w/v Brij-58. The sample was incubated for 1 hour at 4°C while rotating.
Afterwards, cells were homogenized using a tight dounce homogenizer. To remove large
cellular components, the sample was centrifuged for 1 hour at 3000 RPM. Cymal-5 was
then added to the supernatant for a final 1% w/v concentration and incubated overnight
at 4°C while rotating.

Env protein was purified from the cell lysate by affinity chromatography using
CNBr-activated Sepharose 4B beads (GE Healthcare) coupled with anti-HIV Env
antibody (PGT145). CHO cell lysate was flowed over the PGT145 column, followed by
washing with 10 column volumes of wash buffer (PBS pH 7.4, 0.25% w/v cymal-5). The bound Env protein was eluted with 2 column volumes of elution buffer (0.1M glycine pH 2.5, 0.15M NaCl, 0.25% w/v cymal-5), and immediately buffer exchanged into wash buffer using Amicon 100MWCO filter units (EMD Millipore).

3.3.2 Sample Preparation for Electron Microscopy

Purified protein samples that were higher concentration than 1 mg/ml were diluted to 1 mg/ml in HMK100 buffer (50 mM HEPES, 100 mM KAc, 5 mM MgAc, pH 7.6), with 10% glycerol added for storage at -80° C. They were then further diluted into separate tubes to a concentration of 20 μg/ml in HMK100 buffer. Samples were stored on ice until negative staining.

3.3.3 Electron Microscopy and Staining

Negative stain samples were prepared by adding 10 μl of assembled protein to a carbon coated grid. The carbon films were made hydrophilic by exposure to UV light and ozone as described by Burgess et al.(91). The lamp they used is no longer available, so we substituted a Spectroline 11SC-1 Pencil shortwave UV lamp (catalog number 11-992-30; Fischer Scientific, Pittsburgh, PA) and UVP Pen-Ray lamp power supply (catalog number UVP99 0055 01; Fischer Scientific). Grids were treated for 1 hour and used within 4 hours. The sample was removed and the grid stained with 5 drops of 2% uranyl acetate. Electron microscopy was performed using a Philips 420 microscope and images were collected at 49,000x magnification unless otherwise stated.
3.3.4 Data Analysis

Data analysis was performed using the program EMAN2. 2D class averaging was performed for each construct to create 32 class averages. For electron micrographs, the scale is .693 nm per pixel. The particle selection area that we use in EMAN2 is a square with dimensions of 32 pixels by 32 pixels. This gives a side length of 22.176 nm. Specific information about the process of data analysis using EMAN2 can be found in Section 3.4.

3.4 EMAN2 to Perform 2D Class Averaging

3.4.1 Class Averaging

When observing individual particles under the electron microscope, the signal to noise ratio (SNR) is low and dependent on the resolution of staining. There is simply not enough data in a single particle to resolve sub-nanometer structural data. Class averaging vastly increases the SNR by taking images of multiple particles in a variety of similar angles and orientations, and combining them to form a single, higher resolution image. Software is able to map particles that are in similar orientations and group them together. Each grouping of particles is a class and the more data points collected, the larger each class becomes, increasing the SNR and resolution of the final average. With enough particles and ideal imaging conditions, structures with resolutions on the order of 20 Angstroms can be obtained from negative stain images. This allows vastly increased resolution over individual negative stain particles. The class averaging program used in this study is EMAN2, the main screen for which can be seen in Figure 14.
Figure 14: Main screen of EMAN2. Steps in the class averaging process are selected in the left panel, with options for each individual step appearing in the center.

3.4.2 EMAN2 Procedure

After image collection on the electron microscope, the micrographs are loaded into EMAN2 for evaluation, as can be seen in Figure 15. An intensity plot and 2D FFT are shown for each image. At this point, it is determined whether or not to include each individual image in the analysis. If the SNR is too low, then the image should be discarded. Similarly, if the FFT shows that the image is too far out of focus or there is severe astigmatism, the image should be discarded. Images that are acceptable are passed through an evaluation step that adjusts the defocus and background SNR. The results of this step are .hdr images, used in the next step, particle picking.
Figure 15: Defocus and background correction for imported images. The left image is a 2D FFT of the micrograph while the right image shows the corrections to be made. If the FFT shows low signal, strong defocus, or severe astigmatism, the image should be discarded and not included in further analysis.

In order to analyze and average thousands of particles, they must first be individually isolated from the background of the electron micrograph. The particle picker program allows the user to set a box size, determining how large each individual particle should be, as seen in Figure 16. The user can manually choose every single particle or choose a small selection of particles and have the program automatically detect the rest. If the user chooses this automatic particle picking, there are further options about how strict the program is. After the program automatically picks the particles for each image, the user can manually remove particles that are bad or too close to the edge of the image, as seen in Figure 17. Once particle picking is complete, the particle selection and coordinates are written for each individual image.
Figure 16: Particle picking options. Clicking on a micrograph in the left image selects it for particle picking. The right image shows the control panel. The user can change the particle box size and particle picking method. This panel is also used to write the particles to a list to be assembled into a set in a later step.
Figure 17: The left image shows a micrograph where particles have been automatically found. The green circles show individual particles. The dark shadowed circles mark bad particles that have been manually removed by the user. The right image shows every individual particle that has been found by the program. Bad particles can be removed from this window as well. Each particle box is a ~22 nm-sided square.

The user must then assemble the particles into a separate set. Particle picking is done on an image by image basis, but there may be images of different constructs present. The user then selects which images belong to an individual construct and assembles those into a set containing all selected particles. At this point the completed set of particles can be used in class averaging.
Figure 18: Screen to begin Reference Free Class Averaging. The user is able to select the particle set to analyse, number of classes to generate, and number of iterations to run. A higher number of iterations will generate more accurate and refined results at the cost of increased run time.

The next step is to initiate reference free class averaging, as seen in Figure 18. The user is able to control the number of sets produced as well as the number of iterations to run. This is a very resource intensive process for the computer to run and will scale roughly linearly based on the number of particles used in the analysis. After class averaging is completed, all of the sets can be viewed, as seen in Figure 19. Sets can be marked as good or bad. Bad sets tend to consist of similar looking artifacts or empty sections of background that were inadvertently chosen as good particles. After noting good and bad sets, the class averaging can be run again to refine the model.
Removing bad sets and refining the model will give more accurate, higher resolution final results.

Figure 19: The results of Reference Free Class Averaging. The top left image shows the 32 classes created, numbered from 0-31. Note that the majority of the classes are very similar, signifying a valid result. Bad classes such as class 30 may arise from similar microscopy artifacts or debris causing a false positive result. These bad classes can be removed for further refinement. Note that class 0 is selected with a blue border. The upper right image lists all particles included in the selected class 0. The bottom left image shows particles that were initially members of class 0, but were rejected during the final analysis. These particles are not included in the final class average for class 0. Each particle box is a ~22 nm-sided square.

The resulting classes can then be compared between constructs to determine conformational and structural differences. Additionally, if classes of all orientations around the surface of the particle are present, a 3 dimensional model can be created, using the 2D classes to create a full 3D representation of the particle.
3.5 Results

3.5.1 Constructs Observed by Electron Microscopy

A wide range of constructs were utilized in these experiments in order to maximize the chances of finding constructs whose conformations closely mimicked those of the native HIV-1 Env gp trimers. The first construct tested was the BG505 SOSIP RS. This is the gold standard for native like conformations and was used as a baseline and positive control relative to the rest of the constructs tested.

3.5.2 Class Averages

The BG505 SOSIP behaved as expected, showing a native like conformation in the class averaging, as seen in Figure 20. This construct served as a positive control. The CH505 SOSIP and chimera constructs are being compared to the BG505 SOSIP.

![BG505 SOSIP class averages. Note the native like trimer conformation. Each particle box is a ~22 nm-sided square.](image)

The CH505 SOSIP TF construct also showed a native like conformation, as seen in Figure 21. The transmitted founder sample is from the initial infection. Additionally, the CH505 SOSIP samples from week 53, week 78, and week 100 all show native like
conformations, as seen in Figures 22, 23, and 24, respectively. The fact that all of the basic CH505 SOSIP samples show a similar conformation to the BG505 SOSIP is highly suggestive that they will behave in a similar manner throughout further experimentation.

Figure 21: CH505 SOSIP TF class averages. Native like conformation. Each particle box is a ~22 nm-sided square.

Figure 22: CH505 SOSIP week 53 class averages. Native like conformation. Each particle box is a ~22 nm-sided square.
Figure 23: CH505 SOSIP week 78 class averages. Native like conformation. The bad classes, such as 8 and 24 are due to decreased stain contrast for this sample. This resulted in more background and artifact particles being included. Each particle box is a ~22 nm-sided square.

Figure 24: CH505 SOSIP week 100. Native like conformation. Each particle box is a ~22 nm-sided square.

We next tested four separate preparations of the transmitted founder version of the chimera construct, as seen in Figure 25. This construct contains the backbone of BG505 and the CD4 binding site of CH505. The CH505 TFchim SOSIP constructs all
showed native like conformation. This provides strong evidence that the chimera construct is indeed functional and may provide the benefits of using both the BG505 and CH505 within a single construct.

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**Figure 25: CH505 TFchim SOSIP.** Each image is from a separate purification batch. All show native like conformation. Each particle box is a ~22 nm-sided square.

We next tested the weeks 53, 78, and 100 versions of the CH505 chimera, as seen in Figure 26. CH505 SOSIP TFchim week 53, 78, and 100 all showed normal like conformations. This demonstrates that the CH505 did not change significantly over the course of 100 weeks of infection.
Figure 26: CH505 TFchim SOSIP. Upper left image is week 53. Upper right image is week 78. Lower left image is week 100. All show native like conformation. Each particle box is a ~22 nm-sided square.

Finally, we observed the CH505 SOSIP gp150 construct from weeks 53, 78, and 100. This construct contains the transmembrane domain and was purified in detergent. While the gp140 constructs tend to settle on the EM grid in a single orientation, the gp150 constructs settle in a variety of orientations. Most recognizable is the “pineapple” shape when a trimer settles on it side. Due to the large number of orientations present for this construct, the class averaging was less effective. There were fewer particles to fit into a single class, leading to the majority of classes being bad. However, there were enough good particles to form some “pineapple” classes, as can be seen in class 26 of CH505 SOSIP gp150 week 53, class 11 of CH505 SOSIP gp150 week 78, and class 10.
of CH505 gp150 week 100. There are some native like settled trimers present as well, but the detergent is responsible for the wide array of orientations.

Figure 27: CH505 gp150. Upper left is week 53. Note the “pineapple” shape in class 26. Upper right is week 78. Note the “pineapple” shape in class 11. Lower left is week 100. Note the “pineapple” shape in class 10. Each particle box is a ~44 nm-sided square.

3.6 Class Averaging Overview

All of the CH505 SOSIP constructs we tested showed native like conformations. This clearly demonstrates the efficacy of the stabilizing SOSIP modifications. Additionally, the chimera constructs composed of the BG505 backbone and the CH505 CD4 epitope site showed native like conformations. This means that all of these constructs are excellent candidates for future studies testing their ability to react to broadly neutralizing antibodies. The ability to bind to broadly neutralizing antibodies
would make these constructs ideal candidates for vaccine testing and potential future HIV therapies.
4. The Future of FtsZ Experimentation

4.1 The Major Gap in FtsZ Knowledge

While much is known about the form and function of FtsZ, there are still many avenues of study to further our understanding of how FtsZ performs its role in bacterial cell division. The most important future area of study is to determine the substructure of the Z ring. While it has been established that FtsZ forms filaments that arrange at the center of the dividing bacterium to create a Z ring, the actual spacing and arrangement of the filaments remains unknown. Discovering the substructure of the Z ring has further reaching implications than simply knowing that the Z ring looks like. Understanding the arrangement of protofilaments within the Z ring will initiate further experiments and allow creation and refinement of models of FtsZ function. Understanding the structure of the Z ring will give vital clues as to the presence of direct lateral bonds between filaments, the mechanism of FtsZ force generation, the maintenance of tension and force generation during construction, the spacing of filaments as the circumference of the bacterial cell shrinks during septation, and how filament length and spacing affect the dynamics of subunit exchange within filaments. While discovering the substructure of the Z ring will not directly answer all of these questions, it will provide supportive evidence, allowing models to be created, and suggesting further directions of experimentation to actually find the answers. Because understanding the substructure of the Z ring has such far reaching implications in the rest of the FtsZ field, it is the most important question to be answered and the most vital gap in our knowledge of FtsZ.
4.2 Z Ring Substructure

There are two competing models that describe the substructure of the Z ring: the scattered model and the ribbon model. The scattered model posits that the Z ring is greater than 100 nm wide, with pfs 40-50 nm apart, unable to make direct contact with each other (37). The ribbon model states that the Z ring is less than 25 nm wide with 2-5 parallel pfs in direct contact with each other, connected by lateral bonds.

The scattered model was first suggested by a cryoEM study of C. crescentus, which showed short pfs scattered around the Z ring and only rarely in contact with each other (37). Several studies by super-resolution light microscopy have indirectly supported this model by reporting widths of the Z ring ~110 nm (42-44); a ribbon of 2-5 pfs should be only 10-25 nm wide. A recent PALM study of the Z ring in Streptococcus pneumoniae measured the axial width of the Z ring to be 95 nm early in the cell cycle, increasing to 127 nm as the Z ring constricted (45). It should be noted, however, that the most recent and comprehensive PALM studies (42, 45, 46) also measured the thickness of the Z ring in the radial direction. The radial thickness was very close in magnitude to the axial width (71 nm axial width vs 64 nm radial thickness (42); Fig. 7 in (45); 99 nm axial vs 59 nm radial (46)). This large radial width contradicts our expectation that all pfs are tethered to the membrane by FtsA and ZipA, necessitating a Z ring that is radially one subunit thick. It is important to recall that in E. coli the Z ring averages only 2-5 pfs thick (both axial and radial), so there is not enough material to build in the radial direction. This suggests that the actual radial thickness should be ~5 nm. The large radial thickness measured in the PALM images may therefore reflect the achieved resolution (which is worse than the resolution predicted theoretically in the studies). Since the measured axial width was only 7-40 nm more than the radial, the
actual axial width of the Z ring might well be as small as 10-25 nm. Overall, the super-resolution light microscopy does not provide convincing support for the larger width of the scattered model.

A recent cryoEM study by Szwedziak et al (47) provides strong support for the ribbon model in both *C. crescentus* and *E. coli*. Their tomograms showed parallel arrays of pfs encircling the cells at the constriction site; these were clearly ribbons, one pf thick radially, and with variable axial widths. Szwedziak et al (47) also presented clear images of FtsZ rings reconstituted in liposomes with FtsA (their Fig. 5). A typical Z ring comprised a ribbon of 2, 3 or 4 pfs that formed a continuous helical loop around the constriction, with a zone of overlap. In all cases the ribbons were one pf thick, with all pfs apparently linked to the membrane by FtsA. This direct observation by cryoEM provides compelling evidence for the ribbon structure, even if this interpretation was not emphasized in the paper of Szwedziak et al (47).

**4.2.1 Superresolution Light Microscopy**

Epifluorescence microscopy has been the most common and powerful tool for observing the Z ring in living bacteria, but is severely limited when its resolution is compared to the molecular scale. Fluorescence microscopy is diffraction limited to a resolution of 250 nm, far larger than the 5 nm width of a single pf. Additionally, there are only an estimated 5,000 FtsZ subunits in an *E. coli* cell, with only ~2,000 subunits forming the Z ring. This limited amount of FtsZ means that if all of the FtsZ formed into a single, extended superfilament, it would only encircle the dividing bacterium 2.5 times. This limited amount of FtsZ in the Z ring coupled with the resolution limits of fluorescence microscopy have made it extremely difficult to observe the Z ring in high
detail. Fortunately, there are microscopy methods available to bypass this resolution limit and allow greater detail information to be gleaned about the Z ring substructure.

An ideal microscopy technique for investigating Z ring substructure and assembly dynamics in vivo is PALM (PhotoActivated Localization Microscopy (41). PALM is able to achieve 30 nm resolution, roughly 10 times better resolution than is possible with conventional imaging. PALM requires the use of a photoactivatable fluorophore, which is activated by a wavelength of light different than the wavelength used to visualize it. A short pulse of light activates a small number of fluorophores, each of which are likely to be farther apart than 300 nm, and thus can be resolved as separate points. The fluorophores are then excited with the excitation wavelength until they bleach and become nonfunctional. This creates an image of dots, each dot representing a single fluorophore. The process is repeated and eventually an overlaid image of all of the individual fluorophores is created. Each fluorophore in the image is actually a diffuse spread of photons that approximates a Gaussian distribution with a width at half maximum of 250 nm, the resolution of the light microscope. However, the centroid of each point can be calculated, using the Gaussian distribution, to a resolution of ~30 nm. The composite image is crowded with fluorophores closer than 250 nm apart, but because each fluorophore was activated separately, the centroids can be resolved. This is how PALM can achieve such an increased resolution over conventional microscopy. This technique would seem to be ideal for reconstructing images of the individual pfs in a Z ring, as well as for tracking single FtsZ molecules to study assembly and exchange dynamics in vivo.

Despite multiple studies having been conducted using superresolution light microscopy, there are still flaws in the technique and data that have been collected (43,
92-95). The biggest flaw in the microscopy experiments has been the achieved resolution of the technique. While the technique of using Gaussian functions to localize the centroid of a single fluorophore has a resolution limit of ~5 nm, real life experiments have not been able to achieve resolutions anywhere close to this level. Studies performing three dimensional reconstructions of the Z ring are usually resolution limited in the radial direction to ~50 nm, this represents the true resolution of the technique currently. Although studies claim an axial resolution of 20-30 nm, this number cannot be fully trusted until the radial resolution is increased.

All current models propose that the Z ring is one protofilament layer thick in the radial direction, and there has been no convincing evidence to contradict this (42). This would create an actual radial thickness of 5 nm for the Z ring, the thickness of a single subunit. Because the superresolution microscopy studies are seeing a radial depth of at least 50 nm, which should be the depth of a single layer of FtsZ, the practical axial resolution of 20-30 nm cannot be trusted.

An additional issue with current superresolution studies is the fact that not all FtsZ subunits are being observed by the microscope. There are two reasons for this. The first is that it is not possible to activate the fluorophore of every single labeled FtsZ; at best only 30-50% of the fluorophores are activated and imaged. The second issue is that not all FtsZ subunits are actually labeled with a fluorophore. If more than ~30% of the FtsZ in a cell is fluorescently labeled with a C-terminal fluorophore, the cells are no longer able to divide. With a combination of these two issues, only a fraction of the actual FtsZ subunits in the Z ring are collected as data points by superresolution microscopy.
The Erickson lab has recently made advances that allow for a fully functional FtsZ labeled with a fluorophore. An mVenus fluorophore was able to be inserted internally to a loop region of FtsZ, creating a fully functional, fluorescently labeled FtsZ. This development will allow for future superresolution studies to achieve both greater resolution and have vastly increased coverage of the Z ring, solving the issue of incomplete FtsZ labeling. The second issue, inability to observe every FtsZ subunit is more difficult to overcome by light microscopy. Advances in electron microscopy, however, provide a method for direct observation of labeled FtsZ subunits.

4.2.2 Genetically Encoded Electron Microscopy Constructs

Electron Microscopy offers vastly improved resolution over light microscopy, but has traditionally suffered from difficulties in specifically labeling a protein of interest in whole cells. The most common method of labeling is using antibodies to tag the protein of interest. However, there are drawbacks to antibody labeling. The antibody needs to enter the cell to bind to its target, and if there are any off-target interactions, false positive labeling of unintended proteins can occur. It also suffers from the same issue as with fluorescent labeling of FtsZ: antibodies will not bind to 100% of the target protein, some will be missed, giving similar incomplete coverage. The ideal process for visualizing a target protein by electron microscopy would be with a genetically encoded tag that is part of the protein sequence itself, leading to complete labeling. Such a construct has been recently developed.

The laboratory of Alice Ting has created a genetically encoded Ascorbate Peroxidase (APEX) protein that can be added to the N or C terminus of a target protein and allows for visualization by electron microscopy (96). The APEX construct functions by reacting with diaminobenzidine and hydrogen peroxide to form a brown precipitate at
its location. This precipitate then reacts with Osmium Tetroxide and Uranyl Acetate to form electron densities that are observable by EM. This construct essentially acts as a GFP for electron microscopy, allowing specific and complete visualization of a target protein at resolution far exceeding that of light microscopy. The creation of a functional FtsZ-APEX construct will be a valuable step in determining the substructure of the Z ring. Such a construct will bypass the resolution limits of superresolution light microscopy while simultaneously providing far greater coverage of labeling. This will allow for determination of the substructure of the Z ring with greater accuracy than any previous light microscopy studies.

4.3 Further Implications of Understanding Z Ring Substructure

As stated previously, knowing the substructure of the Z ring is an important step in understanding the function of FtsZ in cell division, but will also have far reaching implications in other aspects of FtsZ study. Some of these aspects include the presence or absence of direct lateral bonds between filaments, the mechanism of FtsZ force generation, the maintenance of tension and force generation during construction, the spacing of filaments as the circumference of the bacterial cell shrinks during septation, and how filament length and spacing affect the dynamics of subunit exchange within filaments.

4.3.1 Lateral Bonds Between FtsZ Protofilaments

The presence or absence of lateral bonds between FtsZ protofilaments is a topic of great controversy and is inextricably tied to the debate of the scattered versus ribbon models of Z ring substructure. The presence of lateral bonds would potentially allow for greater stability of groups of protofilaments within the Z ring during constriction. If the
energy generated by protofilament curvature is sufficient to divide the bacterial cell, then lateral bonds may not be necessary. However, if there are other forces involved in the constriction process, then lateral bonds may be required to tether groups of FtsZ protofilaments together to give stability during constriction and force generation.

If the scattered model is indeed representative of the true Z ring substructure, protofilaments would be spaced too far apart to create lateral bonds. This would likely be reflected in an inability of FtsZ protofilaments to create lateral bonds even when placed in close proximity. Conversely, the ribbon model would place neighboring protofilaments in very close proximity, or even touching. While this would not necessitate the presence of direct lateral bonds, it would allow for them to occur. A major supporting factor for the presence of lateral bonds is the fact that tubulin is a homologue of FtsZ. Tubulin forms lateral bonds in sheets and in the curved microtubule wall. The vertical protofilament within a microtubule is analogous to the FtsZ protofilament, with contact between neighboring filaments within the microtubule wall being analogous to these FtsZ lateral interactions. The fact that tubulin readily forms lateral bonds is suggestive that FtsZ might have the ability to do so as well. Unfortunately, there have been no specific sites of lateral bonding identified as of yet.

If a site of direct lateral bonding in FtsZ was discovered, this would then provide some supporting evidence to the ribbon model. It would be a reasonable assumption that the lateral bonding would be biologically relevant and present in the Z ring, otherwise it would seem to serve no apparent purpose. The determination of whether or not FtsZ forms lateral bonds and the locations of those bonds are thus integral to further study and understanding of FtsZ function and Z ring substructure.
4.3.2 Mechanisms of FtsZ Force Generation and Constriction

Knowing the substructure of the Z ring will allow for further refinement of models of FtsZ force generation. The Erickson lab supports the model in which protofilament curvature is the primary mechanism of force generation. Straight FtsZ protofilaments will adopt a highly curved conformation in a generally GTP hydrolysis dependent manner (48, 71, 97-99). This curvature will pull on the membrane attachments uniformly around the circumference of the bacterial cell, creating a net inward pulling force. The force generated by FtsZ is insufficient to completely divide an entire bacterial cell; peptidoglycan remodeling and other downstream factors are required to complete septation, but FtsZ is the primary generator of force (100).

An alternate mechanism of FtsZ force generation is protofilament sliding. The overlapping protofilaments comprising the Z ring would slide past each other, increasing overlap and decreasing the circumference of the protofilament ring. This would create a net inward pulling force on the membrane, leading to constriction. However, this model is dependent on both the substructure of the Z ring and on lateral interactions between neighboring protofilaments. In order for sliding to exert a constrictive force, the filaments must be stabilized after sliding, maintaining the compacted ring conformation. This would likely require lateral bonding, as discussed above. If the protofilaments were not connected by lateral bonds or were too far apart, then there would be nothing to maintain the overlap created by the sliding and the ring would relax. Thus, lateral bonds would play an important role in the sliding mechanism of force generation.

The presence of lateral bonds in this mechanism unfortunately creates an additional issue with the sliding model. As the protofilaments continue to slide and the membrane constricts, the force of the membrane pulling outwards would increase. This
would require stronger bonds between protofilaments to prevent the membrane from snapping back. However, the more lateral bonds that are present, the more difficult it would be to detach the protofilaments from each other to continue sliding. Thus it would create a situation in which the more the ring constricts, the tighter it holds, slowing down the rate of constriction. A potential solution to this problem could involve subunit exchange and protofilament separation, which will be discussed in a further section.

The scattered model of Z ring substructure would not allow for lateral bonds between protofilaments, which would prevent the sliding mechanism of force generation from occurring. The ribbon model would allow for, but not necessarily directly support the sliding mechanism.

4.4 Conclusions

There are many aspects of FtsZ function that are yet to be understood, but the most important of these currently is Z ring substructure. Models of FtsZ force generation, constriction, and subunit exchange are all interdependent on the substructure and spacing of the Z ring. The substructure of the Z ring is also dependent on these other factors, but observation of the substructure of the Z ring is the most direct to observe if new methods are developed. A combination of superresolution light microscopy and novel electron microscopy labeling techniques will be essential to the future of FtsZ study.
References


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Biography

Max Housman was born in California in 1988 and lived there until moving to Tennessee to attend Vanderbilt University for his undergraduate degree. He majored in Molecular and Cellular Biology, graduating with a Bachelor’s degree in 2010. He joined the Cell and Molecular Biology doctoral program at Duke University in 2010 and joined the laboratory of Harold Erickson in the department of Cell Biology. His publications are Srinivasan et al, 2013 (101) and Housman et al, 2016 (102). He has presented his research by being selected to give a talk at the 2015 Cell Biology Department and the 2015 Triangle Cytoskeleton Meeting. He was also selected to give a minisymposium talk at the 2015 ASCB meeting.