Investigation of the structures of ZapA and ZapD from *Escherichia coli* and their roles in bacterial cell division

By

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ABSTRACT

Investigation of the structures of ZapA and ZapD from *Escherichia coli* and their roles in bacterial cell division

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Bacterial cell division is an essential, highly coordinated process that requires multiple proteins. FtsZ is the main scaffolding protein required for division and its mid-cell localization, membrane anchoring, and polymerization are all needed for the formation of the bacterial Z-ring. The Z-ring is a highly dynamic ring-like structure made up of closely associated FtsZ filaments. It acts as the base for recruitment of all downstream proteins into a complex known as the divisome. The bacterial divisome is comprised of 10 essential and 7 non-essential proteins, in *Escherichia coli*, which are recruited to mid-cell in a hierarchical manner and relies on the Z-ring for stability. A group of proteins referred to as the FtsZ associated proteins (ZapA, ZapB, ZapC and ZapD) act to stabilize the Z-ring prior to the on-set of division. This has been visualized *in vitro* as FtsZ filament bundling. Individually these proteins are non-essential, but it is our hypothesis that collectively the FtsZ associated proteins play an essential role in cell division. In this thesis research the crystal structures of ZapA and ZapD are described. ZapA was shown to interact with FtsZ through a charged α-helix located on each of the four protomers in the ZapA tetramer and bundle FtsZ filaments *in vitro*. ZapD was shown to interact with FtsZ through several charged residues located in a proposed binding pocket on each protomer comprising the ZapD dimer. While the FtsZ bundles formed by ZapD appear slightly more organized than those formed in the presence of ZapA, co-bundling and competition assays revealed that they likely
have proximal binding sites on FtsZ. However, ZapA and ZapD did not show any competition for binding FtsZ. The overall findings of this research indicate that ZapA and ZapD have overlapping functions and they have proximal binding sites on FtsZ, while their structures remain distinct from one another. Taken together, the results pave the way for future studies on Zap proteins and open doors for the further analysis of the ZapA-FtsZ and ZapD-FtsZ interactions.
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<tbody>
<tr>
<td>3D-SIM</td>
<td>3D structured illumination microscopy</td>
</tr>
<tr>
<td>CEM</td>
<td>Cryo-electron microscopy</td>
</tr>
<tr>
<td>CET</td>
<td>Cryo-electron tomography</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td><em>EcZapA</em></td>
<td><em>E. coli</em> ZapA</td>
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<tr>
<td><em>EcZapD</em></td>
<td><em>E. coli</em> ZapD</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>NO</td>
<td>Nucleoid occlusion</td>
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<tr>
<td>PALM</td>
<td>Photo-activated light microscopy</td>
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<tr>
<td><em>PaZapA</em></td>
<td><em>Pseudomonas aeruginosa</em> ZapA</td>
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<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>PDB</td>
<td>Protein database</td>
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<tr>
<td>PG</td>
<td>Peptidoglycan</td>
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<tr>
<td>PPIs</td>
<td>Protein-protein interactions</td>
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<tr>
<td>r.m.s.d.</td>
<td>Root-mean-square deviation</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Zap proteins</td>
<td>FtsZ associated proteins</td>
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Chapter 1: Literature Review

1.1 Bacterial cell division

Bacterial cell division is an essential and highly coordinated process that requires multiple proteins. It is under tight regulation to ensure cytokinesis occurs only when environmental conditions are permissive and after cells have matured, elongated and the replicated genetic material has separated to opposite poles of the cell (Figure 1.1). Once bacterial cell division begins, several proteins are recruited to mid-cell in a sequential and interdependent hierarchical manner (1–4). This group of proteins is referred to as the divisome. The divisome coordinates the invagination of the inner- and outer-membranes, the rearrangement and synthesis of septal peptidoglycan, the formation of the septum and the subsequent physical division into two identical daughter cells (4–7).

The study of bacterial cell division is focussed primarily around the rod shaped Gram-negative, γ-proteobacterium, *Escherichia coli*. Other γ-proteobacteria (*Vibrio sp.*, *Pseudomonas sp.*) have been shown to use similar divisional machinery to *E. coli* but unrelated species employ quite different proteins for cell division. Some of the more studied organisms include the Gram-negative α-proteobacterium *Caulobacter crescentus*, the Gram-positive spore-forming bacterium *Bacillus subtilis*, and *Mycobacteria sp.*, which are classified as Gram-positive but whose cell wall does not resemble a typical Gram-positive cell wall. While the individual proteins required for division of these organisms differs, division occurs in two main steps in each: first the scaffolding for protein assembly, the Z-ring (comprised mainly of FtsZ), is formed at the site of division, second, the proteins required for cell wall re-organization and synthesis of the septum
are recruited to the Z-ring (5, 8–10). These two events are distinct and temporally separated in most bacteria.

It is important to note that some bacteria are able to divide without the filamentous protein, FtsZ. For example, *Mycoplasma geneatilium* contains a copy of *ftsZ* within its genome but this can be deleted and cells continue to divide (11). Additionally, *Ureaplasma* and *Calyptoplasma* species do not contain a copy of *ftsZ* within their genomes (12, 13). All three of the organisms are cell-wall-less and have minimal genomes. It is thought that these bacteria have co-evolved with their host organisms, as they are obligate parasites. In these cases the cells divide by adhering to a surface locally at the poles of the cell and separate by the coordinated gliding motility of each daughter cell in opposite directions. While these cells do not require FtsZ for successful division events, most bacteria do. The summary of material in this introduction and the research described in this thesis are of bacterial cell division as it occurs in *E. coli*.

1.2 The divisome

The *E. coli* divisome is comprised of 10 essential and 10 non-essential proteins. These proteins can be divided into four main categories: the proto-ring, the early divisome proteins, the late divisome proteins and the stabilizing proteins. Most essential cell division proteins are designated filamentous temperature sensitive or Fts proteins. The proto-ring is the first protein sub-complex to assemble immediately prior to division and involves interactions between FtsZ (14), FtsA (15), and ZipA (16) (Figure 1.2). FtsZ is the main scaffolding protein required for division and its mid-cell localization, membrane anchoring, and polymerization are all needed for the formation of the bacterial Z-ring. The Z-ring is a ring-like structure acting as the base for recruitment of all downstream divisome proteins and is thought to play a role in generating the
Figure 1.1. Schematic representation of bacterial cell division in *Escherichia coli*. 

[1] Cell elongation and chromosome replication occur simultaneously, [2] chromosomes separate to opposite poles of the cell, the Z-ring assembles at mid-cell, the divisome components are recruited and the membrane begins to invaginate marking the site for septum formation, [3] divisional PG is assembled for the septum at mid-cell, [4] the mother cell physically divides to form two identical daughter cells, completing bacterial cell division.
constrictive force for physical division of one cell into two (17–19). ZipA and FtsA anchor the Z-ring to the membrane and provide stability. While ZipA is a membrane-anchored protein containing a short integral-membrane helix, FtsA is a soluble protein containing a membrane targeting amphipathic helix. Their functions are thought to be overlapping because the over-expression of ZipA can compensate for a temperature-dependant loss of function mutation in FtsA (20). Likewise, a specific mutation in FtsA (FtsA R286W) can bypass the need for ZipA entirely (21). More recently it was shown that FtsA may play a more important structural role in divisome assembly (19, 22, 23). It is an ATPase that can form filaments in vitro (22). These FtsA filaments are thought to associate laterally with the Z-ring and therefore bridge the gap between the Z-ring and the cytoplasmic membrane (19). Under normal conditions, all three of FtsZ, FtsA and ZipA are essential and their mid-cell co-localization are crucial for the recruitment of the early divisome proteins.

The early divisome proteins include FtsK (24), FtsQ (25), FtsL (26), FtsB (27). All four are membrane proteins, while FtsK has a large cytoplasmic domain; FtsQ, FtsL and FtsB are bitopic membrane proteins (Figure 1.2). FtsK is the first recruit among the early divisome proteins and is involved in both chromosome segregation and division (28). FtsK has been referred to as a molecular checkpoint in division because it coordinates the translocation of DNA away from the site of division, after which there is thought to be a conformational change in FtsK allowing for further interaction with divisome components and progression of division. FtsQ, FtsL and FtsB form a sub-complex (FtsQ/L/B) and arrive at mid-cell after FtsK (29). Although the function of the FtsQ/L/B complex has not fully been determined, it has been proposed to act as a stabilizing core for the divisome (2) linking the predominantly cytoplasmic

The late divisome proteins include FtsW (30), FtsI (31), and FtsN (32). FtsW and FtsI form a sub-complex (FtsW/I) (7, 33, 34) and their recruitment depends on all upstream divisome proteins (Figure 1.2). The FtsW/I complex plays a role in generating the divisional peptidoglycan (PG) (35). This is essential in forming the septum and remodelling the PG at the site of division to generate two daughter cells. This complex is thought to act analogously to the RodA/Pbp2 complex, which assembles the PG required during cell elongation prior to division (36). FtsW, was originally proposed to function in a similar manner to RodA, as an essential flippase that translocates lipid II across the cell membrane (33). However, recently this notion has come into question, as MurJ was shown to be the main lipid II flippase in E. coli (37). Given this new development the role of FtsW is unclear, and this essential divisome protein may have a role more specific to septal PG rearrangement rather than septal PG synthesis. FtsI, which acts similarly to Pbp2, is a known transpeptidase (31, 38–40). It interacts directly with FtsW and plays an essential role in divisional PG synthesis, catalyzing the cross-link formation between glycan chains during the formation of a PG network (34). Recently, the structure of the FtsI periplasmic domain was solved revealing a possible second functional domain, although the significance of this domain remains to be shown (41). The last protein recruited to the site of division to complete the divisome is FtsN. FtsN interacts with both early and late stage divisome proteins and its function, while elusive, has been implicated in overall divisome complex stability and in triggering Z-ring constriction (42, 43).

All of the divisome proteins described above are essential in E. coli and therefore are a prominent focus in cell division research. Isogenic mutants of the genes encoding essential
Figure 1.2. A schematic representing the bacterial divisome and the predicted membrane topologies of the individual divisome proteins at mid-cell.

FtsZ (red circles) polymerizes into filaments, anchored to the membrane by FtsA and ZipA, and cross-linked together through Zap proteins. FtsK is a membrane protein with a large cytoplasmic domain, whereas FtsN is a membrane protein with a predominant periplasmic domain. FtsQ, FtsL and FtsB form a sub-complex at the membrane and play an unknown function in cell division. FtsW and FtsI also form a sub-complex within the divisome, thought to play a key role in divisional PG synthesis and assembly.
divisome proteins cannot be generated because their deletion is lethal. However, temperature sensitive mutants can be constructed. These mutants contain a single amino acid substitution in the divisome protein of interest. This substitution typically alters the stability of the protein so that it is non-functional at higher temperatures. When temperature sensitive divisome mutants are grown at permissive temperatures (30°C), cells are unaffected but at non-permissive temperatures (42°C) show a severe filamentation phenotype (Figure 1.3).

1.2.1 Divisome accessory proteins

In addition to the 10 essential divisome proteins, there are 10 accessory proteins that have a crucial but overlapping function in cell division. This group includes the FtsZ associated proteins (ZapA, B, C, D or collectively ‘Zap proteins’), which act to stabilize the Z-ring prior to cell division by increasing lateral interactions between FtsZ filaments (44–48). In addition, GroEL is a molecular chaperone thought to bring divisome components to mid-cell and potentially stabilize the Z-ring as well (49). The FtsE/X sub-complex plays an unknown, salt-dependent role in division and is therefore also considered a divisome accessory protein complex (50, 51). Finally, the PG re-organizing enzyme AmiC (52) and the Tol/Pal protein complex, involved in inner- and outer-membrane invagination, are also involved in bacterial cell division (53).

Individually, these proteins are not essential and the genes encoding these proteins can be deleted from the E. coli genome without causing lethality. For example, removing one of the four Zap proteins causes only a mild filamentation phenotype in a fraction of the cells in a given population, but with the removal of additional Zap proteins, the filamentation becomes more pronounced appearing in more cells and to a further extent (48).
Figure 1.3. The phenotype of filamentous temperature sensitive mutants.
These mutants contain a mutation in a cell division gene, which causes the protein to be unstable at elevated temperatures. This type of conditional mutant is required when studying essential divisome proteins because essential genes cannot be entirely deleted without causing cell death. [A] An *fts* mutant (e.g. *ftsZ84*) can be grown at permissive temperatures (30°C) and division proceeds uninterrupted. [B] When cells are shifted to non-permissive temperatures (42°C) the protein containing the temperature sensitive mutation cannot function normally and cells do not form a functional divisome protein complex, cells do not divide but the chromosome is replicated (54, 55).
1.3 FtsZ: the scaffold for divisome assembly

1.3.1 FtsZ

FtsZ is an essential 40 kDa monomeric divisome protein present in most bacterial cells (14, 56, 57). It is a GTP-binding and GTP-hydrolyzing protein with structural homology to the eukaryotic protein tubulin (58–60). The nucleotide-binding capability of FtsZ is made possible by the self-association of two FtsZ monomers, as the binding pocket forms where the head and tail of the two come together (Figure 1.4) (61). This head-to-tail assembly extends further to allow FtsZ polymerization into filaments, one subunit wide and averaging 30 subunits long (62–65). FtsZ is recruited to mid-cell as the first step in cell division. It interacts extensively with other divisome proteins (66–71) and is thought to act as a scaffold for the assembly of the divisome macromolecular complex in vivo (4, 72, 73).

Structurally, FtsZ is made up of two globular domains separated by a long helix (74, 75). The N-terminal globular domain contributes 6 T-loops (named for their homology to the tubulin GTP binding domain) to the GTP binding domain, where the 7th T-loop comes from the C-terminal domain of the adjacent FtsZ molecule in the filament/oligomer (76) (Figure 1.4). Overall, the GTP-binding domain holds a typical Rossmann fold (77). Across bacterial species the N-terminal domain is not well conserved at the sequence level, except for the GTP-coordinating residues within the T-loops, while the C-terminal domain contains a relatively conserved acidic domain (74). The conserved C-terminal tail has been implicated in interactions with several cytoplasmic FtsZ stabilizing proteins (FtsA, ZipA and Zap proteins) (48, 67, 68, 78).
Figure 1.4. FtsZ filament formation in vitro.

[A] Crystal structure of the FtsZ dimer from Methanococcus jannachii containing GTP (PDB: 1W5A). While other structures of FtsZ exist in the PDB, this structure is the most widely used and sequence conservation suggests that many FtsZ share a very similar structure to this one. The monomers come together in a head to tail fashion to form the GTP binding domain, which includes 6 T-loops from one monomer and the 7th T-loop from the other monomer (large red circle). GTP can bind a site with only 6 T-loops present but GTP hydrolysis does not occur without the involvement of the T7-loop. Additionally, GTP binding increases the affinity FtsZ monomers for one another. The C-terminal tail, implicated in many protein-protein interactions, is partially resolved in this structure and denoted by the red star. [B] FtsZ filaments formed in vitro and imaged by TEM, scale=250 nm, inset scale=25 nm. [C] FtsZ filament assembly occurs in several steps. 1) FtsZ monomers associate to form dimers. These dimers have a higher affinity for each other than monomers. 2) Dimer formation encourages FtsZ self-association and FtsZ polymerizes into filaments in a GTP dependent manner. 3) GTP is hydrolyzed, causing a conformational change in FtsZ and inducing filament curvature. 4) GDP bound FtsZ disassociates from the filaments.
1.3.2 FtsZ filament assembly

The *in vivo* assembly of FtsZ filaments is not well understood, although FtsZ filaments have been studied extensively *in vitro*. FtsZ polymerizes optimally at pH 6.5 in the presence of GTP and MgCl₂ at 30°C, although efficient assembly also occurs at physiological pH (79, 80). FtsZ polymerization is concentration dependent (81), in that polymerization occurs readily past a critical concentration, and is cooperative in nature (82). Therefore, it is thought that once FtsZ is present at sufficient levels at mid-cell FtsZ filaments form quickly. While it has been shown that FtsZ filaments form better in the presence of GTP than GDP, both situations are possible (83). It has, however, been demonstrated that *in vitro* the GDP-bound FtsZ filament is short lived and quickly transitions to monomeric FtsZ (84) (Figure 1.4). This phenomenon has also been shown *in vivo*, where the Z-ring forms at mid-cell and the bound GTP is hydrolyzed to GDP, followed by rapid turnover (84). The half-life of FtsZ within the Z-ring is only a couple of seconds, strongly indicating that it is a highly dynamic structure *in vivo* (84, 85). *In vitro*, FtsZ has been shown to take on many different forms. In a dilute aqueous solution, FtsZ forms single filaments averaging 180 nm in length (86). In the presence of crowding agents, FtsZ can be found in higher order bundled spirals, toroids (rings) (86), helical tubes (64, 87), filament sheets, mini-rings (62), thin ribbons (63), and large disorganized polymer networks (65). Similar higher order structures can be created when introducing Zap proteins to the FtsZ single filament solution (44, 45, 47, 48, 88, 89).

Both the filamentation and bundling of FtsZ can be used to aid in the purification of over-expressed FtsZ from bacterial cells. FtsZ is polymerized from the cell lysate at pH 6.5 by the addition of GTP to a concentration of 1 mM (79). The filaments are then forced into high order bundles by adding CaCl₂ to 20mM. FtsZ, in this reversibly-bundled form, can be sedimented out
Figure 1.5. Schematic of the FtsZ purification strategy.
Cells over-expressing endogenous, un-tagged FtsZ are lysed and the total soluble protein fraction is separated from the insoluble by ultra-centrifugation. Upon the addition of GTP, FtsZ in solution forms filaments; CaCl$_2$ is added causing FtsZ filaments to form large bundles. This solution is centrifuged to pellet the bundled filaments. Supernatant containing non-filamented FtsZ and all other cellular proteins is discarded and upon resuspension in buffer, FtsZ de-polymerizes. This process is repeated to further remove contaminating proteins and select for active FtsZ.
of solution by relatively low speed centrifugation (Figure 1.5). The pellet is resuspended in buffer lacking GTP and CaCl₂, the process is repeated once to exclude inactive FtsZ (79). The product is pure (>95%), active, FtsZ.

1.3.3 The bacterial Z-ring

From the in vitro studies discussed above, two models of Z-ring assembly have been proposed; a model where many short FtsZ filaments associate laterally via Zap proteins to form a bundled band at mid-cell and an alternative model where a helical bundle of FtsZ condenses at mid-cell (68, 87, 90). Studying the oligomeric forms of FtsZ in vitro has yielded much of what we know about the Z-ring, but these findings are inconclusive and remain to be validated in vivo.

The in vivo study of the Z-ring has been limited by currently available microscopic techniques. Conventional transmission electron microscopy (TEM) is not suitable to study the Z-ring, as it is an internal structure and TEM results in a 2D projection image of the 3D cell surface (91, 92). Cryo-electron microscopy (CEM) and cryo-electron tomography (CET) have been used with moderate success in analyzing the Z-ring in the small marine bacterium, C. crescentus and E. coli (17, 19). Initial investigations showed only a few FtsZ filaments scattered randomly at mid-cell, which would not account for the actual amount of polymerized FtsZ present (17). This exclusion is likely due to a combination of background density from the cytoplasm of the cell and limited contrast and resolution due to the electron source and camera used. However, revisiting CEM and CET analysis of whole cells years later revealed continuous rings of FtsZ which were tightly associated with one another laterally at mid-cell (19). A pitfall in using this technique to visualize the Z-ring in vivo is that identification of density at mid-cell as the Z-ring is speculative, as no specific identifier or tag can be used to enhance the contrast of the Z-ring.
Super-resolution fluorescence microscopy methods have been more widely used to observe the Z-ring in vivo. These techniques have resulted in two main models for Z-ring formation in vivo (Figure 1.6). The first is a model where helical bundles of FtsZ condense at mid-cell prior to cell division; this is referred to as helical bundle condensation. The second is that FtsZ filaments do not form a continuous structure at mid-cell and instead are heterogeneously organized in ‘patches’ or a ‘bead-like’ structure (Figure 1.6).

The helical model is supported by three studies. In the first, PALM (photo-activated light microscopy) was used, aided by the fusion of FtsZ to the small photo-convertible protein mEOS2 in E. coli (73). mEOS2 is a preferable photo-convertible fluorescent tag as it is thought to be less disruptive to function than other larger fluorescent tags but it requires UV light for photo-conversion from green to red, which can be damaging to the cell (93). The FtsZ-mEOS2 fusion was unable to complement a temperature sensitive FtsZ mutant at the non-permissive temperature, indicating this FtsZ construct was not fully functional at the same protein levels (73). The results suggest a loosely associated helical bundle of 2-3 filaments had localized to mid-cell.

The second study was performed in C. crescentus, using single-molecule super-resolution microscopy with applied optical astigmatism to improve axial resolution (94). An FtsZ-Dendra2 fusion was used in live-cell imaging and immunofluorescence was used on fixed cells as a control. In contrast to mEOS2, the Dendra2 photo-convertible protein changes from green to red fluorescence upon exposure to blue light (95, 96). The results indicated that FtsZ filaments formed a helical structure, which condensed prior to cell division (94). This differed from the electron microscopy studies where researchers concluded FtsZ filaments were likely tightly associated and highly organized (19).
Figure 1.6. Models for Z-ring formation \textit{in vivo}.

[A] The helical condensation model indicates that long FtsZ filaments (red lines) that can span the circumference of the cell many times are arranged in a loose helical formation prior to divisome assembly. Upon divisome recruitment, Zap proteins (blue ovals) act to bring rungs of the helical FtsZ filament closer together to form a ring-like structure at mid-cell. [B] The patchy band model proposes that many short FtsZ filaments arrange around the circumferences of the cells, either in a patchy band (depicted) or in a continuous band (not shown). These short filaments associate through Zap proteins to form a ring at mid-cell.
The third study employed PALM in *C. crescentus* using the FtsZ-\textit{Dendra2} fusion, and supports both the helical condensation model and the patchy heterogeneous FtsZ organization (97). FtsZ localization was typically patchy at mid-cell and organized into a ring-like structure, (presumed by the authors to be helical-bundled FtsZ) only during constriction (94). One additional study supports the patchy FtsZ localization, referred to as a ‘bead-like’ organization (98). This work used 3D-structured illumination microscopy (3D-SIM), a technique that increases both the lateral and axial resolution by 2-fold compared to conventional wide-field fluorescence microscopy. Although the authors conclude a patchy/bead-like arrangement, their results actually show both patchy/bead-like and continuous ring-shaped localizations of FtsZ (98).

The \textit{in vivo} organization of the Z-ring remains elusive, but it can be concluded with some certainty that more than one FtsZ filament localizes to mid-cell during division, whether it be a helical bundle or in patches. This indicates that FtsZ filament lateral associations play an integral role in Z-ring assembly.

1.3.4 Z-ring dynamics

The bacterial Z-ring is highly dynamic. As discussed above, individual FtsZ filaments undergo rapid turnover \textit{in vivo} and \textit{in vitro}. Although the complete composition of the Z-ring is unknown, several proteins have been shown to localize to mid-cell during Z-ring formation and dissolution, and are thought to contribute to its overall structure, stability and mid-cell placement. Negative regulators act to ensure FtsZ stability forms a ring-structure only at mid-cell and that the Z-ring is disassembled following successful membrane invagination. Positive
regulators play a role in stabilizing the Z-ring by increasing lateral interactions and decreasing the rate of FtsZ filament depolymerization.

1.3.5 Negative regulators

The Min system involves the efforts of the three main proteins, MinC, MinD and MinE, and acts to inhibit septal ring formation at the poles in *E. coli* (Figure 1.7) (99). These work together in oscillatory concert, where MinC oscillates throughout the cell and is activated by MinD, a protein primarily located at the poles of the cell (100, 101). Activated MinC inhibits polymerization of FtsZ into stable filaments by disrupting the GDP-FtsZ interaction after GTP hydrolysis (102, 103). Moreover, MinE is present in a ring conformation closer to mid-cell, where it binds to MinD preventing MinC activation (101). This effectively regulates the topology of the Min systems’ negative regulation on FtsZ, restricting inhibition to the poles of the cell (104). The actions of this system, therefore, prevent the polymerization of FtsZ at the poles and allow for the proper placement of the division apparatus at mid-cell.

The second cell division regulatory system is nucleoid occlusion (NO) (105). NO temporally controls division. Z-ring formation is prevented in areas of the cell containing replicated genetic material by a DNA bound regulatory protein, SlmA (*Synthetically lethal mutant A*) (Figure 1.8) (106). SlmA binds to DNA sequence close to the origin of replication and is activated upon binding (106). Activated SlmA has been shown to interact directly with FtsZ and inhibit FtsZ assembly into filaments (107, 108).

The Min and NO regulatory systems are constitutively expressed and without them cells do not divide. Since FtsZ would be able polymerize anywhere in the cell, a complete and active
Figure 1.7. Schematic illustrating the coordination of the Min System.
MinC oscillates throughout the cell and is only active at the poles of the cell. MinE, represented by the black mid-cell patch is more concentrated closer to mid-cell. MinD is present throughout the cell but is only active at the poles of the cell, represented by the red patches.
Z-ring is unable to form. In addition to these coordinated systems there are several inhibitory, degradative and disassembling proteins that also act on FtsZ and the Z-ring.

ClpXP is a protease complex in *E. coli* (109). The ClpX component forms a hexameric ATPase which acts to recognize FtsZ and provides energy for degradation (110, 111). ClpP is the protease component of the complex (109). This complex is thought to function as part of the regular cell-division machinery by disassembling the Z-ring following physical cell division (110). This is supported by the higher affinity of ClpXP for FtsZ filaments than monomeric FtsZ (110). Another protein that acts to destabilize the Z-ring after division is ZapE (112). ZapE is an ATPase shown to alter FtsZ filament dynamics *in vivo* and *in vitro*, although its precise role in division is unclear (112). In yet another regulatory pathway, upon DNA damage and the induction of the SOS response, cell division inhibitor SulA is expressed (113). SulA works by binding to (and sequestering) FtsZ, thereby preventing polymerization (114).

1.3.6 Positive regulators

Z-ring formation in *E. coli* is modulated by the extreme C-terminal region of FtsZ and its interactions with proto-ring components and Zap proteins (115, 116). FtsA and ZipA promote filament assembly at mid-cell by anchoring FtsZ to the membrane and increasing filament-filament interactions (39, 117). In the absence of FtsA and ZipA, FtsZ filaments would be in constant flux of polymerization and depolymerization, whereas FtsZ filament formation is favoured in their presence. Zap proteins, on the other hand, act to promote FtsZ stability through lateral filament-filament interactions, referred to as FtsZ bundling, and further cross-link bundled FtsZ (Figure 1.9). Although they are also thought to also interact with the C-terminal region of
Figure 1.8. Cartoon showing the action of SlmA.
SlmA is inactive in its unbound state in the cytoplasm. SlmA is activated upon binding DNA (purple line). Once active, SlmA can bind and sequester FtsZ, preventing its polymerization at DNA dense regions.
FtsZ, this has not been shown experimentally for all Zap proteins (only shown for ZapA and ZapD) (48, 68).

1.4 FtsZ Associated Proteins (Zap proteins)

Zap proteins are small cytoplasmic proteins acting early in cell division. Although they are non-essential individually, it is possible that the combined and over-lapping functions of these proteins play an essential role in stabilizing the Z-ring prior to cell division. Presently, there are 4 known Zap proteins: ZapA, ZapB, ZapC and ZapD. ZapA, C, and D can be grouped together as they are proposed to function in similar ways, while ZapB has a separate but related function (Figure 1.9). Not included in this list is ZapE, which acts after cell division and whose function is unrelated to ZapA-D (112).

1.4.1 ZapA, ZapC and ZapD

ZapA was the first of the Zap proteins to be discovered. It was initially identified in the sporulating bacterium *Bacillus subtilis* and was found to localize to mid-cell and enhance FtsZ bundling *in vivo* and *in vitro* (118). ZapA is a well conserved protein; a copy exists in most Gram-negative and some Gram-positive bacteria (44). The structure of the dimeric ZapA from *Pseudomonas aeruginosa* (*PaZapA*) was solved in 2004, providing more insight to the function of ZapA as an FtsZ bundling protein (88). Upon investigation in *E. coli*, it was found that *EcZapA* (ZapA in *E. coli*) functions as a tetramer to bundle FtsZ filaments at mid-cell (119). Although this multi-species approach to studying ZapA had shown that it tends to function similarly in various bacteria, focus then shifted to *EcZapA* alone. *In vitro*, FtsZ filaments are bundled by ZapA into highly-ordered bundles of varying width (44, 90, 120, 121). This
Figure 1.9. Diagram depicting the bundling and cross-linking abilities of Zap proteins. [A] FtsZ (red circles) polymerizes into short filaments, [B] ZapA, C or D (blue rectangles) act to increase the lateral interactions between FtsZ filaments and stabilize them resulting in longer filaments. [C] ZapB (purple hexagons) interacts with ZapA to cross-link FtsZ filaments indirectly.
interaction affected FtsZ by decreasing its GTPase activity, as measured by a phosphate release assay (44). This was also evident in that FtsZ filaments bundled by ZapA are very long (~500 nm), indicating ZapA stabilizes FtsZ filaments through inhibition of GTPase activity, thereby slowing the depolymerisation process following GTP hydrolysis. It is thought that this affect on GTPase activity is secondary to bundling (119). ZapA brings FtsZ filaments close together, potentially covering the GTP binding domain. Since it is likely that a conformational change in FtsZ is required for GTP hydrolysis, the stabilized filament may not be able to catalyze conversion to GDP because ZapA stabilizes it in a protein network (119). The general area for interaction between FtsZ and ZapA was elucidated by bacterial two-hybrid assay to be the conserved C-terminal tail of FtsZ and residues 20-48 of PaZapA, which would position ZapA over the GTP binding domain in bundles and support the aforementioned hypothesis (68) (Figure 1.10). However, the inconsistency seen between two-hybrid assays performed to define other protein-protein interactions indicates that these results should be validated through a secondary method (122).

*In vivo* analysis of a zapA-deleted strain of *E. coli* revealed a role for ZapA in bundling FtsZ helices into a coherent Z-ring at mid-cell (90). Cells lacking ZapA often had uncondensed helical FtsZ, while complementation with exogenous ZapA restored proper Z-ring formation (90). Conversely, a super-resolution microscopic study argues that ZapA merely functions to position the Z-ring and properly align it perpendicular to the elongation plane, as opposed to bundling FtsZ filaments (123). ZapA was also shown to interact directly with ZapB and acts to recruit ZapB to mid-cell (120).

Little is known of the structures and functions of ZapC and ZapD. Both have been shown to localize to mid-cell prior to cell constriction and are implicated in Z-ring stability (45, 46, 48).
Figure 1.10. ZapA dimer from *Pseudomonas aeruginosa*.
Crystal structure (PDB: 1W2E) with the predicted area of interaction with FtsZ highlighted in red.
ZapC and ZapD interact directly with FtsZ in yeast protein-interaction platform experiments, bundle FtsZ filaments \textit{in vitro} and decrease the GTPase activity of FtsZ, just as ZapA does (45, 48). \textit{In vivo} deletion of either gene leads to helical formations of FtsZ at mid-cell and ectopic divisional sites (46, 48). Differences between the two proteins include their oligomeric states, extent of elongation upon gene deletion and conservation across species. ZapC appears to be monomeric when analyzed by size exclusion chromatography, while ZapD is dimeric when analyzed by the same technique (45, 48). A ZapC variant (L22P) has been generated which artificially forms dimers (45). This ZapC variant did not interact and bundle FtsZ filaments efficiently, indicating residues surrounding L22 may be involved in FtsZ binding (45). Morphologically, ΔzapC cells are moderately elongated, while ΔzapD cells do not show significant elongation (46, 48). These results indicate that the roles of these Zap proteins may be different, or that the proteins are multifunctional. It should be noted that the \textit{zap}-gene deletions used in these studies came from a transposon insertion library and the exact site of insertion is unknown, which means the mutation can have downstream affects by disrupting a proximal gene or there could be multiple insertions at different sites throughout the genome (124). Finally, with respect to conservation throughout bacterial lineages, both ZapC and ZapD are limited to \(\gamma\)-proteobacteria.

Varying deletions of \textit{zap}A, \textit{zap}C and \textit{zap}D have revealed their potentially overlapping and redundant roles. Single-gene deletions show varying degrees of cell filamentation, Δ\textit{zap}A being the longest, followed by Δ\textit{zap}C and finally Δ\textit{zap}D cells being the shortest. Δ\textit{zap}AD cells show much longer cells than Δ\textit{zap}A alone (48). Similarly, Δ\textit{zap}AC cells show even greater cell elongation (48). Interestingly, Δ\textit{zap}CD cells do not show any more elongation than Δ\textit{zap}C cells (48). Finally, a triple-gene deletion mutant is longer than any single or double mutant (48).
Taken together these results indicate an overlapping role between ZapA and ZapD and between ZapA and ZapC but not between ZapC and ZapD. However, further investigation is required to reveal the mechanisms behind these elongation phenotypes.

1.4.2 ZapB

ZapB is a small coiled-coil protein and the structure of the *E. coli* ZapB protein is known (47). It differs from the other Zap proteins because it does not interact directly with FtsZ. Rather, ZapB interacts with ZapA and is proposed to play a role in Z-ring formation by cross-linking FtsZ-bound ZapA, to form an ‘inner-ring’ and begin constriction (Figure 1.9C) (120). This dual ring was illustrated by confocal microscopy (120). ZapB was found in a tighter ring just inside of the FtsZ-ZapA colocalization (Figure 1.9C) (120). *In vitro*, ZapB can form polymers and higher order bundles (47). This indicates ZapB may play an important structural role as well. Although the FtsZ interaction appears to differ between ZapB and other Zap proteins, the *in vivo* consequence for *zapB* gene deletion is very similar; cell elongation, helical FtsZ morphology and ectopic division sites (47).

ZapB has a proposed secondary role in coordinating chromosome segregation and cell division. It binds to the DNA bound protein MatP, which acts ensure full segregation of DNA into daughter cells prior to cell constriction (125). FtsK also plays a role in coordinating DNA segregation/transport and cell division, and so it stands to reason that ZapB and FtsK may interact at the site of division, but this has yet to be investigated.
1.5 Bacterial protein complexes

The importance of protein complexes and understanding how they work is illustrated in many biological processes. DNA synthesis, transcription, translation, protein modification, protein degradation, signal transduction, chemotaxis, protein and molecular transport all involve the cooperation of protein complexes, whether transient or maintained (126–130). These protein assemblies combine their functions and act as one cohesive unit. Therefore, it makes sense that proteins with shared functions will have more interactions between them than proteins involved in completely different processes. The lack of physical compartmentalization in bacteria has indirectly brought upon the need for these organized networks of related proteins. By functioning as one unit, each process is made more efficient by the close proximity of proteins involved in the sequential steps of a single process, akin to an assembly line in a production plant.

The divisome is a particularly large macromolecular protein complex and little is known about how it assembles in vivo. The divisome consists of 20 essential and non-essential proteins and several broad scope interaction studies conducted in E. coli have revealed the great number of interactions between these proteins (69, 131, 132). It is these interactions that are attractive targets for new antibiotics, which would specifically aim to disrupt protein-protein interactions. The multitude of interactions lends to the dynamic nature of the divisome. Some interactions, although essential, are transient and therefore difficult to track and define for drug design purposes. Within the divisome, stable sub-complexes would be the starting point for developing these targeted antibiotics, as more is known about their assembly and they have been shown to have less transient interactions in vivo.
1.5.1 Cell division as an antibiotic target

Bacterial cell division and the divisome complex are attractive targets for new antibiotics for several reasons. The first and most important reason that cell division is a good candidate for drug targeting is that there are very few close homologues to divisome proteins in eukaryotes (133). Therefore, there is not a high risk of interfering with host processes. Secondly, the divisome performs distinct functions in constriction, divisional PG synthesis, PG rearrangement and inner- and outer-membrane invagination. The multi-functionality of this complex provides several different processes that have already been targeted for interference and could be further exploited (Figure 1.11). Lastly, the successful division hinges on the highly coordinated interaction of proto-ring, early divisome and late divisome components (Figure 1.1). Recently, it has been demonstrated that protein-protein interactions within complexes are suitable targets for antibiotic design (134, 135). Therefore, by further studying interactions acting to stabilize the divisome complex we may reveal targets for therapeutics.

Presently, there are two main antibiotic targets with regards to bacterial cell division: divisional PG synthesis (FtsI and FtsW) and FtsZ. In *E. coli*, PG synthesis begins in the cytoplasm with the synthesis of PG precursors UDP-N-acetylglucosamine (136, 137) and UDP-N-acetylmuramic acid (138). The enzyme MurG then uses these precursors to form lipid II (139). Lipid II is transferred across the cytoplasmic membrane by a flippase, MurJ (previously thought to be FtsW) (37, 140). In the periplasm, glycosyltransferases act to elongate the glycan chains attached to lipid II (141) and transpeptidases (E.g. FtsI) cross-link these glycans to form a mesh of PG (142). Classically, transpeptidases have been combatted with β-lactam antibiotics, such as penicillin (31). Many bacteria are now resistant to β-lactams, therefore, in an effort to effectively inhibit FtsI, new derivatives of β-lactams have been generated, for example lactivicin (143).
Figure 1.11. Diagram showing where in bacterial cell division current antibiotics act. Inhibitors of Z-ring formation are targeted directly to FtsZ, either binding to the GTP binding domain or inhibiting polymer formation by blocking monomer-monomer interactions. Inhibitors of septum formation are targeted towards FtsI and FtsW.
FtsZ can be inhibited using several different strategies. For proper Z-ring formation and subsequent cell division, the self-association of FtsZ is required, where monomers come together in a head-to-tail manner (61, 144). The GTP binding domain is formed between two dimers. GTP binds to this pocket and is hydrolyzed to GDP (58, 59, 76). This process continues until FtsZ filaments form, the filaments themselves then associate with one another through Zap proteins, FtsA and ZipA, to form a dynamic Z-ring at mid-cell (78, 117, 118). Each of these steps has been targeted in designing antibiotics against FtsZ. A rational starting point for drug design was with GTP since the structure is known and its interaction with FtsZ has been well defined (145). Non-hydrolyzable analogues of GTP have been created in hopes of clogging up the GTP binding pocket and prevent proper FtsZ filament formation (146). GTP analogues containing a bulky side chain at position C8 have been generated and tested with some success in an effort to prevent FtsZ head to tail assembly (147–149). Similarly, the FtsZ self-association has been targeted through its non-GTP binding domain. This was done initially with the drug 3-methoxybenzamine (150). Small molecule screens have also yielded some promising results in affecting FtsZ assembly with Totarol, Viriditoxin, CCR-II and compound 1/PC190723 (151–154). These inhibitors have been shown to be species specific in their action but none have undergone sufficient testing to make their appearance in clinical use.

1.5.2 Inhibiting protein-protein interactions

The basis for designing antibiotics to interfere with protein-protein interactions (PPIs) is the thorough analysis of the 3D structures of the proteins of interest and site-directed mutagenesis of the predicted interaction sites, followed by analysis of the changes in the PPI (155–157). Within this class of antibiotics are the small-peptide inhibitors. These hold
advantages over traditional small-molecule antibiotics in that they are highly specific and less cross-reactive or detrimental to the host organism (158). Successful small-peptide inhibitors would interfere with processes such as an essential dimerization of the target protein (159). This requires that the 3D structures of each protein have been solved and both are amenable to interaction analysis. In the case of cell division, many of the individual structures have been elucidated by x-ray crystallography, making this method of drug development a realistic one. Although the complete co-crystal structure of FtsZ with another divisome protein has not yet been determined, peptide fragments from FtsZ (C-terminus) and ZipA (C-terminus) from *E. coli* were co-crystallized to show the specific interactions between these polypeptides (67).

1.6 Significance and hypothesis

With the growing number of antibiotic resistant strains of bacteria, there is a need for new classes of antibiotics. Bacterial cell division is an attractive target for this, in that it is an essential process for sustaining prokaryotic life. Overall, the lack of understanding about bacterial cell division and, more specifically, divisome assembly stems from the large number of proteins and protein-protein interactions involved. However, it is known that divisome assembly occurs sequentially, and hinges on the polymerization, filament association and membrane tethering of the dynamic protein FtsZ. This integral aspect of divisome assembly is the basis for this thesis research. By understanding the overlapping roles of Zap proteins in FtsZ filament bundling we can further dissect the dynamics of FtsZ and proto-ring assembly, prior to division. The overarching goal of this work is to reveal potential regions on FtsZ and Zap proteins for targeting small peptide antibiotics. In order to accomplish this, the structures of all proteins of
interest must be determined, the multi-valent interactions between those proteins must be shown and the potential overlap of binding sites should be elucidated.

The long-term goal of this research is to improve our understanding of the fundamental mechanisms that drive bacterial cell division. The aim is to identify potential drug targets to exploit this essential bacterial process in order to fight the growing number of multi-drug resistant strains of bacteria. By focussing on the early stages of cell division, the immediate goal is to elucidate the protein-protein interactions and multi-protein complexes involved in Z-ring formation and stability. The overarching hypothesis is that Zap proteins function synergistically, and that their combined interactions with FtsZ play a crucial role in stabilizing the bacterial Z-ring prior to cell division.
Chapter 2: Crystal structure and site-directed mutational analysis reveals key residues involved in *Escherichia coli* ZapA function

Data from this chapter was published in the following journal article:


Statement of contributions: The crystal screens, data collection and resolution of the *EcZapA* structure were preformed by Dr. Matthew Kimber. All other experiments were performed by Ms. Elyse Roach.
Abstract

FtsZ is an essential cell division protein in *Escherichia coli* and its localization, filamentation and bundling at the mid-cell are required for Z-ring stability. Once assembled, the Z-ring recruits a series of proteins that comprise the bacterial divisome. FtsZ-associated proteins (Zap proteins) stabilize the Z-ring by increasing lateral interactions between individual filaments, bundling FtsZ to provide a scaffold for divisome assembly. The X-ray crystallographic structure of *E. coli* ZapA (*Ec*ZapA) was determined, identifying key structural differences from the existing ZapA structure from *Pseudomonas aeruginosa*, including a charged α-helix on the globular domains of the ZapA tetramer. Key helix residues in EcZapA were modified using site-directed mutagenesis. These ZapA variants significantly decreased FtsZ bundling in protein sedimentation assays when compared to wild-type (WT) ZapA proteins. Electron micrographs of ZapA-bundled FtsZ filaments showed the modified ZapA variants altered the number of FtsZ filaments per bundle. These *in vitro* results were corroborated *in vivo* by expressing the ZapA variants in an *E. coli ΔzapA* strain. *In vivo*, ZapA variants that altered FtsZ bundling showed an elongated phenotype, indicating improper cell division. Our findings highlight the importance of key ZapA residues that influence the extent of FtsZ bundling, and which ultimately affect Z-ring formation in dividing cells.
2.1 Introduction

Bacterial cell division requires the presence, accumulation and mid-cell localization of the Filamentous temperature sensitive protein Z (FtsZ). FtsZ is a 40 kDa monomeric protein with GTPase activity that shows structural and functional homology to the eukaryotic protein tubulin (14, 60, 64, 160, 161). FtsZ binds and hydrolyzes GTP in vivo (162), and also polymerizes to form the Z-ring at mid-cell. The Z-ring forms prior to cell division and acts as a scaffold for the recruitment of all downstream cell division proteins; together these comprise the ‘divisome’ (4, 35, 163, 164). In Escherichia coli the divisome includes 20 essential and non-essential proteins (3, 5, 165). Divisome proteins are recruited in a hierarchal manner and depend on the Z-ring for stability. Included in the divisome are the FtsZ-associated proteins (Zap proteins), ZapA, ZapB, ZapC and ZapD (45, 47, 48, 89, 118, 166). These Zap proteins stabilize the Z-ring by increasing lateral interactions between individual filaments, thus bundling FtsZ to provide a scaffold for divisome assembly (71, 163).

Our knowledge of the functional, structural and biochemical significance of the Zap proteins in E. coli is limited. It has been demonstrated that they are not individually essential in vivo (47, 89, 118). However, due to their potentially overlapping function and temporal recruitment to the Z-ring, it may be the cooperative interaction of all four proteins with FtsZ that has a significant impact on cell division (47, 118). Advances in microscopy have aided in the visualization of FtsZ filaments in vitro (18, 62, 64, 79, 167, 168) and Z-ring formation in vivo (17, 85, 123, 160, 169, 170). Depending on the techniques and bacterial species used, recent super-resolution fluorescence studies suggest that, in vivo, FtsZ filaments may adopt either a dynamic ‘bead-like’ organization (98), loose, overlapping bundles comprised of multiple FtsZ filaments (73, 123), loosely-packed continuous bands of bundled FtsZ filaments (94), or a
combination of the arrangements described above (97). While ZapA and ZapB have been implicated in positioning FtsZ filaments at mid-cell (123), the precise function of the Zap proteins in vivo remains unclear.

ZapA has been shown to bundle FtsZ in vitro, and ZapA-deficient strains exhibit significant elongation, indicating a cell division defect (118, 123, 171). To date, all biochemical studies on the E. coli ZapA protein (EcZapA) have been interpreted in the context of the crystal structure of the tetrameric Pseudomonas aeruginosa ZapA (PaZapA) (44, 88, 119, 171). As these two proteins share only 25% sequence identity (as determined by ClustalW (171)), a structure of E. coli ZapA may aid in advancing understanding about the lateral interactions that bundle FtsZ filaments. Recently, a critical region on E. coli ZapA was identified by a bacterial two hybrid assay (68). This 26 amino acid region is weakly conserved across bacterial species but contains several charged residues that could potentially interact with FtsZ. Here, we report the crystal structure of the EcZapA tetramer showing several key differences from its P. aeruginosa counterpart. Additionally, by targeting charged amino acids on the α-helix of the N-terminal globular domain of EcZapA, we have identified key residues involved in the ZapA-FtsZ interaction that promote filament bundling.

2.2 Experimental procedures

2.2.1 Cloning, site-directed mutagenesis, and construction of Zap deletion strain

Plasmids containing zapA and ftsZ were constructed by amplifying the zapA and ftsZ genes using primers 308F (TATATGAATTCTATGCACCACCACCCCAACCGCATCGA AGGTCGAAGTGTTATGCTGCACAACCGCTCGATATC) and 308R (TATCGAAGCTT
TCATTCAAAGTTTTGTTAGTTTTTTCGGTGATGCGACCTTGT TCTATGG), and 400F (TTTAATACCATGGTGTGTTGAAACATGAACTTACCAATG) and 400R (TATTATAAGCTTTATTAATCAGCTTGCTTACGGAATG), respectively. The zapA gene from E. coli W3110 was amplified and cloned into pBAD24 as an EcoRI-HindIII His6-tag-encoding fragment (sites underlined). The fisZ gene was cloned into pET28a using the NcoI and HindIII cut sites (sites underlined) so as to exclude the plasmid-encoded his-tag. The ΔzapA strain was made using the λ red deletion system as described previously (172, 173). The entire zapA gene was replaced with the cat (chloramphenicol acetyl transferase) region from pKD3 mediated by the λ red operon products from pSIM6 in E. coli DH5α. Site-directed mutagenesis was performed using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) and zapA variant sequences were amplified using the primers listed in Table 2.1.

Table 2.1. Strains, plasmids and primers used in this study.

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<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F' ompT gal dcm lon hsdS₈( rK mK')λ(DE3) pLysS(cmR)*</td>
<td>New England Biolabs</td>
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<tr>
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<td>rph-1IN(rrnD-rrnE)</td>
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<tr>
<td>pEJR029b_D33A</td>
<td>pBAD24 encoding D33A His₆-ZapA from E. coli</td>
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</tr>
<tr>
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<td>pBAD24 encoding D33K His₆-ZapA from E. coli</td>
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<tr>
<td>pEJR029b_R46A</td>
<td>pBAD24 encoding R46A His₆-ZapA from E. coli</td>
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<table>
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<tr>
<th>Primers for Mutagenesis</th>
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<tbody>
<tr>
<td>D22A_F</td>
<td>AACTGCCGCCCTGCCTGACGCGAGGGATGC</td>
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</table>
2.2.2 Protein expression and purification

FtsZ expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (Roche) in an *E. coli* BL21(DE3) pLysS strain at 37°C for 3 h in LB medium supplemented with kanamycin and chloramphenicol. Cell pellets were resuspended in PEM-KOH buffer (50 mM piperazine-N,N'-bis(ethanesulfonic acid)-KOH, 1 mM EDTA, 5 mM MgCl₂, pH 6.5) and lysed by French press. Cell debris was removed by centrifugation at 8,000 × g for 15 min, membranes were removed by centrifugation at 100,000 × g for 1 h. FtsZ was purified by two rounds of calcium cycling as described previously (79) with an additional low speed centrifugation step (8,000 × g for 5 min) before desalting using Amicon® Ultra-15 10,000 NMWL centrifugal filters (Millipore). FtsZ was then stored at 4°C for no longer than 4 days in PEM-KOH buffer. For His₆-ZapA expression, an overnight culture of *E. coli* BL21(DE3) pLysS
cells carrying the pEJR029b plasmid was diluted 1/100 into LB medium supplemented with ampicillin. Cells were grown at 37°C to an OD$_{600}$ of 1.0 (~2 h) and then induced by the addition of 0.2% (v/v) L-arabinose (Sigma). Expression proceeded for 1 h before cell pellets were collected by centrifugation. After pelleting, cells were resuspended in A1 buffer (20 mM Tris, 50 mM NaCl, 20 mM imidazole, pH 7.4) and lysed by French press. Cell debris and membranes were removed as described for FtsZ (64). His$_6$-ZapA was purified by immobilized metal affinity chromatography (IMAC), using the Biologic™ Duoflow Chromatography System (BioRad). Soluble protein fractions were loaded onto an MT2 column (BioRad) packed with Ni-NTA Superflow resin (QIAGEN). After washing with 6 column volumes of A1 buffer containing 75 mM imidazole, His$_6$-ZapA was eluted with a linear gradient of imidazole from 75-500 mM. His$_6$-ZapA fractions were pooled, concentrated, and desalted using Amicon® Ultra-15 3,000 NMWL centrifugal filters (Millipore). Purified His$_6$-ZapA was stored at 4°C in A1 buffer for no longer than 4 weeks with no loss of activity during this period.

2.2.3 Crystallization, data collection, structure determination, and analysis of ZapA

EcZapA was crystallized from 0.5 M ammonium sulfate, 1.0 M lithium sulfate, and 0.1 M sodium citrate, pH 5.6. Crystals grew as hexagonal bipyramids up to 500 μm in length. After the removal of surface water by immersion in paratone N-oil, crystals were frozen in liquid nitrogen. Data was collected at the Canadian Synchrotron Light Source (beam line CMB) and processed in XDS (174). Initial attempts at molecular replacement failed in Phaser (175) with a variety of search models derived from the PaZapA dimer or protomer. Molecular replacement eventually succeeded with a single protomer search model, comprising residues 50-94. This model comprises less than 25% of the asymmetric unit, with a pairwise sequence identity of only
~30% to the target. The top translation function scores for the second copy just exceeded the Z-score of 8.0, which generally indicates a reliable molecular replacement solution. The solution is actually out of sequence register with respect to the final model, and with different offsets in the two protomers. The C-terminal helix appears to act as a generic α-helical model of approximately the correct curvature, capturing the essential structural elements independent of the underlying sequence. This initial model was subjected to autobuild in Phenix (176), which was able to correctly build most of chain A. The rest of the structure was completed manually by rebuilding in Coot (177), and refined in Phenix (176), using a translation-liberation-screw model of the atomic displacement parameters.

2.2.4 FtsZ filamentation and sedimentation assays

FtsZ (4.8 μM) filamentation was performed in PEM-KOH buffer at 30°C upon the addition of GTP to 1 mM; in all cases samples were pre-incubated for 5 min at 30°C before filamentation. Sedimentation assays were performed by titrating His6-ZapA (0.5, 1.0, 2.1, 4.8, 8.1, and 11.4 μM) against a constant concentration of FtsZ (4.8 μM). His6-ZapA and FtsZ were diluted and mixed in PEM-KOH buffer, GTP was added to 1 mM (with the exception of the negative control), and reactions were incubated at 30°C for 5 min. Following incubation an aliquot of total protein was taken from each reaction and mixed with 5× SDS sample buffer for analysis. Samples were sedimented at 10,000 rpm for 15 min (centrifuge: Ependorf 5424)). Previous studies (68, 119) used 80,000 rpm for 10 min (rotor: rotor: MLA-135) to sediment FtsZ-ZapA bundles and we observed no differences in FtsZ-ZapA sedimentation between these conditions (data not shown). Soluble protein was obtained from the supernatant, while pellets were resuspended in an equal volume of PEM-KOH buffer; both were mixed with 5× SDS
sample buffer for analysis. All protein samples were boiled in sample buffer and analyzed by SDS-PAGE followed by Coomassie blue staining using the SimplyBlue™ SafeStain (Invitrogen). We performed densitometry on gels to determine the amounts of soluble and pelleted FtsZ relative to the total protein fractions from each reaction using ImageLab™ Software (BioRad). Pellet fractions were compared to the initial fraction, which was set to 100%. In order to account for the FtsZ that sedimented out of solution (pellet fraction) when not bundled, control reactions were performed containing FtsZ and the ZapA variant alone, without GTP. This amount of sedimentation was then subtracted from all other pellet values. Additionally, FtsZ filamentsed by GTP was sedimented as a control to be sure that unbundled filaments did not pellet out of solution when centrifuged at this speed.

2.2.5 In vivo complementation and immunofluorescence

Chemically competent *E. coli* Δ*zapA* cells were made by standard methods. Each pBAD24 vector containing mutated *zapA* (Table 2.1) was transformed into these cells, after which a single colony was chosen and grown for 16 h. Cultures were then diluted 1/100 in LB medium and allowed to grow to an OD$_{600}$ of 1.0. Expression was then induced by the addition of L-arabinose to 0.1% (w/v). Cells were adhered to a copper 200 mesh electron microscopy grid (Canemco) and imaged by transmission electron microscopy. For each variant, ~20 random fields of view were imaged and >100 cells from two experimental replicates were measured using ImageJ (178). Immunofluorescence was performed as described by Hiraga *et al.* (179) with minor modifications. Cells were prepared and fixed as described previously (179). Cells were then blocked with 10% normal goat serum (Invitrogen) for 30 min at room temperature. The blocking solution was replaced with primary antibody (1:100 rabbit anti-FtsZ [Cedarlane] in 1%
BSA-PBST; PBS with 0.05% [v/v] Tween 20). Slides were incubated for 1 h at room temperature before removing the primary antibody and washing with PBST. Secondary antibody (1:200 goat anti-rabbit conjugated to FITC [Sigma-Adrich] in 1% [w/v] BSA-PBST) was added and incubated for 1 h at room temperature. Secondary antibody was removed and samples were washed with PBST, samples were counterstained with 4',6-diamidino-2-phenylindole (10 µg/mL) for 1 min in the dark and washed with PBST. Coverslips were mounted on the slides with SlowFade® Gold antifade reagent (Invitrogen). Slides were imaged using a Leica DM5000B fluorescent microscope equipped with a Hamamatsu ImagEM EM-CCD digital camera (Quorum Technologies). Images were analyzed using Volocity software (version 6.3, PerkinElmer, Inc.). SDS-PAGE gels were run of all complemented cell samples, gel loading was normalized by OD$_{600}$. Gels were transferred to nitrocellulose membranes and blocked with 2% BSA (w/v). The primary antibody, rabbit anti-FtsZ (Cedarlane) (for FtsZ) or mouse anti-His$_6$ (Clontech) (for His$_6$-ZapA) was bound to the membrane and used as per the manufacturer’s guidelines. Secondary antibody, Goat anti-rabbit (Sigma) (for FtsZ) or rabbit anti-mouse (Sigma) (for His$_6$-ZapA) conjugated to alkaline phosphatase was used at a dilution of 1:20,000. Washes were performed using 1 x TBST in between all steps.

2.2.6 Transmission electron microscopy (TEM)

For protein filaments and bundles, grids were subjected to 7 sec of plasma cleaning in the Solarus™ Advanced Plasma Cleaning System (Gatan). Protein samples were adhered to the grid for 45 sec, washed once with PEM-KOH buffer and once with 0.2 µm filtered MilliQ water, then stained with 2% uranyl acetate for 30 sec and allowed to air dry. For whole cell samples, cells were adhered to a copper grid for 10 sec, stained with 1% uranyl acetate for 7 sec, then washed
once with 0.2 µm filtered MilliQ water and allowed to air dry. Grids were imaged using the FEI Tecnai G2 F20 operated at 120 kV.

2.3 Results

2.3.1 X-ray crystallographic structure of EcZapA

The structure of EcZapA was determined to a resolution of 1.95 Å (Figure 2.1, Table 2.2), with two molecules per asymmetric unit. N-terminal residues 1-3 in chain A are disordered, while residues 1 and 46 in chain B are too poorly defined to reliably model. Similar to PaZapA (38), the structure is organized into two distinct domains, with the first 49 residues forming a globular domain comprised of a two stranded antiparallel β-sheet plus an α-helix, while the C-terminal half of the protein (residues 50-102) forms a single, 14 turn α-helix. The ZapA protomer is organized into a pseudosymmetric tetramer, where chains A and B interact with their doppelgangers A' and B' through a crystallographic two-fold axis (Figure 2.1, B and C). The strongest interaction forms a tight dimer that is stabilized predominantly by interactions mediated by the long C-terminal helix. In EcZapA, the first seven turns of the two helices interact through a coiled-coil type packing arrangement, where the two helices wrap around one another to form a left-handed superhelix; the two helices therefore cross at a 20° angle, and equivalent residues pack against one another laterally. Interactions are also mediated by the N-terminal domains, which pack against both helices so that both the α-helix and β-strands pack onto the second protomer. Note that the two chains of EcZapA differ significantly, with an r.m.s.d. of 2.0 Å; this difference is mostly attributable to significant differences in the twist of the C-terminal helices of the respective chains required to optimize these interactions. Overall, the tight dimer interface (A to B) buries 1670 Å² per protomer (Figure 2.1B). The distal ends of the C-terminal helices splay
Table 2.2. Data collection, model refinement, and final structure statistics for EcZapA.

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
<tr>
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**Asymmetric unit contents**

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</tr>
<tr>
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</tr>
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<tr>
<td>Disallowed (%)</td>
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</tbody>
</table>

*The last shell includes all reflections between 2.00 and 1.95 Å;*  
*R_free calculated using 5% of the data which were chosen randomly;*  
*Calculated using Molprobity.*
Figure 2.1. X-ray crystallographic structure of *E. coli* ZapA.

[A] the *Ec*ZapA protomer is organized as a compact N-terminal domain, with an extended C-terminal tail that is built as a single, elongated α-helix. [B] the *Ec*ZapA tetramer is comprised of two protomers that form a tight dimer, mediated mostly by coiled-coil like interactions between the long αB helix. This dimer interacts through the C-terminal end of this α-helix to form a four-helix bundle. Chain identities referred to in the text are marked. [C] the electrostatic surface of the *Ec*ZapA tetramer highlights the N-terminal α-helix, αA, which forms a prominent ridge of electronegative residues. Inset shows the cartoon representation, as well as residues chosen for more detailed studies. [D] details in the packing of the *Ec*ZapA tetramer vs. [E] packing in the *Pa*ZapA tetramer. Note that the two structures were oriented by superimposing the upper dimer of the structure. While the structures do bear an overall resemblance, they differ in numerous details. In particular, the interactions between protomers of the tight dimer are very different, with *Ec*ZapA using a coiled-coil interaction, while *Pa*ZapA has a ladder of hydrophobic residues that interdigitate. In the latter case, this results in the two protomers of the dimer having a noticeable vertical offset from one another. The N-terminal domains also differ markedly in their orientation relative to the C-terminal helices. In *Ec*ZapA, the N-terminal domains both sit at a similar angle across the two C-terminal helices. In *Pa*ZapA, one N-terminal domain in each dimer shows a marked shift, and interacts with αB at an acute angle. Differences in packing of the helical domains also result in the N-terminal domains at opposite ends of the dimer aligning to the same face in *Ec*ZapA, but being rotated almost 90° around the long axis in *Pa*ZapA (small arrows).
apart from their dimeric mates, packing instead against the other dimer in a four-helix bundle-like arrangement. Residues 103-109, C-terminal to this helix, are also all ordered. They pack in an extended conformation against the C-terminal helix from a related protomer, with the last two residues of the protein interacting with the N-terminal domain of the opposite tetramer. The tetramer is stabilized by burying approximately 1500 Å² per protomer between A and A' (and also B and B'), and a further 920 Å² between symmetry-related copies of chains A and B' (as well as B and A'). PISA predicts the dissociation energy of the tetramer (54.0 kcal/mol) is larger than the combined dissociation energy of the respective dimers (28.6 kcal/mol).

Despite an overall resemblance to the PaZapA structure, the EcZapA structure differs in many important aspects (Figure 2.1, D vs. E). The C-terminal helix of PaZapA exhibits much less extensive interaction between tetramers than EcZapA (five turns of helix vs. nine). This may have functional implications, as the PaZapA tetramer seems capable of dissociating into a dimer. In addition, the C-terminal residues in PaZapA are disordered, and so do not form the extended interactions seen in EcZapA. The interactions between protomers within the dimer are also fundamentally different in PaZapA. In the PaZapA structure, the C-terminal helices pack in parallel, but with the helices offset by one turn, so that each residue packs above the equivalent one (Figure 2.1E). The offset of the two C-terminal helices presents two different interaction surfaces to the N-terminal domain, which then packs into two clearly distinct conformations differing by a large (~40°) rotation. Together, these differences in protomer orientation result in dimers which are structurally different than those in the EcZapA structure (Figure 2.1D), and whose protomers superpose with an r.m.s.d. of 5.7 Å (a difference more typically seen between two proteins that are almost at the limits of detectable relatedness). Note that this asymmetry is
seen in two independent structures (1T3U and 1W2E) in different crystal forms (88) and are highly unlikely to represent an artefact of crystal packing.

2.3.2 ZapA variants show altered FtsZ bundling capability in vitro

Galli and Gerdes reported the N-terminal domain of *E. coli* ZapA alone was capable of interacting with FtsZ, albeit weakly (68). Based on their bacterial two hybrid assay results (68), six residues of ZapA were mutated (Figure 2.1C; inset and Table 2.1) in order to assess their roles in ZapA interactions with FtsZ filaments.

ZapA is proposed to bundle FtsZ by increasing the lateral interactions between filaments (68, 72, 88, 118, 121). This bundling interaction can be studied *in vitro* using a sedimentation assay. In the presence of ZapA, FtsZ filaments come out of solution, while in the absence of ZapA they remain soluble (68, 119). This approach was used to titrate the sedimentation of FtsZ filaments using wild-type (WT) ZapA and ZapA variants generated by site-directed mutagenesis (Table 2.1) along the charged N-terminal α-helix (Figure 2.1C, inset). First, FtsZ was allowed to form filaments in the absence of ZapA, as a control, to ensure proper filament formation (Figure 2.2A). These FtsZ filaments resemble those described previously (60, 72), and are single filaments ~5 nm in width (n=100). Next, ZapA variants were mixed at sub-equimolar (0.5 µM, 1.0 µM and 2.1 µM), equimolar (4.8 µM; Figure 2B), and saturated concentrations (8.1 µM and 11.4 µM) with a constant concentration of FtsZ, and filament sedimentation was monitored by SDS-PAGE (Figure 2.2C). The sedimentation assays presented in this study were conducted at pH 6.5 as previously described (68, 72, 118, 119, 121, 171). However, FtsZ-ZapA bundles have also been reported at the more physiologically relevant pH of 7.5 (80). For each reaction, the
Figure 2.2. FtsZ sedimentation assay and the percent of FtsZ in the pelleted fractions.

[A] purified FtsZ filamented with 1 mM GTP in the absence of ZapA (bar = 250 nm, inset bar = 25 nm). [B] FtsZ filaments bundled with purified ZapA (4.8 µM; bar = 100 nm). [C] FtsZ (4.8 µM) filamented with 1 mM GTP in the presence of WT ZapA or ZapA variants (0.5-11.4 µM). Initial samples (I) were used to assess the total protein content in each reaction. Soluble (S) and pelleted (P) fractions were separated by centrifugation at 10,000 rpm for 15 min, and all three samples (I, S, P) were analyzed by Coomassie-stained SDS-PAGE. Pelleted fractions contain FtsZ filaments that were bundled with ZapA. [D] triplicate FtsZ sedimentation assays were performed for each ZapA variant and Coomassie-stained SDS-PAGE were analyzed by densitometry. The mean percentage of FtsZ in the pelleted fractions were plotted for each concentration of ZapA, where the error bars indicate ± SEM (standard error of the mean). Reactions with equimolar ZapA (4.8 µM) and FtsZ (4.8 µM), lacking GTP, were run as a control for protein precipitation (not shown) and these values were subtracted from each reaction’s sedimentation.
initial ZapA-FtsZ mixture (I) was incubated for 2 min and centrifuged to separate the soluble (S) and pellet (P) fractions (Figure 2.2C). These experiments were performed in triplicate and the pelleted fractions were analyzed by densitometry (Figure 2.2D). For WT ZapA, filamentous FtsZ sedimentation increases with increasing ZapA concentration until equimolar concentrations (Figure 2.2C). At 0.5 µM WT ZapA sediments ~16% of FtsZ filaments in solution compared to ~86% at the physiologically relevant equimolar concentration of 4.8 µM (121). When saturated with ZapA (11.4 µM) ~87% of FtsZ filaments are found in the pellet; this indicates that, at equimolar concentrations, WT ZapA can sufficiently sediment the majority of FtsZ filaments. As residues along the charged helix are altered (Figure 2.1C, inset) clear differences emerge in the ability of the ZapA variants to pellet FtsZ, most notably at equimolar and saturated concentrations (Figure 2.2C). For example, at equimolar concentrations N28A shows a 73% decrease in FtsZ sedimentation compared to WT ZapA (Figure 2.2C). We also generated a D40A ZapA variant, but this construct did not express in E. coli cells and we were not able to purify it. This result is similar to what Pacheco-Gómez et al. reported with mutational analysis of residue 41 of E. coli ZapA (119), indicating these residues may be critical for ZapA proper expression and protein folding in vivo.

To better visualize the differences observed in the sedimentation assays, the densitometric data was plotted as ZapA concentration versus the proportion of FtsZ filaments sedimented (Figure 2D). The sedimentation profile of the D22A ZapA variant closely resembles WT ZapA. However, clear differences are seen in the ability of the other ZapA variants to bundle and sediment FtsZ filaments (Figure 2D). R24A ZapA exhibits a clear shift when compared to WT ZapA, indicating a higher concentration of this variant is required to initiate FtsZ sedimentation. D33A ZapA reaches maximal FtsZ filament pelleting at equimolar
concentrations, similar to WT ZapA, however the maximum proportion of FtsZ in the pellet fraction here is much lower than for WT. In contrast, ZapA variants N28A, D32A, D33K and R46A show differences in both the amount of ZapA protein required to initiate sedimentation of FtsZ filaments and maximal FtsZ filament sedimentation. The most pronounced differences compared to WT are seen with D33A and N28A ZapA variants. ZapA D33A reveals the lowest FtsZ sedimentation at the saturated concentrations, and N28A ZapA shows the lowest FtsZ sedimentation at equimolar concentrations.

Based on the data from the sedimentation assays (Figure 2.2D), EC$_{50}$ values were calculated based on the maximum amount of FtsZ sedimentable (as demonstrated by WT ZapA), which correspond to the concentration of ZapA needed to sediment 50% of the total FtsZ filaments in each reaction (Table 2.3). An increase in EC$_{50}$ signifies that more variant ZapA is required to effectively sediment FtsZ compared to WT, and therefore suggests these ZapA variants bundle FtsZ filaments less efficiently. For WT ZapA and ZapA D22A, similar EC$_{50}$ values of 2.22 µM and 2.67 µM were determined, respectively. Higher EC$_{50}$ values than WT were obtained for ZapA variants R24A, N28A, D32A, D33A, D33K and R46A (Table 2.3).

Table 2.3. Summary of interactions for ZapA variants with FtsZ filaments.

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<th>Max Sed. b</th>
<th>Filaments/Bundle$^c$</th>
<th>Cell Length (µm)$^d$</th>
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<td>9.86±0.76</td>
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<tr>
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<td>2.67</td>
<td>0.93</td>
<td>7.33±0.49</td>
<td>2.81±0.08</td>
</tr>
<tr>
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<td>4.48</td>
<td>0.94</td>
<td>7.13±0.76</td>
<td>2.96±0.15</td>
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<tr>
<td>N28A</td>
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<td>0.75</td>
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<td>3.77±0.25</td>
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<tr>
<td>D32A</td>
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<td>0.71</td>
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<td>3.74±0.17</td>
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<tr>
<td>D33K</td>
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<td>0.78</td>
<td>5.46±0.29</td>
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<tr>
<td>R46A</td>
<td>7.61</td>
<td>0.74</td>
<td>N/A$^e$</td>
<td>3.87±0.20</td>
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</table>
a Describes the concentration of ZapA needed to sediment 50% of FtsZ in each reaction;
b Describes the maximum proportion of FtsZ pelleted by each ZapA variant in the sedimentation assays;
c Determined from transmission electron micrographs, mean ± SEM;
d Measured from transmission electron micrographs, mean ± SEM.
The mean cell length of ΔzapA E. coli is 3.52 µm;
e Data not available as no bundles were observed in transmission electron micrographs.

2.3.3 ZapA variants demonstrate reduced bundling of FtsZ filaments

In order to directly assess the effect of ZapA variants on FtsZ filament bundling, ZapA-FtsZ complexes were visualized by TEM (Figure 2.3A). Visualization of bundling was performed at equimolar concentrations of ZapA and FtsZ, since this concentration revealed the greatest difference in FtsZ sedimentation between ZapA variants (Figure 2.2D) and is also the most physiologically relevant (121). From these micrographs, the number of filaments within each bundle was determined (Figure 2.3B and Table 2.3). A decrease in the number of FtsZ filaments per bundle would indicate a decreased ability of ZapA to bundle FtsZ. At equimolar concentrations (4.8 µM) WT ZapA-FtsZ filaments appear as long twisted bundles of laterally associated FtsZ filaments (Figure 2.3A; WT), matching those described previously (80, 97, 118, 119) with an average of ~10 filaments per bundle (Figure 2.3B and Table 2.3). FtsZ filaments bundled by ZapA variants D22A and R24A appeared less organized than those bundled by WT ZapA and demonstrated an average of ~7 filaments per bundle (Figure 2.3B and Table 2.3). It should also be noted that bundles formed in the presence of R24A ZapA appeared to have fewer lateral associations and were less ordered (Figure 2.3A). Electron micrographs also showed that ZapA N28A formed short twisted bundles of FtsZ filaments when compared to WT ZapA.
Figure 2.3. Transmission electron microscopy reveals differential bundling of FtsZ filaments by ZapA variants.

[A] equimolar concentrations of ZapA and FtsZ, FtsZ was filamented with 1 mM GTP and visualized by TEM (bar = 500 nm, inset bar = 50 nm). [B] TEM images were analyzed and the mean number of filaments per bundle ± SEM were plotted for each ZapA variant (n >50 bundles per ZapA variant), two-tailed t-tests were used to compare ZapA variants to WT, * p-value <0.05, *** p-value <0.0001.
(Figure 2.3A), while ZapA variants D32A, D33A, and D33K produced long, twisted bundles of FtsZ filaments similar to those formed by WT ZapA, though these still contained fewer (~5) FtsZ filaments per bundle (Figure 2.3B and Table 2.3). Surprisingly, when sedimentation reactions containing ZapA variant R46A were imaged, no bundled FtsZ filaments, and very few non-bundled FtsZ filaments, were observed (Figure 2.3A). This result was reproducible over six replicate experiments (data not shown). Based on the sedimentation profile of ZapA R46A, which is similar to that of WT ZapA (Figure 2.2D), it suggests the R46A variant may affect FtsZ polymer assembly or disassembly in vitro.

2.3.4 ZapA variants that demonstrate reduced FtsZ filament bundling are unable to generate normal cell length in vivo

To investigate the effect of the ZapA variants in vivo, a ZapA-deleted strain of E. coli (ΔzapA) was constructed. WT ZapA and ZapA variants were expressed in ΔzapA E. coli. No significant differences in growth were observed between any of these strains (Figure 2.4A). Western blot analysis using an anti-His$_6$ antibody confirmed that the His$_6$-tagged WT and ZapA variants were expressed at similar levels (Figure 2.4B). The ΔzapA E. coli strain exhibited an elongated cell morphology as previously described (123), with a mean cell length of 3.52 μm (Figure 2.4C). The WT ZapA variant restored a normal cell size with a mean cell length of 2.65 μm. As with the in vitro results, ZapA variants D22A and R24A restored cell length to that of WT ZapA, with mean cell lengths of 2.81 μm and 2.96 μm, respectively. However, ZapA variants N28A, D32A, D33A, D33K and R46A did not restore ΔzapA cells to their normal length (Figure 2.4C and Table 2.3). These results indicate that residues D22 and R24 are not critical for FtsZ bundling in vivo, while N28, D32, D33 and R46 are needed to maintain the bundling function of ZapA.
Figure 2.4. Complementation of ∆zapA with WT ZapA and ZapA variants.

_E. coli_ ∆zapA cells were transformed with pBAD24 carrying _zapA_ variants. [A] growth was monitored over 6 h and no significant differences were observed in cell density. [B] Western blot analysis using an anti-His antibody confirmed that ZapA variants were expressed to the same levels during the complementation assays in ∆zapA cells (top panel) and Western blot analysis using an anti-FtsZ antibody (bottom panel) was performed as a loading control for comparison. [C] cells were visualized by TEM and measured (n >100 cells per _E. coli_ strain). Mean cell length values ± SEM were plotted and analyzed by two-tailed t-tests as compared to WT cell lengths, NS = no significant difference, *** p-value <0.001. No significant differences in cell length were observed between WT cells and WT ZapA complemented cells, and between ∆zapA and pBAD24 vector control cells. [D] immunofluorescence was performed to detect differences in FtsZ localization and Z-ring formation among WT cells and ∆zapA cells expressing WT ZapA and ZapA variants. (Scale = 1µm).
The ability of this His\textsubscript{6}-tagged ZapA protein to complement functionality \textit{in vivo} is in contrast to a report by Mohammadi \textit{et al.}, in which the addition of a his-tag rendered ZapA non-functional \textit{in vivo} (121). One notable difference between this construct and the one described previously is the cleavage site between the his-tag and ZapA. The factor XA cleavage site used in this study contains fewer charged amino acids than the enterokinase cleavage site used by Mohammadi \textit{et al.} (2 vs. 5, respectively) (121). This could account for the differences seen in the \textit{in vivo} and \textit{in vitro} analyses of these proteins.

Immunofluorescence was also performed on these cells to monitor Z-ring formation and nucleoid segregation in ΔzapA cells carrying ZapA variants (Figure 2.4D). WT \textit{E. coli} cells divide normally, presenting a single condensed mid-cell localization of FtsZ and two segregated nucleoids destined for each of the daughter cells (Figure 2.4D, top). The ΔzapA cells differ from WT \textit{E. coli} in that elongated cells show multiple localizations of FtsZ and multiple nucleoids. When ΔzapA cells were complemented with WT ZapA and ZapA variants, D22A and R24A closely resemble WT \textit{E. coli}. These variants show normal Z-rings and efficient cell division, indicated by a maximum of two nucleoids in each dividing cell. However, when ΔzapA cells were complemented with ZapA variants N28A, D32A, D33A, D33K and R46A, elongated \textit{E. coli} cells show multiple Z-rings and multiple nucleoids. Additionally, Z-rings in these cells often appeared diffuse, perhaps indicating less FtsZ bundling \textit{in vivo}. Alternately, the diffuse localization pattern of FtsZ may be the result of an inability of these ZapA variants to properly align FtsZ at mid-cell \textit{via} interactions with ZapB (123). While these results are consistent with inefficient cell division, the phenotype is resolved by late exponential phase, suggesting a delay in bacterial cell division as seen previously by Galli and Gerdes (171).
2.4 Discussion

ZapA is proposed to stabilize FtsZ filaments, aiding in Z-ring formation and/or positioning prior to bacterial cell division (30,36,38-40,50). Although ZapA alone is not essential for cell division, it is hypothesized that collectively ZapA, ZapB, ZapC and ZapD are required for FtsZ filament bundling and dynamics in *E. coli* (45, 47, 48, 71, 89, 118). While the ZapA protein is widely conserved across bacterial species, it is not required for the lateral association of FtsZ filaments in some organisms (72). In *E. coli* and *Bacillus subtilis*, ZapA has been shown to be important for FtsZ bundling *in vitro* (169) and the absence of ZapA results in an elongated cell phenotype and abnormal septum formation in *E. coli* cells (118, 123).

Although nearly a decade has passed since the crystallographic structure of ZapA from *P. aeruginosa* was determined, and its role in FtsZ bundling proposed (88), questions remain about the specificity of ZapA-FtsZ interactions and the implication of these interactions on Z-ring architecture and dynamics. The structure of EcZapA should prove helpful in investigating these questions, given that *E. coli* has become the most common model for these studies. While *Pa*ZapA has been used to probe the function and physiological role of ZapA in *E. coli* (119, 121), the details of the structure of EcZapA differ enough to impact the design and interpretation of ZapA structure-function studies. For example, a recent study by Pacheco-Gómez et al. based their mutational analysis of the tetramerization domain of ZapA on the *Pa*ZapA structure, with the hypothesis that ZapA dimer/tetramer equilibrium is important for proper FtsZ bundling (119). Although they succeeded in making a properly folded ZapA dimer, Ile83 packs in a very different fashion in EcZapA than the equivalent residue in *Pa*ZapA, meaning that detailed predictions of the consequences of altering this residue are likely to be inaccurate. Interestingly, the I83E dimer variant of EcZapA interacted with FtsZ, but did not form FtsZ bundled filaments.
in vitro (119). Given (i) the extensive stabilizing interactions within the EcZapA tetramer (Figure 2.1D), and (ii) the predominant tetrameric configuration reported for WT ZapA by sedimentation velocity experiments (119), it is unlikely that native EcZapA dimers are present in sufficient levels to bundle FtsZ, as suggested for PaZapA (88). Therefore, EcZapA likely functions as a tetramer. If the N-terminal domains at both ends of the tetramer need to engage with FtsZ filaments to bundle them, the large observed differences in the orientation of these domains (Figure 2.1D and E) suggest differences in FtsZ bundling between these two species.

Here, the structure of EcZapA (Figure 2.1) was used to better understand the role of ZapA in bundling and stabilizing FtsZ prior to bacterial cell division. Galli and Gerdes recently showed that a 26 amino acid region of ZapA from E. coli was involved in ZapA-FtsZ interactions (68). In EcZapA this region forms a charged α-helix (Figure 2.1A and C). Since this globular head of ZapA is not well conserved compared to the C-terminal tetramerization domain, and ZapA-FtsZ interactions vary in importance and extent between species (169), it is possible that this region may be critical for ZapA-FtsZ interactions in E. coli. Site-directed mutagenesis was used to create point mutations to alter specific amino acids along this charged α-helix. The functional implications of these point mutations at each residue are described in Figure 2.5. ZapA variants at residues D22 and R24 (Figure 2.5; coloured in green) show only minor deviations from WT ZapA-FtsZ interactions. ZapA R24A does not cause a significant change in FtsZ sedimentation at saturating concentrations (Figure 2.2D) and this ZapA variant complemented the ΔzapA strain with the same efficiency as WT ZapA (Figure 2.4). These results suggest that ZapA R24A interacts with FtsZ filaments. However, upon visualizing FtsZ filaments bundled by ZapA R24A it became clear that the ZapA-FtsZ interactions were somewhat altered, as bundles appear as disorganized tangles of filaments as opposed to the laterally organized
Figure 2.5. Mutant phenotypes mapped onto the EcZapA structure.
Orientation as in Figure 1C, but here coloured according to the effect of point mutations at each residue. Residues in green have a relatively weak (though measurable) effect on the EC$_{50}$ (EC$_{50}$ < 5 µM), the number of filaments per bundle (>7) and the cell length (<3 µm). Residues highlighted in red, in contrast, have strong effects on the EC$_{50}$ (EC$_{50}$ > 5 µM), the number of filaments per bundle (<7) and the cell length (>3 µm). The residue highlighted in orange has a weak affect on the EC$_{50}$ (EC$_{50}$ < 5), but nevertheless strongly affects both the number of filaments per bundle (<7) and the cell length (>3 µm).
structures seen with WT ZapA (compare Figure 2.3A WT vs. R24A). Since the R24A ZapA variant appears to interact with FtsZ \textit{in vitro} and \textit{in vivo}, there are two possible explanations for this difference. First, ZapA R24A may have an altered functionality made possible by the removal of a charged residue involved in electrostatic interactions with FtsZ. Second, it is possible the full complementation of the ΔzapA phenotype \textit{in vivo} is a result of the overlapping function of Zap proteins in \textit{E. coli} (72).

ZapA variants with point mutations in the charged α-helix region at residues N28, D33 and R46 (Figure 2.5; coloured in red) required much more ZapA to effectively bundle FtsZ filaments (Figure 2.2D), demonstrated fewer FtsZ filaments per bundle (Figure 2.3B and Table 2.3), and increased cell length in complementation assays (Figure 2.4 and Table 2.3). In sedimentation assays, all of these ZapA variants demonstrated a significant decrease in FtsZ sedimentation at equimolar (p-values <0.02) and saturated (p-values <0.05) concentrations (Figure 2.2C), and we linked this decrease in sedimentation to fewer FtsZ filaments per bundle (Figure 2.3). It is also worth noting that these bundles appear to have adopted an inherent twisted, helical-like conformation, especially when observed as bundles of four FtsZ filaments. It cannot be determined whether this shift in conformation is a result of altered ZapA binding, or if it is a conformation typically seen with lower order FtsZ bundles. For ZapA variant R46A, we note that in the sedimentation assay FtsZ sediments significantly when ZapA is present at a saturating concentration (11.4 μM) (Figure 2.2C). This contradicts the TEM results, where no significant bundling was observed (Figure 2.3A). We hypothesize this is due to a loss of bundling functionality, where ZapA may still be binding FtsZ filaments but in an altered way that does not allow the formation of organized, regularly spaced bundles. This may have
functional implications for residue R46 in the coordination of ZapA in the FtsZ-ZapA interaction.

Interestingly, the D32A ZapA variant (Figure 2.5; coloured in orange) presented only a weak effect on the measured EC$_{50}$ values, but strongly affected both the number of FtsZ filaments per bundle and the complemented cell length, suggesting this residue may be important for enhancing Z-ring stability. Together, our results strongly suggest that the surface exposed, charged residues on the EcZapA N-terminal $\alpha$-helix mediate ZapA-FtsZ interactions to facilitate FtsZ filament bundling and Z-ring stability in dividing bacterial cells. While this area of ZapA had been previously implicated in facilitating the ZapA-FtsZ interaction, this is the first report describing the structure of EcZapA and the effects of altering specific residues in this region on the interaction with FtsZ.
Chapter 3: Structural Determination and Mutational Analysis of ZapD from *Escherichia coli* Elucidates Charged Residues Involved in FtsZ Filament Bundling

Data from this chapter is in preparation for submission to the following journal article:


Statement of contributions: The crystal screens, data collection and resolution of the *Ec*ZapD structure were performed by Mr. Charles Wroblewski and Dr. Matthew Kimber. Ms. Laura Siedel preformed the complementation assays and expression in Wt *E. coli* as part of an undergraduate co-op work term. Dr. Dyanne Brewer processed samples for LC-MS/MS and preformed the *in silico* cross-linking and decoy analysis. All other experiments were performed by Ms. Elyse Roach.
**Abstract**

Bacterial cell division is an essential and highly coordinated process. It requires the polymerizing GTPase FtsZ to form a dynamic ring (Z-ring) at mid-cell. The Z-ring is required for recruitment of downstream cell division proteins and relies on a group of FtsZ associated proteins (Zap proteins) for stability at the onset of division. Zap proteins (ZapA, ZapB, ZapC and ZapD) are small soluble proteins that act to bind and bundle FtsZ filaments. ZapD forms a functional dimer and interacts with the C-terminal tail of FtsZ but little is known about its action in vivo and in vitro. In the current study we present the crystal structure of ZapD from *Escherichia coli* (EcZapD), showing it is a symmetrical dimer with centrally located α-helices flanked by β-sheet domains. Site-directed mutagenesis and in vitro sedimentation assays reveal that residues R116, R221 and R225 participate in a charged FtsZ binding pocket. ZapA and ZapD were shown to not compete for binding but may have proximal binding sites on FtsZ. Overall, these results provide evidence for the areas of interaction between FtsZ and ZapD and support the overlapping role of ZapA and ZapD in *Escherichia coli*. 
3.1 Introduction

Bacterial cell division is an essential and complex process that requires the coordinated assembly of a multi-protein molecular machine termed the ‘divisome’. The divisome is responsible for the constriction of the inner- and outer-membranes, synthesis of septal peptidoglycan and subsequent septum formation. At the molecular level, proteins of the divisome are recruited in a hierarchical manner. The divisome proteins can be divided into three main groups based on their order of assembly: the first is termed the proto-ring, then early divisome proteins, followed by the late divisome proteins (3, 5). The successful assembly of the divisome depends on the formation of the ‘Z-ring’, which is comprised of the 40 kDa bacterial tubulin homologue FtsZ. FtsZ assembles in a GTP-dependent manner and in a head-to-tail fashion into filaments (25, 56, 57, 76). The filaments are anchored to the membrane forming the Z-ring, and act as the scaffold for divisome assembly and provide the driving force for cell constriction (17, 115, 180). Although the localization of the FtsZ-filaments and its requirements for recruiting other divisome proteins are well established, the organization and dynamics of the divisome structure have yet to be fully elucidated.

FtsZ filaments have been shown to be highly dynamic in vivo and in vitro, constantly undergoing polymerization, GTP hydrolysis and de-polymerization (23, 81, 84, 85, 94, 169). A group of proteins thought to stabilize FtsZ filaments prior to cell division are the FtsZ associated proteins (Zap proteins). The Zap proteins are four proteins with predicted overlapping functions; ZapA, ZapB, ZapC and ZapD (45–48, 118). The Zap proteins are amongst the first recruited during divisome assembly and interact with FtsZ to stabilize FtsZ filaments by inhibiting the GTPase activity, thereby slowing the de-polymerization rate, with the exception of ZapB which does not interact directly with FtsZ. Although individually these proteins are not essential for
assembly of the divisome, it is thought that the collective role they play in stabilizing FtsZ filaments prior to cell division is critical. *In vitro* ZapA, ZapC and ZapD bundle FtsZ filaments by increasing the lateral interactions between individual filaments (45, 46, 48, 118). Interactions between Zap proteins (ZapA, ZapC and ZapD) and FtsZ also result in much longer FtsZ filaments because they act to inhibit the GTPase activity of FtsZ *in vitro* (45, 48, 121). ZapB does not interact directly with FtsZ, but instead interacts with ZapA to cross-link FtsZ bound ZapA, which provides another level of filament bundling (68, 123, 171). Studies of ZapA from *E. coli* (*Ec*ZapA) revealed its crystal structure to be significantly different from the previously solved *Pseudomonas aeruginosa* ZapA (*Pa*ZapA) structure. The orientation of the protomers within the tetrameric ZapA structure was different in EcZapA when compared to PaZapA, which revealed possible differences in function between the two proteins (181). ZapA specifically bundles FtsZ filaments by binding the C-terminal tail of FtsZ (68). This binding inhibits the GTPase activity of FtsZ through a charged α-helix located on each of the four promoters comprising the functional tetramer (68, 181). ZapD is thought to bundle FtsZ filaments in a similar manner by binding to the C-terminal tail of FtsZ. When ZapD binds FtsZ it also inhibits its GTPase activity but has been shown to form dimers in solution, in contrast to ZapA (48). The site of FtsZ interaction on ZapD remains to be shown and dissecting this interaction further will provide pertinent information about the function of ZapD and its possible overlapping role with ZapA.

In this study, the crystal structure of ZapD from *E. coli* (*Ec*ZapD) is presented. The structure of EcZapD revealed a series of charged surface exposed residues that could be involved in the FtsZ-ZapD interaction. In addition to the structure of EcZapD, chemical cross-linking of FtsZ and ZapD followed by liquid chromatography tandem mass spectrometry showed the two
proteins cross-linked at several residues. Nine site-directed ZapD variants were generated based on the \textit{Ec}ZapD structure and the cross-linking results. Comparison of the WT ZapD bundling with the ZapD variants bundling ability reveals a potential FtsZ binding pocket containing several charged residues. This region of ZapD appears less ordered than the rest of the ZapD dimer in the crystal structure and may be able to move to accommodate the FtsZ filament when binding. Additionally, we tested the ability of ZapD and ZapA to compete for FtsZ binding and have shown that they likely bind and bundle FtsZ filaments in the same manner.

### 3.2 Experimental procedures

3.2.1 Cloning, site directed mutagenesis and construction of the \textit{zapD} deletion strain

The plasmid containing \textit{zapD} was constructed amplified by PCR primers 311F (TTCATAGAGAATTCACTCATCATCATCATCATCAGGATGAAGGCTGAAGTGGTATGCAGACCCAGGTCTTTTTG) and 311R (TTCCTTGAGGCTTTTTAGCAACAGGCCAGTGACGTTCCCGGTACCT). \textit{E. coli} genomic DNA was used as a template to produce a His\textsubscript{o}-tag-encoding fragment flanked by EcoRI and HindIII cut sites to be cloned into pBAD24. Site directed mutagenesis was performed using the QuikChange\textsuperscript{©} Lightning Site-Directed Mutagenesis Kit (Stratagene) using the primers listed in Table 3.1. The \textit{ΔzapD} strain was made using the λ red deletion system as described previously (172, 173). \textit{zapD} was replaced in its entirety by the \textit{cat} gene from pKD3. Gene replacement was mediated by gene products from pSIM6, when expressed in a temperature dependent manner in \textit{E. coli} W3110. Construction of the \textit{ftsZ} containing plasmid was described previously (181).
3.2.2 Protein expression and purification

FtsZ was expressed and purified as described previously (181). Purified, concentrated FtsZ was stored at 4°C for no longer than 4 days in PEM-KOH buffer (50 mM piperazine-N,N’-bis(ethanesulfonic acid)-KOH, 1 mM EDTA, 5 mM MgCl₂, pH 6.5). His₆-ZapD was expressed in *E. coli* BL21(DE3) pLysS cells carrying the pEJR031 plasmid (Table 3.1). An overnight culture was diluted 1/100 into lysogeny broth (LB) medium supplemented with ampicillin and glucose. Growth continued for 3 h, cells were pelleted and re-suspended in inducing LB medium containing ampicillin and 0.2% (v/v) L-arabinose (Sigma) for induction. Expression proceeded

**Table 3.1. Strains, plasmids and primers used in this study.**

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<td>BL21</td>
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66
for 1 h, after which cells were collected by centrifugation at 5000 × g for 8 min. Cell pellets were re-suspended in A2 buffer (20 mM Tris-HCl, 100 mM KCl, 20 mM imidazole, 1 mM EDTA, 10% glycerol, pH 8.0) and lysed by French Press. Cell debris was removed by low speed centrifugation at 8,000 × g for 15 min, membranes were removed by high speed centrifugation at 100,000 × g for 1 h. His₆-ZapD was purified by immobilized metal affinity chromatography as described previously for His₆-ZapA (181). His₆-ZapD was stable for 8 weeks at 4°C in A2 buffer.
3.2.3 Crystallization, data collection, structure determination and analysis of ZapD

ZapD crystals were grown by sitting drop vapor diffusion. 1 µl of 15 mg/ml ZapD was mixed with an equal volume of a solution of 1.2 M ammonium sulfate, 100 mM HEPES pH 7.5, and 10 % PEG 600, and was then equilibrated against the same solution. Crystals grew as hexagonal prisms, up to 300 µm in length. Crystals were frozen in liquid nitrogen after surface water was removed by immersion in paratone N oil. Data was collected at the Canadian Light Source, beamline 08ID. Crystals were highly variable in quality, with the best of the dozen tested diffracting to 2.4 Å; even the best crystals showed signs of lattice disorder. Data was processed in XDS and scaled in XSCALE (174). Crystals were of the hexagonal space group P6\(_{4}\)22. The structure was determined by molecular replacement using PHASER in PHENIX (175, 176), with the structural genomics derived *Vibrio parahaemolyticus* ZapD (PDB: 2OEZ) serving as a model. The structure was rebuilt in Coot (177), and refined in PHENIX. Structure figures were prepared in PyMol.

3.2.4 *In vitro* protein cross-linking, in-gel digestion and mass spectrometry analysis

Reactions were prepared containing FtsZ (4.8 µM), ZapD (4.8 µM) and the chemical cross-linker gluteraldehyde (0.05% [v/v]) and incubated at room temperature for 5, 10 and 15 minutes. At each time interval samples were stopped by mixing with 5× SDS sample buffer and saved for analysis. Conditions were optimized to use the lowest percentage of gluteraldehyde and the shortest incubation time that showed significant numbers of cross-linked proteins by Coomassie staining (SimplyBlue™ SafeStain, Invitrogen) on an SDS-PAGE gel. In separate control reactions, FtsZ was cross-linked without ZapD and ZapD was cross-linked without FtsZ. Samples from optimized reactions were then run on BioRad© 4-20% pre-cast gels (BioRad). Gel
fragments containing apparent cross-linked FtsZ-ZapD were excised using a clean razor blade and in-gel digestion performed. Briefly, the gel slice was destained with 50 mM ammonium bicarbonate in 50% acetonitrile, cysteines were then reduced with 100 mM DTT and alkylated with 55 mM iodoacetamide. After vacuum centrifugation, the gel slice was then subjected to protease digestion using 20 µl of sequencing grade modified trypsin (20 µg/mL in 100 mM ammonium bicarbonate; Promega, Madison, WI, USA) at 37°C for 12 h. The resulting tryptic peptides were extracted from the gel slices by one aqueous wash (50 µl) followed by 2 washes (75 µl) with 5% formic acid in 50% acetonitrile and the combined solution was concentrated to 10 µl using vacuum centrifugation. (182).

Liquid chromatography–mass spectrometry analyses were performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHD 6530 Q-Tof mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. A C18 column (Agilent AdvanceBio Peptide Map, 100 mm × 2.1 mm 2.7 µm) was used for chromatographic separation with the following solvents water with 0.1% formic acid for A and acetonitrile with 0.1% formic acid for B. The mobile phase gradient was as follows: initial conditions, 2% B increasing to 45% B in 40 min and then to 55% B for 10 min followed by column wash at 95% B and 10 min re-equilibration. The first 2 and last 5 min of gradient were sent to waste and not the spectrometer. The flow rate was maintained at 0.2 mL/min. The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at 350°C with a flow rate of 13 L/min. Nebulizer pressure was 40 psi and the fragmentor was set to 150 V. Nitrogen was used as both nebulizing and drying gas, and collision-induced gas. The mass-to-charge ratio was scanned across the m/z range of 300-2000 m/z in 4 GHz (extended dynamic range positive-ion auto MS/MS mode. Three precursor ions per cycle
were selected for fragmentation. The instrument was externally calibrated with the ESI TuneMix (Agilent). The sample injection volume was 100 µl (183).

3.2.5 FtsZ filamentation, sedimentation assays, co-bundling assays and competition assays

Both FtsZ filamentation and sedimentation assays were performed as described previously for FtsZ and ZapA (181). Briefly, FtsZ and ZapD were mixed together in PEM-KOH buffer, equilibrated at 30°C for 5 min, GTP was then added to a final concentration of 1 mM. Reactions were incubated for 5 min at 30°C to allow for bundling and the soluble and pelleted fractions were separated by low speed centrifugation. For the sedimentation assays, soluble protein fractions were collected from the supernatant of sedimented reactions, where pellet fractions were the result of re-suspension of sedimented material in an equal volume of PEM-KOH buffer. All fractions were mixed with 5× SDS sample buffer for analysis by SDS-PAGE and Coomassie staining (SimplyBlue™ SafeStain, Invitrogen). Densitometric analysis was done using ImageLab™ Software (BioRad) by determining pelleted FtsZ relative to the total FtsZ from each reaction. Negative controls were run with each sedimentation assay containing FtsZ and the ZapD variant without GTP. This amount of FtsZ pelleted was then subtracted from all other pelleted fractions to control for FtsZ coming out of solution without bundling.

Co-bundling assays were performed similarly to sedimentation assays with the addition of ZapA. FtsZ concentration remained constant at 4.8 µM in all reactions. A control reaction containing a sub-equimolar concentration of ZapA (2.1 µM) was performed. For the experimental reactions, ZapA and ZapD were added at the same time as FtsZ and allowed to equilibrate at 30°C for 5 min prior to the addition of GTP to 1 mM. Samples were then centrifuged to separate soluble and pelleted fractions.
For competition assays FtsZ was pre-bundled with 2.1 µM ZapD for 5 min then ZapA was added to 7 different reactions at varying concentrations (0.5, 1.0, 2.1, 4.8, 8.0 and 12.4 µM), including a negative control lacking GTP. A complementary competition assay was run where FtsZ was pre-bundled with 4.8 µM ZapA for 5 min then ZapD was added to 7 different reactions at varying concentrations (0.5, 1.0, 2.1, 4.8, 8.0 and 12.4 µM), including a negative control lacking GTP (4.8 µM each Zap). These gels were run in triplicate and analyzed in the same manner as described for the sedimentation assays.

3.2.6 In vivo characterization and complementation with plasmid constructs containing zapD variants

For the in vivo characterization of ZapD variants, electro-competent E. coli W3110 cells were transformed with each pBAD24 vector containing a variant zapD. For complementation, chemically competent ΔzapD cells were transformed with pBAD24 containing variant zapD genes. In each experiment a single colony was inoculated into liquid LB medium containing ampicillin and glucose and grown overnight. Cells were then washed in 1× PBS and re-suspended at a 1/100 dilution in LB medium supplemented with ampicillin, 0.1% glucose (v/v) and 0.2% arabinose (v/v). After two hours of growth, samples were both taken for analysis by SDS-PAGE/Western immuno-blotting and for cell measurements. For SDS-PAGE and immuno-blotting, cells were mixed with 5× sample buffer and gels were loaded normalized to OD\text{600}. Gels were transferred to nitrocellulose membranes and blocked with 2% BSA (w/v). The primary antibody, mouse anti-His\text{6} (Clontech), was bound to the membrane and used as per the manufacturer’s guidelines. Secondary antibody, Goat anti-mouse conjugated to alkaline phosphatase (Sigma), was used at a dilution of 1:20,000. Washes were performed using 1× TBST in between all steps. Cells were also imaged using a Leica DM2000 LED light microscope.
equipped with a ProgRes CT3 camera (Jenoptik AG). Cell length measurements were
determined using ImageJ (178), measuring >100 cells per sample.

3.2.7 Transmission electron microscopy (TEM)

For imaging FtsZ filaments and FtsZ-ZapD bundles, samples were adhered to plasma-
cleaned grids for 45 sec. After wicking away excess sample, grids were washed once in PEM-
KOH to remove excess un-bound protein, washed in ddH₂O to remove reactive salts, then
stained for 30 sec with 2% uranyl acetate. Grids were imaged using the FEI Tecnai G2 F20
microscope operated at 120 kV.

3.2.8 Gel filtration

Gel filtration was performed using the Biologic™ Duoflow Chromatography System
(BioRad) equipped with a Superdex 200 column. Purified His₆-ZapD variants (2.0 – 6.0 mg/mL)
were loaded onto the column and washed with 2 column volumes of A2 buffer. Fractions
containing dimeric ZapD were collected and saved for analysis by SDS-PAGE and Western
immuno-blot.

3.3 Results

3.3.1 Structure of *Escherichia coli* ZapD

The structure of ZapD was determined by molecular replacement at 2.4 Å resolution to
an R<sub>free</sub> of 0.288 (Table 3.2). The structure has a single molecule in the asymmetric unit, and
despite high overall atomic displacement parameters, all residues are ordered except residues 1
and 2. The ZapD protomer has a central \( \alpha \)-helical domain that can be loosely described as a pair of three helical bundles with a distinct kink in between (Figure 3.1A). The C-terminus forms a seven-stranded jellyroll-like \( \beta \)-sandwich domain, with one \( \beta \)-strand contributed from the N-terminus. This sandwich has topology 1, 4, 7, 2 on one sheet, and 3, 6, 5 in the other. ZapD forms a tight dimer in the crystal, with the \( \alpha \)-helical domains interacting primarily through helices 1 and 2, and strands of the \( \beta \)-sandwich domain packing on helix 7 (Figure 3.1B). Analysis by PISA indicates that this interface buries 1871 Å\(^2\) per protomer with a dissociation free energy of 28.1 kCal/mol, indicating a stable biological interface. ZapD dimers are further organized into large fibre like arrangements in the crystal, with a diameter of 110 Å (Figure 3.1C). However, the contacts between dimers are not strong enough to be unambiguously biologically relevant, the interfaces are poorly conserved, and we have not observed ZapD alone to form fibres by centrifugation or electron microscopy. It would seem that is phenomenon is simply a crystal packing artefact.

Searching the PDB with DALI (184) reveals 2OEZ as the only protein with recognizable similarity to the overall protein, with a Z-score of 27.1, r.m.s.d. of 1.7 Å over 243 residues (Figure 3.1D). This protein is 40 % identical to \textit{E. coli} ZapD, and is the \textit{Vibrio parahaemolyticus} ZapD ortholog. This structure is labeled as a protein of unknown function (DUF1342), and is unpublished. The \( \beta \)-stranded domain resembles little else in the PDB, with the closest structural homologs being domains of viral shell proteins such as the adenovirus hexon protein (3ZIF; Z-score 4.2; 2.8 Å r.m.s.d over 60 residues). The helical subdomain does however have some partial resemblance to various \( \alpha \)-helical bundle proteins, by far the closest of which is an integron cassette protein, VpC_cass2 (3JRT; Z-score 8.5, r.m.s.d. 3.3 Å over 119 residues) with
Figure 3.1. The structure of *E. coli* ZapD.

[A] The structure of the ZapD protomer is coloured by secondary structure progression from the N-(blue) to C-terminus (red). The ZapD structure is organized as a β-sandwich domain attached to an α-helical domain. Note that αC and αD protrude from the rest of the structure. [B] Structure of the ZapD dimer. [C] ZapD packing in the crystal results in large hollow fibres 110 Å in diameter. However, we find no evidence that this organization contributes to ZapD’s *in vivo* functioning. [D] Superposition of *E. coli* ZapD (cyan and white) and ZapD from *V. parahaemolyticus* (blue and wheat; PDB: 2OEZ). A single protomer (blue/cyan) was superimposed by DALI, with the second protomer positioned as it maps to the dimer interface. Note that in the *V. parahaemolyticus* ZapD structure, the αF-αG loop is largely disordered (small arrow). [E] Superposition of VpC_cass2 (3JRT; yellow/orange) on the *E. coli* ZapD structure (cyan/white). Note that VpC_cass2 structure superposes well on the core of the ZapD helical domain. [F] *E. coli* ZapD coloured by sequence conservation score. A multiple sequence alignment of ZapD homologs was mapped onto the ZapD structure using Consurf. Highly conserved residues are shown in magenta, poorly conserved residues are in cyan, residues with intermediate conservation are shown in white. [G] Electrostatic mapped onto the ZapD surface. Blue are electropositive regions, red are electronegative.
Table 3.2. Data collection, model refinement and final structure statistics.

<table>
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<th>Crystallographic data collection statistics</th>
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<td>Space group</td>
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<tr>
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<tr>
<td>$c =$</td>
<td>106.7</td>
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<td>Completeness (last shell)$^a$</td>
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<td>$&lt;I/\sigma(I)&gt;$ (last shell)$^a$</td>
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<tr>
<td>$R_{sym}$ (last shell)$^a$</td>
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</table>

<table>
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<th>X-ray structure refinement statistics</th>
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<tr>
<td>Water molecules</td>
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</tr>
<tr>
<td>Other molecules</td>
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<td>Average ADPs (Å$^2)$</td>
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<tr>
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<td>Ramachandran outliers (%)</td>
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</table>

$^a$The last shell includes all reflections between 2.46 and 2.40 Å

$^b$R$_{free}$ calculated using 5% of data, chosen randomly
αA, αB, αE, αF, αG having at corresponding counterparts (185) (Figure 3.1E). The Z-score likely underestimates the relatedness, as this protein also dimerizes using a very similar geometry to that observed in ZapD, doubling the number of corresponding elements; this broader resemblance suggests an intriguing evolutionary relationship between the two proteins. In the absence of a functional understanding of Vpc_cass2, it is unclear whether there are any functional similarities between the two proteins.

Mapping sequence conservation onto the surface of ZapD using Consurf (186) reveals that the face on which αE and αF are exposed is more conserved than the opposite one (Figure 3.1F). This face is also noticeably more basic than the opposite, less conserved face (Figure 3.1G), with several key conserved arginine residues. This face has two distinct clusters of conserved basic residues, with each site spanning both protomers. We selected several key residues as candidates for site directed mutagenesis, in order to assess whether this was the FtsZ binding site.

3.3.2 Chemical cross-linking of purified ZapD and FtsZ reveals targets for site-directed mutagenesis

ZapD and FtsZ were cross-linked chemically using gluteraldehyde at a concentration of 0.05% (v/v) for 5, 10 and 15 min. The expected result was the appearance of high molecular weight bands that would be the result of ZapD-FtsZ cross-linking. Control reactions containing only ZapD and gluteraldehyde showed only monomeric and dimeric ZapD (Figure 3.2). FtsZ control reactions showed a few higher molecular weight bands (Figure 3.2). When both ZapD and FtsZ were incubated with gluteraldehyde, several additional high molecular weight bands were present that did not appear in either of the control reactions. The higher molecular weight
bands from these reactions (Figure 3.2A, starred) were cut out and an in-gel protein digestion was performed prior to analysis. These samples were individually analyzed by LC-MS/MS Q-TOF to detect and identify peptides and cross-links. Decoy analysis was performed on the experimental data to validate the results (187, 188). In decoy analysis, an in silico digest is performed on the proteins of interest and a library of theoretical peptides is generated and compared to the experimental peptides (187, 188). A series of algorithms is then used to give each experimental peptide a ‘score’, which indicates the confidence of peptide identification (187, 188). A lower score is indicative of lower confidence in peptide identification; more specifically, if the score is >50 there is less than 5% false identification rate (187). ZapD was found cross-linked to itself and to FtsZ in these samples (Figure 3.2, Table 3.3). Residues R116 and R221 on ZapD cross-linked close to the C-terminus of FtsZ and provided attractive targets for investigation because the C-terminal tail was shown to be important for the ZapD-FtsZ interaction previously (48). Residue R225 was cross-linked to FtsZ in more than one sample and was therefore also chosen for further investigation (Table 3.3). Other residues were chosen based on the crystal structure of ZapD, their charge and surface exposure. In order to study the involvement of these residues in the ZapD-FtsZ interaction they were individually mutated to alanine by site directed mutagenesis and the following ZapD variants were created: ZapD R16A, R20A, R56A, E114A, R116A, R124A, R176A, R221A and R225A.

Table 3.3. Chemical cross-linking of purified ZapD and FtsZ.

<table>
<thead>
<tr>
<th>Site on ZapD</th>
<th>Site on FtsZ</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>R116</td>
<td>K380</td>
<td>44</td>
</tr>
<tr>
<td>R20</td>
<td>K14</td>
<td>61</td>
</tr>
<tr>
<td>R70</td>
<td>K155</td>
<td>60</td>
</tr>
<tr>
<td>R221</td>
<td>R319</td>
<td>55</td>
</tr>
<tr>
<td>R225</td>
<td>K51</td>
<td>42</td>
</tr>
</tbody>
</table>
Figure 3.2. Chemical cross-linking of purified FtsZ and ZapD.
Reactions were prepared in the presence (+) or absence (−) of purified FtsZ, ZapD and/or the chemical cross-linker gluteraldehyde (Glut) at a concentration of 0.05% (v/v). Experimental reactions containing all three components were incubated for 5, 10 and 15 min and control reactions were incubated for 15 minutes. The legend at the top of the figure indicates the added components for each reaction and depicted below are a representative [A] silver stained SDS-PAGE gel and [B] an anti-His Western immuno-blot detecting His$_6$-ZapD. Bands designated with a star (*) were cut out for processing and analysis.
3.3.3 Individual ZapD residue mutations affect FtsZ sedimentation

FtsZ sedimentation assays have been used with ZapA, ZapC and ZapD to show how effectively each Zap protein can bundle FtsZ filaments *in vitro*. In the presence of GTP, WT ZapD bundles FtsZ filaments to an increasing extent until saturation is reached. The extent of bundling can be measured using a sedimentation assay where ZapD, FtsZ and GTP are combined together, incubated and the soluble and pelleted fractions are separated by centrifugation. The protein proportions in the soluble and pelleted fractions are determined by SDS-PAGE and Coomassie staining by comparison relative to the total protein input to each reaction (Figure 3.3). A graph summarizing the results from the sedimentation assay provides information about the overall sedimentation profile, maximum FtsZ sedimentation and EC$_{50}$ values. For ZapD, this bundling occurred even when ZapD was present at a low concentration relative to FtsZ (<4.8 µM, sub-equimolar). When present at equimolar concentrations (4.8 µM) FtsZ bundling by WT ZapD was at its maximum. When the ZapD concentration was increased beyond equimolar there was no increase in the amount of FtsZ bundling, indicating the association has reached saturation. This is clearly depicted by representative Coomassie stained gels and the summary of triplicate sedimentation assays, as graphed (Figure 3.3). It was expected that if a ZapD variant had an altered capacity to bundle FtsZ filaments that a change may be seen in the sedimentation assay results. Each ZapD variant was analyzed using this assay. ZapD variants R20A, E114A, R116A, R124A and R176A did not show a significant difference in sedimentation profile when compared to WT ZapD (Figure 3.3B). Additionally, these variants shared a similar maximum FtsZ sedimentation value and EC$_{50}$ value (Table 3.4). Variant R16A showed a similar FtsZ sedimentation profile (Figure 3.3B) to WT ZapD but increased maximum sedimentation and a decreased EC$_{50}$ value (Table 3.4). Three ZapD variants, R56A, R221A and R225A, showed
Figure 3.3. ZapD-mediated FtsZ sedimentation assays.  
[A] Representative Coomassie stained gels showing the sedimentation profiles of FtsZ with WT ZapD and FtsZ with ZapD R225A. Reactions were prepared with 4.8 µM FtsZ in all cases and ZapD was added to varying concentrations as indicated. Fractions of the initial protein input to each reaction (I), the soluble portion after centrifugation (S) and the pelleted portion (P) were then run on gels to be analyzed by densitometry. [B] sedimentation profiles were assessed for WT ZapD and all ZapD variants in triplicate and the resulting densitometric data was graphed as the percentage of FtsZ pelleted, relative to the initial input for each reaction, versus the concentration of ZapD.
significantly decreased maximum FtsZ sedimentation and increased EC$_{50}$ values (Table 3.4), while their sedimentation profiles were comparable to WT ZapD (Figure 3.3B).

Table 3.4. Summary of results from FtsZ bundling and sedimentation by ZapD variants.

<table>
<thead>
<tr>
<th>ZapD Variant</th>
<th>EC$_{50}$ (µM)$^1$</th>
<th>Max Sed. (%)$^2$</th>
<th>Filaments/Bundle$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.86</td>
<td>79.5±12.02</td>
<td>12</td>
</tr>
<tr>
<td>R16A</td>
<td>1.26</td>
<td>96.5±14.52</td>
<td>11</td>
</tr>
<tr>
<td>R20A</td>
<td>2.49</td>
<td>73.6±11.35</td>
<td>12</td>
</tr>
<tr>
<td>R56A</td>
<td>5.92</td>
<td>53.9±7.66</td>
<td>7****</td>
</tr>
<tr>
<td>E114A</td>
<td>1.74</td>
<td>77.9±11.69</td>
<td>16*</td>
</tr>
<tr>
<td>R116A</td>
<td>2.77</td>
<td>73.8±10.74</td>
<td>N/A</td>
</tr>
<tr>
<td>R124A</td>
<td>1.93</td>
<td>92.6±12.94</td>
<td>11</td>
</tr>
<tr>
<td>R176A</td>
<td>2.64</td>
<td>80.6±13.01</td>
<td>11</td>
</tr>
<tr>
<td>R221A</td>
<td>3.19</td>
<td>72.1±10.15</td>
<td>N/A</td>
</tr>
<tr>
<td>R225A</td>
<td>9.08</td>
<td>49.2±7.00</td>
<td>N/A</td>
</tr>
</tbody>
</table>

$^1$ EC$_{50}$ value corresponds to the concentration of ZapD required to sediment 50% of FtsZ in solution.

$^2$ Max sed. is the maximum observed sedimentation of FtsZ in the triplicate FtsZ sedimentation assays.

$^3$ The number of filaments per bundle was counted for each sample from TEM images, n=50.

**** p-value <0.0001 when compared to WT results.

* p-value <0.05 when compared to WT results.

3.3.4 FtsZ bundle morphology

WT ZapD acts to organize FtsZ filaments into highly structured, straight, closely associated bundles with characteristic striations (Figure 3.4A). As with previous studies, if one of the variants has an altered ability to bind and bundle FtsZ filaments, the bundles will look morphologically different from WT ZapD-bundled FtsZ filaments (45, 119, 181). It has been shown that mutating even one residue involved in the protein-protein interaction can significantly affect FtsZ bundling (45, 181). Qualitatively, several ZapD variants showed altered
Figure 3.4. Qualitative and quantitative assessment of FtsZ bundles.
[A] Transmission electron micrographs of FtsZ [4.8 μM] bundled in the presence of WT ZapD and ZapD variants [2.1 μM]. Bar = 100 nm. [B] Histogram summarizing the number of FtsZ filaments per bundle, obtained from >10 fields of view per sample (n=50 bundles per ZapD variant). ***p-value<0.0001, *p-value<0.05 compared to WT ZapD.
FtsZ-bundle morphology compared to WT ZapD (Figure 3.4A). ZapD R56A had noticeably shorter filaments within bundles and bundles are thinner (Figure 3.4B). ZapD R116A and R225A displayed dramatically different FtsZ bundling when compared to WT ZapD. The imaged structures showed little organization, curved bundles, few close FtsZ filament associations and little to no striation pattern associated with the bundles (Figure 3.4A). ZapD R221A resulted in long FtsZ filaments that were highly variable compared to WT ZapD-bundled filaments, demonstrating large areas of disorder. The remaining ZapD variants tested produced FtsZ bundles that were qualitatively comparable to WT ZapD-bundled FtsZ filaments.

Quantitatively, WT ZapD-bundled FtsZ consisted of an average of 12 filaments per bundle as measured by transmission electron microscopy (Table 3.4). The thin bundles produced by ZapD R56A were quantified to contain an average of 7 FtsZ filaments per bundle, whereas FtsZ bundled by ZapD E114A averaged 16 filaments (Table 3.4). Bundles formed in the presence of ZapD variants R116A, R221A and R225A could not be quantified due to lack of close associations. ZapD variants R16A, R20A, E114A, R124A and R176A contained a similar number of filaments per bundle compared to WT.

3.3.5 In vivo complementation of ZapD and dominant negative phenotype

When ΔzapD cells were grown in un-supplemented media they did not show an elongation phenotype relative to WT E. coli cells, consistent with previous experiments (48). Upon complementation with a plasmid encoded WT zapD cells elongated slightly (Figure 3.5A). Expression of ZapD variants did not result in a significant change in cell length in the ΔzapD cells compared to WT ZapD (p-values > 0.05). Additionally, ZapD variants were tested for a
Figure 3.5. Complementation and over expression.
[A] Cell lengths after complementing ΔzapD cells with WT ZapD and ZapD variants. [B] Cell lengths after overexpressing WT ZapD and ZapD variants in WT E. coli W3110 to test for a dominant negative phenotype. [C] Anti-his Western immuno-blots from both the complementation and over-expression experiments to show approximately equal amounts of protein are being expressed. Significance is designated by stars, *** p-value<0.001, ** p-value<0.02, * p-value<0.05, NS=no significant difference.
dominant negative phenotype by expressing them in low levels in *E. coli* W3110 cells containing a functional chromosomal copy of *zapD*. Only one cell containing a ZapD variant showed significant elongation (ZapD E114A, p-value 0.0021) and cells expressing 5 other variants showed a significant decrease in cell length compared to cells expressing endogenous WT ZapD (R16A, R56A, R124A, R176A and R221A, p-values <0.05). However, in both experiments control samples were significantly different from WT ZapD over-expressing samples and therefore the significance of these results is unclear.

### 3.3.6 ZapD variant oligomeric state

ZapD variants were analyzed by gel filtration and compared to WT ZapD to confirm that each amino acid substitution did not disrupt the ZapD dimerization interface. All ZapD variants eluted in peaks corresponding to 44 kDa except ZapD R225A, which eluted slightly earlier (Figure 3.6). This shift in elution profile shifts the molecular weight to ~46 kDa. These results are consistent with what was shown previously (48) and indicate that any deviations from WT ZapD results in experiments were not due to a change in the oligomeric state of that ZapD variant. Instead, a shift from WT ZapD results was a consequence of only the single amino acid substitution made.

### 3.3.7 Co-bundling and competition assays

When comparing the sedimentation assays of FtsZ by ZapA and ZapD, two main differences were observed. The maximum amount of FtsZ sedimented by ZapA, as observed by SDS-PAGE, was higher than the maximum amount of FtsZ sedimented by ZapD (Figure 3.3 vs. Figure 2.2 (181)). The EC$_{50}$ value, which indicates the concentration of Zap required to sediment
Figure 3.6. Absorbance at 280 nm during gel filtration. WT ZapD and each ZapD variant was analyzed for oligomeric state by gel filtration. Peaks overlapping at 85 minutes correspond to the dimeric state of ZapD (~44 KDa).
50% of FtsZ in each assay, was also lower for ZapD than ZapA (1.9 vs. 2.2 μM) (181), suggesting that ZapD has a slightly higher affinity for FtsZ filaments than ZapA.

For co-bundling assays, FtsZ, ZapA and ZapD were mixed at saturating concentrations and sedimentation was assessed by SDS-PAGE. It would be expected that if FtsZ preferentially bound ZapA or ZapD, there would be different proportions of each Zap protein sedimenting in a sedimentation assay. For example, in a reaction containing FtsZ, ZapA, ZapD and GTP, if FtsZ preferentially binds ZapD the pellet fraction would contain FtsZ and more ZapD than ZapA. Control reactions contained FtsZ and ZapA or ZapD (11.4 and 4.8 μM, respectively) alone. In both control reactions FtsZ and the respective Zap protein (ZapA or ZapD) appeared predominantly in the pellet fractions (Figure 3.7A). In a reaction containing ZapA, ZapD and FtsZ (11.4, 4.8 and 4.8 μM, respectively), all three proteins were predominantly in the pellet fraction (Figure 3.7A). There was no apparent increase in FtsZ filament sedimentation compared to sedimentation by each Zap protein individually. Rather, slightly less of each Zap protein was sedimented compared to the control reactions. Since both Zap proteins were added to the reaction at the same time and incubated together for bundle formation they both had the same opportunity to bind and bundle FtsZ. This result suggests that neither ZapA nor ZapD preferentially bind FtsZ filaments.

To corroborate the results from the co-bundling assays, competition assays were performed to determine whether one Zap protein could bind FtsZ filaments after being pre-bundled with the other Zap protein. To test the competition of ZapD with ZapA, FtsZ filaments were incubated with ZapA (4.8 μM) and then amounts of ZapD (2.1, 4.8, 8.0, 12.4 μM) were added. In contrast to the co-bundling assays, FtsZ and ZapA were pre-incubated. After the pre-incubation ZapD was added and incubated further. These reactions were centrifuged to
Figure 3.7. Co-bundling and competition assays.

[A] FtsZ sedimentation assays were preformed by co-incubating FtsZ with ZapA and ZapD at saturating concentrations (11.4 µM and 4.8 µM respectively). Control reactions including either ZapA or ZapD and one containing both protein but lacking GTP were preformed as well. Fractions from the initial reaction (I), the soluble portion (S) and the pelleted portion (P) were run on an SDS-PAGE gel and Coomassie stained as shown here. Competition assays were preformed where FtsZ was pre-incubated with either ZapA [B] or ZapD [C] and the other protein was added at various concentrations (highest to lowest 11.4, 8.0, 4.8, 2.1 µM).
separate soluble and pellet fractions and protein samples were analyzed by SDS-PAGE and Coomassie staining (Figure 3.7B and C). By adding ZapD in increasing concentrations to FtsZ pre-bundled with ZapA, if ZapD competes for binding FtsZ filaments with ZapA then less ZapA should appear in the pellet fraction and more ZapD would be in the pellet fraction. Also, if ZapA and ZapD bundle FtsZ filaments by binding different sites on FtsZ then more FtsZ would sediment with increasing amounts of FtsZ added. ZapA sedimentation in these reactions did not change with any amount of ZapD added (Figure 3.7B). FtsZ filaments pre-bundled with ZapA and then incubated with ZapD also show no significant difference in FtsZ sedimentation (Figure 3.7B). The same reaction was prepared by pre-incubation of FtsZ with ZapD (2.1 µM), followed by the addition of ZapA (2.1, 4.8, 8.0, 12.4 µM). Similarly, ZapA did not affect ZapD sedimentation at any concentration tested (Figure 3.7C). Likewise, the further addition of ZapA did not result in any significant change in FtsZ sedimentation (Figure 3.7C).

3.4 Discussion

The structure of ZapD from *V. parahaemolyticus* has been previously solved in a structural genomics project but it remains unpublished. Recently a short paper, by Son and Lee, was published that described the crystallization of ZapD from *E. coli*, however, a structure of EcZapD was not provided in the paper (189). As shown previously in Chapter 2, the crystal structures of a homologous protein from two different organisms can be different and these differences can provide information about the functionality of the protein of interest (Figure 2.1) (181). For these reasons, we solved the structure of ZapD from *E. coli*. In our crystal screen, ZapD crystals formed in an identical space group to the crystals described by Son and Lee, i.e. hexagonal space group P64 (189). After structure resolution and data analysis using PISA
software, ZapD was shown to be a stable dimer consisting of a central α-helical dimer interface domain and two β-sandwich domains (Figure 3.1). The dimeric state of ZapD was confirmed by gel filtration (Figure 3.6) for WT ZapD and for several site-directed ZapD mutants, which suggests that any differences seen between WT and variants were not a result of disrupting the dimerization interface. One variant (ZapD R225A) showed a slight shift in apparent molecular weight, which was reproducible when analyzed by gel filtration (Figure 3.6, orange line). This shift in size is not large enough to account for a change in oligomeric state and is likely due to a slight conformational change within the β-sheet domain upon mutation of R225 (Figure 3.8).

Further analysis of the EcZapD structure and ZapD-FtsZ cross-linking revealed potential interacting residues. Residues R116 and R221 on ZapD were identified to cross-link near the C-terminus of FtsZ. This is consistent with previous results from work by Durand-Heredia et al. (48). They created several FtsZ truncations and tested them for interaction with full-length ZapD via protein interaction platform in Saccharomyces cerevisiae (48). Their results suggest that ZapD interacts with the C-terminal tail of FtsZ (48).

Of the nine ZapD variants generated, five resulted in different bundle morphologies when compared to WT ZapD. While the differences in FtsZ filaments per bundle observed for ZapD variants R56A and R114A were significant (Figure 3.4B), these phenotypes were less severe than those of ZapD variants R116A, R221A and R225A (Figure 3.4A). We therefore suggest that these three residues play a crucial role in FtsZ filament bundling and are likely to interact with FtsZ.

Residues R116 and R225 are located proximal to one another and are therefore proposed to be involved in forming a binding pocket for FtsZ. For residue R221, the sedimentation profile
for ZapD variant R221A only exhibited a slight difference when compared to WT ZapD. However, the TEM images of FtsZ bundled by ZapD R221A showed drastic bundle changes (Figure 3.4). Consequently, we propose that this ZapD variant is still binding to FtsZ filaments but has lost much of its bundling capability. The FtsZ-ZapD R221A bundles contain uncharacteristically long FtsZ filaments compared to WT (Figure 3.8). Since ZapD is known to inhibit the GTPase activity of FtsZ (48) we suggest that this ability is retained or even enhanced in ZapD variant R221A. FtsZ filaments are associated, albeit loosely, when bundled by ZapD R221A and this is likely due to the multi-residue interaction between these two proteins.

FtsZ-ZapD R56A bundles appeared thinner when imaged by TEM and quantitatively contained significantly fewer FtsZ filaments per bundle when compared to FtsZ-ZapD WT bundles (7 vs. 12). However, the overall organization of these bundles resembled that of WT ZapD bundled FtsZ. Residue R56 is also not located close to the proposed FtsZ binding pocket on ZapD. For these reasons we anticipate this residue, while involved with the bundling process, plays a less important role in FtsZ filament bundling. We hypothesize that residue R56 may be involved in recruiting smaller order bundles of FtsZ-ZapD. This would account for the WT arrangement and appearance of the bundles, as well as the decreased number of FtsZ filaments per bundle. The results from the cross-linking, FtsZ sedimentation and bundle characterization are summarized in Figure 3.9, where residues are labeled according to their proposed involvement in the FtsZ-ZapD interaction.

Overall the competition and co-bundling assays revealed that there is no specific competition for binding of FtsZ between ZapA and ZapD under the *in vitro* conditions tested.
Figure 3.8. Low magnification images of FtsZ filament bundles on EM grids. 
[A] WT ZapD bundled FtsZ filaments (arrows), [B] ZapD R221A bundled FtsZ filaments (arrows). Scale=20 µM.
here. They also showed that the two proteins may have proximal binding sites on FtsZ. This was demonstrated in the competition assays by the fact that neither Zap protein was displaced by the other (Figure 3.7B and C) but also because there was no significant increase in bundling with both Zap proteins present (Figure 3.7A, B and C). In co-bundling assays, ZapA and ZapD were added in excess to FtsZ filaments, the fact that one did not preferentially bundle FtsZ filaments indicated both proteins have similar affinities for FtsZ. These affinities cannot be directly measured because of the nature of FtsZ. It has been reported several times that FtsZ, being a filamentous protein, is not amenable to the techniques normally used to quantitatively measure protein-protein interactions and binding affinities (e.g. surface plasmon resonance or isothermal titration calorimetry). For example, studies of the ZipA-FtsZ interaction using surface plasmon resonance and an isothermal titration calorimeter (67) could only produce meaningful interaction data by using a fragment of FtsZ (residues 367-383). The only study that provides consistent (albeit modest) binding affinity values for FtsZ with another protein investigated the interaction of FtsZ with the SOS-responsive protein SulA (114). However, measurements of these interactions was likely only possible because SulA inhibits the polymerization of FtsZ, leaving only the two monomeric forms of each protein bound to one another in solution.

In contrast to other Zap proteins, ZapD function cannot be examined by genetic complementation methods because cells do not show a phenotype upon deletion of zapD. This had been shown previously with a transposon mutagenesis zapD knockout (48). Mutants constructed by transposon mutagenesis can sometimes cause downstream affects, as the gene disruption is not specific for a single gene. For zapD, two non-essential genes (yacG, a DNA gyrase inhibitor and mutT, an antimutator) are located upstream and one essential gene (coaE, co-enzyme A) is located immediately downstream. The close proximity of zapD to coaE
suggests that mutation by transposon mutagenesis could have downstream affects on cellular function. In order to confirm the results from the transposon-generated zapD mutant in a clean knockout of zapD we specifically removed zapD from the *E. coli* genome by λ-red mediated methods. Our results using a λ-red mediated zapD deletion strain were the same as those reported for the transposon mutant (48) and confirm that regardless of deletion, exogenous expression of ZapA causes cell elongation.

Previous work by Druand-Heredia *et al.* (48) indicates the cellular levels of ZapD are very low (500-800 molecules per cell) (48). Our results showed that when WT ZapD is expressed for complementation or for over-expression, at the lowest levels attainable, cells elongated. This is consistent with a low cellular level of ZapD. It is also possible that the expression of ZapD is tightly temporally controlled during normal cell division and this is lost with the plasmid constructs. ZapD expression may occur only at the onset of Z-ring assembly, consistent with the model for Z-ring formation by helical filament condensation (19, 48, 68, 82). In this model, it is proposed that FtsZ forms a loose helical filament at mid-cell covering the circumference of the cell several times and when division begins the helical filament is condense via an as yet unknown mechanism to form a tight ring structure. In this scenario ZapD would contribute to the helical filament condensation. Upon constitutive expression of ZapD, FtsZ is sequestered away from mid-cell by ZapD and neither the helical filament nor the Z-ring can form.

Our results suggest ZapD binds FtsZ through several charged residues located at the interface between the α-helical domain of one ZapD protomer and the β-sandwich domain of the other protomer. Additionally, our co-bundling and competition assays further support the idea that ZapA and ZapD have overlapping functions and have proximal binding sites on FtsZ. Taken
together, these results lend to the advancement of knowledge about the formation and stabilization of the Z-ring prior to bacterial cell division.
Figure 3.9. Model of the EcZapD dimer with residues labeled according to their proposed importance in the FtsZ-ZapD interaction.

Residues were assessed based on the results of 1) Sedimentation assay, 2) Bundle morphology, 3) Cross-linking. Residues labeled in green (R16, R20, R124 and R176) were unaffected in all 3 cases. The residue labeled in yellow was affected in one experiment (E114, bundle morphology). Residues labeled in orange were affected in 2 of 3 (R56A, Sedimentation and bundle morphology; R116, bundle morphology and cross-linked to FtsZ) and residues labeled in red were significantly different from WT ZapD results in sedimentation assay and bundle morphology and were shown to cross-link to FtsZ (R221 and R225).
Chapter 4: Conclusions

ZapA and ZapD belong to a group of proteins referred to as the FtsZ associated proteins (48, 118). They have been shown to bundle FtsZ filaments in vitro and are proposed to stabilize the Z-ring prior to cell division in vivo (44, 48, 118). While ZapA is widely conserved across Gram-negative bacteria, ZapD is restricted to proteobacteria (48, 118). This difference in conservation indicates that these proteins may have both unique and overlapping functions in cell division. Although the proteins have been shown to function in a similar manner, they show no sequence homology. Despite this, ZapA and ZapD have been proposed to have redundant functions in Escherichia coli. Individually, each Zap protein is non-essential to the proper growth and propagation of E. coli, but collectively they are suspected to play an essential role in cell division. In order to investigate the functions of ZapA and ZapD, both the protein structures and their interactions with FtsZ were investigated in E. coli.

The structure of EcZapA (ZapA from E. coli) was solved to 1.95 Å. When compared to the PaZapA (ZapA from Pseudomonas aeruginosa) (88) there were dramatic differences in the protomer organization within the tetramer. These structural differences suggest that there may be functional differences between EcZapA and PaZapA. Mainly, where PaZapA can exist as a dimer or tetramer, EcZapA is likely to function as a tetramer, based on our analysis of the crystal structure. Differences in functionality could be investigated using a cross-complementation experiment to reveal whether or not exogenous PaZapA expression can complement a zapA E. coli knockout.

Our EcZapA structure and previous bacterial two-hybrid assay results (68) were used to generate a series of ZapA variants by site directed mutagenesis. These variants were tested in both in vitro and in vivo assays. Importantly, the bundling action of ZapA on FtsZ filaments
allows for these proteins to be used in \textit{in vitro} FtsZ sedimentation assays. In these assays, FtsZ filaments are bundled by ZapA and then sedimented out of solution by low speed centrifugation. By testing the ZapA variants in FtsZ sedimentation assays each residue can be investigated for its involvement in FtsZ filament bundling. The bundles formed during FtsZ sedimentation can also be visualized by TEM to identify any morphological changes. The WT ZapA bundled FtsZ filaments averaged 10 filaments per bundle, while affected ZapA variants exhibited decreased FtsZ bundling capacity. To support these findings, \textit{in vivo} complementation and immunofluorescence were used to determine the biological implications of each single amino acid substitution in \textit{EcZapA}. The results of these assays identified four residues involved in the FtsZ binding and bundling function of ZapA. These residues, N28, D32, D33 and R46, are located along a 14-turn $\alpha$-helix located on each of the four protomers. These findings indicate that each \textit{EcZapA} tetramer contains four potential FtsZ binding $\alpha$-helices.

The structure of ZapD from \textit{E. coli} (\textit{EcZapD}) was solved to 2.4 Å. The structure of ZapD from \textit{V. parahaemolyticus} (40% sequence identity) was previously available as deposited in the PDB from a structural genomics project but never formally published in the scientific literature. The \textit{EcZapD} structure consists of two protomers making up the functional dimer. Each protomer can be divided into two domains: a central $\alpha$-helical domain and a jellyroll-like $\beta$-sandwich domain. The $\alpha$-helical domain shares some structural similarity to the integron cassette protein, \textit{VpC\_cass2} (PDB: 3JRT) (185), while the $\beta$-sandwich does not resemble anything else deposited in the PDB. Interestingly, ZapD dimers organized into tube-like structures in the crystals. However, the contacts between dimers were not significant and these tubes were not visualized outside of the crystal. Therefore, it is possible that the tube-like arrangements observed are artefacts of crystal packing; further testing is required to determine if this was a significant
finding. For example, using a buffer with a similar composition to the crystallization buffer and loading various concentrations onto TEM grids for visualization. If ZapD tubes were reproducibly formed in vitro, high-resolution microscopic techniques could be employed to visualize higher order ZapD structures in vivo.

The structural analysis of ZapD was supported with chemical cross-linking and LC-MS/MS of ZapD and FtsZ complexes, which lead to the identification of several candidate residues for further investigation. These residues, R16, R20, R56, E114, R116, R124, R176, R221 and R225, were changed to alanine by site directed mutagenesis resulting in a series of ZapD variants. The ZapD variants were subjected to the same in vitro assays as EcZapA. Morphologically, WT ZapD bundled FtsZ filaments exhibited an average of 12 filaments per bundle. The in vitro assays revealed five residues on ZapD to be involved in the ZapD-FtsZ interaction. Residues R56, R116, R221 and R225 cause a defect in FtsZ filament bundling when mutated to alanine, while residue E114 results in enhanced bundling when mutated to alanine. These residues are proximal to each other on the structure of the EcZapD dimer and could make up an FtsZ-binding pocket.

Unfortunately, investigation of the zapD phenotype is not amenable to the in vivo complementation assays described for ZapA above. As shown previously ZapD is likely a low abundance protein and any exogenous expression leads to disruption of cell division. This is thought to be the result of ZapD sequestering FtsZ or prematurely bundling FtsZ filaments (48).

Since ZapA and ZapD are proposed to have overlapping functions, co-bundling and competition assays were used to determine if they share a binding site on FtsZ and if one could outcompete the other. Results from these assays indicate that ZapA and ZapD likely have
proximal binding sites on FtsZ, as adding ZapD to FtsZ filaments pre-bundled with ZapA did not significantly increase bundling further, and vice versa. This was also supported by co-bundling assays, where both proteins were incorporated into bundles equally, with no preference for either. It was determined that there is no competition between ZapA and ZapD. When FtsZ filaments were pre-bundled with one Zap, neither could be forced out of the bundle by adding the second Zap.

This research has identified the charged residues on ZapA and ZapD responsible for FtsZ binding and filament bundling. Through biochemical and morphological analysis, some of these residues have been found to be more important for the FtsZ-Zap interaction. The key residues involved in the ZapA-FtsZ interaction are charged, these residues are also the most conserved among those comprising the \( \alpha \)-helix on ZapA. This conservation suggests these residues may be important for all ZapA’s, not just EcZapA. Similarly, charged residues on ZapD were found to be important for FtsZ bundling. These charged residues were also well conserved among all ZapD homologues in proteobacteria (Figure 3.1F). It has recently been suggested that ZapA and ZapD interact specifically with the C-terminal tail of FtsZ (48, 68). This leads us to believe that an interaction of charged residues with the C-terminal tail of FtsZ may be conserved in the bundling action of Zap proteins on FtsZ filaments.

In addition to defining FtsZ binding sites on ZapA and ZapD, this research describes the bundling of FtsZ filaments in detail. This initial characterization provides a benchmark for further studies on other FtsZ bundling proteins by providing representative micrographs, and reporting the average number of filaments per bundle, the EC\(_{50}\) values for sedimentation assays, the overall sedimentation profiles of WT ZapA and WT ZapD.
When comparing ZapA-bundled FtsZ filaments and ZapD-bundled FtsZ filaments there was one key difference. The level of organization appeared higher with ZapD-bundled FtsZ filaments than ZapA-bundled FtsZ filaments. Specifically, ZapD-FtsZ bundles had very linearly associated FtsZ filaments, which lay flat on electron microscopy grids. While this bundle morphology was also observed periodically for ZapA, it was not representative of the majority of observations. When measurements were recorded, ZapD-FtsZ bundles averaged 12 filaments per bundle, while ZapA-FtsZ bundles averaged 10 filaments per bundle. It is possible that this difference in bundle arrangement was the result of weak interactions between ZapD dimers, as it was shown in the crystal form of ZapD that dimers do have some affinity for one another in close quarters. This could be determined by immune-gold labeling ZapD and ZapA within bundles, if ZapD dimers were interacting, providing a higher order of organization, there may be a more consistent pattern of ZapD arrangement in bundles compared to ZapA in bundles.

The ZapA-FtsZ sedimentation profile was similar overall to the ZapD-FtsZ sedimentation profile. Both profiles show an increase in sedimentation with increasing Zap concentration until equimolar Zap:FtsZ is reached; beyond this ratio FtsZ sedimentation levels out. Although these profiles appear similar, the EC$_{50}$ values differ. The EC$_{50}$ value indicates the concentration of Zap required to sediment 50% of FtsZ in the reaction. The EC$_{50}$ for ZapA is 2.22 µM and the EC$_{50}$ for ZapD is 1.86 µM. The higher EC$_{50}$ of ZapA indicates more ZapA is required than ZapD to sediment 50% of FtsZ in the reaction. This could be attributed to ZapA’s oligomeric state at varying concentrations. It has been described previously that ZapA tetramerization is required for FtsZ filament bundling (119). Low and Löwe posit that ZapA tetramerization is concentration dependent (88). They found that PaZapA exists in a dimer-tetramer equilibrium, which shifts to tetrameric with increasing concentration (88). While the EcZapA structure described here is
likely to be mainly tetrameric based on the crystal contacts, it is still possible that at low concentrations a proportion of ZapA is dimeric and cannot contribute to FtsZ filament bundling.

Currently, FtsZ sedimentation assays are the primary method used to assess the bundling capability of Zap proteins on FtsZ filaments. Although this can give us important information about interactions when comparing Zap variants to WT, these assays are preformed in vitro in the absence of the other cell division proteins. The Z-ring is highly dynamic and in vivo there are many positive and negative regulators that would be acting on FtsZ while Zap proteins are acting to bundle and stabilize FtsZ filaments. This presents an important caveat of the current model for studying FtsZ-Zap interactions. In vitro Zap proteins act to bundle FtsZ very tightly, into highly organized arrays on FtsZ filaments, as visualized by TEM; in vivo this is not likely the case. This phenomenon would potentially affect the competition assays described in this thesis. While both proteins were able to bind FtsZ filaments with the same strength in vitro it is possible that other FtsZ or Zap protein interacting proteins would change that. For example, ZapA is known to interact with and recruit ZapB to the FtsZ. ZapB’s interaction with ZapA may increase or decrease its interaction strength with FtsZ, allowing for competition for binding with ZapD. For this reason it would be interesting to add other divisome proteins to the in vitro bundling and sedimentation assays to test this hypothesis.

In addition to the absence of other Zap proteins and divisome proteins, the in vitro assays used here do not account for the dynamic nature of FtsZ and dynamic interactions between Zap proteins and FtsZ. All reactions represent a snapshot in the bundling process. While the concentration of Zap present in each reaction may be different, all reactions are observed after 5 min of bundling. This may be especially important when looking at the co-bundling of FtsZ filaments with ZapA and ZapD. While both proteins are equally bound to FtsZ filaments after 5
min, ZapA may be binding first then dissociating while the ZapD then binds and re-organizes the filaments within the bundles. When imaging the FtsZ-ZapA-ZapD co-bundles there was some evidence to support this hypothesis. Figure 4.1 shows highly organized, thick bundles formed when all three proteins are present together. However, it is interesting that FtsZ filaments appear to be fraying away from the bundle (arrows) and there are many individual filaments visible surrounding the bundle. This suggests that there may be some type of filament re-organization when two bundling proteins are present. Further studies could be done to confirm this by preforming sedimentation assays on a time course, to determine whether ZapA and ZapD are acting at different times during bundling. Additionally, the bundles from those sedimentation assays could be imagined by TEM and each Zap protein could be targeting using immuno-gold labeling to determine the involvement of each Zap protein within the bundle. An alignment of ZapA to ZapD in *E. coli* shows the relatively low level of similarity between the two proteins (Figure 4.2).

Overall, this research supports the hypothesis that ZapA and ZapD have overlapping functions and proximal binding sites on FtsZ. These findings could have implications in drug design, as FtsZ is an essential cell division protein. The bundling of FtsZ filaments by Zap proteins at mid-cell may be an essential process but this has yet to be shown. Bacterial cell division has emerged as an attractive target for antibiotic development. It is an essential process for bacteria and its components show little homology to eukaryotic proteins. In the case of Zap proteins and FtsZ, it is the interactions leading to FtsZ filament bundling that would be the target for antibiotics development. By clearly defining the binding sites between Zap proteins and FtsZ, this protein-protein interaction can be exploited and disrupted by specific small peptide inhibitors. For this to be possible the orientation of both proteins during the interaction must be
Figure 4.1. FtsZ filaments bundled by ZapA and ZapD.
Bundles were formed by simultaneous incubation of FtsZ (4.8 µM) with ZapA (11.4 µM) and ZapD (4.8 µM) in the presence of GTP (1 mM); the co-bundling assays are described in Chapter 3. Bundles were separated from soluble proteins by centrifugation and resuspended in 1× PEM-KOH buffer before adhering to copper grids for TEM. Arrows indicate individual FtsZ filaments fraying away from the main bundle, scale=250 nm.
Figure 4.2 Alignment of ZapA to ZapD in E. coli
A simple protein alignment of ZapA and ZapD from E. coli, where the most affected residues in ZapA function are highlighted in red and the most affected residues in ZapD function are highlighted in blue.
known. Here we have described the crystal structures of EcZapA and EcZapD, but the structure of EcFtsZ has not yet been solved. Looking forward, the co-crystal structures of these Zap-FtsZ complexes (EcZapA-EcFtsZ and EcZapD-EcFtsZ) would give us the most pertinent information needed for drug design and further validate our results. The initial trials in generating EcZapA-EcFtsZ and EcZapD-EcFtsZ co-crystals are described in the Appendix. Although these trials have yet to yield substantial crystals containing both proteins of interest, the screens did produce some promising hits for future research.

Zap proteins may have previously been largely ignored in the pursuit of cell division inhibitors but with results showing the highly dynamic nature of FtsZ and the overlapping roles of Zap proteins in stabilizing FtsZ at mid-cell, it is becoming more evident that Zap proteins collectively play a crucial role in bacterial cell division. Taken together, the results presented in this thesis have paved the way for future studies on Zap proteins and opened doors for the further analysis of the ZapA-FtsZ and ZapD-FtsZ interactions.
Appendix

A1 Introduction

FtsZ is a potential target for new antibiotics, particularly with respect to inhibiting protein-protein interactions with FtsZ. These protein-protein interactions can be narrowed down to potential regions through bacterial and yeast two hybrid assays (48, 68, 122), which utilize truncations of one protein against the other full-length protein. Typically these assays will show that some truncations maintain the interaction and some do not, yielding a target region for further investigation. Specific amino acids can then be implicated in the interaction by combining the use of site directed mutagenesis and an interaction based assay (181). This approach can give important information about the nature of the interaction between the two proteins, but protein-protein interactions are often multi-valent (190, 191) and it can be difficult to determine every interacting residue through these techniques. Additionally it leaves questions about how the proteins are oriented with respect to one another. Specifically, in the case of divisome proteins, if there were information about a few proteins orientations, determining additional protein-protein interactions and sub-complex assembly/association would be more efficient. Recently, groups have had success with designing antibiotics to inhibit protein-protein interactions through a structure-based approach (134, 135, 192). By solving the structure of a complex, containing either both proteins or parts of each protein, the multi-valent binding can be accurately and completely defined. Obtaining co-crystal structures of protein complexes can be laborious and unfruitful, as the conditions for crystallization must support the maintenance of both proteins’ tertiary structures, sustain the protein-protein interaction of interest and not cause one of the protein to crystallize out of solution alone. In this ongoing study, we report the troubleshooting of buffering conditions, mixing ratios and the initial crystal screening with the
goal of solving the co-crystal structures of both ZapA-FtsZ and ZapD-FtsZ complexes. With promising hits in the first crystal screens, this research shows promise for future experiments.

A2 Experimental procedures

A2.1 Protein over-expression and purification

FtsZ, His-ZapA and His-ZapD were purified as described previously, in Chapters 2 and 3.

A2.2 Buffers and buffer exchange

In an effort to generate ZapA-FtsZ co-complexes, proteins were initially mixed in a 1:1 (v:v) ratio at maximum concentration (8 mg/mL for His-ZapA and 20 mg/mL for FtsZ) in their own purification buffers (1× buffer A1 for ZapA and 1× PEM for FtsZ). Mixing was also preformed at the molar ratio of 1:1 (w:w) in 1× PEM buffer, 1× buffer A1 and a combination of both. For buffer exchange, proteins were dialyzed against the desired buffer overnight at 4°C with 2 buffer changes. Similarly to ZapA-FtsZ, for ZapD-FtsZ co-complexes, proteins were mixed at a 1:1 (v:v) ratio at maximum concentration (5 mg/mL for His-ZapD and 20 mg/mL for FtsZ) in their own purification buffers (1× buffer A2 for ZapD and 1× PEM for FtsZ) and they were mixed at ZapD:FtsZ molar ratios of 1:4, 1:2, 1:1 and 2:1 (w:w).

A3 Results

A3.1 Protein purifications

FtsZ, His6-ZapA (hereafter ZapA) and His6-ZapD (hereafter ZapD) were purified to >95% purity as determined by SDS-PAGE of purified protein samples followed by Coomassie
staining (Figure A1). Individually, these proteins displayed differences in their stability when stored at 4°C after purification. Immediately after purification, FtsZ was soluble at high concentrations and could readily form filaments in the presence of GTP. However, after incubation for 24 hours or longer at 4°C, FtsZ began to precipitate out of solution and FtsZ filament formation in the presence of GTP was less slightly efficient, as observed via TEM of the filamentation reactions. The precipitation of FtsZ was evident by the appearance of a white precipitate and FtsZ filament formation was visualized by TEM. In order to optimize the FtsZ sample being used in co-complex formation, FtsZ was always expressed and purified the same day as protein mixing. Purified and concentrated ZapA was stable and did not precipitate out of solution until 14 days after storage at 4°C. ZapD was also stable when stored at 4°C and did not precipitate for the entire duration of storage at 4°C (> 8 weeks). However, it should be noted that concentrating ZapD beyond 5 mg/mL proved troublesome, as it begins to precipitate reversibly at concentrations higher than this.

A3.2 Protein stability depends on buffering conditions

FtsZ polymerized well in PEM-KOH buffer at pH 6.5, but when attempting to co-complex FtsZ with other proteins, this was not found to be the optimal buffer. ZapA was in buffer A1 pH 7.5 and ZapD was in buffer A2 pH 8.0, and by mixing these proteins directly with FtsZ in PEM-KOH buffer, the change in pH did not support the stability of all proteins. Upon mixing ZapA and FtsZ, without buffer exchange, proteins were stable on ice for 1 hour until both proteins began to come out of solution. Mixing ZapD and FtsZ with no buffer exchange resulted in immediate precipitation of both proteins; this was reversed by dilution in buffer A2.
Figure A1. SDS-PAGE illustrating the purification of proteins.
[A] Two lanes showing the progressive purification of FtsZ. The first lane is purified FtsZ after one round of Ca^{2+} cycling, lane two is purified FtsZ after a second round of Ca^{2+} cycling. [B] Gel of purified His-ZapA. [C] Gel of purified His-ZapD.
In an attempt to keep proteins soluble when mixed, they were dialyzed into the same buffer prior to mixing. When trying to co-complex FtsZ and ZapA, PEM-KOH and buffer A1 were each tried. While both FtsZ and ZapA were stable in buffer A1, ZapA precipitated when dialyzed into PEM-KOH buffer. During dialysis, some FtsZ precipitated, and this precipitate was removed by centrifugation; the remaining soluble protein remained stable at 4°C for 24 hours. FtsZ and ZapA were mixed at a 1:1 molar ratio, where the concentration of each was 500 mM, 250 mM, or 100 mM. In all three situations, both proteins remained stable after mixing when stored on ice. The solutions containing 500 mM and 250 mM of each protein were used in crystal screens.

For the formation of co-complexes FtsZ and ZapD, proteins were also dialyzed into the same buffer before mixing; PEM-KOH, buffer A2 and buffer A1 were all tested. FtsZ was most stable in buffer A2. After dialysis overnight in buffer A2, FtsZ did not precipitate, in contrast to buffers PEM-KOH and buffer A1. ZapD was also only stable in buffer A2 and it precipitated entirely after dialysis into PEM-KOH and buffer A1. In accordance with these results, buffer A2 was chosen as the buffer for mixing FtsZ and ZapD. Proteins were mixed at ZapD:FtsZ molar ratios of 1:4, 1:2, 1:1 and 2:1 (w:w), where the FtsZ molar concentration was kept at 500 mM and ZapD was used at 125 mM, 250 mM, 500 mM and 1 M, respectively. Solutions that contained high concentrations of ZapD a precipitate formed immediately after mixing. Again, this precipitation was reversed by dilution with buffer A2. Both proteins exhibited the least precipitation in the 1:4 ZapD:FtsZ solution stored on ice, which was then used in crystal screens.
A3.3 Preliminary crystal screens did not give usable co-crystals

Initial screens for both ZapA-FtsZ and ZapD-FtsZ co-complex crystals yielded small crystals. For ZapD-FtsZ, hexagonal crystals formed in 0.1M MES pH 6.5, 1.6M MgSO₄. The crystal ZapD-FtsZ crystals belong to the same hexagonal space group P64 which is the same as the ZapD crystal, which could mean it contains only ZapD, and FtsZ was excluded during crystal formation. Data was collected on these crystals at the Canadian Light Source, beamline 08ID. This data was compared to the ZapD data collected for solving the EcZapD structure and it was determined that these crystals only contained ZapD.

ZapA-FtsZ crystals formed in 0.1M Tris pH 8.5, 0.2M Ammonium phosphate, 50%(v/v) 2-methyl-2,4-pentanediol. These small crystals were of variable shape. During processing they dissolved and therefore no data was collected for them.

A4 Conclusions

The results from the co-crystal screens were negative. However, buffers for the stability of ZapA-FtsZ and ZapD-FtsZ in solution were determined. ZapA and FtsZ are stable together in buffer A1 when stored on ice and ZapD and FtsZ are stable together in buffer A2 when stored on ice. There are many variables that may need to be modified to achieve Zap-FtsZ crystal growth and those will be pursued in future projects. While finding the buffer, temperature, concentration and crystal screening conditions that allow for the growth of a single protein crystal can be laborious; finding those conditions for the growth of co-crystals can be even more a lengthy process. For these reasons, it may be a more viable option to co-complex each Zap with the C-terminal tail of FtsZ, which is thought to be the area of interaction with ZapA and ZapD. Mosyak et al. solved the co-crystal structure of the C-terminal tails of FtsZ and ZipA in complex together.
(67). It had been proposed previously that the C-terminal tails of those two proteins interact. Therefore, despite our setbacks the Khursigara group is pushing forward and will pursue both the co-complex structures of the full-length proteins and each Zap with the C-terminal tail of FtsZ in the future.
References


