Title
Characterization of TubR on the plasmid pBtoxis in Bacillus thuringiensis as well as actin-like protein Gp207 within a Bacillus thuringiensis phage

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Characterization of TubR on the plasmid pBtoxis in *Bacillus thuringiensis* as well as actin-like protein Gp207 within a *Bacillus thuringiensis* phage

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

Paula Patterson

Committee in charge:

Professor Joe Pogliano, Chair
Professor Eric Allen
Professor Kit Pogliano

2008
The Thesis of Paula Patterson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2008
DEDICATION

In dedication to my loving parents without whose guidance and support this work would not be possible. Additionally, I would like to thank Ian Nicastro for his unfailing ability to help me through the inevitable failures one encounters in the field of science.
EPIGRAPH

The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, “hmm…. that’s funny…”

Isaac Asimov

Effort only fully releases its reward after a person refuses to quit.

Napoleon Hill
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>GEMSA</td>
<td>Gel Electromobility Shift Assay</td>
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<td>B. subtilis</td>
<td><em>Bacillus subtilis</em></td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>B thuringiensis</td>
<td><em>Bacillus thuringiensis</em></td>
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<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<tr>
<td>ECL</td>
<td>Enchanced Chemiluminescence</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl 1-thio-β-D-Galactopyranoside</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>Spec</td>
<td>spectinomycin</td>
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<td>adenosine triphosphate</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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ACKNOWLEDGEMENTS

Special thanks to Joe for giving me the opportunity to dive into research in his lab. I’d also like to thank the members of Joe’s lab for being there to keep me company throughout the years and answer the myriad of questions I managed to ask, especially Alan Derman and Rachel Larsen.
ABSTRACT OF THE THESIS

Characterization of TubR on the plasmid pBtoxis in *Bacillus thuringiensis* as well as actin-like protein Gp207 within a *Bacillus thuringiensis* phage

by

Paula Patterson

Master of Science in Biology

University of California, San Diego, 2008

Professor Joseph Pogliano, Chair

One of the major projects contained within this work involves characterization of a DNA binding protein encoded on pBtoxis within *Bacillus thuringiensis*. This protein, TubR, was shown via Western Blotting to directly regulate expression of a downstream gene, *tubZ*, which is related to the class of filaments known as tubulins. TubR’s DNA binding properties were shown via gel electrophoresis mobility shift assays. Together, this TubZ and TubR are responsible for the efficient stability of pBtoxis in daughter cells and spores, but whether this is due to proper replication or segregation is unclear.
Within the *Bacillus thuringiensis* phage 0305phi8-36, gp207 has been classified as a distant actin relative. It is most closely related to MreB. By fusing this gene to GFP and expressing it within both *Escherichia coli* and *Bacillus subtilis*, it was shown via microscopy to form a static filament within the cell. This represents the first characterization of bacterial actin encoded by a bacteriophage and lays the groundwork for studies on the role of cytoskeletal proteins in the life cycle of bacteriophage.
CHAPTER I

Introduction
Actin Polymerization

The proteins examined within this work share the common feature of being related to cytoskeletal elements known as actin and tubulin. Actin’s ability to nucleate and form a polymer within the cell is what allows it to participate in the various roles in which it has been implicated such as cell motility and chemotaxis (1). In vivo, actin exists as free G-actin monomers which must polymerize into F-actin. An actin filament is comprised of a fast growing barbed end (the + end) and a slow growing pointed end (the – end) resulting in a polar filament (Figure 1). The rate of growth at each end depends on the pool of G-actin within the cytoplasm but the filament typically grows from the barbed end and shrinks from the pointed end. G-actin is associated with ATP and binds to the growing filament where the ATP will be hydrolyzed into ADP (2). However, the conversion of ATP to ADP is ten times faster than the release of the generated phosphate so the actin subunit usually contains ADP+Pi. This conversion occurs primarily at the growing barbed end of the filament (3). After depolymerization, the free subunits exchange their ADP for an ATP in order to be recycled (4). Since the rate of growth of the filament is dependent on the critical concentration, or the concentration where the monomer and polymer subunits are in equilibrium, this has implications for directional growth as the pointed end has a critical concentration twelve fold higher than that of the barbed end (5). Since filaments only polymerize when the G-actin pool is greater than the critical concentration, it is evident that the barbed end would be predisposed for polymerization while the pointed end would be predisposed for depolymerization
leading to the phenomenon known as treadmilling which requires a concentration at
which the speed of growth at the (+) end is equal to the rate of depolymerization at the
(-) end (6).

This polymerization is fully dependent on the creation of free barbed ends and
this process can be started through a nucleation complex as well as uncapping or
severing of an existing filament (7). One method associated with creation of free
barbed ends is via the Actin related protein (Arp) 2/3 complex, comprised of seven
conserved polypeptides. The Arp complex serves to as a nucleation point for actin
polymerization and associates with free barbed ends at a cell’s leading edge during
cell motility studies (8). This complex can also form free barbed ends in vitro, in an
artificial environment, through association with the Wiskott-Aldrich syndrome protein
(WASP) family and one such protein, Scar, has been shown to enhance actin
polymerization (9-10). In addition to a nucleation complex, free barbed ends can be
formed via uncapping of an existing filament (11-13) or through severing by gelsolin
or coflin (14-16). It is possible to see how actin could be the subject of hundreds of
regulatory proteins to control its polymerization and depolymerization activities.

After creation of free barbed ends, the actin filament can continue to
polymerize. As mentioned previously, G-actin is the monomeric free floating form and
is often kept bound up within the cytoplasm by proteins such as thymosin beta4 and
profilin (17). However, profilin can also have positive effects in allowing recruitment
of G-actin to a filament in order to speed polymerization (18).
Eukaryotic Actin and its Roles

Actin was discovered within eukaryotic organisms and is known to be involved in a vast number of roles. In addition to cell motility and chemotaxis mentioned previously, actin has been implicated in participating in endocytic processes evidenced by the fact that disrupting actin encoding genes blocks the uptake of endocytic markers. However, the molecular mechanism of this interaction is still poorly understood (19). In a broader context, through interaction with endocytic machinery components dynamin, huntingtin-interacting proteins and intersectin, actin has been more conclusively connected to clathrin-mediated endocytosis and vesicular transport within mammalian cells (20-21). In addition to these roles, actin has been extensively shown to play a role in amoeboid movement, muscle contraction, and movement of intracellular pathogens such as *Listeria monocytogenes* (22-26).

The role of actin has been studied intensively within the organism *Dictyostelium discoideum* and its participation in motility as well as cytokinesis, endocytosis, and morphogenesis has been well established (27). Immunofluorescence techniques have been used to show that the concentration of actin within pseudopodia can significantly increase or decrease in a five second timeframe. The actin density within these protrusions exhibit forward and reverse movement and this change precedes the extension or retraction of the pseudopod. These findings indicate that actin is responsible for the structural integration of the pseudopod (28). Essentially, the polymerization of actin is enough of a force to push the pseudopodia toward a stimulus such as that of a food source (29).
Another well known role of actin is its partnership with myosin during muscle contraction. Actin was first tied to a role in contraction through use of cytochalasins that bind free plus ends of actin and prevent further polymerization (30). Muscle contraction is the result of interaction of myosin heads on sarcomere thick filaments with thin filaments comprised mainly of actin to form a cross-bridge. These filaments move past one another due to ATP hydrolysis initiated by a myosin head (31-32). After completion of this power stroke, the remaining ADP molecule dissociates from the complex causing a contraction of the muscle at which point the actin polymer is prevented from depolymerizing by being capped at both ends by tropomodulin and CapZ (33). Once the vacated site is reoccupied with ATP, actin and myosin no longer bind to one another and the cycle can start anew.

Actin is involved in more than just cellular processes as it has been used to great advantage by many intracellular pathogens. Bacterial pathogens such as *Listeria monocytogenes*, *Shigella flexneri*, *Burkholderia pseudomallei*, *Mycobacterium marinum*, and *Rickettsia* species all use actin to move within the cytoplasm of host cells (34). These pathogens accomplish this by exploiting the host’s pool of actin and activating proteins which result in actin nucleation, specifically the Arp 2/3 complex which was described earlier (35). Each type of intracellular pathogen accomplishes this in a slightly different way. *Shigella* uses IcsA which localizes around the outer membrane in a polarized fashion with the highest concentration being at the site of actin tail polymerization (36). *Mycobacterium* is thought to initiate actin polymerization by recruiting WASP as it localizes at the pole from which actin
polymerizes (37). *Listeria* is the most well studied mammalian pathogen that employs the use of host cell actin polymerization to further infection of neighboring macrophage and other cells. This pathogen uses the protein ActA to nucleate actin formation and has maximal expression at the pole from which the actin tail forms (38). The filament forms by adding actin subunits at the barbed end associated with the surface of the pathogen. However, the tails remain short and are capped on their pointed ends indicating that nucleation and filament length are tightly controlled (39). Such regulated filament formation provides the force needed for the bacterium to project itself out of the host cell surface into a nearby cell to spread infection (40).

**Prokaryotic Actin and its Roles**

Actin had previously been thought to only be present in eukaryotic cells until the protein MreB (murein cluster e, gene B), an actin homologue, was found within prokaryotes (41). Its role was established as a cytoskeletal protein when its crystal structure was discovered to be highly similar to that of eukaryotic actin and it was shown to form filamentous helices beneath the cell membrane *in vivo* (42-43). Mutations in this gene cause rod-shaped cells to become spherical which indicates a possible role in cell shape though it is not essential in all cell types (44). MreB was more conclusively linked to its role as a determinant of cell shape when introduction of an inhibitor, A22, resulted in lateral murein synthesis failure and the same spherical cell shape (45). Another related but nonessential protein within *Bacillus subtilis* is Mbl which is thought to play a similar role in cell shape through insertion of new cell wall
material in a helical manner (46). Within *Caulobacter crescentus* MreB was also shown to have a role in cell shape as depletion resulted in lemon shaped cells. Fluorescent molecule tagging led to the discovery that MreB undergoes directed treadmilling within the cell and this may be responsible for a yet undiscovered role for MreB (47-48).

Since the discovery of MreB, many actin-like filaments have been identified within prokaryotes opening up a new area of research. MamK is another filament forming protein responsible for organization of magnetosomes within magnetotactic bacteria. Electron cryotomography was used to show the existence of filaments along a magnetosome chain in *Magnetospirillum magneticum* and it was shown that in a MamK deletion strain, the magnetosomes were no longer ordered in a straight line but scattered about the cell (49). When MamK was visualized either by a GFP fusion or through immunofluorescence, the localization was found to be in the same area as that of the magnetosomes indicating an interaction between the two (50).

**Plasmid Segregation Systems**

In addition to the actin-like proteins discussed so far, several families exist which are thought to aid in segregation of DNA, specifically low copy number plasmids. It is easy to imagine that during replication and division of a bacterial cell, high copy plasmids are present in great numbers and can easily make their way into a daughter cell through simple diffusion. The real challenge comes when the plasmid is in low copy such as 1-3 copies per cell in which a mechanism must exist in order to
ensure that the plasmid is efficiently segregated into daughter cells. Two well characterized segregation systems for accomplishing this are that of the Walker-Type ATPase family and the ParM family. Both families have similar genetic organization in that they consist of two gene products which act in trans and a centromere-like region that acts in cis. In order for normal partitioning of the plasmid to occur, all three gene products are required and must be functional (51).

Perhaps the most well studied examples of the Walker-Type ATPase family would be the partitioning systems located on plasmids P1 and F within *E.coli*. Both plasmid loci share a similar organization as the centromere-like sites parS and SopC respectively are downstream of the *trans* acting genes parA/SopA and parB/SopB (52 and Figure 2). It has been shown that the B proteins are DNA binding proteins that bind to the centromere-like region to form a complex which is necessary for partitioning (53-55). The A proteins are ATPases which in turn bind to the B proteins and the interaction of all three results in segregation of plasmid DNA. In addition, all three are required to regulate transcription of the *parAB* genes (56-59). Fluorescent molecule tagging as well as mutational analysis led to the discovery that SopA forms dynamic filaments in vivo that are necessary for efficient plasmid segregation. Mutations which caused SopA filaments to lose their dynamic properties greatly decreased efficient levels of plasmid segregation. Time-lapse fluorescence microscopy led to the hypothesis that segregation of the F plasmid by the Sop system is accomplished through SopA filaments providing the driving force to push the plasmids apart. SopA recognizes the plasmid through the SopB/sopC complex
causing filament formation on the plasmids to push them to the quarter cell positions prior to cell division (60). There are other plasmids which contain Walker-Type systems, RK2 and pB171 of *E.coli* to name a couple though many more exist (61-62).

Another family similar to the Walker-Type ATPase family is the actin-like ATPase family typified by ParM of plasmid R1. Like in the previously described systems, R1 contains a repressor protein, ParR, which binds to a centromere-like region, *parC*, and filaments are made by the actin-like protein ParM (63 and Figure 2). Segregating plasmids within a cell localize to the ends of the filament formed though the interaction of ParM with ParR in an ATP dependent manner and new subunits are added to the filament between the old filament and the plasmid (64). In addition to this, it was shown that the filaments display dynamic instability more similar to that of microtubules than to actin (65-66.) Taken together this data led to the current ParM segregation model whereby plasmid replication leads to the formation of ParR-*parC* complexes within the cell which are able to attach to ParM filaments causing them to stabilize. New subunits are continually inserted at the ParM-ParR resulting in bidirectional elongation that pushes plasmids to the opposite ends of the cell. A summary of actin-like and tubulin-like proteins can be seen in Table 1.

In addition to active partitioning systems, a plasmid can also contain a post segregational killing system in order to ensure it is maintained. This is accomplished via a toxin and anti-toxin (TA) system or by antisense RNA. In both systems the antagonistic regulators that neutralize the toxins, whether they are antisense RNAs or proteins, are unstable. In a cell lacking a plasmid, the toxin will no longer be removed.
by the antitoxin, therefore leading to cell death. A prime example of this is the coupled cell division (ccd) locus of the F plasmid. This locus consists of ccdA which is the anti-toxin that inhibits the toxin, ccdB. When the plasmid is present, ccdA forms a complex with ccdB and the toxin is rendered harmless. A cell that does not receive a plasmid is killed by this locus (67). A similar setup can be found on the P1 plasmid and the relevant genes are the Phd (prevents host death) anti-toxin and Doc (death on curing) toxin. Just as with the F plasmid ccd system, a daughter cell will be killed if it does not receive a plasmid to continue to synthesize Phd (68-69). Other examples of this protein TA system are the HigBA of Rts1, ParDE of RK2, ParD of R1, and VapBC of a *Salmonella dublin* virulence plasmid (70-73). These types of systems are not exclusive to plasmids but can also be found on bacterial chromosomes. Examples of chromosomal loci include RelAB, MazEF, and HipBA (74-76 and Table 2).

Protein toxins and anti-toxins are not the only mechanism of post segregational killing as an antisense RNA mechanism can achieve the same result. In this system, the antidote is not a protein but rather a small, unstable transcript that binds to the toxin-encoding RNA and inhibits its translation (77). The most well characterized example of this system is the hok (host killing)/sok (suppressor of killing) locus of plasmid R1. Translation of the toxin, hok, is regulated through antisense RNA of sok as well as secondary structure within its own transcript to prevent its translation and interfere with binding to the antisense RNA (78-79). Another known example of the antisense RNA system is the par locus of plasmid pAD1 (80).
Tubulin

Tubulins make up another group of cytoskeletal proteins which were first discovered in eukaryotic cells. Two tubulin subunits (α and β) bind together to form heterodimers (81-82) that self-assemble into microtubules in vivo, as well in vitro when in the presence of magnesium and GTP. Assembly in vitro requires physiological temperatures and low temperatures (such as 0°C) cause microtubules to disassemble (83). Microtubules are made from a series of protofilaments of α,β tubulin and the tubule has a hollow center. The alternation of the two types of tubulin causes the filament to have polarity and the filament is capable of dynamic instability and treadmilling (84-85). Tubulin filaments have many functions in eukaryotic cells and are primarily concerned with maintenance of cell architecture and motility. Most notably microtubules play a central role in segregation of chromosomes during mitosis (86-87). Prokaryotes also have relatives of tubulin, such as FtsZ and TubZ, and will be discussed in the next section.

Bacillus Cell Division and Sporulation

Bacillus subtilis is the most well characterized member of Bacillus species due to its ability to be genetically manipulated. It is a gram positive soil bacterium with the ability to form a resistant endospore in times of poor conditions (88). The mechanism of cell division for B. subtilis requires a number of proteins, including the proteins FtsZ, MinCD, and Noc. FtsZ is a tubulin homologue that can assemble into polymers and is a GTPase (89-91). At the onset of division, FtsZ is recruited from the cytoplasm
where it forms a ring at the division site and remains associated with the leading edge of the invaginating membrane (92). Levels of FtsZ must be tightly controlled as overexpression leads to the formation of minicells through division at a cell pole and absence results in no cell division (93-94). Likely, FtsZ is also responsible for recruiting other division proteins to the division septum and this is certainly the case with FtsA which has been shown to localize with FtsZ and the ratio of the two proteins is essential for cell division (95-96). Another protein which associates with FtsZ and is essential for division is ZipA (97). Two possible models exist for the mechanism of FtsZ constriction. The first relies on a system of overlapping filaments which slide relative to one another and are driven by a motor protein. The second relies on loss of subunits from the filament at the junction with an anchor (98).

FtsZ is recruited to the site of cell division and its location dictates the site of the future septum so there must be a system in place to define this site to be at mid cell every time. The first system in place to ensure that division occurs at mid cell is the nucleoid occlusion model. This model states that a division site cannot be formed over the nucleoid, the DNA containing regions of a dividing cell. With this check in place, division site favor is granted at regions farthest from the nucleoid leaving only mid cell and the cell poles. (99). The protein thought to be involved in this process is Noc and is hypothesized to be a non-specific DNA binding protein which will coat the segregating chromosomes and prevent assembly of FtsZ in the vicinity of the nucleoid. (100). More recent studies within the organism *E. coli* have taken steps to further elucidate this model and a protein, SlmA, has been identified as a DNA-associated
division inhibitor which prevents FtsZ assembly in regions surrounding the nucleoid (101).

The nucleoid occlusion model only allows for FtsZ assembly at mid cell or at the cell poles but there must be an additional mechanism in place to prevent septum formation at the poles which would lead to production of mini cells. This model relies on the proteins MinCDE which were implicated in the process based on the fact that mutation or deletion of minC, minD, or all three genes allows division to occur at the cell poles when it otherwise would not (102). How this system functions in Bacillus subtilis is currently unclear. In E. coli, the Min system is thought to localize to the cell poles where it can serve as a direct inhibitor of FtsZ polymerization (103-108). With nucleoid occlusion and MinCD working together in B. subtilis, the only region left for FtsZ assembly and thus cell division is at mid-cell.

Division in B. subtilis occurs at mid cell during vegetative growth but in times of famine, the cell follows a sporulation pathway which dictates that septum formation occur at a cell pole. This requires a switch of FtsZ recruitment from midcell to the poles and is accomplished through Spo0A, the master transcriptional regulator for sporulation (109). A septum forms at only one pole through regulation by $\sigma^H$ and there is some evidence that indicates $\sigma^E$ is responsible for blocking septum formation at the opposing pole (110). The septum forms over the forespore chromosome and what lies outside the forespore is then translocated via SpoIIIIE. Its origin is anchored to the pole via binding to SpoOJ/Soj that was recruited to the pole via the proteins RacA and DivIVA. (111-112). Once formation of the polar septum is complete, $\sigma^F$ is activated
within the forespore which in turn activates $\sigma^E$ within the mother cell (113). Engulfment proteins are activated by $\sigma^E$ leading to engulfment of the forespore by the mother cell. This process utilizes SpoIID, SpoIIM, and SpoIIP to degrade the cell wall around the forespore so there is space for the mother cell membrane to move around it. In addition, SpoIIQ which is anchored in the forespore membrane and SpoIIIAH which is anchored in the mother cell membrane interact and are thought to prevent the mother cell membrane from retracting once it begins its migration (114-115). Upon completion of engulfment, yet another sigma factor cascade is initiated. Within the forespore, $\sigma^G$ is activated which initiates formation of the final proteins for the forespore such as the coat proteins as well as activating $\sigma^K$ in the mother cell which results in the eventual lysis of the mother cell to release the completed forespore (116-117). Thus, sporulation relies upon is a series of sigma factor cascades that activate the next necessary step until formation of the endospore is complete (Figure 3).

**Bacillus plasmids and TubZ**

Encoded on the plasmid pBtoxis in *Bacillus subtilis* is the operon tubRZ and these two gene products are thought to constitute another plasmid segregation system. The properties of TubR will be examined in this work and so it is necessary to discuss *Bacilli* bacteria and their plasmids. A well known member of this class is *Bacillus anthracis*, the causative agent of anthrax. *B. anthracis* exerts its pathogenicity via virulence factors located on its plasmids, pOX1 which encodes the various exotoxins while pOX2 encodes factors necessary for the production of a capsule which protects
the bacteria from the immune system (118). There are three major exotoxins produced, the protective agent, the edema factor, and the lethal factor, and all three are necessary for virulence as these combine to form binary toxins (119). *Bacillus anthracis* shows the importance of plasmids to bacterial pathogenesis, as loss of either plasmid (pXO1 or pXO2) results in avirulence.

*Bacillus thuringiensis* is most well known for the crystal toxins encoded by its plasmids. The most well studied of this group is *Bacillus thuringiensis* subsp. *israelensis* which contains its crystal toxin on the 128 kilobase plasmid pBtoxis. Crystal toxins encoded on this plasmid are lethal to the larvae of certain species of mosquitoes, fungus gnats, and blackflies while toxins of other subspecies affect different insects (120). The first half of this work will focus on two genes encoded this plasmid, *tubR* and *tubZ* which are required for this plasmid to be stably maintained.

**Phage**

Viruses that infect bacteria are called bacteriophage and can be either lytic or lysogenic. Each of the pathways begins in the same way with the bacteriophage attaching to the host cell and injecting its DNA into the host through its tail (Figure 4). In the lytic cycle, various genes are turned on and off according to a strict regulation program where immediate early, early, and late genes are regulated in order to promote synthesis and assembly of new viral particles. Once these viral particles are assembled, the host cell is lysed to release new phage into the environment to find and infect new host cells. In the lysogenic cycle, the injected DNA integrates with the host
chromosome to form a prophage or replicates freely as a plasmid. All genes except for a few are usually turned off and the phage DNA effectively lies dormant. The advantage to this dormancy is that each replication of the host cell DNA also replicates the phage DNA ensuring that it is passed on to daughter cells. Eventually, the lysogenic cycle can switch to the lytic cycle (121).

One example of a phage that replicates as a plasmid when it is a lysogen is P1. During its lysogenic phase in E.coli P1 replicates as a plasmid with a copy number of about 2 per chromosome. As a result, it requires a segregation system to be stably maintained. As described earlier, P1 relies upon a ParA type segregation system. However, no other types of cytoskeletal proteins have been characterized from bacteriophage. Later in Chapter III, I will describe the identification and characterization of a new family of actins within phage 0305phi8-36 which infects Bacillus thuringiensis. It is a rather novel and unusual phage and became of interest due to an actin-like protein, gp207, within its 220kb genome.
Figure 1. Modes of Actin Polymerization. Growth of an actin polymer typically occurs from the (+) end and these subunits contain ATP. Shrinkage occurs from the (-) end and these subunits contain ADP. Initial growth requires nucleation. Polymerization can be increased through severing of filaments or decreased by capping proteins.
**Table 1. Actin/Tubulin Homologues and their Function**

<table>
<thead>
<tr>
<th>Protein</th>
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<td>Cell shape, chromosomal segregation</td>
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<tr>
<td>Mbl</td>
<td>Cell shape</td>
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<tr>
<td>MamK</td>
<td>Alignment of magnetosomes</td>
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<td>Plasmid Segregation (Actin-Like ATPase)</td>
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<td>Cell Division</td>
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<td>Plasmid Segregation of pBtoxis</td>
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<td>Gp207</td>
<td>Present in B. Thuringiensis Phage; Unknown</td>
</tr>
</tbody>
</table>
**Figure 2: Plasmid Segregation Systems.** All known plasmid segregation systems usually fall into one of two categories: Walker-Type or Actin-like. The classification is based on the type of ATPase in the system. TubZ is a novel tubulin-like GTPase.
### Table 2. Post Segregational Killing Systems

<table>
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<tr>
<th>Locus</th>
<th>Toxin</th>
<th>Anti-toxin</th>
<th>Mechanism</th>
<th>Source</th>
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<td>ccdB</td>
<td>ccdA</td>
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<td>F plasmid</td>
</tr>
<tr>
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<td>doc</td>
<td>phd</td>
<td>TA proteins</td>
<td>P1 plasmid</td>
</tr>
<tr>
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<td>vapC</td>
<td>vapB</td>
<td>TA proteins</td>
<td>Virulence plasmid</td>
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<tr>
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<td>sok</td>
<td>Antisense RNA</td>
<td>Plasmid R1</td>
</tr>
<tr>
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<td>mazF</td>
<td>mazE</td>
<td>TA proteins</td>
<td>Mycobacterium tuberculosis</td>
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<td>RelE</td>
<td>RelB</td>
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<td>higB</td>
<td>higA</td>
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</tr>
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<td>parE</td>
<td>parD</td>
<td>TA proteins</td>
<td>RK2 plasmid</td>
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<tr>
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<td>hipA</td>
<td>hipB</td>
<td>TA proteins</td>
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<td>RNA II</td>
<td>Antisense RNA</td>
<td>Plasmid pAD1</td>
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</tbody>
</table>

Legend: TA= toxin anti-toxin, RNA=ribonucleic acid
Figure 3. Sporulation in *Bacillus subtilis*. Two main sigma factor cascades occur during sporulation. At completion of polar septation, $\sigma_F$ is activated in the forespore and this in turn activates $\sigma_E$ in the mother cell. At completion of engulfment, a second cascade is initiated where $\sigma_G$ is activated in the forespore which leads to $\sigma_K$ activation in the mother cell. Figure is adapted from (122).
Figure 4. Life Cycle of a Phage. A phage can either enter the lytic cycle where formation of new viral particles is initiated immediately, or the lysogenic cycle which is a dormant stage. Lysogenic phage will eventually enter the lytic cycle.
References


CHAPTER II

TubR Regulates TubZ Levels via Binding to an Upstream region of DNA
Introduction

*Bacillus thuringiensis* is capable of producing crystal toxins during sporulation which are toxic to various insects. Different subspecies synthesize different crystals which in turn are effective against different subsets of insect larvae (1). *Bacillus thuringiensis* subspecies *israelensis* is effective against mosquito and blackfly larvae. Its crystal toxin results from major endotoxin proteins, Cry11A, Cry4A, Cry4B, and Cyt1A that are encoded on the large plasmid pBtoxis (1).

TubZ is a gene on pBtoxis which is of interest due to its distant homology to both FtsZ and tubulin, cytoskeletal proteins of marked importance. This relationship was evident as the protein contains conserved regions common to both FtsZ and tubulin. Roles for FtsZ in cell division have been extensively confirmed (2-4). However, TubZ is likely not a cell division protein as it lacks conserved C-terminal motifs which mediate interactions with other cell division proteins. FtsZ associates with ZipA and FtsA through these sequences (5-6). Such conservation of TubZ to FtsZ but distinct lack of a conserved C-terminal region led to the examination of TubZ to determine if it could form polymers. Fusions of TubZ to GFP were constructed under control of the native promoter and shown via microscopy to form polymers which extend the length of the cell (Figure 2). The observed polymers were shown to be dynamic via FRAP as well as shown to treadmill *in vivo* within *B. thuringiensis* and *E. coli* (7). The presence of polymers in both *B. thuringiensis* and *E. coli* indicated that proteins specific to *B. thuringiensis* were not essential for polymer formation. Additionally, mutation of a conserved region within the T7 loop resulted in polymers
losing their dynamic properties which led to loss of pBtoxis in *B. thuringiensis* when it is normally very stably maintained (7). This indicates a role for TubZ in efficient replication or segregation of pBtoxis.

The role of TubZ in plasmid maintenance was also examined for a role in plasmid replication. This gene in conjunction with an upstream gene, TubR, was reported to be sufficient to create a minireplicon, the minimum region of DNA needed for replication of a plasmid (8).

The ability of TubZ to assemble polymers suggested that it could represent a plasmid segregation system. The upstream TubR contains a helix-turn-helix motif characteristic of DNA binding proteins. Together TubR and TubZ are reminiscent of plasmid partitioning loci that always contain a DNA binding protein and a filament forming ATP or GTPase.

All known plasmid partitioning loci contain two *trans*-acting genes and one *cis*-acting centromere-like site at which the proteins act (9-12). In order for efficient plasmid segregation to occur, all three parts are necessary (13-15). In addition, the levels of each protein are essential for proper function (16-18). The first gene in these systems is usually a Walker-type ATPase or an actin-like ATPase (19). The second gene is a DNA binding protein that recognizes specific sites within a centromere-like region (20-23). For example, SopB binds to the *sopC* region of DNA and serves a point of assembly for SopA. Another example is the well-characterized actin-like ATPase ParM of the R1 plasmid. ParM is responsible for filament formation and ParR is the DNA binding protein which binds *parC*, which is a centromere-like site (27-28).
ParR bound to parC stimulates the ATPase activity of ParM (29). Additionally, ParM assembles filaments that are stable when bound to ATP and unstable when bound to ADP giving it properties of dynamic instability. This data led to a proposed model whereby ParM filaments form spontaneously within the cell cytoplasm but ATP hydrolysis leads to instability and therefore degradation. However, if a filament encounters a plasmid, the ParR-parC complex on the plasmid is able to stabilize the filament and insertional polymerization leads to the separation of the plasmids (30).

TubZ is a GTPase and does not fall into either the Walker-type ATPase or the actin-like ATPase family. If TubZ does indeed represent a plasmid segregation system, it forms a new class of tubulin-like cytoskeletal proteins. It therefore also likely has a DNA binding protein, as in the other segregation systems. Here, I present evidence that TubR is, indeed, a DNA binding protein that regulates expression of TubZ.

Results

TubR Downregulates TubZ Levels In Vivo

To determine if TubR is a DNA binding protein that regulates expression of the tubRZ operon, I began by characterizing TubZ-GFP expression when tubR was deleted. A plasmid was constructed which contained an inframe deletion of tubR in the tubRZ operon and then I measured TubZ-GFP expression by Western blot analysis. These experiments were performed in E.coli since this mutation could not be moved onto the native pBtoxis plasmid. A second compatible plasmid was also placed in the
strain to test if *tubR* could complement the deletion mutation *in trans* when expressed from the arabinose promoter.

Strains containing the wild type or the *tubR* deletion were grown with or without expression of TubR for 3 hours. At this point, samples were collected and normalized so the cell count would be identical in each sample. The samples were lysed and run on a polyacrylamide gel. Proteins from the gel were transferred to a nitrocellulose membrane and a western blot detecting TubZ-GFP was performed (Figure 3) using anti-GFP antibodies. Strains which did not express TubR showed much higher (4.5x) levels of TubZ-GFP expression. When TubR was provided back on a separate inducible plasmid, TubZ-GFP levels were shown to be expressed at normal levels. Controls using empty pBad33 vector lacking the *tubR* gene, as well as strains without a *tubR* deletion were used to show that this regulation in TubZ-GFP levels is a result of increased levels of TubR and not some other factor. This result lends evidence to TubRZ as an operon regulated by TubR. The mechanism of regulation is explored further in Chapter II.

**Purification of TubR**

In order to explore the function of TubR as a possible DNA binding protein, it first had to be purified. The TubR protein was tagged with six histidines at its C-terminus through use of the Pet28 vector. This vector was chosen as the histidine tag can be exploited for purification through use of a nickel resin. Once this plasmid was constructed, it was used to transform BL21 (DE3) cells which were plated on
kanamycin 10µg/mL. To induce expression of TubR, the cells were grown in a 37°C water bath until an OD600 of about 0.8 (this usually took about 4 hours) and then the culture was induced with IPTG (0.01 mM final). After induction the cells were grown for an additional 3 hours at 37 degrees. The culture was collected via high speed centrifugation and stored at -40°C until needed. The lysis and purification steps were carried out as stated in the materials and methods. Imidazole concentrations chosen for purification were based on a purification done using a gradient ranging from 25mM imidazole to 175mM (Figure 4). Imidazole outcompetes the TubR-His for binding to the resin and so its concentration in the solutions is critical to the purification process. After purification, a polyacrylamide electrophoresis gel was run to examine the success of the purification and which fractions contained the most protein (Figure 5). One thing to note is that the product being purified appears to be a lower molecular weight than the induced protein. The purification was done in a cold room but it is possible proteases may be degrading the protein.

**TubR as a DNA Binding Protein**

In order to examine a possible DNA binding region for TubR, three pieces of DNA were obtained for initial testing. All three pieces contained the tubR region of DNA plus a variable amount of DNA upstream. The amount of upstream taken was 600, 400, or 200bp in addition to the 300bp tubR region.

The protein used for the shift assays was first obtained via purification with a nickel column as previously mentioned. The protein was then dialyzed in 50mM
NaPO₄, 300mM NaCl buffer in order to remove imidazole from the sample. The final protein concentration was rather low at 3.02 x 10⁻¹¹ moles/uL. Each DNA fragment was incubated with varying protein concentrations and loaded on a 2% agarose gel. At higher protein concentrations, migration of the DNA fragments was retarded and this was the case for all three DNA fragments used. Detection of the DNA band was done using Ethidium Bromide.

In order to ensure that the small observed shift was due to the protein TubR and not some other factor, a control was done using BSA as the protein instead of TubR. The BSA stock was properly diluted to the same molarity as TubR and the shift assay was carried out as stated in Materials and Methods. Even at the highest concentrations of BSA, no effect on migration of the DNA was detected (Figure 6).

Ideally the DNA fragment used in these types of assays should have been very small, roughly 50bp. Detection of DNA should have been done through a more sensitive method such as using radioactive nucleotides rather than ethidium bromide. Such assays should also be done using polyacrylamide rather than agarose. Eventually, trying to conclusively prove TubR was a DNA binding protein came to an end as the work was published from a different lab. Using DNA labeled with γ⁻³²P, the Federici lab ran assays to determine if TubR binds to DNA and if so what region it binds. They performed a competition assay showing that TubR specifically binds a 48bp sequence upstream of the *tubR* gene. TubZ does not bind to this region of DNA. When TubR and TubZ were both added, an even greater shift of the DNA was detected. Taken
together, their results indicate that TubR is a DNA binding protein with a specific binding site and it forms a complex with TubZ (31).

I also attempted to study the role of TubR \textit{in vivo}. I was specifically interested in trying to determine if I could create a TubR-GFP fusion that was functional, and if so, could I use it as a tag for visualizing the subcellular location of pBtoxis within the cell. If TubR is a DNA binding protein and binds somewhere upstream of \textit{tubR}, a TubR-GFP fusion might form small foci within the cell at the location of pBtoxis plasmid as this plasmid would contain a binding site for TubR. For this reason I constructed a plasmid containing inducible TubR-GFP and transformed it into \textit{Bacillus thuringiensis} to make JP6211. Additionally, an isolate of \textit{Bacillus thuringiensis} lacking pBtoxis was also transformed with the construct to make JP6217. We hypothesized that there would be small discrete GFP foci in JP6211 as TubR-GFP would have a binding site on pBtoxis. We also hypothesized that JP6217 would lack foci due to the lack of pBtoxis and therefore lack of a binding site but did expect to see non-specific coating of the chromosome common to DNA binding proteins. However, foci were seen in both cell types and the number of foci did not differ (Figure 7).

The unexpected result of foci in both strains could be explained by inclusion bodies or by TubR being rendered nonfunctional by the GFP fusion. In order to discern between these two results, a functionality assay was performed to see if TubR-GFP functioned in the cell in the same way as TubR. TubR-GFP was moved into pBAD33, a vector induced by arabinose that would allow for chloramphenicol selection and be compatible with the plasmids containing the \textit{ΔtubRubZ-GFP} and
tubRtubZ-GFP reporters. Cultures were grown with and without induction of TubR or TubR-GFP and normalized so each sample would contain the same amount of cells. Samples were boiled with SDS and loaded on a 12% polyacrylamide gel. A western blot was performed using anti-GFP antibodies and it was shown that TubR-GFP downregulated TubZ-GFP levels (Figure 8). Since this is the same result obtained with TubR as discussed in Chapter II, we conclude that the TubR-GFP fusion is functional and working as intended.

**TubR Overexpression Destabilizes pBtoxis**

In order to gain further evidence that TubR was important for pBtoxis stability, I examined the effect of overproduction of TubR. In many other plasmid segregation systems, overproduction of the DNA binding protein greatly destabilizes the plasmid. If TubR was part of plasmid stability system, then its overproduction might also cause pBtoxis to be lost from the cell.

I therefore measured the effect of overexpression of the *tubR* gene from a multicopy plasmid. A previously made construct containing inducible TubR-GFP was utilized to overexpress TubR. Two strains of *Bacillus thuringiensis*, one containing the pWH1520 empty vector and one containing pWH1520 with TubR-GFP, were grown in LB with or without 0.2% xylose, and cultures were allowed to grow and then sporulate. Sporulated cultures were treated with 65°C heat for 30 minutes to kill vegetative cells. I then plated the spores onto LB with tetracycline 10μg/mL to allow the spores to germinate. Plates were incubated overnight at 30°C and individual
colonies were picked for use in colony PCR to determine if plasmid pBtoxis was still present. A representative example of results can be seen in Figure 9. It was observed that the empty vector control as well as the TubR-GFP strain in uninduced conditions had 0% loss of pBtoxis while 5.2% loss was seen when TubR-GFP was induced (Table 1). Under normal conditions, pBtoxis is stably inherited and no loss is observed so 5.2% destabilization can be considered significant. This result provides additional support for the idea that TubR is important for plasmid stability along with TubZ.

**Discussion**

TubZ was first examined due to its homology to FtsZ and tubulin and its ability to stabilize the plasmid pBtoxis in conjunction with TubR. Levels of TubZ are down-regulated in response to production of TubR and this was shown via detection of TubZ-GFP by a western blot. In order to further analyze TubR and its mechanism of action, it was purified through use of a nickel column and a 6-His tag. DNA binding properties of TubR were examined *in vitro* by incubating DNA and TubR protein and conducting gel electromobility shift assays to determine if the band of DNA shifts due to complex formation with the protein. A shift was observed but more sensitive detection methods were needed to conclusively determine a region of binding. Eventually the binding properties of TubR as well as its region of binding was discovered and published by another lab. To gain additional information about the role of TubR *in vivo*, I created TubR-GFP and demonstrated that it was likely to be functional. I then determined if TubR-GFP could be used to detect the presence of
pBtoxis within the cell. Unfortunately, the TubR-GFP was found to form inclusion bodies. Finally, overexpression of tubR was shown to cause loss of pBtoxis from the cell, providing the first in vivo evidence that TubR is important for pBtoxis stability in vivo. Taken together, these results demonstrate that TubR and TubZ constitute a possible plasmid segregation system though the mechanism is unclear. A possible segregation model of how TubZ causes plasmid partitioning is shown in Figure 10. This model suggests that TubR is responsible for anchoring the (-) end of the TubZ filament to the plasmid. The directional growth of TubZ at the (+) end is thought to be the force pushing the plasmids apart.
Figure 1. TubZ is Distantly Related to Both FtsZ and Tubulin. (A) TubZ is distantly related as it branched off early on in evolutionary history. (B) TubZ shares some of the conserved domains represented in gray in the loop segments characteristic of tubulin and FtsZ. Figure borrowed with permission from (7).
Figure 2. TubZ-GFP Forms Polymers in *Bacillus thuringiensis*. When TubZ was fused to GFP under the native promoter, it was observed to form dynamic polymers. This is a modified version from (7).
Figure 3. TubR Downregulates TubZ-GFP. When *E. coli* has a plasmid containing *tubZ* and not *tubR*, TubZ levels are high. When *tubR* is complemented back, TubZ levels decline. Controls show that induction of pBad33 alone has no effect. Numbers reflect band intensity relative to background.
Figure 4. Imidizole Elution Gradient. A gradient ranging from 75mM to 250mM imidazole was used to determine what concentration was necessary to obtain optimum yield and purity of TubR. Numbers represent imidazole concentration at each step. CL represents cell lysate while MW represents molecular weight ladder.
Figure 5. Purification of TubR-His. This polyacrylamide gel shows the stages of a successful TubR-His purification. Fractions of elute were collected in 1mL increments and run on the gel to determine which fraction contained the most protein. It is apparent that TubR is being induced in the cell lysate (CL+) but a protease appears to be active as what is seen in the wash (W) and elution (E) fractions is a smaller molecular weight. Elution is done using 250mM Imidazole.
Figure 6. TubR is a DNA Binding Protein *in vitro*. TubR is able to shift 100ng of a 1kb DNA fragment containing *tubR* and 600np upstream. BSA is not able to shift this same fragment of DNA indicating that TubR is responsible for the observed shift.
Figure 7. TubR-GFP Forms Foci in *Bacillus thuringiensis*.
Bar, 1µm. (A) *Bacillus thuringiensis* with TubR-GFP shows numerous foci within the cell (B) *Bacillus thuringiensis* lacking pBtoxis also shows numerous foci when TubR-GFP is expressed.
Legend: + = induction with 0.1% arabinose

**Figure 8. TubR-GFP is a Functional Fusion.** A Western Blot detecting GFP shows bands at about 100kDa corresponding to TubZ-GFP. In the presence of TubR-GFP, TubZ-GFP levels are downregulated.
Figure 9. The Plasmid pBtoxis is Destabilized by Overexpression of tubR. The larger PCR product (labeled pBtoxis) was amplified from pBtoxis and the smaller PCR product (labeled chromosome) was amplified from the *B. thuringiensis* chromosome as a control. This is a representative agarose gel from PCR analysis of JP6211 (expressing wild-type TubZ-GFP with TubR overexpressed).
Table 1. Overexpression of *tubR* Leads to Destabilization of pBtoxis. Colony PCR was conducted on germinated spores to examine loss of pBtoxis in *Bacillus thuringiensis* during sporulation condition. Cultures were either induced with 0.1% arabinose or grown without induction.

<table>
<thead>
<tr>
<th></th>
<th>2248+</th>
<th>2248-</th>
<th>6211 +</th>
<th>6211-</th>
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<td>0%</td>
<td>0%</td>
<td>5.2%</td>
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</table>
Figure 10. Model for Plasmid Segregation by TubZ. The (-) end of the TubZ filament attaches to the plasmid via TubR. These subunits are bound to GDP. New subunits are added on to the (+) end and are bound to GTP. Directional growth at the (+) end allows the plasmids to be pushed apart. Borrowed with permission from Joe Pogliano.
Material and Methods

TubR-GFP construction

*TubR* was amplified from pRL196 using PCR and the primers BtTubRKpnstart and BtTubRClaINoStop. The fragment was digested with KpnI and ClaI and ligated into the vector pMutinGFP. *TubR-gfp* was then amplified from this pMutin construct via PCR and the primers TubRKpnStart and BT156-34. The fragment was digested with KpnI and inserted in the vector pWH1520. Plasmid DNA was extracted via miniprep (Qiagen) and correct orientation of *tubR-gfp* in the vector was confirmed via a restriction digest using the enzyme BamHI. Sequencing was conducted by Eton.

Growth of Cultures for Colony PCR

*Bacillus thuringiensis* containing pWH1520 (JP2248) and *Bacillus thuringiensis* containing pWH1520 + *tubR-gfp* (JP6211) were grown in LB with tetracyline 10µg/mL. The cultures were allowed to grow for 48 hours before collection allowing for sporulation after 15 generations. Each strain was grown in both uninduced and induced (0.2% xylose) conditions. After collection, the cultures were heat killed through heating for 30 minutes at 65 degrees Celsius. Spore dilutions were made using 1X Tbase. A volume of 100uL from the $10^{-5}$ and $10^{-6}$ titers were plated on LB tetracyline 10µg/mL which was incubated overnight at 30°C.

Colony PCR

Individual colonies were picked off a plate and suspended in 50uL of deionized water. These suspensions were microwaved for 2 minutes and served as the source of genomic DNA in the PCR reaction. Primers Bt036-1 and Bt036-2 were used to test for
the presence of pBtoxis. A positive control using primers trxBf and trxBr were used to ensure the PCR was working properly.

**His-Tagging**

*TubR* was amplified off of the plasmid pBtoxis via PCR using the primers BtTubR1 and BtTubR2. This fragment was digested with EcoRI and NdeI and ligated into the vector Pet28. Pet28 contains the means to add a six histidine chain to the C-terminus of TubR and the means to induce protein production using IPTG. This tag is useful as it will be bound by a nickel resin and exploited for purification.

**Purification Via Nickel Resin**

Pelleted culture was thawed on ice and resuspended using 5 volumes of Lysis/Wash buffer. This buffer consists of 50mM NaPO4, 300mM NaCl, 10mM Imidazole. The culture was then lysed by adding 1/10 volume lysozyme (20mg/mL) and completing five freeze/thaw cycles. Freeze cycles were done via an ethanol and dry ice bath while thaw cycles were done in a 37 degree Celsius water bath. DNase (10ug/mL final) and MgCl2 (10mM final) were added and one more freeze/thaw cycle was undertaken. The homogenate was then spun in a Sorval centrifuge for 15 min at 4000g. The supernatant was then added to Nickel Resin (Sigma). The supernatant and resin were allowed to incubate with gentle rocking overnight. The resin was then poured into a 10mL BioRad column and allowed to settle for 30 minutes in a cold room kept at 4°C. The level of liquid was allowed to drain to the beadline and the eluate was collected as flowthrough. Three washes of two column volumes were performed. Wash 1 contained 10mM imidazole. Wash 2 and 3 contained 75mM imidazole. Finally, an
elution was performed using two column volumes of elution buffer and the eluate was collected in 1mL fractions. The elution buffer consisted of 50mM NaPO4, 300mM NaCl, 250mM imidazole.

PAGE

A 17% polyacrylamide gel was made and run using Biorad equipment. The samples were boiled at 100 degrees Celsius for 10min with 2x SDS sample buffer before loading. A volume of 15uL from each sample was loaded in each lane and the gel was run at 200V for 45 min. The gel was stained for protein using Coomassie Blue.

GEMSA

All three DNA pieces to be used in the gel electrophoresis mobility shift assay contained the region encoding the likely tubRZ promoter as well as upstream DNA. The largest fragment, 900bp, was made using primers Bt1 and TubR2. The middle fragment, 700bp, was made using primers Bt22 and TubR2. The shortest fragment, 500bp, was made using primers Bt23 and TubR2. Initial shifts were done using 100ng DNA, 1uL of 5mM DTT, and a variable amount of protein ranging from 3.02x10-11 moles up to 3.02 x 10-10 moles. The sample was then diluted with water to a volume of 30uL. 5uL of Loading Dye was added to the sample and it was loaded on an agarose gel in its entirety.

Microscopy

Each strain containing tubR-gfp was grown in liquid LB tetracyline 10µg/mL 0.5% xylose for 3 hours at 30°C. 1mL was pelleted and resuspended in 100uL of LB containing 1 µg/mL of FM 4-64. 3uL of this suspension was placed on a slide with
polylysine and examined for fluorescence. Images were collected using a DeltaVision optical sectioning microscope (Applied Precision). (32)

Construction of Strains for TubR-GFP Functionality Assay

*TubR-gfp* was amplified off plasmid pPAU7 using primers TubRKpnStart and BT156-34. The fragment was digested with KpnI and ligated into pBad33. Plasmid DNA was extracted via miniprep (Qiagen) and correct orientation of *tubR-gfp* was confirmed through a restriction digest using SacI. *E. coli* was transformed with *tubR-gfp* in pBAD33 along with ∆*tubRtubZ* in pMutinGFP. An additional *E. coli* strain was made using the *tubR-gfp* in pBad33 and *tubRtubZ* in pMutinGFP. A control strain contained pBAD33 empty vector and ∆*tubRtubZ* in pMutinGFP. All strains made had ampicillin and chloramphenicol resistance.

Growth of Strains for Functionality Assay

One colony from a plate was used to inoculate 1mL of LB cam30µg/mL amp100µg/mL. This culture was grown for 1 hour at 37 degrees Celsius. 0.4mL of this culture was transferred into 4.6mL LB cam30µg/mL amp100µg/mL either with or without 0.1% arabinose. Cultures were then grown for 3 hours. At this point, samples were collected via centrifugation and resuspended in 50mM HEPES, 150mM NaCL in such a way that each sample would contain the same number of cells.

Western Blotting

A 17% polyacrylamide gel was loaded and run at 200V for 40 minutes. The proteins on the gel were then transferred to a nitrocellulose membrane in a tank blotter (BioRad) at 100V for 1 hour. Membrane was blocked in TBST + 5% milk. TubZ-GFP was
detected using mouse anti-GFP as the primary antibody (1:500 from Zymed) and anti-mouse IgG Cy5 conjugate as the secondary antibody (1:1000 from Jackson ImmunoResearch). Detection was done a Typhoon Imager (GE Healthcare). The TubR-GFP Functionality Assay was using mouse anti-GFP primary antibodies (1:500 from Zymed) and anti-mouse HRP secondary antibodies (1:1000 from Amersham). Detection was done using ECL and film.

**Bacillus thuringiensis** Transformation Protocol

A colony was used to inoculate 250mL LB and the culture was grown to an OD of 0.8 at 37°C. Collection of cells was done in a 1L filtration unit and three 25mL washes were done with cold e-poration buffer pH 7. Collected cells were then resuspended in 4mL cold eporation buffer and used for transformation. DNA was added and the culture was stored on ice for 15minutes before electroporation with a Biorad apparatus. Recovery of cells was done in SOC 10% glycerol for 1 hour at 37°C.

**E. coli** Transformation Protocol

An overnight culture of *E. coli* was grown in 5mL LB at 37°C and 0.5mL of this culture was used to inoculate 500mL LB in a flask. The culture in the flask was grown at 37°C until the culture reached an OD600 of 0.65. The flask was chilled on ice for 30 minutes and collected via high-speed centrifugation in a Sorval. The first two resuspension stages were in ice cold 1mM Hepes pH 7 at with 1 volume and then 0.5 volumes. The last two resuspension stages were in cold 10% glycerol at 1/50 and 1/500 volumes. Each centrifugation step was done for 15min at 4000g. To transform, DNA was added to 40uL cells and electroporated at 25µF and 2.5kV in a BioRad
Gene-pulser Xcell using 0.2-cm cuvettes (Genesee). Recovery of cells was done for one hour in LB at 37°C.
References


CHAPTER III

*Bacillus thuringiensis* phage, 0305phi8-36, and the actin-like protein Gp207
Introduction

Bacteriophage are phage that specifically infect bacteria. Just like normal phage they can engage in either a lytic or lysogenic cycle. Those that follow the lysogenic pathway are often called temperate phage. Each cycle begins with attachment of the bacteriophage to the host and injection of genetic material. In the lytic cycle, the DNA is immediately used to synthesize and assemble new viral particles. In the lysogenic cycle, the DNA integrates into the host chromosome and lies dormant while being passed on to daughter cells when the chromosome in replicated. Unknown factors eventually trigger the DNA to excise itself from the chromosome and enter the lytic cycle.

0305phi8-36 is a bacteriophage whose preferred host is *Bacillus thuringiensis*. This phage has a 220kb genome that was sequenced and the virion proteins were identified (1). It was discovered during soil sampling to identify large, unusual phages of little known varieties (2). However, 0305phi8-36 page are unusual in that they can form plaques only in ultra-dilute gels, are prone to aggregation, and have a long contractile tail which contains three large corkscrew shaped fibers projecting from the baseplate (3) Aggregation properties are important as they are often linked to the potential to cause disease as well as potential for bioremediation (4-5). A new method of *in situ* fluorescence microscopy was employed to show that this *Bacillus thuringiensis* phage does indeed form aggregates (6). Attempts have been made to identify the reason behind these aggregations and it is hypothesized that the bacteriophage have evolved to maintain a high host finding rate in a trade-off with
maintaining high rate of progeny production (7). In this way the formed aggregates seed future growth.

Examining the genome sequence of 0305phi8-36 led to the identification of Gp207 as an actin-like protein. This gene was flanked by two small putative open reading frames which could form an operon with gp207 and contribute to its unknown function for the phage (8). Characterization of Gp207 and its flanking genes is discussed below.

**Results**

When the genome of phage 0305phi8-36 was published, Gp207 was identified as an actin-like protein which led us to take interest in what this protein could be doing for the phage. To understand how Gp207 functions, we constructed a plasmid containing a gfp fusion to the C-terminus of gp207 and transformed *E. coli* with this construct. Gp207-GFP was shown via microscopy to form a filament reminiscent of actin in vivo (Figure 1a). However, this filament appeared to be static as no movement within the cell was detected.

In order to examine the filament further, a *Bacillus subtilis* strain PY79 was transformed with the plasmid containing gp207-gfp. We were unable to get the plasmid into *Bacillus thuringiensis* and so a related *Bacillus* species was chosen for transformation. Though the protein still formed a filament in vivo (Figure 1b) it appeared to be static within the cell as seen with time-lapse photography (Figure 1c). A disadvantage to the vector pMutinGFP is that expression within *E.Coli* and *Bacillus*
subtilis is constitutive, meaning that protein production is always turned on. For this reason, gp207-gfp was moved into a xylose inducible vector so that cellular levels could be controlled. Even at low levels of expression (0.01% xylose) the filament remained static and this prompted us to take another look at the phage genome and examine the upstream and downstream genes.

The next step was to attempt to recreate the system that would make this filament dynamic. For this reason, the upstream and downstream open reading frames were chosen for greater evaluation. Three genes, gp206-gp208, were amplified off genomic DNA using PCR. After amplification, the region was cloned into pDG1664, a vector used for chromosomal integration at the ThrC locus within Bacillus subtilis. The vector was moved into Bacillus subtilis and transformants were screened for integration of the plasmid. Stable isolates were obtained and made competent so that the strain could be transformed with the gp207-gfp inducible construct. Genomic DNA was isolated from transformants in order to ensure the plasmid had not integrated onto the chromosome. This was checked by designing primers that would only form a product if the plasmid had integrated. A positive control was included in the reaction. Recombination was a possibility that had to be checked due to the gp207 region being present on both the chromosome and the plasmid representing a 1kb region of homology. Successful clones were grown on agar pads without antibiotics but with xylose present for induction. Even with the chromosomal integrant, the observed filaments were still static (Figure 2a,b). Such a result left two possibilities. Either the system required to make the filament static had not yet been reconstructed, or the cells
were unable to recognize the phage promoters for the chromosomally integrated genes and they were thus not being transcribed.
Figure 1. Gp207 is a Distant Relative of Known Actin Families. Families of eukaryotic actin, MreB, and MamK are relatively related as they cluster near one another on the tree. Gp207 falls at a distance from these groups indicating its distant relationship.
Figure 2. Gp207-GFP Forms Static Polymers in vivo within *E. coli* and *B. subtilis*

Bar, 1µm. (A) Gp207-GFP expressed in *E. coli* forms filaments in vivo which span the length of the cell. (B) Gp207-GFP expressed in *B. subtilis* (PY79) forms filaments in vivo. (C) The filaments formed within *B. subtilis* are static as indicated by time-lapse photography. Images are taken 12 seconds apart for 1 minute.
Figure 3. Gp207 Filaments Remain Static when Complemented with Gp206-208 via a Chromosomal Integration at ThrC. Gp206-208 was integrated at the ThrC locus in the *Bacillus subtilis* chromosome. An inducible plasmid containing Gp207-GFP allowed for visualization of the filament. Bar, 1µm. (A) Gp207-GFP forms filaments in *Bacillus subtilis* (B) The filaments formed within *Bacillus subtilis* are static as indicated by time-lapse photography. Images are taken 15 seconds apart for 1.25 minutes.
Materials and Methods

GFP Tagging

Gp207 was tagged at its C-terminus with GFP through use of the vector pMutinGFP. Amplification of *gp207* was achieved from genomic DNA via PCR using primers Orf207KPN and Orf207CLA. The PCR product was digested with KPNI and CLAI and then ligated into pMutinGFP. *E.Coli* was transformed with the plasmid and plated on ampicillin 100µg/ml. Plasmid DNA was isolated from successful transformants via miniprep (Invitrogen) and sequenced (GeneWiz).

Inducible Promoter Construction

In order to place Gp207-GFP under inducible control, the region was amplified off the pMutinGFP vector which contained *gp207* as its insert. This was done via PCR using the primers Orf207KPN and Bt156-34. The fragment was digested with KPNI and ligated into pWH1520. *E. coli* was transformed with the plasmid and plated on Ampicillin 100µg/mL. Plasmid DNA was isolated from successful transformants via miniprep (Invitrogen) and sequenced (GeneWiz). Prior to sequencing, a restriction digest with BstEII was done to ensure proper orientation of the insert since this was not a forced cloning. Additionally, a digest with SpeI was done to ensure the culture contained a single plasmid type.

Creation of pDG1664 Construct

Amplification of *gp206-208* was accomplished via PCR using genomic DNA and primers FwdBam208 and RevHind206. The fragment was digested with BamHI and
HinDIII and ligated into pDG1664. *Bacillus subtilis* was transformed with the plasmid and the culture was plated on erm 10µg/mL.

**Screening for pDG1664 Recombination**

Integration success can be determined by patching transformants onto spectinomycin 100µg/ml and erythromycin 10µg/mL. Isolates should be spec sensitive and erm resistant. Colonies showing this phenotype were then patched to minimal media plates with and without threonine. If integration was successful, the bacteria should only grow on minimal media plates that have been supplemented with threonine.

**Growth of Strains on Agar Pads**

Pads containing ¼ LB, 0.5% agar, and 0.5 µg/mL FM 4-64 were made. Strains were placed on these pads from LB tet10 µg/ml plates and allowed to grow at 30 degrees Celsius for 2 hours before examination.

**Bacillus subtilis Transformation Protocol**

A colony was used to inoculate 2mL of SpC. The culture was grown at 37 degrees Celsius for 5 hours and transferred to 18mL of prewarmed SpII. After an hour of growth at 37 degrees Celsius the culture was pelleted for 8minutes at 4000rpm and resuspended in 1.6mL of the supernatant after pelleting. 0.4mL of 50% glycerol was added to this suspension and the cells were ready for transformation. An appropriate amount of DNA was incubated with 0.3mL cells and 0.3mL Tbase-EGTA. The culture was incubated for 30 minutes at 37 degrees Celsius and plated on selective media.
References


CHAPTER IV

Future Experiments
It would be interesting to see if simply getting the *gp207-gfp* plasmid into *Bacillus thuringiensis* would confer dynamic properties on the filament. Even if this was not the case, *B. thuringiensis* with the plasmid could serve as an infection model. The bacteriophage could be obtained to be grown in the lab and used to infect *B. thuringiensis*. In this way, the filament could be observed in process of infection. Ideally, Gp207-GFP would coassemble with wild type Gp207 produced from the phage chromosome and the properties of the filament could be observed during infection. Should dynamic properties be conferred on the filament during infection, this would show that either the necessary genes to reconstitute the system have been provided, or that the filament is only dynamic during active infection.

Additionally gp207 could be mutated on the phage DNA and the titer of the phage could be observed. If it is changed in some way, perhaps the function of the protein relates to the infection process.

Perhaps the protein does not need to be dynamic in order to serve its function. It is possible that a static polymer could serve as a scaffold for movement of viral particles or an anchor for viral assembly.
APPENDIX

List of Strains, Plasmids and Primers
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