The Isolation of Intragenic Suppressors to Point Mutations In the 3' Coding Region of ftsZ of Streptomyces coelicolor

Andrew Joseph Szabo

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The Isolation of Intragenic Suppressors to Point Mutations In the 3' Coding Region of 

ftsZ of Streptomyces coelicolor

By

Andrew J. Szabo

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Duquesne University

2006
Name: Andrew Joseph Szabo

Thesis Title: The Isolation of Intragenic Suppressors to Point Mutations In the 3’ Coding Region of ftsZ of Streptomyces coelicolor

Degree: Master of Science

Date: June 15, 2006

Approved by: ____________________________________________
Dr. Joseph R. McCormick

Approved by: ____________________________________________
Dr. Jana Patton-Vogt

Approved by: ____________________________________________
Dr. Nancy Trun

Approved by: ____________________________________________
Dr. Joseph R. McCormick
Interim Chair, Department of Biological Sciences

Approved by: ____________________________________________
Dr. David Seybert
Dean, Bayer School of Natural and Environmental Sciences
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Abstract

*Streptomyces coelicolor* is an excellent model organism to investigate the function of *ftsZ* because division is dispensable for viability. In *Escherichia coli*, FtsZ is the earliest acting protein in the assembly of the division machine. Although much is known about this protein, there is still a great deal more to learn about the interaction between FtsZ and the rest of the division proteins. FtsZ localizes to form a ring at the division site and then the other proteins assemble on the FtsZ scaffold. The polymerization of FtsZ into protofilaments and Z ring formation is driven by GTP hydrolysis. In *S. coelicolor*, mutations in *ftsZ* often result in hyphae that cannot support division and the formation of uninucleoid spores.

A previously isolated NTG-induced mutant contains a point mutation, an alanine codon changed to a valine at the 275th residue of *ftsZ<sup>Sc</sup>*, resulting in a dominant-negative phenotype. Part of this study focused on isolating intragenic suppressors to this A275V mutation to investigate the function of the C-Terminal domain of the protein. Point mutations elsewhere in the gene have been isolated that must restore partial function of FtsZ. To date, there have been eight intragenic suppressors of A275V isolated, three in this study. Their distribution in FtsZ is scattered, although there seems to be a preference for mutations that alter amino acids near the GTP-binding domain of FtsZ. The belief is that the GTP-binding domain changes conformation due to the A275V mutation and these suppressors restore its function to varying degrees. Also, the highly conserved C-Terminal Core Domain of FtsZ was investigated using a similar intragenic suppressor strategy. During screens for suppressor mutations, in the only isolated potential suppressor a recombination event occurred between the mutated C-Terminal Core Domain and the truncated *ftsZ* of the *ftsZ*-null strain. An event with such a low probability (nearly zero) suggests that the region coding for the extreme C-Terminus of FtsZ is highly important. Finally, I constructed and tested a development-specific promoter tool for the expression of any *ftsZ* allele in aerial filaments alone. As a whole, this study further characterizes this crucial division gene.
Introduction

Cell division is a process that is studied in organisms throughout the prokaryotic and eukaryotic world (Nanninga, 2001). The genes that control this process are essential in the unicellular bacteria *Escherichia coli* and *Bacillus subtilis*, as well as many others. The advantage to studying division in *Streptomyces coelicolor* is that cell division genes are superfluous for growth and viability in the laboratory. This creates a unique environment to be able to manipulate any cell division gene and observe the effect it has on cytokinesis.

Prokaryotic Cell Division Genes

Cell Division research has primarily been carried out in the unicellular, rod-shaped bacteria *E. coli*, *B. subtilis* and *Caulobacter crescentus*. The assembly of cell division proteins at the mid-cell and subsequent division machine formation has been extensively studied (Buddelmeijer and Beckwith, 2002; Lutkenhaus and Addinall, 1997; Margolin, 2000; Rothfield and Justice, 1997). The process requires the essential proteins: FtsZ, FtsA, ZipA, FtsEX, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, FtsN, and AmiC for *E. coli* (Weiss, 2004). Evidence suggests that they are recruited in an ordered fashion to the mid-cell (Figure 1). ZapA is a non-essential protein that also associates with FtsZ during cytokinesis (Gueiros-Filho and Losick, 2002). FtsA and ZipA are believed to tether the FtsZ Ring, which forms at the mid-cell and acts as a scaffold for the other proteins that make-up the division machine, to the membrane (Pichoff and Lutkenhaus, 2005; Liu et
al., 1999). It has also been shown that FtsA-FtsZ interactions can occur between species as long as the sequence homology does not differ a great deal (Ma et al., 1997).

These genes have mostly been identified by temperature-sensitive (conditional) lethal mutations resulting in a filamentous phenotype at non-permissive temperature. Their filamentous temperature sensitive phenotype (Fts) allowed for easy identification of those essential genes (Rothfield and Justice, 1997).

![Figure 1: The Division Machine.](image)

This is a model for the proposed ring created at the mid-cell during cytokinesis of *E. coli*. FtsZ is recruited to the midcell first and establishes the Z ring. The other proteins are then joined individually to the ring. The arrows indicate the order (from top to bottom) each protein localized to the ring. This structure then contracts and splits the cell in two (adapted from Weiss, 2004).
**Streptomyces coelicolor**

*S. coelicolor* is a gram positive, filamentous, sporulating soil bacterium that grows in a branching network. The entire 8.7 Mb genome has been sequenced and characterized (Bentley *et al.*, 2002; Redenbach, 1996). The life cycle of *S. coelicolor* is comparable to that of certain filamentous fungi. In the early stages of growth, the mycelium embeds into the substrate. As growth continues, there is an induction of multigenomic aerial hyphae. These filaments grow away from the surface of the colony. As the hyphae mature, they undergo synchronous septation and form a long chain of uninucleoid cells that will eventually become uninucleoid spores (Chater, 1993).

In addition to the utilizing *S. coelicolor* to understand microbial development, it is also a good model organism for cell division research because division genes are dispensable for growth. As a result, there are visual screens that make it easy to identify division mutants. Some division mutants, such as *ftsZ* and *ftsQ*, overproduce the blue-pigmented antibiotic actinorhodin on minimal glucose medium, while the wild type colonies remain slightly pigmented (McCormick *et al.*, 1994; McCormick and Losick, 1996). Also, a grey-white colony screen has been developed on soya flour and mannitol (SFM) medium. The aerial mycelium of division and developmental mutants appear white and the wild type sporulating colonies appear grey due to a pigment associated with spores (Hopwood, 1999).
Cell Division in *Streptomyces coelicolor*

To date, the earliest acting division protein in *E. coli* is FtsZ (Dai and Lutkenhaus, 1991). In *S. coelicolor* a similar division machine is created in hyphal filament before cytokinesis. Homologs of *ftsL, ftsI, ftsQ, ftsW, ftsK*, and *divIC* have been characterized (Bennett and McCormick, 2001; manuscripts in preparation, McCormick Lab). However, there are no known protein homologs for FtsA, ZipA, ZapA, or FtsN, as shown by sequence analysis. FtsA and ZipA bind FtsZ and interact early in the division process of *E. coli*. Since *S. coelicolor* lacks these two proteins, FtsZ may bind or interact with FtsK (the next known homolog). However, the *ftsK*-null has no obvious phenotype, suggesting that this may not be the case (McCormick, unpublished).

The major difference between *S. coelicolor* division and other prokaryotic model organisms is that null mutations result in viable a organism (McCormick *et al*., 1994; McCormick and Losick, 1996). The McCormick laboratory has already created null mutants for *ftsZ, ftsK, ftsQ, ftsL, ftsI, ftsW* and *divIC*. The resulting strains were still viable, but were unable to divide at all (Δ*ftsZ*), unable to divide as efficiently as wild type (Δ*ftsQ, ΔftsL, ΔftsI, ΔftsW* and ΔdivIC) or essentially wild type (Δ*ftsK*). Since FtsZ is essential for septation, colonies can form a mycelium, but cannot divide or sporulate.

The ability of *S. coelicolor* to grow, but not divide without certain division proteins has already been used to investigate how FtsQ acts in the division sequence (Bennett and McCormick, 2001). The initial *ftsQ* mutant caused a near complete block in division in vegetative filaments and subsequent sporulation. However, intergenic suppressor mutations in the *S. coelicolor* chromosome were isolated, partially restoring division in the *ftsQ*-null. The Bennett and McCormick study (2001) mapped the
suppressor mutations to two independent regions in the chromosome. Furthermore, these two regions were well separated from each other, and the division and cell wall cluster (which contains *ftsQ*). The McCormick laboratory has also found intragenic suppressors of a point mutation in another region of *ftsZ* using an *E. coli* mutator strain for mutagenesis and the grey-white screen on SFM medium (C. Ciccone Master’s Thesis, 2003; Morris and McCormick, unpublished).

FtsZ

FtsZ is not only the earliest acting protein in the division machine, it also is required for proper formation of the rest of the divisome (Hale and de Boer, 1997). If the concentration of FtsZ is too low in *E. coli*, it will result in a reduced number of rings and therefore a lack of division in the filaments. On the other hand, modest overproduction of FtsZ in *E. coli* results in multiple ring formation (at the poles) and division into mini-cells (Ma and Margolin, 1999).

FtsZ is the prokaryotic homolog to tubulin (Erickson, 1995). This claim is based on four unique characteristics of FtsZ: 1) it has the same “signature” sequence as tubulin (Bi and Lutkenhaus, 1992; de Boer *et al.*, 1992; RayChaudhuri and Park, 1992), 2) it localizes to the mid-cell during the division process (much like the eukaryotic cytoskeletal protein actin; however FtsZ shows more homology to another cytoskeletal protein, tubulin), 3) it binds and hydrolyzes GTP and 4) its polymerization into filaments is GTP dependant (Erickson, 1995). Although the sequence homology over the entire protein is weak, the crystal structure similarity makes the FtsZ-tubulin homolog case stronger (Lowe, and Amos, 1998).
FtsZ has three domains: the N-Terminal, the C-Terminal and the C-Terminal Core Domain (Lowe and Amos, 1998; Ma and Margolin, 1999). Figure 2 shows the arrangement of the three domains.

![Diagram of FtsZ domains](image)

**Figure 2: A diagram of the three domains of FtsZ.**

The FtsZ protein of most bacteria has three important regions. The first is the N-Terminal domain, which contains a GTPase binding site. The second is a C-Terminal domain of unknown function that is separated from the C-Terminal Core Domain by a variable linker region. (Note that the diagram is not drawn to scale).

According to Lowe and Amos (1998), the N-Terminus is the most conserved, and contains the GTPase domain. There is currently no known function of the C-Terminal domain. The C-Terminal Core Domain is highly conserved and is found in most bacteria where it functions in the binding of FtsA and ZipA (membrane tethering of the Z ring). The C-Terminal Core Domain is separated from the rest of the conserved regions of the C-Terminus by the linker region. The linker region is variable in length and sequence from species to species. However, it is usually rich in proline and glutamine. Its specific function is not yet known.

Part of my work focused on suppressing a mutation ftsZ(A275V) that occurs in C-Terminal region and inactivates division. This project should shed light on the unknown
function of the C-Terminal Domain or at least how it acts during polymerization of FtsZ. The other part of my project centered on the C-Terminal Core Domain, which is involved in protein-protein interactions with the recruited members of the divisome beginning with FtsA in many prokaryotes and ZipA in prokaryotes closely related to *E. coli*. This is most important to my work because the some of the point mutations I am trying to suppress in this project occur in the coding region for the extreme C-Terminus. There are no obvious homologs of FtsA or ZipA in *S. coelicolor* and it is hypothesized that the C-Terminal Core Domain has another function. The hope is to find out how FtsZ interacts with itself during polymerization and if this region shares functional similarities with the other bacteria known to have interacting proteins in this region.

**Intragenic Suppression of ftsZ(A275V) in the C-Terminus**

Justin Morris originally isolated the mutant containing the *ftsZ*25 allele [referred to hereafter as *ftsZ*(A275V) as in Morris's Master's Thesis] using 1-methyl-3-nitro-1-nitrosoguanidine (NTG) chemical mutagenesis (Morris Master's Thesis, 2002). This dominant-negative mutation completely blocks division in aerial filaments. Initially, structure-directed mutagenesis was tried to suppress the *ftsZ*(A275V) mutation (Ciccone Master's Thesis, 2003). The change of *ftsZ*(L259V) was used to try this strategy. This site was chosen because of the proximity to the original *ftsZ*(A275V) mutation and valine was chosen because this amino acid occurs at the analogous position in *E. coli*. The structure-directed mutagenesis failed using the *ftsZ*(L259V) mutation to suppress *ftsZ*(A275V) (Ciccone Master's Thesis, 2003). This is surprising because valine occurs at both positions in the wild type *ftsZ* sequence in *E. coli*. Ciccone then created a
mutagenized library of potential intragenic suppressor mutations using an *E. coli* mutator strain (C. Ciccone Master’s Thesis, 2003). She partially screened this library for her thesis, however the screen was not exhaustive. Only two mutants were found in the Ciccone study. Additionally, neither suppressor mutation was isolated more than once, nor was the true revertant ever found in the Ciccone study or in any trial ever attempted by Morris, where 3 more suppressors were isolated. I have isolated three more suppressor mutants and repeatedly hit the two independent mutations more than once. I am sure the screen is not exhaustive (since the true revertant was not isolated), but the repeat hits argue that the screen is closer to being finished.

**Intragenic Suppression of C-Terminal Core Domain Mutants**

The C-Terminal Core Domain of FtsZ is highly conserved throughout the bacterial world. In other bacteria, like *E. coli*, the C-Terminal Core Domain is the region of FtsZ that interacts with the division proteins FtsA and ZipA. Obvious homologs for these proteins are not in the *S. coelicolor* genome, yet the region for interaction is conserved (Figure 3). The focus of part of my thesis was to address why there was conservation in this region if the function is not necessarily conserved.

In his research, Morris found that mutations in multiple amino acids in the C-Terminal Core Domain of *ftsZ* are essential for proper division (Morris Master's Thesis, 2002). I focused my study on *ftsZ* with point mutations in the codons for the final 3 amino acids of the C-Terminal Core Domain. A dominant-negative phenotype (lack of division and sporulation) in the presence of a wild type copy of the gene, or at least a
reduction in division in aerial filaments, is displayed in these mutants (Morris Master’s Thesis, 2002; Morris and McCormick, unpublished).

**Figure 3: Conservation of the C-Terminal Core Domain of FtsZ.**

The C-Terminal Core Domain is highly conserved and is found in FtsZ of most bacteria. In red are the residues that are identical between the organisms, whereas the blue residues are chemically conserved. The Core Domain is separated from the rest of the conserved regions of the C-Terminus by the linker region. Most importantly, the C-Terminal Core Domain is involved in protein-protein interactions with the recruited members of the division machine beginning with FtsA in most prokaryotes (more widely conserved) and ZipA in prokaryotes closely related to *E. coli*. Although there is no known homolog of FtsA or ZipA in *S. coelicolor*, the C-Terminal Core Domain is still conserved. (Note that the diagram is not drawn to scale).
Mutations in the three codons targeted in this study are: a null, dominant-negative phenotype (F397A); a null, partial dominant-negative phenotype (L398A); or a null, recessive to wild type (K399A) (Morris Master’s Thesis, 2002). I have created libraries of potential intragenic suppressors to these mutations using the same *E. coli* mutator strain as Carla Ciccone used in the previously mentioned study. Evaluation of the C-Terminal Core Domain mutants supports the idea that the conservation of this region is still extremely important for the function of FtsZ.

**Observing *ftsZ* Allelic Phenotypes in Aerial Filaments Alone**

Our laboratory typically uses defective sporulation as an assay for division. Because the sporulation phenotype is the potential combined result of a defect in vegetative cross-wall formation and in sporulation septation, I have created a tool in the hope of directly viewing the phenotypes of independent *ftsZ* alleles solely in the aerial filaments of *S. coelicolor*. Since this study focuses on finding mutations that restore division in aerial filaments, a major goal was to eventually see how the different mutant alleles of *ftsZ* function in aerial filaments devoid of division.

Normally, *ftsZ* is expressed from three promoters. Previous studies have shown that the *ftsZ2p* promoter is dispensable for vegetative growth and septation, but essential for sporulation (Flärdh *et al.*, 2000). Following the future directions presented in the Flärdh *et al.* study, I designed a construct (pAJ14, Table 3) that could place any allele of *ftsZ* under the specific control of the aerial filament sporulation promoter, *ftsZ2p*. This construct could be used in conjunction with the strain of *S. coelicolor* K101 (*ftsZΔ2p*, Table 2) to directly observe how the different alleles function during sporulation and
septation in aerial filaments (Grantcharova et al., 2003). Vegetative cross-wall formation is normal in K101, but sporulation septa are not produced in aerial filaments (Grantcharova et al., 2003). Alleles of $ftsZ$ that affect aerial filament division or the mutants that restore division in defective $ftsZ$ genes can be cleanly viewed directly in $S. coelicolor$ aerial filaments using this new tool. This construct, and the biochemical data from future tests, will help clarify the work started in this thesis or completed in previous studies (Ciccone Master's Thesis, 2003; Morris Master's Thesis, 2002).

**Thesis Summary**

My research centered on the analysis of $ftsZ$ and its role in cell division. FtsZ is the earliest known protein involved in the division process (Addinall et al., 1997). I investigated how both the C-Terminal domain and the C-Terminal Core Domain function during division using intragenic suppressor mutation analysis of point mutations known to disrupt division. A mutation in the C-Terminal domain called $ftsZ$(A275V) yields a dominant-negative phenotype and also does not support division (Morris Master's Thesis, 2002). In the C-Terminal Core Domain, independent mutations of each of the final three amino acids also inhibit division to varying degrees (Morris Master's Thesis, 2002). By isolating intragenic suppressors I intended to understand the defect in $ftsZ$ in the strains with single point mutations. I had hoped to be able to add to the knowledge of how FtsZ functions as a single monomer or in a protofilament. A focus on FtsZ interaction with other proteins would require an extragenic suppression study.
Materials and Methods

Bacterial Strains and Plasmids

All plasmids used in this study were constructed in *E. coli*. Table 1 lists all *E. coli* strains used in these steps. The strain TG1 was used for most plasmid propagation; LL308 was used for pCMC22 (Sambrook *et al.*, 2001; Zengel *et al.*, 1980). All interspecies conjugation experiments between *E. coli* and *S. coelicolor* were carried out using ET12567/pUZ8002 as the donor strain (MacNeil *et al.*, 1992). The recipient strains were M145, MT1110, HU133 and AZ10. Table 2 lists all *S. coelicolor* strains used or created in this study. All *S. coelicolor* strains used in this study are derivatives of the wild type strain A3(2) (Hopwood *et al.*, 1985). The prototrophic SCP1’SCP2’ strain M145 was the parental strain of the *ftsZ*-null strain HU133 (McCormick *et al.*, 1994) and the *ftsZ*-null strain AZ10 (this study, see below). The *ftsZ*-null strain AZ10 was created by PCR-targeted gene replacement using the *E. coli* strain BW25113 (Datsenko *et al.*, 2000) containing plasmid pIJ790 (Gust *et al.*, 2003). The strains AZ11, AZ12, AZ13 and AZ14 are the control strains created for the C-Terminal Core Domain mutant screenings. The strain K101 (Grantcharova *et al.*, 2003) is lacking a sporulation-specific promoter for the division gene *ftsZ* (*ftsZΔ2p*). Plasmids that were used or created in this study are listed in Table 3.
Table 1: *E. coli* Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL308</td>
<td>Δ(pro-lac) recA nalA supE thi / F′ pro+ lacI lacZΔM15</td>
<td>Zengel <em>et al.</em>, 1980</td>
</tr>
<tr>
<td>ET12567</td>
<td>dam-13::Tn9 dcm-6 hsdM hsdR recF143 zjj201::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtlI glnV44 F′</td>
<td>MacNeil <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>TG1</td>
<td>Δ(pro-lac) hsdD15 supE thi-1 / F′ traD36 pro+ lacI lacZΔM15</td>
<td>Sambrook <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>BW25113</td>
<td>K12 Derivative: ΔaraBAD ΔrhaBAD</td>
<td>Datsenko <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>XL-1 RED</td>
<td>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT:: Tn10 (Tet)</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

Table 2: *S. coelicolor* Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
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<td>M145</td>
<td>Prototrophic SCP1 SCP2</td>
<td>Hopwood <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>MT1110</td>
<td>Prototrophic SCP1 SCP2</td>
<td>Kieser <em>et al.</em>, 2000</td>
</tr>
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<td>HG25</td>
<td>fitsZ(A275V) - MT1110 Background</td>
<td>Morris, Master’s Thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2002</td>
</tr>
<tr>
<td>HU133</td>
<td>ΔfitsZ::aphl - M145 Background</td>
<td>McCormick <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>K101</td>
<td>fitsZΔ2p - J1915 Background</td>
<td>Grantcharova <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>AZ10</td>
<td>ΔfitsZ::hyg - M145 Background</td>
<td>This Study</td>
</tr>
<tr>
<td>AZ11</td>
<td>pCMC22/AZ10 (wild type control)</td>
<td>This Study</td>
</tr>
<tr>
<td>AZ12</td>
<td>pAJ6/AZ10</td>
<td>This Study</td>
</tr>
<tr>
<td>AZ13</td>
<td>pAJ7/AZ10</td>
<td>This Study</td>
</tr>
</tbody>
</table>
Table 3: Plasmids Used or Created in This Study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptiona</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II SK(+)</td>
<td>Commercial Cloning Vector, ( \text{amp}^R )</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>PCR-Product “TA” Cloning Vector, ( \text{amp}^R )</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSET152</td>
<td>Mobilizable Integrating Vector for \textit{Streptomyces}, ( \text{apra}^R )</td>
<td>Bierman \textit{et al.}, 1992</td>
</tr>
<tr>
<td>pJR125</td>
<td>pBluescript derivative with ( \text{ftsZ}_{sc} ) and native promoters</td>
<td>McCormick and Losick, 1996</td>
</tr>
<tr>
<td>pIJ790</td>
<td>( \lambda)-RED (( \text{gam, bet, exo} ) cat araC rep101)\textsuperscript{18}</td>
<td>Gust \textit{et al.}, 2003</td>
</tr>
<tr>
<td>pUZ8002</td>
<td>RK2 derivative which is a non-transmissible helper plasmid in \textit{E. coli}, ( \text{kan}^R )</td>
<td>Kieser \textit{et al.}, 2000</td>
</tr>
<tr>
<td>C69</td>
<td>Cosmid 69 containing the entire \textit{S. coelicolor} division and cell wall cluster, ( \text{amp}^R \ \text{kan}^R )</td>
<td>Redenbach \textit{et al.}, 1996</td>
</tr>
<tr>
<td>pCMC15</td>
<td>pSET152 containing ( \text{ftsZ}(A275V) ) with native promoters</td>
<td>Ciccone, Master’s Thesis, 2003</td>
</tr>
<tr>
<td>pCMC16</td>
<td>pSET152 derivative containing ( \text{ftsZ}(L259V) ) with native promoters</td>
<td>Ciccone, Master’s Thesis, 2003</td>
</tr>
<tr>
<td>pCMC22</td>
<td>pSET152 derivative with ( \text{ftsZ}_{sc} ) and native promoters</td>
<td>Ciccone, Master’s Thesis, 2003</td>
</tr>
<tr>
<td>pJR86</td>
<td>pBluescript II derivative containing the \textit{Bcl I} fragment of ( \text{ftsZ} ) including the promoter region</td>
<td>McCormick \textit{et al.}, 1994</td>
</tr>
<tr>
<td>pJWM24</td>
<td>pCMC22 derivative containing ( \text{ftsZ}(K399A) )</td>
<td>Morris, Master’s Thesis, 2002</td>
</tr>
<tr>
<td>pJWM32</td>
<td>pCMC22 derivative containing ( \text{ftsZ}(L398A) )</td>
<td>Morris, Master’s Thesis, 2002</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pJWM42</td>
<td>pCMC22 derivative containing <em>ftsZ</em>(F397A)</td>
<td>Morris, Master’s Thesis, 2002</td>
</tr>
<tr>
<td>pJWM59</td>
<td>pCMC22 derivative containing <em>ftsZ</em>(G101S, A275V)</td>
<td>Morris, unpublished</td>
</tr>
<tr>
<td>pKC1053</td>
<td>XylE, hyg cassette</td>
<td>Kuhstoss and Rao, 1991</td>
</tr>
<tr>
<td>pKLM2</td>
<td><em>hyg</em> cloned into pKC1053 without XylE, in the same orientation as it was originally in pKC1053</td>
<td>McConahy, unpublished Bennett and McCormick, unpublished</td>
</tr>
<tr>
<td>pAJ6</td>
<td>Asc I-EcoRI fragment of pJWM42 cloned into pCMC22 digested with the same enzymes, resulting in a mobile, integrating, <em>apra</em> ( R ) plasmid containing <em>ftsZ</em>(F397A)</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ7</td>
<td>constructed similar to pAJ6; containing <em>ftsZ</em>(L398A) of pJWM32</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ8</td>
<td>constructed similar to pAJ6; containing <em>ftsZ</em>(K399A) of pJWM24</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ12</td>
<td>pCR2.1 derivative of the PCR product of pJR86 fragment deleting <em>ftsZIp</em> and <em>ftsZ3p</em>, <em>amp</em> ( R )</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ13</td>
<td>C69 derivative with ( \DeltaftsZ::hyg ), constructed by PCR targeting gene replacement</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ14</td>
<td>pCMC22 derivative containing the Xba I-Asc I fragment of pAJ12, the tool for aerial expression of <em>ftsZ</em>, <em>apra</em> ( R )</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ15</td>
<td>Suppressor of <em>ftsZ</em>(A275V): <em>ftsZ</em>(T157A, A275V) isolated from 15A library</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ16</td>
<td>Suppressor of <em>ftsZ</em>(A275V): <em>ftsZ</em>(L32F, A275V) isolated from 15A library</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ17</td>
<td>Recombination event resulting in wild type ( ftsZ ) from ( ftsZ(F398A) ): isolated from a screen of 7Red Library</td>
<td>This Study</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pAJ18</td>
<td>Suppressor of ( ftsZ(A275V) ): ( ftsZ(T157A, A275V) ) isolated from 15A Library (2(^{\text{nd}}) isolate, different tube in library)</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ19</td>
<td>Suppressor of ( ftsZ(A275V) ): ( ftsZ(L32F, A275V) ) isolated from 15A library (2(^{\text{nd}}) isolate, different tube in library)</td>
<td>This Study</td>
</tr>
<tr>
<td>pAJ22</td>
<td>Suppressor of ( ftsZ(A275V) ): ( ftsZ(A23T, A275V) ) isolated from the 15B library</td>
<td>This Study</td>
</tr>
</tbody>
</table>

*Restriction enzyme sites with a superscript 0 (i.e. \( \text{Acc}65 \, 1^{\circ} \)) were rendered blunt by treatment with Klenow fragment of DNA Pol I.*

### Media and Growth Conditions

Strains of *E. coli* used for regular plasmid propagation were grown at 37°C on solid or liquid LB. The *E. coli* strain BW25113/pIJ790/C69 was grown in liquid SOB (Hanahan, 1983) at 30°C. XL-1 Red mutagenesis was carried out in SOC medium (Hanahan, 1983) according to the suggested Stratagene protocol, except that growth was at 30°C. Except where otherwise noted, antibiotic concentrations were: ampicillin at 100 \( \mu \text{g/ml} \), apramycin at 50 \( \mu \text{g/ml} \), kanamycin at 25 \( \mu \text{g/ml} \), chloramphenicol 25 \( \mu \text{g/ml} \), hygromycin at 50 \( \mu \text{g/ml} \), and carbenicillin at 100 \( \mu \text{g/ml} \).

Strains of *Streptomyces* were grown on minimal medium supplemented with 0.5% glucose or R2YE agar (Kieser *et al.*, 2000). Liquid cultures of *Streptomyces* were grown in Super YEME, which is YEME as described in Hopwood *et al.* (1985) supplemented.
with 3.3% Glycine and 0.5% MgCl$_2$. Antibiotic concentrations for *Streptomyces* were: nalidixic acid at 20 µg/ml, apramycin at 25 µg/ml and hygromycin at 50 µg/ml (except in transformation overlays of protoplasts the concentration was 200 µg/ml). For conjugation with *S. coelicolor* spores, 2X YT was used for washing glycerol from frozen spore stocks (Hopwood, 1999).

Soya flour and mannitol (SFM) medium (Kieser et al., 2000) was used to support growth of *E. coli* and *Streptomyces* during interspecies conjugations. Antibiotic concentrations in this medium were: nalidixic acid at 20 µg/ml to counter-select *E. coli* donors and apramycin at 25 µg/ml to select *S. coelicolor* transconjugants.

**DNA Techniques**

Plasmid DNA was isolated from *E. coli* using the Quick Spin Miniprep Kit (QIAGEN) or using Alkaline Lysis Extraction (Sambrook et al., 1989). Chromosomal DNA was prepared from *Streptomyces* using the Wizard Genomic DNA Purification Kit (Promega). Restriction enzymes were used according manufacturer’s instructions. Calf-Intestinal Phosphatase (CIP) was used to dephosphorylate all vectors used in ligation reactions. The Gel Extraction Kit (QIAGEN) was used to purify restriction fragments after agarose gel electrophoresis. Products of ligation reactions were used to transform either chemically or electro-competent TG1 cells.
DNA Amplifications

During the creation of AZ10, the hyg gene was amplified from pKLM2 (Table 3) using the polymerase chain reaction (PCR) using the oligonucleotide primers oZ57 (5′-GGGGGCACTCATGCTTTCCGGCGGCCGGCGACACGTAACCTACCTACCAAAACAATGCC-3′) and oZ60 (5′-TCACGGGGTGCCTGCTTCATCCAAGGAAGTCCGGGTCAGGCGGCCGGGGCGGTGTC-3). This results in a mutagenic PCR product containing 40 bases of homology to ftsZ flanking hyg. When required, the ftsZ gene contained in the various plasmid constructs was amplified before being sequenced for a potential suppressor mutation. The primers used to amplify ftsZ were 25Z03 (5′-CATATGGCAGCACCACAGCTACCC-3′) or 25ZF01 (5′-CTCGAGGGGAGGGCTCGTGACGCTG-3′) forward primers and M13R (5′-CAGGAAACAGCTATGAC-3′) or 25ZR01 (5′-CGAGGTGAGCTTCCTCGACGGGAC-3′) as reverse primers. All PCR reactions were conducted in a Biometra Thermocycler unit. The 50 μl reaction contained manufacturer's 1x amplification buffer, 1x Enhancer (Invitrogen), 0.3 mM dNTP’s, 1 mM MgSO₄, 0.5 μM primers, 50 ng of DNA template, and 1U of Pfx (Invitrogen). The amplification of hyg followed the program listed in the REDIRECT manual (Gust et al., 2002). When fragments were cloned using the TA cloning vector pCR2.1, Taq DNA Polymerase (NE Biolabs) was added to amplify PCR reaction products of the ftsZ gene. The reaction program was run as follows: 2 minutes at 94°C, 30 cycles (45 seconds at 94°C, 45 seconds at 55°C, 90 seconds at 72°C), 5 minutes at 72°C. Upon completion of
all PCR reactions, the PCR Purification Kit (QIAGEN) was used to remove excess primers and yield clean product.

To create pAJ12, the Bcl I fragment of ftsZ contained on pJR86 was used as template in 2 separate extension reactions. First, it was extended deleting ftsZ3p with the paired primers oZ207x (5′-TCTAGAAAAAGAAAAACGGGAGGTTC-3′) and oZ110B (5′-CTAACGCTGGATCCGGGTTACCAGTGTGCTG-3′). In second extension reaction, with the other paired set of primers oZ102B (5′-GGTAACCCGATCCAGCTTAGGGTTCCGGTC-3′) and oZ474 (5′-GGTCGTTGGGGATGACGATG-3′), it was deleted for ftsZ1p and replaced the "-10" hexamer with a BamH I site (TAAACTT changed to GGATCC). The extension reaction conditions were: 15 cycles of (40 seconds at 94°C, 40 seconds at 55°C, 15 seconds at 72°C). Next 1 µl of each extension reaction was amplified in a standard 50 µl reaction with the outside oligonucleotides (oZ207x and oZ474) for 28 cycles of (40 seconds at 94°C, 40 seconds at 55°C, 80 seconds at 72°C) followed by the addition of a single, non-template A using Taq polymerase (2 cycles of 72°C for 10 minutes).

**Sequencing**

In order to sequence ftsZ in its entirety in pAJ6, pAJ7, pAJ8, and plasmids containing potential suppressor mutations, the primers ftsZ493 (5′-GTCGTTGGGGATGACGA-3′), ftsZ651 (5′-GAAGTCGGTCATGTTCCGAG-3′), ftsZ872 (5′-CGAGGCCAACATCATCTTC-3′), ftsZ923 (5′-TCACGGTGACCGCACCT-3′), ftsZ132 (5′-CAGGCGCTTGATGAGCGA-3′) and ftsZ161 (5′-GTCGACTTAGGTCTGCTGTC-
3′) were used in conjunction with oZ207x and oZ474 (both described above in the DNA Amplification section) to verify the sequence of the promoter region of pAJ12 (ftsZ2p alone). The sequencing reactions were carried out in a Biometra Thermocycler unit in accordance with the Thermo Sequenase Big Dye-Terminator cycle sequencing kit (ABI). Reactions were analyzed using an ABI Prism 310 Genetic Analyzer and compared to the wild type S. coelicolor ftsZ sequence (GenBank accession number U10879).

**Construction of pAJ13**

In the following procedure, the mutagenic PCR fragment containing hyg with 40 base extensions homologous to ftsZ (described above) was inserted into ftsZ borne by cosmid C69 using PCR-targeted gene replacement resulting in a ΔftsZ::hyg genotype and the cosmid was called pAJ13 (REDIRECT: Gust et al., 2002). Fifty ml of SOB containing kanamycin (50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (25 μg/ml) and 0.01 M L-arabinose was inoculated with 2.5 ml of an overnight LB culture of BW25113/pIJ790/C69 containing the same antibiotics grown at 30°C. The culture was grown for 3-4 hours at 30°C shaking at 200 rpm until reaching an OD<sub>600</sub> of ~0.6. The cells were centrifuged at 5000 rpm for 10 minutes at room temperature and quickly resuspended in 50 ml of ice cold 10% glycerol. Again the cells were centrifuged as above and resuspended in 25 ml of ice cold 10% glycerol. Finally, the cells were centrifuged a third time and resuspended in the final drop of glycerol. The mutagenic hyg PCR product (~ 100 ng in 2 μl) was mixed with 50 μl of the resuspended cell suspension and electroporated in a 0.2 cm ice-cold electroporation cuvette using a BioRad MicroPulser set to 200 Ω, 25 μF, and 2.5 kV. Immediately, 1ml of ice cold LB was
added and the cell suspension was incubated at 37°C for 1 hour. The culture was then spread onto LB plates containing kanamycin (50 µg/ml), ampicillin (100 µg/ml), and hygromycin (50 µg/ml), and incubated overnight at 37°C. The resulting colonies were streaked for isolated colonies and grown in liquid LB carbenicillin (100 µg/ml), kanamycin (50 µg/ml), and hygromycin (50 µg/ml) at 37°C overnight. Plasmid DNA from resulting colonies was then prepared and digested with Sac I to confirm. The fragments obtained for pAJ13 were: 14.7 Kb and 12.7 Kb (seen as a doublet), 6.7 Kb, 5.2 Kb and 5.1 Kb (seen as a doublet), 2.7 Kb and 2.6 Kb (seen as a doublet) and 2.1 Kb. The fragments obtained for the C69 digest were: 14.7 Kb and 12.7 Kb (seen as a doublet), 6.7 Kb, 5.2 Kb and 5.1 Kb (seen as a doublet), 2.7 Kb and 2.6 Kb (seen as a doublet). The presence of the 2.1 Kb insert was enough to confirm the plasmid's identity (ΔftsZ::hyg). Sequence analysis was not performed on this construct. The cosmid that resulted was identified as pAJ13 (Table3).

Transformation of *S. coelicolor* with Plasmid DNA Prepared from *E. coli*

Plasmid DNA prepared from *E. coli* must be isolated from a methylation-deficient strain before transforming *S. coelicolor*. Therefore, plasmids were passed through the strain ET12567 (*dam*⁻*dcm*⁻) prior to the interspecies transformation (Table 1).

The transformation of *S. coelicolor* was carried out according to the conventional method (Hopwood *et al.*, 1985). In short, 30 ml of Super YEME was inoculated with 200 µl of *S. coelicolor* spores (M145 or MT1110 in this study) and grown at 30°C for 36-48 hours. The mycelia were washed with 10.3% sucrose (3 times) and then resuspended in a lysozyme solution (2 mg/ml) in P-Buffer Complete (Kieser *et al.*, 2000). This was
incubated at 30°C for 90 minutes, triturating 3 times every 15 minutes. The protoplasts that resulted were filtered through sterile cotton, centrifuged, and resuspended in 100 µl of P-Buffer (per set of 3 platings). The plasmid DNA was alkaline denatured prior to transformation (Kieser et al., 2001). This DNA was then added simultaneously with 500 µl P-Buffer + 25% PEG 1000 (Polyethylene Glycol 1000) to each 100 µl aliquot of protoplasts. The transformation mixtures were plated on R2YE medium and incubated at 30°C. After 16-20 hours, the plates were overlayed with the appropriate antibiotics to select for transformants and incubation continued at 30°C. Individual colonies formed within 5 days and subsequently restreaked 3 times on minimal glucose with antibiotic.

**Construction of AZ10**

In order to create a new ftsZ-null strain AZ10 (Table 2) with a larger deletion of ftsZ, the cosmid pAJ13 was used to transform wild type *S. coelicolor* strain M145 to hygromycin resistance. The individual candidates were preliminarily identified by the characteristic blue halo phenotype associated with ftsZ mutants. The candidates were also screened for kanamycin sensitivity to identify clones resulting from double crossover events during the recombination (Figure 4).
Figure 4: Creation of the *S. coelicolor* ftsZ-null Strain AZ10

The introduction of pAJ13 (carrying ΔftsZ::hyg) to the chromosome of M145 occurred through recombination between the wild type chromosome and the homology contained in pAJ13. This effectively deleted the majority of ftsZ, leaving behind only 13 base pairs to maintain wild type expression of the downstream genes. The strain yields the characteristic blue halo phenotype observed for all ftsZ-null mutants.

The resulting strain was called AZ10 (ΔftsZ::hyg) and only 13 base pairs of ftsZ remained in order to maintain the proper expression of the downstream genes. This strain is used for a background to screen the XL-1 RED mutant libraries of the C-Terminal Core Domain mutants in pAJ6, 7, and 8 (described below).
Construction of pAJ6, 7, and 8

The first step to test if intragenic suppressors could be isolated in the C-Terminal Core Domain of FtsZ required making constructs capable of integrating into the chromosome that contain point mutations in \textit{ftsZ}. Through \textit{Asc} I-\textit{EcoR} I restriction enzyme digestion, and subsequent ligations, I was able to fuse the 5' end of wild type \textit{ftsZ} found in pCMC22 to a DNA segment that has point mutations in the extreme 3' end created by alanine scanning mutagenesis and contained in pJWM24, 32, and 42 (C. Ciccone Master’s Thesis 2003; Morris Master's Thesis, 2002). The backbone of pCMC22 contains \textit{int} $\phi c31$, and \textit{attP} for site-specific integration into the \textit{S. coelicolor} chromosome. In addition, pCMC22 also contains \textit{oriT} to deliver the plasmid by interspecies conjugation from \textit{E. coli} and an apramycin-resistance gene to select for transconjugants (Figure 5).

Three independent constructs were made. The first was pAJ6, which contains a point mutation in the codon for residue 397, changing a phenylalanine to an alanine (F397A). The second construct was pAJ7, which contains a point mutation in the codon for residue 398, changing a leucine to an alanine (L398A). Finally, pAJ8 was created, which contains a point mutation in the codon for residue 399, changing a lysine to an alanine (K399A) (Table 3).
Figure 5: Construction of pAJ6, 7, and 8 - Plasmids Containing Point Mutations in the Region Coding for the C-Terminal Core Domain of FtsZ

Shown is the construction of the three plasmids capable of integrating into the chromosome that contain independent point mutations in the C-Terminal Core Domain of ftsZ. The first was pAJ6, which contains a point mutation at residue 397, changing a phenylalanine to an alanine (F397A). The second construct was pAJ7, changing a leucine to alanine (L398A). Finally, pAJ8 was created changing a lysine at residue 399 to alanine. Each was constructed by substituting the Asc I-EcoRI fragments from the top two plasmids.
XL-1 RED Mutagenesis and Mutagenized Library Creation

In order to isolate intragenic suppressors, the plasmids pAJ6, 7, and 8 were propagated in the *E. coli* mutator strain XL-1 RED according to the manufacturer's instructions (Stratagene, Table 1). In brief, SOC medium was pre-warmed to 42°C while the commercially prepared XL-1 RED competent cells were thawed on ice for 30 minutes. These cells were split into two 100 µl aliquots and treated with 1.7 µl of 1.42 M β-mercaptoethanol. After a 10-minute incubation (mixed every 2 minutes by gently tapping), 75 ng of plasmid DNA was added to each aliquot. Next, these mixtures were subjected to a 30-minute ice incubation, followed by a 42°C heat shock for 47 seconds. The mixture was then cooled on ice for 2 minutes. Immediately, 900 µl of the pre-warmed SOC was added to the cells, mixed, and incubated for 1 hour at 37°C with aeration. The cells were plated on LB apramycin (30 µg/ml) and incubated at 30°C. This temperature is lower than the Stratagene protocol, but more efficient as determined by a pilot experiment.

Approximately 72 hours later, approximately 250 total colonies were apparent on each transformation plate. These colonies were pooled, concentrated by centrifugation and resuspended in 2 ml LB with apramycin (30 µg/ml) and 1.4 ml of 50% glycerol. Aliquots were stored at -80°C. One hundred and twenty-five µl of this XL-1 RED library was diluted into 25 ml SOC apramycin (30 µg/ml) culture (1:200 dilution), and grown overnight at 30°C with shaking. Plasmid DNA from the entire sample of the first overnight culture of the initial pooled XL-1 RED transformants was prepared. A sample was digested with *Eco*RI-*Bam*HI in order to confirm that the plasmid DNA remained intact during the transformation.
Next, 50 ng (contained in 2.5 µl) of DNA prepared from the first overnight culture of XL-1 RED transformants was transformed into ET12567/pUZ8002 electrocompetent cells. The cells were plated and incubated overnight on LB apramycin (50 µg/ml), kanamycin (25 µg/ml) at 37°C. Approximately 10,000 transformants were pooled, washed, and resuspended in 10 ml of 20% glycerol. This yielded eleven 1 ml aliquots of potential mutants in an E. coli strain that can be conjugated into S. coelicolor. All libraries were stored at -80°C.

Mutagenized libraries were created for ftsZ(A275V) in this same manner (Ciccone Master's Thesis, 2003). The two independent libraries were prepared and named 15A and 15B. Both libraries were used to screen for suppressor mutations in this study.

**Interspecies Conjugation from E. coli to S. coelicolor**

The introduction of plasmid DNA from E. coli to the chromosome of S. coelicolor was accomplished by interspecies conjugation (Kieser et al., 2000). Overnight cultures of ET12567/pUZ8002/pAJ"X" were grown at 37°C in LB apramycin (30 µg/ml), kanamycin (25 µg/ml), and chloramphenicol (25 µg/ml). They were diluted 1:100 in LB with antibiotic selection and grown to an OD$_{600}$ between 0.4 and 0.6. Next, 10 ml cells were centrifuged and washed twice with LB and resuspended in a final volume of 500 µl. Simultaneously, S. coelicolor mycelial fragments of the ftsZ-null mutant recipient were initially prepared by mechanical maceration (McCormick, personal communication). This process involved picking several (10-25) colonies of the ftsZ-null strain and smashing each with a sterile stick in 0.85% sterile saline. Approximately 500 µl of this fragmented mycelia are then mixed with 100 µl of washed E. coli cells. The mixture was
spread on SFM plates containing no antibiotic, and allowed to grow overnight at 30°C. The next day, the plates were overlayed with 3 milliliters of antibiotics. To select transconjugants and counter-select the \textit{E. coli} donor. The final concentrations of antibiotics in a 25 ml plate were apramycin (25 µg/ml) and nalidixic acid (20 µg/ml). The plates were incubated at 30°C until transconjugants were observed (5-7 days later). This procedure was inefficient with a division mutant.

Conjugation from \textit{E. coli} to germinated \textit{S. coelicolor} spores from a wild type strain was also accomplished. Here, 100 µl of a thawed spore stock was washed by adding to 900 µl of 2X YT to remove the glycerol from the spores (Hopwood, 1999). After centrifugation for 5 minutes, the pellet was resuspended in 500 µl of 2X YT, and heat shocked at 50°C for 10 minutes and then allowed to cool. Next 500 µl of the \textit{E. coli} cells were mixed to the spores, and centrifuged for 2 minutes. The supernatant was removed, and the pellet of \textit{S. coelicolor} spores and \textit{E. coli} cells was resuspended in the remaining liquid. The entire resuspension was plated on SFM, incubated and treated with antibiotics as described above.

**Improving the Efficiency of Interspecies Conjugation with Division Mutants**

The mechanical maceration method described above proved to be quite inefficient for obtaining sufficient transconjugants for potential intragenic suppressor mutations. I tested a few new methods to increase the efficiency of fragmentation, and thus of producing more transconjugants. These experiments were necessary in order to expedite my screening efforts.
The first method tested was an attempt to permeate the thick cell wall of a gram-positive bacterium by brief lysozyme treatments combined with sonication to produce mycelial fragments. A 30 ml culture of an ftsZ-null mutant strain (HU133, Table 2) was grown in Super YEME for 75 hours at 30°C and the mycelia were washed twice with 10.3% sucrose. An increase in time of growth was used because the ftsZ-null mutant grows much poorer than wild type and I wanted to have a comparable amount of material to work with in the experiments. The pellet was resuspended directly in a lysozyme solution.

Subsequently, in the initial experiment, cells were exposed to 2 mg/ml lysozyme for incubation times of 0 minutes, 10 minutes, 20 minutes, and 30 minutes. A two-fold dilution series starting with 2 mg/ml lysozyme and ending with 0.0039 mg/ml lysozyme was used to test the effects of the lysozyme on S. coelicolor. A 0 mg/ml solution was used as a control for the experiment. All lysozyme treatments following the initial trial were incubated at 30°C for 10 minutes.

Next, the “Cup” Sonic Dismembrator 60 (Fisher Scientific) was employed for the sonication treatment of cells exposed to lysozyme. The lysozyme treated samples were subjected to sonication at 15 watts for varying times. The time points for sonication were: 0 minutes (control), 2 minutes, 5 minutes, and 10 minutes.

Approximately 100 µl of each of the sonicated and lysozyme treated cells were plated on SFM. The plates were incubated at 30°C for 5-7 days. The results were recorded and plating efficiency of the fragments was then determined by manual counts.

Another approach to increase the fragmentation of mycelium was tested by subjecting the mycelium to pressures that would fragment the mycelium, but leave viable
fragments using the French Pressure Cell (Thermo Spectronic). The parts of the French Press were autoclaved in order to ensure sterility throughout the experiment. A 30 ml culture of a wild type strain (M145, Table 2) and an \textit{ftsZ}-null strain (AZ10, Table 2) were grown in Super YEME at 30°C for 36-48 hours. The cultures were pelleted, washed twice with 10.3% sucrose, resuspended in 20% glycerol, and split into three 4 ml aliquots for passage through the French Press. The experimental cell pressures were: 0, 500, 1,000, 5,000, and 10,000 psi. After passing samples through the French Press at the different pressures, the mycelial fragments were plated in a dilution series (10\(^{-2}\), 10\(^{-3}\), and 10\(^{-6}\)) on SFM and allowed to grow at 30°C for 5 days. The resulting colonies were counted and a relative fragmentation efficiency was determined.

**Construction of pAJ14**

The upstream regulatory region of \textit{ftsZ} was constructed to contain only \textit{ftsZ2p} by performing 2 separate extension reactions and using these overlapping products as template in a PCR reaction (see above). The PCR product obtained from the extension-amplification reactions, designed to delete \textit{ftsZ1p} and \textit{ftsZ3p}, was cloned into pCR2.1 following the manufacturer's protocol (Invitrogen). The extension reactions replaced \textit{ftsZ3p} with an \textit{Xba} I restriction site, and \textit{ftsZ1p} with a \textit{Bam} H I restriction site. The presence of the PCR construct in pCR2.1 was verified by restriction digest with \textit{Eco} R I and named pAJ12 (Table 3). The \textit{ftsZ} fragment and altered promoter region contained in pAJ12 were verified by sequence analysis.

Next, the wild type fragment containing all 3 promoters of pCMC22 was replaced with an analogous fragment containing only \textit{ftsZ2p} from pAJ12. Using the \textit{Xba} I site
introduced in the PCR reaction described above, and the Asc I site contained in the ftsZ fragment on pAJ12, the ftsZ2p construct was cloned into pCMC22 (Xba I-Asc I). This plasmid containing ftsZ' expressed only from ftsZ2p was named pAJ14 (Table 3). The cloning procedure left both restriction sites, Xba I and Asc I, intact in pAJ14. Future ftsZ alleles will be able to be cloned into this construct.

**Phase-Contrast Microscopy**

All *S. coelicolor* strains viewed using phase-contrast microscopy were grown on SFM media supplemented with the proper antibiotics. The cover slips were inserted in the media at 45° angles, and inoculated at the base adjacent to where the slip contacts the medium (McCormick and Losick, 1996). The inoculated plates containing the cover slips were incubated at 30°C and grown for 5 days. Individual cover slips were removed and mounted in 10 µl of 50% glycerol. A Nikon Microphot-SA microscope was used to view the *S. coelicolor* under 100X oil immersion. A Nikon UFX-DX camera with Kodak 100 T-Max black and white film was used to capture the images. The processed negatives were scanned into digital form using an Epson Perfection 3490 Photo Flatbed Scanner. The images were imported into Adobe Photoshop CS (Version 8.0) for figure construction.

**Constructing an FtsZ Homology Model**

Using the computer program Deep View: Swiss PDB Viewer an FtsZ homology model was created (Guex and Peitsch, 1997; http://www.expasy.org/spdbv/). This
homology model was created based on the *Mycobacterium tuberculosis* FtsZ because the amino acid sequence for *M. tuberculosis* is 82% identical to that of *S. coelicolor*. 
Results

Testing Alternative Methods to Fragment Division Mutant Strains

My thesis focused on the isolation of intragenic suppressors to point mutations in the C-Terminal domain and the C-Terminal Core Domain of FtsZ. To do this, a mutagenized \textit{ftsZ} was introduced into the chromosome of \textit{S. coelicolor} by interspecies conjugation with \textit{E. coli}. The largest problem encountered in this project was that interspecies conjugation was very inefficient when using a division mutant strain as a recipient. To resolve this, I had to develop a new conjugation protocol different than the one typically used for a wild type strain, which uses spores (Kieser \textit{et al.}, 2000). Division mutants do not produce spores so mechanical maceration was usually used to prepare the mycelia for conjugation. Mechanical maceration is nothing more than smashing individual colonies into small fragments with sterile wooden applicators. I attempted to increase the efficiency of \textit{S. coelicolor} mycelia fragment production to give more \textit{S. coelicolor} recipient.

In pilot experiments for the first method, I tested lysozyme treatment followed by gentle sonication using the mycelia of the \textit{ftsZ}-null strain HU133 obtained from a 30 ml liquid culture (Table 4). Initially, lysozyme treatment was at such a high concentration (2 mg/ml), that all \textit{S. coelicolor} growth was eliminated. The non-treated filaments and the sample of sonicated material did grow, and often produced a full lawn of colonies. For most combinations of lysozyme and sonication tested, colony forming units were decreased rather than increased. Eventually, the lysozyme was diluted to 0.004 mg/ml, and sonicated for 5 minutes producing equal growth to the material treated with 0.008
mg/ml of lysozyme and no sonication. Only this lysozyme concentration lead to approximately 530 colonies per plate, observed when 4-fold more of the sonicated-lysozyme treated sample was plated. This was the highest colony count reached during any lysozyme-sonication trials. Comparing this to non-lysozyme treated (0 mg/ml), sonicated \textit{S. coelicolor}, which produced 495 colonies, meant the lysozyme-sonication treatments were not increasing the fragmentation to a level that sufficiently made this method any better than the published or mechanical maceration method. Data from single plate colony counts for a representative experiment under the lysozyme-sonication trials is contained in Table 4. The sample was diluted 5-fold and equal amounts of material were plated for each condition.

<table>
<thead>
<tr>
<th>LYSOZYME CONCENTRATION</th>
<th>ZERO MINUTES OF SONICATION</th>
<th>2 MINUTES OF SONICATION</th>
<th>FIVE MINUTES OF SONICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg/ml</td>
<td>350 colonies</td>
<td>464 colonies</td>
<td>493 colonies</td>
</tr>
<tr>
<td>63 µg/ml</td>
<td>132 colonies</td>
<td>106 colonies</td>
<td>125 colonies</td>
</tr>
<tr>
<td>31 µg/ml</td>
<td>144 colonies</td>
<td>175 colonies</td>
<td>210 colonies</td>
</tr>
<tr>
<td>16 µg/ml</td>
<td>212 colonies</td>
<td>346 colonies</td>
<td>304 colonies</td>
</tr>
<tr>
<td>8 µg/ml</td>
<td>290 colonies</td>
<td>439 colonies</td>
<td>533 colonies</td>
</tr>
</tbody>
</table>

I attempted to use the French Press apparatus to fragment the \textit{S. coelicolor} mycelia. This method used a pressurized capsule to break the mycelia into viable fragments. These experiments were all conducted at room temperature. I used liquid grown mycelium of a wild type strain (M145, Table 2) and the \textit{ftsZ-null} strain AZ10 (Table 2), to compare the French Press treatment results to the lysozyme-sonication experiments. Initially, the French Press was used at low cell pressures (CP) of 0, 500,
and 1000 psi. The results were dramatically higher for the treated material such that a dilution series had to be plated to obtain an accurate colony count. The 500 psi trial produced approximately $5 \times 10^8$ colony forming units (cfu) compared to the untreated sample that produced $2.8 \times 10^8$ cfu. This is only approximately a 2-fold increase, but it is certainly not a decrease. Further experiments showed that colony counts could be increased 60 to 70-fold when compared to the untreated sample by increasing the cell pressure to 5,000-psi. An increase to 10,000-psi did not increase the colony count beyond 60 to 70-fold. An example of experiments with a $ftsZ$-null strain AZ10 can be seen in Figure 6 while data from single plate colony counts in the French Press experiments are found in Table 5. As a result of these tests, my screens using an $ftsZ$-null strain were all conducted using fragmented mycelium from this French Press method once it was established.

![Figure 6: French Press Fragmentation Experiment Results](image)

**Figure 6: French Press Fragmentation Experiment Results**

Liquid-grown cells of the $ftsZ$-null strain AZ10 were washed and resuspended in an isotonic solution and treated by passage through a French Pressure cell. Dilutions were made and equal aliquots were plated. These are the results of passing *S. coelicolor* mycelia through the French Press apparatus plated on SFM agar after 5 days of growth at 30°C. All plates are arranged with the $10^1$ dilution atop the pyramid, a $10^3$ in the lower left and the $10^6$ occupies the lower right. In the control (A), no treatment, the growth is spotty and sub-par. In the 5,000-psi test (B), the growth increase is quite evident, and comparisons between the $10^6$ dilution plates can be drawn; the $>50$-fold increase is apparent. In the 10,000 psi test (C), no significant increase is seen. Therefore, the 5,000 psi became the condition at which the fragmentation for conjugation should take place to combat the conjugation inefficiencies that plagued our laboratory.
Table 5: French Press Fragmentation Experiment Data for the \textit{ftsZ-null} Strain AZ10

<table>
<thead>
<tr>
<th>PRESSURE (PSI)</th>
<th>(10^3) PLATE*</th>
<th>(10^3) PLATE</th>
<th>(10^6) PLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.0 (\times) 10^3 cfu</td>
<td>2.7 (\times) 10^3 cfu</td>
<td>1.0 (\times) 10^6 cfu</td>
</tr>
<tr>
<td>5,000</td>
<td>TMTC</td>
<td>(\sim)8.0 (\times) 10^5 cfu</td>
<td>(\sim)7.5 (\times) 10^7 cfu</td>
</tr>
<tr>
<td>10,000</td>
<td>TMTC</td>
<td>(\sim)7.0 (\times) 10^5 cfu</td>
<td>(\sim)6.5 (\times) 10^7 cfu</td>
</tr>
</tbody>
</table>

*Indicates Plates that had "Too Many To Count."

cfu: colony forming units

In order to determine if French Pressed material could be stored at -20°C and thawed for use at any time, I did control experiments to see if plating efficiency changed with a freeze-thaw cycle. If the material was used within 1-2 weeks, the same high number of colonies were observed on the control plates. However, after approximately 2 months, the efficiency when conjugating with the freeze-thaw method decreased. I then prepared and used a fresh batch of cells and regained the high efficiency of conjugation witnessed in the early trials. As a result, all \textit{S. coelicolor} used in conjugation experiments with division mutants now employ the French Press technique at 5,000 psi in order to increase conjugation efficiency, and the cells are never subjected to the freeze-thaw method.

**Creating a Strain Containing a Larger \textit{ftsZ} Deletion**

In order to screen the XL-1 RED mutagenized libraries for suppressor mutations to alanine substitutions in the C-Terminal Core Domain, a new \textit{ftsZ-null} strain had to be constructed. This was necessary to combat a high background level of recombination that I discovered when controls for the assay were attempted with HU133, the original
published ftsZ-null strain. In previous studies with these same alleles, minimal problems occurred when characterizing the phenotypes of the alanine substitutions when ftsZ was contained on a low-copy number, autonomously replicating plasmid (Morris, personal communication). However, this study used ftsZ-containing plasmids that integrate by site-specific recombination into the chromosome. Originally, I introduced plasmids pAJ6, 7 and 8, containing the alanine substitutions of ftsZ into HU133 to create the control strains for my experiment. Surprisingly, rather than 100% of the transconjugants being defective for cell division, 65-80% of the transconjugants appeared wild type even though they should have only contained a single altered copy of ftsZ. The 3’ end of ftsZ’ remaining in HU133 (approximately 180 base pairs) codes for the C-Terminal 60 amino acids of FtsZ. Since this region covers where my mutations are, the truncated wild type ftsZ must have been recombining with the mutant ftsZ and yielding a wild type copy in S. coelicolor (Figure 7). In order to test this possibility, I extracted the chromosome from several S. coelicolor sporulating transconjugants, recovered the integrated plasmid by transforming E. coli and sequenced 2 independent candidates to check ftsZ. Both candidates were found to be wild type; recombination between ‘ftsZ’ must be restoring the sequence to the mutant ftsZ.

In order to keep the truncated ftsZ in the chromosome from recombining with the incoming altered ftsZ carried on the plasmids, I created a new ftsZ-null with a larger deletion and named the strain AZ10 (Table 2). First, ftsZ on cosmid C69 was deleted and replaced by hyg using a mutagenic PCR product in E. coli to create pAJ13.
Figure 7: A Diagram of a Recombination Event Between `ftsZ` of HU133 and C-Terminal Core Domain Mutations Borne by Integrating Plasmids

A. The introduced `ftsZ` is shown recombining with the remaining `ftsZ` of wild type *S. coelicolor*. This would result in an `ftsZ` copy in the chromosome instead of the mutant `ftsZ`. A high background of `ftsZ` made it impossible to screen a library of mutants for a restoration of wild type phenotype in HU133. Mutations in the C-Terminal Core Domain are indicated by an asterisk (*).

B. This diagram represents the result of the deletion-insertion mutation for `ftsZ` of AZ10. This `ftsZ-null` mutant strain that I created replaces `ftsZ` with *hyg*, leaving only 13 base pairs of the wild type gene.
Using an *E. coli* strain expressing the λ RED recombination function, this mutagenic PCR product was used to recombine cosmid C69 that contains the *S. coelicolor* division and cell wall cluster. Hygromycin resistant recombinants were checked by restriction enzyme digestion. The resulting deletion removed all but the final 13 base pairs of *ftsZ*. The remaining bases of *ftsZ* were left because they contain the Shine-Dalgarno sequence and start codon for the next downstream gene. One cosmid with the appropriate digestion pattern was named pAJ13.

Using pAJ13, *S. coelicolor* was transformed to hygromycin resistance. Transformants were selected for hygromycin resistance and screened for kanamycin sensitivity to identify marker replacement (Figure 4). The new *ftsZ*-null strain was verified by genetic complementation with *ftsZ*+ contained on pCMC22 (AZ11, Figure 9), which was previously used in genetic complementation analysis of the *ftsZ*-null mutant HU133. AZ10 was not characterized to the nucleotide level because other *ftsZ*-null strains were already published. This strain was only created for screening for intragenic suppressors. This larger deletion was predicted to reduce the chance for recombination to essentially zero. The possibility is minimal that a homologous recombination event could occur within those 13 bases of homology. AZ10 (Δ*ftsZ::hyg*) is shown next to the original *ftsZ*- null, HU133 (Δ*ftsZ::aph*) for comparison (Figure 8) and with the parental wild type strain M145 (Figure 9).
Figure 8: A Pair of $ftsZ$-null Strains - HU133 and AZ10 versus The Wild Type Strain M145

Shown here are the two $ftsZ$-null strains, HU133 (A.) and AZ10 (B.) and the wild type strain M145 (C.), grown on minimal glucose medium for 5 days. HU133 ($\Delta ftsZ::aphI$) is the published $ftsZ$-null (McCormick et al., 1994) used for most experiments in the McCormick Laboratory. AZ10 ($\Delta ftsZ::hyg$) is the new $ftsZ$-null created in this study to screen for C-Terminal Core Domain suppressor mutations. The characteristic blue-halo phenotype, resulting from the overproduction of the blue-pigmented antibiotic actinorhodin, is present in both. In fact, the strains are indistinguishable on minimal glucose media as well as other tested media. The wild type strain has been added for comparison, stressing the phenotypic differences.

Attempts to Isolate Suppressors to Point Mutations in the Region Coding for the C-Terminal Core Domain of FtsZ

The plasmid pCMC22, containing $ftsZ^+$, was first introduced into AZ10 to create a control strain that all transconjugants could be compared to in the visual screen. This also demonstrated genetic complementation and showed that the division defect was a result of the insertion-deletion mutation. This strain was named AZ11 (Figure 9, Table 2). Subsequently, the plasmids containing the three C-Terminal Core Domain point mutations (pAJ6, 7, and 8) were introduced to AZ10 in order to establish controls for the experiment. Now, 100% of the transconjugants have defects in division, as was previously seen with autonomously replicating plasmids (Morris Master's Thesis, 2002).
These controls ensure that the screen will not have a background of transconjugants that appear wild type (as was the case in HU133).

**Figure 9: The Control Strains for the C-Terminal Core Domain Mutant Screens**

The strains shown above are the controls for the screening of the XL-1 RED mutant libraries for pAJ6, 7, and 8 grown for 5 days at 30°C on SFM medium. The strains are: M145, *ftsZ*⁺; AZ10, Δ*ftsZ::hyg*; AZ11, *ftsZ*/Δ*ftsZ*; AZ12, *ftsZ*(F397A)/Δ*ftsZ*; AZ13, *ftsZ*(L398A)/Δ*ftsZ*; AZ14, *ftsZ*(K399A)/Δ*ftsZ*. Each strain (AZ12, 13, and 14) looks different from AZ10 and from AZ11. This ensures that transconjugants that appear wild type are not necessarily recombinants, and strains containing pAJ6, 7, and 8 have phenotypes different from AZ10 prior to XL-1 RED mutagenesis. For example, AZ14 is the least defective for division, the aerial mycelia are very white compared to M145, which has aerial mycelia that are very grey. This allele probably does not effect vegetative division, but does effect sporulation septation. However, AZ12 and AZ13 most likely have a defect in both types of division resulting in the dramatic phenotype observed above.
Approximately 50 transconjugants for each control were obtained and all appeared to have the same phenotype. Representative resulting strains were named AZ12, 13, and 14, respectively. These phenotypes can be observed in Figure 9.

In this newly developed ΔftsZ background, AZ10, I screened the XL-1 RED mutagenized libraries for pAJ6, 7, and 8 looking for transconjugants that appeared grey, similar to AZ11 (Figure 9). In total, I screened 2,688 transconjugants for XL-1 RED mutagenized pAJ6, 2,401 transconjugants for XL-1 RED mutagenized pAJ7, and 851 transconjugants for XL-1 RED mutagenized pAJ8. The reason for the differences in the number screened is because of 2 limiting factors. First, the XL-1 RED libraries were created sequentially. I originally isolated the pAJ6 library and conducted tests with this in order to establish an efficient protocol for screening. This task proved harder than initially thought and therefore set back the other library screenings accordingly. The XL1-RED mutagenized pAJ6 library was screened using mechanically macerated AZ10 as the recipient strain until the superior French Press method was established. Then the screening of all libraries was expedited.

After screening each library, I only isolated 1 grey transconjugant that remained grey upon re-streaking. This was isolated from the XL-1RED mutagenized pAJ7 mutant library, \(ftsZ\text{L398A}\). Under phase-contrast microscopy, the potential suppressor contained on pAJ17 (Table 3) created spore chains and appeared to function like wild type. The chromosomal DNA was extracted from \(S.\ coelicolor\) and used to transform \(E.\ coli\) to apramycin resistance. The plasmid was reintroduced to AZ10 to check for phenotypic linkage to the plasmid-encoded gene. The resulting wild type phenotype was caused by the plasmid. Upon sequence analysis, the \(ftsZ\) contained on pAJ17 was in fact
the wild type sequence. Even though only 13 bases of homology are present in AZ10, this wild type \( ftsZ \) must have arrived through homologous recombination. The revertant to wild type would have had a 2 base change, from the alanine (GCG) to the leucine (CTG), a transversion and a transition. This double point mutation is even less likely to occur than the recombination event even though only 13 bases of the gene remained. Despite these efforts, recombination still occurred in my screens, albeit at a drastically reduced level (from 65-80% witnessed in HU133 to 1 in 5,940 with AZ10). The libraries I created can be further screened in the future to search for other mutations.

**Isolation of Intragenic Suppressors of \( ftsZ(A275V) \)**

Another part of my project continued a previous study. I have isolated 3 novel suppressors of the dominant-negative point mutation called \( ftsZ(A275V) \). A strain containing \( ftsZ(A275V) \) is unable to divide and has a white aerial mycelium. By random visual screening of the XL-1 RED mutant library created by Ciccone, suppressors were identified by transconjugants with a grey phenotype (Figure 10; Ciccone, Master’s Thesis). The plasmids were introduced to \( S. coelicolor \) by interspecies conjugation from \( E. coli \). The plasmids integrate into the \( S. coelicolor \) chromosome through site-specific recombination. Transconjugants were selected for apramycin resistance and the \( E. coli \) donors were counter-selected with naladixic acid. Previously, 5 suppressor mutations were isolated for the original mutation from this screen. These point mutations resulted in the amino acid changes \( ftsZ(L32P), ftsZ(A52T), ftsZ(R83C), ftsZ(G101S), \) and \( ftsZ(I225V), \) in addition to the original \( ftsZ(A275V) \). They restore partial division in aerial filaments (Ciccone Master’s Thesis, 2003; Morris and McCormick, unpublished).
Figure 10: A Representative Plate of the Visual Screen of Transconjugants forSuppressor Mutants

Shown is a 5-day-old plate of transconjugants grown on SFM medium. This is typical of the results obtained when conjugations are performed recipient *S. coelicolor* mycelia generated with the French Press. Out of the hundreds of colonies only a few have grey aerial mycelium (the rest are white, or of uncharacteristic color). The grey colonies examples of potential candidates and are indicated with an arrow. This is an example of the visual screen used in my study.

In conjunction with previous studies (Ciccone Master's Thesis, 2003; Morris Master’s Thesis, 2002), I screened approximately 13,000 transconjugants using the French Press and mechanical maceration methods of preparing recipient cells. From these 13,000, I isolated approximately 80 potential suppressor mutants based on the visual screen. I narrowed those potential suppressor mutants down to 30 candidates by
picking the ones that appeared to be isolated from other colonies. From those 30, I have isolated three novel suppressor mutations \textit{ftsZ}(A23T), \textit{ftsZ}(L32F) and \textit{ftsZ}(T157A). I also screened one mutant that was not linked to the plasmid and may be an intergenic suppressor mutation. That strain is named 15B-12 (Table 2) and was stored for potential further investigation. A typical plate from a transconjugant screen is shown in Figure 10, while the pigment differences in the grey aerial mycelia between strains can be visualized in Figure 11. Analyses of the remaining strains that contain an altered \textit{ftsZ} allele were not completed and were stored at -80°C for future analysis.

The three mutations I isolated, \textit{ftsZ}(A23T), \textit{ftsZ}(L32F), and \textit{ftsZ}(T157A), are suppressors of the original mutation \textit{ftsZ}(A275V). The suppressor mutations are borne on plasmids named pAJ22, pAJ16 and pAJ15, respectively (Table 3). The codon changes for all three were transitions. For the change from an alanine at position 23 to a threonine, the codon GCC changed to ACC. For the change from leucine at position 32 to a phenylalanine, the codon CTC changed to TTC. For the change from a threonine at position 157 to an alanine, the codon ACC changed to GCC. Interestingly, I isolated the \textit{ftsZ} allele containing both \textit{ftsZ}(L32F) and \textit{ftsZ}(T157A) mutations twice, from different vials within the frozen library and they may represent siblings. Plasmids from these second isolates were called pAJ19 and pAJ18, respectively (Table 3). The codon changes are the same transitions in these plasmids as well. The fact that I have found duplicated mutations within the screen suggests that the possibility of isolating more novel suppressors is decreasing.
Figure 11: Visual Phenotypes of Suppressor Mutants

Shown are the macroscopic (plate) phenotypes of the wild type, the division mutant \([ftsZ(A275V)]\), and the suppressor mutants. These were grown on SFM media for 5 days at 30°C. Grey pigmentation of aerial mycelium is associated with spore formation. The aerial mycelium of the strain expressing \(ftsZ(A275V)\) is white. The best suppressor isolated, \(ftsZ(G101S, A275V)\), is included here for comparison.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Suppressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type <em>S. coelicolor</em></td>
<td>(ftsZ(A275V, T157A)/\DeltaftsZ::neo)</td>
</tr>
<tr>
<td>(ftsZ/\DeltaftsZ::neo)</td>
<td>(ftsZ(A275V, L32F)/\DeltaftsZ::neo)</td>
</tr>
<tr>
<td>(ftsZ(A275V)/\DeltaftsZ::neo)</td>
<td>(ftsZ(A275V, G101S)/\DeltaftsZ::neo)</td>
</tr>
</tbody>
</table>
The suppressor mutations all resulted in the characteristically grey-pigmented aerial mycelia known to be indicative of wild type spore formation. The HU133 (ΔftsZ::aph I) background and HU133/pCMC15 [ftsZ(A275V)] control strains show very different phenotypes given that these are plated on two different media (Figure 8A versus Figure 11). For completeness, the previously isolated strain containing ftsZ(A275V, G101S) was used for comparison because this was the best suppressor isolated to date. This suppressor mutation most closely restores a strain with ftsZ(A275V) to wild type division (Ciccone Master’s Thesis, 2003). Visually, the new suppressors I have isolated yield the grey-pigment in the aerial mycelium to the same degree as the ftsZ(A275V, G101S) mutation.

The major difference between a strain with ftsZ(A275V, G101S), and one with ftsZ(A275V, L32F) or ftsZ(A275V, T157A) can be seen when phase-contrast microscopy is employed to visualize division during spore formation (Figure 12). The aerial hyphae of the wild type strain divides and produced evenly-sized spores. The strain with ftsZ(A275V, G101S) made elongated, misshapen spores. This was the closest phenotype to wild type of the isolated suppressor strains. Strains containing the 2 suppressors I have found in this study, ftsZ(A275V, L32F) and ftsZ(A275V, T157A), restored division to the coiling stage of development (Figure 12). This phenotype is similar to several developmental mutants blocked for sporulation septation and a subset of other division mutants when grown on certain media. This phenotype is consistent with the interpretation that FtsZ functions sufficiently such that division in vegetative hyphae has been restored but division in the aerial hyphae has not.
Figure 12: Phase-Contrast Microscopy of Strains Containing $\textit{ftsZ}$(A275V) Suppressor Mutations

Shown are phase-contrast images of aerial filaments to demonstrate the division phenotypes. All strains were grown on SFM medium and photographed after 5 days of growth. The wild type $\textit{S. coelicolor}$ shows normal division (sporulation). Division is defective in the strain containing the $\textit{ftsZ}$(A275V) mutation. Division is restored in the positive control, $\textit{ftsZ}^+$ complementing the $\textit{ftsZ}$-null strain ($\Delta\textit{ftsZ}::\textit{neo}$). The T157A mutation partially restores division and many loosely coiled filaments are observed. The L32F mutation partially restores division and some tightly coiled filaments are present, as well as some misshapen, oblong spores (not shown). Division is also restored nearing the wild type status with the G101S suppressor mutation. This is the best suppressor candidate found to date.
During the final screening for suppressor mutations to $ftsZ(A275V)$ I isolated the suppressor mutation $ftsZ(A23T, A275V)$. It was originally identified using the visual screen for grey pigments in the same manner as the other suppressor mutants. Although images of this suppressor on a plate for phenotypic comparison were not obtained, phase contrast images were taken and can be seen in Figure 13. This strain restores division in the filaments well and the division sites are highlighted with arrows. I would argue that this mutation suppresses the original $ftsZ(A275V)$ mutation to nearly the same degree as $ftsZ(G101S, A275V)$.

![Figure 13: Phase-Contrast Image of Suppressor Mutation $ftsZ(A23T, A275V)$](image)

Shown is a phase-contrast image of the suppressor mutation $ftsZ(A23T, A275V)$ grown on SFM medium for 5 days. Wild type M145 is shown next to the division defective strain, the $ftsZ$-null strain HU133 containing $ftsZ(A275V)$. The loss of sporulation septation is apparent. The introduction of $ftsZ^+$ complements the $ftsZ$-null strain HU133, restoring division. The final panel shows $ftsZ(A23T, A275V)$, the most recently discovered suppressor of the mutation $ftsZ(A275V)$. The arrows point to the division sites along the filament. The spore chain shown consists of partially elongated spores and some regularly shaped spores. Nonetheless, these are spores and that is characteristic of partial restoration of division to the filament.
Figure 14 shows a structural model for *S. coelicolor* FtsZ based on the crystal structure of *Mycobacterium tuberculosis* FtsZ (Leung et al., 2000). The placement of the mutations show the even distribution of substitutions over FtsZ. The screen for intragenic suppressors of ftsZ(A275V) is not exhausted because a true revertant has not been isolated. It is narrowing however, since the same mutation has been isolated more than once in the library. These results are providing valuable insight into the important areas for function of the unknown C-Terminal Domain. Suppressors are located in non-conserved residues, but in regions near or adjacent to the conserved residues for GTP binding.

**Building and Testing an Allele-Specific Expression System for Aerial Filaments**

The major target of this study is to find mutations that restore division to aerial mycelia that express a mutant ftsZ. Currently the observed division phenotype is a combination of the defect in vegetative cross-wall formation and in sporulation septation. To visualize how division is affected in aerial filaments alone, I have created a construct called pAJ14 as the preliminary step to view different ftsZ alleles under the control of the sporulation promoter ftsZ2p (Flärdh et al., 2000). This matable construct contains a version of wild type ftsZ under the control of ftsZ2p alone. The sequence established in this construct is shown in Figure 15.
Figure 14: Locations of Suppressor Mutations of \textit{ftsZ}(A275V)

A. A diagram depicting the locations of the suppressor alterations relative to the original \textit{ftsZ}(A275V) mutation designated in \textit{RED}. Additionally, a structure directed change L259V, which was originally chosen as the site most likely to restore division based on the crystal structure of FtsZ (\textit{Methanococcus jannaschii}) is shown. The mutation L259V did not suppress the \textit{ftsZ}(A275V) phenotype as predicted. The other mutations listed do suppress the original \textit{ftsZ}(A275V) to varying degrees.

B. A homology model for the crystal structure of \textit{S. coelicolor} FtsZ based on the \textit{Mycobacterium tuberculosis} FtsZ was created using the "Deep View: Swiss PDB Viewer" computer program (Guex and Peitsch, 1997; \url{http://www.expasy.org/spdbv/}). The amino acid sequences are 82\% identical between FtsZ of \textit{S. coelicolor} and \textit{M. tuberculosis}. In the crystal structure, the suppressor alterations are highlighted in \textit{RED}, and the original \textit{ftsZ}(A275V) is highlighted in \textit{GOLD}. The L259V alteration that does not suppress is highlighted in \textit{BROWN}. This structure displays of the suppressor mutations in relation to the GTP-binding site. This region is important for the polymerization of FtsZ. Suppressor \textit{ftsZ}(G101S) is located in the GTP-binding site, immediately adjacent to the tubulin/FtsZ signature sequence. The disruption in division witnessed for the original \textit{ftsZ}(A275V) mutation may be linked to conformational changes in the GTP-binding site. The suppressor mutations may alleviate this change and restore function to the GTP-binding site thereby restoring division to varying degrees.

\begin{verbatim}
TCTAGA AAAGAAAAACGGGGAGTTCCGCGGTGTTCGGTTGAACGTCGGCCACTTGGTGACT
TAGTTG CCTGTTCGGAGAGTGCCAAGAGACAGACACTGCTAACCAGGATCCAGCTTT
AGGGTTCCGCGCCTACGGGACCCTCAATCGGATGTCGGCGGTCGCGGGGGCATCAGTCGC
AGGCGCCGGACGCATGCAGGCTCAGGCTGGCAGGCTGGCAGGCGGACGCGCGCTGGCG
GCACCGAGAACTACCTCGCAATTACATCGGCTTGTGGCCGGCGGGCACGTCTGCTT
TGCCATCAGCGGATGTCAGGCTGTCTCAAGGCCGTCAGTTGATAAGGCCTGGCGGAC
CCGACCGCAGGGCGCCTGTGATTGACGAGGCACGGCAAGCTCAAGTCGAGTCCGGCG
CTACCCGGGACTCGGCGCCGGAGAACCCCGGCGTACGCGCGCAAGCCCGCCCGAGAC
CACCGGCAGGAGATCGAGGAGGCTTGAAGGGGGCCGACATGGTCTTCTYGATGACGCGG
TGAGGGCGGCGGACCCGGGAGGCGCGCGG
\end{verbatim}

Figure 15: The Sequence of \textit{ftsZ2p} Only - The Promoter Region of pAJ14

This is the sequence left after construction of pAJ14 deleted the promoters \textit{ftsZ3p} and \textit{ftsZ1p}, leaving only \textit{ftsZ2p} to control the expression of \textit{ftsZ}. Highlighted in \textit{RED} are the sequences for the \textit{Xba I} site and \textit{BamH I} site that effectively deleted the \textit{ftsZ3p} and the -10 region of \textit{ftsZ1p}, respectively. Highlighted with \textbf{BOLD UNDERLINED} text are the -35 and -10 regions of \textit{ftsZ2p}. Highlighted in \textbf{BLUE} is the region of \textit{ftsZ} that is included in pAJ14, ending with the Asc I site (\textbf{BLUE}).
The $ftsZ'$ on pAJ14 is capable of complementing the strain K101 ($\DeltaftsZ2p$, Table 2), which is defective in sporulation septation due to the lack of $ftsZ2p$ (Figure 16). However, K101 has wild type $ftsZ$ expression from $ftsZ1p$ and $ftsZ3p$ in vegetative filaments, as well as septation in the vegetative filaments (Grantcharova et al., 2003). Using this construct will further the knowledge of the exact method of operation for any $ftsZ$ allele specifically in aerial filaments. Future $ftsZ$ alleles will be able to be cloned into pAJ14, which is just like any other pCMC22 derivative (Table 3) and conjugated into K101 to directly visualize the phenotype in aerial hyphae.

![Figure 16: Complementing the $ftsZ\Delta2p$ Strain with the $ftsZ2p$ Only Construct - pAJ14](image)

Shown is the $ftsZ\Delta2p$ strain K101 and a complemented version using the $ftsZ2p$-$ftsZ'$ construct pAJ14 on SFM medium, grown for 5 days. As can be seen, division is lost in the aerial filaments of K101. Division during sporulation is restored when the construct pAJ14, carrying $ftsZ2p$ only, is introduced to the $ftsZ\Delta2p$ strain. In the future, the tool known as pAJ14 will be used to express any $ftsZ$ allele in aerial filaments alone.
Discussion

Cell division in prokaryotes is an increasingly popular topic of study for many bacterial cell biologists. *S. coelicolor* is a great model organism in which to dissect this complex process. A main reason for this is that the entire 8.7 Mb genome has been sequenced and characterized (Bentley *et al*., 2002; Redenbach, 1996). Additionally, the division genes that are essential for division in other model organisms, such as *E. coli* or *B. subtilis*, are dispensable for growth in *S. coelicolor* (McCormick *et al*., 1994; McCormick and Losick, 1996). Simple visual screens on two separate types of media allow easy identification of division mutants and suppressor mutants that restore wild type phenotype (Figures 8-10). The *ftsZ* and *ftsQ* division mutants overproduce the blue-pigmented antibiotic actinorhodin on minimal glucose medium yielding a “blue halo” phenotype (McCormick *et al*., 1994; McCormick and Losick, 1996). Also, aerial hyphae of wild type *S. coelicolor* produce grey-pigmented spores on soya flour and mannitol (SFM) medium, whereas division mutants cannot sporulate and the aerial mycelium of colonies remains white. This provides an easy grey-white screen for division (Hopwood, 1999).

Taking advantage of these characteristics of *S. coelicolor*, I have further analyzed *ftsZ* in an attempt to increase our understanding of its function. To date, FtsZ is the earliest known protein that localizes to the division site (Addinall *et al*., 1997; Dai and Lutkenhaus, 1991). Besides that, FtsZ localization is necessary for the proper formation of the rest of the division machinery into a ring (Hale and de Boer, 1997). FtsZ is the prokaryotic homolog of tubulin, although the sequence homology over the entire protein
is weak, the crystal structure is superimposable with that of tubulin (Erickson, 1995; Lowe and Amos, 1998; Erickson, 1998). Moreover, FtsZ and tubulin share a highly conserved tubulin signature sequence motif: GGGTG(T/S)G (Erickson, 1998).

Specifically, I focused on identifying the importance of the C-Terminal Domain and the C-Terminal Core Domain (Figure 2). The N-Terminal Domain is known to house the GTPase function that allows FtsZ to polymerize into filaments (Lowe and Amos, 1998; Wang et al., 1997). The specific function of the C-Terminal domain is unknown, but the C-Terminal Core Domain is essential for protein-protein interactions with the division machinery. FtsA and ZipA are recruited early in the division cycle and bind directly to the C-Terminal Core Domain of FtsZ in E. coli (Hale and de Boer, 1997; Ma and Margolin, 1999). However, S. coelicolor does not have any obvious homologs for FtsA and ZipA, but the sequence of the C-Terminal Core Domain is still conserved, as it is throughout many of the prokaryotes (Figure 3).

I focused my work on the these two less understood portions of FtsZ in S. coelicolor. My approach to this study centers on the isolation of intragenic suppressor mutations to individual point mutations in either the C-Terminal domain or the C-Terminal Core Domain of FtsZ. Deletions or mutations in ftsZ in the region coding for the C-Terminal Core Domain are known to cause disruptions of the FtsZ-FtsA and the FtsZ-FtsZ interactions in C. crescentus, E. coli, B. subtilis, S. aureus and loss of division in S. coelicolor (Din et al., 1998; Ma and Margolin, 1999; Wang et al., 1997; Yan et al., 2000; Morris Master's Thesis, 2002). By identifying suppressor mutations within ftsZ that restore its ability to produce a functional FtsZ, I hoped to demonstrate how FtsZ interacts with itself during polymerization. Interaction with other members of the
division machinery would require an extragenic suppression study. Intragenic suppressor mutations could provide the answer to just how FtsZ folds and functions during polymerization. Additionally, this study could help suggest the location of the C-Terminal Core Domain because that region of the protein is not ordered in the crystal structure.

Isolating intragenic suppressors to any mutant \textit{ftsZ} library proved to be a difficult challenge. One mutation for which I attempted to isolate suppressors was the dominant negative mutation \textit{ftsZ}(A275V), partially characterized previously (Ciccone Master's Thesis, 2003; Morris Master's Thesis, 2002). The mutation \textit{ftsZ}(A275V) is located in the HC3 helix (the third alpha helix of the C-Terminal domain) of FtsZ (\textit{Methanococcus jannaschii}), near the bottom interface involved in FtsZ monomer-monomer interactions (Figure 17).

Based on the \textit{M. jannaschii} crystal structure provided by Lowe and Amos (1998) it is possible that the A275 is interacting with the 5 amino acids that are also contained within a hydrophobic pocket in this region of the proteins (Morris and McCormick, unpublished). In \textit{M. jannaschii} and \textit{E. coli}, the corresponding amino acid to A275 of \textit{S. coelicolor} is a valine; the mutation \textit{ftsZ}(A275V) actually introduces an amino acid that is present in the wild type \textit{ftsZ} of other organisms (Erickson, 1998). Figure 17 shows the crystal structure given by the 1998 Lowe and Amos study, as well as a closer look at HC3 inside the hydrophobic pocket (this being \textit{S. coelicolor} FtsZ as modeled from the \textit{M. jannaschii} FtsZ shown in part A). How a conservative residue change in that position of the protein drastically effects its function is unknown.
Figure 17: FtsZ Helix HC3 and the Hydrophobic Pocket

A. This is the crystal structure of *M. jannaschii* FtsZ given in the Lowe and Amos study (1998). The helix containing A275, HC3, is clearly labeled in the C-Terminal domain (left hand portion of the figure) and is highlighted by the arrow. This is where the A275 is located in *S. coelicolor* FtsZ. The C-Terminal (RED) and N-Terminal (GREEN) domains are separated by the long helix H5 (YELLOW and located in the center of the figure).

B. A close look at the hydrophobic pocket where A275 (green) is located in a homology model of *S. coelicolor* FtsZ created in this study based on the *M. jannaschii* FtsZ. The residue in blue is the leucine at position 259, a focus of prior site directed mutagenesis experiments hypothesized to suppress the original *ftsZ*(A275V) mutation based on location and comparison with *E. coli* FtsZ (C. Ciccone, Master’s Thesis).

The introduction of the slightly larger valine in *S. coelicolor* FtsZ in this position within the hydrophobic pocket could create steric problems and disrupt the function of FtsZ. Although in other organisms the actual residue is a valine, the increase in size from the alanine to valine in *S. coelicolor* may disrupt proper folding of the protein. The *ftsZ*(A275V) may also disrupt the bottom interface of FtsZ and prevent the next monomer from polymerizing. FtsZ is thought to assemble by adding one subunit at a time to the
ever growing "+ end," just like the mechanism seen in tubulin polymerization (Scheffers and Driessen, 2001). Any malformation introduced to the bottom of one subunit may prevent the binding of the next subunit in line. Alternatively, the \textit{ftsZ}(A275V) mutation may alter the conformation and disrupt the GTP-binding domain of FtsZ. GTP hydrolysis is essential in the formation of protofilaments (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1998). Without a functional GTP-binding domain, FtsZ cannot polymerize into filaments and complete division. Finally, the A275V mutation may not allow FtsZ protofilaments to bind laterally to other protofilaments when creating the division ring or bind to another protein (hereto unknown) that is essential for the division machine to function properly.

Previously (Ciccone Master's Thesis, 2003), site-directed mutagenesis was used to attempt to suppress \textit{ftsZ}(A275V) by creating \textit{ftsZ}(L259V, A275V). This structure-directed approach did not work although the secondary mutation was chemically identical to the FtsZ of \textit{E. coli} and chosen based on the \textit{M. jannaschii} crystal structure. The search for random intragenic suppressors using the awesome power of screening brought about some interesting results. I would have thought that most suppressor mutations would occur in, or with close relation to, the hydrophobic pocket that houses the \textit{ftsZ}(A275V) mutation. Based on the conformation shift hypothesis, I thought the original conformation shift would be corrected by another slight conformation shift that brings FtsZ back to functional, or nearly wild type status. As shown in Figure 14, this is not the case. In fact, most of the suppressor mutations are found in the N-Terminal domain, on the opposite side of the predicted crystal structure and many are located near the GTP-binding pocket. Additionally, most mutations are found in loops, not in conserved
residues, but adjacent to conserved residues. Interestingly, the best suppressor mutation isolated to date is \textit{ftsZ}(G101S), and it is found directly adjacent to the GTP-binding domain of FtsZ. This implicates the GTP-binding hypothesis detailed above. I believe that the \textit{ftsZ}(A275V) introduces a shift in the tertiary structure of the protein, such that the GTP-binding site is skewed, and left unable to bind or hydrolyze GTP. The suppressor mutations all restore this binding site to varying degrees of normalcy.

Another key region of FtsZ seems to be near the leucine at position 32. This residue was changed to both a proline and a phenylalanine in order to suppress the \textit{ftsZ}(A275V) mutation. Although these are two very different amino acids, they may be responsible for adding a kink in the structure that restores proper conformation. Proline is known to introduce kinks due to its imino ring and phenylalanine has a bulky benzene ring in its side chain capable of altering the tertiary structure. Not only that, but the \textit{ftsZ}(L32F) was isolated twice, in two independent conjugation trials from the XL-1 RED mutagenized pCMC15 library. It is possible that these isolates are siblings given that the 2 vials are aliquots of a much larger homogenous library. This mutation is located in the N-Terminal domain in a position that may effect the conformation of the GTP-binding pocket. However, this position may be dispensable in FtsZ function because it can be so easily manipulated to compensate for the loss of function created by \textit{ftsZ}(A275V), but further analysis is required.

Additionally, the suppressor mutation \textit{ftsZ}(T157A) was isolated twice, in two independent conjugation trials from two independent vials in the XL-1 RED mutagenized pCMC15 mutant library. Again the possibility exists that these might be siblings in the large library. This residue is located in the region close to where the N-Terminal and C-
Terminal domains of FtsZ are linked together (Figure 14). If it is possible that the original \textit{ftsZ}(A275V) mutation twisted the GTP-binding site out of conformation, a counter-twist to the entire domain may offset the change, and yield a functional FtsZ. The mutation \textit{ftsZ}(T157A) may be in the right place to introduce such a dynamic shift in domains.

I also isolated \textit{ftsZ}(A23T, A275V) and this mutation suppresses the dominant negative \textit{ftsZ}(A275V). This mutation is very close in the primary structure of FtsZ to \textit{ftsZ}(L32F) and \textit{ftsZ}(L32P), which are also suppressor mutations of \textit{ftsZ}(A275V). In \textit{ftsZ}(A23T), division of aerial filaments is restored to a degree where coiling and misshapen spores are visible. Given its location in the crystal structure, I believe this suppressor mutation also fits with the conformation shift and relief hypothesis.

Based on the intragenic suppression results, the GTP-binding site disruption leading to the loss of hydrolysis seems the most likely to account for why the suppressor mutations are evenly distributed throughout the protein. The next step in this project would be to perform biochemical analysis using GTP-binding and GTP-hydrolysis assays. To do these studies, active protein must be isolated. This has yet to be accomplished, even for the wild type protein, let alone for a protein from a double mutant strain. However, these tests will be used to distinguish the defect of \textit{ftsZ}(A275V) has by disrupting the GTP-binding domain (hydrolysis), inhibiting polymerization, and effectively eliminating division.

If any mutations in this study effect how FtsZ binds to other members of the division machinery, or other subunits in the filament, the C-Terminal Core Domain mutants may yield outstanding candidates to use for probing these hypothetical
interactions. In other organisms, this region is highly conserved and essential for division (Ma and Margolin, 1999). The sequence similarities were highlighted in Figure 3. The C-Terminal Core Domain is the region known to directly bind and interact with the essential division proteins FtsA and ZipA in *E. coli* (Hale and de Boer, 1997; Ma and Margolin, 1999). FtsA and ZipA play a key role in tethering FtsZ to the membrane, and facilitate division by supporting the scaffolding the FtsZ ring creates for other members of the machine to bind (Pichoff and Lutkenhaus, 2005; Liu *et al*., 1999). Additionally, the number of molecules of FtsZ in an *E. coli* cell outnumbers the number of FtsA molecules present (Vicente *et al*., 2006). There is about a 5-fold difference between the levels of FtsZ and FtsA (Ma *et al*., 1997). The fact that 80% of FtsZ is not bound by FtsA or ZipA in *E. coli* suggests the possibility that the C-Terminal Core Domain might have more than one role and might do more than bind FtsA. All FtsZ molecules have C-Terminal Core Domains, but only one fifth of the monomers actually bind FtsA. This proportional difference in *E. coli*, in part, is the foundation for part of my project.

Obvious homologs of the essential division proteins FtsA and ZipA of *E. coli* do not exist in *S. coelicolor*, but the sequence in FtsZ where they bind is highly conserved. Prior to this study, it was my belief that functional homologs existed for FtsA and ZipA and that the C-Terminal Core Domain is responsible for protein-protein interactions in *S. coelicolor*.

Using the intragenic suppression method outlined in previous studies, I hoped to find mutations that restored function to three point mutations in the final three amino acids that code for the C-Terminal Core Domain in the 399 amino acid protein. This will only work if the C-Terminal Core Domain of FtsZ interacts with FtsZ. Because the C-
Terminal Core Domain is not part of the crystal structure, it was difficult to anticipate where suppressor mutations are most likely to be found within FtsZ. One of the mutations studied was \textit{ftsZ}(F397A). This residue change results in a null phenotype for division and is dominant-negative when in the presence of the wild type gene. Another mutation analyzed in this study was \textit{ftsZ}(L398A). This mutation also produced a null phenotype for division, and is partially dominant negative. Lastly, I looked at the mutation \textit{ftsZ}(K399A). Although this mutation also interfered with division in aerial filaments, \textit{ftsZ}(K399A) is recessive to the wild type, not dominant negative like the other two analyzed in this study. Justin Morris first showed the phenotypes associated with these three independent point mutations (Morris and McCormick, unpublished), but suppression studies were not attempted until this study.

I was unable to isolate any intragenic suppressor mutations of any of the above-mentioned point mutations in the C-Terminal Core Domain. In fact, the only potential suppressor mutant obtained in any of the independent XL-1 RED mutagenized library screenings was a recombinant. I only screened a limited amount of transconjugants. If the an intragenic suppressor mutation is a rare event, a more robust screen would have to be conducted.

One prediction about the C-Terminal Core Domain mutations is that they might affect the ability of FtsZ to polymerize, but almost certainly not in the same way as \textit{ftsZ}(A275V). It is possible that this predominantly hydrophobic tail of FtsZ reaches back on itself and stabilizes the interaction between FtsZ monomers, an intrasubunit mechanism. On the other hand, the C-Terminal Core Domain may act like a string connecting two adjacent subunits in a protofilament, an intersubunit mechanism. Perhaps
this attachment stabilizes the GTP-binding site and helps in the hydrolysis of GTP, an essential step in protofilament assembly (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1998).

Another possibility is that the introduction of the smaller alanine to each of the three positions in the C-Terminal Core Domain disrupts how the region folds in vivo. Although alanines generally do not have this effect, the possibility remains. Since the extreme C-Terminus of FtsZ is not ordered in the crystal structure of FtsZ (Lowe and Amos, 1998), it is possible that it does not fold in a single formed structure. The introduction of a non-polar, hydrophobic amino acid with a very small (to minimize steric hindrance) side chain, like alanine could be why the interaction is eliminated. Leucine and phenylalanine have non-polar side chains, and perhaps that is why these two are the point mutations that exhibit the dominant negative phenotypes. The alanine is not able to compensate, and in fact ruins, the hydrophobic tail interactions. Replacing the positively charged lysine, the final residue of FtsZ, with a non-polar side chain (in an already non-polar environment) may actually put less of a stress on the protein as a whole. This results in the recessive phenotype, not the dominant negative as shown in the others.

On the other hand, the fact that suppressors were not isolated may indicate that the C-Terminal Core Domain may still function in protein-protein interactions. However, if an FtsZ-binding protein does exist, the most likely region for association with FtsZ is not the C-Terminal Core Domain. Although there is no homolog known for FtsA or ZipA, the essential division proteins that bind the C-Terminal Core Domain in E. coli, perhaps a functional homolog exists that aids in the division process. An unknown protein may bind the C-Terminal Core Domain of FtsZ in S. coelicolor and tether the
protein scaffolding to the membrane of the filament in order to establish the division apparatus. There is biochemical evidence for a protein that binds to FtsZ in *S. coelicolor*, but the protein most likely binds to a region other than the C-Terminal Core Domain (B. McGourty Master's Thesis, 2001). The FtsZ-binding protein remains to be isolated and identified, but I think the point mutations to the coding region of the extreme C-Terminus of FtsZ introduced in this study do inhibit the interaction between FtsZ and the unknown protein(s). I think the loss of this interaction eliminates division, and that is why no intragenic suppressor mutation of any of the three independent point mutations was easily found in this study. Either I failed to screen enough colonies, or an intragenic suppression event is impossible. Perhaps this region is so important for the protein-protein interaction that a recombination event was forced to occur in order to restore division. If this is the case, no amount of screening any of the three independent XL-1 RED mutagenized libraries will yield a suppressor mutation.

The future experiments of the *ftsZ* C-Terminal domain project will focus on two major topics. First, the biochemical characterization of altered FtsZs will be done to determine if FtsZ polymerization, GTP-binding and/or GTPase activity are affected. To this date, however, no McCormick Laboratory member has been able to purify functional FtsZ protein over-expressed in *E. coli*. Secondly, using the tool pAJ14 the effects of some, or all, mutations of *ftsZ* that effect division can be clearly analyzed to see the division phenotype in aerial hyphae. This plasmid only contains the developmental promoter for *ftsZ* expression *ftsZ2p*. This tool gives one the opportunity to have the most thorough analysis of the exact effect of any *ftsZ* allele in the sporulation process. Upon
completion, the combined studies will give a better understanding for the role of the C-Terminal domain in division and sporulation.
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