

Investigating the Key cell division proteins FtsZ and SepF in *Streptomyces coelicolor*

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Abstract

Understanding the regulation of cell division in *Streptomyces coelicolor* is key to gaining insight into how the complex life cycle of this bacterium is regulated. To date, researchers have established a key cell division protein FtsZ, yet little is known as to how its function is controlled and which proteins associate with FtsZ to form septum. In this thesis I therefore investigate several proteins in *S.coelicolor* to gain a better understanding of their role in cell division. In **Chapter 3** I used the basic binding assay; Bacterial Two Hybrid, to test Interactions between Proteins involved in Cell division, Polar growth and DNA Segregation. Whilst known protein interactions were confirmed; many new binding partners were discovered between a range of proteins involved in all stages of the life cycle. This included confirming SepF2 can bind the divisome initiating protein FtsZ. Furthermore, SepF2 was shown to bind cytokinesis protein SsgB and polar growth protein ParH. In **Chapter 4** I present the initial characterisation of the potential SepF homologs to investigate their role within *S.coelicolor* cell division. I use bioinformatics and *in vitro* techniques to characterise SepF2. SepF2 shares great sequence identity to its homologs but is not predicted to be able to bind lipid membranes. When viewed using microscopy, It forms polymers of long coil structures which take on the appearance of a spring. In **Chapter 5** I attempt to gain a better insight into the biochemical properties of FtsZ from *S.coelicolor*. I use bioinformatics to generate a three dimensional model of FtsZ which shares conserved features with homologs in other bacterium. I use protein assays to understand the dynamics and polymerisation properties of FtsZ. The protein is able to self associate using the ligand Magnesium and requires Guanine Tri Phosphate (GTP) for disassembly of polymers. In **Chapter 6** I characterise SepF2 (SCO2079) a potential SepF homolog found within the cell division gene cluster. I generate a three dimensional structure of the C-terminal domain of SepF2 which appears to share key residues required for FtsZ binding and dimer formation with its homolog in *Bacillus subtilis*. Using Analytical Gel Filtration and buffer optimisation I stabilise the formation of a truncated variant of SepF2 into dimers. Overall, I have found that SepF2 in *S.coelicolor* appears to play a crucial role in several stages of the bacterium's life cycle as it binds to proteins linked to growth, cell division and DNA segregation. The work reported in this thesis contributes to improving our understanding around the regulation of the life cycle of *S.coelicolor*, in particular gaining insight into two key proteins FtsZ and SepF2.

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1 Introduction

Cell division is essential in the life cycle of all organisms including those belonging to the Prokaryotic kingdom. In order to survive a cell needs to divide and reproduce progeny which are an exact replica of the parent cell. Therefore, the process is tightly regulated, involving a sequence of steps and checkpoints; co-ordinated by specialist cytokinesis machinery (*Bai et al, 2021., Errington et al, 2012*). It fundamentally involves the replication of chromosomal DNA, which is segregated to each pole in rod shaped bacteria, followed by the formation of a middle septum; the location of division (*Dessen et al, 2010., Donachie, 1993*). The components and mechanisms employed by this cytokinesis machinery are governed by the morphology of the organism (*Margolin, 2009*). There is huge diversity in the cell shapes of the bacterial phyla, as members occupy all environments, and have evolved their structure to suit the selective pressures of their specific niche (*Young, 2006*). Despite the variation in cell shape; the majority of bacteria use the same basic cell division components to undergo binary fission. Model organisms like the rod shaped *Escherichia coli* and *Bacillus subtilis* have increased our understanding of this symmetrical division (*Donachie, 1993*). However, not all bacteria adopt binary fission for cell division, with *Streptomyces* being one such organism. The elaborate life cycle of this bacterial species has led to the evolution of multiple fission of developed aerial hyphae to generate numerous spore cells (*Jakimowicz et al, 2012*).

To ensure the integrity of the bacterial species is maintained; cell division will only take place once the chromosomal DNA has been replicated and segregated; minimising the risk of producing progeny with fragmented chromosomes (*Bai et al, 2021; Errington et al, 2012*). There is also an additional requirement for the single cell to extend in size. The replication, segregation and cell extension events have dedicated proteins which co-ordinate their respective tasks. The basic components of the cell division machinery are conserved amongst eubacteria, with variations being reflections of an individual's morphology or habitat *Figure 1* (*Cueto et al, 2015*).

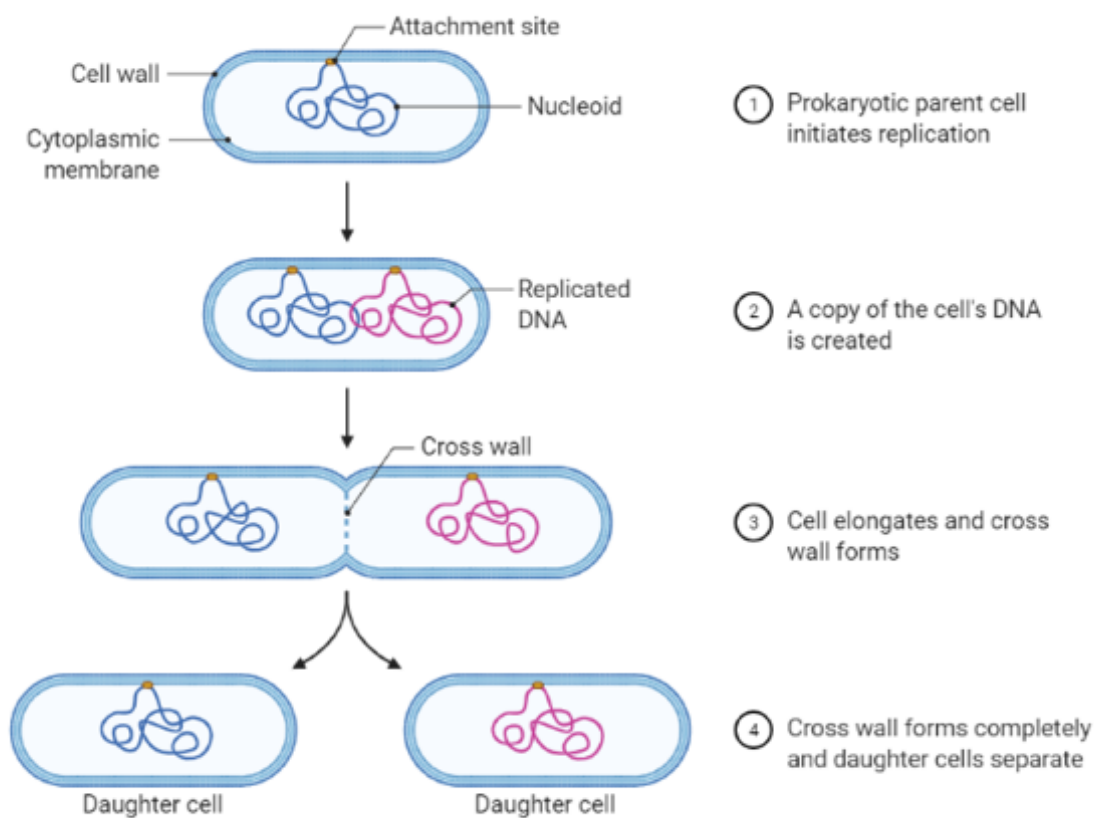


Figure 1. The events that take place for cell division to occur 1) Initiation of DNA replication 2) Replication of DNA completed 3) Cell elongation and septum formation 3) Two progeny produced [Adapted from Biorender 2022]

place at the mid-cell to generate two progeny, each with a copy of the replicated chromosome (Errington *et al* 2012). This crucial life cycle event is coordinated by a multiple protein complex called the divisome. The main component of the divisome is FtsZ; a homolog of eukaryotic tubulin, which forms a ring at the mid cell to mark the site of septum formation (Blaauwen *et al*, 2017; Adams *et al*, 2009). Once the Z ring has been formed by the GTP dependent polymerisation of FtsZ, additional components of the divisome are recruited. Figure 1.1, highlights the major components of the divisome. FtsZ is anchored to the cell membrane via FtsA and ZipA in model rod-shaped organisms where nucleoid occlusion or the MinD system localise FtsZ to the mid-cell. In *Streptomyces* cell division requires Ftsz, SepF and SsgB (Kelemen, 2017).

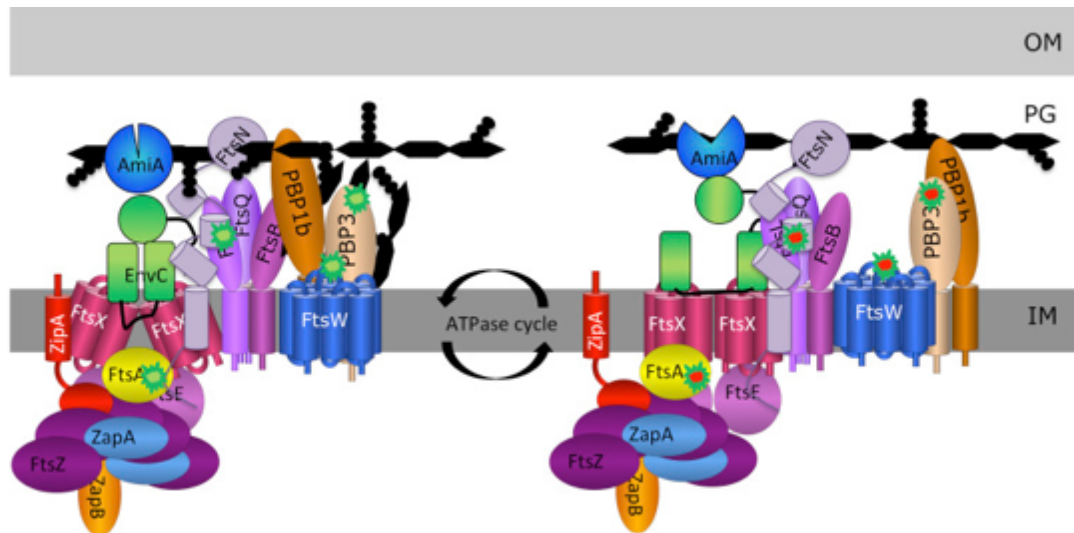


Figure 1.1. The current view of the divisome components in model rod-shaped bacteria. Early proteins FtsZ, FtsA, ZipA and ZapA form at the membrane prior to late protein recruitment including FtsI and FtsW required for septal wall synthesis. A conformational change in proteins including FtsA, FtsN and FtsX along with ATP hydrolysis aids maturation of the divisome [Adapted from Blaauwen et al, 2017]

1.1.1 FtsZ

FtsZ is the first protein which assembles at the site of cell division and is crucial for the recruitment of the other divisome components. The protein structure of FtsZ from *Methanococcus jannaschii* suggests it adopts a fold which is similar to eukaryotic tubulin with a GTPase domain at the N terminus and a four stranded beta sheet at the C terminal separated by a central helix (Opitz et al, 2020; Lowe et al, 1998). Despite the very low level of sequence identity between FtsZ and its eukaryotic counterpart, FtsZ polymerises using the same mechanism after hydrolysing GTP with the motif GGGTGS/TG of one of the two loop regions facilitating nucleotide binding for GTP hydrolysis (Driessen et al, 2001). The GTPase domain is sufficient for polymerisation of FtsZ via the head to tail contact of individual FtsZ proteins into protofilaments. These filaments then bundle using lateral interactions, facilitated by a flexible unstructured region of the C-terminal (Cueto et al, 2015). The fully formed Z-ring localises at the mid point of the cell to form a scaffold and recruit additional protein components of the divisome. The Z-ring is a discontinuous structure formed from many single polymers held by lateral connections rendering it dynamic due to individual polymer and monomer turnover (Blaauwen et al, 2017). The final stages of cell division involve the invagination of the cell membrane and the production of peptidoglycan at the developing septum, which in *E.coli* will constrict

generating two identical daughter cells (Lutkenhaus *et al*, 1991; Buss *et al*, 2010). Recently, it has been shown the z-ring acts as a scaffold and treadmill for the peptidoglycan machinery (Huang *et al*, 2017).

1.1.2 Z-ring regulators

The divisome complex is regulated by a number of proteins to help position and stabilise the Z-ring correctly at the mid-cell. Positive regulators, such as the actin homologue FtsA, bind to the C-terminus of FtsZ; an important region for binding modulators of its activity (White *et al*, 2021., Buske *et al*, 2012). FtsZ is also negatively regulated by two disparate systems; the min and nucleoid exclusion systems to ensure septum formation is central to the cell and doesn't occur over the chromosome (Lutkenhaus, 2007).

1.1.2.1 Positive regulators

Positive regulators of FtsZ work to stabilise the Z-ring by attaching it to the cell membrane. The model organism *E.coli* has two trans-membrane proteins FtsA and ZipA which act as membrane anchors by binding to the central hub; a conserved region on FtsZ (Lutkenhaus *et al*, 2002). This tethering to the membrane of FtsZ keeps the divisome in position for cell division to occur. Recent research has suggested FtsA and ZipA can regulate each other as they can interact (Margolin *et al*, 2018).

ZipA contains a short N-terminal periplasmic end followed by a single transmembrane domain with the C-terminal domain containing the FtsZ binding site (Cueto *et al*, 2015). FtsA, unlike ZipA, which is only found in gammaproteobacteria, is widely conserved amongst both Gram-negative and Gram-positive bacteria (Chaudhuri *et al*, 1999). It attaches to the cellular membrane via an amphipathic helix at its C-terminal and interacts with the Z-ring by contacting individual FtsZ proteins (Garcia-Lara *et al* 1999; Lutkenhaus *et al*, 2005). In *E.coli* FtsA is essential for the positioning and polymerisation of FtsZ into Z-rings; whereas *B.subtilis* are still capable of forming septa when FtsA isn't present (Harry *et al*, 2005., Lutkenhaus *et al*, 2002).

An additional protein; SepF complements the role of FtsA in *B.subtilis* and appears to share the ability to stabilise Z-ring maturation. Genes encoding both *FtsA* and *SepF* are located within the cell division cluster, upstream of the gene for *FtsZ* (Errington *et al*, 2006). Deletion of the *sepF* gene results in septum formation defects and *ftsA-sepF* double mutants are not viable, suggesting SepF is involved in Z-ring stabilisation. Mutations in C-terminal residues of FtsZ which have been shown to facilitate FtsA binding, also abolish its interaction with SepF (Bezouwien *et al*, 2012). SepF is also capable

of binding the lipid bilayer through an amphipathic helix found at its N-terminal which was confirmed with a truncated protein variant lacking residues 1-12. Moreover, leucine at position 7 is key for this interaction as a mutation at this position in the full length protein also renders it incapable of binding lipids (Celik *et al*, 2013).

1.1.2.2 Negative regulators

The correct positioning of the septum is under the control of two disparate systems which work simultaneously; the *min* and nucleoid exclusion systems (Lutkenhaus, 2007) *Figure 1.1.2.2.1*. The *min* system in *E.coli* prevents septum formation at the cell poles and consists of three proteins; Min C, D and E. Min C, once activated by membrane bound MinD, inhibits cell division by antagonising FtsZ and is found throughout the cytosol. During cell division, the third protein MinE dimerises and oscillates from pole to pole to inhibit MinCD. Thus, MinC is not found at the mid-cell, where the highest concentration of MinE is found, *Figure 1.1.2.2.1*. (De Boer *et al*, 2001). This in turn means FtsZ is not antagonised at the mid-cell and accumulates there. In *B.subtilis* oscillation of MinCD is not observed, and the protein MinE is not present (Edwards *et al*, 1998). Instead, DivIVA, a coiled-coil protein, aids the central z-ring formation by sequestering MinCD at the poles facilitated by the adapter protein MinJ (Baarle *et al*, 2009). DivIVA forms higher-order assemblies facilitated by its coiled-coil structure which allows for its specific association with negatively curved cell membranes (Errington *et al*, 2009).

A second system, known as nucleoid exclusion, prevents cell division in the proximity of the chromosome, *Figure 1.1.2.2.2*. Nucleoid exclusion factors; SlmA and Noc in *E.coli* and *B.subtilis* respectively, prevent FtsZ polymerisation which would intersect the chromosome (Bernhardt *et al*, 2017., Errington *et al*, 2004). Two separate studies have concluded SlmA targets FtsZ, but with their findings disagreeing on the mechanism. The first, suggested SlmA acts by depolymerising FtsZ, whereas the second concluded it disrupts FtsZ higher assembly (Bernhardt *et al*, 2011., Tonthat *et al*, 2011). Although both Noc and SlmA bind the nucleoid, Noc executes its function on an alternative target to FtsZ (Errington *et al*, 2012). It has been shown to bind the cell membrane, sequestering the nucleoid (Adams *et al*, 2015).

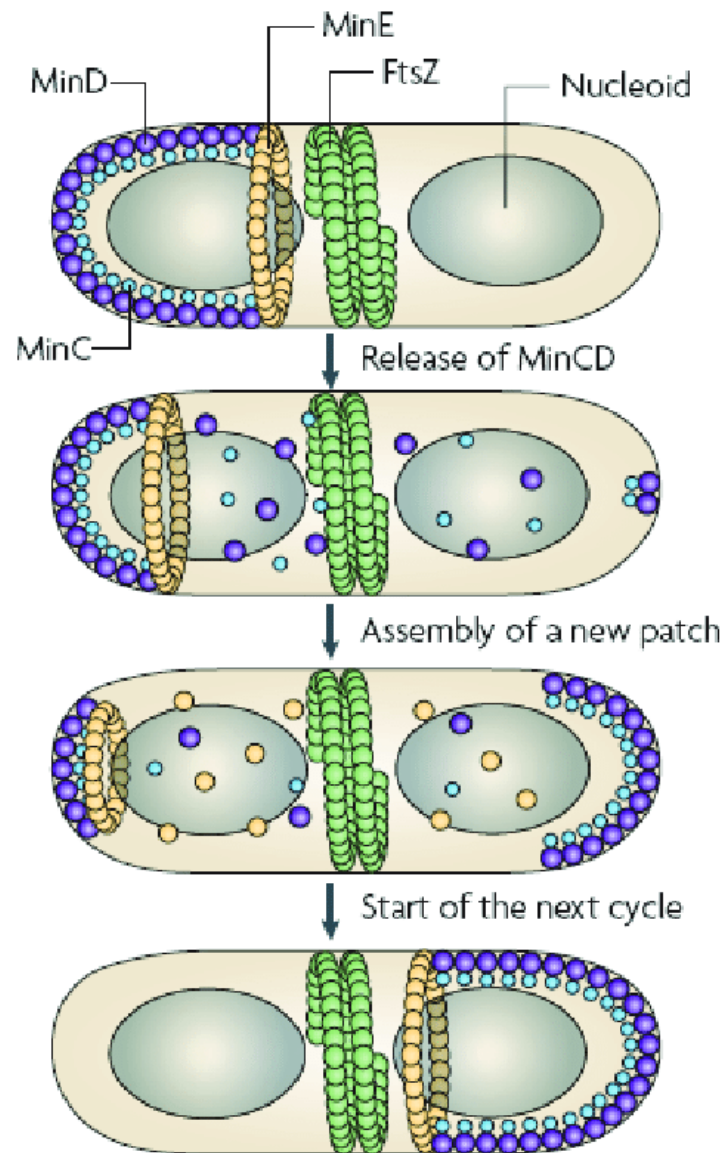


Figure 1.1.2.2.1. The MinCDE system in *E. coli*. MinD (blue) binds the membrane with MinC. ATP hydrolysis to MinD-ADP releases it from the membrane. MinD-ATP assembles at membranes distant to oscillating MinE (orange), creating a MinCD gradient which is lowest at mid-cell. FtsZ (green) assembles where the lowest concentration of MinCD is; at the mid-cell. [Adapted from Baarle et al, 2009]

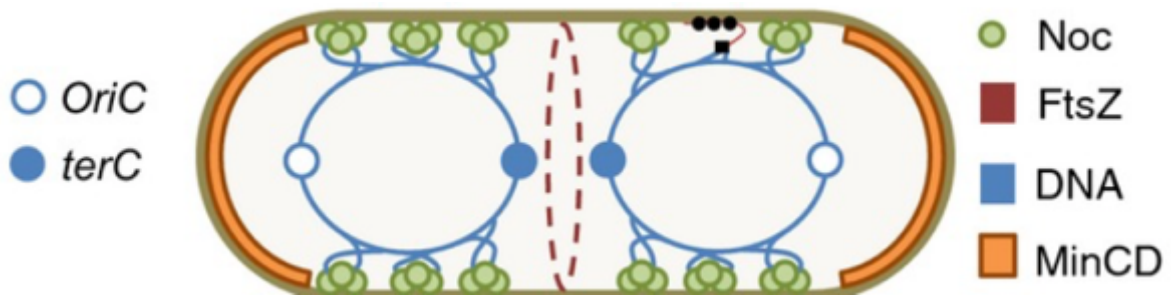


Figure 1.1.2.2.2. The Nucleoid exclusion system in *B. subtilis*. Noc (green) binds the membrane and DNA to hold it away from the mid-cell where FtsZ forms a z-ring [Adapted from Errington et al, 2015]

1.1.3 Z-ring Maturation

Once the Z-ring has formed by the GTP dependent polymerisation of FtsZ and anchored to the membrane, additional proteins are recruited to form the complete divisome (*de Boer et al, 2010*). This second step in cytokinesis occurs after a lag and adds the essential proteins to the complex (*Beckworth et al, 2005*). In *E. coli* these extra proteins are added to the Z-ring in a step wise process, *Figure 1.1.3.1*.

The first protein couple recruited is FtsE and FtsX which together form an ABC transporter with a membrane associating domain and ATPase, respectively. The pair work to remove lipoproteins from the membrane and absence of them leads to a defect in cell division (*Schmidt et al, 2004; Reddy et al, 2007*). The second protein to join is a DNA translocase FtsK which sits within the membrane via four transmembrane spanning segments. It provides an essential link between division and chromosome segregation as FtsK translocates DNA away from the septum by binding KOPS sequences on the chromosome (*Bigot et al, 2005*).

Following on from the previous protein recruitment, is the acquisition of a triplet of proteins FtsQ, FtsL and FtsB. All three have no enzymatic capability and serve a role to link the peptidoglycan biosynthetic machinery to the divisome. FtsQ has a large periplasmic domain and a membrane embedded region while the other two are much smaller but still contain a transmembrane segment (*Beckwith et al, 2005*). The tri-part recruit FtsI and FtsW which are proteins involved in the late stages of cell division (*Lutkenhaus et al, 2012*).

FtsW and FtsI are both members of a SEDS (shape, Division and Sporulation) protein pair. FtsI is a PBP (Penicillin Binding Protein) Transpeptidase called PBP3. The PBP3 enzyme both synthesises and crosslinks peptidoglycan using transglycosylation and transpeptidation to form a double layer of

peptidoglycan (Dessen et al, 2010). FtsW is thought to transport the peptidoglycan precursors required for FtsI (Holtje, 1998).

The final protein recruited is FtsN which is thought to signal that maturation of the Z-ring is complete and so septation should occur. Its structure is of a bitopic protein with a transmembrane domain connecting a large periplasmic part to a smaller cytoplasmic section. A SPOR domain at the C-terminal binds to peptidoglycan specific to the septum (Gerding et al, 2009).

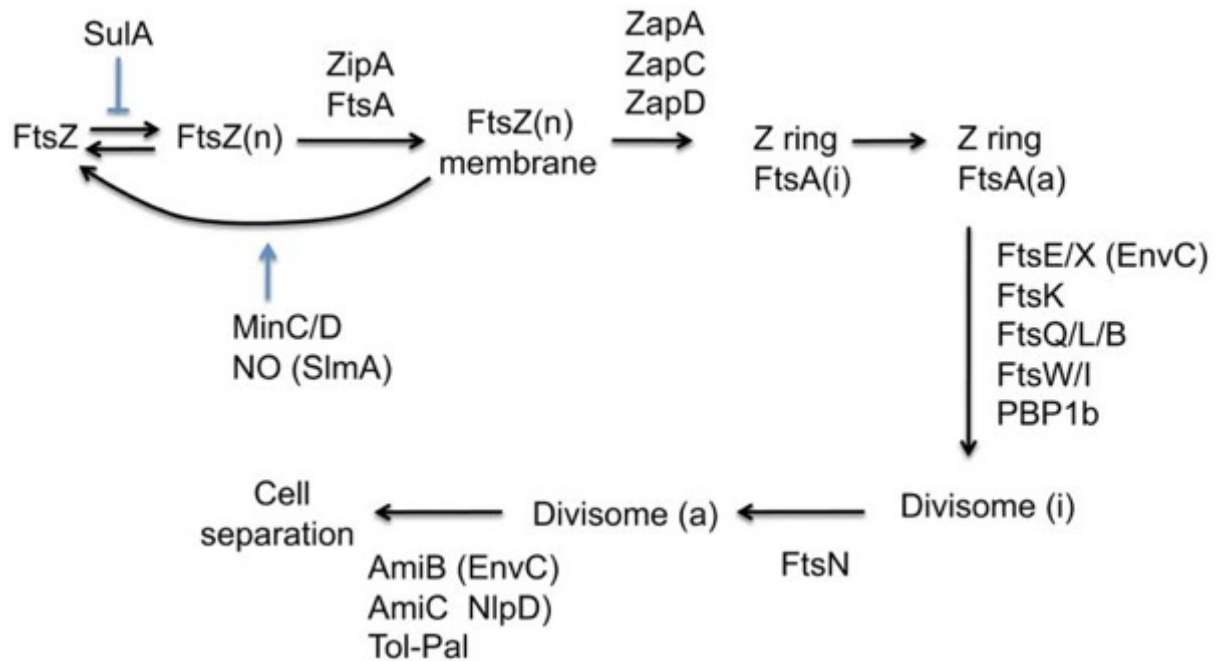


Figure 1.1.3.1. Z-ring maturation in *E. coli*. FtsZ polymers (FtsZ[n]) are anchored to the membrane by FtsA. Active FtsA recruits the remaining proteins to form the Divisome. a=active i=inactive. [Adapted from Du et al, 2012]

1.2 Bacterial Growth

Bacterial cell wall growth can take place at different locations, which contribute to maintaining their shape and morphology (Bai et al, 2021). Cell wall growth is due to incorporation of peptidoglycan into the cell wall. Peptidoglycan synthesis is a complex process, with the process requiring different protein complexes at cell division and cell elongation, Figure 1.2.1. In both the process begins in the cytosol where lipid II is synthesized at the inner leaflet of the cytoplasmic membrane and is transported to the periplasmic side by flippase MurJ. Lipid II is then utilized by Peptidoglycan synthases

PBPs, Peptidoglycan glycosyltransferases (GTases) and transpeptidases (TPases) to cross link the glycans and form peptide crosslinks. Penicillin-binding proteins (PBPs) of class A (also called aPBPs) catalyse both reactions (Benett 2020).

The first type of bacterial growth takes place at lateral sites of rod-shaped bacteria, whereby new cell wall incorporation is driven by the actin homologue MreB. The second mechanism of growth is utilised by bacteria which exhibit cellular elongation at the poles, which is controlled by DivIVA (Flardh et al, 2003). The third location where new cell wall synthesis takes place is the division site. This involves the incorporation of peptidoglycan at the mid cell to complete cell division. This process is governed by FtsZ which co-ordinates the divisome machinery (Burby et al, 2020).

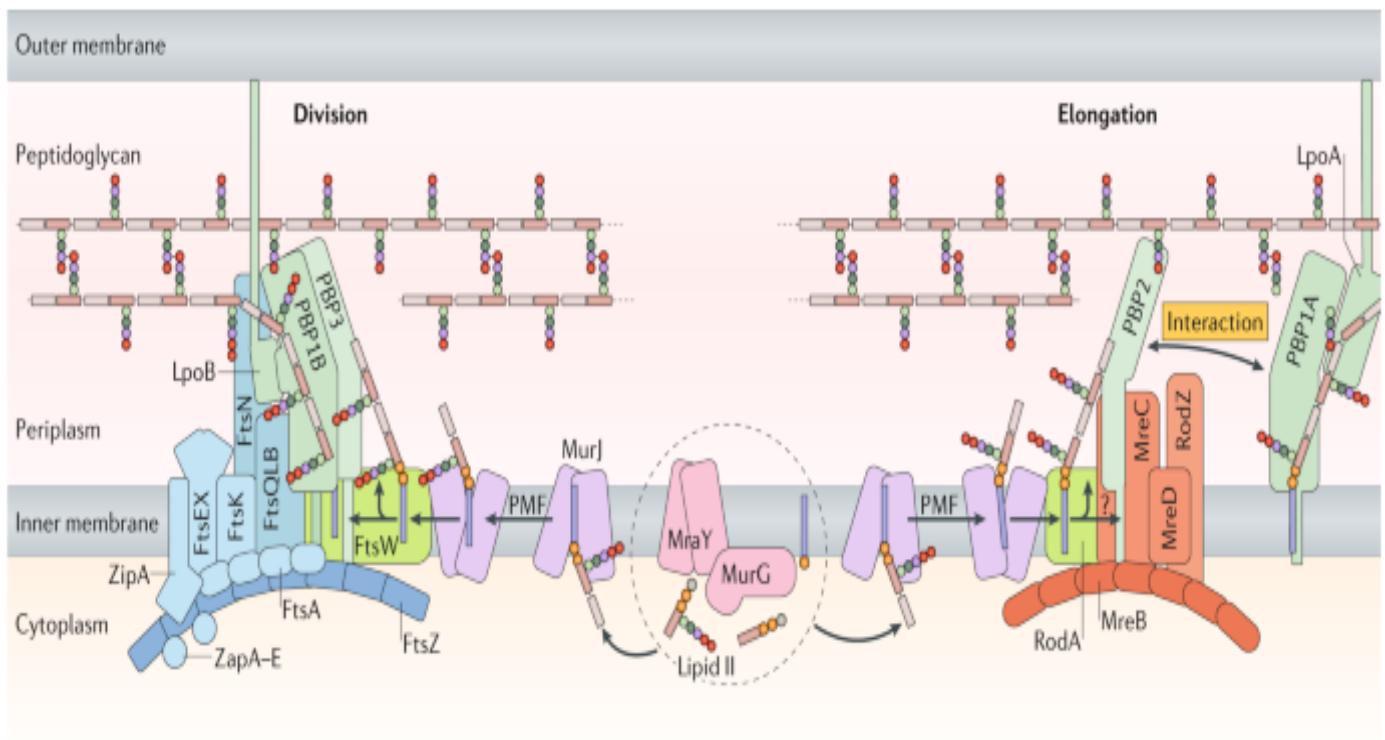


Figure 1.2.1. The process of peptidoglycan synthesis at cell division and cell elongation. Lipid II is synthesized in the cytosol and transported via Flippase MurJ to the periplasm where it is synthesized into glycan chains by Peptidoglycan glycosyltransferases (GTases). Transpeptidases (TPases) form peptide crosslinks between the glycan chains with Penicillin-binding proteins (PBPs) of class A (also called aPBPs) catalysing both of the latter reactions. [Adapted from Errington, 2020]

1.2.1 Lateral Growth

A homolog of eukaryote actin; MreB directs the pattern of new wall growth in rod-shaped cells by interacting with peptidoglycan synthesising enzymes (*Amos et al, 2001*). A non-dividing cell has peptidoglycan incorporated into the side walls with MreB forming actin like cables to facilitate this cell elongation (*Park et al, 2008*). The double helical cables, dissociate during cell division and form an outer ring around the septum marking Z-ring. This targets cell wall synthesis to the mid-cell rather than the lateral walls.

The distribution of MreB homologues amongst rod-shaped bacteria seems to correlate to an individual's morphology. Many rod-shaped bacteria require MreB for correct cell shape, but some require more MreB proteins than others. MreB is not found in spherical bacteria or those which grow by tip extension (*Margolin et al, 2009*). The current model, *Figure 1.2.1.1*, by which MreB cables facilitate cell elongation has been suggested due to multiple studies on the protein. MreB polymerises into filaments using ATP, which form antiparallel double filaments and eventually complex sheets. These patches and filaments contact the cell membrane and rotate unidirectionally to direct sidewall synthesis (*Furchtgott et al, 2011*).

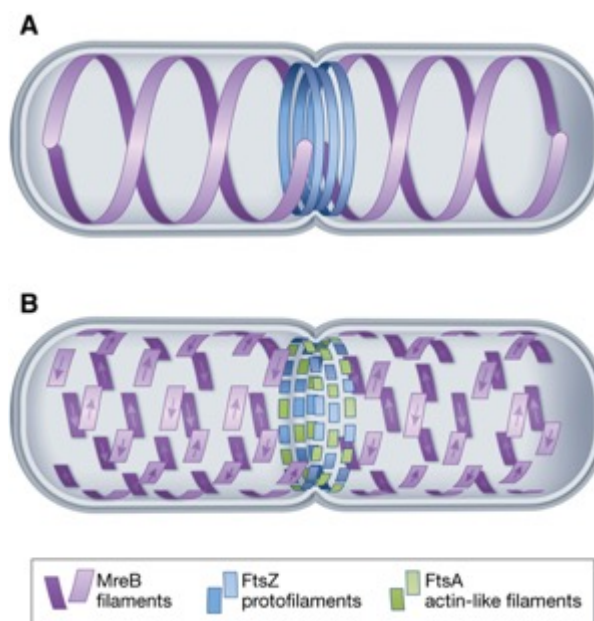


Figure 1.2.1.1 (A) MreB (purple) spiral pattern of filaments controlling cell shape made up of patches of MreB polymers **(B)** [Adapted from Margolin et al, 2012]

1.2.2 Polar Growth

The majority of bacteria, including *E.coli* and *B.subtilis* divide by binary fission after undergoing cell elongation by lateral growth. However, some bacteria grow by polar growth including Gram-positive *Actinobacteria* (Errington *et al*, 2003). The *Actinobacteria* genera include cells which form cocci and rod shaped as well as branched cells. Despite the range of diverse morphologies, all grow by tip extension using DivIVA (Flärdh *et al*, 2019., Brun *et al*, 2011). DivIVA is a highly conserved protein which carries out a range of tasks in bacteria, most notably its involvement in Z-ring localisation in rod-shaped cells, such as *B.subtilis*. Although, DivIVA localises to the cell poles in the bacteria which have the protein, it either serves to control cell division or growth. A knock out of DivIVA in *Corynebacterium glutamicum* resulted in the formation of circular cells due to the disruption of polar growth. This highlights its role in cell wall growth patterning in this bacterium (Flärdh *et al*, 2008). The protein is thought to control cell wall growth by directing the growth machinery; interacting with Penicillin binding protein 3 (PBP3). The PBP3 enzyme both synthesises and crosslinks peptidoglycan using transglycosylation and transpeptidation to form a double layer of peptidoglycan (Dessen *et al*, 2010).

Homologs of DivIVA are absent from the Gram-negative bacteria which utilise polar growth. This discovery suggests polar growth has evolved independently in Gram+ and Gram- bacteria and they use another mechanism. Further studies need to be conducted to find the exact proteins implicated in the cell elongation of these bacteria (Brun *et al*, 2011).

1.3 Chromosome Segregation

To ensure both cells of a dividing pair are identical, they need to contain a copy of the chromosome each. Therefore, prior to cell division, the chromosomal DNA must be replicated and segregated to the poles of the paternal cell. This process is highly organised and coordinated with cell division to guarantee DNA is not sliced by the incorrect placement of the septum or due to poor segregation (Lutkenhaus *et al*, 2012., Leach *et al*, 2018). Chromosome segregation in rod-shaped bacteria such as *E.coli*, is regulated by the tripartite ParA, ParB and *parS* found at the plasmid partitioning (Par) locus on the genome (Koonin *et al*, 2000).

The Par proteins are a part of the ParA/MinD superfamily with the Par members involved in chromosome segregation and the Min proteins having a role in septum localisation (Adam *et al*, 2020). Despite their different functions all can be classed as Walker A cytoskeletal P loop ATPases (ParAs) with a characteristic GXGGXHKTS motif to bind ATP. Par systems comprise of an ATPase and a protein which binds to a specific region on DNA (Gerdes *et al*, 2002). ATP dependent polymerisation of ParA

forms protofilaments to drive the translocation of ParB DNA binding protein to the cell poles. ParB binds to the *parS* gene locus, which resides near to the origin of chromosomal replication. Thus, the repositioning of the chromosome has parallels with the eukaryotic mitotic system (Gerdes *et al*, 2010).

1.4 Streptomyces

Streptomyces are Gram-positive bacteria belonging to the phylum *Actinobacteria*, which occupy areas of decaying matter such as soil and sediments. These filamentous bacteria have a uniquely high content of G + C DNA and produce a wealth of secondary metabolites, which are of importance to medicine and biotechnology, including antibiotics and antimicrobials (Hopwood, 2007). The majority of antibiotics in use today have originated from *Streptomyces*. Furthermore, *Streptomyces* also have an important role ecologically in the recycling of fungi and plant materials (Chater, 2016). A lot of the secondary metabolites that they are capable of producing are regulated by genetic pathways that need to be switched on, for example biosynthetic gene clusters. A lot of research into this species of bacteria has focused on unlocking these pathways and switching on production of the metabolites.

Their life cycle and cell structure are complex and resembles that of filamentous fungi (Araujo *et al*, 2012). The complexity of the *Actinomycete* life cycle and the fact cell division is not required for their survival has a huge impact on how it is conducted. This is highlighted by the presence of actinomycete specific proteins, alongside the basic cytokinesis machinery, for effectively co-ordinating division (Jakimowicz *et al*, 2012). *Streptomyces coelicolor* is the most widely studied member of the genera and has become a model organism to delve into the regulation of cell cycle events including the gene expression of the proteins controlling aerial hyphae growth and sporulation. These studies are important as *Mycobacterium*; which includes pathogens that cause tuberculosis and leprosy, is also in the genus *Actinobacteria*. Therefore research into *S.coelicolor* can help to improve our understanding of the bacterium involved in these diseases and lead to developing therapies to control them.

Recently, studies have been conducted in *Streptomyces venezuelae* due to its fast growth rate and ease of genetic manipulation. It also sporulates in liquid media rendering it an ideal host for developmental studies (Cho *et al*, 2021). This is in contrast to *Streptomyces coelicolor* which has a much slower growth rate and clumps into mycelial aggregates in liquid growth media. Furthermore, a wealth of tools for genetic manipulation have been developed for use in *Streptomyces venezuelae* including the latest techniques like CRISPR-Cas9 gene editing.

1.4.1 Life Cycle

The life cycle of *Streptomyces* is highly complex and vastly different to that of the typical rod-shaped bacteria, such as *E.coli*, which undergo binary fission. A single *Streptomyces* spore cell will germinate into germ tubes which develop into branching vegetative hyphae. This then forms a network of mycelium which uptakes nutrients from the environment. Growth is exponential and occurs via extension at the hyphal tips with branches forming along the hyphae. Once the nutrient source has depleted, the vegetative phase forms extending aerial hyphae, which undergo multiple fission to generate numerous single cell spores for replication, *Figure 1.4.1.1* (Lopez-Garcia et al, 2012). The switch between vegetative and aerial hyphal growth is controlled by a number of genes with the latter stage being linked to secondary metabolite production.

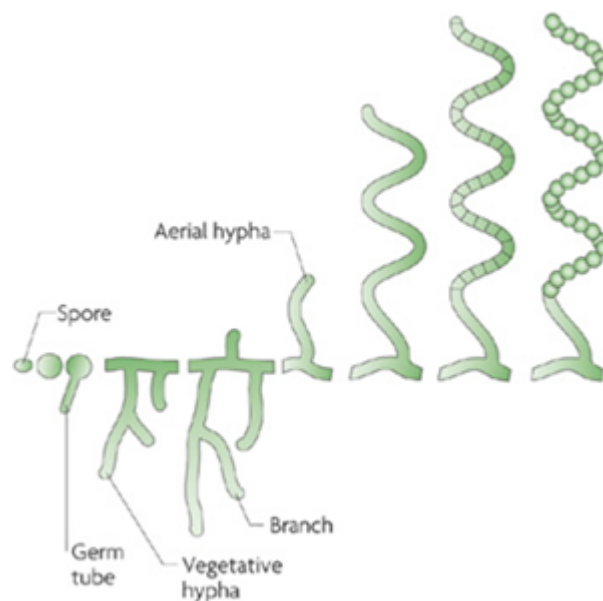


Figure 1.4.1.1. The life cycle of *Streptomyces*; a single spore germinates and develops into a germ tube and hypha which branch to form the vegetative mycelium. Upon nutrient depletion, growth switches to aerial hyphae and eventually they differentiate into prespore compartments and mature spores for dispersal. [Adapted from Buttner et al, 2009]

Cell division which generates progeny only occurs in the second growth phase when aerial hyphae have fully developed. However, vegetative mycelium are separated into compartments by an irregular pattern of cross-walls which do not constrict. These cross walls are made up of DNA

impermeable membranes (Willemse *et al*, 2016). Each compartment contains several copies of the chromosome and communication is possible throughout the hyphae even though they are segmented. Growth is achieved predominantly due to branching at the lateral walls. Colonies suspended at this stage display a 'bald' phenotype, with a smooth appearance, due to the blocking of the *Bld* genes which are required for the initial stages of aerial hyphae growth (Merrick, 1976).

Once the nutrient source has been exhausted, the mycelial biomass is recycled by lysis to aid in the formation of aerial hyphae (Miguélez *et al*, 1999). Sporulation occurs once aerial hyphae have elongated by apical tip extension. Aerial hyphae growth and subsequent sporulation is initiated by a cascade of proteins in response to external factors such as nutrient depletion (Chater, 2003). The two set of proteins involved in aerial hyphae formation and sporulation are encoded by the bald (*bld*) and white (*whi*) genes respectively (Bechthold *et al*, 2015). Many of these genes are sigma factors or transcription factors required to promote the transcription of downstream effectors (Buttner *et al*, 2000). These cascades exist to coordinate polar growth and sporulation, with cross-talk between the proteins involved in chromosome segregation, septum localisation and apical tip extension. It is also during this stage in the life cycle that antibiotics are produced as a form of protection for the colony. Polar growth occurs at the hyphal tips, generating multigenomic hyphae which give a white fluffy appearance (Celler *et al*, 2016).

The final stage in the life cycle is the generation of multiple individual uni-genomic spores which disperse into the environment. Once chromosomal DNA has been aligned the mature aerial hyphae undergo cell division to produce prespores due to the regular placement of septa. As in other bacteria, FtsZ promotes the assembly of the Z-ring to recruit the divisome components needed for cytokinesis. The maturation of the spores is highlighted by a grey appearance and signals the completion of the life cycle (Barka *et al*, 2016).

1.4.2 Polar Growth

Streptomyces belong to a group of bacteria which grow using tip extension at the poles of the cell and so undergo polar growth for cell elongation. This apical tip extension is conducted by a polarisome protein complex the tip organising centre (TIPOC) (Flärdh, *et al*, 2019). This protein assembly is required to facilitate polar growth whilst maintaining the structural rigidity of the hypha (Ausmees, 2013). Growth is controlled by a collection of coiled-coil proteins including DivIVA, Scy (*Streptomyces* Cytoskeletal element) and FilP (Filament-like protein) which share a common overall architecture, (Herron *et al*, 2011). All three have two coiled-coil domains, separated by a flexible linker

region, allowing subunit oligomerisation into higher order assemblies (Burkhard *et al*, 2001). The first coiled-coil segment has a canonical heptad repeat structure with an apolar residue located at the first and fourth position. The second segment differs between the proteins in terms of length and residue composition *Figure 1.4.2.1*.

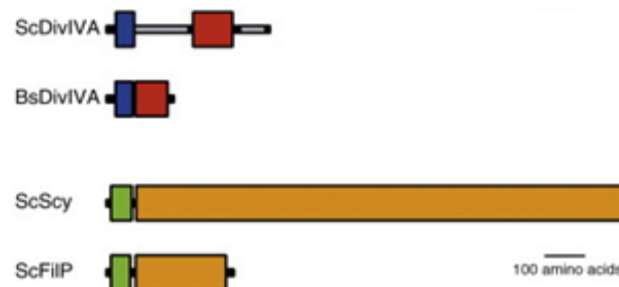


Figure 1.4.2.1: The overall domain architecture of coiled-coil proteins affecting hyphal growth in actinobacteria, with coiled-coil domains shown as boxes. *B.subtilis* DivIVA is compared to *S.coelicolor* DivIVA; ScDivIVA has a longer linker region. ScScy is compared to ScFilP; both have a canonical heptad repeat in the first domain and a longer 51 repeat in the second. The second coiled-coil domain is longer in ScScy. [Adapted from Flärdh, 2010]

The main orchestrator of the TIPOC is Scy, which provides a platform to assemble the proteins required in polar growth. Scy is a large Intermediate Filament (IF) like protein due to it sharing common structural and sequential features with IFs from eukaryotes. Both have coiled-coil domains formed by alpha helices coiling together. Scy localises at the hyphal tips, forming coiled-coil filaments, to facilitate and coordinate the association of the other TIPOC components. Several homologs of Scy have been identified but only amongst filamentous *Actinomycetes*. The second coiled-coil domain of Scy is relatively large, with a novel 51 residue repeat, rendering it capable of forming long filaments to facilitate protein interactions (Holmes *et al*, 2013).

This first suggestion of Scy being implemented in polar growth was when it was found to localise to hyphal tips in *S. coelicolor* (Holmes *et al*, 2013). Null mutants of *scy* in *S.coelicolor* have impaired polarised growth with TEM showing distinct variations in hyphal width and tip symmetry between the wild-type and mutant. The mutant strain also has irregular chromosome segregation suggesting Scy is essential for maintaining the TIPOC which has been shown to coordinate polar growth with cell division and chromosome segregation (Ditkowski *et al*, 2013). Higher-order organisation of Scy into filaments is thought to facilitate its interaction with other known TIPOC

components including DivIVA. Furthermore, proteins not directly related to polar growth, including ParA have been shown to be regulated by their interactions with Scy (Ditkowski *et al*, 2013). Therefore, Scy performs a scaffolding role at hyphal tips to recruit and organise other proteins with its long CC51 domain providing a platform for interaction.

DivIVA acts as a marker at hyphal tips to promote growth and was the first protein of the TIPOC to be characterised. DivIVA is an essential polarity regulator for tip extension and branching in *S.coelicolor* and is a homolog of the *B.subtilis* DivIVA sharing a genetic location downstream of *ftsZ* (Cha *et al*, 1997). DivIVA of *S.coelicolor* has a similar domain organisation to its homolog from *B.subtilis*, but with a longer linker region between the two coiled-coil domains. Partial deletion of DivIVA in *S.coelicolor* resulted in a phenotype resembling those seen in mutant fungi lacking tip growth proteins. Hyphae became kinked and curled with an irregular pattern of branching. Moreover, overproduction of DivIVA caused hyperbranching and bulging of hyphal tips (Flårdh *et al*, 2003). Recent studies have concluded DivIVA marks the site of hyphal branches and is responsible for recruiting other essential regulators of polar growth, including the cell wall synthesising enzyme PBP3. It has also been shown that the lipid composition of the membrane determines the affinity DivIVA has for the membrane (Flårdh *et al*, 2020). Therefore it plays an integral role of controlling polar growth (Breukink *et al*, 2017).

The final protein required to form the TIPOC complex is the Intermediate Filament like protein FilP which forms a stress bearing structure to support tip growth as apical tip extension is dynamic, rendering the hyphal tip flexible and vulnerable (Gooday, 1990). FilP is recruited to the TIPOC by DivIVA and assembles into a network of crosslinked cytoskeletal filaments (Ausmees *et al*, 2008). The overall domain organisation in FilP reflects that found in Scy but with a shorter second coiled coil region. Both Scy and FilP have been proposed to be IF like and class as members of the newly emerged bacterial IF family (Kelemen, 2017). The first member identified was Crescentin from *Caulobacter crescentus* and AbpS from *Streptomyces reticuli*, has been shown to form dimers and higher order homotetramers via interactions between its two alpha-helical domains (Ausmees, 2006; Schrempf *et al*, 2003).

FilP forms a variety of branching filamentous networks, to provide a “stress bearing” structure, within the growing hyphal tips of *Streptomyces*. FilP was first localised to the tip and curved membrane of hyphae with null mutants of FilP having impaired growth as aerial hyphae are less rigid when mechanically probed with Atomic force microscopy (Ausmees *et al*, 2008). It is now known that FilP is capable of forming an interconnected network *in vivo*, with its dynamics governed by DivIVA (Ausmees, 2013). This suggests FilP is a cytoskeletal element which maintains the integrity of growing

hyphae while new peptidoglycan is being incorporated at the tips. Once growth has terminated, Flp expression is repressed by WhiA and WhiB resulting in its dispersal throughout the hyphae (Bibb *et al*, 2016) This is another example of where the TIPOC is involved in coordinating polar growth with cell division.

1.4.3 DNA segregation

As mentioned previously chromosome replication and segregation needs to be coordinated with growth and cell division to ensure all progeny are identical. The majority of bacteria divide by binary fission to produce two daughter cells, each with a single copy of the chromosome. *Streptomyces* and other filamentous bacterium have an elaborate life cycle involving multiple growth stages. The alignment of numerous copies of the chromosome along growing hyphae is coordinated with polar growth, to guarantee the high number of chromosomes replicated are positioned correctly for sporulation to produce multiple spores.

Replication of DNA in mycelium and aerial hyphae seems to be unsynchronised and multiple chromosomes are generated which reside in the hyphae. Model organisms segregate DNA using the Par proteins, whereby the ATPase ParA drives the relocation of DNA bound ParB to the poles. *S.coelicolor* has itself a partitioning system and deletion of the *parAB* genes seems to exclusively effect segregation in aerial hyphae (Dedrick *et al*, 2009). ParA is recruited to the hyphal tips by Scy where its polymerisation is initiated (Donczew *et al*, 2016). It forms long filaments along the hyphae, contacting its partner ParB to align the chromosomes and position one chromosome for each prespore, (Ditkowski *et al*, 2013., Chater *et al*, 2005).

Prior to sporulation the 'arm' regions of the chromosomes are separated from the 'core' regions. This is facilitated by ParB, the Structural Maintenance of Chromosomes (SMC) protein Condensin and *Streptomyces* specific HU protein HupS (Dulawa *et al*, 2021). Once sporulation is initiated, the 'arm' regions are brought into close proximity with the 'core' region to allow for one chromosome for each spore.

1.4.4 Cell Division

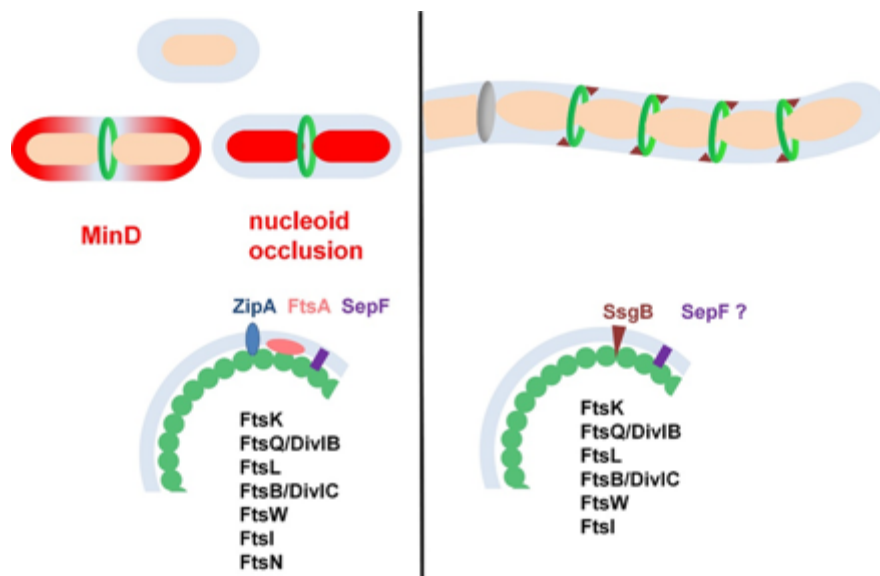


Figure 1.4.4.1. The divisome components in model rod-shaped bacteria (left) and *S. coelicolor* (right). The typical composition includes FtsZ (green) anchored to the membrane via FtsA, this component is missing in *Streptomyces*. FtsZ, SepF and SsgB are all required for cytokinesis in *S. coelicolor*. [Adapted from Kelemen, 2017]

Streptomyces do not require cell division for survival, which sets them apart from other bacteria. During vegetative growth single layered non peptidoglycan containing cross walls are formed at irregular positions, as the hyphae develop, to generate a collection of interconnected compartments (Champness *et al*, 1994; Celler *et al*, 2016). The vegetative septa remain intact with FtsZ being the only protein shown to be required for septum formation. Null mutants of all the divisome components do not effect viability and a null mutant of FtsZ is still capable of forming vegetative septa (Flärdh *et al*, 2003). This may be due to the fact that the hyphal cross walls are formed with asynchrony and are not to be constricted. Therefore, proteins for Z-ring stabilisation, localisation and constriction such as FtsA, are not needed in *Streptomyces* mycelium. The mechanism of FtsZ recruitment is still to be elucidated.

Unlike vegetative growth, sporulation specific cell division is synchronised to produce multiple regularly spaced septum which go on to be fully constrict and generate spores. This is predominantly due to FtsZ monomers which form an organised assembly along aerial hyphae appearing as a ladder of evenly distributed Z-rings (Angert *et al*, 1997). While null mutants of FtsZ are still capable of

vegetative division, they are unable to perform sporulation specific division. This suggests FtsZ is crucial in the shift from vegetative to aerial hyphae formation (Buttner *et al*, 2000).

1.4.4.1 FtsZ

The initiation of cell division in both rod-shaped bacteria and *S.coelicolor* is controlled by the polymerisation of FtsZ. While FtsZ is crucial to the viability of many bacterium it is dispensable in *Streptomyces* even though vegetative cross walls and sporulation specific septa do not form. In fact, several proteins of the divisome that are essential to cell division in other bacteria are not required for *Streptomyces* cell division, making it an ideal organism for studying cytokinesis Figure 1.4.4.1. (Andreu *et al*, 2004).

Once aerial hyphae are fully developed, and the linear chromosome has been aligned, septum can begin to form. FtsZ polymerises into a series of regularly spaced Z-rings, which give a ladder-like appearance and mark the site of future septa. The regularity and synchronisation of the rings is highly controlled including the upregulation of the *FtsZ* gene. Initially spiral shaped FtsZ intermediates are remodelled into the regular Z-ring spacing due to the rise in available FtsZ (Flärdh *et al* 2005). Once the Z-rings are aligned, sporulation septa rapidly appear and constrict to give rise to a chain of unigenomic spores. Like rod-shaped bacterium, constriction of the cell membrane to produce a peptidoglycan cell wall is due to the recruitment of additional divisome components by FtsZ.

1.4.4.2 Z-ring regulation

Z-ring formation does not appear to be controlled by negative systems, like the min and nucleoid occlusion mechanisms of rod-shaped bacteria. Although the *S. coelicolor* genome encode a homolog of DivIVA, it is involved in tip extension rather than septum localisation (Flärdh *et al*, 2008). There are no homologs of Noc, SlmA or minC and minE and the essential anchoring components of the divisome, found universally amongst the bacterial phyla are also missing from the *Streptomyces* genome (McCormick, 2009). Instead, Z-ring positioning is positively regulated by proteins which locate to the division site (Margolin *et al*, 2015). Alternative *Actinomycete* specific proteins recruit and stabilise FtsZ polymerisation including SsgA-like proteins (SALPS) and membrane associated dynamins A and B (Schlimpert *et al*, 2017).

SsgB is a small membrane associated protein of the divisome complex which directly recruits FtsZ and promotes its polymerisation Figure 1.4.4.1. (Borst *et al*, 2011). Its binding partner SsgA

controls septum location as it positions SsgB, thus promoting Z-ring uniformity along the aerial hyphae. Previous studies involving null mutants of both *ssgB* and *ssgA* produced colonies that unable to sporulate (Noens *et al*, 2005). Furthermore, both SsgB and FtsZ proteins colocalise during sporulation in *S.coelicolor* to produce a near identical pattern (Borst *et al*, 2011). Even though it is unknown what aids the positioning of SsgB and SsgA, a model for the control of Z-ring maturation by the SALPS has been proposed. It suggests young aerial hyphae have regular foci of SsgA while FtsZ and SsgB are dispersed. This is followed by the colocalization of SsgA and SsgB at alternate sides of the hyphae. FtsZ concentration is upregulated prior to sporulation specific division where it forms long spiral structures. These spirals may attach to the SsgB foci to tether the spirals which eventually form Z-rings *Figure 1.4.2.2.1*. The current model suggests chromosome segregation, divisome formation and polar growth are synchronised. In young aerial hyphae, SsgA positions itself on the chromosome while ParA is restricted to the tip. In early division, SsgA and SsgB associate and ParA extends filaments, prior to ParB contact. FtsZ assembles, first as spiral-like filaments and then as Z-rings, with SsgB recruiting FtsZ.

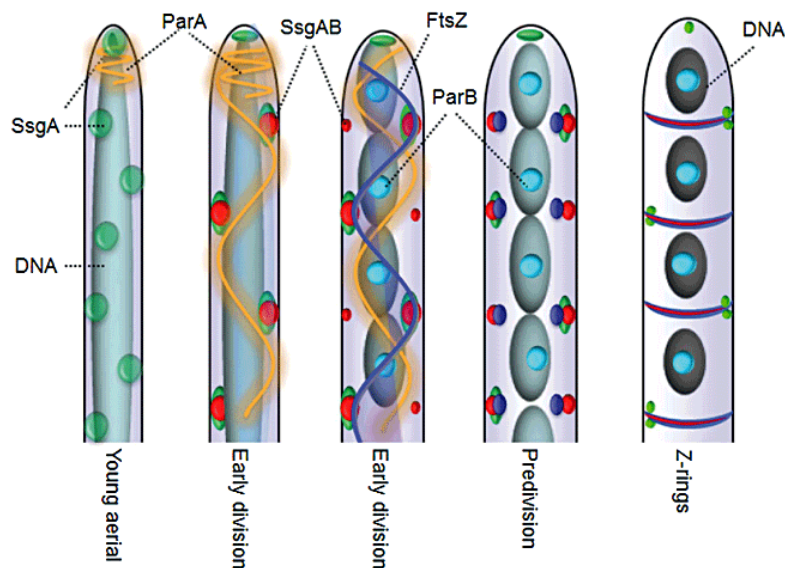


Figure 1.4.2.2.1. A representation of the events involved in Z-ring localisation and chromosome segregation in *Streptomyces*. In young aerial hyphae, SsgA positions itself on the chromosome while ParA is restricted to the tip. In early division, SsgA and SsgB associate and ParA extends filaments, prior to ParB contact. FtsZ assembles, first as spiral-like filaments and then as Z-rings, with SsgB recruiting FtsZ. [Adapted from Borst *et al*, 2011]]

Recently, two dynamin-like proteins DynA and DynB have been implicated in the stabilisation of Z-rings within *Streptomyces*. Dynamins are large GTPases found within Eukaryotes and several bacteria also encode homologs. DynA and DynB were shown to localise to septa in *Streptomyces venezuelae* and mutations in both had unstable Z-rings which in turn led to a lack of septum synthesis. As a result, long spore compartments with multiple chromosomes were observed in the aerial hyphae. This suggests DynA and DynB have a role in supporting septum synthesis in *S. venezuelae* (Schlimpert et al, 2017).

FtsZ filaments are stabilised in other bacteria by membrane anchoring proteins like FtsA, ZipA and SepF. *Streptomyces* lack FtsA and ZipA but do encode for three SepF homologs with the gene of one (SCO2079) being in the conserved position within the division and cell wall (dcw) gene cluster between *ftsZ* and *divIVA*. *Mycobacteria*, who also belong to the genus *Actinobacteria*, have a SepF homolog which has been found to interact with FtsZ by forming ring-like structures. Deletion of *M. tuberculosis* SepF led to a complete block of cell division indicating its importance in septum formation (Casonato et al, 2015). However, these potential SepF proteins have not been fully investigated within *Streptomyces* to confirm whether they do stabilise the divisome by attaching it to the membrane. To date, sequence alignments of all three homologs have shown they share the conserved C-terminal region found in the *B. subtilis* protein, including the residues needed for polymerisation and FtsZ binding. Furthermore, two out of the three SepF proteins in *S. venezuelae* colocalised with FtsZ and one interacted with FtsZ directly (Schlimpert et al, 2017). These results indicate *Streptomyces* Z-rings may be stabilised by a network of proteins and more research needs to be carried out to elude the mechanism.

1.5 Aims

Cell division in bacteria is essential and involves a number of proteins which form the divisome. The divisome formation and the initiation of cell division is co-ordinated with other life cycle events including growth and chromosome segregation to ensure all progeny are identical and capable of survival. A lot of research has highlighted the specific roles of these proteins including FtsZ, ZipA and SepF in model bacterium *B. subtilis* and *E. coli*. FtsZ is the main orchestrator of the process of septum formation and recruits the stabilising proteins FtsA, ZipA and SepF. *Streptomyces* has a complex elaborate life cycle compared to *E. coli* or *B. subtilis*; despite this many of the proteins involved in cell division are conserved. Single spores germinate into branching vegetative hyphae forming a network of mycelium. Upon nutrient depletion aerial hyphae grow by polar growth with the chromosome aligning ready for cell division by sporulation. Much research has concentrated on the

effects of FtsZ; it is indispensable and aligns uniformly along hyphae. Yet little is known about the regulation of FtsZ in *S.coelicolor*.

I will attempt to understand how FtsZ and SepF are regulated in *Streptomyces* by identifying proteins which may stabilise them. I will look into the biochemical properties of both and try to uncover the communication network between all the stages of the *S.coelicolor* life cycle including polar growth, chromosome segregation and cell division.

Chapter 3:

- I generate BTH constructs expressing truncated versions of SepF2 and FtsZ of *S.coelicolor*.
- I confirm known protein binding partners using Bacterial Two Hybrid assays.
- I probe into potential binding partners of cell division proteins using Bacterial Two Hybrid assays.

Chapter 4:

- I used further bioinformatics to compare the three SepF homologues from *S.coelicolor* to those from other bacteria.
- I optimised the overproduction and purification of SepF and SepF2 in *S.coelicolor*.
- I characterised the phenotype of a truncated version of SepF from *S.coelicolor* using microscopy.

Chapter 5:

- I perform bioinformatic analysis of FtsZ homologs.
- I generate a protein model of FtsZ from *S.coelicolor*.
- I optimise overproduction and purification of FtsZ from *S.coelicolor*.

Chapter 6:

- I generate a protein model of SepFTR from *S.coelicolor*.
- I optimise the stabilisation of purified SepFTR into dimers.
- I prepare the protein for crystallisation trials.

2 Materials and Methods

2.1 Media

Table 2.1.1. Media used in this study

Media	Purpose	Components	Preparation
Lennox Broth (LB) Agar	Growing <i>E.coli</i> on solid media	Tryptone 16g Yeast Extract 8g Sodium Chloride 8g Glucose 1.6g Agar 16g Deionised Water 1600mL	All ingredients except the Agar were dissolved in the deionised water. Four 500mL Duran Bottles had 4g Agar and 400mL dissolved media added prior to autoclaving.
Lennox Broth (LB)	Growing <i>E.coli</i>	Tryptone 16g Yeast Extract 8g Sodium Chloride 8g Glucose 1.6g Deionised Water 1600mL	All ingredients were dissolved in the deionised water and dispensed into 10mL, 50mL or 500mL aliquots in universals, 250mL or 2L flasks respectively prior to autoclaving.

2.2 Bacterial Strains and Plasmids

Table 2.2.1 Bacterial strains used in this study

Strain	Genotype	Reference or source
DH5α	F^- , <i>endA1</i> , <i>glnV44</i> , <i>thi-1</i> , <i>recA1</i> , <i>relA1</i> , <i>gyrA96</i> , <i>deoR</i> , <i>nupG</i> , $\Phi 80dlacZ\Delta M15$, $\Delta(lacZYA-$ <i>argF</i>) <i>U169</i> , <i>hsdR17</i> ($r_K^- m_K^+$)	(Hanahan, 1983)
BL21 Rosetta2 (DE3) pLysS	F^- <i>ompT</i> <i>hsdS_B</i> ($r_B^- m_B^-$) <i>gal dcm</i> (DE3) pLysSRARE2 (Cam ^R)	(Crosson et al, 2005)
BL21 (DE3) pLysS	F^- , <i>dcm</i> , <i>ompT</i> , <i>lon</i> , <i>hsdS_B</i> ($r_B^- m_B^-$), <i>gal</i> , λ (DE3), pLysS(cm ^R)	(Studier and Moffatt, 1986)
BTH101	F^- , <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galk16</i> , <i>rpsL1</i> (<i>Str</i> ^r), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i>	(Karimova et al., 2000)

Table 2.2.2 Plasmids used in this study

Plasmid	Genotype	Reference or source
pET28a	<i>ori</i> pBR322, T7 Promoter, His•Tag coding sequence, <i>lacI</i> , <i>kan</i> , <i>ori</i> f1	Novogen
pUT18C	pUC19 derivative with T18 domain upstream of MCS.	(Karimova et al., 2000)
pKT25	pSU40 derivative with T25 domain upstream of MCS.	(Karimova et al., 2000)
pKT	pSU40 derivative with T25 domain downstream of MCS.	(Karimova et al., 2000)
pUT18	pUC19 derivative with T18 domain downstream of MCS.	(Karimova et al., 2000)

Table 2.2.3 Plasmid constructs

Plasmid	Genotype	Reference or source
pET28a:FtsZ	pEt28a carrying His-tagged FtsZ for protein overproduction.	This Study
pET28a:FtsZTR	pEt28a carrying His-tagged FtsZTR for protein overproduction.	This Study
pET28a:SepF	pEt28a carrying His-tagged SepF for protein overproduction.	This Study
pET28a:SepFTR	pEt28a carrying His-tagged SepFTR for protein overproduction.	This Study
pUTC-SepFC	Expressing T18-SepFC fusion	This Study
pUT-SepFC	Expressing SepFC-T18 fusion	This Study
pKT-SepFC	Expressing T25-SepFC fusion	This Study
pKNT-SepFC	Expressing SepFC-T25 fusion	This Study
pUTC-FtsZC	Expressing T18-FtsZC fusion	This Study
pUT-FtsZC	Expressing FtsZC-T18 fusion	This Study
pKT-FtsZC	Expressing T25-FtsZC fusion	This Study
pKNT-FtsZC	Expressing FtsZC-T25 fusion	This Study
pUTC-SsgB	Expressing T18-SsgB fusion	This Study
pUT-SsgB	Expressing SsgB-T18 fusion	This Study
pKT-SsgB	Expressing T25-SsgB fusion	This Study
pKNT-SsgB	Expressing SsgB-T25 fusion	This Study
pUT18c-Scy	Expressing T18-Scy translational fusion	(Walshaw et al., 2010)
pKT25-Scy	Expressing T25-Scy translational fusion	(Walshaw et al., 2010)
pUT18c-DivIVA	Expressing T18-DivIVA translational fusion	(Holmes et al., 2013)
pKT25-DivIVA	Expressing T25-DivIVA translational fusion	(Holmes et al., 2013)

pUT18c-FilP	Expressing T18-FilP translational fusion	(Holmes et al., 2013)
pKT25-FilP	Expressing T25-FilP translational fusion	(Holmes et al., 2013)

2.3 Bacterial growth conditions

2.3.1 *E.coli* strains

E.coli strains were grown in LB liquid media or on LB solid media and incubated at 37°C.

2.4 Antibiotics

Antibiotic	Stock Concentration (mg/mL)	Final Concentration (µg/mL)
Ampicillin	100	100
Chloramphenicol	25	25
Hygromycin	50	50
Kanamycin	100	50

2.5 General Molecular Biology Methods

2.5.1 Plasmid DNA Isolation

A 50mL liquid LB media with relevant antibiotic was inoculated with a single colony of *E.coli* DH5 which contained the relevant plasmid. The culture was incubated overnight at 37°C, shaking at 250rpm prior to collection by centrifugation at 4°C for 5 minutes at 5000rpm. The resulting cell pellet was washed in 40mL of solution one and centrifuged for a further 5 minutes, 4°C at 5000rpm. The cells were then resuspended in 1mL of solution 1, followed by mixing by rotation of the homogenous cells with 2mL of solution 2. After incubation on ice for 4 minutes and the addition of 1.5mL of solution 3, the lysate was shaken vigorously and left on ice for 10 minutes. Another centrifugation at 4°C and 5000rpm for 10 minutes was performed before the supernatant was moved for extraction using 500l phenol/chloroform. This was followed by vortexing for 30 seconds and centrifugation for 5 minutes at 5000rpm at 4°C. The upper phase was removed and 500l chloroform added before vortexing for another 30 seconds and centrifugation at 5000rpm for 3 minutes at 4°C. The upper phase was collected and incubated for 1 hour at 37°C with 5µL of 30mg/mL RNase. A second phenol/chloroform extraction was carried out and the resulting upper phase mixed with 6mL Isopropanol (stored at -20°C)

to allow DNA precipitation by incubation on ice for 20 minutes. DNA was collected by centrifugation for 15 minutes at 4°C at 5000rpm and the pellet was resuspended in 600µL sterile deionised water. 200µL phenol/ chloroform was added, followed by vortexing for 1 minute and centrifugation at 5000rpm for 5 minutes. The upper phase was transferred to a 2mL Eppendorf and 400µL chloroform added prior to 30 seconds vortexing and 3 minutes of centrifugation at 13,500 rpm. The upper phase was divided equally between two eppendorfs and 30µL 3M sodium acetate pH 5.5 added to each. This was followed by the addition of 900µL 100% Ethanol (stored at -20°C) and the eppendorfs were inverted to allow mixing. Precipitation was encouraged by incubation on ice and the supernatant discarded after a 15 minute centrifugation at 4°C. 70% Ethanol (stored at -20°C) was used to wash the pellet which was left to air dry after centrifugation at 13,500rpm for 5 minutes at 4°C. An appropriate volume of sterile deionised water was added to each Eppendorf to resuspend the pellet. Both, aliquots were combined and stored at -20°C.

2.5.2 Agarose gel electrophoresis analysis of DNA

Agarose gels were prepared using 1x TAE buffer supplemented with ethidium bromide (0.5µg/mL) at a range of 0.7% to 1% range and were set into Bio-Rad trays. DNA samples were loaded into the cast gels after mixing with 1x loading dye and run at a range of 30 – 50 Volts. A DNA marker which had been digested with *HindIII* and *EcoRI* was also loaded to allow size estimation of bands viewed under Ultra Violet light.

2.5.3 Polymerase Chain Reaction

Polymerase Chain reactions (PCR) were used to generate fragments for cloning and for the confirmation of fragments that had been ligated into plasmids. The reactions were carried out using a BioRAD DNA Engine Thermal Cycler.

Table 2.5.3.1 Conditions for PCR reactions

PCR Purpose	Reaction Components	PCR Cycle Conditions
Generation of Fragments for cloning	1 x Phusion GC Buffer, 200M dNTPS, 1.5mM MgCl ₂ , 3% DMSO, 1M Reverse Primer, 1M Forward Primer, 0.02U/L Phusion DNA Polymerase (Thermofisher)	Initial Denaturation 98°C 2 minutes Denaturation 98°C 20 seconds Primer annealing XC for 30 seconds Extension 72°C for B seconds Go To Step Two 24 times Final Extension 72°C for 5 minutes Final Step 20°C for 5 minutes

Confirmation of Ligated Fragments	1 x Go Taq polymerase Buffer, 200M dNTPs, 2.5mM MgCl ₂ , 5% DMSO, 1M Reverse Primer, 1M Forward Primer, 0.02U/L Go Taq DNA Polymerase	Initial Denaturation 96°C 2 minutes Denaturation 92°C 20 seconds Primer annealing XC for 30 seconds Extension 72°C for B seconds Go To Step Two 30 times Final Extension 72°C for 5 minutes Final Step 20°C for 5 minutes
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X- Primer annealing temperature calculated according to DNA polymerase used and the calculator at www.thermofisher.com/tmcalculator

B- Extension time calculated with respect to the length of the product and the reaction rate of the DNA polymerase used

Table 2.5.3.2 Primer Sequences

Name	Oligonucleotide Sequence
SepF Trunc Xba Nde FRW	GGTCATCTAGAGCATATGTCCGAACGAGAGCCCTACCG
FtsZ Trunc Xba Nde FRW	GGTCATCTAGAGCATATGGGGTCTCTCGGCCAAGCG
FtsZ UTC REV	GGATCAGAATTCTCTCACTTCAGGAAGTCCGGCACG
FtsZ UT REV	GGATCAGAATTCGACTTCAGGAAGTCCGGCACG
SepF UT REV	GGTCAGAATTCGAGCTCTGGTTGAAGAACCCGCC
SepF UTC REV	GGATCAGAATTCTCTCAGCTCTGGTTGAAGAACCCGC
SsgB Xba Nde FRW	GGATCATCTAGAGCATATGAACACCACGGTCAGCTGCG
SsgB UTC REV	GGATCAGAATTCCTAGCTTTCCGCCAGGATGTGC
SsgB UT rev	GGTCAGAATTCGAGCTTTCCGCCAGGATGTGCG

2.5.4 Restriction Digest

The preparation of DNA fragments for integration into plasmids and subsequent generation of linearised plasmids for which the fragment would be ligated into were carried out using a restriction digest with appropriate enzymes. A 100µL volume of digestion mixture was prepared using 1µL of each appropriate restriction enzymes in 1 X digestion buffer. The reaction was incubated overnight 37°C and stopped by heating to 65°C and then analysed using agarose gel electrophoresis.

2.5.5 DNA isolation by agarose gel extraction

DNA fragments to be obtained were separated using agarose electrophoresis and excised using a razor to cut the gel when viewed using Ultra Violet light (310nm). The extracted gel was purified using the Qiagen QIAquick Gel Extraction Kit. to obtain DNA which was dissolved in sterile dH₂O to kept at -20°C.

2.5.6 Ligation

The ligation of DNA fragments into linear vectors was performed using either a 1:3 or 1:6 molar ratio of plasmid to insert in a total volume of 11µL containing dH₂O. The reaction was incubated at 65°C for 3 minutes prior to the addition of 1µL T4 DNA Ligase and 3L 5x Ligase buffer. The Ligation was then incubated overnight at 4°C.

2.5.7 Transformation of competent *E.coli* Cells

2.5.7.1 Electroporation

Appropriate antibiotics were added to 10mL LB which was then inoculated with a single colony of *E.coli*. The culture was incubated overnight at 37°C shaking 250 rpm, 500µL of which was used to inoculate 50 mL LB, supplemented with antibiotics. The new culture was incubated at 37°C, shaking 250 rpm for 4 hours and subjected to centrifugation at 5000 rpm and 4°C to harvest the cells. The cell pellet was washed with 10% glycerol and resuspended in 100µL 10% glycerol. A 45µL volume of competent cells was mixed with 1µL plasmid or 4µL ligation reaction in a 0.2cm cuvette on ice and subjected to electroporation. After which the reaction was mixed with 500µL LB and incubated at 37°C, shaking 250 rpm for 1 hour 30 minutes.

2.5.7.2 Chemical Competence

Appropriate antibiotics were added to 10mL LB which was then inoculated with a single colony of *E.coli*. The culture was incubated overnight at 37°C shaking 250 rpm, 500uL of which was used to inoculate 50 mL LB, supplemented with antibiotics. The new culture was incubated at 37C, shaking 250 rpm for 2 hours 40 minutes and subjected to centrifugation at 5000 rpm and 4°C to harvest the cells. The cell pellet was washed by centrifugation at 5000 rpm, 4°C with 10mM NaCl and the resulting cell pellet was incubated for 40 minutes on ice after resuspension in 30mM CaCl₂/10mM RbCl₂. After the final centrifugation at 4°C and 5000 rpm the competent cells were prepared by resuspension of the pellet in 150°L 30mM CaCl₂/10mM RbCl₂. A 50 L volume of cells was mixed with 1µL plasmid or 4µL Ligation reaction and incubated on ice for 40 minutes. After incubation the cells were heated to

42°C for 1 minute and returned to ice before being mixed with 500µL LB. The reaction was incubated for 1 hour 30 minute at 37°C, shaking 250 rpm.

2.6 Protein Purification

2.6.1 Cell Lysis

Proteins were overproduced under optimal conditions for each target protein as detailed in the results section and collected in 500mL Beckmann centrifuge tubes for 5 minutes, 20,000rpm at 4°C in rotor Beckmann JA 10 500 and Beckmann centrifuge Avanti J20 (both Beckmann). The resulting cell pellet was resuspended in 30mL lysis buffer and subjected to either two cycles of a French press at 1000 psi or a cell disruptor at 30Kpsi pressure. Lysate was centrifuged in an Avanti J20 centrifuge using the rotor JLA 25 50 in 50mL centrifuge tubes for 30 minutes at 7,500rpm at 4°C. The supernatant and cell pellet fractions were used for either purification under native or denaturing conditions respectively.

2.6.2 Purification under Native Conditions

Phosphate Buffers

Binding Buffer 50mM NaHPO, 300mM NaCL, 10mM imidazole pH8

Elution Buffer 50mM NaHPO, 300mM NaCL, 300mM imidazole pH8

An Amersham AKTA FRC FPLC machine was used for purification with a 5mL NI-NTA preppacked column, both from GE Healthcare. Solutions and lysate were filtered using 0.2 micron filters prior to loading onto the column. The column was equilibrated with 25 mL each of dH₂O and binding buffer at 1 mL/min. After which, the cell extract was introduced to the column, along with binding buffer at a speed of 0.2 mL/min. The UV absorbance at 280 nm was measured throughout. Following sample loading the column was washed with binding buffer at a total volume of 10 mL and 25 mL of a buffer mixture containing 6% Elution buffer (27 mM imidazole) at 0.5 mL/min. The protein was eluted using an increasing gradient of elution buffer to reach 100% at 50 minutes. Fractions containing purified proteins were identified using SDS-PAGE.

2.6.3 Purification under Denaturing Conditions

Phosphate Buffers

Non-denaturing buffer Tris 50mM, 300mM NaCL, 10mM Imidazole, 10mM MgCl₂ pH8

Buffer A 8M Urea, 0.1M NaH₂PO₄, 0.01M Tris pH8

Buffer B 8M Urea, 0.1M NaH₂PO₄, 0.01M Tris pH6

Buffer C 8M Urea, 0.1M NaH₂PO₄, 0.01M Tris pH4.5

Ni-NTA column washed with 20mL dH₂O and equilibrated with 10mL 8M Urea Buffer A. Pellet fraction was resuspended in 6mL 8M Urea Buffer A at room temperature and sonicated for three cycles of 40 seconds with a minute break in between each. The resulting solution was agitated for 1 hour and centrifuged 10,000 rpm, room temperature for 15 minutes. The solution was filtered using a 0.2 micron filter prior to loading onto the column. A wash of the column with 8mL 8M Urea Buffer A and 8mL 8M Urea Buffer B respectively. Protein was eluted with 11mL 8M Urea Buffer C and analysed using SDS-PAGE.

2.6.4 Dialysis

Dialysis of proteins was performed using Spectra/Por molecularporois membrane tubing using volumes of 1 mL. Tubing was cut to 5 cm and secured to a 2 mL microcentrifuge tubing, containing the protein:buffer solution, using an O-ring. This was then placed into a float and the membrane submerged into the desired buffer of either 500mL or 1L. The buffer was stirred at room temperature for at least 2 hours before exchange. The buffer was exchanged twice to allow three lots of dialysis.

2.7 SDS-PAGE

SDS-PAGE gels were cast using the ATTO system and submerged in an ATTO tank using 1x SDS-PAGE running buffer. The percentage of acrylamide in the resolving gel was adjusted depending on the size of protein analysed. Protein loading dye was added to the protein sample to give a 1x buffer dilution. Following this the protein sample was heated at 95°C for 5 minutes to denature the protein. Upon sample loading with an appropriate protein marker, electrophoresis was run for 1 hour at 180V. Gels were stained with Colloidal Coomassie Blue R250 for 1 hour with agitation. Destaining of gels was performed by boiling gels in dH₂O for 5 minutes which allowed viewing using a Bio-Rad trans-illuminator.

2.8 Silver Staining

Silver staining of SDS-PAGE gels was carried out using several steps, with three 20 second washes with dH₂O after each stage. First the gel was fixed in 150 mL of 50% ETOH, 5% Acetic acid and 0.05% Formaldehyde for 1 hour with gentle rocking. This was subsequently washed three times (20 minutes for each wash) with a 50:50 ETOH to dH₂O solution. The gel was sensitised for 1 minute using 0.02% Sodium Thiosulphate. Next the 0.1% Silver Nitrate, 0.08% Formaldehyde stain was added for 30

minutes. Development of the gel could then take place using 2% Sodium Carbonate, 0.04% Formaldehyde and 0.01% Sodium Thiosulphate. Development was halted using 5% Acetic Acid solution.

2.9 Transmission Electron Microscopy

Images of proteins were collected on an 8 Mp AMT XR81 camera after viewing using a JEOL JEM-2010 Electron Microscope fitted with a tungsten cathode filament. To obtain samples; proteins were diluted to 0.05mg/mL in 50mM Tris-HCL, pH 8, 50mM NaCl, 10% glycerol, 0.4M L-arginine and 2% glycine. Polymerisation of proteins was encouraged by incubation at 30°C for 20 minutes and applied to carbon coated grids. This was negatively stained using freshly prepared 2% ammonium molybdate, pH 7.0, on a carbon coated 400-mesh copper grid (Agar Scientific).

3 Interactions between Proteins involved in Cell division, Polar growth and DNA Segregation

3.1 Introduction

Bacterial cell division is performed by a multi-protein complex known as the divisome which marks the site of septum formation, facilitates the synthesis of a new peptidoglycan wall and provides the constricting force to complete the separation of the two new cells (*Beckwith et al, 2005*). To ensure the integrity of the bacterial species is maintained; cell division will only take place once the chromosomal DNA has been replicated and segregated; minimising the risk of producing progeny with fragmented chromosomes (*Errington et al, 2012*). There is also an additional requirement for the single cell to extend in size to a minimum length. The replication, segregation and cell extension events have dedicated proteins which co-ordinate their respective tasks. The basic components of the cell division machinery are conserved amongst eubacteria, with variations being reflections of an individual's morphology or habitat (*Cueto et al, 2015*).

The main orchestrator of septum formation is the GTPase FtsZ which initiates cell division by polymerising into a ring structure. FtsZ monomers consist of two domains linked by a central helix. The motif GGGTGS/TG and two loop regions facilitate nucleotide binding for GTP hydrolysis (*Driessen et al, 2001*). *In Vitro*, FtsZ polymerises into one-stranded protofilaments which associate to make a 2-4 filament thick Z-ring. As polymerisation is required for the nucleoside hydrolysis activity of FtsZ, it can be described as a self-activating GTPase (*Ma et al, 1996; Lutkenhaus et al, 1996*).

A fully formed Z-ring recruits the additional divisome components with FtsA and ZipA being required in the rod-shaped bacterium, *E.coli* for the initiation of cell division (*Harry et al, 2005., Lutkenhaus et al, 2002*). Both FtsA and ZipA provide stabilisation of FtsZ polymers by tethering to the lipid membrane using either an amphipathic helix or transmembrane alpha helix respectively. FtsA is essential in *E.coli* but *B.subtilis* have evolved the membrane binding protein SepF which can compensate for the loss of FtsA.

The genome of *S. coelicolor* also encodes for three homologs of SepF with the gene of *sepF2* (SCO2079) being located in the conserved position within the division and cell wall (dcw) gene cluster between *ftsZ* and *divIVA*. As *Streptomyces* do not have an FtsA protein for stabilisation of a developing septa; one of the three SepF homologs may perform a tethering role. Previous work in the Kelemen laboratory has suggested SepF was needed for sporulation as septum do not form in the aerial hyphae of a *sepF2* null mutants (*Tan PhD Thesis 2019., Cassettari PhD Thesis 2021*) To investigate whether FtsZ is attached to the membrane via SepF in *Streptomyces*, experiments were designed to

establish if the two proteins interact. The SepF homolog encoded by the gene at SCO2079 was chosen, as its location on the genome is shared with that of the *B.subtilis* SepF.

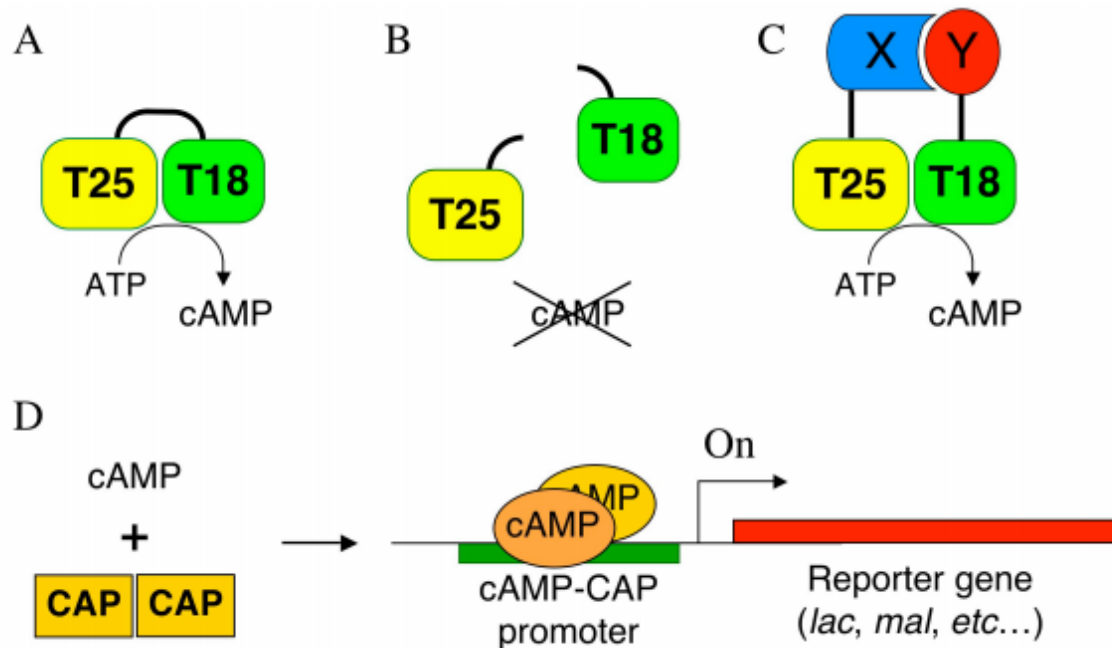


Figure 3.1.1 The principle of the BATCH bacterial hybrid system. A) The adenylate cyclase of *Bordetella pertussis* consists of the fragments T25 and T18 which convert ATP to cAMP. B) The T25 and T18 fragments can not produce cAMP when not complemented. C) T25 and T18 fragments attached to proteins of interest X and Y respectively. If X and Y interacted cAMP is generated. D) cAMP forms a complex with CAP which regulates the activity the reporter gene.

Further to the potential interactions between FtsZ and SepF, other proteins involved in cell division and polar growth were also tested to develop a better understanding of how both life cycle events are coordinated. Previously, Z-ring positioning was found to be positively regulated by proteins which locate to the division site (Margolin *et al*, 2015). These include alternative *Actinomyces* specific proteins which recruit and stabilise FtsZ polymerisation including SsgA-like proteins (SALPS) SsgB and SsgA (Schlimpert *et al*, 2017). Therefore, interactions between SsgB, SepF2, and FtsZ were tested. Additionally, ParH which was previously identified in our laboratory was investigated for interactions with SepF2, FtsZ and SsgB. The partitioning system of *S.coelicolor* consists of the proteins ParA and ParB whereby ParA is recruited to the hyphal tips by Scy. Polymerisation of ParA is initiated to form long filaments along the hyphae which contact ParB to align the chromosomes and position one chromosome for each prespore (Ditkowski *et al*, 2013., Chater *et al*, 2005). The ParA like protein ParH has an effect on septum placement prior to sporulation. Finally, the Interfilament like protein FilP

which forms a stress bearing structure to support hyphal tip growth was tested with several other protein including FtsZ, SepF and SsgB (Flärdh et al, 2019., Gooday, 1990).

To test the potential binding partners of FtsZ and SepF, the bacterial two-hybrid (BTH) system BATCH was employed as a simple and quick method to detect protein-protein interactions *in vivo*. The basic principle of BATCH is the re-formation of the adenylate cyclase from *Bordetella pertussis* if the two suspected binding partners under investigation interact, Figure 3.1.1.

This subsequently leads to the generation of cyclic adenylate mono phosphate (cAMP) which forms a transcriptional activator when in complex with CAP; Catabolite activator protein. The cAMP/CAP pair regulates the activity of several *E.coli* genes including the reporter gene in the assay, allowing detection on appropriate media. The two domains, T25 and T18, of adenylate cyclase are inactive when separated and can be fused to the individual proteins of a potential interaction pair. Once co-

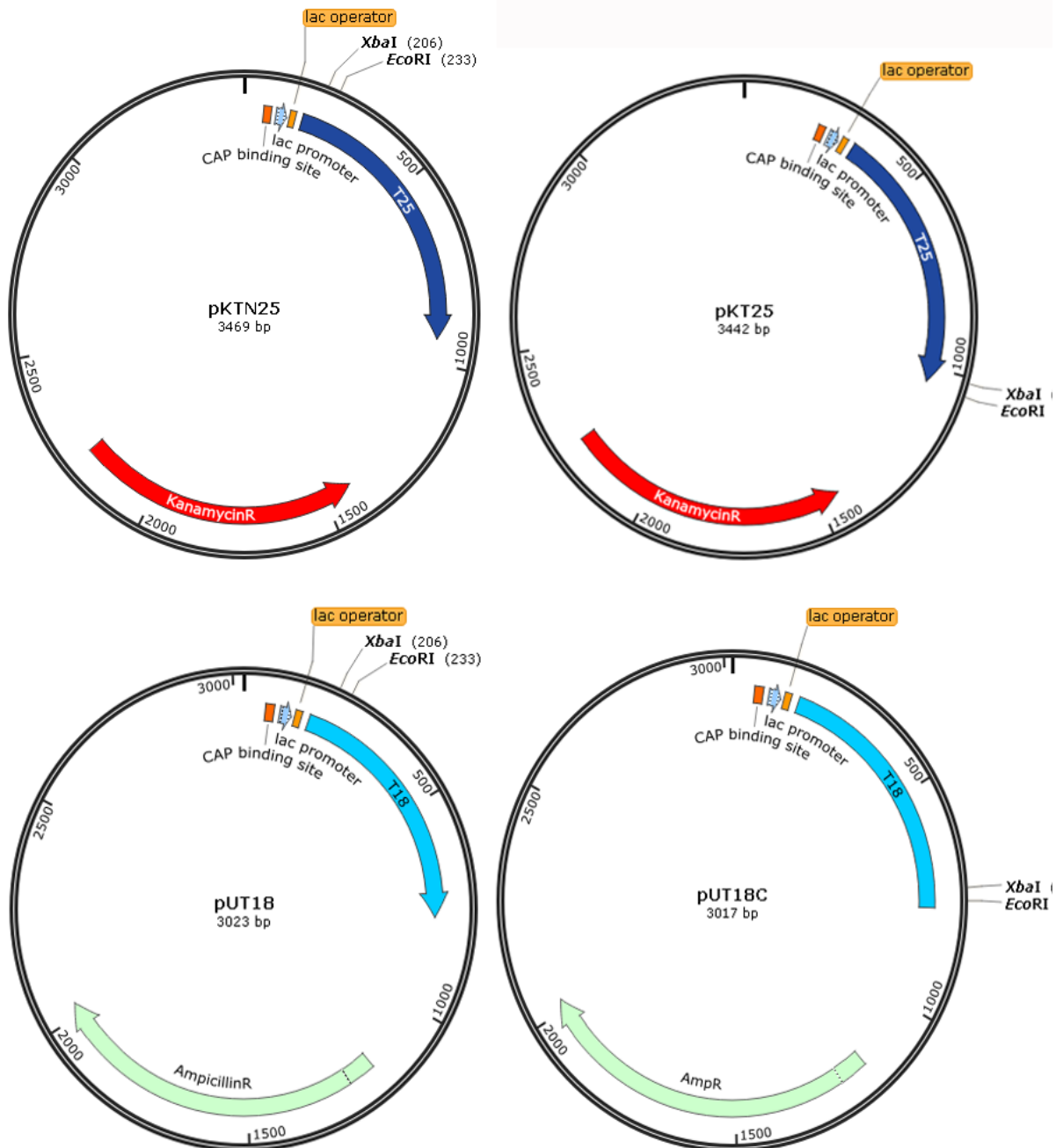


Figure 3.1.2. The four plasmids used in the Bacterial Two Hybrid System. pKTN25 encodes the T25 fragment to allow fusion at the C-terminal of a protein. pKT25 encodes the T25 fragment to allow fusion at the N-terminal of a protein. pUT18 encodes the T18 fragment to allow fusion at the C-terminal of a protein. pUT18C encodes the T18 fragment to allow fusion at the N-terminal of a protein.

transformed into the adenylate cyclase deficient *E.coli* strain BTH101, cyclic AMP production will resume if the proteins bind in such a way that adenylate cyclase is reactivated. The downstream reporter gene *LacZ* is then activated by cAMP/CAP to allow metabolism of Lactose as the main carbon source. The bacteria are grown on LB Agar which is lacking glucose but has been supplemented with the Lactose analogue X-Gal. Another protein encoded by *LacZ* is B-galactosidase which breaks down X-Gal into a blue insoluble product. Therefore, if two proteins form a complex; this can be confirmed by the generation of blue colonies. The four vectors pUT18C, pUT18, pKNT25 and pKT25 can be used to fuse either the T25 or T18 domains by cloning the genes downstream or upstream of the domain encoding genes, using the multiple cloning site (MCS). This leads to either a N or C terminally fused tag on the protein of interest, *Figure 3.1.2*.

In this Chapter, we attempt to create a better picture as to the interaction network of proteins involved in the cell division stage of *S.coelicolor* life cycle.

- I generate BTH constructs expressing truncated versions of SepF2 and FtsZ of *S.coelicolor*.
- I confirm known protein binding partners using Bacterial Two Hybrid assays.
- I probe into potential binding partners of cell division proteins using Bacterial Two Hybrid assays.

3.2. Construction of Plasmids for Bacterial Two Hybrid Analysis

3.2.1 PCR amplification of *SepF* Truncated and *FtsZ* Truncated genes

PCR products were generated for both the *sepF2* and *ftsZ* truncated genes; to allow their respective protein fusions to either the T25 or T18 domains at their C and N terminus. The SepF2 Truncated PCR1 fragment was amplified using 4A10 cosmid DNA as template and primers SepF trunc Xba Nde Forward and SepF UT Reverse. This generated a DNA fragment lacking a stop codon to allow translation of the relevant tag, T18 or T25 at the C-terminus of SepF2. SepF Truncated PCR2 was amplified using primers SepF trunc Xba Nde Forward and SepF UTC Reverse from the 4A10 cosmid. This produced a construct with a stop codon as the tag would be fused to the N-terminus of SepFTR. The FtsZ Truncated PCR1 and FtsZ Truncated PCR2 fragments were also amplified from 4A10 cosmid DNA. The primers FtsZ trunc Xba Nde Forward and FtsZ UT Reverse were used for FtsZ Truncated PCR1 to produce a fragment without a stop codon. FtsZ Truncated PCR2 was generated using primers FtsZ trunc Xba Nde Forward and FtsZ UTC Reverse to produce a construct with a stop codon. All PCR products were digested with EcoRI and XbaI before loading onto a 0.7% preparative agarose gel. Bands corresponding to the

expected sizes of 294bp and 228bp were excised and the DNA purified using QIAQuick gel extraction Kit, *Figure 3.2.1.1*. The purified DNA was analysed on a 0.7% agarose gel to confirm the quantity of the recovered fragment. The predicted length of truncated *sepF* and *ftsZ* gene fragments are 294bp and 228bp, respectively.

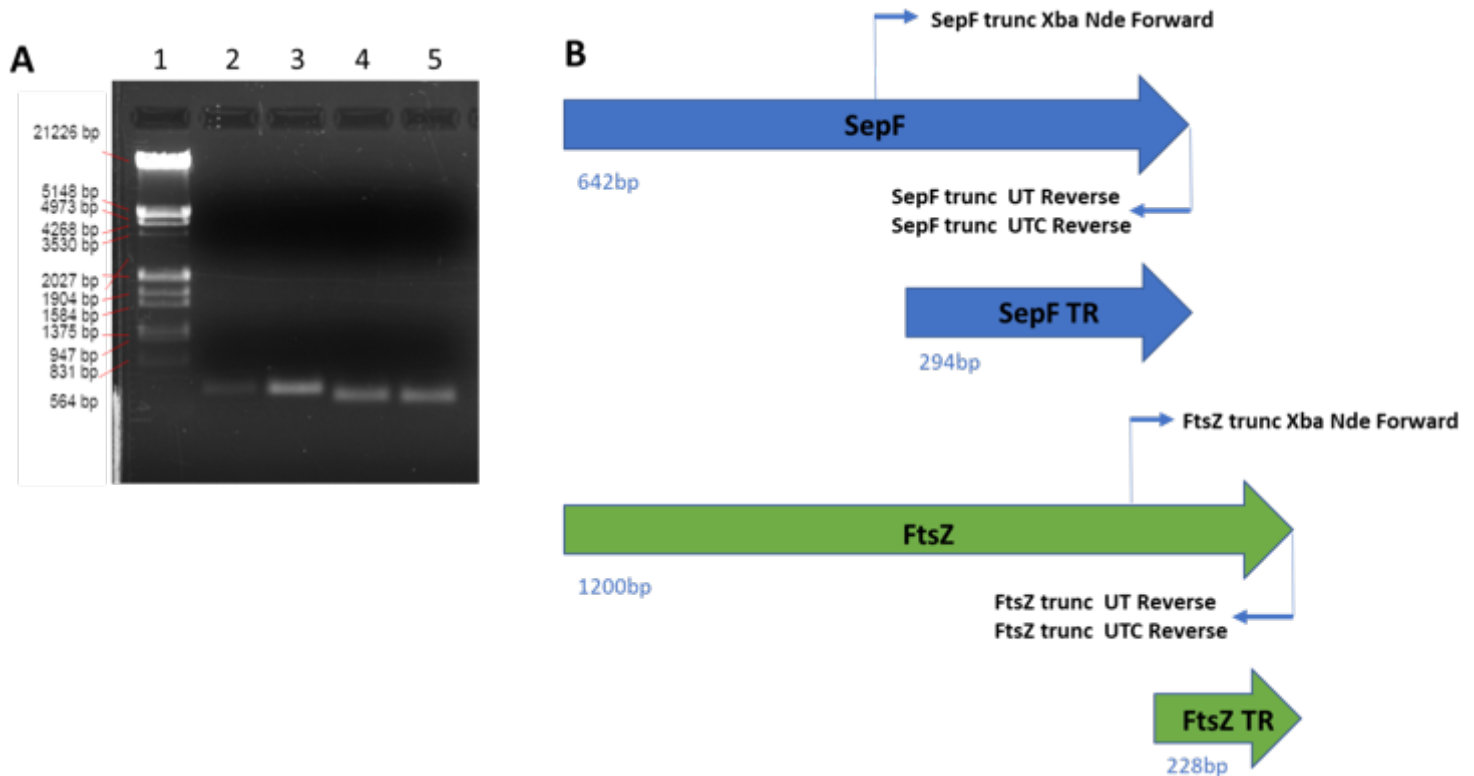


Figure 3.2.1.1. Confirmation of the generated PCR product by gel electrophoresis. (A) Lane 1, λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left. Lanes 2 and 3, *SepF* Truncated PCR1 and PCR2 product. Lanes 3 and 4, *FtsZ* Truncated PCR1 and PCR2. (B) PCR primers and products used for generating the truncated gene products.

3.2.2 Ligation

Four ligation reactions were performed for both the *ftsZ* and *sepF* truncated sets of gene fragments to insert them into a relevant plasmid. *FtsZ* Truncated PCR1 and *SepF* Truncated PCR2 products were ligated with pUT and pKNT where T25 and T18 would be fused to the C-terminus. *FtsZ* Truncated PCR1 and *SepF* Truncated PCR2 gene fragments were ligated with pUTC and pKT to allow fusion of either T25 or T18 to the N-terminus. The ligation mixes were transformed into DH5 α competent cells and transformants were selected using LB agar plates supplemented with ampicillin or kanamycin. Each plate was incubated overnight at 37° C, producing colonies in a range of 20-100.

3.2.3 Colony PCR to confirm transformants carrying the correct inserts

Individual colonies for each plasmid were streaked onto grid lined plates and grown overnight at 37 °C *Figure 3.2.3.1*. The colonies were screened using colony PCR to confirm successful ligations of plasmids with the *sepFTR* or *ftsZTR* genes. To amplify the gene for SepFTR; SepF Trunc Xba Nde forward

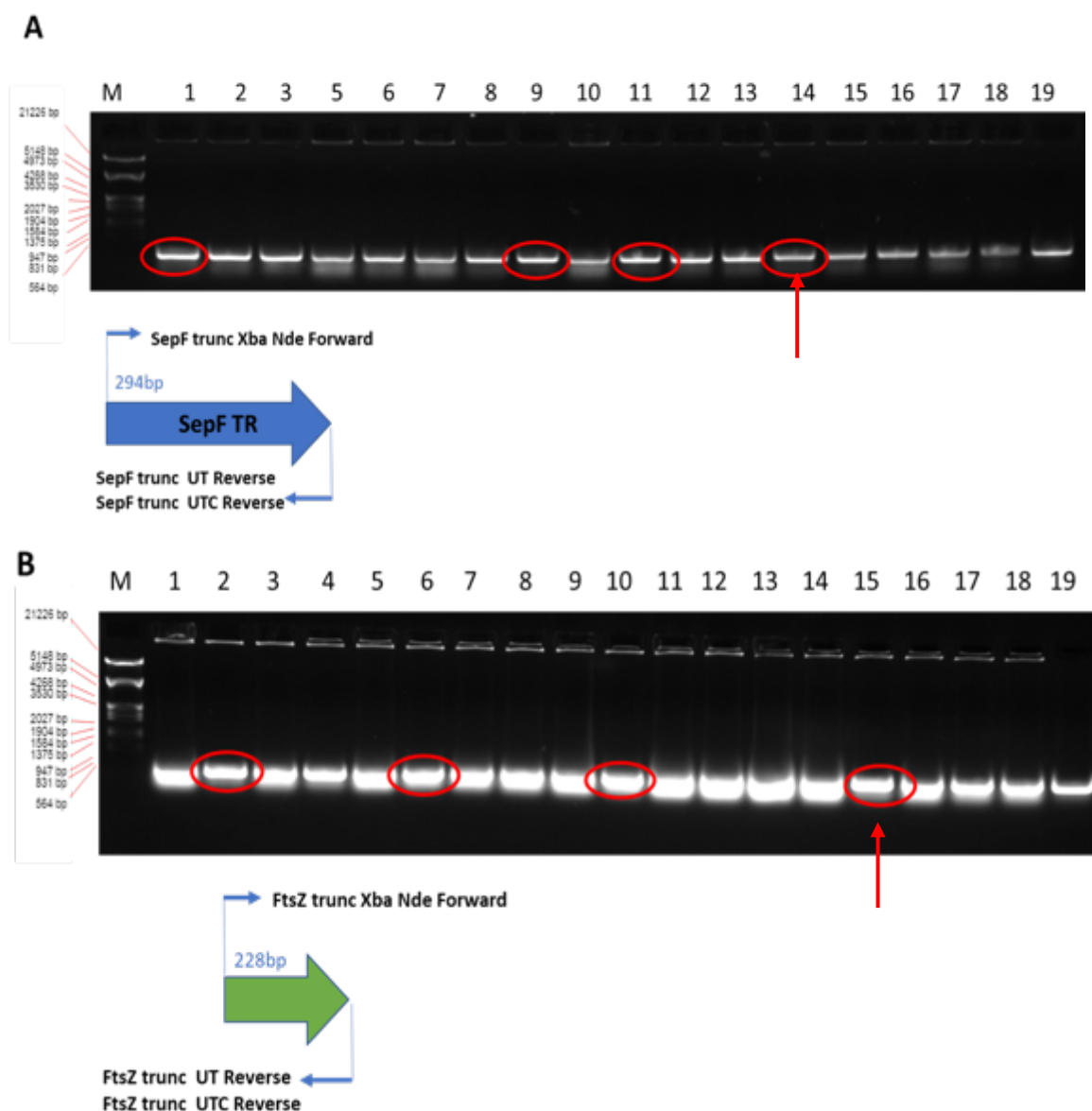


Figure 3.2.3.1. Gel analysis of colony PCR for *SepFTR* and *FtsZTR*. Lane M contains λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left and colony numbers are labelled for each lane A) 1-4, 5-9, 10-13 and 14-18 contain individual colonies with *FtsZTR* pUT, *FtsZTR* pKNT, *FtsZTR* pUTC and *FtsZTR* pKT respectively. Lane 19 is a control which contains 4A10 template DNA. B) 1-3, 4-8, 9-12 and 13-17 contain individual colonies with *SepFTR* pUT, *SepFTR* pKNT, *SepFTR* pUTC and *SepFTR* pKT respectively. Lane 19 is a control which contains 4A10 template DNA. Colonies that were used for further BTH screening are highlighted in red circles.

and SepF UT reverse primers were used. For the FtsZTR clones the primers FtsZ Trunc Xba Nde forward and FtsZ UT reverse were used. The PCR samples were analysed on 0.7% agarose gels at 50 V, alongside a positive control, to confirm all the screened colonies for successful transformation of a plasmid with correct gene insert, *Figure 3.2.3.1.* Colonies shown with a red arrow were confirmed with DNA sequencing.

3.2.4 PCR amplification of *SsgB* gene

PCR products were generated for the *ssgB* gene; to allow the protein fusion to either the T25 or T18 domains at the C and N terminus. The *SsgB* PCR1 fragment was amplified using M145 chromosomal DNA as template and primers *SsgB* Xba Nde Forward and *SsgB* UT Reverse. This generated a DNA fragment lacking a stop codon to allow translation of the relevant tag, T18 or T25 at the C-terminus of *SsgB*. *SsgB* PCR2 was amplified using primers *SsgB* Xba Nde Forward and *SsgB* UTC Reverse from the M145 chromosomal DNA. This produced a construct with a stop codon as the tag would be fused to the N-terminus of *SsgB*. All PCR products were digested with *EcoRI* and *XbaI* before loading onto a 0.7% preparative agarose gel. Bands corresponding to the expected size of 420bp were excised and

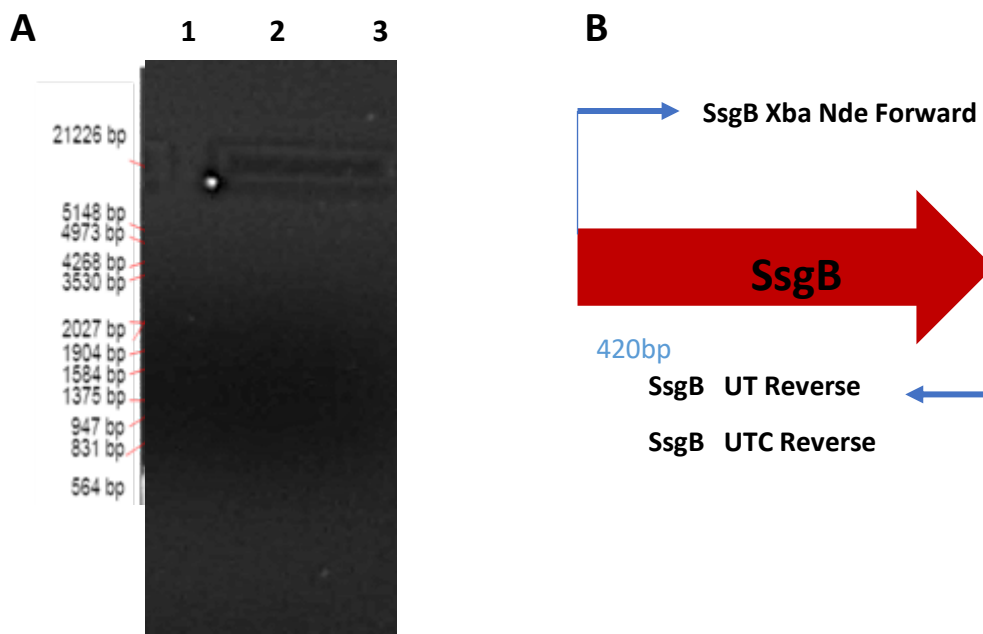


Figure 3.2.4.1. Confirmation of the generated PCR product by gel electrophoresis. (A) Lane 1, λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left. Lanes 2 and 3, *SsgB* PCR1 and PCR2 product.. (B) PCR primers and products used for generating the gene products.

the DNA purified using QIAQuick gel extraction Kit, *Figure 3.2.4.1*. The purified DNA was analysed on a 0.7% agarose gel to confirm the quantity of the recovered fragment of 420bp.

3.2.5 Ligation

Four ligation reactions were performed for SsgB gene fragments to insert them into a relevant plasmid. SsgB PCR1 was ligated with pUT and pKNT where T25 and T18 would be fused to the C-terminus. SsgB PCR2 was ligated with pUTC and pKT to allow fusion of either T25 or T18 to the N-terminus. The ligation mixes were transformed into DH5 α competent cells and transformants were selected using LB agar plates supplemented with ampicillin or kanamycin. Each plate was incubated overnight at 37° C, producing colonies in a range of 20-100.

3.2.6 Colony PCR to confirm transformants carrying the correct inserts

Individual colonies for each plasmid were streaked onto grid lined plates and grown overnight at 37 °C. The colonies were screened using colony PCR to confirm successful ligations of plasmids with the *ssgB* gene. To amplify the gene for SsgB; SsgB Xba Nde forward and SsgB UT reverse primers were used. The PCR samples were analysed on 0.7% agarose gels at 50 V, alongside a positive control, to confirm all the screened colonies for successful transformation of a plasmid with correct gene insert, *Figure 3.2.6.1*. The colony shown with a green arrow was confirmed by DNA sequencing.

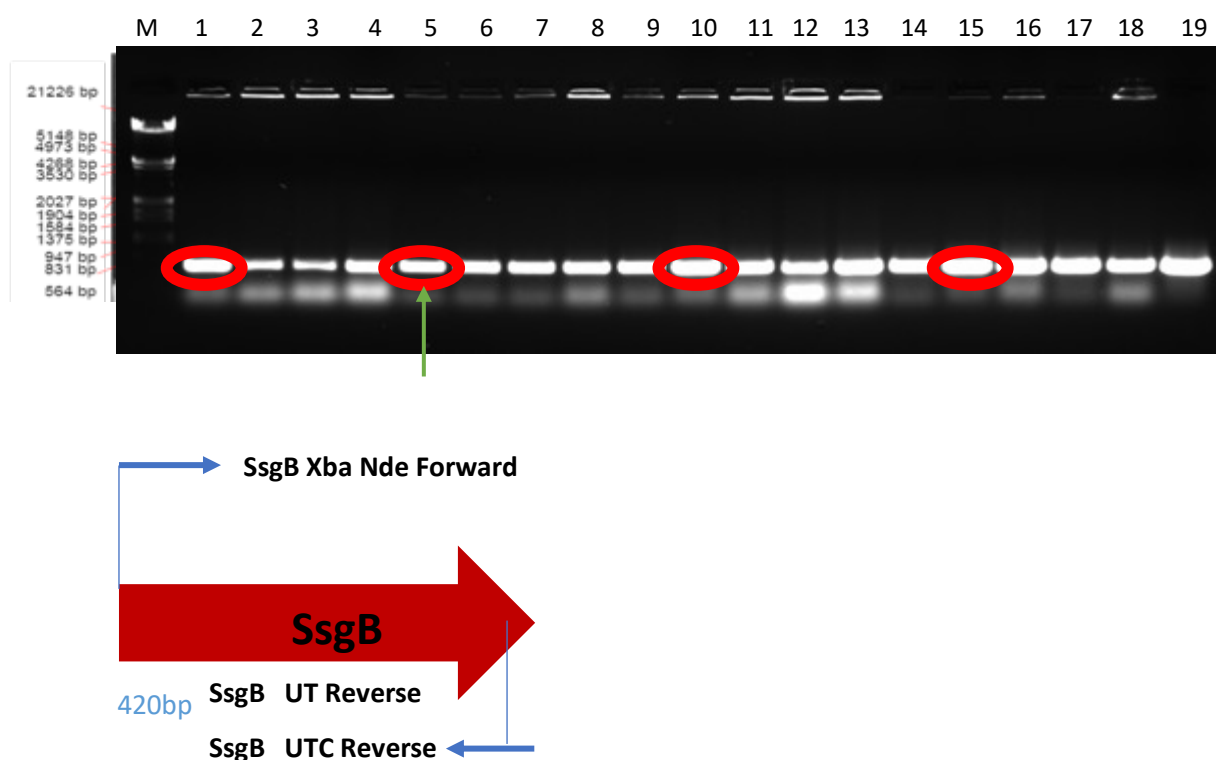


Figure 3.2.6.1. Gel analysis of colony PCR for SsgB. Lane M contains λ ladder digested with EcoRI and HindIII as a size marker. Sizes of λ DNA fragments are indicated on the left and colony numbers are labelled for each lane 1-4, 5-9, 10-13 and 14-18 contain individual colonies with SsgB pUT, SsgB pKNT, SsgB pUTC and SsgB pKT respectively. Lane 19 is a control which contains M145 template DNA.. Colonies that were used for further BTH screening are highlighted in red circles.

3.3 Testing of constructs via Bacterial Two Hybrid Assay

3.3 Bacterial Two Hybrid Analysis of Constructs

The constructs created and ones previously generated for other research, including FilP, Scy, and ParH were co-transformed into adenylate cyclase deficient BTH101 *E.coli* cells to test 140 different potential protein interactions, *Table 3.3.1*. Three colonies from successful transformants were streaked onto LB Agar plates which contained no glucose along with positive and negative controls. Plates were incubated at 30°C and monitored at 24 and 48 hours to identify positive interaction when the streaks turned blue.

For quantification of the positive and negative interactions the blue intensity was classified as either strong positive, weak positive or negative. This was achieved by taking camera images of the plates, inverting the image and selecting a 35 x 25 pixel area on each streak. The selected area had the background image removed by subtracting the negative control and the signal intensity was measured to be classed as strong (>8000) weak (1501 – 7999) or negative (<1500). Original plate images of all constructs tested are in the appendix.

Table 3.3.1. *S.coelicolor* proteins tested in BTH assay.

Polar Growth	Chromosome segregation	Septum Formation
FilP	ParA	FtsZ
Scy	ParH	SepF
DivIVA		SsgB

3.4 Conformation of protein interactions involved in cell division

Several proteins essential for cell division of *S.coelicolor* have been shown to have the potential for interaction in separate studies. It was previously established that the SALP protein SsgB

recruits and positions FtsZ for septum formation this was confirmed with several tested orientations of the proteins, *Figure 3.4.1* , *Appendix Figure 9.1.1*.(Borst et al, 2011).

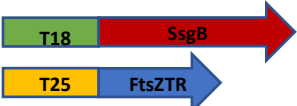
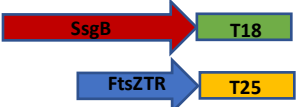
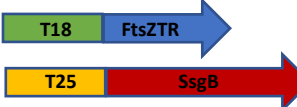
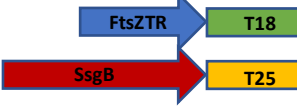
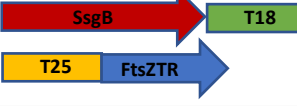
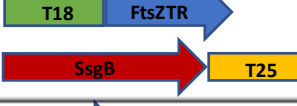
Constructs Tested	Strength of Interaction
	weak
	strong
	weak
	strong
	strong
	weak
	strong
	strong

Figure 3.4.1. The interaction of SsgB with FtsZTR. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SsgB (red arrow), FtsZTR (blue arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

Interactions between *S.coelicolor* FtsZ and the potential Z-ring stabilising protein SepF2 were analysed. However, truncated FtsZ produced positives with truncated SepF2 but not with the full length SepF2 protein, *Figure 3.4.2*, *Appendix Figure 9.1.2* . This is surprising as protein interactions between this pair have been confirmed in other bacteria with SepF being essential for FtsZ stabilisation in *B.subtilis* (Errington et al, 2011). The interaction studies of the SepF homolog suggest there is a

potential for the N- terminus to effect protein binding. This is highlighted by the fact fewer interactions were detected with the full length SepF in comparison to the truncated version.

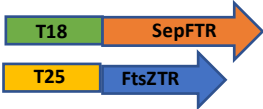
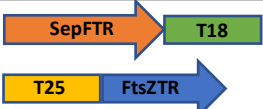
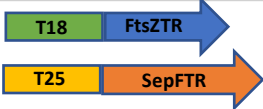
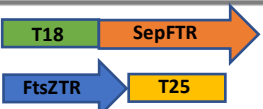
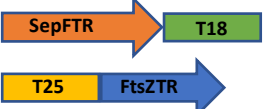
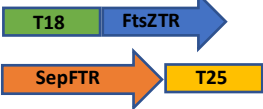
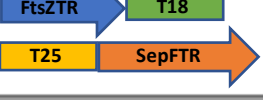
Constructs Tested	Strength of Interaction
	none
	strong
	strong
	strong
	strong
	weak
	strong
	none

Figure 3.4.2. The interaction of SepFTR with FtsZTR. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SepFTR (orange arrow), FtsZTR (blue arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

3.5 Self interactions of proteins

Full length and truncated SepF constructs were tested for self-interaction. Full length SepF produced a strong interaction with itself when the T18 and T25 domains were on opposite terminals of the protein, *Figure 3.5.1, Appendix Figure 9.1.3*. This suggests this is the orientation which allows

SepF self-assembly into higher order structures. Truncated SepF produced strong interactions with itself in all orientations, *Figure 3.5.1. Appendix Figure 9.1.3*. This is unsurprising as the truncated SepF consists of the C-terminal domain only which has been shown to be essential for the polymerisation of SepF in *B.subtilis* (Celik et al, 2013).

A	Constructs Tested	Strength of Interaction
		weak
		strong
		weak
		strong

B	Constructs Tested	Strength of Interaction
		strong
		strong
		strong
		strong

Figure 3.5.1. A) The interaction of SepF with itself. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SepF (orange arrow) with T18 (green) or T25 (yellow) at the C or N terminal. **B)** The interaction of SepFTR with itself. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SepFTR (orange arrow) with T18 (green) or T25 (yellow) at the C or N terminal

FtsZ was tested for self interaction but the truncated construct produced no positives, *Appendix Figure 9.1.4..* This could be accounted for by the fact the C-terminal domains are not suspected to be near one another within a polymerised FtsZ filament, hence lack of the N-terminus of FtsZ prevent polymerisation or dimerization of the FtsZTR protein.

SsgB self-interaction was confirmed with all orientations tested and confirms previous studies *Figure 3.5.2, Appendix Figure 9.1.5. (Borst et al, 2011).*

Constructs Tested	Strength of Interaction
	weak
	strong
	strong
	strong

Figure 3.5.2. The interaction of SsgB with itself. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SsgB (red arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

3.6 New interactions between *S.coelicolor* cell division proteins

SsgB is a positive regulator of FtsZ within *S.coelicolor* as it recruits and positions FtsZ along aerial hyphae to aid in Z-ring positioning (Borst et al, 2011). When tested for interaction of SsgB with SepF2; strong positives were produced with truncated SepF. These results suggest SepF is also needed for the correct localisation of FtsZ and the process is more complex than previously thought, Figure 3.6.1, Appendix Figure 9.1.6.

Constructs Tested	Strength of Interaction
	weak
	strong
	weak
	strong
	strong
	weak
	strong
	strong

Figure 3.6.1. The interaction of SepFTR with SsgB. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as SepFTR (orange arrow), SsgB (red arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

3.7 Proteins involved in separate cell cycle events communicate

The crucial cell cycle events of cell division, chromosome segregation and polar growth are highly regulated in *S.coelicolor*. Each of these processes occurs at different time points with check points put in place. However, our study has highlighted the potential for communication between the proteins thought to be dedicated to each process.

Recent research in the Kelemen laboratory has identified another Par like protein which may perform a role in chromosome segregation. The partitioning system of *S.coelicolor* consists of the proteins ParA and ParB whereby ParA is recruited to the hyphal tips by Scy. Polymerisation of ParA is initiated to form long filaments along the hyphae which contact ParB to align the chromosomes and position one chromosome for each prespore (Ditkowski *et al*, 2013., Chater *et al*, 2005). A ParA like protein ParH was identified in our laboratory (Gillespie, 2019 PhD Thesis) which has an effect on septum placement prior to sporulation. Interactions were tested between ParH and several other proteins involved in cell division. ParH was able to bind with SsgB and SepF2 but the same was not found for ParH with FtsZ. SepF was found to bind ParH in multiple orientations of the respective proteins. The strongest association was found when the tags were positioned on opposite sides *Figure 3.7.1, Appendix Figure 9.1.7*. An association between the DNA binding protein ParH, which is thought to control chromosome partitioning in *S.coelicolor*, was produced in one orientation of the protein with SsgB, *Figure 3.7.2. Appendix Figure 9.1.8*.

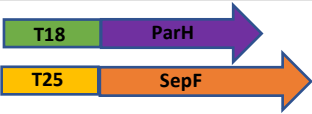
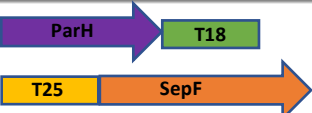
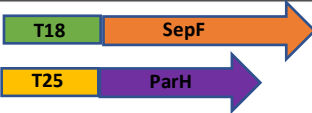
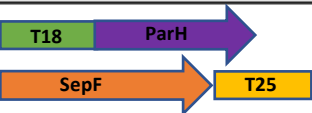
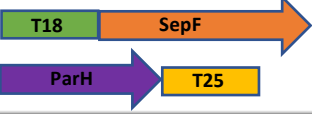
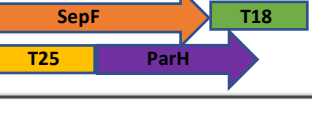
Constructs Tested	Strength of Interaction
	weak
	weak
	weak
	weak
	weak
	strong
	weak
	weak

Figure 3.7.1. The interaction of ParH with SepF. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as ParH (purple arrow), SepF (orange arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

These results shed light on a previously unexplored network of communication between cell division and chromosome organisation. More research needs to be carried out to probe into why proteins involved in sporulation specific division would need to cross talk to DNA segregating regulators like ParH.

Aswell as the previously mentioned interaction between ParH and SepF2TR there was also communication between ParH with the TIPOC protein FilP. This supports previous research which has suggested there is a link between the cell cycle events, with ParA interacting with the main orchestrator of the TIPOC, Scy (Ditkowski *et al*, 2013., Chater *et al*, 2005). However, interplay between

Constructs Tested	Strength of Interaction
	none
	none
	none
	none
	none
	none
	strong
	none

Figure 3.7.2. The interaction of ParH with SsgB. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as ParH (purple arrow), SsgB (red arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

the TIPOC proteins controlling polar growth and those involved in septum formation has not been detected before. Cooperation was detected between the proteins involved in the polar growth of the hyphal tips and those involved in the positive regulation of the Z-ring. This included a strong association of SsgB with the intermediate filament homolog FilP which performs a stress bearing role at the growing tip end, *Figure 3.7.3.Appendix Figure 9.1.9*. In fact, both SsgB and SepFTR also were able to form protein: protein binding to FilP. This is a novel and exciting discovery as polar growth and cell division are separated in time and space.

A	Constructs Tested	Strength of Interaction
		weak
		weak
		weak
		weak
		weak
		weak
		weak
		weak
B	Constructs Tested	Strength of Interaction
		none
		weak
		strong
		weak
		strong
		weak
		weak
		none

Figure 3.7.3. A) The interaction of SepFTR with FilP. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as FilP (pink arrow), SepFTR (orange arrow) with T18 (green) or T25 (yellow) at the C or N terminal. **B)** The interaction of SsgB with FilP. The results of the bacterial two hybrid assays were visually quantified into strong positive, weak positive and negative. Gene constructs tested are shown as FilP (pink arrow), SsgB (red arrow) with T18 (green) or T25 (yellow) at the C or N terminal.

3.8 summary

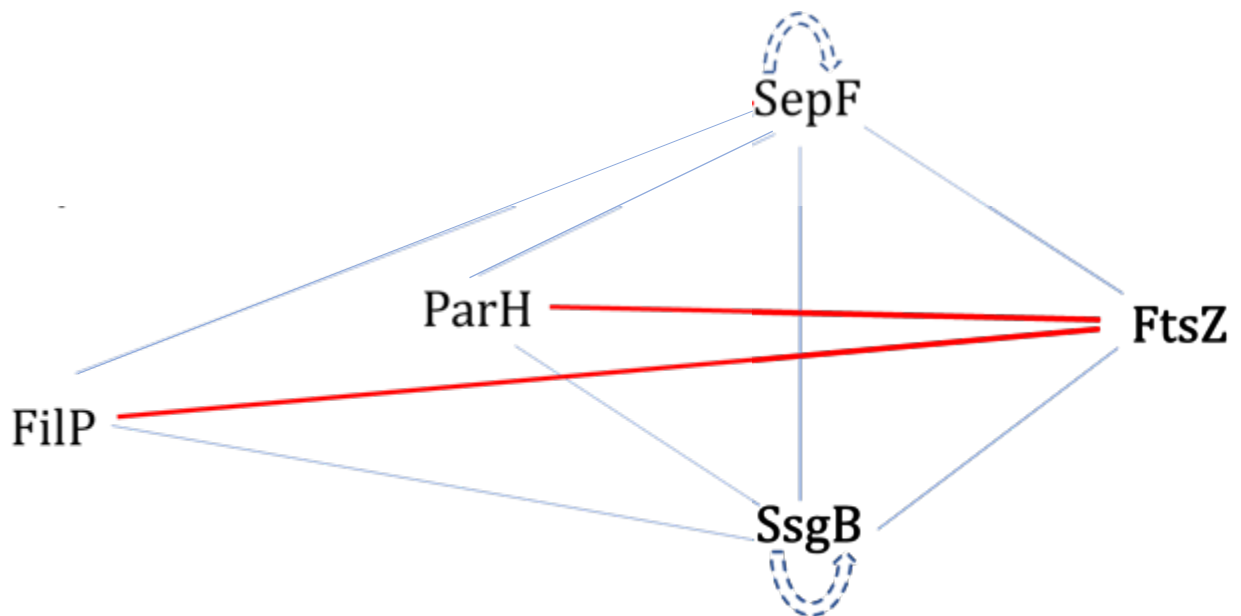


Figure 3.8.1. Diagram to represent BTH assay results. Red lines and blue lines indicate negative and positive interactions respectively.

In this chapter we have investigated the potential interactions between proteins involved in several stages of *S.coelicolor* life cycle. We utilised the bacterial two hybrid assay BATCH to probe into the binding partners of several proteins known to be essential for polar growth, cell division and DNA segregation. Whilst known protein interactions were confirmed; many new binding partners were discovered, *Figure 3.8.1.*

We first analysed interactions between the key cytoskeletal proteins for the cell division of *S.coelicolor*. FtsZ is well established as an initiator of cell division in the majority of bacterium, including *Streptomyces* (Adams *et al*, 2009). A wealth of research has highlighted the binding partners of FtsZ in model organisms such as *E.coli* and *B.subtilis*, but little is known about how it is regulated in *Streptomyces*. Here we demonstrated that FtsZ is capable of interacting with SepF. SepF is known to act as a membrane anchor to stabilise the Z-ring within *B.subtilis* (Errington *et al*, 2011). Our results indicate septum formation is stabilised by SepF within *S.coelicolor*. Previous research has shown the *Streptomyces* specific SALP protein SsgB recruits and positions FtsZ for Z-ring initiation. This interaction was confirmed with several tested orientations of the proteins which validates the reliability of our assay (Borst *et al*, 2011).

Next we analysed the self oligomerisation of the cytokinesis proteins FtsZ, SepF and SsgB. The self interaction of the GTPase FtsZ has been well studied in other bacterium. The Z-ring is formed due

to the polymerisation of single FtsZ monomers to form protofilaments (*Driessen et al, 2001*). However, FtsZ self interaction was not detected with our assay. This is due to fact our FtsZ construct is a truncated version of the protein lacking the N-terminal domain which is crucial for polymerisation in *Staphylococcus aureus* (*Andreu et al, 2017*). SepF has been shown to oligomerise into ring structures in *B.subtilis* (*Celik et al, 2013*). We also observed SepF self-binding using a truncated protein consisting of the C-terminal region; known to be implicated in SepF polymerisation. SsgB is an important regulator and initiator of septum formation in *Streptomyces* (*Borst et al, 2011*). When tested for self interaction we observed a positive result which has not been demonstrated before.

Then we looked into multiple interactions between the three cell division components. SsgB is a cell division protein which recruits FtsZ along with its partner protein SsgA. We looked into the potential interaction of SsgB with the suspected membrane anchor SepF. Our results indicate that in addition to the established FtsZ-SsgB interaction, SepF can also bind SsgB. This suggests that the regulation of cell division is more complex than previously thought, including either a tri-partite SepF-FtsZ-SsgB interaction, or alternatively some competition amongst these three proteins.

New interactions were discovered between a range of proteins involved in all stages of the life cycle. These included FilP of the TIPOC complex, chromosome segregating machinery and cell division. ParH is an ATPase linked to a DNA binding protein that might be involved in chromosome segregation (*Gillespie, 2019 PhD thesis*). Our study highlights the communication between ParH and the cytokinesis proteins SsgB and SepF. This is a new and exciting result as no previous research paper has suggested cross talk between these two life cycle stages. In fact, communication was also uncovered between the TIPOC protein FilP and proteins involved in cell division, including SsgB and SepF. This result was unexpected as Polar growth dominates active growth phase and then there is a switch from growth to division where sporulation takes place. Therefore, a direct link between proteins involved in the separate phases of the life cycle is exciting and surprising. We have established a direct link between chromosome organising machinery and cell division machinery which has never been done before in *Streptomyces*

This Chapter has highlighted the link between polar growth and cell division which is unsurprising as these two key life cycle events need to be carried out efficiently to ensure each resulting spore is capable of germination.

The BTH assay is a basic tool to probe into protein: protein binding and therefore these results have limitations. For example, I can not confirm if the proteins folded correctly into their biologically relevant three dimensional structure. Therefore any interactions could be due to amino acids that are not normally exposed on the protein surface. Additionally just because two proteins bind *in vitro*

doesn't confirm they bind *in vivo* if they are not expressed at the same point in the life cycle or are present in the same part of the cell. Further research needs to be carried out to validate these results including *in vivo* microscopy analysis including co-localisation of the binding partners at least at some stages during development. In addition, *in vitro* biochemical protein: protein binding assays such as ultracentrifugation and co-elution of proteins after Ni-affinity purification can further characterise the interactions. In particular, studies need to be conducted to elucidate how the N-terminus of SepF effect its three-dimensional structure, and as a consequence, interactions with other proteins.

4 SepF: A Biochemical Analysis

4.1 Introduction

The divisome complex is regulated by a number of proteins to help position and stabilise the Z-ring correctly at the mid-cell. Positive regulators bind to the C-terminus of FtsZ; an important region for binding modulators of its activity (Buske *et al*, 2012). This includes the actin homolog FtsA; a transmembrane protein which is found conserved amongst Gram - negative bacteria. In *E.coli* FtsA is essential for the positioning and polymerisation of FtsZ into Z-rings; whereas *B.subtilis* is still capable of forming septa when FtsA isn't present (Harry *et al*, 2005., Lutkenhaus *et al*, 2002). An additional protein; SepF complements the role of FtsA in Gram – positive and cyanobacteria (Errington *et al*, 2006). SepF appears to share the ability to stabilise Z-ring maturation with FtsA as it binds FtsZ via its C-terminus and associates with the lipid bilayer through an N-terminal amphipathic helix (Celik *et al*, 2013).

Errington *et al* first identified SepF as a potential cell division protein in *B.subtilis* by deleting the conserved *ylm* locus upstream of *divIVA*; encoding an essential protein involved in septum placement. This resulted in an elongation of cells and deformed septa when viewed by light and electron microscopy respectively. Further investigations using single gene deletions highlighted that *ylmF* alone, renamed *sepF*, was responsible for the phenotype. The newly discovered SepF localised to division septa and was capable of both self interaction and interaction with FtsZ (Errington *et al*, 2006).

SepF of *B. subtilis* was later purified and refolded from *E.coli* (Singh *et al*, 2008). It was found to promote the assembly of FtsZ protofilaments into bundles when viewed by Transmission Electron Microscopy (Singh *et al*, 2008). Independently, the Errington group also purified the SepF protein and confirmed its capability to influence FtsZ polymerisation into long tubular structures. Furthermore, large ring structures of 50nm were formed under physiological conditions (Errington *et al*, 2011). Recently, studies into SepF have shown the thickness of the division septum is controlled by the SepF polymers. SepF homologs were isolated from a number of species and found to have ring diameters ranging from 19 to 44nm which correlated to the thickness of the septa of the bacteria (Wenzel *et al*, 2021).

A number of studies have tried to establish which residues of FtsZ and SepF facilitate their interaction. Mutations in C-terminal residues of FtsZ; which have been shown to facilitate FtsA binding, including P372 and F374, also abolish its interaction with SepF (Bezouwien *et al*, 2012). The association

between FtsZ and SepF is thought to be due to the protein fold of FtsZ as eleven individual mutations in the last 16 amino acids of the C-terminus inhibit SepF binding. Additionally the *E.coli* FtsZ is able to bind *B.subtilis* SepF, which isn't an *in vivo* interaction (Bezouwen *et al*, 2012).

Two separate studies have suggested that the C-terminal end of SepF is essential for FtsZ binding. Bezouwen *et al* found two mutations of the SepF C-terminus of *B.subtilis* at positions 98 and 124 which prevent SepF associating with FtsZ using pull-down experiments. This was supported by Celik *et al* when error-prone PCR was used to isolate 10 mutants all with individual mutations in their C-terminal domain. All SepF mutants were unviable when introduced into FtsA null mutants of *B.subtilis* (Celik *et al*, 2013). In contrast a deletion of the complete N-terminal region of SepF which had a wild type C-terminus were able to partner FtsZ. However these mutants were unable to self-associate, indicating the N-terminus is implicated in the higher order assembly of the SepF structure.

The proposed crystal structure of *B.subtilis* SepF C-terminal domain was solved by molecular replacement (Celik *et al*, 2013). A single SepF monomer forms a tight dimer with β -sheets at the interface. Crystal packing shows the polymer is assembled from dimers with α -helices on the exterior of the dimers facilitating interactions. Transmission electron microscopy analysis of the truncated SepF used for crystallisation revealed individual rings at pH 10 which associated to form a tube at lower pH. A mutation in a Glycine at position 109 which lies at the interface between dimers to a lysine destroys polymerisation and renders the mutants incapable of ring formation (Celik *et al*, 2013).

Actinomycetes, including *Streptomyces* do not have the membrane anchoring proteins FtsA or ZipA found in other bacteria to stabilise FtsZ. However, their genomes do contain potential *sepF* genes encoding proteins that could carry out the role of attaching the Z-ring to the membrane. A study into the *M.tuberculosis* SepF has indicated its importance in septum formation (Casonato *et al*, 2015). Deletion of the *sepF* gene led to a complete block of cell division with the bacteria (Casonato *et al*, 2015). A more recent study has confirmed SepF promotes the polymerisation of FtsZ in *M.smegmatis* and a single point mutation of residue G51 rendering it incapable of stabilising FtsZ (Bhattacharya *et al*, 2018). Schlimpert *et al* have shown a SepF homolog in *S.venezuelae* binds FtsZ (Schlimpert *et al*, 2017). All studies suggest SepF is essential for the proper formation of the Z-ring in *Actinomycetes*.

S.coelicolor has an elaborate life cycle which resembles that of a filamentous fungi and so essential events are highly regulated by a plethora of proteins and pathways. Throughout its life cycle two separate forms of growth occur; vegetative and polar with these being coordinated with cell division. FtsZ is not essential for the viability of *S.coelicolor* as it is only required for the late stages of life where regular septation occurs during sporulation. *S.coelicolor* seem to encode three potential SepF homologs with one encoded within the cell division cluster. Yet none of these have been fully

characterised and neither has their role within the bacterium been investigated. It would be unsurprising if cell division required multiple Z-ring anchoring proteins to stabilise septum production.

In this chapter we present the initial characterisation of SepF to investigate its role within *S.coelicolor* cell division:

- I used further bioinformatics to compare the three SepF homologues from *S.coelicolor* to those from other bacteria.
- I optimised the overproduction and purification of SepF and SepF2 in *S.coelicolor*.
- I characterised the phenotype of a truncated version of SepF from *S.coelicolor* using microscopy.

4.2 Comparison of SepF homologs in *S.coelicolor*

The *S.coelicolor* genome encode for three potential SepF homologs, *Figure 4.2.1*. The three SepF proteins have unique SCO and Uniprot numbers for identification. Their amino acid length, molecular weight and PI are highlighted in Table 4.1.1. The gene for SepF2 (SCO2079) is located within the cell division gene cluster, a feature conserved with that of *B.subtilis*, *Figure 4.2.1*. Both *B.subtilis* and *S.coelicolor* have the genes encoding FtsZ and DivIVA downstream and upstream of the gene encoding SepF2, respectively.

Table 4.2.1. Comparison of the three SepF homologs from *S.coelicolor*.

Protein	SCO number	Uniprot	Length Amino Acids	Molecular Weight (Kda)	PI
SepF1	1749	Q9EWX7	146	15.9	4.56
SepF2	2079	Q9S2X2	213	23.7	5.21
SepF3	5967	Q93JG0	136	14.7	5.17

A multiple sequence alignment was performed using the SepF sequence from *B.subtilis* (Uniprot 031728) and the three *S.coelicolor* SepF homologs SepF1, SepF2 and SepF3 encoded by the

genes SCO1749, SCO2079 and SCO5967 respectively. The genome of *S.coelicolor* is very large and often the genes encoding proteins are found duplicated in multiple locations. This is reflected in the number of SepF genes but isn't the case for the other essential cell division proteins including FtsZ. This could reflect the level of regulation required in the different types of hyphal compartmentalisation; vegetative and sporulation specific. However, only one of the genes; *sepF2* (SCO2079) is found within the cell division gene cluster upstream of *ftsZ*.

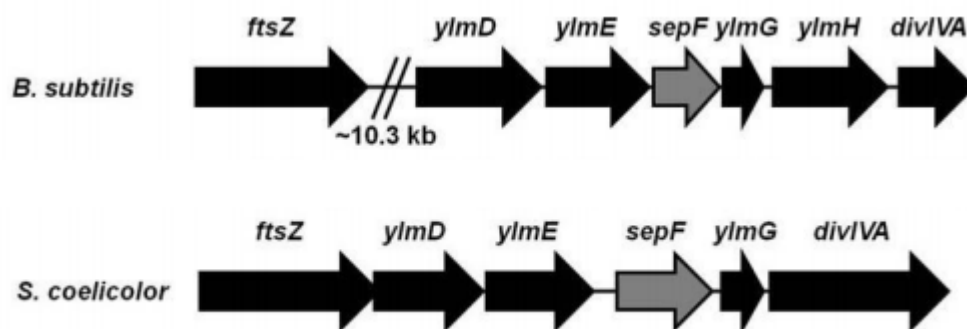


Figure 4.2.1. Gene organisation surrounding *sepF2* of *S.coelicolor* and *sepF* of *B.subtilis*.

Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to perform the multiple sequence alignment of the three *S.coelicolor* SepF homologs to the 151 amino acid long SepF from *B.subtilis*. The greatest level of sequence identity between *S.coelicolor* and the *B.subtilis* SepF was with SepF2 (SCO2079) at 31% even though it is longer in length at 213 amino acids. Furthermore, the shared sequence overall has the greatest coverage at 70%. In contrast, both SepF1 (SCO1749) and SepF3 (SCO5967) have a similar size to the *B.subtilis* *sepF* at 146 and 136 amino acids respectively but have less sequence identity, Table 4.2.2. Interestingly, the SepF from *M.tuberculosis* has relatively high sequence identity to the *B.subtilis* SepF at 33%. However, the overall sequence coverage is 49% and therefore the *B.subtilis* SepF seems to be more highly similar to SepF2 (SCO2079). Initial analysis indicates little conservation of amino acids between the *B.subtilis* SepF and SepF2 as sequence identity is 31%. This could reflect the variation in polypeptide length between the two proteins as SepF2 is 62 residues longer with the N-terminal domain consisting of the majority of the additional amino acids.

Table 4.2.2. Comparison of the SepF from *B.subtilis* to the three SepF homologs from *S.coelicolor* and SepF from *M.tuberculosis* .

Protein	Organism	Uniprot	Length Amino Acid	Molecula Weight (Kda)	PI	% Identity to <i>B.subtilis</i> SepF	% Coverage to <i>B.subtilis</i> SepF
SepF1	<i>S.coelicolor</i>	Q9EWX7	146	15.9	4.56	29	52
SepF2	<i>S.coelicolor</i>	Q9S2X2	213	23.7	5.21	31	70
SepF3	<i>S.coelicolor</i>	Q93JG0	136	14.7	5.17	29	45
SepF	<i>B.subtilis</i>	O31728	151	17.4	5.15	100	100
SepF	<i>M.tuberculosis</i>	P9WGJ5	218	25.3	5.40	33	49

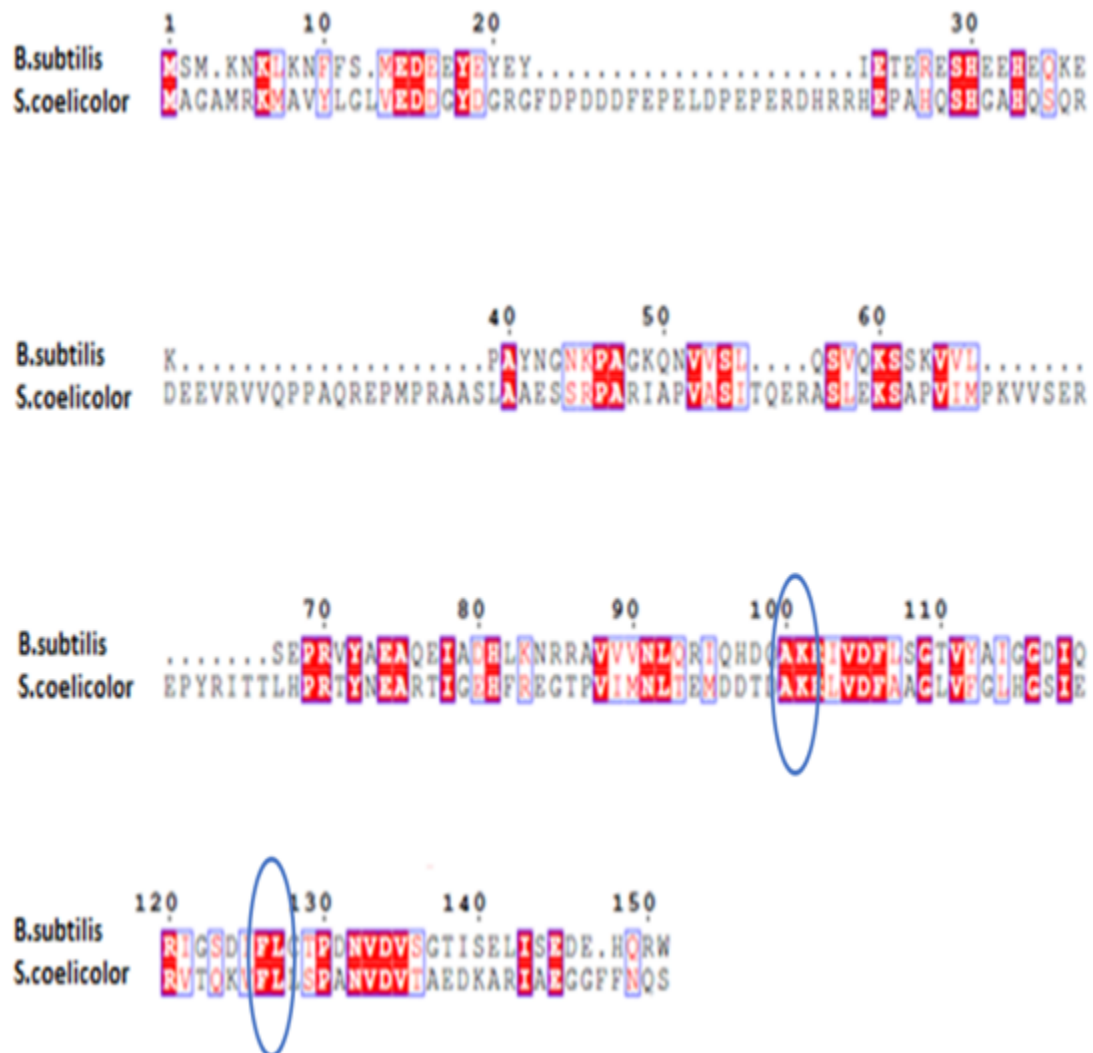


Figure 4.2.2 : Protein sequence alignment of the SepF from *B. subtilis* to SepF2 (SCO2079) from *S. coelicolor*. Conserved residues are highlighted using boxes with a red background and similar residues are shown using boxes with no background. The sequence alignment was generated using T-coffee (<https://www.ebi.ac.uk/Tools/msa/tcoffee/>) and Esprpt (<http://esprpt.ibcp.fr/ESPrpt/ESPrpt/>) software. Residues essential for FtsZ binding are indicated with a ring.

CLUSTAL O(1.2.4) multiple sequence alignment

```

|031728| B.sub      -MSMKNKLKNFFS-MEDEEY EY EY IETER-----ESHEEHEQKE 37
|Q93JG0| SepF1      -MGSVRKASAWLGLVDDNDDERYDDDDYSEGPESGDA----- 37
|Q9EWX7| SepF2      MAGAMRKMAVYVGLVEDDGYDGRGFDPPDDDFEPELDPEPERDHRHREPAHQSHGAHQSQR 60
|Q9S2X2| SepF3      -----MKS GEPV-NSHDVTDEQW 17

|031728| B.sub      K-PAYNGNK-----PAGKQNVVSLQSVQ 59
|Q93JG0| SepF1      ----wV-----TDPRVKVASDVAEE 53
|Q9EWX7| SepF2      DEEVRVVQPPAQREPM PRAASLAAESSRPARIAPVASITQERASLEKSAPVIMPKVVSER 120
|Q9S2X2| SepF3      E-GLAQVVPLRGRDAWPS-----AVGHRAMPEA-ETE 47
                                     : . .

|031728| B.sub      KSSKVVLSEPRVYAEAEQEIADHLKNRRRAVVNLQRIQHDQAKRIVDFLSGTVYAIGGDIQ 119
|Q93JG0| SepF1      KGRRIATVTPDSFRDARAIGELFRDGVPIVNL TAMEGTD AKRVVDFAAGLIFGLRGSIE 113
|Q9EWX7| SepF2      EPYRITTLHPRTYNEARTIGE HFRGTPVIMNLTEMDDTDAKRLVDFAAGLVFGLHGSIE 180
|Q9S2X2| SepF3      RRRRFVVL RINVFADAREVAETLMAGIPVLLDL TSAEGEVAKRVLDFSTGVVFG LASGMH 107
. :.. :*: :. : *::* : ***:** :* :... :...

|031728| B.sub      RIGSDIFLCTPDNVDVSGTISELISEDEHQRW- 151
|Q93JG0| SepF1      RVSTRVFL LSPADTQVISGESAAHRSDGFFNQ S 146
|Q9EWX7| SepF2      RVTQKVFL LSPANVDVTAEDKARIAEGGFFNQ S 213
|Q9S2X2| SepF3      RVD RNVFL LTPAGTEV NGLMESAAGVPGV---- 136
*: **: * :.* . .

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Figure 4.2.3 Protein sequence alignment of the SepF from *B.subtilis* (O31728) to the three SepF homologs from *S.coelicolor*. Conserved residues are highlighted using an asterisk (*), those important for FtsZ binding and for polymerisation are highlighted with a red or green dot respectively. The sequence alignment was generated using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

A comparison between the three *S.coelicolor* SepF proteins was also performed using T-coffee (<https://www.ebi.ac.uk/Tools/msa/tcoffee/>) and Esript (<http://esript.ibcp.fr/ESript/ESript/>) software, Figure 4.2.2. SepF2 (SCO2079) which is the most like the *B.subtilis* SepF, shares 59% and 38% sequence identity with SepF1 (SCO1749) and SepF3 (SCO5967) respectively. Despite the difference in sequence identity both SepF1 (SCO1749) and SepF3 (SCO5967) share approximately 40% overall sequence. The alignment shows that most of the shared sequence is within the C-terminal; a region which is important for the *B.subtilis* SepF interacting with FtsZ (Figure 4.2.3). In fact, two out of the three residues required for FtsZ binding including A98 and F124 are conserved within the SepF proteins from *S.coelicolor* (Bezouwen *et al*, 2012). This suggests they may be able to interact with FtsZ to stabilise the Z-ring.

The N-terminal of *B.subtilis* SepF consists of an amphipathic helix which enable the anchoring of FtsZ to the lipid membrane (Celik *et al*, 2013). The software amphiseek (<https://omictools.com/amphipaseek-tool>) was used to predict whether the three potential SepF homologs from *S.coelicolor* have a membrane attaching helix. The amino acid sequence of each

protein was input into the software and a graph showing the likelihood of a membrane attaching region was produced. The red line giving a positive number, and therefore above the zero line, indicates a lipid binding helix. However, none of the proteins are expected to contain an amphipathic helix region, *Figure 4.2.4*. Moreover their amino acid sequence does not contain a Leucine at position seven which has been shown essential in *B.subtilis* for SepF binding lipids. Therefore if the SepF homologs of *S.coelicolor* are needed for the stabilisation of FtsZ they may attach to the membrane using a different mechanism which could require additional binding partners.

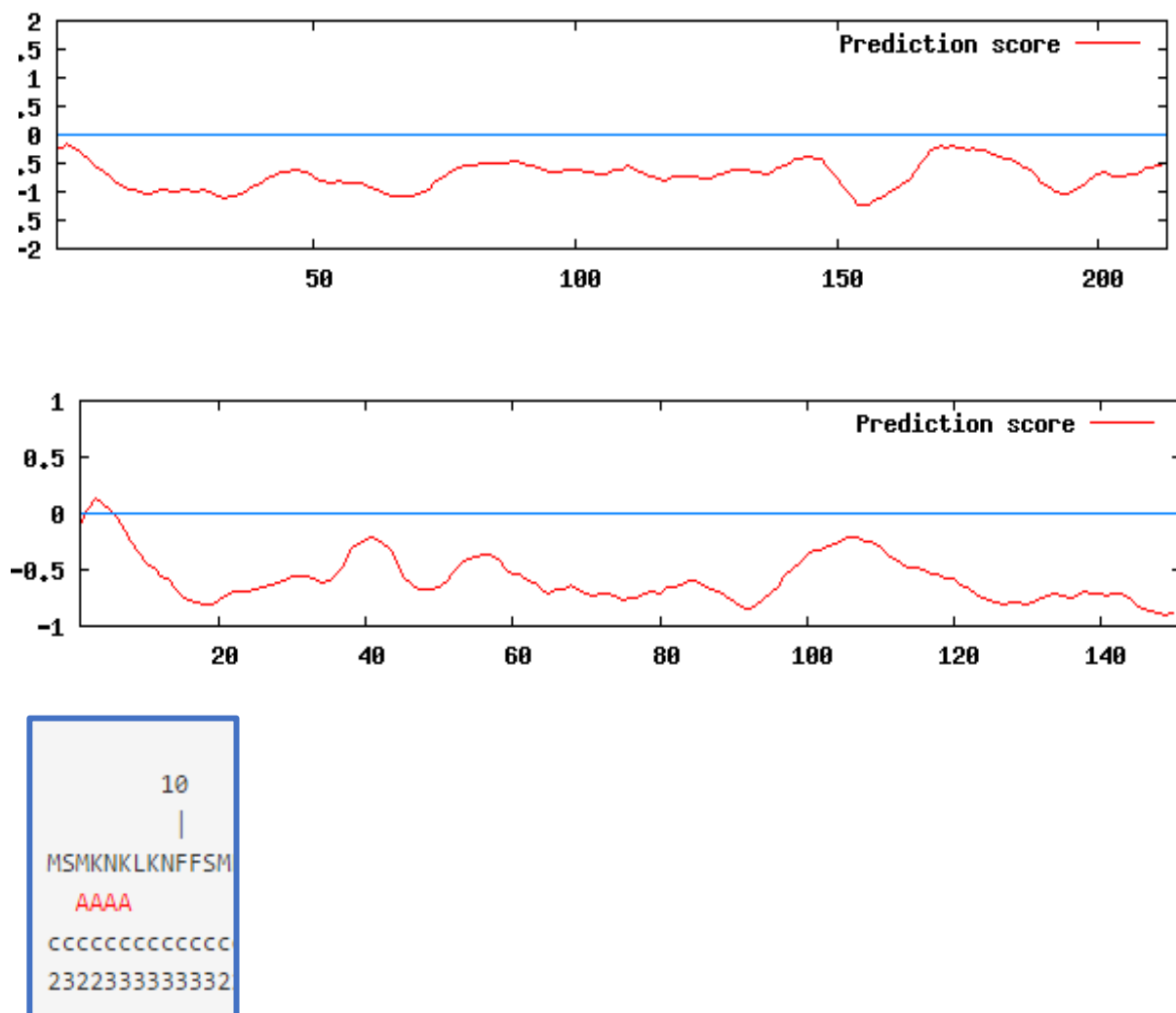


Figure 4.2.4. amphipathic helix prediction using the software amphiseek (<https://omictools.com/amphipaseek-tool>). (Top) SepF2 of *S.coelicolor* (Bottom) SepF of *B.subtilis*. Residues 1-13 shown in a box highlight the amphipathic helix (red letter A) of *B.subtilis*.

4.3 Optimisation of the protein production of *S.coelicolor* SepF homologs

To purify proteins from *S.coelicolor*, a heterologous host expression system based on expressing proteins in *E.coli* was used. The *Streptomyces* gene of interest was previously cloned into the vector pET28a which was subsequently transformed into the appropriate competent *E.coli* cells (Figure 4.3.1). The pET vector system is a well-utilised expression system and pET28a has a relatively small copy number of approximately 45 copies per *E.coli* cell. It has a T7 polymerase promoter gene upstream of the gene of interest, so expression is driven by T7 polymerase. All genes were cloned into the plasmid via *NdeI* and *EcoRI* restriction sites which introduced a thrombin cleavable N-terminal polyhistidine-Tag for purification. The vector also allows for easy selection due to the presence of a kanamycin resistance gene. A T7 promoter drives the expression of the gene from the ATG start site which is integrated into the *NdeI* restriction site (Studier *et al* 1990). Induction of the gene of interest is initiated by addition of isopropyl β -D-1 thiogalactopyranoside (IPTG).

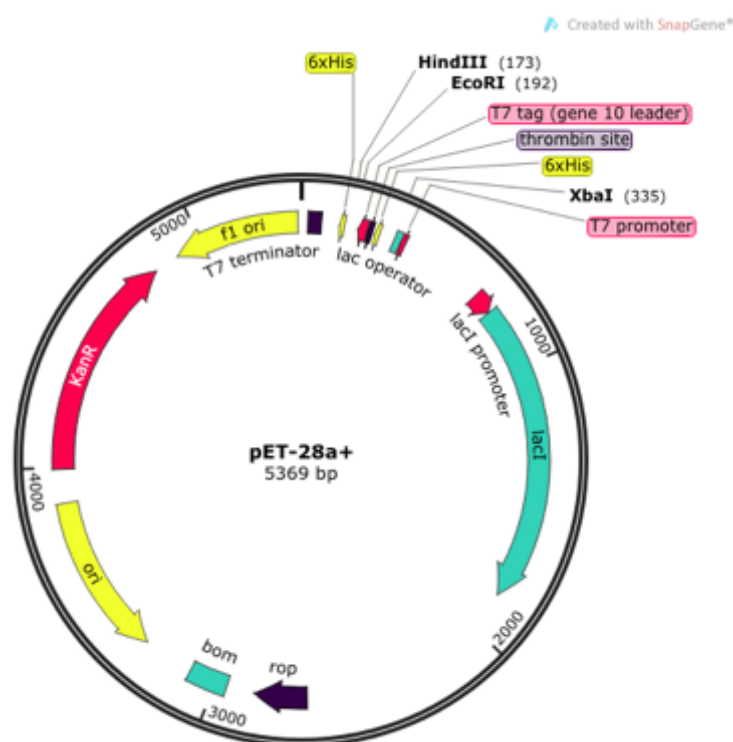


Figure 4.3.1. pET-28a+ plasmid map. The plasmid encodes the T7 promoter, LacI promoter, LacI gene, Ori, and the kanamycin resistance gene KanR (Snapgene).

Initially the *E.coli* strain BL21(DE3)pLysS was used to express the three *S.coelicolor* SepF homologs; SepF1, SepF2 and SepF3. This *E.coli* strain is lacking the proteases *lon* and *ompT* which would degrade misfolded proteins and encodes the T7 RNA polymerase needed for expression of the gene via a lambda DE3 prophage. In addition, the T7 lysozyme is present which inhibits basal expression levels of the target gene (Walker *et al*, 1996). However initial expression trials were unsuccessful using a range of conditions including variations in temperature and inducer concentration, Figure 4.3.2.

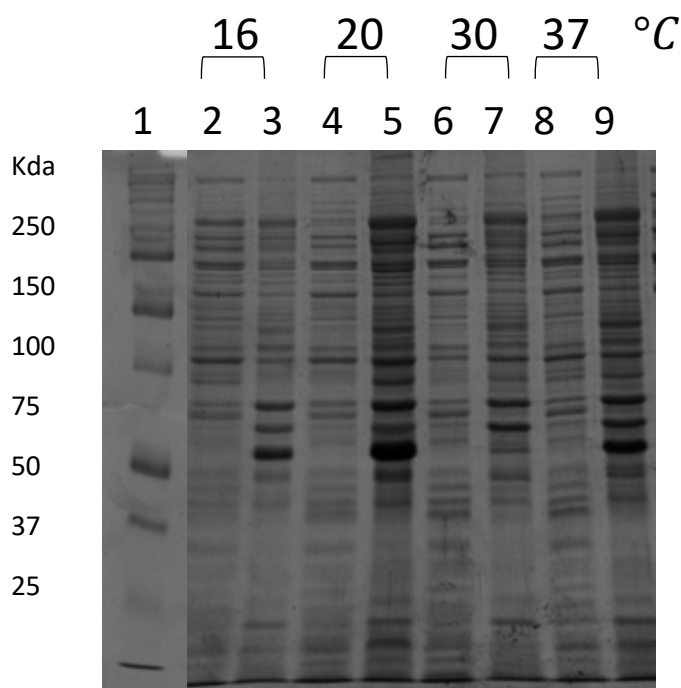


Figure 4.3.2. 15% SDS PAGE analysis of SepF2 overexpression trials using 1 mM IPTG. Lanes 2,4,6,8 show soluble fractions, Lanes 3,5,7,9 show insoluble fractions respectively. Temperatures (°C) used for growth are shown above. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1. Stained with Coomassie blue.

To determine whether the protein was not detected using SDS PAGE analysis, a different staining technique was used. Invision His-Tag staining can detect proteins which consist of a Histidine Tag when present within an SDS PAGE gel. It was possible SepF2 was masked by other proteins or was not expressed. A 15% SDS PAGE gel was prepared with a protein extract from *E.coli* BL21 which had been transformed with pET28a:SepF2. As a control purified FlpP; a protein purified from *S.coelicolor* which also has a His Tag, was loaded onto the gel. The gel was stained with Invision staining technique and imaged using a UV transilluminator which releases a wavelength at 560nm. The stain consists of a fluorescent dye conjugated to Ni²⁺ which binds to the histidine domain of the His-tag. The result

showed FilP was visible, but no other proteins were seen suggesting SepF2 either had no His Tag or wasn't expressed, *Figure 4.3.3*.

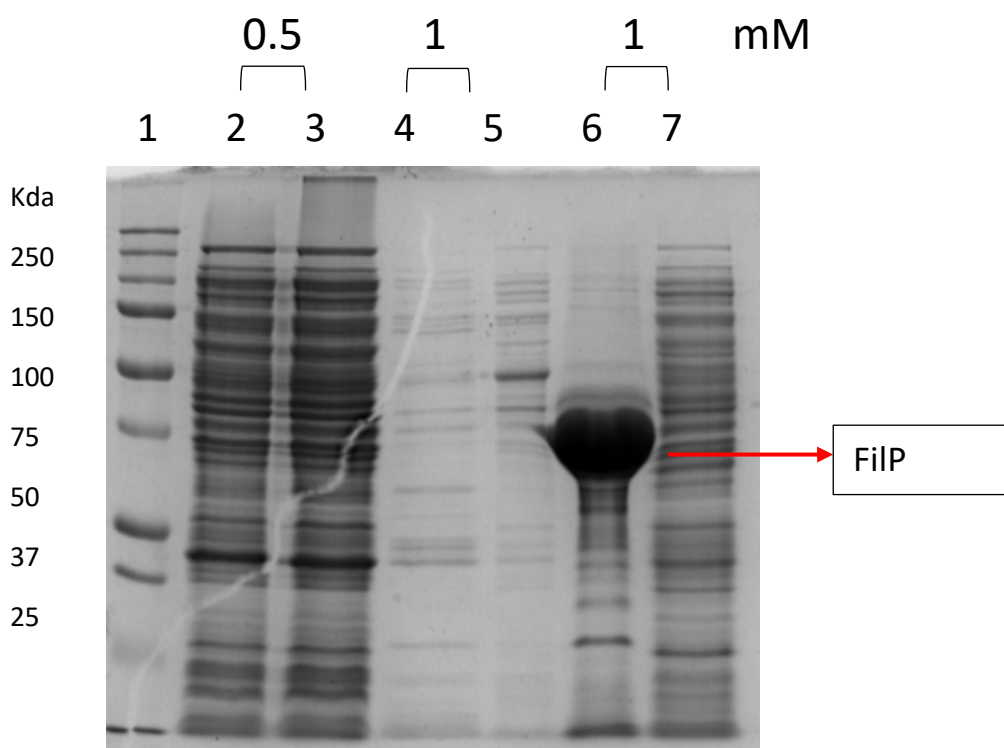


Figure 4.3.3. 15% SDS PAGE analysis of SepF2 overexpression trials at 37 (°C) . Lanes 2,4,6, show soluble fractions , Lanes 3,5,7, show insoluble fractions respectively. Lanes 2-5 show SepF2 Lanes 6 and 7 show FilP. IPTG concentrations (mM) use for induction are shown above. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1. Stained with Invision His Tag stain and Coomassie blue.

When the construct expressing *sepF2* gene was made, DNA sequencing confirmed the base pair sequence was as expected. Upon inspection of the amino acid sequence of SepF2 it became apparent that it contained rare codons which can not be recognised by *E.coli*. Therefore pET28a:SepF2 was transformed into the BL21 derivative Rosetta 2 which supplies the tRNAs required to translate the rare codons (*Nouri et al, 2016*). The highest level of protein expression was achieved at 37°C growth conditions with 1mM IPTG induction.

4.4 Purification of His-SepF2

The construct pET28-SepF2 produces N-terminally His-tagged SepF2 protein with a molecular weight of 25.9kDa and a PI of 5.81, *Figure 4.4.1*. The production of SepF2 was started using a single

colony of BL21 Rosetta2 carrying pET28a:SepF2 which was inoculated into 10mL LB containing kanamycin. The culture was grown overnight at 37°C and subcultured into 50mL of LB containing kanamycin and grown for 3.5 hours prior to induction with 1mM IPTG. Cells were grown at 37°C collected 20 hours after induction by centrifugation and washed with before being resuspended in 30 mL Non-denaturing buffer (Tris 50mM, 300mM NaCl, 10mM Imidazole, 10mM MgCl₂ pH8). The cells were lysed using two cycles of a high-pressure French press at 1000 psi and centrifuged again. Even though, the optimised protein production yielded soluble protein, SepF2 was purified under denaturing conditions using a Ni-NTA column as higher levels of insoluble proteins are produced. The resulting cell pellet was resuspended in 6mL 8M Urea Buffer and sonicated to improve the viscosity before being agitated for an hour. Following a final centrifugation to remove and sedimentation the solution was loaded onto a pre-equilibrated column. Once loaded the column was washed with 8mL 8M Urea Buffer A and 8mL 8M Urea Buffer B to remove the undesired proteins from the column. The bound protein was eluted using buffer 8mL 8M Urea Buffer C and collected as 1mL aliquots labelled E1-E10. The elutions, wash fractions and initial cell lysate was analysed on a 12% SDS-PAGE and showed elutions contained protein at 25.9Kda, *Figure 4.4.1*.

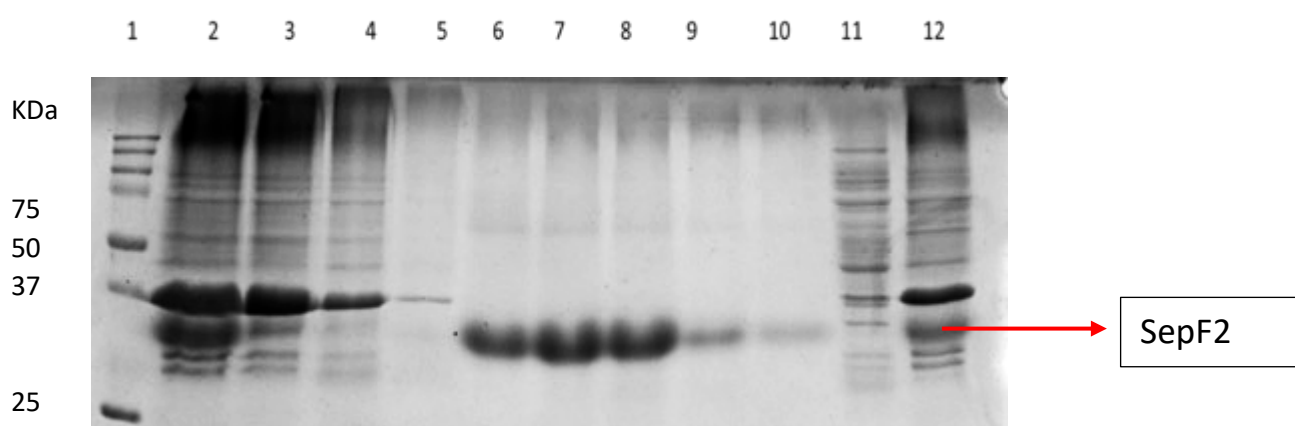


Figure 4.4.1. 15% SDS PAGE analysis of His-SepF2 purification. Lanes 2-12 show Pre Load, Flow Through, Wash 1, Wash 2, elutions 1- 5, soluble fraction control and insoluble fraction control respectively. The control is full length SepF. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1.

4.5 Generation of His-SepFTR

Previous research on SepF and in particular its structure has been carried out using a truncated fragment of the gene (*Celik et al, 2013*). Therefore, a truncated version of SepF2 (SepFTR) was

generated and cloned into pET28a so that the experimental conditions used in other studies to probe into the proteins structure and dynamics could be directly compared. The SepF2 Truncated PCR1 fragment was amplified using 4A10 cosmid DNA as template and primers SepF trunc Xba Nde Forward and SepF UT Reverse. SepF Truncated PCR2 was amplified using primers SepF trunc Xba Nde Forward and SepF UTC Reverse from the 4A10 cosmid. This produced a construct with a stop codon as the polyhistidine tag would be fused to the N-terminus of SepFTR. All PCR products were digested with EcoRI and XbaI before loading onto a 0.7% preparative agarose gel. Bands corresponding to the expected sizes of 294bp were excised and the DNA purified using QIAQuick gel extraction Kit, *Figure 4.5.3.1*. The purified DNA was analysed on a 0.7% agarose gel to confirm the quantity of the recovered fragment. The predicted length of truncated *sepF2* gene fragment of 294bp.

4.5.2 Ligation

SepFTR was cloned into the *NdeI* and *EcoRI* sites of pET28a to generate a N-terminally His-tagged SepFTR using ligation. The ligation mix was transformed into DH5 α competent cells and transformants were selected using LB agar plates supplemented with ampicillin or kanamycin. Each plate was incubated overnight at 37° C, producing colonies in a range of 20-100.

4.5.3 Colony PCR to confirm transformants carrying the correct inserts

Individual colonies were streaked onto grid lined plates and grown overnight at 37° C. The colonies were screened using colony PCR to confirm successful ligations of the plasmid with the SepFTR gene. To amplify the gene SepFTR; SepF Trunc Xba Nde Forward and SepF UT reverse primers were used. The PCR samples were analysed on 0.7% agarose gels at 50 V, alongside a positive control, to confirm all the screened colonies for successful transformation of a plasmid with correct gene insert, *Figure 4.5.3.1*.

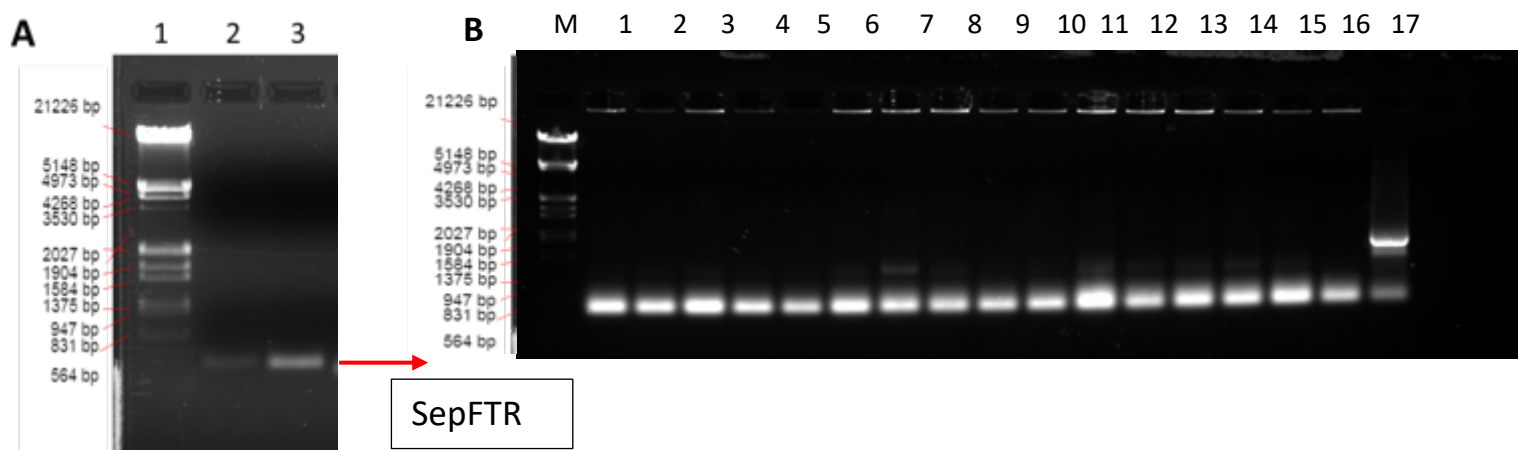


Figure 4.5.3.1. Confirmation of the generated PCR product by gel electrophoresis. (A) Lane 1, λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left. Lanes 2 and 3, *SepF* Truncated PCR1 and PCR2 product. (B) Gel analysis of colony PCR for *SepFTR*. Lane M contains λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left and colony numbers are labelled for each lane 1-16 contains individual colonies of pET28a:*SepFTR*, lane 17 contains a control of *SepF2*.

4.5.4 Purification of His-SepFTR

The crystal structure of *SepF* from *B.subtilis* was solved using a truncated version consisting of amino acids 61 to 140 (Celik *et al*, 2013). Therefore the *SepF2* gene was PCR amplified using primers *SepF* Trunc Xba Nde FRW and *SepF* UT REV to generate a truncated version of *SepF2* (*SepFTR*). *SepFTR* was cloned into the *NdeI* and *EcoRI* sites of pET28a to generate a N-terminally His-tagged *SepFTR* protein with a molecular weight of 13.2kDa and a PI of 6.3. As the overexpression of *SepF2* had been optimised, as shown earlier in this chapter, the same protocol was used for large scale overexpression of *SepFTR*. *SepFTR* was purified under denaturing conditions using a Ni-NTA column as higher levels of insoluble proteins are produced. The resulting cell pellet was resuspended in 6mL 8M Urea Buffer and sonicated to improve the viscosity before being agitated for an hour. Following a final centrifugation to remove and sedimentation the solution was loaded onto a pre-equilibrated column. Once loaded the column was washed with 8mL 8M Urea Buffer A and 8mL 8M Urea Buffer B to remove the undesired proteins from the column. The bound protein was eluted using buffer 8mL 8M Urea Buffer C and collected as 1mL aliquots labelled E1-E10. The elutions, wash fractions and initial cell lysate

was analysed on a 15% SDS-PAGE and showed eluted protein at expected size of 13.2Kda, *Figure 4.5.4.1.*

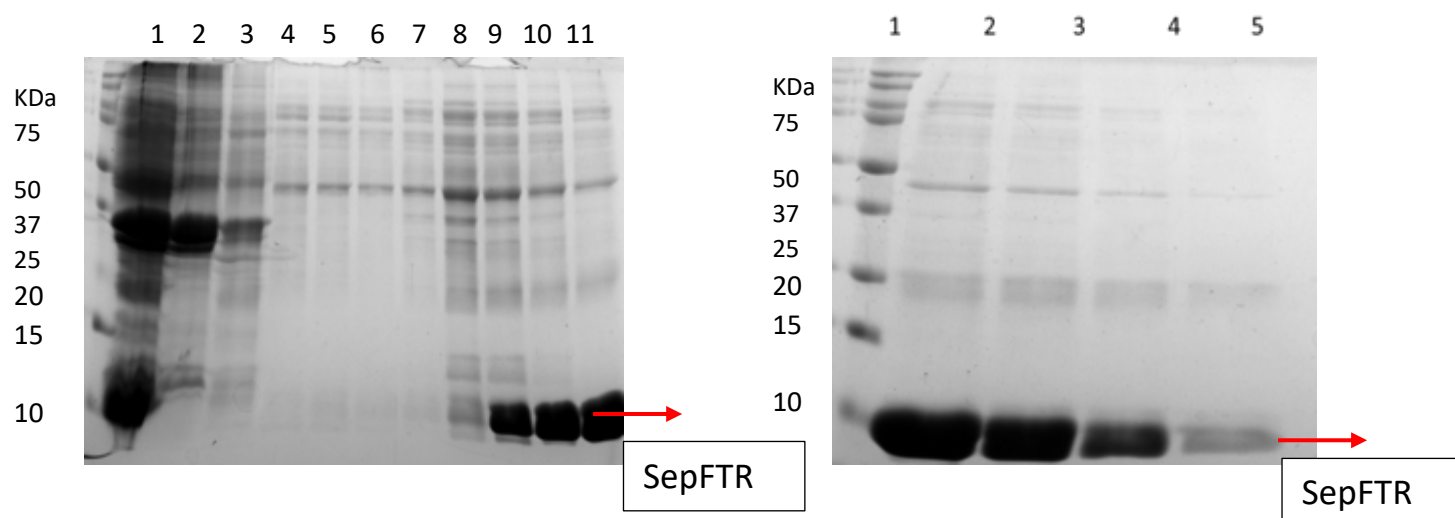


Figure 4.5.4.1. 15% SDS PAGE analysis of His-SepF2TR purification. (Left) Lanes 1-11 show Pre Load 1, Wash 1, Wash 2 and elutions 1- 8 respectively. (Right) Lanes 2-5 show elutions 9-11 and Wash 3 respectively. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1(Right)

4.6 Dialysis of His-SepF proteins

Initial attempts to dialyse the SepF proteins we used HEPES buffer (50mM HEPES, 50mM KCL, pH 7.2), however we found that SepF2 and SepFTR precipitated under these conditions, in spite of the fact that the buffer's pH was well above the pIs of the proteins. This could have happened either because some of the proteins were purified from the insoluble fraction of the cell lysate and therefore were not in their native form or because this protein forms higher order assemblies particularly at high protein concentrations. We sought to test for a buffer composition that would allow for the dialysis of the His-SepF proteins to remove the imidazole present from the elution buffer, Table 4.6.1.1, *Figure 4.6.1.1.*

Firstly, a 500µL solution was prepared using a 1:4 mixture of eluted protein to 8M Urea Buffer C and dialysed against the HEPES buffer. It is thought the step wise dialysis will allow the protein more time to refold without forming an aggregate. However, no soluble protein was achieved after the completion of dialysis.

Next arginine was added to the buffer and a 500µL solution of eluted protein was dialysed against the HEPES buffer. Research has shown the addition of arginine suppressed protein aggregation as the guanidinium group of arginine contacts tryptophan side chains of the protein (*Arakawa et al, 2004*). Although this method yielded more protein, very little was recovered.

Finally, the concentration of KCL was increased to 100mM as the addition of salts is believed to maintain the ionic strength of the solution. However, no protein appeared to be recovered after this dialysis was complete.

A search of the literature on SepF purification was conducted to discover an alternative buffer suitable for the dialysis of SepF proteins. Previously the SepF of *B.subtilis* was purified and refolded in

Table 4.6.1.1. Comparison of the Buffers used to dialyse SepFTR.

Condition	Buffer	Volume of protein solution	Composition Of Fraction	precipitation	% Recovered
1	50mM HEPES 50mM KCL pH7.2	500	Protein	Yes	10
2	50mM HEPES 50mM KCL pH7.2	500	1:4 Protein to Urea	Yes	10
3	50mM HEPES 50mM KCL 50mM Arginine pH7.2	500	1:4 Protein to Urea	Yes	10
4	50mM HEPES 100mM KCL pH7.2	500	1:4 Protein to Urea	Yes	10
5	50mM Tris-HCL 50mM NaCl 10% glycerol 0.4M L-arginine 2% glycine pH 8	500	1:4 Protein to Urea	No	25

a stabilising buffer (50mM Tris-HCL, pH 8, 50mM NaCl, 10% glycerol, 0.4M L-arginine and 2% glycine) (*Singh et al, 2008*). A 500µL fraction of purified His-SepFTR was dialysed against 500mL of stabilising buffer at room temperature. After three buffer exchanges soluble protein was recovered and analysed using 12% SDS PAGE, *Figure 4.6.1.1*. The protein was detected at the suspected molecular weight of 13.2Kda.

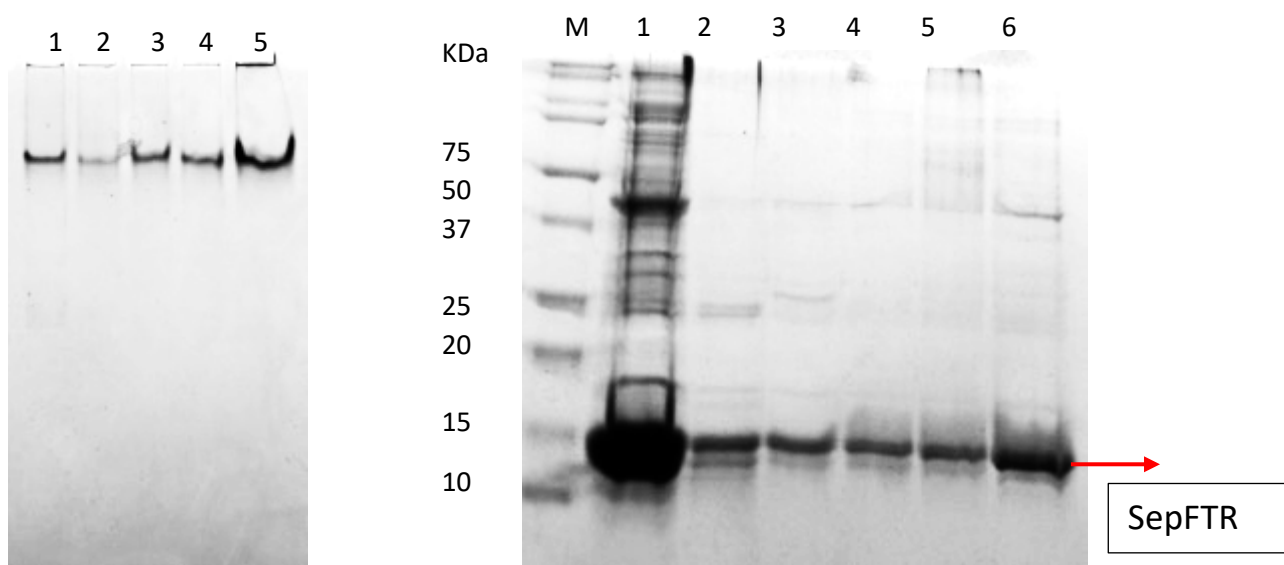


Figure 4.6.1.1. (Left) 15% NATIVE PAGE analysis of His-SepFTR protein dialysis trials. Lanes 1-5 show His-SepFTR dialysed in conditions 1-5 (Shown in table 5.1.1.1) respectively. (Right) 12% SDS PAGE analysis of His-SepFTR protein dialysis trials. Lane 1 shows undialysed His-SepFTR and lanes 2-5 show the insoluble His-SepFTR dialysed in conditions 1-4 respectively. Lane 6 shows His-SepFTR dialysed in condition 5. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane labelled M.

4.7 Transmission Electron Microscopy of SepFTR

Previous research using truncated SepF from *B.subtilis* has revealed individual rings using Electron Microscopy at pH 10 these then associated to form a tube at lower pH (Celik *et al*, 2013; Errington *et al*, 2011)). We attempted to view our truncated SepF2 from *S.coelicolor* after dialysis using 50mM Tris-HCL, 50mM NaCl, 10% glycerol, 0.4M L-arginine, 2% glycine, pH=8.0. A 10 μ L aliquot of purified and dialysed His-SepFTR was incubated at 30°C for 20 minutes to encourage polymerisation of proteins and 5 μ L applied to carbon coated grids. This was negatively stained using freshly prepared 2% ammonium molybdate, pH 7.0 and analysed by Emily Alcock using Transmission Electron Microscopy (TEM), Figure 4.7.1.

The TEM revealed SepFTR forms rings and long tubular structures which appear to stretch like a spring. This suggests the *S.coelicolor* SepF2 forms higher order assemblies facilitated by the C-terminal region. These findings support the previously created structural model of *S.coelicolor* which

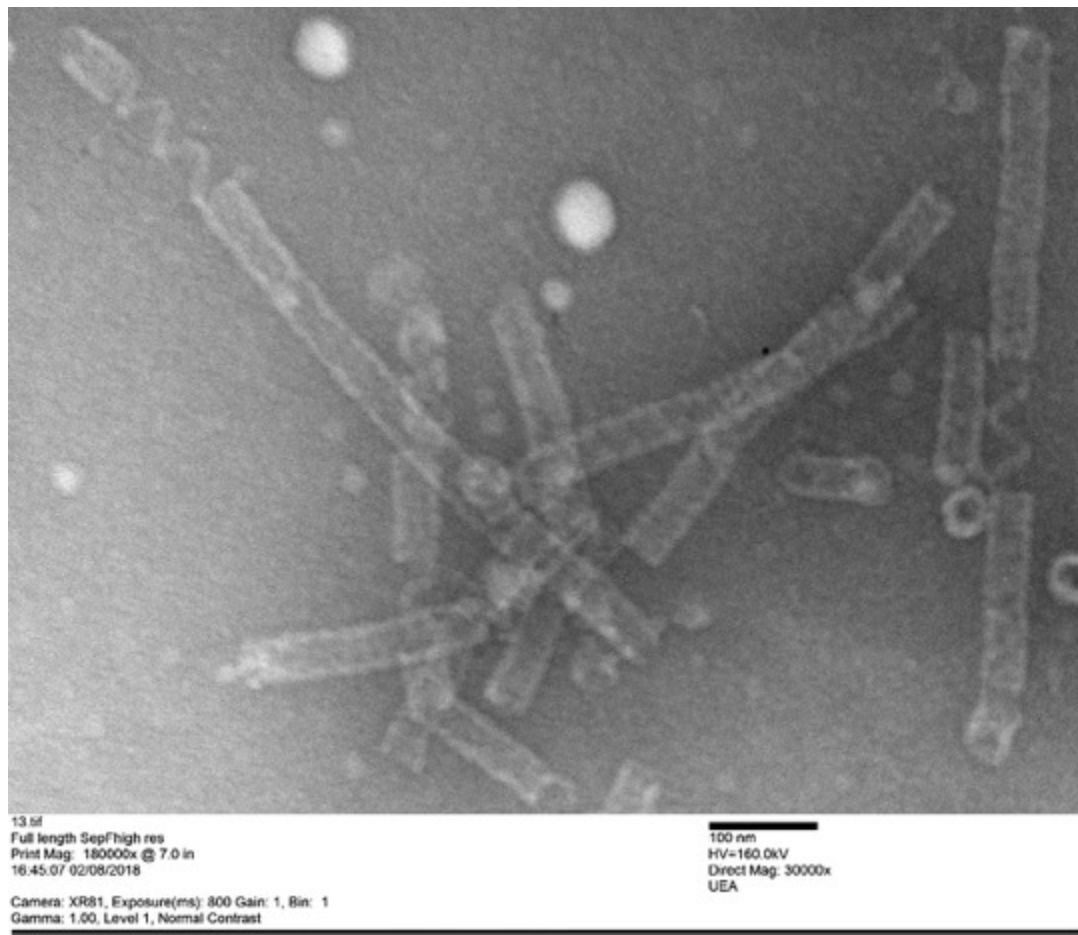


Figure 4.7.1. TEM image of SepFTR in buffer 50mM Tris-HCL, 50mM NaCl, 10% glycerol. 0.4M L-arginine, 2% glycine, pH=8.0.

closely resembles the *B.subtilis* homo dimer formed from residues 61 to 140. SepFTR is able to polymerise into rings and therefore could perform a stabilising role for FtsZ like *B.subtilis* SepF.

Measurements of the springs and rings of SepFTR were taken, *Figure 4.7.2*. The width of an individual spring measures 43.5nm and an individual ring 43.5nm.

Although, tubes and individual rings of *B.subtilis* SepF have been observed previously, no spring like structures have been seen (*Errington et al, 2011*). One possibility for this is that the SepF from *B.subtilis* is able to form these structures but it hasn't been viewed. Alternatively, the *S.coelicolor* SepF could be unique and needs to form a polymer that is flexible .

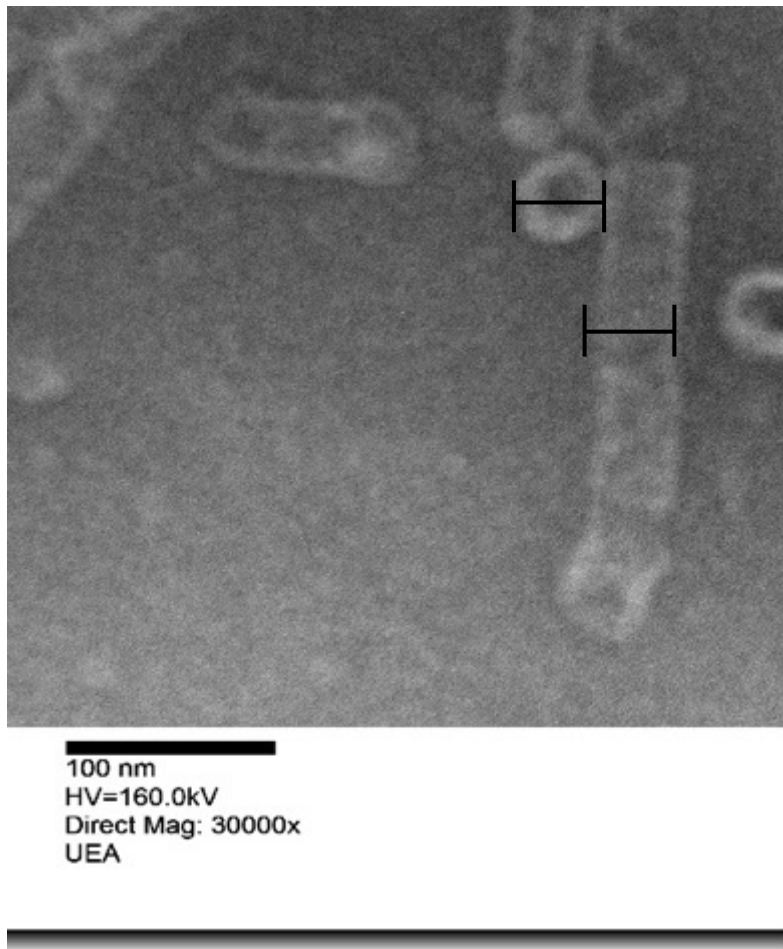


Figure 4.7.2. TEM image of SepFTR in buffer 50mM Tris-HCL, 50mM NaCl, 10% glycerol. 0.4M L-arginine, 2% glycine, pH=8.0. The width of a ring and spring labelled.

4.8 Summary

In, this Chapter we aimed to investigate the three potential SepF proteins of *S.coelicolor* to probe into their function, structure and role in the bacterium. We initially performed bioinformatics of the proteins using numerous software and this included a multiple sequence alignment of the three SepF of *S.coelicolor* with SepF homologs in other bacteria. We found the *S.coelicolor* SepF2 shares the amino acid residues required for the binding of the protein to FtsZ with the *B.subtilis* protein. However none of the homologs are predicted to be able to bind lipid membranes.

An attempt to purify the three SepF homologs from *S.coelicolor* was made using the heterologous host *E.coli*. The full length *sepF* genes and a truncated version of *sepFTR* was successfully cloned into a pET28a construct for purification and the production of the proteins was optimised to

find the ideal conditions for large scale overproduction. SepF2 and truncated SepF2 was purified using a Histidine tag via metal affinity chromatography under denaturing conditions. This was followed by the optimisation of the dialysis of the proteins into buffers which kept the proteins stable for future assays.

Finally, Transmission Electron Microscopy was used to visualise truncated SepF2 in stabilising buffer. The protein appeared to create a spring-like structure that is compatible with the previously observed multiple ring structure of the *B.subtilis* SepFTR, which stacked to produce a tubular structure. The size of the rings and spring measures a width of 43.5nm which is close to the 50nm observed in *B.subtilis* (Errington *et al*, 2011). It is thought this higher order structure is produced due to interactions of the C-terminal residues which was also found with the *B.subtilis* SepF.

The research carried out in this chapter has limitations. The bioinformatics included suggests SepF2 of *S.coelicolor* has the amino acid residues for binding FtsZ but this needs to be confirmed further using mutation studies whereby a version of the SepF2 protein is expressed that has substituted residues and it would be subjected to binding studies with FtsZ. Furthermore, only one program was used to predict if SepF2 can bind lipid membranes and so more programs would need to be utilised for a better prediction, alongside lipid binding experiments using artificial membranes.

5 FtsZ: A Biochemical Analysis

5.1 Introduction

A crucial process in bacterial cell division is the formation of the Z-ring at the future site of septation, which is initiated by the protein FtsZ. This well characterised eukaryote tubulin homolog is conserved across most prokaryotes with early studies highlighting its importance. FtsZ polymerises into a ring at the division site and recruits other cell division proteins (*Lutkenhaus et al, 1991*). It serves as a cytoskeletal protein and shares the ability to hydrolyse GTP with its eukaryotic counterpart. Recent work has focused on understanding the dynamics of FtsZ polymerisation, but the exact mechanism is still under debate.

The assembly of the Z-ring requires a switch from FtsZ concentration being at the poles to the mid-cell. Prior to this transition, FtsZ forms short membrane anchored assemblies that move along the circumference of the cell. This was determined by using fluorescently labelled FtsZ in *E.coli* (*Walker et al, 2020*). This step is then followed by Z-ring formation which is governed by positioning systems like the Min system and additional proteins.

FtsZ monomers consist of several domains; the GTPase domain, a conserved C-terminal peptide with a disordered C-terminal linker (CTL) between the latter two. The motif GGGTGS/TG and two loop regions within the protein CTL facilitate nucleotide binding for GTP hydrolysis (*Amos et al, 1998*). Fluorescence spectroscopy has shown Loop T3 undergoes a conformational change upon the loss of the γ -phosphate. While Loop T7 is thought to contact a nucleotide of an adjacent monomer as mutations in this region inhibit GTPase activity in *E.coli* FtsZ (*Matsui et al, 2014; Andreu et al, 2001*). This suggests for GTP hydrolysis to occur, two FtsZ monomers must form a dimer to provide the active site. This is a contrast to tubulin which exists as a preformed heterodimer.

In vivo, FtsZ polymerises into one-stranded protofilaments which associate to make a 2-4 filament thick Z-ring. As polymerisation is required for the nucleoside hydrolysis activity of FtsZ, it can be described as a self-activating GTPase (*Ma et al, 1996; Lutkenhaus et al, 1996*). The exact structure of the Z-ring has been difficult to determine which has led to *in vitro* studies of FtsZ polymerisation. Individual FtsZ monomers are highly dynamic, with a turnover time of approximately 10-30 seconds within the Z-ring (*Anderson et al, 2004*). *In vitro*, the dynamics of FtsZ and its polymeric state are determined by many factors, one of which is the GTPase domain activity. Deletion of this domain results in a longer turnover time of FtsZ monomers impacting polymer dynamics (*Levin et al, 2003*).

Furthermore, different experimental conditions have led to the presence of protofilaments sheets and strands (Erickson *et al*, 1996). This has made it difficult to determine the steps required in FtsZ polymerisation. Recently, deletion of the CTL *in vivo* results in a change in Z-ring morphology and its dynamics, suggesting the region is crucial for FtsZ polymerisation and interfilament interactions (Barrows *et al*, 2020; Buske *et al*, 2013).

FtsZ has been shown to form single stranded protofilaments in the presence of GTP and self-associates into oligomers, when in the presence of Mg ions alone (Andreu *et al*, 2004). Numerous studies have tried to identify the role of GTP in protofilament formation. The GTPase activity is Mg ion dependent and high levels of KCL increase nucleoside hydrolysis (Gómez-Puertas *et al*, 2009). The current view is that GTP binding promotes polymerisation and its subsequent hydrolysis is required to regulate the dynamic behaviour of the Z-ring as protofilament disassembly occurs upon γ -phosphate release. Evidence for this has come from fluorescence recovery after photobleaching (FRAP) studies which is further supported by the fact mutations inhibiting hydrolysis but not binding stabilise polymers (Mingorance *et al*, 2010).

FtsZ is regulated by several proteins which bind to the C-terminal including the membrane anchoring proteins FtsA, SepF and ZapA. These proteins effect localisation and polymer dynamics. Studies into ZapA, a coiled coil protein, indicate it aligns FtsZ polymers into a condensed Z-ring (Loose *et al*, 2019). Additionally, FtsA anchors FtsZ to the membrane and effects treadmilling supported by the fact *in vitro* FtsZ co-assembles with FtsA (Bisson-Filho *et al*, 2017). However, membrane anchor SepF doesn't effect FtsZ filaments dyanmics (Barger *et al*, 2020). Deletion of the final 16 amino acids of FtsZ or a mutation of Proline 372 abolishes SepF binding to FtsZ in *B.subtilis* (Bezouwen *et al*, 2012). Recently mutation studies of FtsZ have highlighted key residues which contribute the polymerisation and dynamics of the protein. These include Isoleucine 375 and Glycine 314 which contribute to binding ZapD and ZipA respectively (Huang *et al*, 2017., Cameron *et al*, 2021)

Recently, several studies have shown FtsZ undergoes a process known as treadmilling, whereby subunits are added and subsequently removed from either end of a filament resulting in a movement of FtsZ polymers in a direction (Loose *et al*, 2014). Treadmilling, requires a local concentration of FtsZ, GTP hydrolysis and membrane association via the previously mentioned proteins. It is thought a conformational switch occurs between FtsZ self activating assembly to treadmilling. Furthermore, FtsZ treadmilling is correlated with the PG enzymes that drive constriction of the cell membrane (Cruz *et al*, 2019).

Streptomyces including *S.coelicolor* utilise FtsZ for the regulation of sporulation specific cell division. Localisation studies using fluorescently tagged FtsZ have confirmed the formation of a regular

array of Z-rings upon the length of aerial hyphae prior to sporulation. Deletion of *ftsZ* in *S. coelicolor* blocks septum formation but aerial hyphae growth can still occur (McCormick *et al*, 1994). Unlike other bacteria, *S.coelicolor* is still viable without FtsZ which has made it a useful organism for studying cell cycle events; particularly cell division. Despite numerous studies into the role of FtsZ in *Streptomyces*, the structure of FtsZ and the mechanism of polymerisation of the protein are unknown. Characterisation of the *S.coelicolor* FtsZ could help to elude the mechanism and structure of FtsZ in other bacterium.

In this chapter we will attempt to gain a better insight into the biochemical properties of FtsZ from *S.coelicolor* and suggest what implications this has on its intracellular function.

- I perform bioinformatic analysis of FtsZ homologs.
- I generate a protein model of FtsZ from *S.coelicolor*.
- I optimise overproduction and purification of FtsZ from *S.coelicolor*.
- I investigate the polymerisation properties of FtsZ using simple biochemical assays.

5.2 Biochemical Analysis of *S.coelicolor* FtsZ

A multiple sequence alignment was performed using the FtsZ sequence from *B.subtilis* (Uniprot P17865), the FtsZ protein of *M.tuberculosis* (Uniprot P9WN95) and the FtsZ protein from *S.coelicolor* (Uniprot P45500), *Table 5.2.1*.

Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to perform the multiple sequence alignment of the three FtsZ homologs from *B.subtilis*, *S.coelicolor* and *M.tuberculosis*. The greatest level of sequence identity was between *S.coelicolor* and the *B.subtilis* FtsZ at 62% and there are similar in size at 399 and 382 amino acids respectively. Interestingly, the FtsZ from *M.tuberculosis* has relatively high sequence identity to the *B.subtilis* FtsZ at 59%. Furthermore, the overall sequence coverage is 88% and therefore the *B.subtilis* FtsZ seems to be highly similar to both the *S.coelicolor* and *M.tuberculosis* FtsZ.

Table 5.2.1. Comparison of FtsZ homolog from *S.coelicolor* to other organisms.

Organism	Uniprot	Length Amino Acids	Molecular Weight (Kda)	PI	% Identity to <i>B.subtilis</i> FtsZ	% Coverage to <i>B.subtilis</i> FtsZ
<i>B.subtilis</i>	P17865	382	40.4	5.01	100	100
<i>S.coelicolor</i>	P45500	399	41.1	4.47	62	85
<i>M.tuberculosis</i>	P9WN95	379	38.8	4.55	59	88

A comparison between the *FtsZ* proteins of *S.coelicolor* and *B.subtilis* was also performed using T-coffee (<https://www.ebi.ac.uk/Tools/msa/tcoffee/>) and Esript (<http://esript.ibcp.fr/ESript/ESript/>) software, *Figure 5.2.1*. The alignment shows that most of the shared sequence is within the N-terminal but the region which is important for the binding of FtsZ regulators, the c-terminus, shares little similarity. However, the proline residue 372 required for

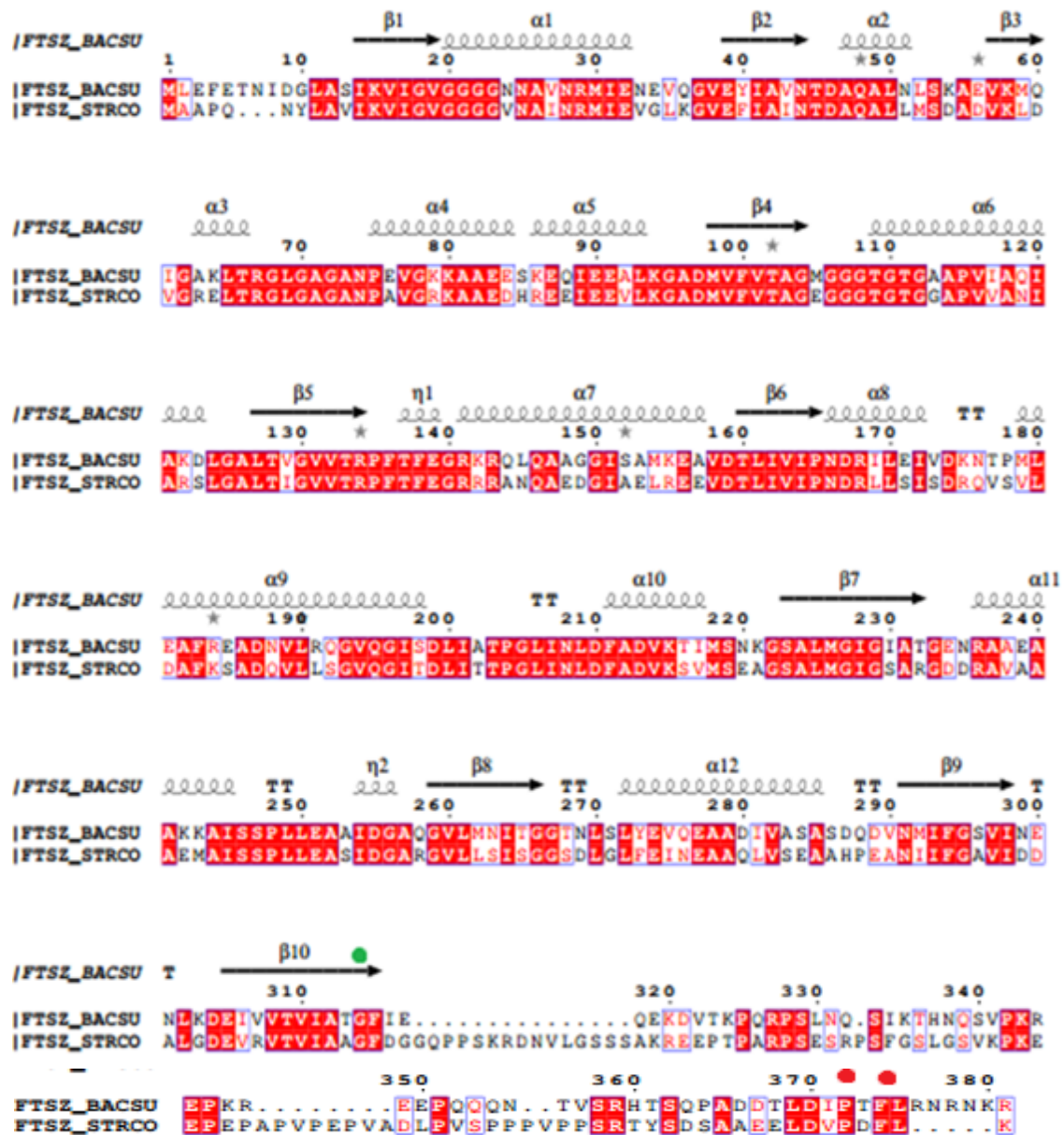


Figure 5.2.1. Protein sequence alignment of the *FtsZ* from *B.subtilis* to *FtsZ* from *S.coelicolor*. Conserved residues are highlighted using boxes with a red background and similar residues are shown using boxes with no background. The sequence alignment was generated using T-coffee (<https://www.ebi.ac.uk/Tools/msa/tcoffee/>) and Esript (<http://esript.ibcp.fr/ESript/ESript/>) software. Residues essential for the binding of FtsZ regulators are highlighted with a red or green circle.

binding SepF in *B.subtilis* , the isoleucine residue 375 for binding ZapD and the glycine residue 314 for binding ZipA is conserved with the FtsZ protein from *S.coelicolor*.

A protein model of FtsZ from *S.coelicolor* was created to predict the three dimensional protein structure of the protein using the software Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>). The sequence of *S.coelicolor* FtsZ was input in FASTA format (Uniprot P45500) and a template protein of FtsZ from *Thermobifida fusca* YX-ER1 (PDB: 4e6e) was used. Both proteins share 93.91% sequence identity and a template alignment was produced, *Figure 5.2.2*. The software generated a 3D protein structure diagram from the alignment which shows a monomer consisting of alpha-helices, beta-sheets and loop regions. The conserved TG motif is represented as a ball and stick.

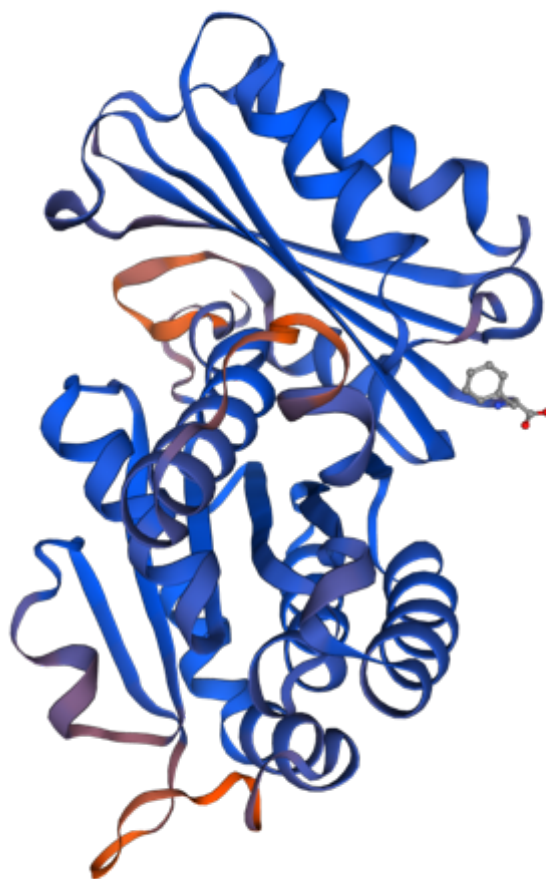


Figure 5.2.2. Three dimensional ribbon diagram of a model of FtsZ from *S.coelicolor*. Shown are beta-sheets, alpha helices, loop regions and the TG motif as ball and stick. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 4e6e.

The protein model data shows the model consists of amino acid residues from 1 to 312, therefore is missing the remaining 88 residues of the C-terminal. The conserved Glycine residue at position 314 (*B.subtilis*) 310 (*S.coelicolor*) is shown on Figure 5.2.3, which has been shown to be important for the binding of ZipA (Cameron *et al*, 2021). The alignment data also shows the model of FtsZ from *S.coelicolor* shares the beta-sheets and alpha helices from FtsZ from *Thermobifida fusca* YX-ER1 (PDB: 4e6e).

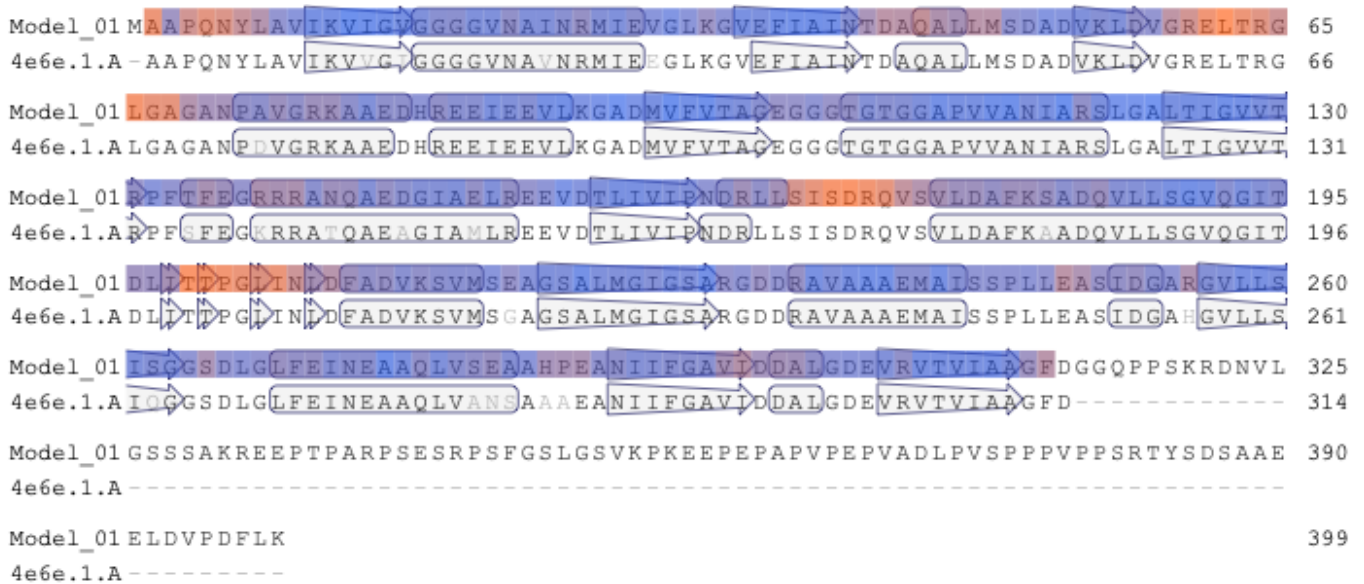


Figure 5.2.3. Model alignment data of a model of FtsZ from *S.coelicolor*. Shown are amino acid alignment from the Model (model_01) and FtsZ from *Thermobifida fusca* YX-ER1 (PDB: 4e6e) beta-sheets are shown as arrows and alpha helices as rings around the residues. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 4e6e.

The GTPase domain of FtsZ in *E.coli* has been identified and is made up of residues N207, D209 and D212 of region known as the 'synergy' loop (Erickson *et al*, 2001). The protein model of FtsZ from *S.coelicolor* also has a loop region with these residues conserved, which suggests GTP binds there, Figure 5.2.4.

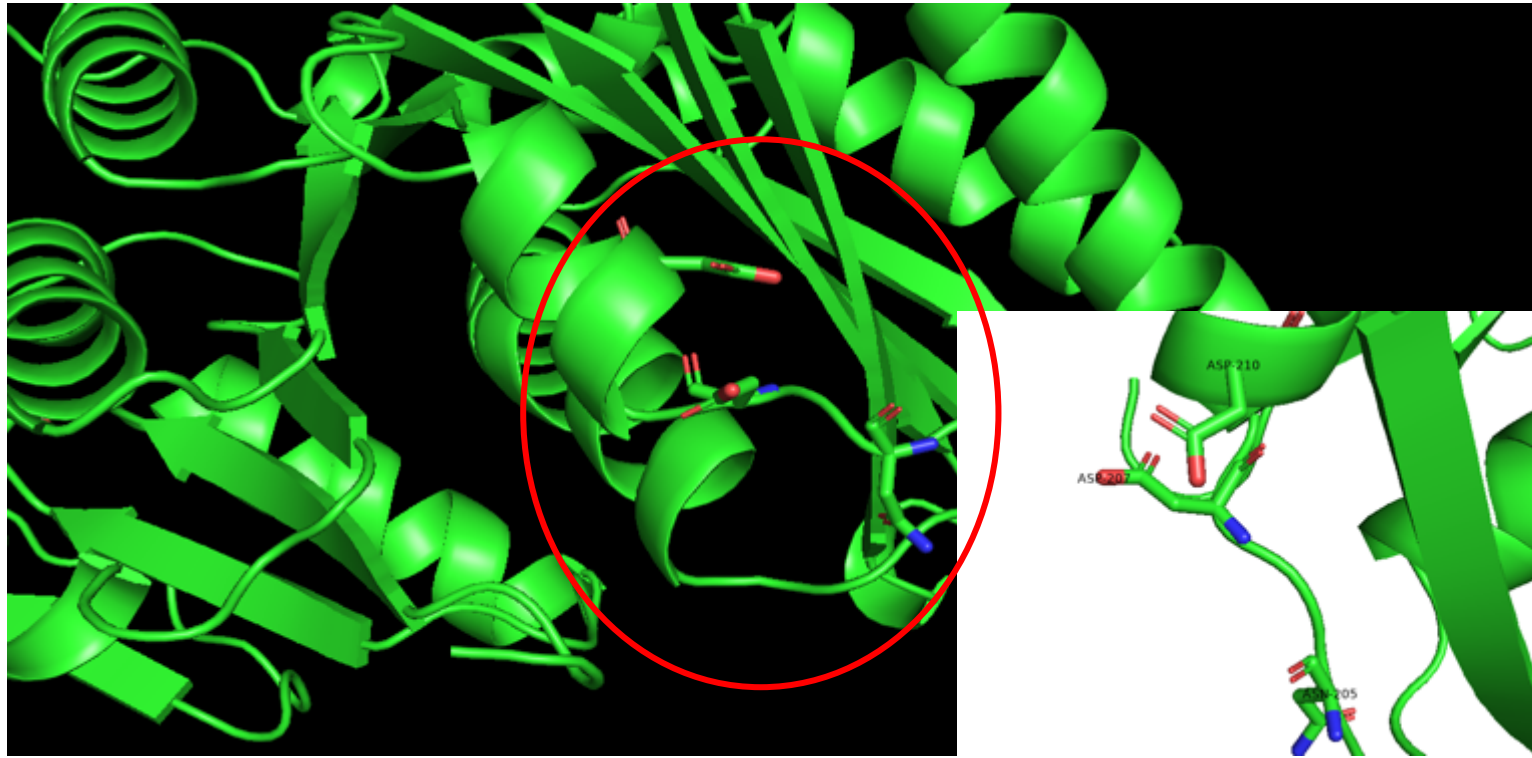


Figure 5.2.4. Three dimensional ribbon diagram of a model of FtsZ from *S.coelicolor*. Shown is the GTP binding site (red circle) of the Model (model_01) created using FtsZ from *Thermobifida fusca* YX-ER1 (PDB: 4e6e) Inserted is the residues that make up the GTP binding site, N205, D207, D210. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 4e6e.

5.3 Optimisation of the protein production of *S.coelicolor* FtsZ

To purify proteins from *S.coelicolor*, a heterologous host expression system based on expressing proteins in *E.coli* was used. The *Streptomyces* gene of interest was previously cloned into the vector pET28a which was subsequently transformed into the appropriate competent *E.coli* cells (Figure 5.3.1). All genes were cloned into the plasmid via *NdeI* and *EcoRI* restriction sites which introduced a thrombin cleavable N-terminal polyhistidine-Tag for purification. Induction of the gene of interest is initiated by addition of isopropyl β -D-1 thiogalactopyranoside (IPTG).

The *E.coli* strain BL21(DE3)pLysS was used to express the *S.coelicolor* FtsZ . Initial expression trials were successful, a range of conditions including variations in temperature and inducer concentration were tested, Figure 5.3.2.

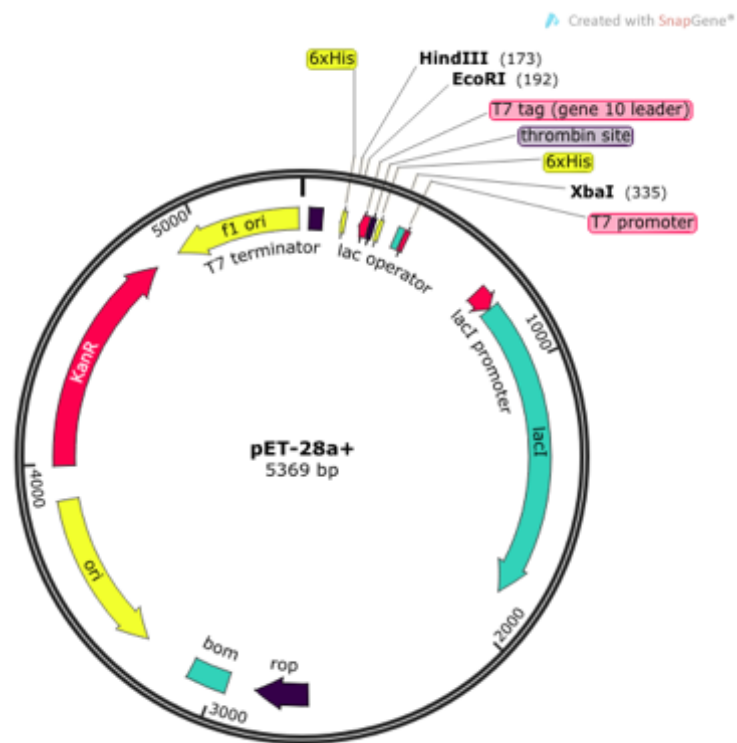


Figure 5.3.1. *pET-28a+* plasmid map. The plasmid encodes the T7 promoter, *LacI* promoter, *LacI* gene, *Ori*, and the kanamycin resistance gene *KanR* (Snapgene).

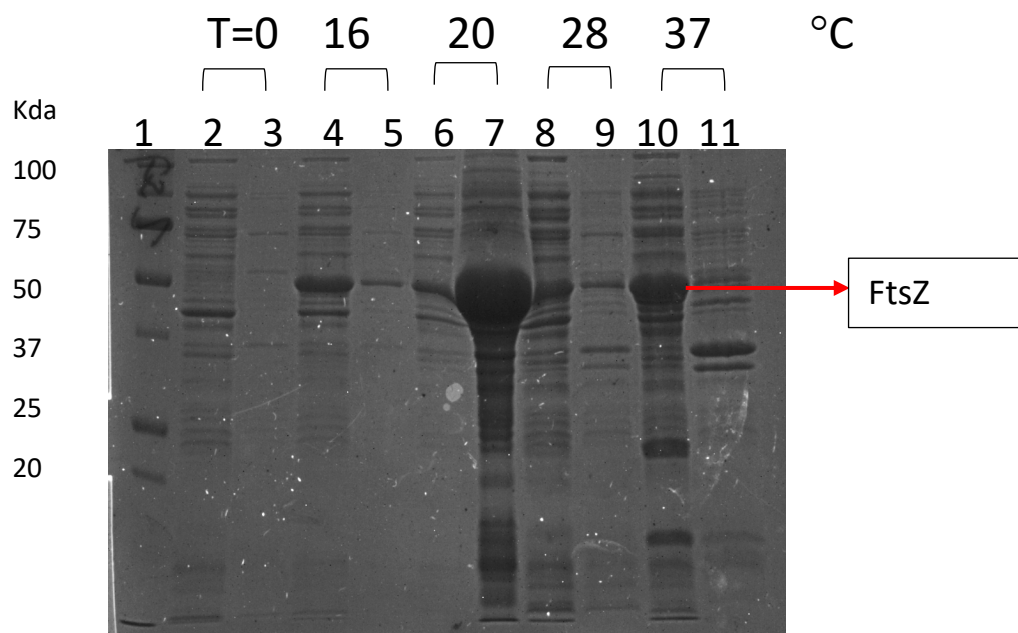


Figure 5.3.2. 15% SDS PAGE analysis of FtsZ overexpression trials using 1 mM IPTG. Lanes 2,4,6,8,10 show soluble fractions , Lanes 3,5,7,9,11 show insoluble fractions respectively. Temperatures ($^{\circ}\text{C}$) use for growth are shown above. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1. Stained with Coomassie blue. Lane 2 and 3 shows expression level before induction.

5.3.2 Purification of His-FtsZ

To purify FtsZ the *ftsZ* gene was cloned into the *NdeI* and *EcoRI* sites of pET28a to generate a N-terminally His-tagged FtsZ protein with a molecular weight of 43.3kDa and a PI of 4.73. The production of FtsZ was started using a single colony of BL21(DE3)pLysS carrying pET28a:FtsZ which was inoculated into 10mL LB containing Kanamycin. The culture was grown overnight at 37°C and subcultured into 500mL of LB containing Kanamycin and grown for 3.5 hours at 37°C prior to induction with 1mM IPTG. After induction cells were grown at 28°C Cells were collected 20 hours after induction by centrifugation and washed with before being resuspended in 30 mL Non-denaturing buffer (Tris 50mM, 300mM NaCl, 10mM Imidazole, 10mM MgCl₂ pH8). The cells were lysed using two cycles of a high-pressure French press at 1000 psi and centrifuged again. The resulting clear lysate was loaded onto a Ni-NTA column and purified using an Amersham AKTA FRC FPLC machine. Fractions containing

purified proteins were detected using 280 nm UV absorbance and identified using 12% SDS-PAGE, *Figure 5.3.2.1.*

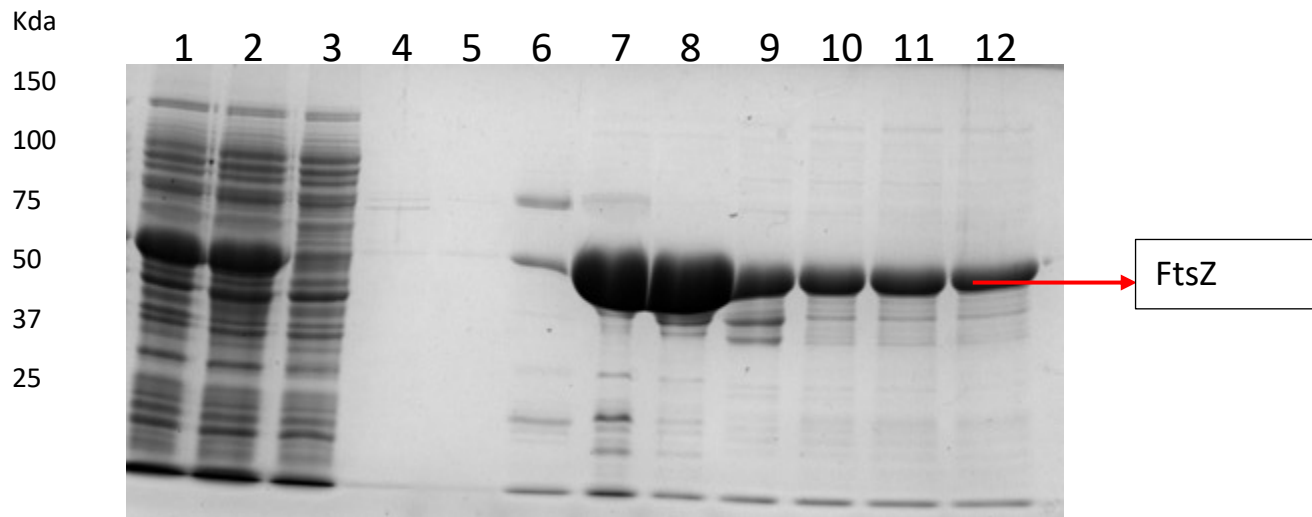


Figure 5.3.2.1. 12% SDS PAGE analysis of His-FtsZ purification. Lanes 1-12 show Pre Load, Flow Through, Wash 1, Wash 2, elutions 1- 8.

5.3.3 Dialysis of FtsZ

Protein elutions of 1mL were dialysed against 500mL HEPES buffer (50mM HEPES, 50mM KCL, pH 7.2) and soluble protein was achieved after completion and detected at expected molecular weight 43.3KDa, *Figure 5.3.3.1.*

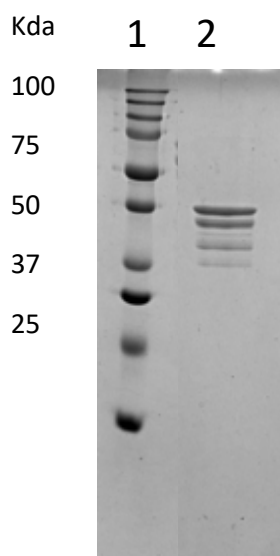


Figure 5.3.3.1. 12% SDS PAGE analysis of His-FtsZ Dialysis in HEPES buffer (50mM HEPES, 50mM KCL, pH 7.2) . Lanes 1 and 2 show Bio-Rad Precision Protein Plus Molecular Weight Marker and His-FtsZ dialysed.

5.4 Generation of His-FtsZTR

A truncated version of *FtsZ* (*FtsZTR*) was generated and cloned into pET28a. The protein would consist of the c-terminal region where regulators bind *FtsZ* but be lacking the n-terminal region. A lack of n-terminal region is suspected to stop polymerisation. This theory can be tested using *FtsZTR*. The *FtsZ* Truncated PCR1 fragment was amplified using 4A10 cosmid. The primers *FtsZ* trunc Xba Nde Forward and *FtsZ* UT Reverse were used for *FtsZ* Truncated PCR1 to produce a fragment without a stop codon. *FtsZ* Truncated PCR2 was generated using primers *FtsZ* trunc Xba Nde Forward and *FtsZ* UTC Reverse to produce a construct with a stop codon as the polyhistidine tag would be fused to the N-terminus of *FtsZTR*. All PCR products were digested with *EcoRI* and *XbaI* before loading onto a 0.7% preparative agarose gel. Bands corresponding to the expected sizes of 228bp were excised and the DNA purified using QIAQuick gel extraction Kit, *Figure 5.4.1.1*. The purified DNA was analysed on a 0.7% agarose gel to confirm the quantity of the recovered fragment. The predicted length of truncated *FtsZTR* gene fragment of 294bp.

5.4.2 Ligation

FtsZTR was cloned into the *NdeI* and *EcoRI* sites of pET28a to generate a N-terminally His-tagged FtsZTR using ligation. The ligation mix was transformed into DH5 α competent cells and transformants were selected using LB agar plates supplemented with ampicillin or kanamycin. Each plate was incubated overnight at 37° C, producing colonies in a range of 20-100.

5.4.3 Colony PCR to confirm transformants carrying the correct inserts

Individual colonies were streaked onto grid lined plates and grown overnight at 37° C. The colonies were screened using colony PCR to confirm successful ligations of the plasmid with the FtsZTR gene. To amplify the gene FtsZ trunc Xba Nde Forward and FtsZ UTC Reverse were used. The PCR samples were analysed on 0.7% agarose gels at 50 V, alongside a positive control, to confirm all the screened colonies for successful transformation of a plasmid with correct gene insert, *Figure 5.4.1.1*.

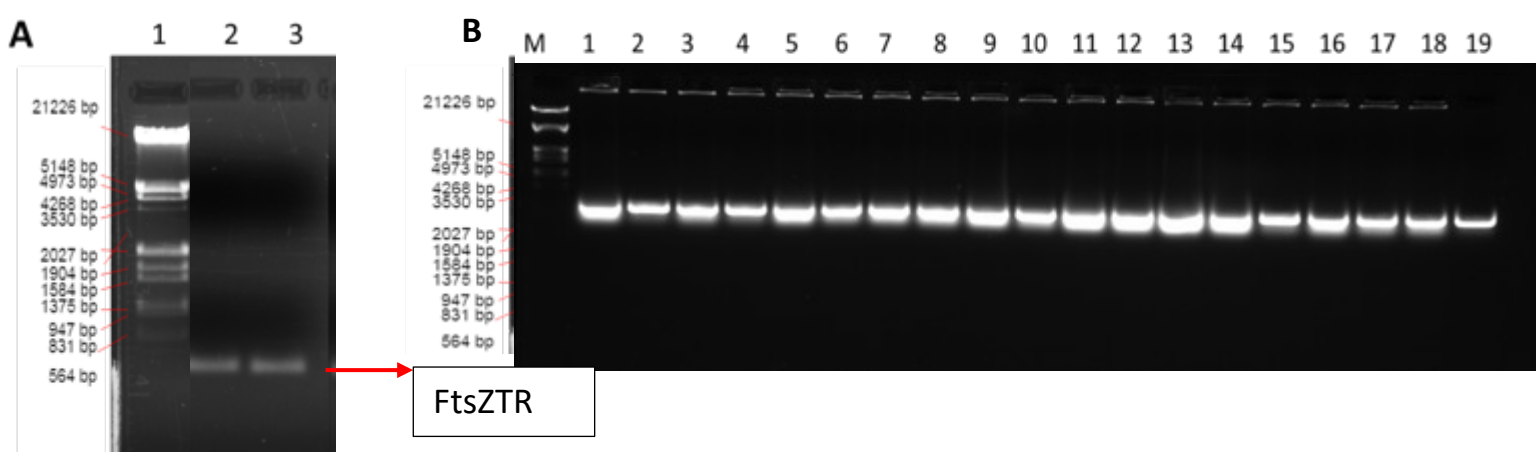


Figure 5.4.1.1. Confirmation of the generated PCR product by gel electrophoresis. (A) Lane 1, λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left. Lanes 2 and 3, FtsZTR Truncated PCR1 and PCR2 product. (B) Gel analysis of colony PCR for FtsZTR. Lane M contains λ ladder digested with *EcoRI* and *HindIII* as a size marker. Sizes of λ DNA fragments are indicated on the left and colony numbers are labelled for each lane 1-19 contains individual colonies of pET28a:FtsZTR.

The *E.coli* strain BL21(DE3)pLysS was used to express the *S.coelicolor* FtsZTR . Initial expression trials were unsuccessful, a range of conditions including variations in temperature and expression strain were tested, *Figure 5.4.1.2*.

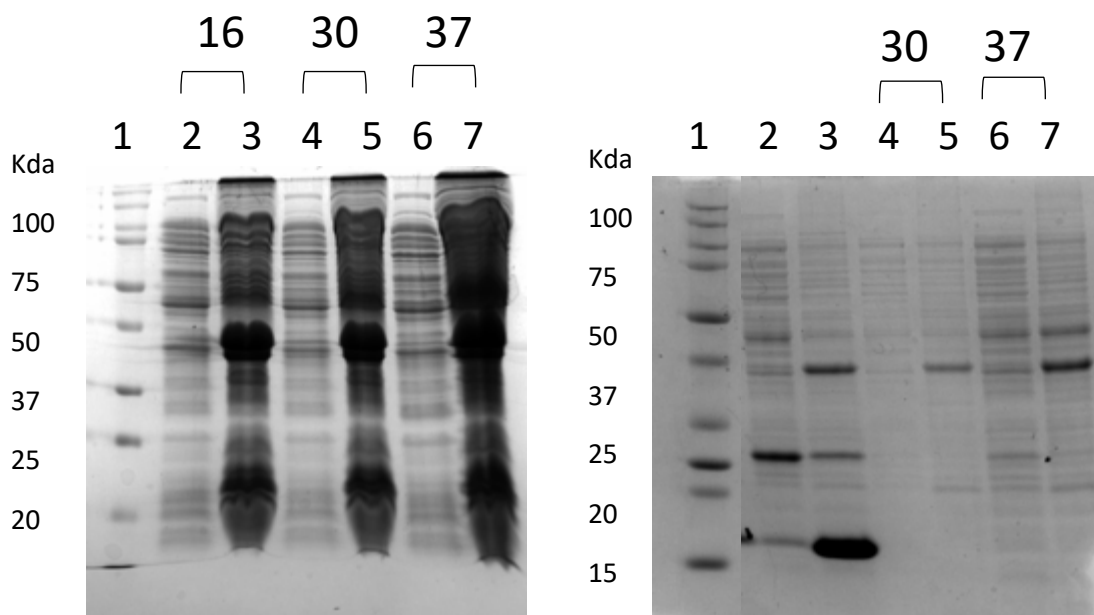


Figure 5.4.1.2. (Left) 12% SDS PAGE analysis of FtsZTR overexpression trials in BL21 using 1 mM IPTG. Lanes 2,4,6 show soluble fractions , Lanes 3,5,7, show insoluble fractions respectively. Temperatures (°C) use for growth are shown above. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1. Stained with Coomassie blue. (Right) 12% SDS PAGE analysis of FtsTR overexpression in BL21 Rosetta2. Lanes 2,4,6 show soluble fractions , Lanes 3,5,7, show insoluble fractions respectively. Temperatures (°C) use for growth are shown above. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1. Stained with Coomassie blue. Lane 2 and 3 shows overexpression of SepFTR.

Overexpression of FtsZTR in BL21(DE3)pLysS produced no clear protein band in a 12% SDS PAGE analysis of a protein extract from *E.coli* BL21 which had been transformed with pET28a:FtsZTR. This suggests the protein had no His-Tag or had been degraded by proteolysis. A further overexpression trial was tested in BL21 derivative Rosetta 2 which supplies the tRNAs required to translate the rare codons (Nouri et al, 2016). A control was loaded onto the gel of pET28-SepFTR overexpression in the Rosetta 2 strain which showed a clear band. No bands were detected at the expected 8.44Kda for FtszTR. This suggests the protein either is missing a His-Tag, was subjected to proteolysis or the band is too small to be detected.

5.5 FtsZ Polymerises in the Presence of Mg alone

The exact mechanism of FtsZ polymerisation is still unknown but Mg ions and GTP are thought to be required. To attempt to elude the dynamics of the polymerisation of *S.coelicolor* FtsZ protein, cell pelleting assays were conducted. The theory behind cell pelleting assays is that a protein is incubated with required co factors or on its own for a set time period and temperature. After which, ultracentrifugation separates polymers into the insoluble (pellet) fraction. Several conditions were set up to investigate the role of Mg and GTP in the polymerisation of FtsZ *in vitro*. Reactions were set up with His-FtsZ, His-FtsZ and GTP, His-FtsZ and Mg and His-FtsZ with both Mg and GTP. Samples were incubated at 30°C for 20 minutes in HEPES buffer with the addition of DTT 2-Mercaptoethanol, prior to centrifugation at 105,000g at 4°C. The supernatant was separated, and the cell pellet resuspended in the reaction buffer. All fractions were analysed using 12% SDS-PAGE, Figure 5.5.1.

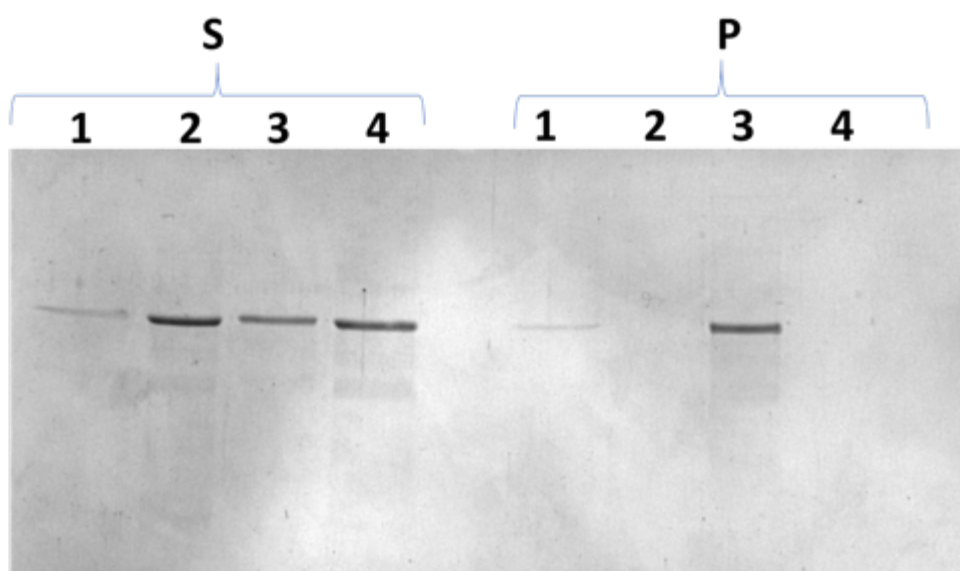


Figure 5.5.1. 12% SDS PAGE analysis of His-FtsZ pelleting assay. Lanes 1-4 show FtsZ with MgCl₂, GTP (1) GTP (2) MgCl₂ (3) and FtsZ alone (4). S and P represent soluble and pellet fractions respectively.

The pellet assay confirms that no polymerisation is detected when FtsZ is lacking cofactors. This is expected as FtsZ from *B. subtilis* has been shown to require Mg²⁺ ions and GTP *in vitro* to form oligomers (Gómez-Puertas *et al*, 2009). Interestingly the addition of GTP without Mg²⁺ did not promote FtsZ filament formation. This could be explained by the fact that FtsZ requires Mg²⁺ ions for GTPase activity. *S.coelicolor* seems to share the ability to self-activate itself in the presence of Mg ions with

B.subtilis (Andreu et al, 2004). In fact, the greatest amount of pelleting was achieved when FtsZ was incubated with Mg ions only. This is surprising as only a small level of polymerisation occurs when both Mg and GTP are present with FtsZ. This suggests GTP hydrolysis is not essential for the polymerisation of FtsZ and perhaps it is required for the regulation of the dynamics of FtsZ filaments. Pelleting assays assume the cell pellet is due to a high order structure of a protein. However, from the pelleting assay alone it is not possible to distinguish the exact type of structure which might have formed, whether that be a dimer, oligomer or polymer. It is also possible that the protein has formed a precipitate due to the composition of the buffer.

5.6 Summary

In, this Chapter we aimed to investigate FtsZ of *S.coelicolor* to probe into its function, structure and role in the bacterium. We initially performed bioinformatics of the protein using numerous software and this included a multiple sequence alignment of the genome to FtsZ in other bacterium. We found the *S.coelicolor* FtsZ shares the amino acid residues required for the binding of regulators with the *B.subtilis* protein. Futhermore a predicted structural model was generated to suggest the tertiary fold of the protein.

We then set out to purify FtsZ from *S.coelicolor* using the heterologous host *E.coli*. The *ftsZ* gene was successfully cloned into a pET28a construct for purification and the production of the protein was optimised to find the ideal conditions for large scale overproduction. FtsZ was purified using a Histidine tag via metal affinity chromatography from solution under native conditions. This was followed by dialysis of the protein into buffers which kept FtsZ stable for future assays.

Next the oligomerisation and polymerisation of FtsZ was investigated using a pelleting assay. This technique allowed us to show that FtsZ forms oligomers in the presence of GTP with the addition of MgCl₂. FtsZ is also able to form higher order assemblies when GTP is absent but Mg ions are still present. In fact, our assay suggests there is a greater level of FtsZ polymerisation when FtsZ has only Mg ions in the reaction buffer compared to Mg and GTP. This indicates that GTP has a negative effect on filament formation. These results support previous research where FtsZ has self assembled with the addition of Mg and GTP hydrolysis has led to the disassembly of oligomers.

The research carried out in this chapter has limitations. The bioinformatics included suggests FtsZ of *S.coelicolor* has the amino acid residues for binding regulators like SepF but this needs to be confirmed further using mutation studies whereby a version of the FtsZ protein is expressed that has

substituted residues and it would be subjected to binding studies with SepF2. Furthermore, the pelleting assay used to investigate the oligomerisation of FtsZ needs to be confirmed further with microscopic analysis of the polymers as the pellets obtained may be protein aggregates rather than FtsZ filaments.

6 SepF Structural Analysis

6.1 Introduction

The cell division protein SepF was first identified in *B.subtilis* as a membrane bound stabilising protein, which complements FtsA in the correct positioning of the divisome co-ordinator FtsZ. The divisome complex forms at the mid- cell due to the polymerisation of FtsZ into a Z-ring. It is regulated by a number of proteins with positive regulators bind to the C-terminus of FtsZ; an important region for binding modulators of its activity (Buske *et al*, 2012). This includes the actin homolog FtsA; a transmembrane protein which is found conserved amongst Gram - negative bacteria. In *E.coli* FtsA is essential for the positioning and polymerisation of FtsZ into Z-rings; whereas *B.subtilis* are still capable of forming septa when FtsA isn't present due to the complementation by SepF (Harry *et al*, 2005., Lutkenhaus *et al*, 2002). It is now known that SepF homologs are present in other bacterium, including actinomycetes and archaea, and are essential for their septum formation.

Celik *et al*, solved the crystal structure of the *B.subtilis* SepF C-terminal domain and found a single monomer forms a tight dimer with a β -sheets at the interface and α -helices on the exterior of the dimers facilitating interactions (Celik *et al*, 2013). Further studies have suggested the C-terminal end of SepF is essential for FtsZ binding. Bezouwen *et al* found two mutations at A98 and F124 of the SepF C-terminus of *B.subtilis* which prevent SepF associating with FtsZ. This was supported by Celik *et al* when Error-prone PCR was used to isolate 10 mutants all with individual mutations in their C-terminal domain. All SepF mutants were unviable when introduced into FtsA null mutants of *B.subtilis* (Celik *et al*, 2013).

A recent study has further supported the interaction of FtsZ and SepF; as a small peptide fragment of FtsZ was co-crystallised with SepF (Alzari *et al*, 2019).. The SepF and FtsZ proteins of *Corynebacterium glutamicum* were crystallised as a complex using the C-terminus of SepF and a ten peptide region of the C-terminus of FtsZ. The structure revealed a homodimer of SepF, consisting of a central four-stranded β -sheet where two FtsZ peptides bound in pockets. This suggests a SepF dimer helps to polymerise FtsZ by holding two FtsZ monomers in close proximity. Furthermore, a comparison of EM images of FtsZ alone and FtsZ in the presence of SepF demonstrated the promotion of FtsZ polymerisation by SepF (Alzari *et al*, 2019).

Archaea divide by binary fission and lack many divisome components except FtsZ. The only membrane tethering protein appears to be SepF homologs. Pende *et al*, solved the crystal structure

of the *Methanobrevibacter smithi* (*M.smithi*) SepF alone and in complex with the C-terminal of FtsZ (FtsZCTD). SepF forms a dimer consisting of a 5 stranded β -sheet with 2 α -helices on the exterior, similar to that found in *B.subtilis*. SepF interacts with the C-terminal of FtsZ using specific residues Arginine 130, Lysine 115 and Arginine 118. Despite the evolutionary distance between bacteria and archaea, the SepF-FtsZ interaction is highly conserved (Pende *et al*, 2021).

Actinomycetes, including *Streptomyces* do not use the membrane anchoring proteins FtsA or ZipA found in other bacteria to stabilise FtsZ. However, their genomes do contain potential *sepF* genes, with some species having more than one SepF homolog. These proteins could carry out the role of tethering the Z-ring to the membrane. Furthermore, many of those bacteria that have SepF homologs have at least one which is encoded in the cell division gene cluster, near to *ftsZ*. A study into the *M.tuberculosis* SepF has indicated its importance in septum formation (Casonato *et al*, 2015). Deletion of the *sepF* gene led to a complete block of cell division with the bacteria hence was lethal (Casonato *et al*, 2015). A more recent study has confirmed SepF promotes the polymerisation of FtsZ in *M.smegmatis* and a single point mutation of a Glycine residue 51 rendering it incapable of stabilising FtsZ (Bhattacharya *et al*, 2018). Both independent studies suggest SepF is essential for the proper formation of the Z-ring in *Mycobacterium*.

S.coelicolor has an elaborate life cycle which resembles that of a filamentous fungi and so essential events are highly regulated by a plethora of proteins and pathways. Throughout its life cycle two separate forms of growth occur; vegetative and polar with these being coordinated with cell division. FtsZ is not essential for the viability of *S.coelicolor* as it is only required for the late stages of life where sporulation septation occurs. *S.coelicolor* encodes three potential SepF homologs with one being within the essential cell division cluster.

In previous chapters, we have confirmed SepF2 and a truncated version consisting of the C-terminal domain SepFTR interacted with FtsZTR using bacterial two hybrid assays. We have also seen SepFTR is capable of forming higher order assemblies in stabilising buffer when viewed using TEM Yet the three dimensional structure of SepF2 or SepFTR have not been solved.

In this chapter, we present an attempt to uncover the structure of SepFTR using biochemical techniques and protein crystallisation trials.

- I generate a protein model of SepFTR from *S.coelicolor*.
- I optimise the stabilisation of purified SepFTR into dimers.
- I prepare the protein for crystallisation trials.

6.2 SepFTR Protein Model

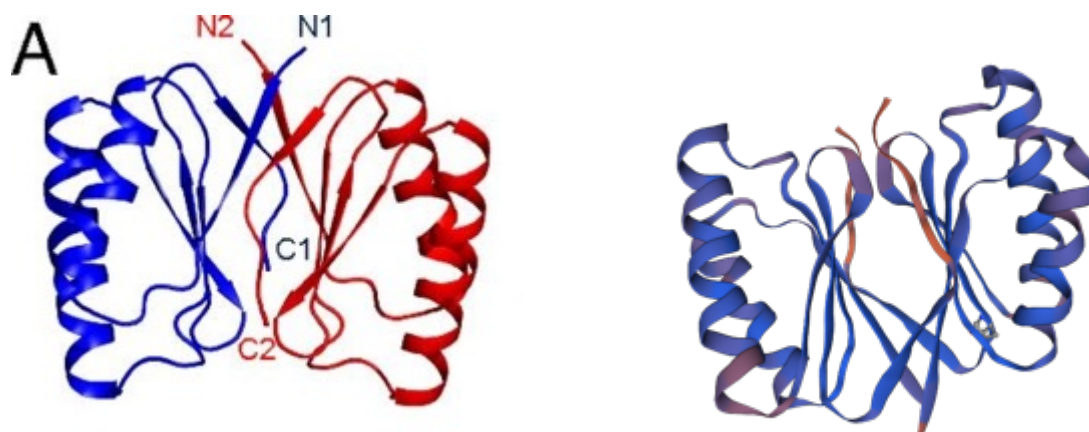


Figure 6.2.1. (Left) Crystal Structure of *B.subtilis* SepF residues 61-140. (Right) Three dimensional ribbon diagram of a model of SepF2TR from *S.coelicolor*. Shown are beta-sheets, alpha helices of a predicted homo-dimer. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 3zii.

A protein model of SepFTR from *S.coelicolor* was created to predict the three dimensional protein structure of the protein using the software Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>). The sequence of *S.coelicolor* SepF2 C-terminal (residues 61-213) was input in FASTA format (Uniprot Q9S2X2) and a template protein of the c-terminus of SepF from *B.subtilis* (PDB: 3zih) was used. Both proteins share 32.97% sequence identity and a template alignment was produced, Figure 6.2.1. The software generated a 3D protein structure diagram from the alignment which shows a homo-dimer consisting of 2 alpha-helices and 5 beta-sheets per monomer. Interestingly SepFTR is predicted to form a homo-dimer, like *B.subtilis* SepF via β -sheets at the interface. There is also a Glycine residue at position 109, which has been shown to be essential for polymerisation and ring formation (Celik et al, 2013). This suggests SepF2 may be capable of forming a polymer like *B.subtilis* SepF by α -helices on the exterior of the dimers facilitating interactions.

The protein model data shows the model consists of amino acid residues from 108 to 201, therefore is missing the residues of the N-terminal. The alignment data shows the model of SepF from *S.coelicolor* shares the beta-sheets and alpha helices from SepF from *.subtilis* (PDB: 3zih), Figure 6.2.2.

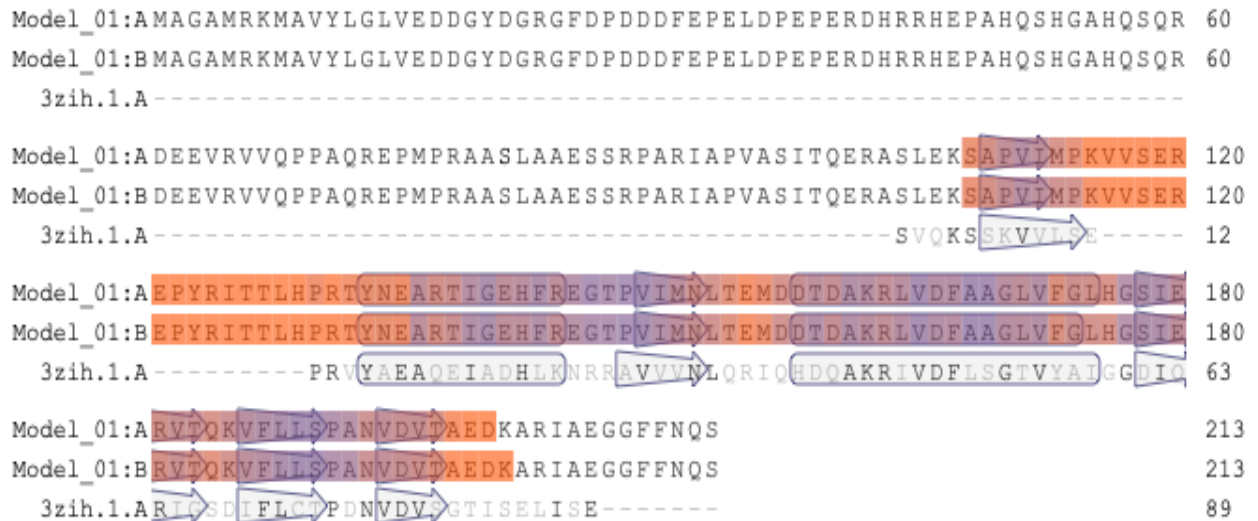


Figure 6.2.2. Model alignment data of a model of SepF from *S.coelicolor*. Shown are amino acid alignment from the Model (model_01) and SepF from *B.subtilis* (PDB: 3zih) beta-sheets are shown as arrows and alpha helices as rings around the residues. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 3zih.

The FtsZ binding site of SepF in *Archea* has been identified and is made up of residues K115, R118 and R130 (Pendel et al, 2021). The protein model of SepF from *S.coelicolor* also has region with two of the residues conserved, which suggests FtsZ binds there, Figure 6.2.3.

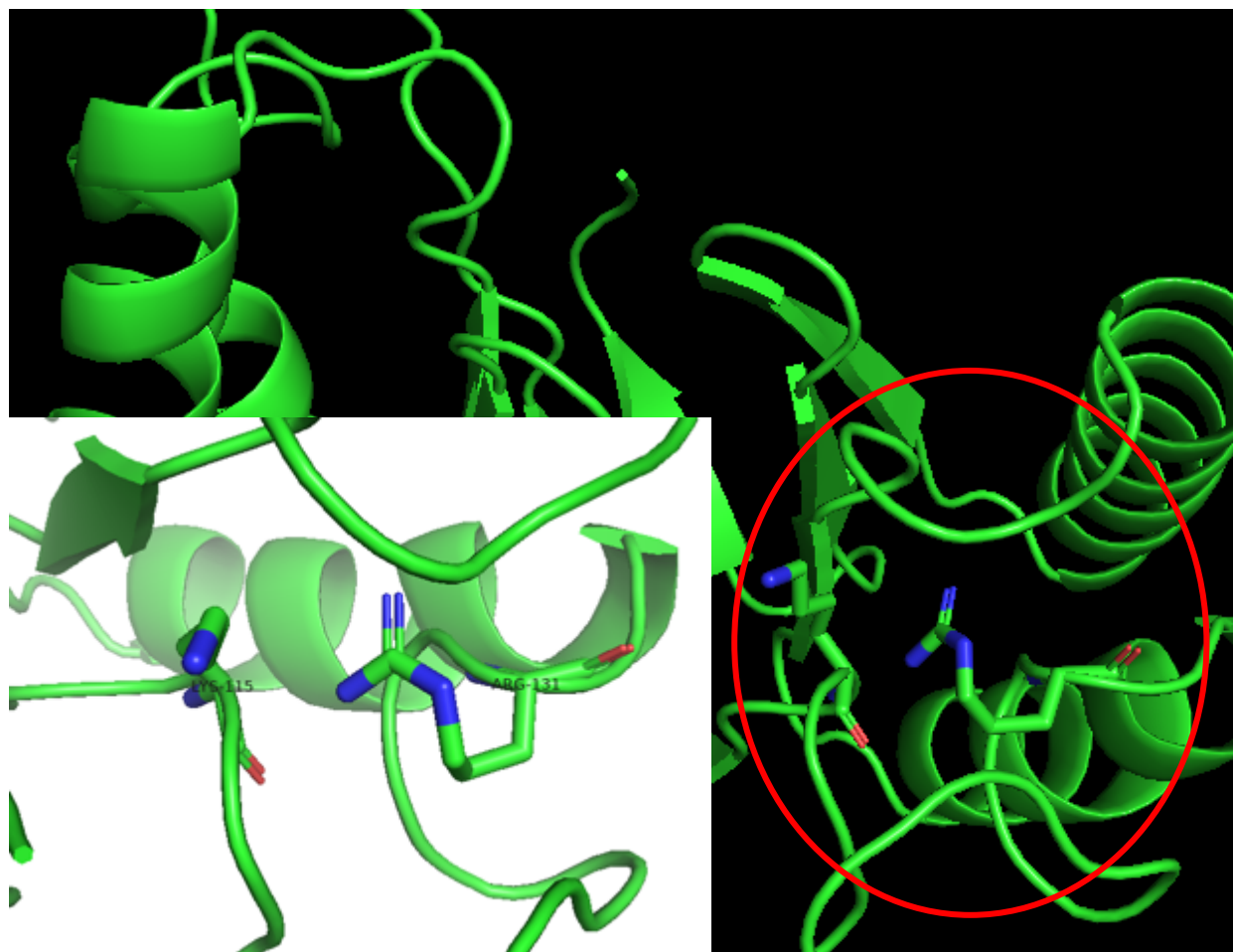


Figure 6.2.3. Three dimensional ribbon diagram of a model of SepF from *S.coelicolor*. Shown is the FtsZ binding site (red circle) of the Model (model_01) created using SepF from *B.subtilis* (PDB: 3zih) Inserted is the residues that make up the FtsZ binding site, K115, R130. Generated using Swiss-Model (<http://swissmodel.expasy.org/interactive/5qdMb6/models>) and PDB 3zih.

6.3 Optimisation of SepFTR Single order assembly

In the previous chapter, SepFTR was shown to form a large polymer when in stabilising buffer (50mM Tris-HCL, pH 8, 50mM NaCl, 10% glycerol, 0.4M L-arginine and 2% glycine). To be able to perform crystallisation studies on SepFTR, it was necessary to find a buffer which would keep SepF2TR in a smaller subunit size of a dimer or a monomer. A look at the literature suggested SepFTR from *B.subtilis* was found to form individual rings at pH 10 which associated to form a tube at lower pH.

Therefore a buffer was designed which had a higher pH of pH 10 but still contained the composition which would keep SepFTR stable. Buffer A consisted of 50mM Tris, 50mM NaCl, 0.4M Arginine, 2% Glycine pH10. A second buffer was also tested which had been shown to be successful in the purification and crystallisation of SepFTR from *B.subtilis* (Celik *et al*, 2013). Buffer B consisted of 25mM Tris, 100mM NaCl pH 8.5, Table 6.3.1.

A 500µL of aliquot (0.05mg/mL) of purified SepFTR was dialysed against either buffer A or B. The dialysed fractions were then analysed using Native PAGE. A single band was seen on the gel representing SepFTR dialysed in buffer A, whereas multiple bands appearing as a smear on the gel were produced from SepFTR dialysed in Buffer B. This suggests SepFTR forms one type of structure in buffer A, however it is not known whether this is a monomer, dimer or higher order assembly, Figure 6.3.1.

Table 6.3.1. Comparison of the Buffers used to dialyse SepF2TR.

Condition	Buffer	Volume of protein solution	Composition Of Fraction	precipitation	% Recovered
A	50mM Tris-HCL 50mM NaCl 10% glycerol 0.4M L-arginine 2% glycine pH 10	500	Protein	Yes	20
B	25mM Tris-HCL 100mM NaCl pH 8.5	500	Protein	Yes	10

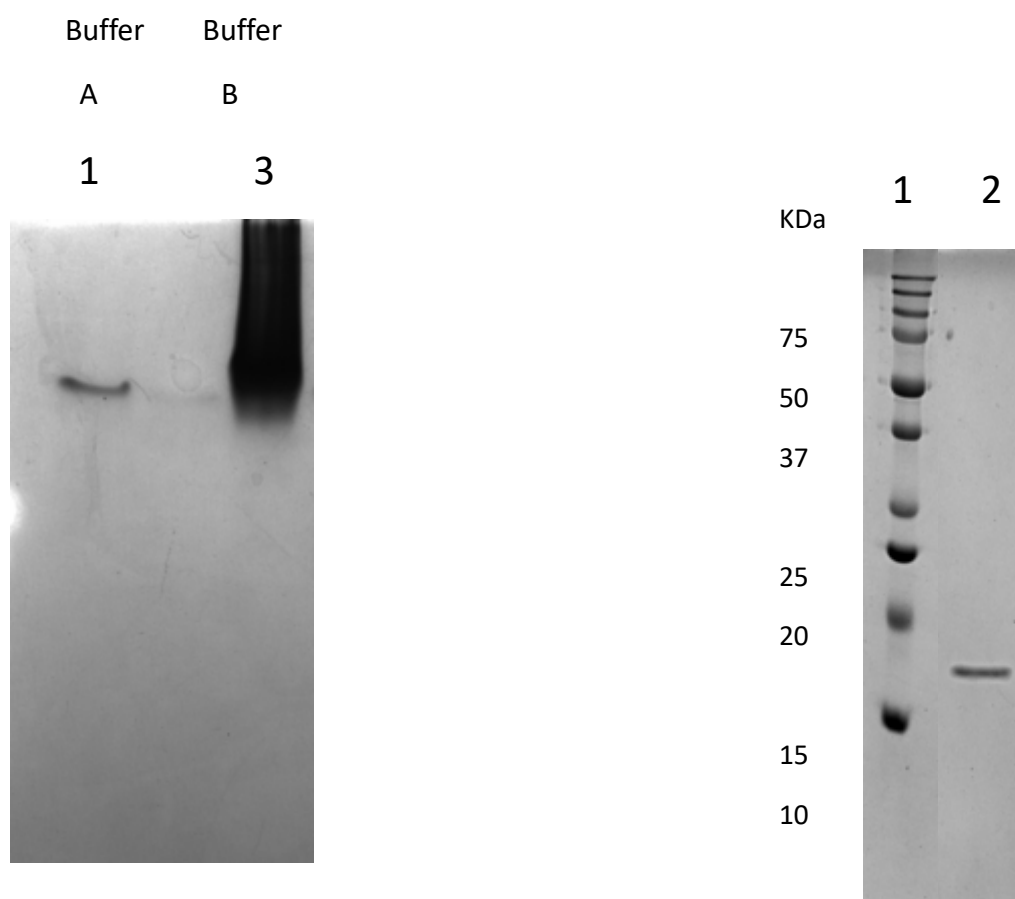


Figure 6.3.1. (Left) 15% NATIVE PAGE analysis of His-SepFTR protein dialysis trials. Lane 1 and 3 shows dialysed His-SepFTR Buffers A and B (Shown in table 6.3.1) respectively. (Right) 12% SDS PAGE analysis of dialysed His-SepFTR in Buffer A. Bio-Rad Precision Protein Plus Molecular Weight Marker in Lane 1.

To elude what structure SepFTR forms in Buffer A, Analytical Size Exclusion Chromatography (aSEC) was utilised. aSEC separates molecules based on their molecular weight. Firstly, the production of SepFTR was started using a single colony of BL21 Rosetta2 carrying pET28a:SepFTR which was inoculated into 10mL LB medium containing kanamycin and chlorophenicol. The culture was grown overnight at 37°C and subcultured into two times 500mL of LB in 2L fasks containing kanamycin and chlorophenicol, each was grown for 3.5 hours prior to induction with 1mM IPTG. Cells were collected 20 hours after induction by centrifugation before being resuspended in 30 mL non-denaturing buffer (Tris 50mM, 300mM NaCl, 10mM Imidazole, 10mM MgCl₂ pH8). The cells were lysed using two cycles of a high-pressure French press at 1000 psi and centrifuged again. SepFTR was purified under denaturing conditions using a Ni-NTA column as higher levels of insoluble proteins are produced. The resulting cell pellet was resuspended in 6mL 8M Urea Buffer and sonicated to improve the viscosity before being agitated for an hour. Following a final centrifugation to remove any sedimentation, the solution was filtered through a 0.45µm filter before being loaded onto a pre-equilibrated column. Once loaded the Ni-NTA column was washed with 8mL 8M Urea Buffer A and 8mL 8M Urea Buffer B to remove the undesired proteins from the column. The bound protein was eluted using buffer 8mL 8M Urea Buffer C and collected as 1mL aliquots labelled E1-E10. The elution's, wash fractions and

initial cell lysate was analysed on a 15% SDS-PAGE and showed eluted protein at expected size of 13.2Kda, *Figure 6.3.2*.

Ten 1mL fractions of purified His-SepFTR were dialysed against 500mL of Buffer A (Table 6.3.1.) at room temperature. After three buffer exchanges soluble protein was recovered and analysed using 12% SDS PAGE, *Figure 6.3.3*. The protein was detected at the suspected molecular weight of 13.2Kda. The protein concentration was found to be 1.1 mg/mL using a nanodrop.

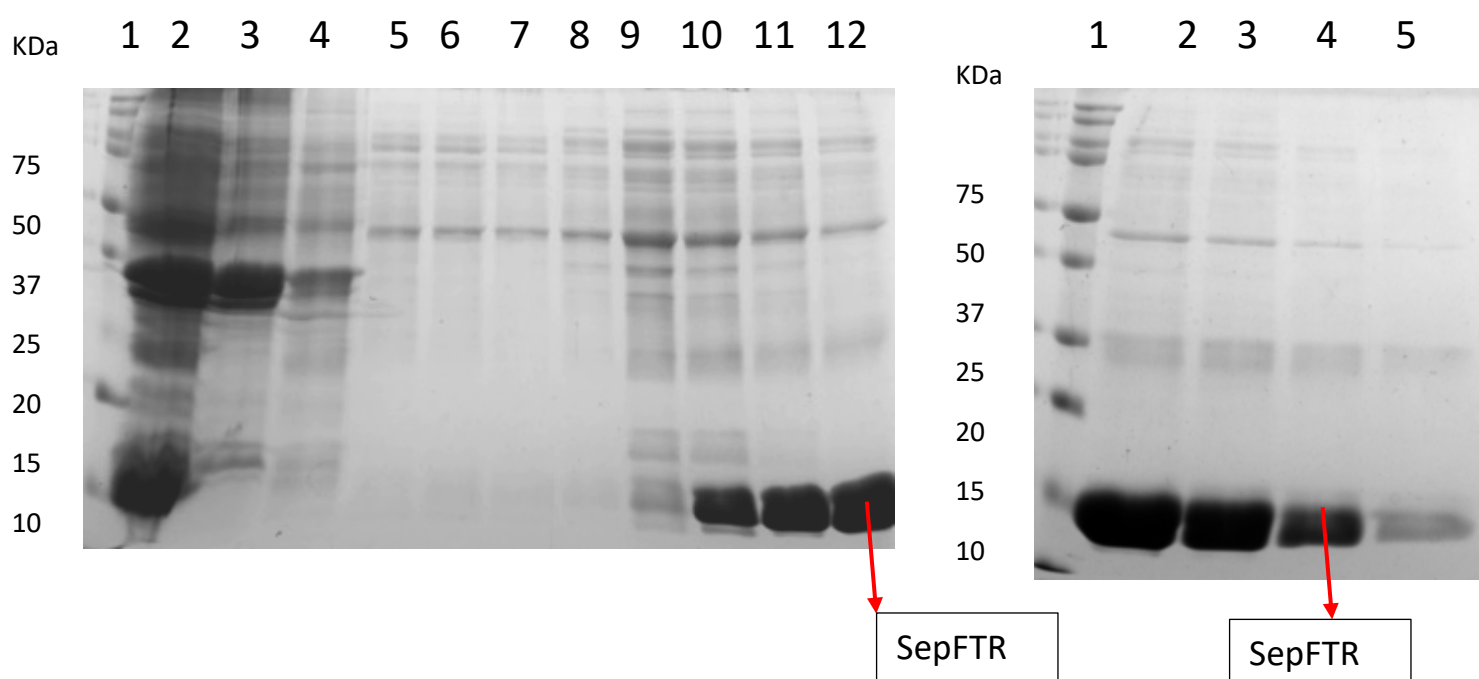


Figure 6.3.2. 12% SDS PAGE analysis of His-SepFTR protein purification. (Left) Lanes 2 - 5 shows preload, flow through, wash 1 and 2 respectively. Lanes 6 - 12 shows elutions 1 to 7. (Right) Lanes 2 - 4 shows elutions 8 to 10. Lane 11 shows a final wash. Lane 1 in both has Bio-Rad Precision Protein Plus Molecular Weight Marker.

A 1mL aliquot of the protein sample was loaded onto the analytical size exclusion chromatography column using gel filtration buffer (50mM Tris, 150mM NaCL pH 8). AKTA Pure was used to perform the aSEC flowing 0.8mL per minute and UV at 300nm was used to detect the protein.

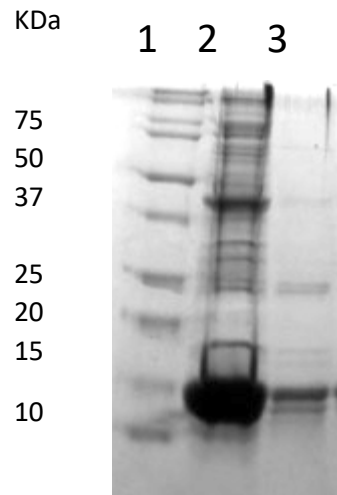


Figure 6.3.3. 12% SDS PAGE analysis of His-SepFTR after dialysis. Lane 2 shows protein before dialysis. Lanes 3 shows protein after dialysis. Lane 1 has Bio-Rad Precision Protein Plus Molecular Weight Marker.

Several peaks were observed, comparison of the peak data to a standard calibration of the column using proteins of known molecular weight, suggests the protein eluted at fraction 12 with a molecular weight of 26Kda, therefore a dimer. The collected 1mL fractions were analysed using 15% SDS-PAGE as the extinction coefficient of the protein is low at 160mAU. This suggests protein will be poorly detected by UV. The SDS-PAGE analysis confirmed fractions 8-12 contained SepFTR, Figure 6.3.4.

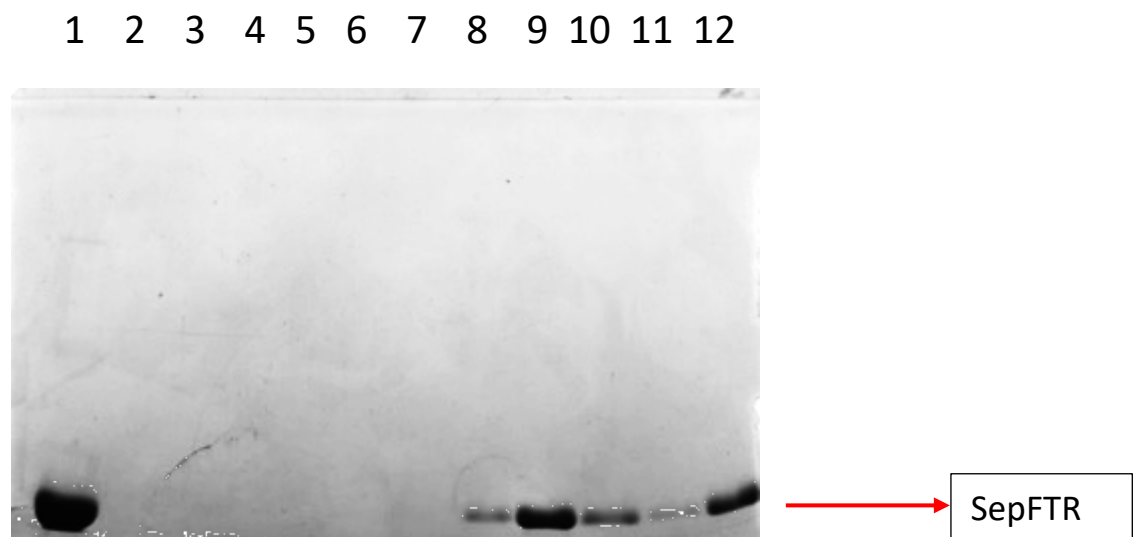


Figure 6.3.4. 15% SDS PAGE analysis of His-SepFTR after aSEC. Lane 1 shows concentrated protein before aSEC. Lanes 2 - 12 shows elutions 2 to 12.

6.4 Preparative Analytical Size Exclusion Chromatography

As the oligomeric state of SepFTR appeared to be a dimer in Buffer A (50mM Tris, 50mM NaCl, 0.4M Arginine, 2% Glycine pH10); preparative aSEC was performed to prepare the protein for crystallisation trials. The remaining 8 mL of protein solution was loaded onto an aSEC using AKTA Pure, a Superdex 75 10 300 GL column and gel filtration buffer. The gel filtration was completed three times with 2mL fractions being collected. The peak data suggests a major peak at elution 12.33 (26kDa) corresponding to a dimer structure. The other peaks were at elutions 8.54 (100kDa) and 18.51 (40Da) suggesting protein at molecular weight of a higher order assembly and a tetramer, *Figure 6.4.1*.

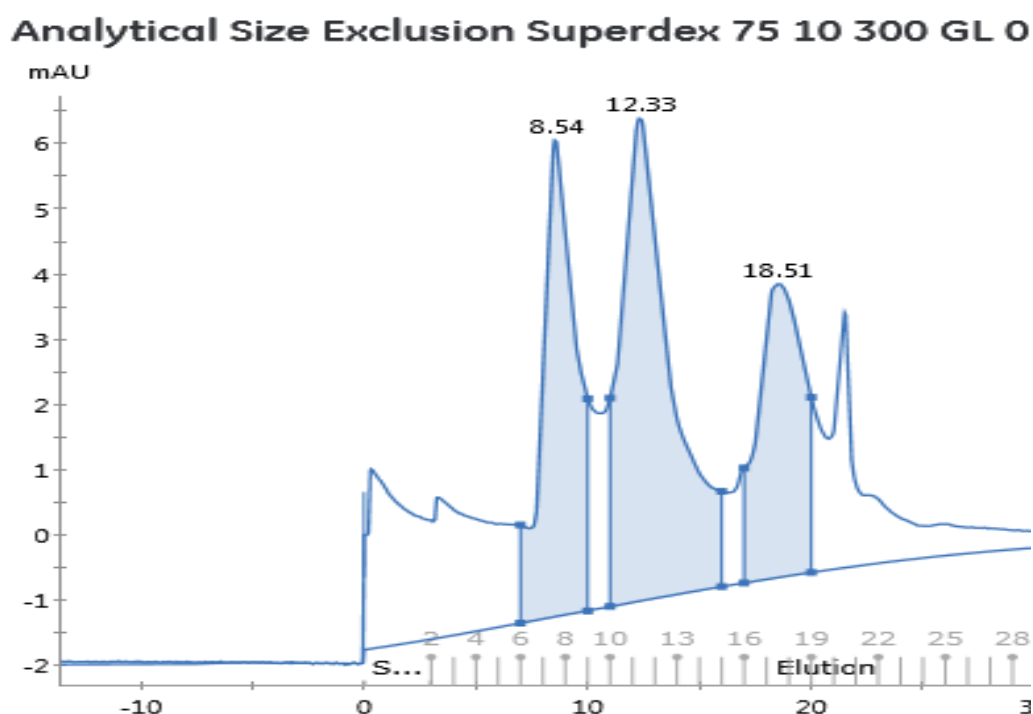


Figure 6.4.1. Graphical read out from analysis of His-SepFTR after aSEC. Major peaks are shown and highlighted in blue.

Fractions 13-16 were pooled together to yield 48mL of protein sample which was concentrated to 8.125mg/mL at a volume of 150 μ L using a 3000Da spin column concentrator. Concentrating was performed in a step wise manner, by centrifugation at 4,500rpm in 5-minute implements for a total of 3 hours. The protein concentration was tested after each 1mL reduction in volume using a nanodrop, *Table 6.4.1*.

Table 6.4.1. Volume and corresponding concentration of SepFTR.

Volume of protein solution	Concentration	Absorbance 280nm
48mL	0.375mg/ml	0.06
18mL	0.500mg/ml	0.08
2.5mL	1.063mg/ml	0.17
0.8mL	2.010mg/ml	0.34
0.35mL	3.69mg/mL	0.59
0.150mL	8.125mg/mL	1.30

Once complete saturation of the protein solution was achieved at 8.125mg/mL, crystallisation trials were set up. A small aliquot of protein was mixed with a buffer and left at 25°C for a week. No crystals were observed in the four buffer conditions tested.

6. 5 Summary

In this Chapter, we aimed to discover more about the structure of SepF from *S.coelicolor*. We initially performed bioinformatics of the protein to generate a predicted structural model which suggests the tertiary fold of the protein. SepFTR forms a homo-dimer consisting of alpha-helices and beta-sheets. The similarity of *S.coelicolor* SepF to the homolog from *B.subtilis* suggests it may be capable of forming a polymer with α -helices on the exterior of the dimers facilitating interactions.

We then set out to purify SepFTR from *S.coelicolor* using the heterologous host *E.coli*. The *SepFTR* gene was successfully cloned into a pET28a construct (Chapter 4) for purification and the production of the protein was optimised to find the ideal conditions for large scale overproduction. SepFTR was purified using a Histidine tag via metal affinity chromatography from solution under denaturing conditions. This was followed by dialysis of the protein into a buffer which kept SepFTR stable for future assays.

Next, different buffer conditions were tested to produce stable protein which was in the smallest possible oligomeric state. To confirm our buffer had resulted in single order assembly of SepFTR, we utilised analytical size exclusion chromatography. Although several peaks were observed, a major peak at fraction 12 consisted of protein with a molecular weight of 26kDa corresponding to a dimer. Finally, preparative analytical size exclusion chromatography was used to isolate the dimer form of SepFTR. The protein was then concentrated until fully saturated and subjected to crystallisation trials.

The research carried out in this chapter has limitations. Although a dimer form of SepFTR was detected using analytical size exclusion chromatography I was unable to crystallise the protein. Further reading of the literature as to what conditions were used previously to obtain crystals would help in planning for future experiments.

7 Discussion

It is still not fully known how the individual life cycle events of *Streptomyces* are coordinated. Single unigenomic spores germinate into vegetative which form a network of mycelium utilising the resources of the surrounding environment. There is a switch to aerial hyphae growth upon nutrient depletion with filamentous like hyphae that branch and grow at the poles, before sporulation occurs to produce numerous progeny. The highly complex life cycle of *Streptomyces*, involving multiple and contrasting growth and division stages is thought to involve a high degree of regulatory networks. We currently have uncovered a range of proteins which are dedicated and specific to each of the stages including growth, DNA segregation and Cell division. Yet we still do not fully understand the extent of proteins needed or the interplay between proteins involved in the separate cell cycle events (McCormick *et al*, 2009).

To date our understanding of the *Streptomyces* life cycle has come from studies of *S.coelicolor*. Many of the proteins involved are similar to eukaryotic cytoskeletal proteins. Research has mainly focused on the coiled-coil components of the TIPOC, including Scy, DivIVA and FilP which assemble at the tip of hyphae to control polar growth (Holmes *et al*, 2013). Each protein has a specific role associated to it to control apical tip extension and stabilise the growing hyphae. We know that there is cross talk between the TIPOC and DNA segregating proteins as Scy recruits the DNA partitioning protein ParA to the hyphal tips to facilitate the alignment of chromosomal DNA via its partner ParB (Ditkowski *et al*, 2013). However, we are yet to uncover how the chromosomes are uniformly placed. Furthermore, a wealth of research has highlighted the essential role of FtsZ in sporulation specific cell division. FtsZ forms a ladder arrangement along aerial hyphae to control septum formation and its deletion is not lethal (Angert *et al*, 1997). Despite our understanding of the role of FtsZ we are still unclear as to how it is placed uniformly along hyphae or how it is stabilised to form the Z-ring. A high level of information about how the genes are regulated to control the life cycle of *S.coelicolor* is known. A cascade of transcription factors including the *Bld* and *Whi* genes allow the switch between vegetative and aerial growth to occur and subsequently initiate sporulation. However, it is only recently becoming clear that regulation at a protein level occurs in addition to the regulation of genes.

As mentioned previously, the final stage in the life cycle is the generation of multiple individual uni-genomic spores which disperse into the environment. Once chromosomal DNA has been aligned the mature aerial hyphae undergo cell division to produce prespores due to the regular placement of septa. As in other bacteria, FtsZ promotes the assembly of the Z-ring to recruit the divisome components needed for cytokinesis. This is due to the FtsZ monomers forming an organised

assembly along aerial hyphae appearing as a ladder of evenly distributed z-rings (Angert *et al*, 1997). While null mutants of FtsZ are still capable of vegetative division, they are unable to perform sporulation specific division. This suggests FtsZ is crucial in the shift from vegetative to aerial hyphae formation (Buttner *et al*, 2000). The tubulin homolog FtsZ has been well characterised in other bacterium with its structure showing a GTPase domain sufficient for polymerisation into the fully formed Z-ring (Cueto *et al*, 2015). Positive and negative regulators act upon FtsZ. In the majority of bacterium, the proteins SepF, FtsA and ZipA help to position and stabilise the Z-ring correctly at the mid-cell. Despite our understanding of the importance of FtsZ in sporulation specific division within *Streptomyces*, it is still unclear to which proteins promote and regulate FtsZ polymerisation.

In this project, I attempted to uncover how the individual stages of the *Streptomyces* life cycle are co-ordinated by the communication between proteins involved. We investigated the potential interactions between proteins involved in several stages of the *S.coelicolor* life cycle including Polar growth, Cell division and DNA segregation. I also focused on the protein FtsZ which is essential for septum formation prior to the multiple fission of aerial hyphae. This included the investigation into a possible candidate protein which may stabilise the Z-ring during cell division; SepF2 (SCO2079) was discovered from analysis of the *S.coelicolor* genome.

FtsZ

I initially performed bioinformatics of the FtsZ using numerous software and this included a multiple sequence alignment of the gene to FtsZ in other bacterium. We found that the *S.coelicolor* FtsZ is highly similar to FtsZ from *B.subtilis* as it shares 65% of the gene sequence. Despite this, the region which has been shown to be important for the binding of regulators is poorly conserved. However, the amino acid residues required for the binding of stabilising proteins to the *B.subtilis* FtsZ; proline 372, isoleucine 375 for binding ZapD and the glycine 314 for binding ZipA is shared with our FtsZ (Huang *et al*, 2017., Cameron *et al*, 2021).

To further probe into the similarities between the *B.subtilis* and *S.coelicolor* FtsZ, a predicted structural model was generated to suggest the tertiary fold of the *S.coelicolor* protein. The FtsZ forms a monomer consisting of alpha helices, beta-sheets and loop regions. The loop regions of FtsZ in *B.subtilis* have shown to be important in the GTPase and subsequent polymerisation activity of the protein. It is unknown if the predicted loop regions of *S.coelicolor* FtsZ could also be implemented in the polymerisation mechanism. The GT motif which is required for nucleotide binding is conserved between the two proteins and additionally the monomer has two domains linked by a central helix which is also shared between both FtsZ proteins. Furthermore, the 'synergy' loop region identified in *E.coli* made up of residues N207, D209 and D212 is conserved in *S.coelicolor* (Erickson *et al*, 2001).

To better understand the structure of FtsZ we decided to purify the protein using *E.coli* cells as a host. An overexpression construct was generated using the FtsZ gene and pET28a as an expression vector. Once the optimal conditions for the production of soluble FtsZ was determined, it was overproduced to a large scale and purified using nickel affinity chromatography. After purification FtsZ was dialysed in 500mL HEPES buffer (50mM HEPES, 50mM KCL, pH 7.2) to allow biochemical analysis.

FtsZ is a Tubulin homolog with GTPase activity regulating its oligomerisation and dynamics. Previous work has highlighted a contrast between the polymerisation methods of FtsZ to Tubulin; as FtsZ requires dimerisation to form the GTPase active site whereas Tubulin exists as a heterodimer. Numerous studies have disputed the polymerisation of FtsZ; some concluding that GTPase activity is required while others have suggested nucleotide hydrolysis promotes depolymerisation. Furthermore, FtsZ has been shown to self-associate without GTP into oligomers, but it is unclear as to whether these can form a higher order structure. The discrepancies mean the formation of the Z-ring due to FtsZ assembly is poorly understood. However, it is clear that GTPase activity is Mg ion dependent and high levels of KCL increase nucleoside hydrolysis (*Gómez-Puertas et al, 2009*). The current view is that GTP binding promotes polymerisation and its subsequent hydrolysis is required to regulate the dynamic behaviour of the Z-ring as protofilament disassembly occurs upon pi release, known as treadmilling (*Loose et al, 2014*).

To try to uncover a clearer picture of FtsZ dynamics and oligomerisation I utilised a basic cell pelleting assay. Several conditions were set up to investigate the role of Mg and GTP in the polymerisation of FtsZ *in vitro*. As expected FtsZ was unable to oligomerise on its own and required co factors. The FtsZ from *S.coelicolor* was able to self-associate in the presence of Mg ions alone; supporting the research into the *B.subtilis* FtsZ. The addition of GTP to FtsZ led to no polymerisation without Mg. This could be due to GTPase activity requiring Mg ions, which has been shown previously. Interestingly, the greatest amount of cell pellet was generated when FtsZ was incubated with Mg alone. One possible explanation for this could be that the binding of Mg induces a structural change which allow oligomerisation of FtsZ monomers. The binding of GTP could be required as a regulation of the polymerisation process. As a cell pelleting assay is basic it is difficult to interpret the results and much more research needs to be conducted.

Interactions between Proteins involved in Cell division, Polar growth and DNA Segregation

Next I wanted to gain a better understanding of the role of FtsZ in the cell division and life cycle of *S.coelicolor*. This would give a context to the proteins interactions, regulation and function within the bacterium. The bacterial two hybrid assay BATCH was utilised to confirm known protein interactions and uncover previously unknown protein partners. FtsZ is a key protein in sporulation specific cell division within *Streptomyces* as it forms the Z-ring to mark the septum site. Previous research has shown the *Streptomyces* specific SALP protein SsgB recruits and positions FtsZ for Z-ring initiation. This interaction was confirmed with several tested orientations of the proteins which validates the reliability of the assay (Borst *et al*, 2011). Despite this well-established protein interaction, very little is known about how FtsZ is regulated in *Streptomyces*. One particular poorly understood process is the stabilisation of the Z-ring. Several proteins have been identified in other bacterium; including ZipA and FtsA, which anchor FtsZ to the cell membrane. The *S.coelicolor* genome lacks potential ZipA and FtsA proteins but encodes for three homologs of SepF; a membrane anchoring protein in *B.subtilis*. The gene of *SepF2* (SCO2079) is located in a conserved position within the division and cell wall (dcw) gene cluster between *ftsZ* and *DivIVA*. Therefore, interactions between FtsZ and *SepF2* were investigated. FtsZ was able to bind a truncated version of *SepF2* consisting of the C-terminal domain only. This suggests *SepF2* may be capable of attaching FtsZ to the cell membrane but the N-terminal region inhibits the interaction. This is an exciting result as cell division in *S.coelicolor* is more complex than other bacterium due to the formation of multiple Z-rings upon an aerial hyphae. Therefore, the placement, stabilisation and regulation of FtsZ is essential. Here we have shown that the FtsZ of *Streptomyces* may also be stabilised in a similar way to other bacterium despite the differences in cell division.

To further analyse the polymerisation of FtsZ, self oligomerisation of the protein was tested. As mentioned earlier, previous research has shown that FtsZ can self associate in other bacterium and the cell pelleting assay suggested the *S.coelicolor* can do the same. However, no oligomerisation of FtsZ was detected. This may be accounted for by the fact the FtsZ construct used was a truncated version which lacks the N-terminal domain which has been shown to be crucial for polymerisation in *Staphylococcus aureus* (Andreu *et al*, 2017).

To gain a better understanding into the proteins which are likely to be important for cell division, more self-oligomerisation tests were performed. The self-association of SsgB and *SepF2* were analysed. SsgB was able to interact with itself which validates previous research. Interestingly, the truncated *SepF2* construct was capable of self-binding, this suggests the structure of the *SepF2* in *S.coelicolor* is similar to that of *B.subtilis* as the C-terminal domain has been shown to be essential for

the polymerisation of SepF in *B.subtilis* (Celik et al, 2013). These results suggest oligomerisation of cell division proteins is important in the execution of the process. We performed more BTH assays to create a better picture of how the cell division proteins SsgB, FtsZ and SepF2 cross-talk. Interestingly, SsgB was able to bind to SepF2; this suggests the life cycle event of cell division in *Streptomyces* has a complex network of protein communication. However, it is unclear as to what role the protein pair may play in the process.

Recently, it has become apparent that there is not only cross communication between proteins involved in each stage of the life cycle, but proteins involved in each stage cross-talk to others which are essential in another stage. ParA which is needed to segregate the chromosomal DNA interacts with the main orchestrator of the TIPOC, Scy (Ditkowski et al, 2013., Chater et al, 2005). Our research suggests this link between stages is more complex than previously thought. Kelemen et al, have found another Par like protein ParH which has an effect on septum placement prior to sporulation. Interactions were tested between ParH and several other proteins involved in Cell division. ParH was able to bind with SsgB and SepF2 but the same was not found for ParH with FtsZ. Furthermore, ParH was also able to bind members of the TIPOC, including the stress bearing intermediate filament homolog FilP; linking polar growth to DNA segregation once again.

The network of protein cooperation became more complex as we detected binding between the proteins involved in the polar growth of the hyphal tips and those involved in the positive regulation of the Z-ring. This included a strong association of SsgB and SepFTR with the intermediate filament homolog FilP. This is a novel and exciting discovery as polar growth and cell division are separated in time and space. These results shed light on a previously unexplored network of communication between cell division, chromosome organisation and polar growth. However, the fact these proteins are able to form complexes does not suggest they do within the cell; as they may not be present in the same location or functioning at the same time. Further *In Vitro* experiments including microscopy and protein: protein binding assays would build a clearer picture of the communication network of proteins involved in the life cycle of *Streptomyces*.

SepF

Next, I turned my attention to uncovering how FtsZ is stabilised in *S.coelicolor*. Positive regulators of FtsZ bind to its c-terminal and some conserved residues, including Proline 372 facilitate this interaction. In *E.coli* two trans-membrane proteins FtsA and ZipA act as membrane anchors by binding to the central hub; the conserved region on FtsZ which includes Proline 372 (Lutkenhaus et al, 2002). FtsA is an actin homolog consisting of transmembrane amphipathic helix at its C-terminal to attach to the cellular membrane. *B.subtilis* also use FtsA to anchor the Z-ring to the cellular membrane,

but are still capable of forming septa when FtsA isn't present as they have an additional protein SepF which can perform the role. SepF is also capable of binding the lipid bilayer through an amphipathic helix found at its N-terminal and is located within the cell division gene cluster alongside FtsA and FtsZ. The genome of *Streptomyces*, does not encode for FtsA or ZipA but there are three homologs of SepF. It could be possible that one of these proteins binds to FtsZ and attaches it to the cellular membrane during septum formation.

I initially performed bioinformatics of the three SepF homologs; sepF1, sepF2 and sepF3 encoded by the genes SC01749, SC02079 and SC05967 respectively. This included a multiple sequence alignment of the three genes to SepF in other bacterium and an alignment of them together. There is a variation of length between the three proteins, both SepF1 (SC01749) and SepF3 (SC05967) have a similar size to the *B.subtilis* sepF at 146 and 136 amino acids. SepF2 (SC02079) is longer at 213 residues. Despite the variation, all three share 40% overall sequence coverage with the majority of the shared sequence within the C-terminal; a region which is important for the *B.subtilis* SepF interacting with FtsZ. Only SepF2 is found within the cell division protein cluster on the genome of *S.coelicolor* like the SepF of *B.subtilis*. Therefore we focused on this protein for further biochemical analysis.

I found that the *S.coelicolor* SepF2 is highly similar to FtsZ from *B.subtilis* as it shares 31% of the gene sequence. Even though, this sequence identity may be low, the overall coverage is relatively high at 70%. The length of the *S.coelicolor* SepF2 protein is predicted to be 213, which is 67 residues more than that of the *B.subtilis* protein. Therefore the differences could be accounted for by the extra length of the *S.coelicolor* N-terminal domain. The region which has been shown to be important for the binding of FtsZ; the C-terminal is conserved between the two proteins. The residues required for FtsZ binding including A98 and F124 are conserved with the SepF proteins from *S.coelicolor* (Bezouwen *et al*, 2012). This suggests they may be able to interact with FtsZ to stabilise the Z-ring.

SepF is a membrane anchoring protein which consists of an N-terminal amphipathic helix and a C-terminal FtsZ binding region. The proposed crystal structure of *B.subtilis* SepF C-terminal domain shows a single SepF monomer forms a tight dimer with β -sheets at the interface. Crystal packing suggests the polymer is assembled from dimers with α -helices on the exterior of the dimers facilitating interactions. Electron Microscopy analysis of the truncated SepF used for crystallisation revealed individual rings at pH 10 which associated to form a tube at lower pH. A mutation in a Glycine at position 109 which lies at the interface between dimers to an arginine destroys polymerisation and renders the mutants incapable of ring formation (Celik *et al*, 2013). Mycobacterium, a member of the *Actinomycete* genre, also use SepF for FtsZ stabilisation. Deletion of the *SepF* gene led to a complete

block of cell division within *M.tuberculosis* and SepF promotes the polymerisation of FtsZ in *M.smegmatis*. Therefore, one of the SepF homologs is likely to perform a similar role in *Streptomyces*.

A crucial feature of SepF is its ability to attach FtsZ to the cellular membrane via its N-terminal amphipathic helix. Therefore, we attempted to predict whether the three SepF homologs are able to attach to the lipid membrane. The software amphiseek was used and suggests that known of the three SepF proteins of *S.coelicolor* can attach to the membrane via an amphipathic helix. This is interesting as all three are highly conserved in the N-terminal FtsZ binding region but not the C-terminus, which has been implicated in membrane interactions. If the SepF homologs of *S.coelicolor* are needed for the stabilisation of FtsZ they may attach to the membrane using a different mechanism which could require additional binding partners. Perhaps the extra length of the N-terminal of SepF2 protein could be involved in the membrane anchoring.

Recently, a newly discovered actinobacterial specific protein called SepH has been identified in *S. venezuelae*. SepH stimulates the assembly of the z- ring by interacting with FtsZ to promote protofilament formation (Schlimpert et al, 2021). Furthermore, two dynamin like proteins; DynA and DynB stabilise the Z-ring through a network of interactions during sporulation specific division. This includes FtsZ, SsgB and SepF in *S. venezuelae* (Schlimpert et al, 2017). It is thought SepF is anchored to the membrane via binding of DynA and DynB which in turn bind SsgB. SsgB is a protein which recruits FtsZ during the initiation of cell division to align the multi Z-rings along the aerial hyphae with its partner protein SsgA (Borst et al, 2011). This model suggests why SepF is not predicted to bind the cell membrane as the interaction is facilitated by a multi protein network.

Next I generated a predicted structural model to suggest the tertiary fold of the SepF2 *S.coelicolor* protein. The Crystal structure of the *B.subtilis* SepF was created using a C-terminal truncate of the full length protein. Therefore, only residues 61-213 were input into the model making software which produced a homo-dimer consisting of alpha-helices and beta-sheets. Interestingly the *B.subtilis* SepF also forms a homo-dimer via β -sheets at the interface. This suggests SepF2 may be capable of forming a polymer like *B.subtilis* SepF by α -helices on the exterior of the dimers facilitating interactions.

To better understand the structure of the three SepF homologs we decided to purify the proteins using *E.coli* cells as a host. An overexpression construct was generated using the three SepF genes and pET28a as an expression vector. As previous work on the *B.subtilis* SepF has involved a truncated protein, I also generated a SepFTR construct. Once the optimal conditions for the production of all was determined, they were overproduced to a large scale and purified using nickel affinity chromatography. SepF2 and SepFTR were purified under denaturing conditions and SepF3 was

purified from solution under native conditions. After purification initial dialysis in 500mL HEPES buffer (50mM HEPES, 50mM KCL, pH 7.2) was unsuccessful. Therefore, a stabilising buffer (50mM Tris-HCL, pH 8, 50mM NaCl, 10% glycerol, 0.4M L-arginine and 2% glycine) was utilised to refold the SepFTR and SepF2 proteins (Singh *et al*, 2008).

Finally, Transmission Electron Microscopy was used to visualise truncated SepF2 in stabilising buffer. Previous research of the *B.subtilis* SepF protein has shown it promotes the assembly of FtsZ protofilaments into bundles when viewed by Transmission Electron Microscopy (Singh *et al*, 2008). Furthermore, large ring structures consisting of SepF at 50nM were formed under physiological conditions (Errington *et al*, 2011). The purified SepFTR protein appeared to create multiple ring structures when viewed. These rings stacked to produce a tubular structure which visually looks like a spring with a width of 45.3nM which is close to the 50nM observed in *B.subtilis* (Errington *et al*, 2011). It is thought this higher order structure is produced due to interactions of the C-terminal residues which was also found with the *B.subtilis* SepF. This is promising as the SepF2 from *S.coelicolor* is behaving in a similar manner to the SepF from *B.subtilis*. The tubular appearance of SepF was observed by Errington *et al*, at low pH which reflects the conditions of our stabilising buffer; pH 8. Individual ring structures have only been viewed at the higher pH of 10. Therefore, it would be useful to repeat the experiment at a higher pH.

The self association of SepF homo-dimers is thought to be due to residues in the alpha helices. The proposed structural model and the formation of rings by the SepFTR protein indicate that the *S.coelicolor* protein can polymerise. The N-terminal of *B.subtilis* SepF is thought to be involved in the higher order assembly of SepF as mutants lacking this region can't self associate. Here, we have shown the SepF2 of *S.coelicolor* is able to self associate even though it has a much longer N-terminal. The FtsZ binding site of SepF in *Archea* has been identified and is made up of residues K115, R118 and R130 (Pendel *et al*, 2021). The protein model of SepF2 from *S.coelicolor* also has a region with two of the residues conserved, which suggests FtsZ binds there.

To gain a better understanding as to how the structure of SepF implicates on its function within *S.coelicolor*, an attempt was made to gain crystals of the truncated SepF2 protein. Purified SepFTR was refolded and dialysed using stabilising buffer with a pH of 10. Analytical Size Exclusion Chromatography was utilised to determine the molecular weight of the different protein structures present within the refolded protein sample. The major structure detected was a dimer at 26kDa, confirmed using a comparison of the data to a calibration curve for the column and 15% SDS PAGE analysis of the eluted protein samples. Subsequently, Preparative Size Exclusion Chromatography was performed on the remaining protein sample and eluted protein collected. The major peak was again

detected at the weight associated with a dimer structure of SepFTR. This result coupled with the three dimensional structural model created using pymol suggests SepF2 from *S.coelicolor* forms a dimer like the SepF from *B.subtilis* (Celik *et al*, 2013). Subsequently SepF2 is highly likely to be involved in stabilising the FtsZ ring.

A lot more research needs to be conducted to understand how SepF forms rings and subsequently tubes. Furthermore, the protein FtsZ needs to be introduced to SepF to investigate whether the *S.coelicolor* SepF can promote FtsZ polymerisation.

Future Work

Previous research into FtsZ has highlighted discrepancies between *in vivo* and *in vitro* polymerisation of FtsZ. These are unsurprising as experimental conditions *in vitro* do not represent the conditions present in the cytoplasm. It is known the assembly of protein complexes, including actin and tubulin, are greatly influenced by the concentration of macromolecules. To date, much of the work has been carried out using buffer and macromolecules compositions which differ greatly from those found in a cellular environment.

Several techniques can be used to reflect the crowded volume of a cell when performing *in vitro* experiments with cytoskeletal proteins. Firstly, the macromolecular content present can be increased using unrelated macromolecules such as Dextran and cyanmethemoglobin. Secondly, the use of physiological buffers, which mimic the ionic environment of the cytoplasm can be used. They contain potassium at high and chloride ions at low concentrations with a pH of 7.4. Thirdly, it can be useful to use additional enzymes which regenerate GDP to GTP or GTP analogs which hydrolyse at a slower rate. This promotes the stability of polymers allowing experiments to be carried out in a reasonable time-scale. This is very important in the case of FtsZ polymerisation studies as potassium chloride increases the GTPase activity of FtsZ.

FtsZ is anchored to the membrane by additional proteins including FtsA and SepF in *B.subtilis*. This provides stability to the Z-ring and ensures efficient cell wall constriction is achieved. It can therefore be useful to study FtsZ interactions and polymerisation in membrane like systems. One way this is achieved is to use nanodiscs which are membrane protein scaffolds which form a ring containing phospholipids. This technique has been used to study the interaction of ZipA with FtsZ but is only useful when the FtsZ anchoring protein is known for the organism. This problem can be overcome by creating FtsZ variants which can attach to the membrane. FtsZ-mts, with an amphipathic helix at their C-terminus were incorporated into lipid vesicles and deformed the liposomes into tubules. This provides evidence of FtsZ as a key protein in the constriction of membranes.

The division of Bacterial Cells is essential and is regulated by multiple proteins and transcription factors. A major protein complex involved in the process is the divisome, with the Tubulin homolog FtsZ initiating its formation and recruiting additional proteins. To date, *in vivo* experiments have been challenging as single protofilaments are difficult to view and FtsZ forms a variety of polymerisation structures during *in vitro* experiments. This has led to a lack of clarity with the evidence needed to suggest a mechanism of polymerisation. Future experiments should focus on studying FtsZ dynamics in conditions which reflect the cellular interior. This would include using biophysical buffers, additional macromolecules and a GTP regeneration system to reflect the cytoplasm.

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9 Appendix

9.1 Figures

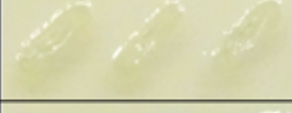
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27 pUTC/T18-FtsZTR pKT/ T25-SsgB		31 pUTC/T18-FtsZTR pKNT/ SsgB-T25	
28 pUT/ FtsZTR-T18 pKNT/ SsgB-T25		32 pUT/ FtsZTR-T18 pKT/ T25-SsgB	

Figure 9.1.1. BTH assay of SsgB and FtsZTR.

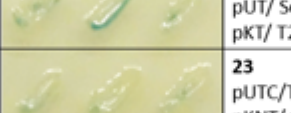
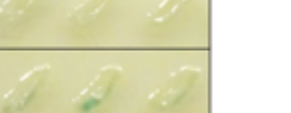
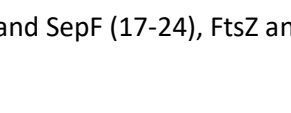
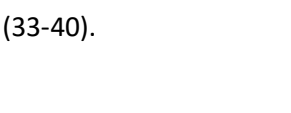
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18 pUT/ SepF-T18 pKNT/ FtsZTR-T25		22 pUT/ SepF-T18 pKT/ T25-FtsZTR	
19 pUTC/T18-FtsZTR pKT/ T25-SepF		23 pUTC/T18-FtsZTR pKNT/ SepF-T25	
20 pUT/ FtsZTR-T18 pKNT/ SepF-T25		24 pUT/ FtsZTR-T18 pKT/ T25-SepF	
33 pUTC/T18-SepFTR pKT/ T25-FtsZTR		37 pUTC/T18-SepFTR pKNT/ FtsZTR-T25	
34 pUT/ SepFTR-T18 pKNT/ FtsZTR-T25		38 pUT/ SepFTR-T18 pKT/ T25-FtsZTR	
35 pUTC/T18-FtsZTR pKT/ T25-SepFTR		39 pUTC/T18-FtsZTR pKNT/SepFTR-T25	
36 pUT/ FtsZTR-T18 pKNT/SepFTR-T25		40 pUT/ FtsZTR-T18 pKT/ T25-SepFTR	

Figure 9.1.2. BTH assay of FtsZ and SepF (17-24), FtsZ and SepFTR (33-40).

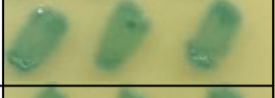
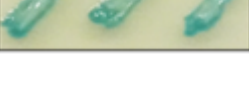
121 pUTC/ T18-SepFTR pKNT/ T25-SepFTR		Positive control	
122 pUT/ SepFTR-T18 pKNT/ SepFTR-T25		Negative control	
123 pUTC/ T18-SepFTR pKT/ T25-SepFTR		1 pUTC/ T18-SepF pKT/ T25-SepF	
124 pUT/ SepFTR-T18 pKNT/ SepFTR-T25		2 pUT/ SepF-T18 pKNT/ SepF-T25	
		3 pUTC/ T18-SepF pKNT/ SepF-T25	
		4 pUT/ SepF-T18 pKT/ T25-SepF	

Figure 9.1.3. BTH assay of SepFTR (121-124) and SepF (1-4) self interaction.

125 pUTC/ T18-FtsZTR pKT/ FtsZTR-T25	
126 pUT/ FtsZTR-T18 pKNT/ T25-FtsZTR	
127 pUTC/ T18-FtsZTR pKT/ FtsZTR-T25	
128 pUT/ FtsZTR-T18 pKNT/ T25-FtsZTR	

Figure 9.1.4. BTH assay of FtsZTR self interaction.

5 pUTC/ T18-SsgB pKT/ T25-SsgB	
6 pUT/ SsgB-T18 pKNT/ SsgB-T25	
7 pUTC/ T18-SsgB pKNT/ SsgB-T25	
8 pUT/ SsgB-T18 pKT/ T25-SsgB	

Figure 9.1.5. BTH assay of SsgB self interaction.

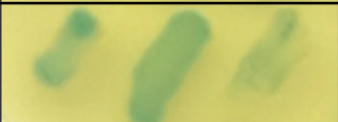
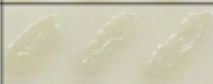
73 pUTC/ T18-SsgB pKNT/ T25-SepFTR		77 pUTC/ T18-SsgB pKT/ SepFTR-T25	
74 pUT/ SsgB-T18 pKNT/ SepFTR-T25		78 pUT/ SsgB-T18 pKNT/ T25-SepFTR	
75 pUTC/ T18-SepFTR pKT/ T25-SsgB		79 pUTC/ T18-SepFTR pKT/ SsgB-T25	
76 pUT/ SepFTR-T18 pKNT/ SsgB-T25		80 pUT/ SepFTR-T18 pKNT/ T25-SsgB	

Figure 9.1.6.. BTH assay of SsgB and SepFTR.

57 pUTC/ T18-ParH pKT/ T25-FtsZTR		61 pUTC/ T18-ParH pKNT/ FtsZTR-T25	
58 pUT/ ParH-T18 pKNT/ FtsZTR-T25		62 pUT/ ParH-T18 pKT/ T25-FtsZTR	
59 pUTC/T18-FtsZTR pKT/ T25-ParH		63 pUTC/T18-FtsZTR pKNT/ ParH-T25	
60 pUT/ FtsZTR-T18 pKNT/ ParH-T25		64 pUT/ FtsZTR-T18 pKT/ T25-ParH	

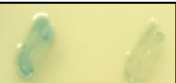
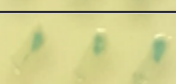
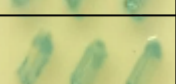
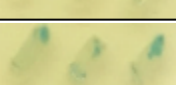
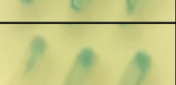
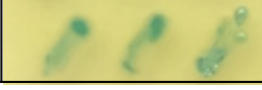
81 pUTC/ T18-ParH pKNT/ T25-SepF		85 pUTC/ T18-ParH pKT/ SepF-T25	
82 pUT/ ParH-T18 pKNT/ SeF-T25		86 pUT/ ParH-T18 pKNT/ T25-SepF	
83 pUTC/ T18-SepF pKT/ T25-ParH		87 pUTC/ T18-SepF pKT/ ParH-T25	
84 pUT/ SepF-T18 pKNT/ ParH-T25		88 pUT/ SepF-T18 pKNT/ T25-ParH	

Figure 9.1.7. BTH assay of ParH and FtsZTR (57-64) ParH and SepF (81-88).

49 pUTC/ T18-ParH pKT/ T25-SsgB		53 pUTC/ T18-ParH pKNT/ SsgB-T25	
50 pUT/ ParH-T18 pKNT/ SsgB-T25		54 pUT/ ParH-T18 pKT/ T25-SsgB	
51 pUTC/ T18-SsgB pKT/ T25-ParH		55 pUTC/ T18-SsgB pKNT/ ParH-T25	
52 pUT/ SsgB-T18 pKNT/ ParH-T25		56 pUT/ SsgB-T18 pKT/ T25-ParH	

Figure 9.1.8. BTH assay of *ParH* and *SsgB*.

66 pUT/ FliP-T18 pKNT/ SepFTR-T25	
67 pUTC/ T18-SepFTR pKT/ T25-FliP	
68 pUT/ SepFTR-T18 pKNT/ FliP-T25	
69 pUTC/ T18-FliP pKT/ SepFTR-T25	
70 pUT/ FliP-T18 pKNT/ T25-SepFTR	
71 pUTC/ T18-SepFTR pKT/ FliP-T25	
72 pUT/ SepFTR-T18 pKNT/ T25-FliP	

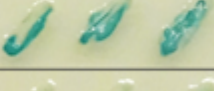
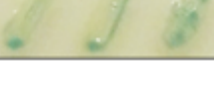
41 pUTC/ T18-FilP pKT/ T25-SsgB		45 pUTC/ T18-FilP pKNT/ SsgB-T25	
42 pUT/ FilP-T18 pKNT/ SsgB-T25		46 pUT/ FilP-T18 pKT/ T25-SsgB	
43 pUTC/T18-SsgB pKT/ T25-FilP		47 pUTC/T18-SsgB pKNT/ FilP-T25	
44 pUT/ SsgB-T18 pKNT/ FilP-T25		48 pUT/ SsgB-T18 pKT/ T25-FilP	

Figure 9.1.9. BTH assay of *FilP* and *SepFTR* (66-72) *FilP* and *SsgB* (41-48).