DEVELOPMENT OF FLUORESCENCE TECHNOLOGY FOR *S. COELICOLOR*

DEVELOPMENT OF FLUORESCENCE TECHNOLOGY FOR USE IN *STREPTOMYCES COELICOLOR*

By

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ABSTRACT

The growing problem of antibiotic resistance has prompted the need for new and novel antimicrobial therapies. The bacterial cell division pathway holds great promise for the development of novel broad-spectrum antibiotics as the majority of the proteins are essential for viability. The wealth of information regarding bacterial cell division has come from studies of the model organisms *Escherichia coli* and *Bacillus subtilis*. Although much has been elucidated regarding this pathway, the functions of many individual proteins remain unsolved.

An important model organism for the investigation of cell division is *Streptomyces coelicolor*. The mycelial *Streptomyces* are sporulating, Gram-positive bacteria that grow in long branching networks of filamentous cells much like filamentous fungi. The normally essential process of cell division is dispensable for growth and viability of *S. coelicolor*. More interestingly, there are two different modes of cell division in this organism, one for vegetative growth and one is utilized for synchronous septation during sporulation. It is still unclear how developmental regulators control this switch, but advancements in fluorescence microscopy have shed some insight into the cell division process by allowing direct visualization of many cellular components and their dynamics.

To better understand bacterial cell division and its regulation in *S. coelicolor*, three additional fluorescent proteins (FPs), including mRFP, CyPet and YPet, have been established in this work. An mRFP shuttle vector was constructed and the utility of mRFP was tested by translationally fusing it to a tip-localizing protein, DivIVA. This work demonstrated that mRFP is functional and an efficient marker for localized proteins. Also, established in this work is a two-colour fluorescence reporter system, which includes the fluorescent proteins CyPet and YPet that can be used to study co-localization and protein-protein interactions within cells. Future plans are to use co-localization of FP fusions and fluorescence resonance energy transfer (FRET) between CyPet and YPet to investigate the assembly of protein complexes within the cells, such as those involved in cell division. These studies will reveal critical information that is needed for the development of drugs that have novel mechanisms of action.

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ABBREVIATIONS

ABC	ATP-binding cassette
Amp	Ampicillin
Bp	Base pair
CyPet/YPet	Cyan or yellow fluorescent protein for energy transfer
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
FP	Fluorescent protein
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
Kb	Kilobase
LB	Luria broth
MCS	Multiple cloning site
mRFP	Monomeric red fluorescent protein
MS	Mannitol soy flour
NO	Nucleoid occlusion
PBP	Penicillin binding protein
PG	Peptidoglycan
SALP	SsgA-like protein
SEDS	Shape, Elongation, Division and Sporulation
TSB	Tryptone soya broth

1.0 INTRODUCTION

1.1 The need for novel antimicrobials

The treatment of infectious disease has been revolutionized by the discovery and development of antibiotics. However, the added selective pressure has resulted in a rapid emergence of resistance to all commercially available antibiotics (Coates and Hu, 2007; Hawkey, 2008). Due to the time and costs related to the development of novel antibiotics, many of the new drugs produced in recent years are only slight variants of existing overused antibiotics (Coates and Hu, 2007; Lock and Harry, 2008). As such, their introduction into a clinical setting has translated to the rapid emergence of resistance since only small modifications to resistance mechanisms are required. This suggests that there is a pressing need to discover new drugs with novel modes of action to combat bacterial diseases as the incidence of multi-drug resistance continues to rise in hospitals around the world.

1.2 Targeting the cell division apparatus

Most drugs used to treat bacterial infections inhibit one of four critical processes: nucleic acid synthesis, protein biosynthesis, cell-wall synthesis, or folate coenzyme biosynthesis (Walsh, 2003). To date, there are no commercially available antibiotics that specifically target the essential cell division process, but it represents an attractive area with great potential for novel drug discovery as it is essential for the growth and viability of most bacteria (Barak and Wilkinson, 2007; Lock and Harry, 2008). Cell division proteins are highly conserved among bacteria and the disruption of one or more division

proteins often result in cell death (Goehring and Beckwith, 2005; Margolin, 2005; Harry *et al.,* 2006; Lutkenhaus, 2007; Lock and Harry, 2008; Hett and Rubin, 2008). Furthermore, an intricate web of interactions between the different division proteins is required for the completion of septation. Some of the interactions are essential for both the recruitment and proper functioning of these proteins, suggesting that the design of novel inhibitors to disrupt protein-protein interactions can be as effective as inhibiting a specific protein (Hale and de Boer, 1997; Jensen *et al.,* 2005; Lock and Harry, 2008). Clearly, the inhibition of cell division is of great therapeutic potential but a greater fundamental understanding of the functions of the individual proteins within the division process in bacteria is needed for the design of novel antimicrobial therapies.

1.3 Bacterial cell division

Cell division is a fundamental process that is essential for the propagation of all living species (Harry *et al.,* 2006). In bacteria, cell division is an asexual event known as binary fission where a single parental cell divides into two equal daughter cells (Margolin, 2005). This duplication process is highly complex and involves precise coordination of events both spatially and temporally. Proper regulation of DNA replication and segregation, cytoplasmic partitioning, and formation of new cell wall are required to produce two morphologically and genetically identical progeny cells (Barak and Wilkinson, 2007; Hett and Rubin, 2008).

Cell division has been studied most extensively in rod-shaped bacteria such as *Escherichia coli* and *Bacillus subtilis* (Goehring and Beckwith, 2005; Harry *et al.,* 2006; Barak and Wilkinson, 2007). The first cell division proteins were identified when researchers looked for temperature sensitive *E. coli* mutants that were affected in growth and replication. The work led to the isolation of conditional mutations that produced characteristic long filamentous cells at nonpermissive temperatures, indicating that these cells were unable to divide (Hirota *et al.,* 1968; Van De Putte *et al.,* 1964). Consequently, many of the division genes were given the *fts* designation (filamentation thermosensitive).

The earliest known step of bacterial cell division is the assembly of FtsZ into a ring structure, referred to as the Z ring (Figure 1.1; Bi and Lutkenhaus, 1991). This ring marks the future division site, usually at midcell, and act as a scaffold for the recruitment of the late division proteins (Barak and Wilkinson, 2007). In *E. coli*, the recruitment of division proteins is essentially linear and hierarchical with the early division proteins FtsZ, FtsA, and ZipA localizing at the midcell first to form the Z ring and to mark the division site. Subsequently, the late division proteins are recruited in a linear hierarchy of dependence to the division site, where downstream proteins require the presence of all upstream proteins for localization (Buddelmeijer and Beckwith, 2002). This linearity is in sharp contrast to cell division in *B. subtilis*, where the assembly of the division apparatus occurs in two steps. Once the Z ring has formed with the localization of FtsA, ZapA, SepF and FtsZ, the remaining cell division proteins are then recruited and localize interdependently to the division site (Errington *et al.*, 2003). Regardless of the mode of recruitment, the complement of division proteins arrives at the site of division to form a 'divisome' so that cell division can be completed (Kawai and Ogasawara, 2006).

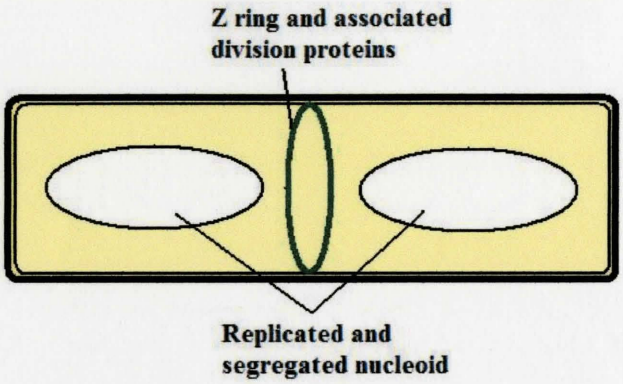

Figure 1.1. Assembly of the Z ring at midcell during cell division. The association of FtsZ monomers to form the Z ring marks the initial cell division event. This ring is often placed at the future division site, usually at midcell between the segregated chromosomes, and functions to recruit other division proteins necessary for septation.

1.4 Overview of the late cell division proteins

After the formation and stabilization of the Z ring by the early division proteins, the remaining proteins are recruited so that cell division can be completed (Table 1.1). One of the earliest division proteins to be recruited by the Z ring is FtsK. It is a multifunctional membrane protein which has roles in both Z-ring stabilization and chromosome segregation (Bigot *et al.,* 2004). Next, FtsQ, FtsL, and FtsB first associate to form a tripartite protein complex, then assemble into the Z ring. The role of this multiprotein complex is still unclear but it may act to bridge the cytoplasmic and periplasmic components (Buddelmeijer and Beckwith, 2004). The periplasmic proteins FtsW and FtsI are next to join the Z ring. These two proteins are thought to be vital for the synthesis of septal peptidoglycan (PG) since FtsI is a septation-specific transpeptidase (PBP3) and the multi-spanning membrane protein FtsW is believed to act as a transporter of PG precursors (Holtje, 1998). FtsN, a protein which binds PG and has a weak

homology to an amidase, is one of the late recruits to the division site; however, its exact role is still unknown (Addinall *et al.,* 1997; Ursinus *et al.,* 2004). The remaining proteins that are recruited to the division site include FtsE, FtsX, AmiC, and EnvC. FtsE and FtsX belong to the ABC transporter family and participate directly in the process of cell division (Schmidt *et al.,* 2004). However, recent evidence suggests they may have roles in Z ring assembly and stabilization rather than transport (Vicente *et al.,* 2006). Finally, AmiC and EnvC are PG-degrading enzymes required for the separation of daughter cells (Bernhardt and de Boer, 2003; 2004).

Table1.1. Bacterial cell division proteins encoded by *E. coli*, *B. subtilis*, and *S. coelicolor*.

Protein	Function in *E. coli* and/or *B. subtilis*	*E. coli*	*B. subtilis*	*S. coelicolor*	Function in *S. coelicolor*	References
FtsA	Stabilize Z ring – act as membrane tether, homolog of actin	+	+	-		
FtsB	Complex with FtsL and FtsQ, May bridge cytoplasmic with periplasmic components	+	DivIC	DivIC	Coordinating symmetrical ingrowth of the invaginating septum	Bennett *et al.*, 2007
FtsE	Could be involved in Z ring stability, ABC transporter family	+	+	-		
FtsI	Synthesize septal PG, encode septum-specific PBP3, cognate transpeptidase of FtsW	+	+	+	Transpeptidase (PBP 3), Interacts with FtsW, may substitute for lack of ZipA or FtsA	Mistry *et al.*, 2008
FtsK	Bifunctional: N terminus for cell division, Z ring stability, and C terminus for DNA segregation – recruits TopoIV and XerCD	+	SpoIIIE	+	Role in chromosome segregation during sporulation	Wang *et al.*, 2007
FtsL	Possible role in Z ring constriction, complex with FtsB and FtsQ	+	+	+	Coordinating symmetrical ingrowth of the invaginating septum	Bennett *et al.*, 2007
FtsN	Possible role: spans periplasm and shuttles PBPs, weak homology to amidase, binds PG	+	-	-		
FtsQ	Chaperone - stabilize other division proteins, complex with FtsB and FtsL	+	DivIB	+	Unknown, but mutants were blocked at a later stage in cell division	McCormick and Losick, 1996; Mistry *et al.*, 2008
FtsW	Member of SEDS family, possible transporter of PG precursors, or a communication channel between cytoplasm and periplasm	+	+	+	Interacts with FtsZ and FtsI, may substitute for lack of ZipA or FtsA	Mistry *et al.*, 2008
FtsX	Unknown, ABC transporter family	+	?	-		
FtsZ	Z ring assembly, GTPase, tubulin homolog, recruit division proteins to septum	+	+	+	Z ring assembly, GTPase, tubulin homolog, recruit division proteins to septum	McCormick *et al.*, 1994
ZipA	Stabilize Z ring – act as membrane tether	+	ZapA	?		
SepF/YlmA	Similar roles as FtsA	?	+	SCO2079?		
EzrA	Prevent FtsZ assembly	-	+	-		
SlmA	Binds DNA, sequesters FtsZ monomers and prevent their assembly into Z ring	+	Noc	?		
MinC	Promote FtsZ disassembly, interfere with FtsZ lateral interactions, interacts with MinD	+	+	-		
MinD	ATPase, enhances MinC activity, binds both MinC and MinE	+	+	-		
MinE	Topological factor, binds and induces ATPase activity of MinD, and oscillates MinCD between cell poles	+	-	-		
DivIVA	Recruits MinCD to the cell poles	-	+	+	Essential for coordinating hyphal tip growth and morphogenesis (not involved in cell division in *S. coelicolor*)	Flardh, 2003b
AmiC	Amidase, hydrolyzes septal PG to separate progeny cells	+	?	SsgE-F	Controls spore separation	Noens *et al.*, 2005; 2007; Traag and van Wezel., 2008
EnvC	Hydrolase, hydrolyzes septal PG to separate progeny cells	+	?	SsgE-F	Controls spore separation	Noens *et al.*, 2005; 2007; Traag and van Wezel., 2008
ZapB[1]	Division factor that stimulates Z-ring assembly	+	?	?		
GpsB[2]	With EzrA, helps co-ordinate switch between lateral and septal cell wall synthesis by re-localization of PBP1	?	+	?		

+, present; -, absent;?, unknown
Function in *E. coli* and *B. subtilis* reviewed by Harry *et al.*, 2006; Barak and Wilkinson, 2007, unless otherwise noted
[1,2]Recently described cell division factors in *E. coli* (Ebersbach *et al.*, 2008) and *B. subtilis* (Claessen *et al.*, 2008)

1.5 The major cell division protein FtsZ

FtsZ is a highly conserved division protein and its presence in almost all bacteria reflects its pivotal role in cell division (Harry *et al.,* 2006; Vicente *et al.,* 2006). The FtsZ structure comprise of 4 domains (Figure 1.2): a variable N terminus, a conserved core region, a variable spacer and a conserved C terminus (Margolin, 2005). The N terminus and spacer domains are variable in length and their functions are still unknown. GTP binding and hydrolysis is contained within the core region (Olivia *et al.,* 2004). The C terminus is necessary for interacting with other division proteins (Ma and Margolin, 1999). The three-dimensional structure of FtsZ was revealed to be very similar to that of eukaryotic tubulin, the building blocks of microtubules in eukaryotes (Lowe and Amos, 1998, Nogales *et al.,* 1998). Like tubulin, FtsZ is thought to assemble into polymers in a GTP-dependent fashion prior to the assembly of Z rings. In support of this, it has been shown that FtsZ can self-assemble into linear head-to-tail polymers upon GTP binding (Mukherjee and Lutkenhaus, 1994). As well, electron microscopy has revealed that the head-to-tail polymers resemble protofilaments of microtubules found in eukaryotes (Olivia *et al.,* 2003; 2004). The energy released from GTP hydrolysis is thought to drive Z-ring constriction which leads to the invagination of the cell membrane (Ryan and Shapiro, 2003). This can be supported by the fact that GTP-bound FtsZ protofilaments are relatively straight and rigid whereas GDP-bound protofilaments are more labile and adopt a curved conformation (Lu *et al.,* 2000).

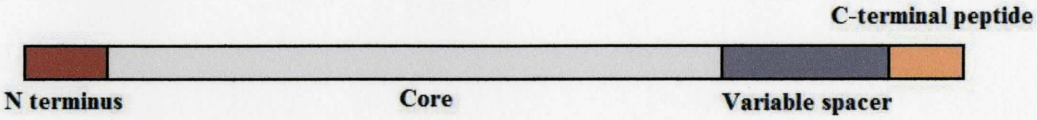

Figure 1.2. The domain structures of FtsZ. FtsZ comprise of 4 domains: a variable N terminus of unknown function, a conserved core region for GTP binding and hydrolysis, a variable spacer of unknown function, and a conserved C terminus for binding to other division proteins.

1.6 Modulators of Z-ring assembly

1.6.1 Proteins affecting polymerization

Assembly of the Z ring is tightly controlled and dependent on the fine balance between rapid FtsZ polymerization and depolymerization. In *E. coli* and *B. subtilis*, there are ~15,000 copies of FtsZ per cell, but only ~30% of this cellular pool is needed for Z-ring formation, indicating that inhibitors must exist to regulate the assembly of Z rings (Stricker *et al.,* 2002). Indeed, numerous proteins that can interact directly with FtsZ to modulate its dynamic behaviour have been identified (Barak and Wilkinson, 2007; Harry *et al.,* 2006). Many of these regulatory proteins include positive regulators like ZapA (Gueiros-Filho and Losick, 2002), SepF/YlmF (Hamoen *et al.,* 2006; Ishikawa *et al.,* 2006), ZipA and FtsA (Pichoff and Lutkenhaus, 2002); and negative regulators such as EzrA (Haeusser *et al.,* 2004), SulA (Bi and Lutkenhaus, 1993), and MinCD (Bi and Lutkenhaus, 1993; Hu *et al.*, 1999). The positive regulators can act to promote the bundling of the FtsZ protofilaments and to stabilize the Z ring at the membrane. In contrast, the negative regulators function to either prevent FtsZ association or to disassemble preformed filaments. Together, these accessory proteins act in a concerted

manner to prevent aberrant Z-ring formation and to coordinate proper ring assembly at the right time and place.

1.6.2 Tethering the Z ring to the membrane

Several proteins have been identified that have a role in stabilizing the Z ring at the division site. In *E. coli*, FtsA and ZipA are essential for cell division because they act as membrane anchors that tether the Z ring to the inner cytoplasmic membrane (Ohashi *et al.*, 2002; Pichoff and Lutkenhaus, 2005). In *B. subtilis*, a protein called SepF is a more recent addition to the bacterial divisome (Hamoen *et al.*, 2006; Ishikawa *et al.*, 2006). Ishikawa *et al.*, (2006) found that an *ftsA*-null mutant could be complemented by the overexpression of SepF. This has brought up the notion that FtsA and SepF have redundant roles *B. subtilis*, and that the combination of SepF & FtsA in *B. subtilis* may be equivalent to that of ZipA & FtsA in *E. coli* for Z-ring stabilization at the membrane.

1.7 Regulation of Z-ring placement

The cell must establish the midcell as the site at which cell division is to take place prior to the actual division. Two overlapping mechanisms, Nucleoid Occlusion and the Min System, control this precise placement (Figure 1.3; de Boer *et al.,* 1989; Woldringh *et al.,* 1991). These mechanisms act mainly to prevent FtsZ polymerization at sites other than midcell, and allowing Z ring to form only at midcell by default.

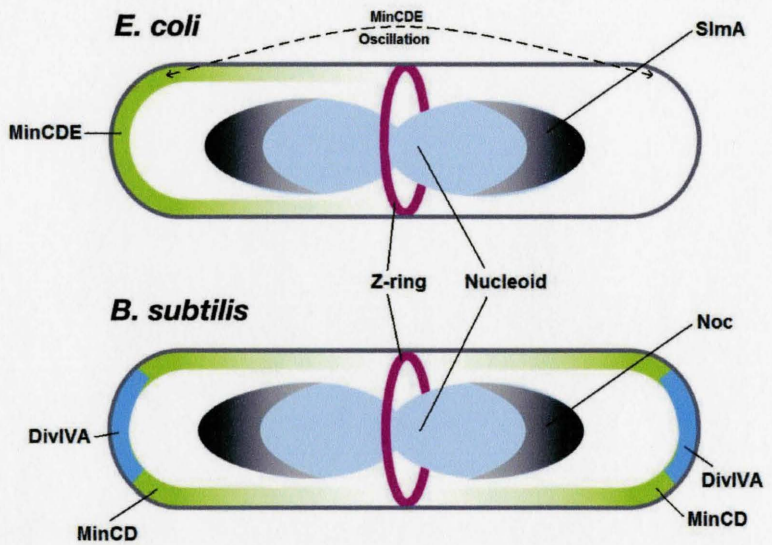

Figure 1.3. Regulation of Z-ring placement in *E. coli* and *B. subtilis*. Both NO and the Min system function in concert to limit cell division only at midcell. Localization of MinCD is dependent on MinE in *E. coli*, and DivIVA in *B. subtilis*. SlmA and Noc participate in NO in *E. coli* and *B. subtilis*, respectively. Adapted from (Lutkenhaus, 2007).

1.7.1 Nucleoid Occlusion

Nucleoid occlusion (NO) is a poorly understood phenomenon, which acts to prevent Z-ring formation over the space that is occupied by the nucleoid (Woldringh *et al.,* 1991). Thus, Z rings can only form in areas with little or no DNA. Once chromosome segregation is well under way, the Z ring is free to assemble at the midcell (Margolin, 2005). Proteins that are involved in this mechanism include unrelated DNA-binding proteins SlmA in *E. coli* (Bernhardt and de Boer, 2005) and Noc in *B. subtilis* (Wu and Errington, 2004). Results presented by these two groups have suggested that these proteins associate with the nucleoid and sequester the monomeric FtsZ molecules to prevent their local assembly into Z rings (Wu and Errington, 2004; Bernhardt and de Boer, 2005).

1.7.2 The Min System

NO alone limits potential division sites to the midcell and the cell poles. Therefore, a second mechanism is required to suppress division at the DNA-free pole regions. This task is carried out by the Min System, which involves the proteins MinC, MinD and MinE (de Boer *et al.,* 1989). MinC and MinD associate with each other to synergistically inhibit FtsZ polymerization. In *E. coli*, the topological factor MinE, functions to oscillate MinCD back-and-forth between the cell poles to limit their localization only to those positions (Hale *et al.,* 2001). *B. subtilis* lacks the MinE protein but contains a pole-localizing protein, DivIVA, which recruits and stabilizes MinCD to the cell poles (Edwards and Errington, 1997). Although this mechanism is slightly

different in the two organisms, the end result remains the same: Z-ring formation is inhibited at the cell poles, leaving the only available area for Z-ring assembly at midcell.

While much has been elucidated regarding the assembly and placement of the divisome, relatively little is known about the exact functions of many individual proteins. Therefore, a greater understanding of these proteins is highly beneficial to the design of effective inhibitors. A great candidate for the investigation of cell division is the bacterium *Streptomyces coelicolor*; the best-characterized bacterium in its genus and an important model system for studying antibiotic production and multi-cellular development (Bentley *et al.,* 2002). Cell division in *S. coelicolor* is very different from most other bacteria as it is not essential for viability making it an ideal system for the investigation of cell division (McCormick *et al.,* 1994; McCormick and Losick, 1996).

1.8 Streptomyces: an unusual mode of growth

Streptomyces are soil-dwelling, mycelial bacteria that grow in long branching networks of cells. The growth and developmental biology of these high GC Gram-positive bacteria, which involves substantial cell-type specialization, differs from most other bacteria and resembles that of filamentous fungi (Flardh, 2003a). Their life cycle (Figure 1.4) begins with a single spore, which upon germination gives rise to germ tubes that grow outwards and away from the spore. This outward growth of cells is mediated exclusively by tip extension – that is, new cell wall is incorporated only at the tip of growing cells and not at the lateral walls, as is the case in most bacteria (Flardh, 2003a). These cells will grow and branch to produce a dense network of long multigenomic cells, collectively known as the 'substrate mycelium'. After about 24 hours of growth, morphological differentiation is initiated and a second cell type, known as the 'aerial mycelium', emerge from the colony surface and grow upwards into the air. The substrate mycelium will eventually die, whereas the aerial cells mature and undergo multiple septations (or cell division events) simultaneously to give rise to chains of unigenomic spores. Eventually the spores mature, indicated by a grey spore pigment, and are released as free spores and the growth cycle begins anew (Chater, 2001; Willey *et al.*, 2006).

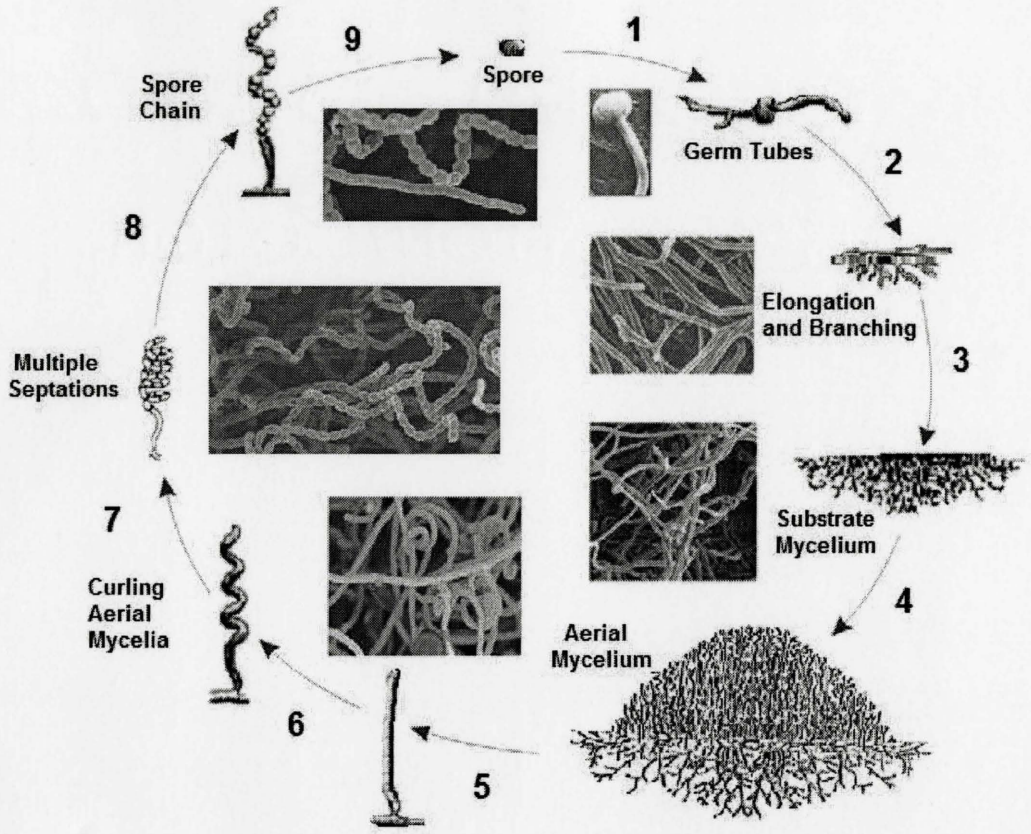

Figure 1.4. The *Streptomyces* life cycle. (1) Emergence of germ tubes after spore germination. (2) Elongation and branching of germ tubes by tip extension. (3) The resulting filamentous cells form a dense substrate mycelium. (4,5) Aerial cells begin to emerge after ~24 hours and grow upwards into the air. (6) Maturation and curling of the aerial hyphae. (7) Multiple cell division events occurring along the length of the filament, simultaneously and uniformly. (8) A chain of unigenomic spores is produced. (9) Resulting mature free spores. Adapted from Jakimowicz (2007).

1.9 Cell division is different in Streptomyces

Like most bacteria, *Streptomyces* use the same basic cell division machinery for division. This can be accounted for due to the presence of known bacterial division proteins, such as FtsZ, FtsQ, FtsW, etc. (Table 1.1) in the *Streptomyces* genome sequences (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). However, different from most bacteria *Streptomyces* use two different modes of cell division (Figure 1.5), and both are dispensable for growth and viability (McCormick *et al.*, 1994; McCormick and Losick, 1996). The first mode occurs at irregular intervals along the length of the substrate cells to produce thin septal crosswalls that act as a physical barrier between adjacent daughter cells. These daughter cells are multinucleated and do not physically separate from one another after cell division. In contrast, the second mode occurs during sporulation of the aerial mycelium where multiple division events occur uniformly and synchronously to produce a spore chain (Grantcharova *et al.*, 2005). Autolysis of the cell wall between the spores will then take place to produce individual spores. Deletions of *ftsZ* or *ftsQ* in *S. coelicolor* result in cells that are completely (or at least substantially) blocked in septation, but otherwise viable – that is, they grow vegetative cells that are devoid of crosswalls and produce aerial hyphae that do not go on to produce spores (McCormick *et al.*, 1994; McCormick and Losick, 1996). Hence, cell division is not an essential process for growth and viability in *Streptomyces*.

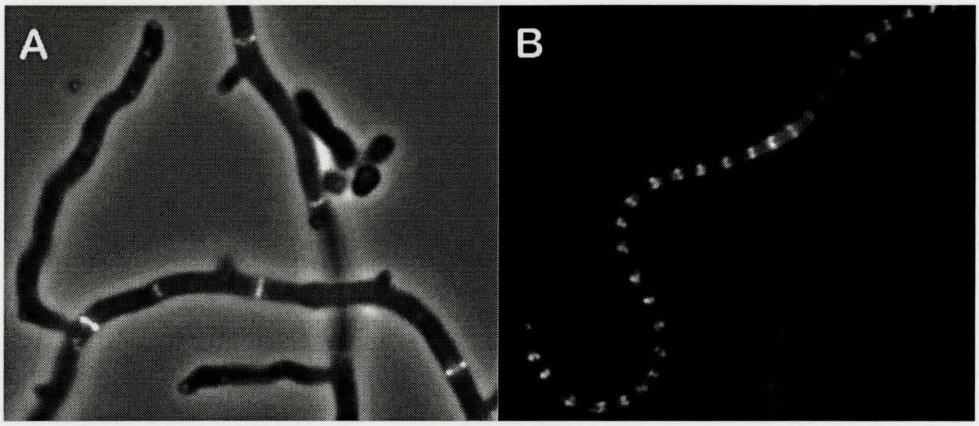

Figure 1.5. Cell division in the two *S. coelicolor* cell types: vegetative and aerial hyphae. These cells contain the FtsZ-eGFP fusion to allow for visualization of Z ring formation in substrate hyphae (A) and aerial hyphae (B). Infrequent FtsZ rings are seen in the vegetative substrate hyphae as compared to the regularly spaced rings in the sporulating aerial hyphae. Adapted from Grantcharova *et al.,* (2005).

In addition to the two different modes of cell division, *Streptomyces* also lack regulatory proteins that influence the assembly and disassembly of FtsZ, such as FtsA & ZipA from *E. coli* and ZapA & EzrA from *B. subtilis*. The two major negative regulatory mechanisms, the Min System and Nucleoid Occlusion, used for precise Z-ring positioning in *E. coli* and *B. subtilis* are also absent in *Streptomyces* (Flardh, 2003a). Therefore, *Streptomyces* must possess unique regulatory proteins in order to control Z-ring assembly and placement. Indeed, the rigorous investigation of cell division in *S. coelicolor* in recent years has led to the discovery of a plethora of actinomycete-specific proteins that are involved in this process (Table 1.2: Noens *et al.,* 2005; 2007; Del Sol *et al.,* 2006; Tseng *et al.,* 2006; Ausmees *et al.,* 2007).

Table 1.2. Sporulation-specific cell division proteins encoded by *S. coelicolor*.

Protein	Phenotype of mutant	Function	References
SsgA	Deletion mutants fail to sporulate, while overexpression results in hyperseptation	Activator of sporulation-specific cell division, septum site selection	Noens *et al.*, 2007
SsgB	Large colonies with longer than normal mycelial filaments	Cessation of mycelial growth before onset of sporulation	Noens *et al.*, 2005
SsgC	Aberrant DNA segregation, irregular spores	Septum site initiation, DNA segregation, may be antagonist of SsgA	Noens *et al.*, 2005
SsgD	Aberrant spore wall	Synthesis of lateral cell wall	Noens *et al.*, 2005
SsgE	Massive amounts of single spores	Controls timing of spore dissociation	Noens *et al.*, 2005
SsgF	Short spore chains	Controls spore separation	Noens *et al.*, 2005
SsgG	Aberrant spore sizes, spores exactly 2x, 3x or 4x normal size	Septum site selection	Noens *et al.*, 2005
CrgA	Overexpression results in failure to sporulate	Inhibition of Z ring formation	Del Sol *et al.*, 2006
ShyA	Altered spore chain morphology	Coordinate septation and spore chain formation	Tseng *et al.*, 2006
SmeA/SffA	Pleiotropic effects: aberrant chromosome segregation, spore wall, spore separation	Influence septum placement, DNA segregation, thickening of spore wall, separation of spores	Ausmees *et al.*, 2007

A family of proteins called the SsgA-like proteins (SALPs), which are present only in sporulating actinomycetes, have various effects on sporulation septation (Table 1.2; Traag and van Wezel, 2008). In the *S. coelicolor* genome seven SALPs, SsgA-G, have been identified (van Wezel and Vijgenboom, 2004). These proteins play critical roles at various stages of sporulation; from septum site selection to separation of the mature spores (Figure 1.6, Noens *et al.*, 2005; 2007). In this model, SsgB is responsible for regulating the cessation of mycelial growth before the onset of sporulation, and the remaining SALPs are involved in septum site selection (SsgA/G), septum growth (SsgA/B), DNA segregation (SsgC), spore wall synthesis (SsgD), and spore separation (SsgE/F) (Noens *et al.*, 2005). Since SALPs do not possess enzymatic activity it is likely that these proteins regulate the activity and localization of cell wall synthesis enzymes (Noens *et al.*, 2007; Traag and van Wezel, 2008).

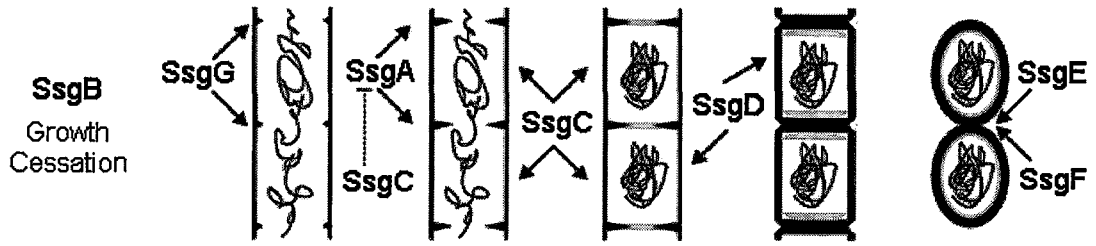

Figure 1.6. A model detailing the proposed functions of the SALPs in controlling sporulation-specific cell division in *Streptomyces*. In this model, SsgG is somehow responsible for septum site selection, whereas the remaining SALPs are involved in septum growth (SsgA), DNA segregation (SsgC), spore wall synthesis (SsgD), and spore separation (SsgE-F). Adapted from Traag and van Wezel (2008).

Apart from the SALPs, other actinomycete-specific proteins have been described that have effects on sporulation-specific cell division. These proteins include ShyA, SmeA, SffA and the CrgA-like proteins (Ausmees *et al.*, 2007; Del Sol *et al.*, 2006; Tseng et *al.*, 2006). Cells lacking ShyA were able to sporulate normally but displayed hyperseptation (Tseng *et al.*, 2006). SmeA and SffA deletion mutants had effects on chromosome segregation, placement of septa during sporulation, and spore maturation (Ausmees *et al.*, 2007). The CrgA-like proteins play a pivotal role in the inhibition of Z ring formation because overexpression led to the inhibition of sporulation (Del Sol *et al.*, 2006).

In addition to having unique division proteins, many conserved cell division proteins serve different roles in *Streptomyces*. For example, DivIVA in *B. subtilis* and other Gram-positive bacteria regulates cell division at the cell poles. However, this protein in *Streptomyces* is found at hyphal tips but is somehow involved in coordinating tip extension and not cell division (Flardh, 2003b). The molecular basis of tip extension

in prokaryotes is still unknown, but it is obvious that cell wall synthesis enzymes like penicillin binding proteins (PBPs) must be present at these apical growth zones. However, the regulated placement of these proteins to the apex of growing cells is still unclear. The identification of the tip localizing DivIVA in *Streptomyces* has shed some insight into this process (Flardh, 2003b). Consistent with its role in tip growth, DivIVA-eGFP forms discrete foci that localize mainly to the tips of growing cells (Flardh, 2003b). The large fluorescent foci observed at the mycelial tips indicate the presence of large numbers of DivIVA molecules. These proteins may oligomerize to form higher-order structures, such as a scaffold that function to recruit other proteins required for tip growth. Therefore, DivIVA appears to be only one component of a larger apical protein complex required for the co-ordination of tip growth. More recently the role of DivIVA-like proteins in *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* have also been shown to regulate polar cell wall synthesis, suggesting that apical growth may be conserved among members of the actinomycetes (Letek *et al.*, 2008; Kang *et al.*, 2008).

1.10 The use of fluorescent proteins to study cell division

It is becoming increasing apparent that the precise subcellular localization of proteins is critical to their function. The popularization of cell biology techniques in recent years, such as the use of FP tags to visualize individual proteins at the subcellular level, has provided much insight into the complexities of the prokaryotic cell. The continual optimization of FPs has led to the development of numerous variants that are good marker for localized proteins in bacteria (Meyer and Dworkin, 2007). For example, the pole-to-pole oscillation of the *E. coli* Min proteins, and the dynamic assembly of the *B. subtilis* MreB cytoskeleton have been observed *in vivo* with the aid of FPs (Raskin *et al.,* 1999; Carballido-Lopez and Errington, 2003). In addition, fluorescence technology in the last decade has offered critical insights into the dynamics and cellular localization of FtsZ (Vicente *et al.,* 2006). With the aid of the eGFP, FtsZ was found to assemble into highly mobile intermediate helices or spirals *in vivo* that act as precursors and reorganize to form the Z ring prior to cell division (Ben-Yehuda and Losick, 2002; Thanedar and Margolin, 2004; Grantcharova *et al.,* 2005; Michie *et al.,* 2006). Furthermore, Stricker *et al.,* (2002) used fluorescence recovery after photobleaching (FRAP) to show that the Z ring is a highly dynamic structure with continual exchange of subunits between the ring and the monomers within the cytoplasm.

1.11 Research barriers

The dynamic regulation of cell division in *Streptomyces* emphasizes an intriguing process that requires further attention. Many unique division proteins have been identified, but exactly how they alter the cell division process remains elusive. A major challenge is to assess the numerous and intricate interactions between the different cell division proteins as they occur within the cells. Although most interactions that have been established through *in vitro* two-hybrid experiments do provide insight into function, they do not necessarily concur with what happens within the cell. *In vitro* isolation may force these proteins to interact or form aggregates and thus not reflect exactly how they behave within the cells. To better understand the exact role of division proteins in *S. coelicolor*, it is necessary to determine how, when and where these proteins are distributed within the cell with respect to each other. Unfortunately, the tools for multi-colour tracking of different proteins simultaneously in *S. coelicolor* are not yet available.

Although much has been elucidated about cell division using FPs, only a single FP, the eGFP, has been used for studies in *S. coelicolor*. The initial use of the GFP as a reporter in *S. coelicolor* failed due to the difference in codon usage between the relatively AT-rich *gfp* gene and the GC-rich streptomycete genome, which resulted in poor translation of the GFP (Sun *et al.,* 1999). Nevertheless, since then, the eGFP - a GC-rich variant of the original *gfp* (Haas *et al.,* 1996), has proven itself to be an invaluable tool for biological investigation of *S. coelicolor* (Sun *et al.,* 1999). Since its introduction into *S. coelicolor* by Sun and coworkers (1999), it has rapidly become the preferred tool in the field, finding applications in the study of cell type specific gene expression (Kelemen *et*

al., 2001; O'Connor *et al.*, 2002), chromosome segregation (Jakimowicz *et al.*, 2005; 2006; Mazza *et al.*, 2006), and cell division (Flardh, 2003a, b; Grantcharova *et al.*, 2005; Del Sol *et al.*, 2006). However, having only one fluorescent tag limits our studies to only one gene or one protein at a time. Furthermore, *S. coelicolor* exhibits high levels of autofluorescence in the green spectral region, making it difficult to distinguish between real fluorescent signals and background autofluorescence. Therefore, additional FPs are needed to supplement the already established eGFP and facilitate future analyses of the complex cell division process.

1.12 Research objectives

FP tools promise to be useful in the study of cellular functions in *Streptomyces* as it will allow for direct visualization of protein dynamics, formation of protein complexes, analysis of co-localization and protein-protein interactions. However, the tools for such analyses are lacking as only a single FP is currently available for use in *S. coelicolor*. Therefore, the primary goal of this work is to increase the number of available FPs for use in *S. coelicolor*.

The optimization of various parental FPs, such as the GFP to eGFP, has led to the development of much improved variants predicted to work well in *S. coelicolor* because they have been improved for intensity and have a higher-GC content than their parents. To begin, we have chosen three FPs: mRFP, CyPet and YPet. We were particularly interested in these FPs because, in conjunction with the eGFP, they represent a good spectral range (from green to red) for analysis as these spectrally distinct variants will allow for co-localization studies via multi-colour tracking. An interesting possibility with the establishment of these three FPs is the development of fluorescence resonance energy transfer (FRET; Festy *et al.,* 2007), a technique previously unavailable to *Streptomyces* due to the limited number of FPs. FRET has been used extensively in other organisms, including *E. coli* and *B. subtilis*, to investigate protein-protein interactions (Sourjik and Berg, 2002; Kidane and Graumann, 2005). Therefore, once established, the three additional FPs will offer several donor/acceptor pairs that are suitable for FRET, including the eGFP/mRFP and CyPet/YPet pairs (Peter *et al.,* 2005; Nguyen and Daugherty, 2005).

In order to test the utility of the three chosen FPs, translational fusions to DivIVA, a protein involved in driving tip growth, will be constructed and fluorescence patterns accessed. DivIVA localization has been well studied using fluorescence microscopy through translational fusion to the eGFP (Flardh, 2003b). By comparing the localization of our fusion proteins to these previously established localization patterns, we will determine if these FPs will express and fluoresce in *S. coelicolor* in a manner suitable for routine genetic analysis.

2.0 MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Cultivation conditions for the different bacterial strains (Table 2.1) were performed as described previously for *E. coli* (Sambrook *et al.*, 1989) and *Streptomyces* (Kieser *et al.*, 2000). *E. coli* strains XL1 Blue and ET12567/pUZ8002 were cultivated in liquid Luria-Broth (LB) or on solid LB agar medium at 37°C. XL1 Blue cells were used for plasmid propagations and general DNA recombinant methods, while ET12567/pUZ8002 was used for conjugation with *Streptomyces* (Flett *et al.*, 1997). *S. coelicolor* strain M145 was cultivated on solid mannitol soy flour (MS) agar, or in yeast extract (R2YE) solid medium at 30°C. All media were supplemented with the appropriate antibiotics with final concentrations of 50μg/mL apramycin, 25μg/mL nalidixic acid, 25μg/mL kanamycin, and 25μg/mL chloramphenicol.

For the introduction of plasmids (Table 2.1) into *S. coelicolor*, the plasmids were first transformed into the methylation-deficient *E. coli* strain ET12567/pUZ8002 and then transferred into *S. coelicolor* via conjugation (Flett *et al.*, 1997; Kieser *et al.*, 2000). Following conjugation, exconjugants were selected by overlaying the plates with nalidixic acid (25μg/mL) and apramycin (50μg/mL). Single colonies of exconjugants were restreaked, twice, on R2YE supplemented with nalidixic acid (25μg/mL) and apramycin (50μg/mL) to ensure the integrity of the strain. A single colony was then streaked onto R2YE without any antibiotics for the preparation of a spores stock (Kieser *et al.*, 2000).

Table 2.1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference
Plasmids		
pBluescript II KS(+)	Amp^r, cloning vector	Sambrook *et al.*, 1989
pIJ8660	eGFP	Sun *et al.*, 1999
pMU-2	mRFP	This work
pRdiv-1	DivIVA-mRFP fusion	This work
pMU-4	CyPet and YPet	This work
pMU-4A	DivIVA-CyPet fusion	This work
pMU-4B	DivIVA-YPet fusion	This work
pMU-4C	DivIVA-CyPet and DivIVA-YPet Fusions	This work
Streptomyces coelicolor		
M145	Prototroph $SCP1^-$, $SCP2^-$	Kieser *et al.*, 2000
KN1	M145 containing pMU-2	This work
KN2	M145 containing pRdiv-1	This work
KN3	M145 containing pMU-4	This work
KN4	M145 containing pMU-4A	This work
KN5	M145 containing pMU-4B	This work
KN6	M145 containing pMU-4C	This work
Escherichia coli		
XL1 Blue	*recA1 endA1 gyrA96 thi-1 glnV44 hsdR17 supE44 relA1 lac [F' proAB lacIq Z_M15 Tn10 (Tetr)*	Stratagene
ET12567/pUZ8002	*dam::Tn9 dcm6 hsdM hsdR recF143 galK2 galT22 zjj201::Tn10 ara14 lacY1 syl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44 pUZ8002*	Flett *et al.*, 1997

2.2 Recombinant DNA methods

S. coelicolor strain M145, and *E. coli* strains XL1 Blue and ET12567/pUZ8002 were grown as described previously (Kieser *et al.*, 2000; Sambrook *et al.*, 1989). Genomic DNA was isolated from *S. coelicolor* M145 as described (Kieser *et al.*, 2000) and used as the template for the amplification of the *divIVA* gene. General molecular techniques were performed as previously described (Kieser *et al.*, 2000; Sambrook *et al.*, 1989). Plasmid DNA was extracted from overnight *E. coli* cultures either by using or the Qiagen QIAprep Spin Miniprep Kit or the Qiagen Plasmid Midi Kit (Qiagen Inc., Mississauga, ON).

All restriction enzymes were obtained and used as suggested by the manufacturer, New England Biolabs (Pickering, ON), unless otherwise specified.

Ligation reactions were performed using T4 DNA ligase (New England Biolabs) and were left at room temperature overnight. *E. coli* transformations were performed via heat shock at 42°C for 45-60 seconds and incubated at 37°C for at least 1 hour before plating.

PCR reactions, digestion reactions, and gel-purified products were all run on 0.8% agarose in 1x TBE at ~120V for roughly 1.5-2.0 hours. Molecular markers used were the 1.0 kb ready to use DNA ladder (United Bioinformatica Inc., Calgary, AB) and 100 bp DNA ladder (New England Biolabs). Agarose gels were stained in ethidium bromide, and DNA fragments were excised from the agarose gels and purified using the Qiagen

QIAEX II Gel Extraction Kit (Qiagen Inc., Mississauga, ON) according to manufacturer's instructions.

2.3 Gene amplification

The genes encoding mRFP, DivIVA, CyPet and YPet were amplified via Polymerase Chain reaction (PCR). *divIVA* was amplified from *S. coelicolor* M145 chromosomal DNA, whereas *mRFP*, *CyPet* and *YPet* were cloned from pmRFP-C1 (Clontech), pLNCyPetMAMM, and pLNYPetMAMM (obtained from Dr. Ray Truant, McMaster University, Hamilton, ON), respectively. Primers used in all PCR reactions (Table 2.2) were synthesized at the MOBIX facility (McMaster University, Hamilton, ON). After PCR, the *mRFP*, *divIVA*, *CyPet* and *YPet* coding sequences were sequenced at the MOBIX facility.

All PCR amplifications were performed by a touchdown PCR program with varying annealing temperatures for each gene. PCR reactions were set up according to general procedures in a 50μL sample volume and carried out using either *Pfu* or *Vent* polymerase (Fermentas).

Generally, reactions were set up as follows: all reagents except for the polymerase were added. A hot start is performed where reactions were incubated at 95°C for 5 minutes prior to the addition of DNA polymerase. The PCR program was designed to have at least 30 cycles with decreasing annealing temperatures, and elongation time varied based on gene length and polymerase used. A sample PCR program, used to amplify YPet, is shown below.

1) 1x 95°C, 5 minutes (hot start)
2) Polymerase added (*Vent* polymerase)
3) 3x 95°C, 30 seconds; 55°C, 30 seconds; 72°C, 1 minute
4) 3x 95°C, 30 seconds; 53°C, 30 seconds; 72°C, 1 minute
5) 3x 95°C, 30 seconds; 51°C, 30 seconds; 72°C, 1 minute
6) 3x 95°C, 30 seconds; 49°C, 30 seconds; 72°C, 1 minute
7) 20x 95°C, 30 seconds; 47°C, 30 seconds; 72°C, 1 minute
8) 1x 72°C, 5 minutes
9) Hold at 4°C

Table 2.2. Primers and oligonucleotides. The forward and reverse primers used to PCR-amplify various genes, and oligonucleotides used for the multiple cloning sites in pMU-4.

Gene	Size (~bp)	Primer Name	Primer Sequence (5'→3')
mRFP	750	ML14633 (Forward)	GTCGCCCATATGGCCTCCTCCGAG GAC
		ML14634 (Reverse)	TATGATGCGGCCGCTCAGTTAT CTAGATCCGGT
divIVA[1]	1310	ML15034 (Forward)	TGATCATCGTCTACATCCTGA
		ML15035 (Reverse)	CGTACTACCATATGGTTGTCGTC CTCGTCCA
divIVA[2]	1310	ML20634 (Forward)	TACATCCAGATCTCGATCGTGAG
		ML20635 (Reverse)	CGTACTACACTAGTGTTGTCGTC CTCGTC
CyPet	750	ML19865 (Forward)	GATCCAGATATCGCCACCATGGT GA
		ML19866 (Reverse)	TTATTGGATCCATTTATTATTTGT ACAG
YPet	750	ML19863 (Forward)	GTCGCCCATATGGTGAGCAAAGGC
		ML19864 (Reverse)	TCGATGGCGGCCGCTTTACTTATA GA
*MCS1	30	ML20182	GATCTACGTCTAGACGGGCCGTAT ACCCACCGGAT
		ML20183	ATCCGGTGGGTATACGGCCCGTCT AGACGTA
*MCS2	40	ML20180	GATCCCTAACTAGTCAGTCGAATA TTCGTCAGAGGCCTGATCA
		ML20181	TATGATCAGGCCTCTGACGAATAT TCGACTGACTAGTTAGG

[1] *divIVA* gene cloned into pMU-2 to produce pRdiv-1
[2] *divIVA* gene used to make translational fusions to CyPet and YPet
*MCS1/2 = multiple cloning sites upstream of CyPet, and YPet in pMU-4, respectively

2.4 Plasmid construction

2.4.1 pMU-2

To construct pMU-2, *mRFP* was PCR amplified from the plasmid pmRFP-C1 (Clontech). *Pfu* polymerase (Fermentas) and the primers ML14633 and ML14634 (Table 2.2) generated a ~750bp fragment. The PCR amplified fragment included an upstream *Nde*I restriction site a downstream *Not*I restriction site. These sites were specifically designed so that they were suitable for the replacement of the *egfp* gene in the plasmid pIJ8660 (Sun *et al.*, 1999). The fragment was purified from the agarose gel and ligated to *Eco*RV-digested pBluescript using T4 DNA ligase (Fermentas). The gene was excised out of pBluescript as an *Nde*I-*Not*I fragment, gel-purified, and ligated to pIJ8660 cut with the same enzymes to generate pMU-2 (Nguyen *et al.*, 2007).

2.4.2 pRdiv-1

pRdiv-1 is the plasmid construct which contains the *S. coelicolor divIVA* gene translationally fused at its C terminus to *mRFP*. To construct this plasmid, the *divIVA* coding region and its upstream regulatory region (~1300 bp) was amplified from *S. coelicolor* M145 chromosomal DNA using *Pfu* polymerase (Fermentas) and primers ML15034 and ML15035 (Table 2.2). The reverse primer was designed so that the stop codon of *divIVA* was replaced with an *Nde*I restriction site in the PCR product. The amplified DNA was ligated to pBluescript, and subsequently excised as a *Hind*III-*Nde*I fragment. This fragment was produced by first cutting with *Hind*III, end-filled with *Klenow* polymerase (New England Biolabs), and then cut with *Nde*I. The *Hind*III-*Nde*I

divIVA fragment was ligated into pMU-2 (Nguyen *et al.*, 2007), cut with *Eco*RV and *Nde*I, to generate pRdiv-1 (Nguyen *et al.*, 2007).

2.4.3 pMU-4

To construct this plasmid, pMU-2 was used as the backbone. The coding sequences for *CyPet* and *YPet* (both ~750 bp) were PCR amplified from their respective templates (pLNCyPetMAMM and pLNYPetMAMM) using *Vent* DNA polymerase (Fermentas). The primers used were ML19865 and ML19866 for *CyPet* and ML19863 and ML19864 for *YPet* (Table 2.2). The primers were designed such that the PCR amplified *CyPet* fragment contained an N-terminal *Eco*RV site and C-terminal *Bam*HI site. Likewise, the *YPet* fragment contained an N-terminal *Nde*I site and a C-terminal *Not*I site. After cloning into pBluescript and the integrity of the coding sequences were verified (as above), the fragments were introduced into the proper backbone plasmid. The restriction sites on the ~750bp *YPet* fragment were suitable for the replacement of the *mRFP* gene in the plasmid pMU-2 (Nguyen *et al.*, 2007), to yield pMU-2b. Subsequently, the *CyPet* fragment was introduced upstream of *YPet* in pMU-2b by using the *Eco*RV and *Bam*HI sites to generate pMU-2c. The next step was to introduce a unique MCS upstream of both *CyPet* and *YPet* in pMU-2c. Pairs of oligos were ordered such that when annealed together would yield a double stranded DNA fragment which contained designed restriction sites. The oligos were annealed in 1 mL of annealing buffer which consisted of 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-Cl pH8.0, dH_20, and varying concentrations of oligos (from 0.001-0.01 mM). The reactions were then placed

into a heat block, heat up to 95°C where it remained for 2 minutes before letting the temperature go back down to room temperature by turning off the heat block.

For MCS1 (to be placed upstream of *CyPet*), the oligos ML20182 and ML20183 (Table 2.2) were annealed together to form a dsDNA fragment, which contained the restriction sites *Bgl*II, *Xba*I, *BstZ17*I and *Eco*RV (5'→3'). In between the restriction sites is a 3-6 nucleotide spacer, as suggested by the NEB catalog, to allow for maximum cutting efficiency. This MCS1 fragment was ligated into pMU-2c that had been cut with *Bgl*II and *Eco*RV to yield pMU-2d. Similarly, ML20180 and ML20181 (Table 2.2) were annealed together to form MCS2 (to be placed upstream of *YPet*), which contained the restriction sites *Bam*HI, *Spe*I, *Ssp*I, *Stu*I, and *Nde*I (5'→3'). This fragment was then introduced into pMU-2d to generate pMU-4.

2.5 Constructing divIVA-CyPet and divVIA-YPet translational fusions

To construct translational fusions, where the *S. coelicolor divIVA* gene is translationally fused at its C terminus to CyPet or YPet, the *divIVA* coding region and its promoter region was amplified from *S. coelicolor* M145 chromosomal DNA using *Pfu* polymerase (Fermentas) and primers ML20634 and ML20635 (Table 2.2). The PCR amplified *divIVA* fragment included an N-terminal *Bgl*II restriction site a C-terminal *Spe*I restriction site.

The specific restriction sites chosen for the two upstream MCSs allowed for the insertion of this *Bgl*II-*Spe*I *divIVA* fragment into pMU-4 at two different positions so that it is translationally fused to both *CyPet* and *YPet*. The restriction sites in MCS1 have overhang sequences that are compatible to the restriction sites in MCS2. Therefore, the same *Bgl*II-*Spe*I *divIVA* fragment was inserted upstream of *CyPet* in pMU-4 via *Bgl*II and *Xba*I, and upstream of *YPet* via *Bam*HI and *Spe*I.

2.6 Fluorescence microscopy

For visualization of the fusion proteins, *S. coelicolor* strains carrying the appropriate plasmids were grown on glass coverslips inserted at a 45° angle in solid R2YE medium as described previously (Schwedock *et al.*, 1997). After the desired incubation at 30°C, coverslips with cells were mounted onto glass slides containing 15 µL of 50% glycerol. The edges of the coverslips were sealed with clear nail polish to prevent movement. For the time-course experiments, spores were pre-germinated in double strength germination medium and then grown in Tryptone Soya Broth (TSB) for 2 to 24 hours (Kieser *et al.*, 2000; Flardh, 2003b). About 15-20 µL of germinated spores were removed from liquid culture and mounted directly onto glass slides coated with 1% agarose in phosphate-buffered saline (PBS) and covered with coverslips (Flardh, 2003b). Cells were visualized by differential interference contrast (DIC) microscopy and fluorescence deconvolution microscopy.

Fluorescence microscopy was performed at the McMaster University Biophotonics Facility (McMaster University, Hamilton, ON) using the Leica DMI 6000B widefield fluorescence microscope. This microscope was equipped with the appropriate filter sets required for this study (Texas Red, Cyan, Yellow, EGFP, and DAPI filter sets), and connected to a Hamamatsu 1394 ORCA-ER digital camera, and Volocity software for data acquisition (Version 3 for mRFP work and updated to Version 4 for CyPet and YPet work; Improvision). All images were acquired by first obtaining the differential interference contrast (DIC) image using a 100x objective. The same field of view was then illuminated using the appropriate filter set and fluorescent images were acquired after 300-500 ms exposure. Fluorescence images were subjected to deconvolution to improve the signal to noise ratio. Acquired images in TIFF format were analyzed using the Iterative Deconvolution Algorithm from Volocity deconvolution software (Versions 3 and 4 as above; Improvision) and processed using Adobe Photoshop 7.0.

3.0 RESULTS AND DISCUSSION

3.1 Construction of an mRFP shuttle vector for Streptomyces

The introduction of the eGFP into *S. coelicolor* (Sun *et al.,* 1999) has allowed for greater understanding of growth and development of this organism. Since then, the eGFP has been accepted as an indispensable tool in the field, finding multiple applications (Kelemen *et al.*, 2001; O'Connor *et al.*, 2002; Del Sol *et al.*, 2006; Flardh, 2003b; Grantcharova *et al.*, 2005; Mazza *et al.*, 2006; Jakimowicz *et al.*, 2005; 2006; 2007). Although the eGFP has proven itself to be an invaluable tool for biological investigation of *S. coelicolor*, having only one fluorescent tag limits fluorescence studies to one gene or protein at a time. To facilitate future analyses of this complex organism, alternative fluorescent proteins that will accompany or substitute the already established eGFP are required. By extending the spectrum of available colours, multi-colour tracking of fusion proteins will be possible. The newly adapted fluorescent proteins will be used for localization and co-localization to study many aspects of cellular function.

The most attractive candidate to supplement the already established eGFP in *S. coelicolor* is the monomeric red fluorescent protein (mRFP) that was developed from DsRed (Campbell *et al.,* 2002; Shaner *et al.,* 2004). The mRFP matures much faster than DsRed, therefore, will be very useful in time sensitive experiments. The red-shifted mRFP will also confer greater tissue penetration and be less toxic to cells during imaging since its excitation maxima is outside of the UV range. Greater spectral separation from autofluorescence and other FPs should also result due to its red-shifted nature (Campbell

et al., 2002). To apply *mRFP* to protein localization in *S. coelicolor*, the vector pMU-2 (Figure 3.1; Nguyen *et al.,* 2007) was constructed by replacing the *egfp* gene from pIJ8660 (Sun *et al.*, 1999) with an *mRFP* gene generated by PCR. In pMU-2 the *mRFP* gene is flanked by two transcription terminators, *tfd* and t_0 from phage fd and phage λ, respectively. Upstream of the *mRFP* coding region is a MCS which has several unique restriction sites for the introduction of cloned promoters or genes. pMU-2 also carries an apramycin-resistance gene, *acc(3)IV*, the pUC18 origin of replication for growth in *E. coli*, an RK2 origin of transfer for direct conjugation from *E. coli* to *Streptomyces*, and the ϕC31 *int* gene and *attP* site for site-specific integration of the vector into the *attB* attachment site in the *Streptomyces* chromosome.

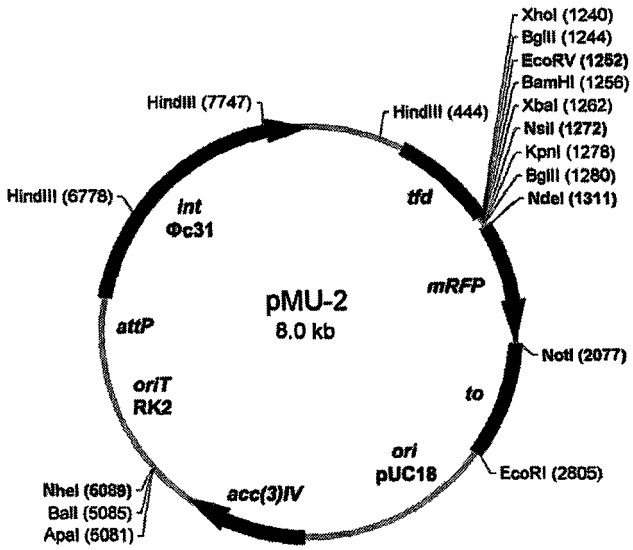

Figure 3.1. Restriction map of pMU-2. The vector contains: *tfd*, major transcription terminator of phage fd; *to*, transcription terminator from phage λ; *aac(3)IV*, apramycin-resistance gene selectable in *E. coli* and streptomycetes; *ori* pUC18, origin of replication from pUC18; and *oriT* RK2, origin of transfer from plasmid RK2; and *int* ΦC31 and *attP*, the integrase gene and attachment site of the temperate phage ΦC31, respectively. From Nguyen *et al.*, (2007).

The successful construction of pMU-2 will facilitate future studies of protein localization and gene expression. For protein localization, genes of interest can be introduced into pMU-2 as translational fusions to mRFP. Once introduced into *S. coelicolor*, the plasmid will integrate into the *Streptomyces* chromosome, and the expression of the fusion protein will be driven by the natural upstream regulatory region of the target gene.

3.2 Testing the functionality of mRFP in Streptomyces

To test the utility of mRFP in *Streptomyces*, the *divIVA* gene along with its promoter region was PCR-amplified and inserted into pMU-2, such that it was translationally fused to the mRFP-encoding gene. The resulting vector, pRdiv-1 (Nguyen *et al.*, 2007), was introduced into *S. coelicolor* strain M145 via conjugation and subjected to fluorescence microscopy. Spores bearing pRdiv-1 were grown on glass coverslips in solid medium as described previously (Schwedock *et al.*, 1997). Coverslips containing *S. coelicolor* cells were mounted onto glass slides and imaged first by differential interference contrast (DIC) microscopy. The fluorescence images were obtained after 300-500ms exposure and subjected to deconvolution to improve the signal to noise ratio.

The images in Figure 3.2 (Nguyen *et al.*, 2007) illustrate the localization of DivIVA-mRFP in *S. coelicolor* during vegetative growth. A cluster of growing hyphal cells, including numerous tips, can be observed in the DIC image (Figure 3.2A). Upon fluorescence illumination of this field, more than 20 small red fluorescent foci can be seen (Figure 3.2B). When the two images (DIC and fluorescence) were superimposed, it showed clear positioning of the foci at the hyphal tips of the growing cells (Figure 3.2C). Clear red fluorescence was found mostly at the tips, however, smaller spots of weak fluorescence were also observed along the length of the hyphae. It has been suggested that these weaker fluorescence spots probably correspond to nascent branch points (Flardh, 2003b). Since DivIVA is essential for tip growth, it is highly possible that it is also required for the initiation of lateral outgrowths (i.e. the establishment new branches). Indeed, lateral branches have been shown to emerge from areas on the hyphal length

where DivIVA has accumulated (Klas Flardh, personal communications). Control cells, containing pMU-2 lacking an inserted gene, did not exhibit any tip fluorescence (data not shown), indicating that the mRFP signal was linked to DivIVA expression. These data are consistent with previous observations made by Flardh using the DivIVA-eGFP fusion (Flardh, 2003b).

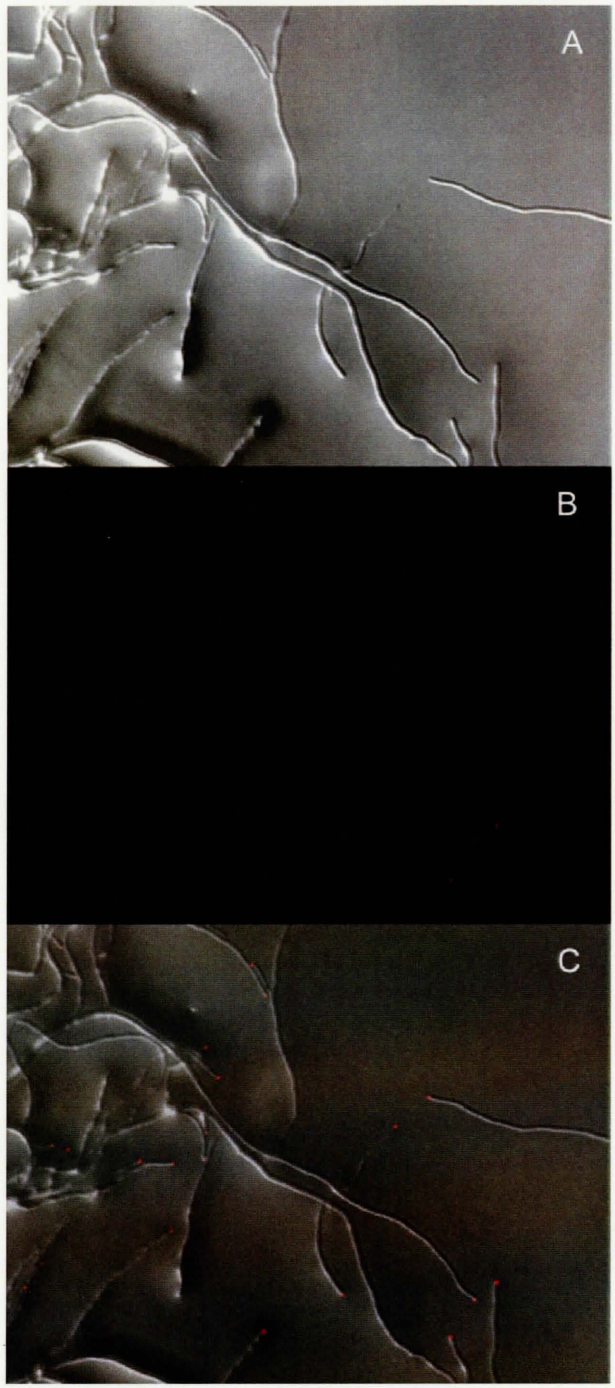

Figure 3.2. Subcellular localization of DivIVA-mRFP in *S. coelicolor*. (A) A DIC image depicting live *S. coelicolor* cells carrying the pRdiv-1 plasmid. (B) The fluorescence image taken when the same field of view was illuminated. (C) A merged image (DIC+fluorescence) showing the tip localization of DivIVA-mRFP in *S. coelicolor*. From Nguyen *et al.*, (2007).

3.3 Time-course experiments to monitor DivIVA-mRFP localization

Time-course experiments carried out by Flardh and coworkers, using the DivIVA-eGFP fusion, revealed that germinating spores express *divIVA* prior to the emergence of a germ tube. The DivVIA proteins then organize into one or two distinct spots within in the spore. From these spots germ tubes emerged and the DivIVA focus is associated with the tip of this growing cell from the earliest stages throughout growth (Flardh, 2003b). To determine whether the DivIVA-mRFP fusion could reproduce this behaviour, spores were germinated in liquid medium and examined as a function of time. Spores of *S. coelicolor* containing DivIVA-mRFP were germinated and grown in liquid medium, mounted on glass slides, and imaged at various stages of growth (Figure 3.3; Nguyen *et al.*, 2007). In dormant spores, red fluorescent foci were absent (Figure 3.3A), but after approximately two hours of germination at least one clear red fluorescent focus was observed in each of the spores; most likely the future sites for germ tube emergence (Figure 3.3B). After four hours, the emergence of germ tubes was clearly visible and the leading edge of each outgrowth exhibited a red fluorescent focus (Figure 3.3C). Eight hours following germination, distinct hyphal cells with red fluorescence mainly at their tips could be seen (Figure 3.3D). These tip-associated foci were highly stable with continued growth and branching (Figure 3.3E-F). A control containing only pMU-2 did not display similar fluorescence signals, indicating that the mRFP signal was linked to DivIVA expression (data not shown).

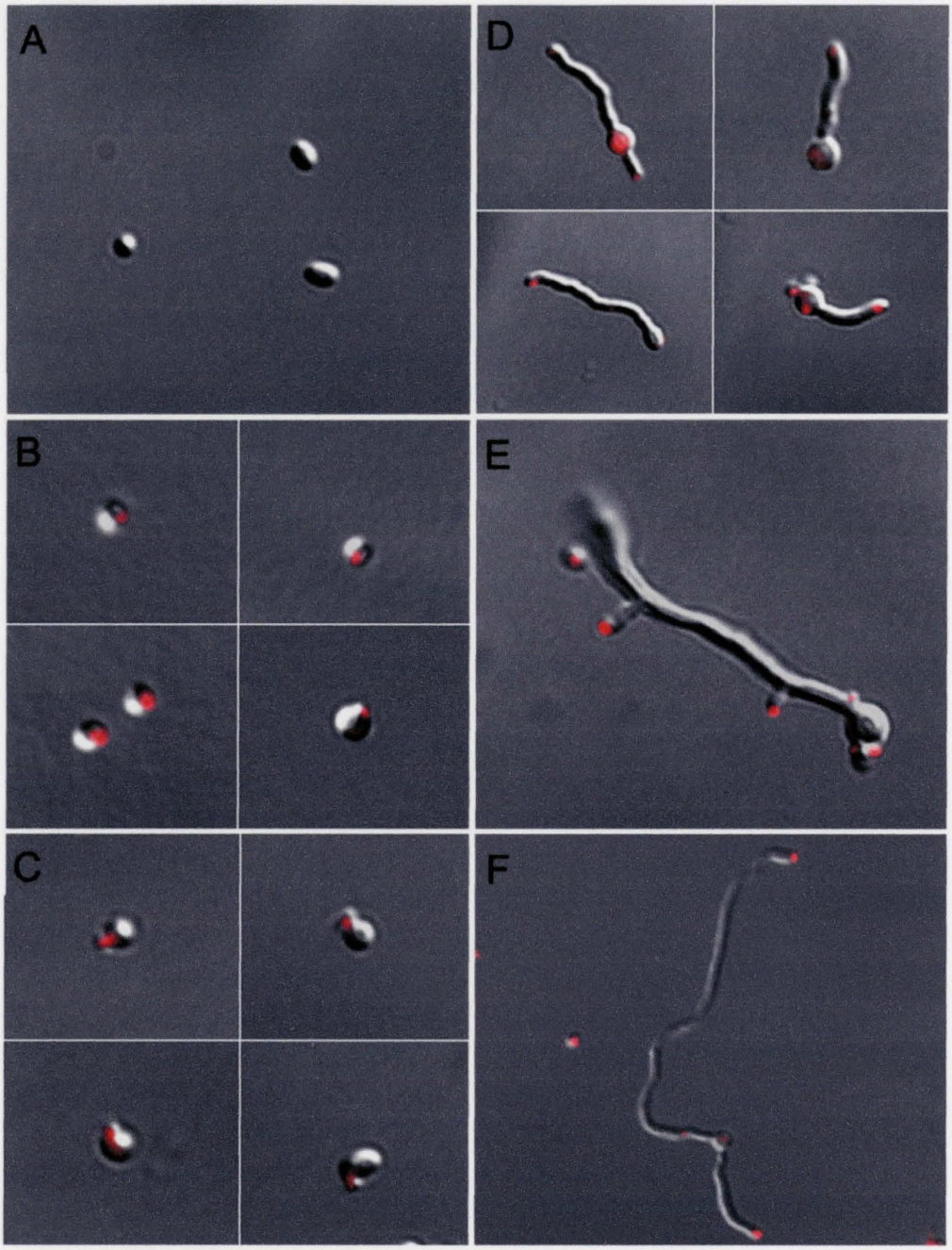

Figure 3.3. A time-course showing the emergence and propagation of the DivIVA-mRFP signal in *S. coelicolor* cells during spore germination. *S. coelicolor* spores bearing the pRdiv-1 plasmid were germinated and imaged at different stages of growth. Images in panels A, B, C, D, E, and F correspond to images of spores germinated for 0, 2, 4, 8, 12 and 20 hours, respectively. All images shown are overlay of fluorescence images on DIC images. From Nguyen *et al.,* (2007).

These results reproduced exactly the observations made by Flardh and coworkers (2003b) with the DivIVA-eGFP fusion, suggesting that mRFP was fully functional and relatively benign to protein function in *S. coelicolor*. The time-course experiments showed that mRFP could be detected early and that the integrity of its signal was maintained throughout growth. The fact that mRFP matures more than ten times faster than its parent DsRed (Campbell *et al.*, 2002) was instrumental for our observations in the earliest stages of sporulation. Most importantly, the result of this work established mRFP as a remarkably good reporter for sub-cellular localization of proteins in *S. coelicolor*. The mRFP together with the eGFP will allow for multi-colour tracking, co-localization and examination of protein-protein interactions through FRET (Peter *et al.*, 2005).

3.4 Testing the utility of other FPs in S. coelicolor

The extension of the spectrum of available colours to red wavelengths (mRFP) provided a distinct label that can now be used for multicolour tracking in *S. coelicolor*. However, GFP's cyan and yellow variants have found more widespread use as markers for localized proteins and gene expression (Shaner *et al.*, 2007). Two FPs in particular have sparked great interest due to their recent optimization. This pair includes a cyan FP, known as CyPet, and a yellow FP called YPet (Nguyen and Daugherty, 2005). These FPs hold great promise as spectrally distinct companions or substitutes for the already established eGFP and mRFP in *S. coelicolor*. An additional benefit is the fact that they have been specifically engineered for FRET, a technique our lab intends to employ in the near future. Together, these FPs will broaden the range of FP applications in *Streptomyces*.

3.4.1 Construction of a CyPet/YPet shuttle vector for Streptomyces

To apply *CyPet* and *YPet* to protein localization in *S. coelicolor*, the vector pMU-4 was constructed (Figure 3.4). This vector contains *CyPet* and *YPet* in head-to-tail orientation between two major transcription terminators. Upstream of both *CyPet* and *YPet*, unique MCSs were introduced so that target genes or promoters can be inserted. The design of the MCSs was very specific because in addition to having unique cut sites, the two MCSs contain restriction sites with compatible ends. For example, the *Bgl*II site upstream of CyPet has compatible ends with *Bam*HI upstream of YPet; *Xba*I is compatible with *Spe*I, and so forth. This is extremely useful as it will allow for easier construction of fusion proteins, removal of either *CyPet* or *YPet*, and swapping of target genes/promoters if necessary.

The shuttle vector was built by first replacing *mRFP* in pMU-2 (Nguyen *et al.*, 2007) with *YPet*. *CyPet* and the two MCSs were then introduced to create pMU-4. Since pMU-4 has the same backbone as pMU-2, all the features remain the same: it has two transcription terminators, *tfd* and t_0 from phage fd and phage λ, respectively; an apramycin-resistance gene, *acc(3)IV*; the pUC18 origin of replication for growth in *E. coli*; an RK2 origin of transfer for direct conjugation from *E. coli* to *Streptomyces*; and the ϕC31 *int* gene and *attP* site for site-specific integration of the vector into the *Streptomyces* chromosome.

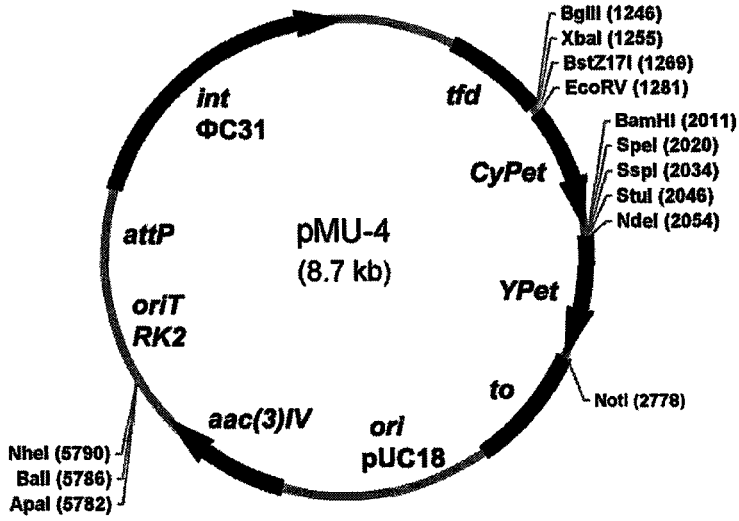

Figure 3.4. The plasmid pMU-4 built to test the utility of CyPet and YPet in *S. coelicolor*. *CyPet* and *YPet* were cloned between the two major transcription terminators: *tfd* from phage fd, and *to* from phage λ. This plasmid contains an apramycin-resistance cassette selectable in both *E. coli* and streptomyces, *aac(3)IV;* an origin of replication from pUC18, *ori* pUC18; an origin of transfer from plasmid RK2, *oriT* RK2; and the *int* ΦC31 integrase gene and the *attP* attachment site of the temperate phage ΦC31. Unique MCSs were introduced for the cloning of target genes and/or promoters.

3.4.2 Testing the utility of CyPet and YPet in Streptomyces

Like mRFP, the utility of CyPet and YPet has not been demonstrated previously in *S. coelicolor*. To test their functionality in this organism, the DivIVA protein was used as a fusion partner due to its robust localization at the tip of growing cells. The *divIVA* gene and its upstream promoter region was PCR-amplified and cloned into pMU-4 to create three different constructs (Figure 3.5). One of the constructs shown (Figure 3.5A) refers to the backbone vector containing only the promoterless CyPet and YPet (i.e. pMU-4) which will be used as a control to measure background fluorescence. The other three constructs contain DivIVA translationally fused to the different FPs. In pMU-4A, the

divIVA gene was cloned into pMU-4 so that *CyPet* was translationally fused to its C terminus, and *YPet* was left without a fusion partner. The opposite was true for construct pMU-4B, where *YPet* was fused to *divIVA* and *CyPet* was partnerless. In the last construct, pMU-4C, both *CyPet* and *YPet* had their own copy of the *divIVA* gene fused to them.

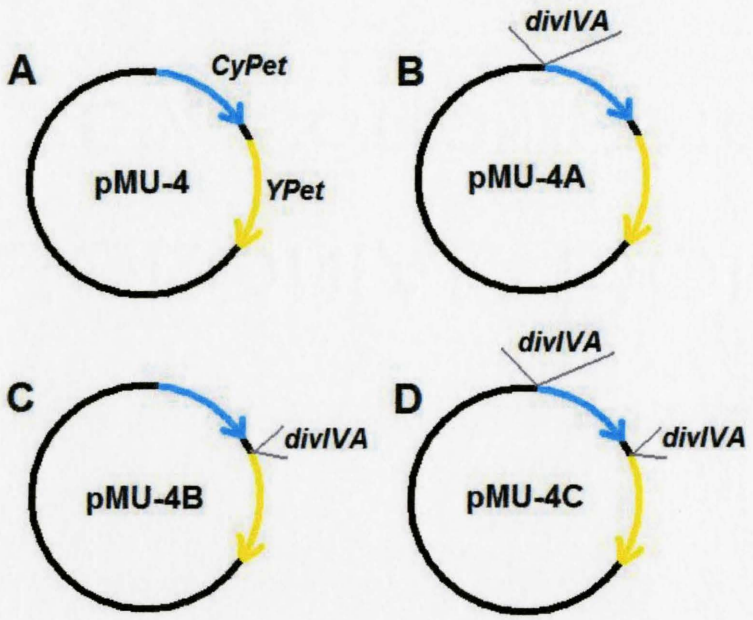

Figure 3.5. The different translational fusions constructed to test the functionality of CyPet and YPet in *S. coelicolor*. One of the constructs (A) is a simplistic representation of pMU-4, which was used as a control for measuring autofluorescence. The other three constructs contain DivIVA translationally fused to either CyPet (B), or YPet (B) or both (D). Each plasmid was introduced into *S. coelicolor* and imaged under the appropriate channels.

Prepared *S. coelicolor* cells harbouring each of the four different plasmids were imaged using the wide-field fluorescence microscope. *S. coelicolor* cells which contained the pMU-4A plasmid showed strong fluorescent signals at the tips under the cyan filter channel (Figure 3.6C) but not under the yellow filter channel. When cells containing the pMU-4B plasmid were imaged, localized tip fluorescence was observed in the yellow channel (Figure 3.6D) but not the cyan channel. In contrast, distinct tip fluorescence was not observed, but delocalized autofluorescence was observed, in control cells that contained only the backbone pMU-4 plasmid (Figure 3.6AB). These observations were consistent with what was expected since only those FPs that contained a fusion partner fluoresced under the appropriate filter channels. The control cells only showed delocalized autofluorescence because the two FPs in the pMU-4 vector were promoterless. The obtained results clearly indicated that the DivIVA-CyPet and DivIVA-YPet fusions were expressed and functional in *S. coelicolor* and that the FPs did not interfere with the function of DivIVA, and vice versa. Therefore, two additional fluorescent proteins can now be used in *S. coelicolor* bringing the overall total to four.

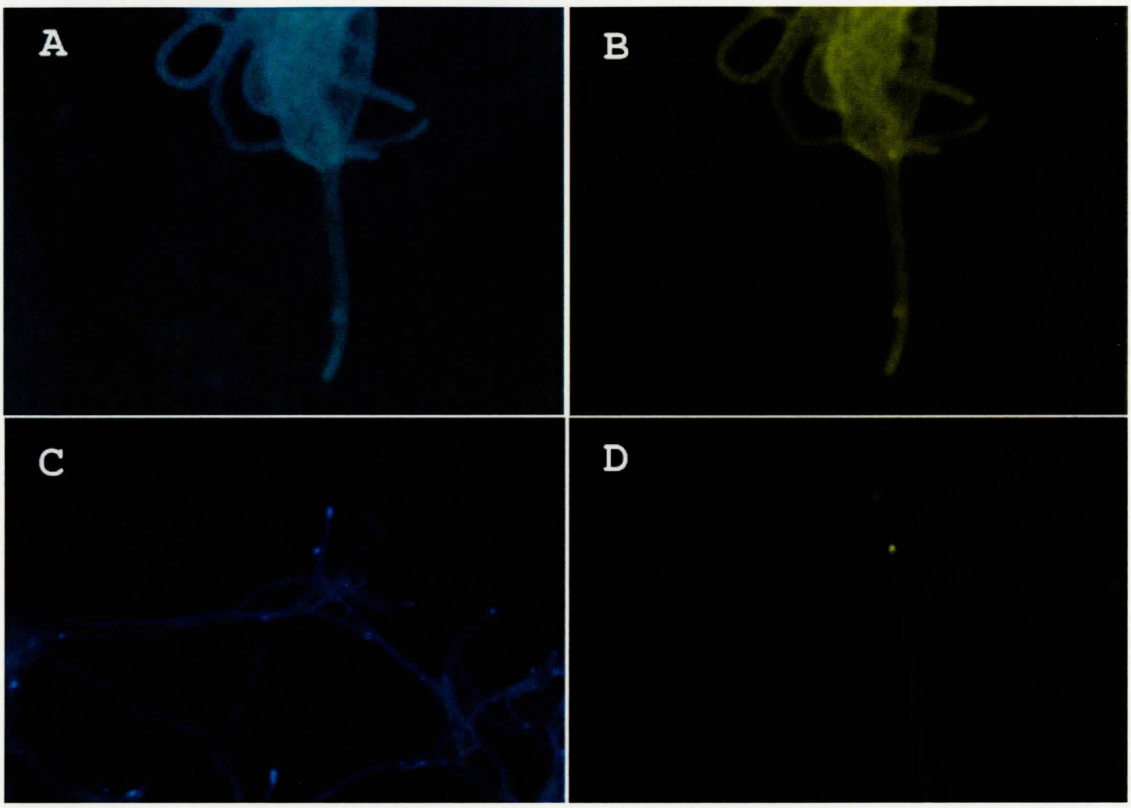

Figure 3.6. Fluorescence images of *S. coelicolor* cells harbouring either the DivIVA-CyPet or the DivIVA-YPet translational fusion. Images of cells containing the backbone pMU-4 vector were taken under the cyan (A) and yellow (B) channels to measure autofluorescence. (C) Cells containing the pMU-4A plasmid (DivIVA-CyPet) were imaged using the cyan filter. (D) Cells containing the pMU-4B plasmid (DivIVA-YPet) were imaged using the yellow filter. Only those cells containing FPs with DivIVA as a fusion partner showed tip fluorescence under the respective filter channels.

3.4.3 Testing the utility of pMU-4 for co-localization

One of the main purposes for increasing the number of FPs in *S. coelicolor* is to use them for multi-colour tracking of different proteins. Due to the limited availability of FPs in this organism, co-localization has never been performed. Since pMU-4 contains both CyPet and YPet, and *divIVA* is also cloned, a convenient opportunity was presented for testing co-localization using these FPs.

S. coelicolor cells that contain the pMU-4C plasmid were imaged using the wide-field fluorescence microscope under the cyan and yellow channels (Figure 3.7AB). When the same field of view was illuminated, strong tip fluorescence was present under both cyan and yellow channels indicating that DivIVA-CyPet and DivIVA-YPet co-localized to the tips of growing cells. Control cells containing pMU-4 without *divIVA* did not show any tip fluorescence (data not shown). It is imperative that there is no overlap in excitation between the two different FPs during co-localization work. The results that we have obtained suggested that CyPet and YPet are spectrally distinct – that is, the excitation of one does not affect the other, and their fluorescent signals do not overlap in the two channels. Therefore, the fluorescence signals from CyPet and YPet can be distinguished, suggesting that they are useful for co-localization work.

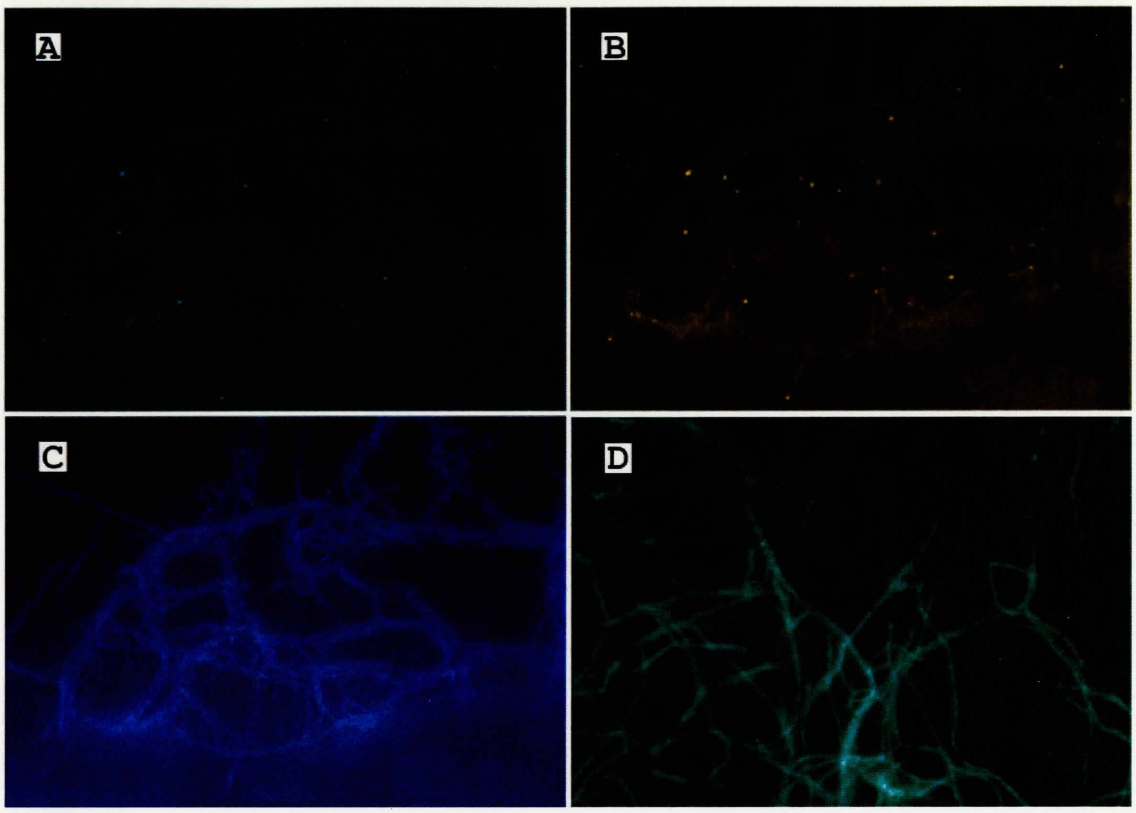

Figure 3.7. Co-localization of DivIVA-CyPet and DivIVA-YPet in *S. coelicolor*. Images of cells containing the pMU-4C vector (both DivIVA-CyPet and DivIVA-YPet are present in the same strain) were taken under the cyan channel (A), yellow channel (B) and the control DAPI channel (C). (D) Wild type *S. coelicolor* cells imaged under the cyan channel. Tip co-localization was observed for the two fusion proteins - DivIVA-CyPet and DivIVA-YPet, whereas the tip fluorescence was absent in control cells.

3.4.4 Verification of the CyPet and YPet fluorescence signals

To confirm that these were in fact true fluorescent signals and not merely background autofluorescence, an image was also taken under the DAPI channel (DAPI is a fluorescent stain that is excited by UV light and emits blue light). Since the cells were not stained with DAPI, this image showed a high level delocalized background fluorescence, but no specific localized tip fluorescence (Figure 3.7C). To further confirm this tip fluorescence, images of wild-type *S. coelicolor* cells that did not contain any of the FP plasmids were also taken (Figure 3.7D). Similar to the DAPI channel, these images showed delocalized fluorescence that is unlike the tip fluorescence observed with the fusion proteins. Therefore, the observed tip fluorescence must be from the DivIVA-FP fusions.

3.5 A comparison of the four FPs in S. coelicolor

In our general experience, it was observed that the level of autofluorescence was much lower and more uniform in the red spectral region as compared to the green spectral region. Overcoming the disadvantage of high autofluorescence is of great benefit as the low and uniform autofluorescence will allow for more sensitive and reliable detection of protein localization in *Streptomyces*. In addition, the red-shifted nature of mRFP should be less toxic to cells during fluorescence imaging than the eGFP. Therefore, it suggests the possibility that for some applications, the mRFP may prove superior to the eGFP.

Overall, when comparing relative brightness, YPet consistently gave the strongest fluorescent signals. Following closely behind is the eGFP, which gave fluorescent signals that were more intense than CyPet and mRFP. Although YPet and eGFP were bright, they also had the highest levels of autofluorescence. This was problematic as it was more difficult to distinguish between the autofluorescence and the signals from the FPs. CyPet displayed lower levels of autofluorescence, while mRFP had the least amount. Clearly, there are drawbacks to each FP and not all are optimal for all applications, so it is up to the user's discretion to pick the right one for the right application.

3.6 Summary and implications for future work

Great effort has been devoted to overcoming the drawbacks of using FPs as fluorescent labels. These efforts have resulted in wavelength-shifted and enhanced FP mutants with great spectral diversity. The large colour variety can be classified into several main groups: green FPs, yellow FPs, red FPs and other FPs of different hues, from blue to orange. From the results presented here, it is obvious that FPs from all of the main groups are now represented in *S. coelicolor*. This will be of tremendous impact as they will pave the way for future use of multi-colour imaging and FRET.

3.7 Short term research goals

The next step of this work is to establish more FP tools, namely FRET, as the utility of FRET has not been demonstrated in *Streptomyces*. FRET is a powerful technique that will allow for the detection of both intermolecular and intramolecular protein interactions, such as dimerization and protein folding, at nanometer resolution in living cells (Piston and Kremers, 2007). Since FRET can only occur over very short distances, up to ~8-10nm (Festy *et al.*, 2007), it proved to be a highly useful tool for the investigation of protein-protein interactions in *E. coli* and *B. subtilis* (Kidane and Graumann, 2005; Sourjik and Berg, 2002).

Several FP pairs useful for FRET are now available in *S. coelicolor* due to the increased number of FPs. The eGFP/mRFP pair has been shown to be good for energy transfer (Peter *et al.*, 2005), but a superior pair includes CyPet and YPet because they have been optimized specifically for FRET (Nguyen and Daugherty, 2005), and will likely give the best chances for detecting energy transfer in *S. coelicolor*. Furthermore, the different pMU-4 constructs created for testing the utility of CyPet/YPet and co-localization studies will be useful for FRET analysis as they already have fusion partners. The large fluorescence foci observed during the DivIVA-FP work has led to the belief that a large concentration of DivIVA molecules is present at the tips of these cells. It is possible that these proteins are associating with each other in order to carry out their function. Previous work with *B. subtilis* DivIVA has shown that DivIVA can form higher ordered structures such as filaments (Muchova *et al.*, 2002) and two-dimensional networks (Stahlberg *et al.*, 2004). Therefore, the well characterized subcellular

localization of DivIVA and its possible oligomeric state makes it a great candidate to test FRET in *S. coelicolor*.

3.8 Long term research goals

3.8.1 Defining the core divisome

Our goal in this pursuit is to add to the understanding of cell division using *S. coelicolor* as the model organism. By examining cell division in this organism it will help to define the core division pathway for bacterial cell division and how it has been adapted to fit each species. This will be established by the comparison of a wide range of microbes, including *S. coelicolor*, whose cell division is already under investigation. In the long term, this information will be key to the identification and optimization of novel drugs that target cell division. It may be possible, through identification of essential division proteins, to target antimicrobials to specific micro-organisms. For example, *S. coelicolor* is related to many pathogenic actinomycetes, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*; therefore, it may be possible to identify drug targets to proteins specific to actinomycetes.

3.8.2 Understanding the different modes of cell division in S. coelicolor

Of tremendous impact to *Streptomyces* research is the understanding of the regulation required for the two types of cell division that occur during the streptomycete lifecycle. Since *S. coelicolor* employs two different modes of cell division, it is believed that there are two separate complements of FtsZ modulators, one for each of the two cell types. In support of this Flardh *et al.* have revealed that *ftsZ* is under the regulation of three promoters, and the upregulation of *ftsZ* expression is cell-type specific (Flardh *et al.*, 2000). Also, an FtsZ mutant that only affected sporulation septation and not vegetative septation was isolated (Grantcharova *et al.*, 2003), suggesting that there are different regulatory proteins in the two cell types, and that only one of those groups was able to modulate the mutant FtsZ but not the other. Finally, the recent identification of sporulation-specific cell division proteins, including the SALPs, CrgA, ShyA, and SmeA, provides further evidence for the differences in cell division in the two cell types (Ausmees *et al.*, 2007; Del Sol *et al.*, 2006; Noens *et al.*, 2005; 2007; Tseng et *al.*, 2006). Therefore, one of our ultimate goals is to use the different FPs for multi-colour tracking and co-localization of the different division proteins, as they will no doubt offer new insight into this complex process.

3.9 Conclusion

The inhibition of cell division is of great therapeutic potential but a greater fundamental understanding of the functions of the individual proteins within the division process in bacteria is needed for the design of novel antimicrobial therapies. The more information available about the bacterial cell division pathway, the greater our chances are at target driven approaches to identify novel inhibitors.

The rigorous investigation of cell division in recent years has provided much insight into the spatial and temporal coordination of this process in model bacteria. The basis of cell division involves a multiprotein apparatus called the divisome. Central to this divisome is the contractile Z ring that encircles the cell at approximately midcell. Proteins that influence the assembly and disassembly of the Z ring, and the two important negative regulatory mechanisms involved in its precise placement have been described. However, significant variations in FtsZ assembly dynamics have been observed *Streptomyces*. Many unique proteins have been identified, but exactly how these regulators alter the cell division process remains elusive. *S. coelicolor* is a worthwhile model organism for the investigation of cell division because this process is not essential for growth and viability of this organism, making it easier to obtain division mutants. Also, *S. coelicolor* possesses unique cell division proteins that may participate in novel division mechanisms that are yet to be uncovered, and that these mechanisms may stand in for the two missing regulatory mechanisms (NO and Min). Since *S. coelicolor* is closely related to pathogenic bacteria, the information gained from studying this organism

may provide better understanding of the pathogenic organisms so that novel antimicrobial agents can be identified.

The regulation of cell division in *Streptomyces* emphasizes an intriguing process that requires further attention. Modern fluorescence microscopy has provided new opportunities to dissect the cell division process further. In particular, the use of FPs has allowed for the direct visualization of protein localization in live cells, and provided a large influx of information regarding protein function. Therefore, the use of FPs is necessary to elucidate the exact involvement of individual division proteins in *S. coelicolor*.

This work has led to the establishment of three additional FPs - mRFP, CyPet and YPet - in *Streptomyces*. These new FPs promise to be useful in the study of cell division in *Streptomyces* as they will allow for direct visualization of individual or multiprotein dynamics, analysis of co-localization, and protein-protein interactions via FRET. The use of these FP tools will lead to a greater understanding of cell division not only in *S. coelicolor* but will also have a significant impact in the study of many important pathogenic bacteria (like *Mycobacteria*) for which these tools are not yet available. Therefore, FP tools promise a wealth of information that is needed for the development of novel antimicrobials that will keep us ahead in the race against antibiotic resistance.

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