Sporulation septation in Streptomyces as a model to investigate bacterial cell division.

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Sporulation septation in *Streptomyces* as a model to investigate bacterial cell division

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ACKNOWLDGEMENT

"IN THE NAME OF ALLAH MOST GRACIOUS AND MOST MERCIFUL"

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الاهداء الـى روح ابي رحمه الله

Abstract

Streptomyces are interesting gram positive filamentous bacteria and have been studied mostly in the context of antibiotic production. This system is controlled by specific networks of genes through regulatory signals that combine to regulate both morphological and physiological differentiation in the organism. But it is in the context of growth and cell division that they are also fascinating. FtsW is one of four **S**hape, **E**longation, **D**ivision and **S**porulation (SEDS) family proteins encoded in the genome of *S. coelicolor*. In this study the effect of *ftsW* complementation, and its overexpression in *S. coelicolor*, and role of other related proteins was studied. Complementation of an *ftsW* mutant resulted in a sporulating phenotype confirming that the white phenotype was due to *ftsW* disruption, and *ftsW* function was restored by the complementing plasmid. Macroscopically, *ftsW or ftsI* complementation or overexpression in *S. coelicolor* does not show any difference in phenotype. In addition, the microscopic analysis revealed that there is no effect of *ftsW or ftsI* overexpression on the sporulation septation and chromosomal condensation of aerial hyphae. FtsW localization was investigated revealing a diffuse distribution of fluorescence in the aerial hyphae of an *ftsW* complemented strain, due to *in vivo* proteolytic cleavage of the FtsW-mCherry translational fusion protein, precluding any inference of FtsW localisation itself. Also mycobacterial orthologs of FtsW and FtsI cannot replace the function of their related streptomycete proteins. By construction of triple mutants of *ftsW*, *sfr* and *rodA2* SEDS genes in one strain of *S. coelicolor*, results showed that these genes are dispensable for growth and survival. No difference was observed between the triple mutant strain that exhibited a white non-sporulating phenotype, and the *ftsW* single mutant phenotype. An absence of sporulation septa in both the triple mutant and *ftsW* single mutant aerial hyphae, while an *sfr*/*rodA2*double mutant has normal sporulation septa, confirming that FtsW is required specifically for sporulation septation. In contrast and from the results of fluorescence microscopical analysis of the vegetative mycelium of the triple mutant strain and the wild type strain, a similar staining pattern of vegetative septa were observed, suggesting that these genes (*ftsW*, *sfr* and *rodA2*) are not required for vegetative septation in *S. coelicolor*. In addition, in order to understand the cell division mechanism in *S. coelicolor* more clearly, the **B**acterial **A**denylate **C**yclase **T**wo-**H**ybrid (BACTH) system used to study protein-protein interactions. Proteins tested were FtsW, FtsI, FtsZ, FtsQ, CrgA, Sfr, MreB, RodA, RodA2, penicillin binding proteins PBP1, PBP2 and PBP3) in several combinations of protein pairs. Notably, the results showed a strong interaction between CrgA and FtsQ, and also with other cell division proteins, suggesting a

central role for CrgA in the cell division process. According to this significant results, alignment of the *S. coelicolor* CrgA sequence with orthologs from other *Actinobacteria* was carried out, revealing only four amino acids G_{40}, W_{45}, N_{65} and W_{83} that are well conserved. After site directed mutagenesis to modify *S. coelicolor* CrgA, this revealed that the amino acids N_{65} and W_{83} are required for interaction of the protein with itself and PBP2.

ABBREVIATIONS

ATP	Adenosine Triphosphate
bp	Base pair(s)
CIAP	Calf Intestinal Alkaline Phosphatase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleoside Triphosphate
EDTA	Ethylene Diamine Tetraacetic acid
g	Gram(s)
h	Hour(s)
HMW	High Molecular Weight
IPTG	Isopropyl-β-*D*-thiogalactoside
Kbp	Kilobase pair(s)
kDa	Kilodalton(s)
L	or l Litre
Mb	Megabase(s)
min	Minute(s)
MW	Molecular weight
μ	Micro
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBP(s)	Penicillin Binding Protein(s)
rpm	Revolutions per minute
s	Second(s)
SDS	Sodium Dodecyl Sulphate
SEDS	Shape, Elongation, Division and Sporulation
TBE	Tris Borate EDTA
TES	*N*-tris(hydroxymethyl)-*2*-aminoethanesulphonic acid
Tris	Tris(hydroxymethyl)amino-methane
V	Volt(s)
W	Watt(s)
X-gal	5-bromo-4-chloro-3-indolyl-β-*D*-galactoside

Contents

Chapter 1: Introduction

The streptomycetes are important soil bacteria that have had a great impact for the pharmaceutical industry due to their ability to synthesise antibiotics. The importance of *Streptomyces* also comes as a result of their morphological differentiation underpinned by complex gene regulation. *Streptomyces* are gram positive bacteria that are natural produces of antibiotics, such as actinomycin that was the first antibiotic discovered from this bacterium (Zhou *et al.*, 2011). The first isolation of the streptomycin was in 1943 from *S. griseus*, which has been used as a broad range antibiotic to treat several bacterial diseases such as tuberculosis and plague (Distler *et al.*, 1992, Mansouri and Piepersberg, 1991).

The life cycle of *Streptomyces* begins with an immobile spore which germinates and forms the substrate mycelium (vegetative); this will grow and extend through the substrate seeking nutrients and subsequently forming branching hyphae which subsequently start extending into the air forming an aerial mycelium - this aerial mycelium could be considered as the end and the beginning of the cycle as it can form the spores that start the process again (Flärdh and Buttner, 2009). *Streptomyces* are considered as being a model system to study the molecular mechanisms of morphological differentiation such as the regulation of cell-cycle progression, cell growth, sporulation, cell wall assembly, extracellular signaling and its mutual effect on secondary metabolism (Flärdh and Buttner, 2009). Another beneficial factor of the *Streptomyces* is their involvement in carbon recycling process due to their ubiquity in soil and being able to degrade the insoluble remains of several organisms. The complexity and the variability of gene regulation in *Streptomyces* bacteria is also an important feature enabling them to adjust to a wide range of environmental conditions (Zhou *et al.*, 2011). The 65 sigma factors that can associate with RNA polymerase in *S. coelicolor* A3 (2) are central players in the gene regulation networks (Bentley *et al.*, 2002); these sigma factors are regulated by very specific anti- and anti-anti-sigma factors, often encoded by gene clusters in the chromosome (Bentley *et al.*, 2002, Zhou *et al.*, 2011). The secretion systems of *Streptomyces* enable the release of a large number of proteins. There are at least three secretion systems; the general secretary (Sec) pathway, the ESX-1/type VII (Esx) secretion system, and finally the twin-arginine translocation (Tat) pathway. Each one of these systems has its own secretion function as the first (Sec) is responsible for secreting unfolded proteins, the second pathway (Esx) has the ability to secrete specific small proteins, whereas the third pathway (Tat) is responsible for transporting pre-folded proteins and some enzymes (Chater *et al.*, 2009). Most *Streptomyces* are non-pathogenic, while a small number have the ability to

cause diseases in humans such as *S. somaliensis* and *S. sudanensis* (Hassan *et al.*, 2001, Quintana *et al.*, 2008), and others such as *S. scabeis*, *S. turgidiscabies* and *S. acidiscabies* are the causative agents of some diseases for plants like potato scab (Bignell *et al.*, 2010, Lerat *et al.*, 2009).

1.1 General features of *Streptomyces*

1.1.1 Classification of Actinobacteria

Streptomyces are classified under the class of Actinobacteria which is one of the largest groups of gram-positive bacteria. This class contains a high amount of guanine plus cytosine (G+C) in their DNA (Stackebrandt *et al.*, 1997). The DNA base composition of organisms in this group usually is above 50 % G+C (there are some exceptions) (Krasil'nikov, 1949).

1.1.2 Classification of *Streptomyces*

The first classification of *Streptomyces* species was based mostly on their morphological and phylogenetic properties and then some other classification characteristics and properties were added such the response to gram stain. The sum of this classification was shown in a classification diagram published by Woese, C.R in the year 1987 (Woese, 1987). Modern categorization of *Streptomyces* includes several approaches. Chemotaxonomy depends on the detection of the chemical composition of the bacterial cell; some of these chemicals were mentioned by Colwell in the early seventies such as peptidoglycan, polar lipids and fatty acids, isoprenoid quinones, cytochromes, and the base composition of DNA (Colwell, 1970). Another classification strategy depends on experimental results that focus on the similarities between the DNA strains of any two studied species (Stackebrandt *et al.*, 1997). One of the most recent strategies added the sequence similarities of 16s rRNA and rDNA to the classification process (Stackebrandt *et al.*, 1980, Stackebrandt *et al.*, 1997).

1.2 Life cycle of *Streptomyces*

Streptomyces have very complex life cycles; one of the first investigations on the life cycle of *Streptomyces* was that published in the early fifties by Mcgregor using the Robinow HC1-Giemsa method of nuclear staining. This study concluded that there are four stages; the first was the division phase, then the primary mycelium, this followed by the secondary mycelium, and the final stage was the spore formation (McGregor, 1954). At the initial stage (division phase) the bacteria produce side branches by initiating growth of a few germ tubes in certain environmental

conditions, these tubes having the ability to grow and elongate to form the irregularly septated vegetative mycelium after repeated branch formation. The hyphae can attach to the substrate and penetrate the medium due to the specific action of the extracellular hydrolytic enzymes. At this stage the secondary mycelium starts developing and forming the aerial hyphae that terminate in the form of chains of uninucleate spores (Daniel and Errington, 2003, Flärdh, 2003a, Flärdh, 2003b, McGregor, 1954).

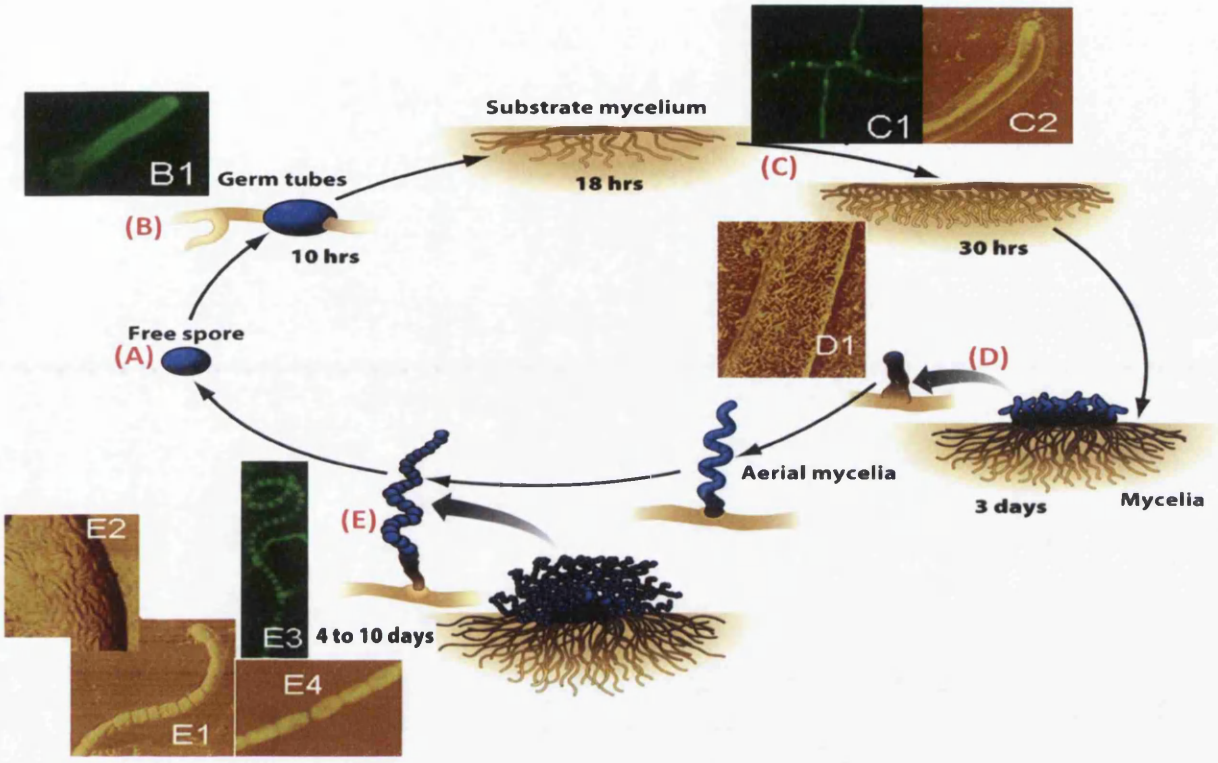


Figure 1.1: The streptomycete life cycle illustrated by that of *S. coelicolor*. After dispersal, in favourable conditions, a spore (A) can germinate (B). Fluo-vancomycin staining of an emerging germ-tube (B1) reveals that synthesis of new peptidoglycan, and hence cell growth, occurs primarily at the tip. A substrate mycelium grows by tip extension and sub-apical branching (C): fluo-WGA (wheat germ agglutinin) staining reveals infrequent cross-wall formation in the substrate mycelium (C1). Atomic-force microscopy (AFM) reveals that the surface of substrate hyphae is relatively smooth (C2), without the decoration that is characteristic of aerial hyphae. Moreover an extracellular matrix is deposited as the tip grows, allowing the hyphae to adhere to the substrate. Growth of an aerial hypha is away from the nutrient source (D), fuelled in part by cannibalization of the substrate hyphae; antibiotic biosynthesis at this stage in development is believed to protect the nutrients released by breakdown of the substrate mycelium from other competing microorganisms. Young aerial hyphae are decorated with an irregular fibrous layer (AFM image, D1) specified by chaplin and rodlin genes. After growth cessation, the aerial hyphae differentiate to form spore chains (E). This involves highly coordinated multiple septation, revealed by fluo-WGA staining (E3), and by the appearance of regular-spaced spore compartments revealed by AFM (E1). The surface of the spore-compartments consists of a dense layer of well-organised fibres specified by the chaplin and rodlin genes (AFM image, E2). Older spores have characteristic surface concavities (AFM image, E4), indicating dehydration and loss of turgor.

At the differentiation stage, along with aerial hyphae formation, there are other changes to *Streptomyces* colonies such as the production of extracellular enzymes (Champness, 1988). In certain environmental conditions the aerial mycelium undergoes two kinds of degeneration; autolysis and physiological cell death (Miguélez *et al.*, 1999). During autolysis the cell wall undergoes an early degradation by uncontrolled murein hydrolysis. This happens very quickly and can impact several hyphae in the colony. The second event, physiological death, usually occurs to a greater proportion of substrate hyphae and takes longer than the first event. In addition the cellular contents have less chance to leak through the longer-maintained cell wall. This helps the colony to utilize and degrade the cellular contents without affecting the general organization of that colony. Furthermore, at this stage (physiological death), the dead hyphae play a role in supporting the aerial hyphae to extend towards the air and away from the medium. Antibiotic secretion occurs during this vegetative and aerial development in order to prevent the consumption of the nutrients by any other micro-organisms. There is a specific network of gene regulation controlling these processes. For instance, the development of aerial hyphae is regulated by several *bld* genes that encode regulatory proteins (the exception is *bld*A that encodes a tRNA) (Kelemen and Buttner, 1998, Lawlor *et al.*, 1987). Mutations of the *bld* genes are pleiotropic and prevent the production of antibiotics as well as affecting carbon catabolite repression and cell-cell signaling (Kelemen and Buttner, 1998).

When the aerial hyphae mature the apical compartments start forming a spiral syncytium that contains multiple copies of the genome. At this stage the aerial hyphae stop growing and undergo multiple septation generating chains of unigenomic pre-spore compartments. The pre-spore compartments subsequently metamorphose into thick-walled pigmented spores. A group of around eight regulatory genes are responsible for controlling spore formation; these regulatory genes are called the *whi* genes (*whi* A, *whi* B, *whi* D, *whi* E, *whi* G, *whi* J, *whi* H, *whi* I) exclusively for the white aerial mycelial phenotype (Chater and Chandra, 2006, Kieser *et al.*, 2000). Some other changes have been observed during reproductive growth. The vegetative mycelium growing in moist substrates have a hydrophilic cell surface, while the aerial hyphae possess a hydrophobic layer (Del Sol *et al.*, 2007, Elliot *et al.*, 2003). This hydrophobic sheath plays a role in protecting from dehydration. The extended aerial hyphae help in distributing the spores by the wind and other possible ways of dispersion such as via some insects or animals, contrasting to the immobile vegetative mycelium.

1.3 Ecology of *Streptomyces*

Streptomyces are very diverse and highly distributed organisms that have the ability to decompose any organic matter. Soil is one of the favoured environments along with other habitats such as fresh and deep sea water (Colquhoun *et al.*, 2000, Kieser *et al.*, 2000). *Streptomyces* living in soil increases the fertility of the soil as they can adapt and live for long time as saprophytes; also they have the ability to degrade complex organic materials such as cellulose, lignin and chitin (McCarthy and Williams, 1992). *Streptomyces* prefer alkaline and the neutral soils rather than acidic ones as they can develop better in these environments (Alexander, 1977). The complexity of soil as well as the constant changes of its biological, physical and chemical characteristics presents a challenging environment, and they employ many strategies to sustain themselves in this complicated environment. Proliferation and sporulation are kinds of these strategies as well as the secretion of hydrolytic enzymes that have the ability to metabolize recalcitrant molecules. *Streptomyces* also produce antibiotics as one of their strategies to maintain their population; this strategy along with the benefits of morphological development of *Streptomyces* allows them to inhibit the growth of the other microorganisms and defend their nutrition source (Challis and Hopwood, 2003). Although *Streptomyces* are saprophytic species some of them can colonize the rhizosphere of some plants and roots in a very close association (Castillo *et al.*, 2002, Tokala *et al.*, 2002). In association with human health, just a few species of *Streptomyces* are found to be causative of disease, such as *S. somaliensis* that causes some subcutaneous diseases (Hassan *et al.*, 2001, Quintana *et al.*, 2008).

1.4 Physiology of *Streptomyces*

Streptomyces can sustain themselves in severe conditions associated with the soil's physicochemical changes, low levels of nutrients along with a high abundance of other microorganisms that compete for nutrient sources. This kind of sustainability is due to the adaptability of these species. The complexity of the life cycle of *Streptomyces* and production of secondary metabolites is controlled by specific signalling molecules that behave as bacterial hormones ((D'Alia *et al.*, 2011). Whereas primary metabolism is responsible for the growth of *Streptomyces*, secondary metabolism is part of the adaptation properties of these bacteria to environmental stresses.

1.4.1 Primary metabolism

Streptomyces primary metabolism is highly dependent on carbon compounds from plants in the soil and consequently there is a deficiency of some other compounds such as the nitrogen and phosphate. Access to a variety of carbon compounds increases ability of *Streptomyces* to adopt different carbohydrate catabolic pathways (Hodgson, 2000). These pathways are inducible and mainly subject to carbon catabolite repression (CCR) (Angell *et al.*, 1994, Kwakman and Postma, 1994). CCR in some kinds of bacteria is phosphotransferase dependent, whereas in *Streptomyces* the CCR relies on ATP-dependent glucose kinase (GLKA) (Kieser *et al.*, 2000). For nitrogen assimilation *Streptomyces* use two pathways; glutamate synthase (GS) and glutamate: 2-oxoglutarat transaminase (GOGAT) (Fisher and Wray, 1989, Kieser *et al.*, 2000). The GS pathway requires ATP in order to assimilate ammonia into glutamate (Fisher and Wray, 1989). The presence of GS along with GOGAT pathways is due to nitrogen shortage in the soil. Biosynthesis of amino acids and their catabolic pathways in *Streptomyces* species are similar to those in most bacteria. A notable difference is arginine catabolism; this can be catabolised to γ-guanidinobutyramide and γ-guannidinobutyrate (Kieser *et al.*, 2000).

1.4.2 Secondary metabolism

Secondary metabolism is regulated and generally overlaps with morphological differentiation in *Streptomyces*. The relationships between physiological and morphological differentiation in *Streptomyces* species have been recognized via experimental observations of different mutants that affect the development of both processes. Secondary metabolism is stimulated by different factors including environmental stresses, and regulated by specific pathways and some specific regulatory signals. The *bld* mutants of *S. coelicolor* that are affected in the production of antibiotics and the formation of the aerial hyphae are the best example of the link in regulation of morphological and the physiological differentiation (Pope *et al.*, 2003, White and Bibb, 1997). γ-butyrolactones are regulators produced by some *Streptomyces* species that function as pleiotropic regulators of morphological and physiological differentiation (Eritt *et al.*, 1982, Takano, 2006). γ-butyrolactone regulators play their role through the modulation of repressor activity of γ-butyrolactones-receptor proteins that behave as repressors of morphological and chemical differentiation. The classic example of a γ-butyrolactone is A- factor (2Risocapryloyl-3R-hydroxymethyl-4-butanolide) (Horinouchi, 2002). This factor is produced just before commencement of differentiation (Bibb, 2005, Horinouchi, 2002). The role of A factor in

regulation of antibiotic (eg: streptomycin) production happens through the binding to its receptor protein ArpA by which the ArpA protein is released from his promoter region of the *adpA* gene encoding a transcriptional activator protein (AdpA). This activator protein binds later to the promoters of the strR gene encoding a pathway-specific transcriptional activator for streptomycin synthesis (Mistry, 2008). These processes are shown in (Fig 1.2).

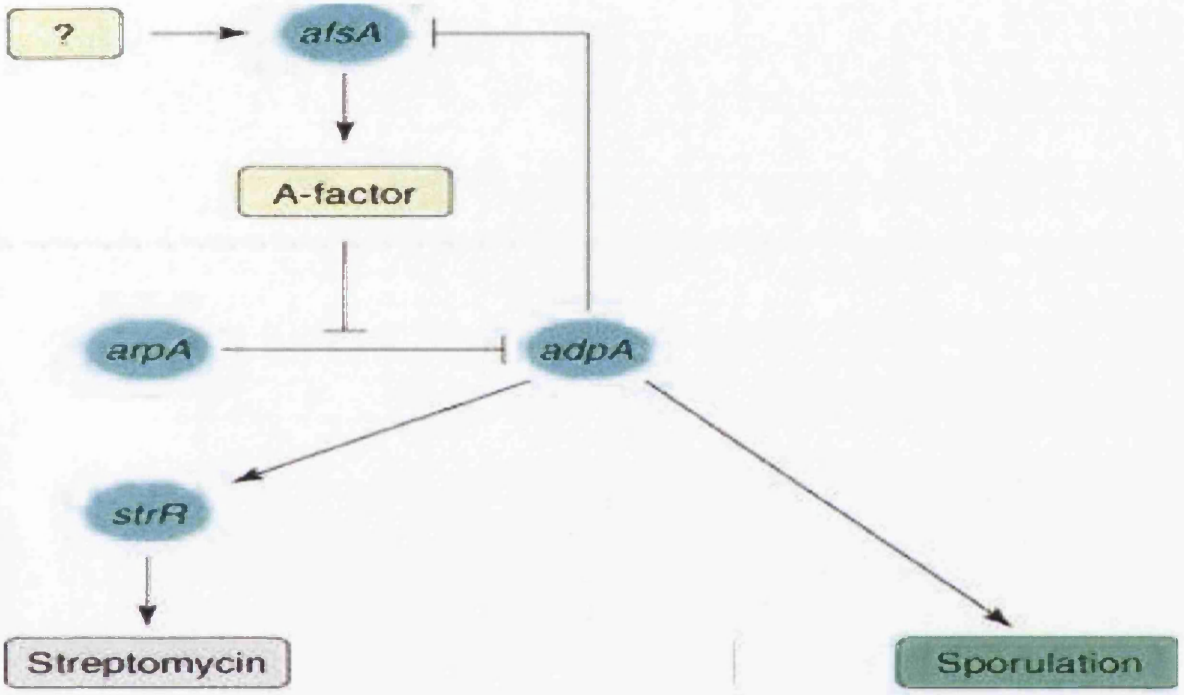
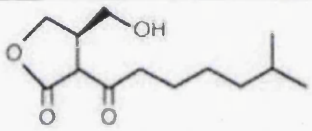
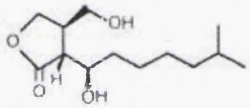
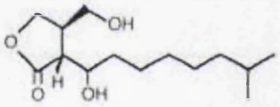
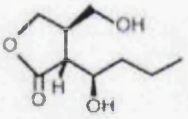
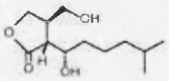


Figure 1.2: Diagram of the role of A factor of *S. griseus*, in this diagram undefined signals, indicated by ('?') can trigger A factor synthesis facilitated somehow by AfsA. When the A-factor reaches a specific concentration it binds and start releasing the A- factor receptor protein (ArpA) from its promoter region of transcriptional activator encoding gene *adpA*. AdpA also start binding to promoter regions of many genes needed for physiological and morphological differentiation. The *strR* gene encoding the pathway-specific transcriptional activator for streptomycin biosynthesis genes is activated by AdpA (Bibb, 2005).

Different, γ-butyrolactones are involved in the regulation of secondary metabolism in other *Streptomyces* species. Some of those *Streptomyces* regulators are included in Table 1.1.

Table 1.1: Chemical structures of γ-butyrolactones from *Streptomyces* species(Horinouchi, 2002).

Chemical structure of γ-butyrolactone	Producing organism	Biological activity
 ***A-factor***	*S. griseus*	Streptomycin Grixazone Sporulation
 **SCB1 [(2*R*,3*R*,1′*R*)-2-(1′-hydroxy-6-methylheptyl)-3 hydroxymethylbutanolide**	*S. coelicolor*	Actinorhodin Undecylprodigiosin
	*S. bikiniensis,* *S. cyaneofuscatus*	Anthracycline Morphological differentiation
 **IM-2 [(2R,3R,1′R)-2-1′-hydroxybutyl-3-hydroxymethyl γ-butanolide**	*S. lavendulae*	Showdomycin Minimycin
 **VB virginiae butanolides**	*S. virginiae*	Virginiamycin

Recently, three A-factors having different function from the original A-factor (homologues) (SCB1, SCB2 and SCB3) were determined in *S. coelicolor*. SCB1 (*S. coelicolor* butanolide 1) is the most abundant factor that has the ability to stimulate the undecylprodigiosin and actinorhodin biosynthesis (Bibb, 2005, Horinouchi, 2002, Takano, 2006).

Streptomyces are well known producers of secondary metabolites including enzyme inhibitors, immunosuppressants, antitumor agents, anthelminthic agents, herbicides, antibacterial agents, and antifungal agents (Paradkar *et al.*, 2003, Watve *et al.*, 2001). Over ten thousand of these bioactive compounds are produced by the Actinomycetes, with 8700 out of the 10000 being antimicrobial agents. Also *Streptomyces* are responsible for production of around 7600 of the bioactive compounds, accounting for 74% of the total produced by Actinomycetes (Berdy, 2005). Analysis of the genome sequences of *S. coelicolor*, *S. avermitilis* and *S. griseus* disclosed 23, 30 and 34 gene clusters for the biosynthesis of secondary metabolites respectively (Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Ohnishi *et al.*, 2008).

Streptomyces coelicolor A3 (2) is affected by the limitation of several nutrients which then leads to the generation of nutrient-stress responses, mediated by several regulators such as PhoP, GlnR, and AfsR regulators. These regulators combine at the molecular level to play a significant role in the control of the secondary metabolite biosynthesis and differentiation. Furthermore, the use of chitin or N-acetylglucosamine can control secondary metabolism through a specific mechanism facilitated by DasR. In *S. coelicolor*, PhoP controls secondary metabolism via the attachment to a PHO box in the afss promoter that overlaps with the AfsR binding site. As a result, the afss promoter helps to integrate the PhoP-mediated response to the limitations of phosphate and the AfsR-mediated responses to some of the unknown environmental stimuli. The overall regulation of phosphate and nitrogen utilization pathways prevents nutritional imbalances. The transport of N-acetylglucosamine by the NagE2 permease and the regulation of secondary metabolism depend on the balance of the phosphorylated/dephosphorylated proteins of the N-acetylglucosamine phosphotransferase system (Martín *et al.*, 2011).

1.5 Genomics of *Streptomyces*

Studying the genetics of *Streptomyces* started as early as in the 1950s (Hopwood, 1999, Hopwood, 2007), but complete genome sequences of representative species weren't published until the years 2002, and 2003 (Bentley *et al.*, 2002, Ikeda *et al.*, 2003). From the year 2002 to the year 2008 four completed genome sequences were available online for *S. avermitilis*, *S. griseus*, *S. coelicolor*, and *S. scabies* (Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Ohnishi *et al.*, 2008). The sizes of these chromosomes are in the range of 8 Mb to 10 Mb which is considered to be large compared to the chromosomes of many unicellular bacteria that have about the half the size in DNA content and gene numbers (Bao and Cohen, 2001). Chromosomes of *Streptomyces* have been divided into two regions according to the distribution of gene types. These two regions are: the central core region which contains essential genes such as those required for cell division, transcription, translation, DNA replication and amino acid biosynthesis, while the other region is the pair of arms coding for non- essential genes such as gas vesicle proteins, and secondary metabolites (Bao and Cohen, 2001). Up to the year 2011 there were around 32 sequenced *Streptomyces* genomes, and these sequences are available to researchers online (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi): out of these 32 genomes there are only nine strains with their complete sequences as mentioned in Table 1.2, while the other 23 genomes are recorded as draft sequences.

Table 1.2: The *Streptomyces* Genome Projects

Organism	Chromosome Size (Mb)	ORF	GC%	Ref Seq/GenBank Accession	References
S. avermitilis	9.0	7580	70	NC_003155	(Ikeda *et al.*, 2003)
S.bingchenggensis	11.9	10023	71	CP002047	(Wang *et al.*, 2010)
S. cf. griseus	8.7	7265	72	NZ_ADFC00000000	(Grubbs *et al.*, 2011)
S. coelicolor	8.7	7768	72	NC_003888	(Bentley *et al.*, 2002)
S. flavogriseus	7.3	6298	71	CP002475	
S. griseus	8.5	7136	72	NC_010572	(Ohnishi *et al.*, 2008)
S. scabies	10.1	8746	71	NC_013929	(Yaxley, 2009)
S. sp. Tü6067	7.4	6466	73	NZ_AFHJ01000000	(Erxleben *et al.*, 2011)
S. venezuelae	8.2	7453	72	FR845719	

Each one of these nine fully sequenced *Streptomyces* species has its own specific properties; the most studied one is *S. coelicolor* and this can produce different antibiotics such as methylenomycin, undecylprodigiosin, actinorhodin and CDA (calcium-dependent antibiotic) (Kieser *et al.*, 2000). The first genetic map of *S. coelicolor* was published at the end of 1950's after the construction and genetic analysis of auxotrophic and antibiotic-production mutants. Since then and by developing genetic tools the number of mapped genes increased progressively. Soon after, scientists all over the world started accumulating genetic maps of several other *Streptomyces* species such as *S. clavuligeurs*, *S. venezuelae*, *S. lividans*, *S. griseus*, and *S. ambofaciens* via the use of easily scorable phenotypic markers such as auxotrophy (Paradkar *et al.*, 2003).

In 1992 the first detailed genetic map was published combined with another physical map of *S. coelicolor* containing around 170 loci on the map. Furthermore, chromosome linearity of some *Streptomyces* species was also revealed in several studies that used pulsed field gel electrophoresis (PFGE) analysis of the genomes (LeBlond *et al.*, 1993, Paradkar *et al.*, 2003). Following that, genome sequencing also confirmed the linearity of the chromosomes ((Ikeda *et al.*, 2003, Merrick, 1976, Ohnishi *et al.*, 2008). Chromosome linearity in *Streptomyces* species was thought of as having originated by single-crossover recombination between a primarily circular chromosome and a linear plasmid (Ventura *et al.*, 2007, Volff and Altenbuchner, 2002). In *Streptomyces* the ends of the chromosomes contains terminal inverted repeats (TIRs), which are bound with a protein known as terminal protei., This binding takes place at free 5' ends (Bao and Cohen, 2001, Wang *et al.*, 1999, Yang and Losick, 2001). Terminal proteins have also been proposed to anchor the DNA to the membrane when the chromosomes start replicating (Bao and Cohen, 2001). The terminal inverted proteins are extremely different in size according to the species (Ikeda *et al.*, 2003, Ohnishi *et al.*, 2008, Ventura *et al.*, 2007).

1.5.1 Composition of the *S. coelicolor* genome

The *S. coelicolor* genome consists of 8,667,507 bp of DNA sequence (Bentley *et al.*, 2002). The physical locations of some of the genes are shown in Fig. 1.3. *S. coelicolor* has the ability to secrete as many as 819 proteins in its natural soil habitat, and among these 819 there are 13 chitinases, chitosanases, 3 amylases and 8 celluloses. The chromosomal ends of *S. coelicolor* are known to be highly unstable. Large deletions and recombination can generate the unstable mutant phenotypes of the *S. coelicolor* at frequency of more than 0.1% (Volff and Altenbuchner,

2002). *Streptomyces* harbour a considerable diversity of indigenous plasmids that rang from small covalently circular (CCC) to the giant linear plasmids (Keen *et al.*, 1988, Kinashi *et al.*, 1994).

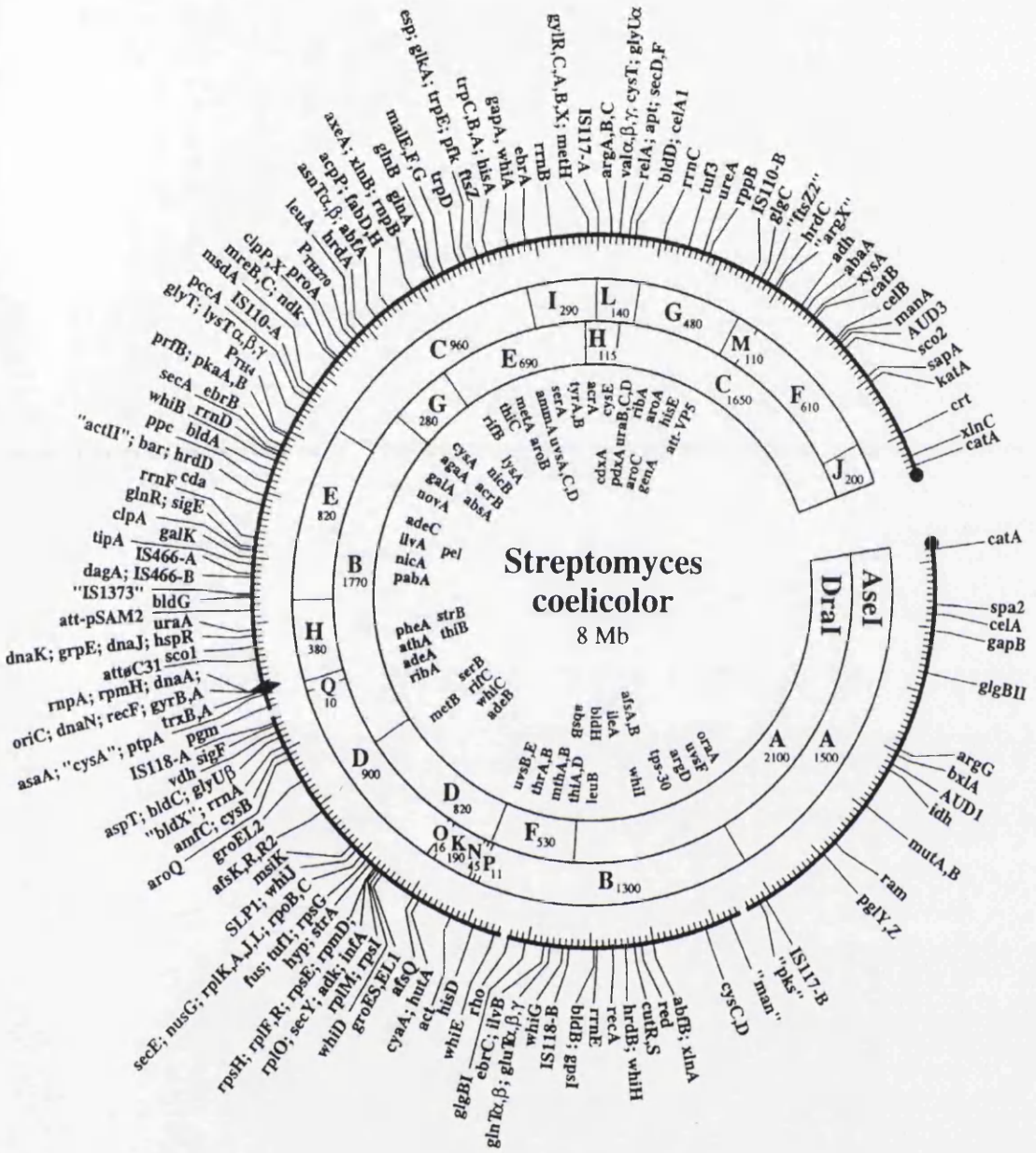


Figure 1.3: Combined genetic and physical map of the *S. coelicolor* chromosome. The positions of markers on the outside of the circle come from their locations on the cosmid contig. Markers inside the circle have been mapped only genetically; their approximate positions relative to the physically mapped markers were determined by interpolation (Hopwood, 1999, Redenbach *et al.*, 1996).

1.5.2 Properties of the genomes of sequenced *Streptomyces*

Streptomyces genomes are widely studied along with phylogenetic analysis of 16s rRNA sequences. Most common features of those genomes are:

1- Their chromosomes are linear and are rich in GC bases (guanine, cytosine), which is thought to be produced by mutational biases (Muto and Osawa, 1987). Their linearity is clearly different from other bacterial species and other primitive living single-celled organisms that are known to have circular chromosomes. *Streptomyces* linear chromosomes are changeable and experience a number of rearrangements including those related to DNA deletion and amplifications (Bey *et al.*, 2000, Yang *et al.*, 2002). The advantages of a chromosomal linearity are unclear (Hopwood, 2006, Kirby, 2011), but may be useful for sustaining a large size of genome. Moreover, the linearity may contribute to genetic variability which may have played a major role in the evolution of specific traits in *Streptomyces* including the secretion of extracellular enzymes, secondary metabolism, and morphological differentiation (Volff and Altenbuchner, 2002). Most of those processes are regulated by specific genes situated in the two arm regions. The presence of a high GC content in *Streptomyces* DNA is likely to have been of significance in control of codon usage for translational optimization and gene expression (Knight *et al.*, 2001). In *S. coelicolor* A3(2), the *bld*A gene plays a role in encoding the tRNA species that is necessary for translation of the rare Leu TTA codon competently (Leskiw *et al.*, 1991); this codon is found to be associated with genes involved in processes such as secondary metabolism and morphological differentiation (Leskiw *et al.*, 2006, Takano *et al.*, 2003).

2- Another feature of *Streptomyces* genomes is the presence of the duplicated genes that respond to distinctstimuli and function in a 'tissue specific' manner (Bentley *et al.*, 2002, Doroghazi and Buckley, 2010). Specific to *S. coelicolor* A3 (2) genomes, there are around 65 sigma factors; most of them are used in the transcriptional regulation in response to different environmental conditions (Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Raivio and Silhavy, 2001).

3- A common feature is a large number of gene clusters devoted to secondary metabolism and that many of these clusters have no known products. Typically the number of chemically distinct compounds produced by a given species grown under lab conditions represents a third to a half of the apparent number predicted by 'mining' genome sequences to be potentially produced. For example, the *S. griseus* genome has around 30 gene clusters for secondary metabolism, but only a small number of these clusters are associated with the biosynthesis of known compounds such as

terpenoids, hopanoids, and carotenoids (Ohnishi *et al.*, 2008). In *S. bingchenggensis*, there are around 23 gene clusters reported, but only a few are involved in the production of known compounds such as bingchamide, and milbemycin (Wang *et al.*, 2010). The important thing to mention is that most of these genes clusters are located in the chromosome arms, in the subtelomeric regions (Zhou *et al.*, 2011).

1.6 Morphogenesis of *Streptomyces*

The developmental programme of *Streptomyces* can be separated into two stages based on the phenotypes of two classes of developmental mutant of the model species, *S. coelicolor*. The first stage is the formation of aerial hyphae as indicated by bald (*bld*) mutants that lack these aerial structures and have a smooth colony appearance (Kelemen and Buttner, 1998). The second stage is the subsequent differentiation of the aerial hyphae into spore chains: mutants defective in this stage are named white (*whi*) mutants as they cannot form mature grey spores (Flärdh *et al.*, 1999).

1.6.1 Formation of Aerial Hyphae –The bld Cascade

In *S. coelicolor*, the development of aerial hyphae is a complex process that is controlled by *bld* genes (Kelemen and Buttner, 1998). *bld* mutants are conditionally affected in development of aerial hyphae and production of antibiotics. Furthermore, they are also affected in carbon catabolite repression (Champness, 1988, Chater and Chandra, 2006, Pope *et al.*, 2003). The *bld*A gene is one of the best studied genes and it encodes a tRNA. This tRNA can translate the rare leucine codon (UUA) (Leskiw *et al.*, 2006, Leskiw *et al.*, 1991). In *Streptomyces* genomes the TTA codon contributes to around 2 to 3% of total codons (Chater and Chandra, 2006, Chater and Chandra, 2008). The leucyl tRNA is involved in many phases of secondary metabolism along with morphological differentiation, and its role as a regulator of the translation process is indicated by the phenotypes of *bld*A mutants (Kataoka *et al.*, 1999, Kwak *et al.*, 1996, Leskiw *et al.*, 2006). At the beginning of morphological and physiological differentiation there is a sequential build up of active *bld*A tRNA (Kataoka *et al.*, 1999, Leskiw *et al.*, 1993, Trepanier *et al.*, 1997). Expression of the *adpA* gene in several *Streptomyces* species underlines the role that the *bldA* gene plays in the formation of aerial hyphae; the *adpA* gene contains a TTA codon and it encodes an Ara C-like transcriptional regulator of differentiation (Kwak et al., 1996, Nguyen et al., 2003, Takano et al., 2003). The *adpA* gene of *S. griseus* and *bldH* gene of *S. coelicolor* are orthologs (Nguyen *et al.*, 2003, Takano *et al.*, 2003).

bldB mutants fail to sporulate and produce antibiotics on media with poor carbon sources, whereas other *bld* mutants have the ability to restore their developmental phenotype if they grow in a media with minimal amounts of carbon sources (Champness, 1988, Merrick, 1976, Pope *et al.*, 2003). *bldD* is a DNA binding protein that can behave as an auto-repressor and blocks the promoters of two sigma factors encoding genes:, *bldN* and *whiG* (Elliot *et al.*, 2001, Elliot and Leskiw, 1999). The first (*bldN*), encodes an extracytoplasmic function (ECF) sigma factor, that was shown by Bibb *et al.*, 2000 as essential for the transcription of *bldM* gene that is essential for the aerial growth (Bibb *et al.*, 2000, Molle and Buttner, 2002, Ryding *et al.*, 1999). A DNA binding protein related to the MerR transcriptional activators is encoded by *bldC*, while the *bldG* gene encodes an anti-anti-sigma factor (Bignell *et al.*, 2006, Bignell *et al.*, 2000, Hunt *et al.*, 2005). *bldK* encodes subunits of an oligopeptide-permease, and as a consequence *bldK* mutants are resistant to the toxic tripeptide bialaphos (Nodwell *et al.*, 2003). *bldJ* is believed to be required in the formation of the extracellular oligopeptide. This extracellular oligopeptide behaves like a signaling molecule (Nodwell and Losick, 1998).

Table 1.3: *bld* genes discovered in *S. coelicolor*

Gene	Gene product	Function	Reference(s)
bldA	UUA leucyl tRNA	Translation of rare TTA codons	(Lawlor *et al.*, 1987, Leskiw *et al.*, 1991)
bldB	Small DNA binding protein	Putative transcription factor	(Eccleston *et al.*, 2002, Pope *et al.*, 1998)
bldC	DNA binding protein	Member of MerR family of transcription regulators	(Hunt *et al.*, 2005, Merrick, 1976)
bldD	Small DNA binding protein	Transcription regulator (Repressor protein)	(Elliot *et al.*, 1998, Elliot *et al.*, 2001)
bldG	Like ant-anti sigma factor	Unknown	(Bignell *et al.*, 2000, Champness, 1988)
bldH(*adpA*)	Activator/repressor family protein	Unknown	(Nguyen *et al.*, 2003, Takano *et al.*, 2003)
bldJ(*bld*261)	Unknown	Required for production of signal 1, an extracellular oligopeptide in *bld* signalling cascade	(Nodwell and Losick, 1998)
bldK	Oligopeptide permease	Oligopeptide importer	(Nodwell *et al.*, 2003)
bldL	Unknown	Unknown	(Nodwell *et al.*, 1999)
bldM(*whiK*)	Orphan response regulator	Activation of the chp genes that encodes chaplin proteins	(Molle and Buttner, 2002)
bldN(*whiN*)	Extracytoplasmic function (ECF) sigma factor	Require for transcription of one of two promoters of *bld*M	(Bibb *et al.*, 2000)

bld mutants are also known of having the ability to show some extracellular complementation that results from the dispersion of one or more substances from one strain to another (donor and recipient respectively) (Willey *et al.*, 1991, Willey *et al.*, 1993). This complementation has specific pattern in different *bld* mutants as they are always develop in one direction and creates a specific hierarchical pattern according to the mutant classes. The sequential complementation of the *Streptomyces* species mutants is interpreted as an intercellular signaling pathway; each pathway involves at least five extracellular signals which then leads to the production of SapB as shown in Fig 1.4.

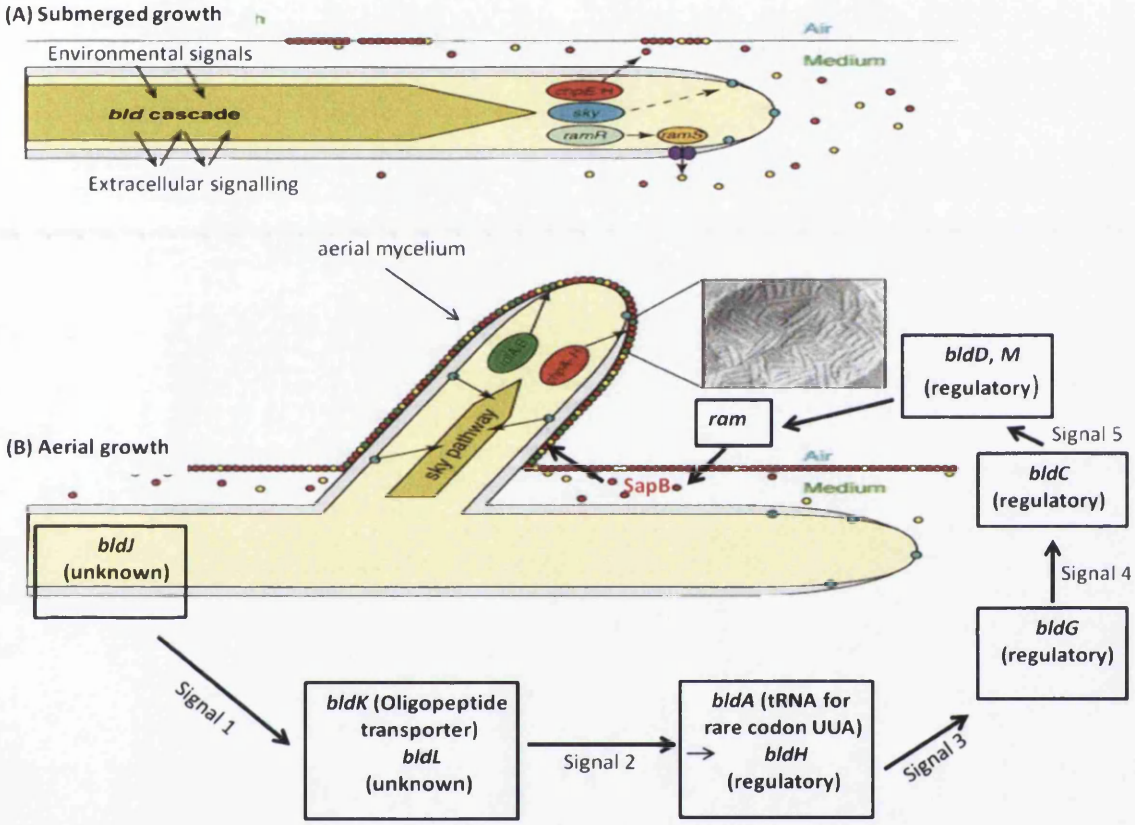


Figure 1.4: Extracellular signal exchange that leads to the growth of the aerial hyphae in *S. coelicolor*. The lower part depicts how substrate hyphae in different physiological states under *bld* gene control may produce signals that act in a cascade leading to production of SapB, which is thought to allow formation of aerial hyphae (one is illustrated near the top of the figure) (Kroos and Maddock, 2003).

Some of *bld* mutants, such as *bldB*, *bldI* and *bldN*, were found not to fit the suggested hierarchical signaling cascade (Bibb *et al.*, 2000, Willey *et al.*, 1993). The visible characteristics of the *bld* mutants that result from the interaction between their genetic makeup and the environment depend on the medium they grow on. Some of these mutants have the ability produce aerial hyphae along with the sporulation, while they cannot restore the process of antibiotic production when they grow on minimal medium that may contain alternative carbon sources such as mannitol instead of glucose (Willey *et al.*, 1991). Any possible faults in aerial hyphae formation that may result from the *bld* mutations can be relieved by application of purified SapB. The resulting aerial hyphae lack some of the normal features as so are defective in the sporulation phase (Tillotson *et al.*, 2002, Willey *et al.*, 1993, Willey *et al.*, 2005). The molecular basis of SapB function is based on its properties as a small secreted lanthionine-containing lantibiotic-like hydrophobic peptide. This peptide acts as a biological surfactant that can release surface tension at the air-water interface. This surface gives an extra advantage to the hyphae by allowing them to escape from the watery environment and grow straight (Kodani *et al.*, 2004, Tillotson *et al.*, 2002). SapB has a specific characteristic of not having any antibiotic activity and this feature makes it different to other lantibiotics. Some fungi such as the Schizophyllum commune produce related peptides (SapT) that have a similar activity in being able to produce and erect aerial structures (Tillotson *et al.*, 2002). These similarities clearly show the importance of the hydrophobic peptide as a surfactant instead of being a signaling molecule. SapB is the product of post translational modification from the *ramS* gene cluster (rapid aerial mycelium) that contains the ramCSAB operon and *ramR* (Kodani *et al.*, 2004, Ma and Kendall, 1994). Development of aerial hyphae can be accelerated by overexpression of the ram cluster, while deletion of *ramR* or *ramS* leads to a reduction of aerial hyphae formation (Ma and Kendall, 1994). Each of these *ram* genes has its specific function; *ramA* and *ramB* encode components of an ABC transporter that has a high probability of being involved in SapB export. The *ramS* encodes a 42 amino acid peptide precursor of SapB. This peptide is modified by the *ramC* gene product to produce the active peptide 21 amino acids. RamC shows homology at the amino and carboxy terminal domains respectively with specific serine/threonine kinases and with lantibiotic processing proteins (Hudson *et al.*, 2002, Kodani *et al.*, 2004). The components of the medium play a conditional role on the characteristics of most *bld* mutants. The production of SapB and the extracellular

complementation cascade propose that checkpoint controls that involve most *bld* genes occur subject to specific nutritional conditions (Claessen *et al.*, 2006, Kelemen and Buttner, 1998).

There is another gene cluster recently discovered and found to be regulated by the RamR regulator, and this gene cluster has the ability to control development of aerial hyphae and sporulation. It consists of a four gene operon (ragABKR), encoding two subunits of an ABC transporter (products of ragA and ragB). Epistasis analysis by overexpression of the operon in *bld* and *ram* mutants showed that the rag operon can activate the sporulation process in *S. coelicolor* independent of the SapB pathway (Paolo *et al.*, 2006).

S. coelicolor hydrophobic aerial proteins are a family of secreted proteins that have a significant role in the hydrophobic characteristics of the aerial hyphae (Claessen *et al.*, 2003, Elliot *et al.*, 2003). These chaplin and rodlin proteins can form a hydrophobic sheath on the surface of aerial hyphae and the spores (Claessen *et al.*, 2004, Elliot and Talbot, 2004). In *S. coelicolor* there are eight chaplin proteins that share a hydrophobic domain of 40 amino acids. The chaplin genes are characteristic of sporulating actinomycetes including *S. coelicolor* and *S. avermitilis* (Elliot and Talbot, 2004). The effect that these chaplins play on colony morphology depends on how many chaplin are expressed; deletion of just single chaplin gene does not cause any noticeable effect, while the loss of four to eight of them may lead to delay in formation of the aerial hyphae and sporulation. On the other hand deletion of all chaplins leads to a gross defect in aerial hyphae formation as well as losing the hydrophobic surface property. Defects in aerial hyphae formation because of loss of chaplins is not related to the media components (Claessen *et al.*, 2004, Elliot *et al.*, 2003). The chaplins are surface active proteins and have the ability to reduce the surface tension of the air-water interface (Claessen *et al.*, 2003). Capstick *et al.*, 2007, established that synthesis of SapB and the chaplins leads to normal growth of aerial hyphae in rich medium, while just the SapB-independent pathway can do so on a minimal medium (Capstick *et al.*, 2007).

1.6.2 Transformation of aerial hyphae into spores

After the formation of the aerial hyphae, the apical regions of each hypha start forming a spiral syncytium that contains many genome copies. These apical regions undergo multiple septation resulting in chains of unigenomic pre-spore compartments and, ulitimately, grey-pigmented spores. *whi* genes are extremely important for this stage of sporulation, and mutation of these genes causes obvious defects in sporulation. These *whi* genes can be subdivided into two

kinds of sporulation genes: early and late sporulation genes. The early sporulation genes are listed in Table 1.4.

Table 1.4: Early regulatory genes affecting sporulation in *S. coelicolor*

Genes	Product	Reference(s)
whiA	Unknown (conserved among gram-positivebacteria)	(Ainsa *et al.*, 2000)
whiB	Small putative DNA binding protein (specific to actinomycetes)	(Molle and Buttner, 2002 Soliveri *et al.*, 1992)
whiG	Sigma factor (similar to σFliA of *E. coli*)	(Chater, 1989, Tan *et al.*, 1998)
whiH	GntR-like regulatory protein	(Ryding *et al.*, 1998)
whiI	Response regulator	(Aínsa *et al.*, 2002)
whiJ	Small protein with lambda repressor-like DNA binding domain	(Chater and Chandra, 2006)

Mutants of early sporulation genes fail to form the unigenomic compartments, while the late sporulation gene mutants have the ability to form recognizable spores, but these spores are defective in pigmentation or irregular in size (Chater, 1972, Chater, 2000, Hopwood *et al.*, 1970). There are six *whi* genes discovered, three of them (*whiG*, *whiI*, and *whiH*) encode homologs of well-known proteins, and the other three (*whiA*, *whiB*, and *whiJ*) encodeing other proteins that are not from well-known families of the proteins (Chater, 1972, Chater, 2000). *whiG* encodes an RNA polymerase sigma factor similar to those important for the formation of the flagella and other structures in unicellular bacteria (Chater, 1989, Chater, 2000, Tan *et al.*, 1998). *WhiG*'s role in differentiation of aerial hyphae is evident from the *whiG* mutant's phenotype that has straight hyphae with several vegetative septa (Chater, 1989, Flärdh *et al.*, 1999). Termination of aerial hyphal growth is suggested to be controlled by *whiA* and *whiB* gene products. *whiA* encodes a protein of unknown function that is conserved among the gram positive bacteria (Ainsa *et al.*, 2000). *whiB* encodes a small putative DNA-binding protein that belongs to a family of transcription factors specific to actinomycetes (Soliveri *et al.*, 1992). In the *S. coelicolor* genome there are approximately ten genes that encode *whiB*-like proteins, and this includes the late sporulation gene *whiD* (Molle and Buttner, 2002, Soliveri *et al.*, 1993). The *whiI* response regulator lacks several functional amino acid residues in its predicted phosphorylation pocket and there is no associated kinase gene. These findings suggest that *whi*I may function in a phosphorylation-independent manner (Ainsa *et al.*, 2000, Tian *et al.*, 2007). *WhiI* mutants share some similarities with *whiH* mutants: they both produce loosely coiled aerial hyphae without spores. There are also similarities with *whiG*, *whiA*, and *whiB* mutants that all lack DNA condensation (Aínsa *et al.*, 2002, Flärdh *et al.*, 1999). *WhiI* and *whiH* expression depends on the availability of the *whiG* sigma factor, and also both have autoregulatory activity with evidence that these genes may repress the expression of each other (Aínsa *et al.*, 2002, Ryding *et al.*, 1998). *WhiJ* mutants are pale grey, and the gene product has a lambda repressor-like DNA-binding domain at its N-terminus (Chater and Chandra, 2006).

Table 1.5: late regulatory genes affecting sporulation in *S. coelicolor*

genes	product	Reference(s)
whiD	WhiB-like protein	(Molle and Buttner, 2002)
whiE	Type II polyketide synthases	(Davis and Chater, 2006 Kelemen and Buttner, 1998)
whiL	Unknown	(Ryding *et al.*, 1999)
whiM	Unknown	(Ryding *et al.*, 1999)
whiO	Unknown	(Ryding *et al.*, 1999)

whiD as one of the late sporulation genes (Table 1.5) encodes a protein containing an oxygen-sensitive 4Fe-4S cluster and is one of the *whiB*-like family of the transcription factors (Jakimowicz *et al.*, 2005). The spores from *whiD* mutants are irregular in their size and shape, and also the wall of the spores is variable in thickness (Chater, 1972, McVITTIE, 1974). Eight genes together form a cluster in the complex *whiE* locus; these genes encode homologues of several enzymes involved in the synthesis of aromatic polyketides (Davis and Chater, 2006). The *whiE* locus consists of two transcription units, and these are expressed most during sporulation, dependent on the early *whi* genes (Kelemen and Buttner, 1998). Grey pigment is not produced in the whiE mutants (Chater, 1972, Davis and Chater, 2006, McVITTIE, 1974).

1.6.3 Other gene families that have impact on the development of the aerial hyphae

New gene families have been discovered in recent years that have impact on the development of the aerial hyphae. Some of these genes are listed in Table 1.6.

Table 1.6: Other genes that affect the development of aerial hyphae

genes	product	Referenc(s)
ssgA-G	members of SsgA-like proteins (SALPs) family	(Noens *et al.*, 2005)
sigF	RNA polymerase sigma factor	(Potúčková *et al.*, 2006)
crgA	Small membrane protein	(Del Sol *et al.*, 2006, Del Sol *et al.* 2003)
smeA	Small membrane protein	(Ausmees *et al.*, 2007)
sff	Member of SpoIIIE/FtsK-like protein family	(Ausmees *et al.*, 2007)

The *ssgA*-like genes encode the SsgA-like protein members (SALPs); these genes are found mostly in the sporulating Actinomycetes. There are seven paralogues of the SsgA-G, each one of those seven has its role in the process of sporulation, and their role starts from the initiation of the septa to the autolytic cleavage of spore wall peptidoglycan. SsgA and SsgB are found to be essential for the specific cell division that occurs during the sporulation process, SsgC–G are responsible for correct DNA condensation and segregation, spore wall synthesis is regulated by SsgD, SsgE, SsgF has a role in autolytic spore separation, while the SsgG controls the exact septum localization (van Wezel and Vijgenboom, 2004). SsgA was found to impact on cell division in both *S. coelicolor* and *S. griseus* (Kawamoto and Ensign, 1995). *ssgA* mutants in both bacteria have normal vegetative septa but both are defective in sporulation septation (Jiang and Kendrick, 2000). There is evidence of *ssgA* expression in submerged cultures of *S. coelicolor* even though under these conditions there is no sporulation (Kawamoto *et al.*, 1997, Van Wezel *et al.*, 2000a, van Wezel *et al.*, 2000b). In *S. coelicolor* the *ssgA* gene is regulated through the upstream-located *ssgR* (an *iclR*-type regulatory gene) in a *whi*-independent manner (Traag *et al.*, 2004, Yamazaki *et al.*, 2003). *ssgA* and *ssgR* gene transcription in *S. coelicolor* is regulated developmentally and activated towards the start of sporulation (Traag *et al.*, 2004). On the other hand, it was found that the overexpression of this gene may result in extensive septation that appears a thick and irregular (Kawamoto *et al.*, 1997, Van Wezel *et al.*, 2000a). An *ssgB* mutant is defective in sporulation grows as large white colonies (Keijser *et al.*, 2006, Kormanec and Sevcikova, 2002). The formation of aerial hyphae was found to be associated with the expression of SsgB and is dependent on sigH (σH) (Kormanec and Sevcikova, 2002).

sigF encodes an RNA polymerase sigma factor (Potúčková *et al.*, 2006). The mutant of this gene can produce sensitive spores with small, irregular thin walls. The expression of this gene is regulated mainly at the level of transcription, and depends on six *whi* genes (Kelemen *et al.*, 2006, Potúčková *et al.*, 2006). The *smeA* gene encodes a small membrane protein, while sffA encodes a member of SpoIIIE/FtsK-like protein family. *smeA* and *sffA* gene analysis shows that both genes are involved in the late stages of the sporulation process such as division septum formation, segregation and condensation of the chromosomes, spore pigmentation and separation, and spore wall maturation. *smeA* and *sffA* gene transcription occurs at the start of sporulation cell division and just after the stage of Z- ring formation. *smeA* gene expression was found to be dependent on *whi* gene products (Ausmees *et al.*, 2007). The gene *crgA* also encodes a small

membrane protein that has a role in the coordination of growth and cell division (Del Sol *et al.*, 2003). A *crgA* mutant of *S. coelicolor* exhibits early growth of the aerial hyphae as well as increased production of actinorhodin, while the overexpression of this gene blocks the the sporulation septation process (Del Sol *et al.*, 2003). The expression of this gene is controlled developmentally and it reaches its maximum expression at the time of differentiation. CrgA overexpression prevents the formation of the Z-ring, the initial stage in sporulation septation (Del Sol *et al.*, 2006). FtsI, FtsQ and FtsW proteins of *S. coelicolor* were also studied and found to have an impact specifically on sporulation septation (McCormick and Losick, 1996, McCormick *et al.*, 2006).

1.7 The process of cell division

Cell division is one of the most fundamental processes of most if not all organisms. In bacteria this process starts simply by duplicating the contents of the cell followed by the formation of the septum via the inward growth of the cell wall and cytoplasmic membrane that lead to the formation of two new cells. In fact this process is not simple as it seems; it is a highly regulated by specific proteins at each stage. For example there are around 15 known proteins controlling septum formation in *E. coli* species: these proteins are FtsZ, FtsA, ZipA, ZapA, FtsE, FtsX, FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI, FtsN, AmiC and EnvC (Bernhardt and de Boer, 2004) (Weiss, 2004). Out of those 15 proteins there are just 10 (FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI, and FtsN) that are known to to be essential for cell division (Goehring *et al.*, 2005, Vicente and Rico, 2006). Isolation of conditional mutants was the way that the role of these cell division genes was identified. This method depends on the isolation of mutants that can grow as filaments at specific temperatures that then led to the common name of these genes, *fts*, which stands for filamentous temperature sensitive phenotype.

Table 1.7: Cell division proteins of *E. coli* and *B. subtilis* (Ebersbach et al., 2008, Errington, (2003), Goehring et al., 2005, Harry et al., 2006b).

E. coli Protein	Homologue in *B. subtilis*	Product	Function	Homologue in *S. coelicolor*
Early division (Z-ring associated proteins				
FtsZ	**FtsZ**	Tubulin homologue	Z-ring scaffold formation	**FtsZ**
FtsA	FtsA	Actin superfamily member	Z-ring stabilization, recruitment of late division proteins	NP
ZipA	NP	Unknown	Z-ring stabilization	NP
ZapA	ZapA	Unknown	Positive regulator of Z-ring formation	NP
ZapB	?	Unknown, Homodimeric anti-parallel coiled-coil protein	Z-ring assembly	?
?	EzrA	Unknown	Negative regulator of Z-ring assembly	NP
Late division proteins				
FtsE	FtsE	Similar to ABC transporter	Unknown	FtsE
FtsX	?	Similar to ABC transporter	Unknown	FtsX
FtsK	SpoIIIE^{S}	Unknown	Chromosome segregation	SCO5750
FtsQ	DivIB	Unknown	Unknown	FtsQ
FtsL	**FtsL**	Contain putative leucine zipper coiled-coil regions	Unknown	FtsL
FtsB (formerly YgbQ)	**DivIC**	Unknown	Unknown	DivIVC
FtsW	**FtsW**^{N} (YlaO) & SpoVE^{S}	Polytopic membrane protein of SEDS family	Probably in recruitment of cognate PBPs	FtsW/SCO3846 Sfr/SCO5302
FtsI	Pbp2B & SpoVD^{S}	Division specific trans peptidase	Septal cell wall synthesis	SCO2090
FtsN	NP	Contains murein binding domain	Unknown	NP
AmiC	?	Amidase	Daughter cell separation	?
EnvC	?	Lipoprotein murein hydrolytic activity	Daughter cell separation	?
NP	SepF (=YlmF)	Unknown	Unknown, FtsZ interacting protein	?
Z- ring positioning				
MinC	MinC	Unknown	FtsZ polymerization inhibitor	NP
MinD	MinD	ParA family ATPase	Min oscillator (*E. coli*), Localization of MinC at the cell poles (*B. subtilis*)	SCO5006 SCO3557
MinE	NP	Unknown	Topological regulator of Min system	NP
NP	**DivIVA**	Unknown	Topological specificity of Min system	DivIVA
SlmA	Noc^{F}	Unknown	Negative regulator of FtsZ assembly, prevents Z ring formation over nucleoid (Nucleoid occlusion)	?
SulA	YneA^{F}	Unknown		?

Essential proteins indicated in bold letters; NP: Not Present; ^{F} Functional homologue (structurally unrelated); ^{N} Function is not verified yet; ? : Other homologues may exist; ^{S} Sporulation Specific and not required for vegetative septation.

Not all of the genes are present in the process of the cell division in all kinds of bacteria. On the other hand, some specific genes are found to be involved in the mechanism of cell division of all bacteria. Normally, those genes are grouped and linked to genes encoding peptidoglycan synthesis enzymes forming the cluster of the genes that is known as the division cell wall cluster (*dcw*) (Vicente *et al.*, 1998).

Cell division starts with the formation of the Z ring through the polymerization of the protein FtsZ at the site of the expected septum that in *E. coli* is orgainised to be exactly in the middle of the cell (Erickson *et al.*, 1996, Romberg and Levin, 2003). The formed Z ring works as a mark for the division site and provides the required scaffold for the rest of the proteins needed for the division process. The assembly of division proteins formed at the site of the scaffold along with the Z ring is known as the divisome that drives the division process. Up to now the complete functions of the individual divisome proteins are not known yet. Of known function are, for example, FtsA, ZipA, ZapA that support Z ring assembly. Other known functions include the coordination of septation with the chromosome segregation (FtsK), employing and stabilizing the late divisome proteins at the site of the division, the formation of the peptidoglycan cell wall (FtsI, FtsW), and the hydrolysis of peptidoglycan to separate the new formed cells (AmiC, EnvC) (Errington, (2003) , Harry et al., 2006a).

1.7.1 Divisome proteins and their association

FtsZ is the first protein involved in cell division that locates at the site of division; this protein is found in virtually all bacterial species (Harry et al., 2006a). FtsZ has some similarity to tubulin as it binds and hydrolyzes GTP (Romberg and Levin, 2003). FtsZ has similar features as tubulin polymers; it reversibly accumulates into dynamic filamentous structures. This similarity supports the proposed cytoskeletal function of FtsZ (Mukherjee *et al.*, 1993, Mukherjee and Lutkenhaus, 1998). Translational fusions with GFP combined with immunofluorescence microscopy indicates that the gathering of the Z ring at the midcell results from the reorganization of FtsZ helices (Anderson *et al.*, 2004, Stricker *et al.*, 2002, Thanedar and Margolin, 2004).

The cytoplasmic protein FtsA is one of the first proteins employed to form the Z ring (Fig. 1.5). The sequence and the structure oft FtsA are similar to the ATPase superfamily of proteins as both contain actin, sugar kinase and the Hsp70 family of heat shock proteins (Bork *et al.*, 1992). FtsA plays a role in the formation and stabilization of the Z ring through the interaction with the C-terminus of FtsZ - this was shown clearly in yeast two-hybrid interaction studies (Di Lallo *et*

al., 2003, Din *et al.*, 1998, Ma *et al.*, 1996). The role of the FtsA differs from one kind of bacterium to another; it is considered to be essential in *E. coli*, while it's not essential in *B. subtilis* (Harry et al., 2006a).

ZipA is the second protein employed at the site of division in *E. coli.* This protein was found to be essential for division in *E. coli*, although it is considred to be poorly conserved protein as it can be founded only in the γ-proteobacteria (Margolin, 2006). ZipA interacts directly with FtsZ and locates at the Z ring in an FtsZ-dependent, FtsA-independent manner (Hale and de Boer, 1997, Hale and De Boer, 1999).

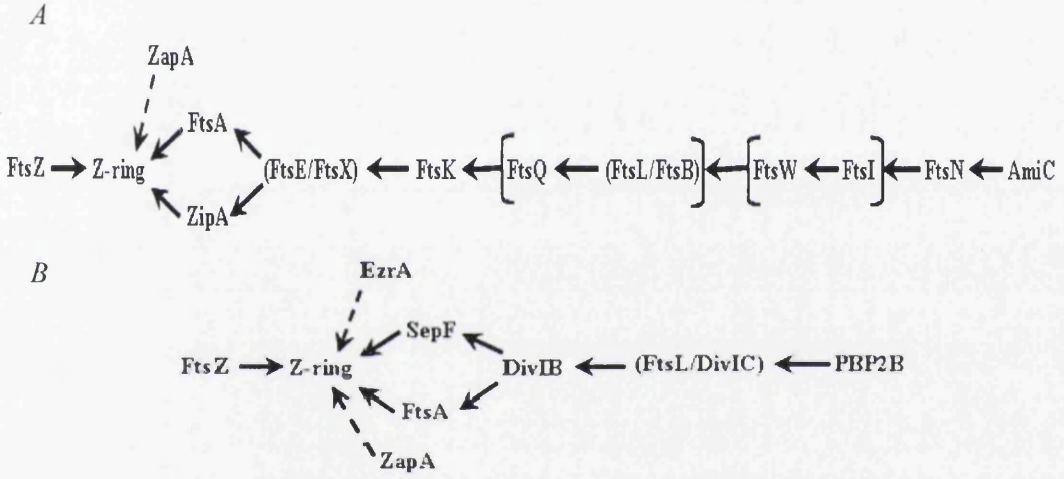


Figure 1.5: Divisome assembly pathway in *E. coli* and *B. subtilis*. (A) In *E. coli* a proto-ring is formed by interaction between three proteins (FtsZ, FtsA and ZipA) assembling on the cytoplasmic membrane, that is followed by the addition of FtsK to form the cytoplasmic ring. At a late assembly stage additional elements forming a periplasmic connector (FtsQ, FtsB and FtsL) and the proteins of the ring involved in manufacturing septal peptidoglycan (FtsW and FtsI) are added, followed by FtsN, as a ring protruding into the periplasm and connecting with the peptidoglycan layer. FtsL and FtsB, presented in small bracket can contact with each other independently of the FtsQ prior to forming a complex (present in big bracket) with FtsQ. (B) In *B. subtilis* the divisome assembles in a much more concerted manner. Similar to *E. coli*, a proto-ring is formed by interaction of FtsZ, FtsA, SepF and EzrA. Once the Z ring has formed the late recruited proteins (DivIB, FtsL, DivIC and PBP 2P) are localized interdependently, forming the divisome. Dashed arrows indicate that a protein is not required for downstream recruitment but is involved in forming or stabilizing the Z ring. In *B. subtilis* there is no apparent homologue of FtsN and the FtsK homologue (SpoIIIE) is not required for division. (Errington, (2003) , Goehring et al., 2006, Harry et al., 2006a)

Despite the essential role that FtsA and ZipA play in septum formation, the Z ring was found to be formed in the presence of the either ZipA or FtsA, while it cannot be formed in the absence of both; this reflects the unclear role of these two proteins in the construction of the Z ring (Pichoff and Lutkenhaus, 2002). In recent years, a study showed that the protein SepF (YlmF) acts as an FtsZ interacting protein in *B. subtilis*; this study showed also that SepF (YlmF) locates at the site of the division in FtsZ dependent manner (Hamoen *et al.*, 2005, Ishikawa *et al.*, 2006).

There is a negative regulator in *B. subtilis* known as EzrA protein, which is a transmembrane protein similar to ZipA protein. This EzrA protein is found in some of the gram-positive bacteria that have low content of guanine + cytosine nucleotides. It is also founded to be distributed through the plasma membrane and concentrates at the cytokinetic ring in an FtsZ-dependent manner during cell division. It is also founded that the loss of the EzrA protein leads to the formation of multiple Z rings that are distributed at the middle and the poles of the cell (Levin *et al.*, 1999). EzrA protein interacts directly with FtsZ *in vitro* to prevent the polymerization of the FtsZ (Haeusser *et al.*, 2004). This interaction was then recently to reduce the ability of the FtsZ to bind to GTP; and minor interaction can also accelerate the rate of GTP hydrolysis by the protein EzrA. These EzrA interactions are not preferred for FtsZ polymerization (Chung *et al.*, 2007). It has been suggested recently that the EzrA protein can act as a positive regulator of cell division as a low level of EzrA expression results in a filamentous phenotype (Chung *et al.*, 2004).

FtsE and FtsX are proteins related to the ABC transport superfamily and are employed in the cell division process and exactly at the site of division. These two proteins have the ability to interact with each other forming a complex; this interaction requires FtsZ, FtsA and ZipA to be localized at the midcell (Schmidt *et al.*, 2004). There is also another small cytoplasmic protein that has the ability to interact with the FtsZ known as the ZapA (Z ring associated protein; this protein was found to be not dispensible for cell division as well as not being required for the localization of the downstream division proteins (Gueiros-Filho and Losick, 2002). ZapB is another that protein can interact with the FtsZ; this protein is required to sustain the length of the cell and promote the gathering of the Z ring. This protein requires the availability of FtsZ, while it does not require FtsA, ZipA or FtsI to localize the site of division (Ebersbach *et al.*, 2008). The exact function of the ZapB has not been revealed yet.

FtsK is another protein involved in the process of the division; this protein is a large polytopic membrane protein that has several functions and contains three different domains that

are responsible for different functions of the protein (Begg *et al.*, 1995, Bigot *et al.*, 2004). FtsK requires the availability of FtsZ, FtsA and ZipA to take place at the site of division in *E. coli* as well as the availability of three other bitopic proteins FtsQ, FtsL and FtsB later in time to correctly localize (Weiss, 2004). These three bitopic proteins each contain a short N-terminal cytoplasmic region, a single transmembrane segment and a large C-terminal periplasmic domain and form a complex just before their localization to the division site (Buddelmeijer and Beckwith, 2004, Chen and Beckwith, 2008, Chen *et al.*, 1999, Daniel *et al.*, 2006).

FtsQ is another protein that has different roles according to the species; it is found to be essential for the survival in *E.coli*, whereas its homologue (DivIB) in *B. subtilis* is essential for survival of the cell just at high temperatures (Katis *et al.*, 2000). This protein (FtsQ), requires FtsZ, FtsA, ZipA and FtsK to find its location in the Z ring (Chen and Beckwith, 2008). Several recent studies that built on the mutational analysis of the FtsQ in *E.coli* and its homologue DivIB in *B. subtilis* have shown that there are three functional subdomains (α, β and γ in *B. subtilis*) which are involved in cell division through the interaction with other cell divisome proteins; this show the importance of their availability in the cell division process (D'Ulisse *et al.*, 2007, Robson and King, 2006, Van Den Ent *et al.*, 2008, Wadsworth *et al.*, 2008).

FtsL and FtsB are other proteins found to be related to the process of cell division in several organisms such as the *E. coli*. Their periplasmic domains contain putative leucine zipper coiled-coil motifs that play a major role in protein- protein interactions (Buddelmeijer and Beckwith, 2002, Daniel and Errington, 2002, Ghigo and Beckwith, 2000). In *B. subtilis* there are four proteins FtsL, DivIC, DivIB, and PBP 2B that founded to be interdependent for assembly unlike the linear protein assembly pathway in *E.coli*. (Errington, (2003)).

FtsW and FtsI (PBP3) are other proteins employed at the site of division (Vicente and Rico, 2006). The first protein (FtsW) belongs to a large polytopic family of membrane proteins that have a role in defining the shape of the cell, elongation, division and sporulation (SEDS) (Henriques *et al.*, 2002, Ikeda *et al.*, 1989). RodA is a member of the SEDS proteins in *E.coli* that has a role in the cell elongation (Errington, (2003)). FtsW has been shown to function as a transporter of lipid-linked cell wall precursors across the cell membrane (Mohammadi *et al.*, 2011). FtsI (PBP3) and the homologue PBP 2B, in *E.coli* and *B. subtilis* respectively, are a sort of

class B penicillin-binding proteins (PBPs) (Daniel and Errington, 2002, Errington, (2003) , Harry, 2001). This protein and its homologue are founded to be required for the synthesis of peptidoglycan. The localisation of FtsI requires the availability of FtsZ, FtsA, FtsQ, FtsW and FtsL (Harry et al., 2006a, Mercer and Weiss, 2002).

Another protein that localizes at the site of the division in some bacteria is FtsN, this protein is found only in the enteric bacteria and Haemophilus spp. (Errington, (2003)) Localization of FtsN requires FtsZ, FtsA, FtsI and FtsQ (Addinall *et al.*, 2003). FtsN has no clear function despite the limited sequences that suggest a possible role of the FtsN to hydrolysis the cell wall (Errington, (2003)).

The last two periplasmic proteins AmiC and EnvC have been studied in *E.coli* and localize at the division ring (Bernhardt and de Boer, 2004, Bernhardt and De Boer, 2003). AmiC localization depends on FtsN and it has the ability to degrade the septal cell wall that leads to the separation of two daughter cells (Bernhardt and De Boer, 2003). While EnvC has a role in daughter cell separation as it has a murein hydrolysis activity (Bernhardt and de Boer, 2004).

Past studies of the *E. coli* divisome proteins indicated that their assembly pathway is in a linear order (Buddelmeijer and Beckwith, 2002). On the other hand, more recent studies show that protein assemblies are recruited in a hierarchical manner (Aarsman *et al.*, 2005, Goehring *et al.*, 2006, Goehring *et al.*, 2005). This explains that during the assembly of the Z ring there is a proto-ring formed beforehand, this proto-ring results from the assembly of FtsZ, FtsA and ZipA. FtsK is then employed to form the cytoplasmic ring. FtsQ, FtsL and FtsB incorporate at a later stage to the cytoplasmic ring that acts as a connecter between the late proteins and the cytoplasmic ring. Then FtsW and FtsI proteins are added. The final stage involves the recruitment of the FtsN to connect the ring with peptidoglycan synthesis (Goehring *et al.*, 2006, Vicente and Rico, 2006).

1.7.2 Irregular Septation during Sporulation

Some bacterial species like *B. subtilis* undergo another process during the sporulation cycle and this process is assumed to be similar to midcell division; it is called asymmetric septation and controlled by very specific proteins (Bigot *et al.*, 2004, Levin and Grossman, 1998). The mechanism starts with the relocalization of the Z-ring from the mid-cell to polar sites; this happen via a spiral intermediate (Ben-Yehuda and Losick, 2002). The transfer of the Z-ring position is mediated by the activity of a polytopic membrane protein called SpoIIE and under the control of

Spo0A (Barak and Youngman, 1996, Khvorova *et al.*, 1998). The role of SpoIIE protein in asymmetric septation was shown by fluorescence microscopy that revealed how the asymmetric septum located in the form of a ring (Arigoni *et al.*, 1995); the protein can activate bipolar Z-ring formation, and stabilize the ring when it is formed. Another study suggests that the SpoIIE protein acts as a membrane anchor for the Z ring (Khvorova *et al.*, 1998). Once both rings are formed, all the division machinery involved in midcell division assembles at the poles of the cell (Barak and Wilkinson, 2007).

1.7.3 Spatial Control of Z-ring Positioning

The placement and the timing of the septum placement are very important steps in cell division. The control of these steps is achieved through the regulation of Z ring assembly. In *E.coli* and *B. subtilis* there are two mechanisms for the accurate positioning of the Z ring: these mechanisms are the Min system and nucleoid occlusion. The first can prevent asymmetric septation during vegetative growth, while the second (nucleoid occlusion) inhibits premature Z-ring assembly at the midcell (Fig. 1.8) (Barak and Wilkinson, 2007, Errington, (2003) , Harry et al., 2006a, Margolin, 2001, Rothfield et al., 2005b).

(I) Nucleoid Occlusion

This regulation appears at the nonpolar regions where the nucleoid prevents septum formation in its area; this mechanism is named nucleoid occlusion (Fig. 1.6C) (Woldringh *et al.*, 1991). Nucleoid occlusion impacts on Z-ring assembly (Harry et al., 2006a). Two genes (noc and slmA) have been identified in *B. subtilis* and *E. coli*, respectively, which encode specific effectors involved in nucleoid occlusion in both species (Bernhardt and De Boer, 2005, Wu and Errington, 2004). Noc and SlmA proteins belong to completely different families of DNA binding proteins. These two proteins have a quite similar role in the prevention of FtsZ polymerization in the area of the nucleoid despite the differences in their sequences (Barak and Wilkinson, 2007, Bernhardt and De Boer, 2005, Harry et al., 2006a, Wu and Errington, 2004).

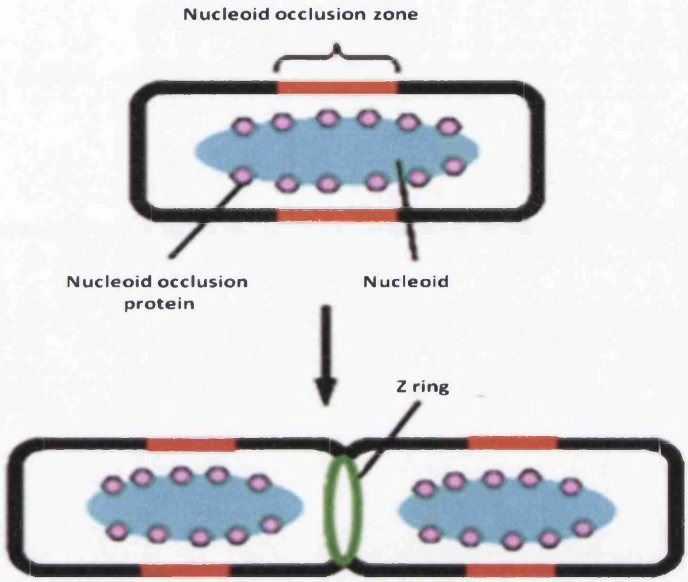


Figure 1.6: In the nucleoid occlusion system of both the bacteria, nucleoid occlusion proteins (Noc or SlmA, small pink ovals), associated with the nucleoid form a nucleoid occlusion zone (red line) in the vicinity of of the nucleoid (light blue) and block cell division. (Harry et al., 2006a, Rothfield et al., 2005a).

1.8 Role of SEDS Proteins and their cognate PBPs in Cell Wall Synthesis

In 2005 it was revealed that SEDS family proteins may have a role in the mechanism of cell wall synthesis; this was as a result of studying the components of cell wall that contain glycan polymers. The glycan polymers are unified by the peptide side chains bonds to form peptidoglycan. Peptidoglycan synthesis starts in the cytoplasm and, after its synthesis; UDP-MurNAc-pentapeptide is linked to undecaprenyl phosphate in order to form the lipid I intermediate at the cytoplasmic membrane. After the formation of lipid I, lipid II is produced when N-acetylglucosamine (GlcNAc) from UDP-GlcNAc is added to lipid I. Lipid II is then transported across the membrane to the periplasmic space by a specific mechanism that involves the SEDS proteins. Cell wall synthesis happens outside the cytoplasmic membrane. In a rod shaped bacteria, wall synthesis appears in the modes of elongation and division and these modes are catalyzed by various protein complexes (Nanninga, 1998, Scheffers and Pinho, 2005). SEDS family proteins and PBP are parts of the protein complexes involved in cell wall synthesis. As the SEDS family proteins are polytopic membrane proteins they are involved in cell shape determination as well as other mechanisms such as the elongation, division and sporulation(Daniel and Errington, 2003, Henriques *et al.*, 2002). The gene encoding a member of the SEDS family proteins is located close to a gene encoding a member of class B High Molecular Weight (HMW) PBPs (Fig. 1.7 A, B and C), and the respective proteins work in conjunction with each other (Datta *et al.*, 2006, Hara *et al.*, 1997, Matsuzawa *et al.*, 1989, Mercer and Weiss, 2002). RodA and PBP2 are among the best pair examples since they are responsible for the cell elongation, while FtsW and FtsI (PBP3) are responsible for cell division in *E. coli* species (Hara *et al.*, 1997, Matsuzawa *et al.*, 1989, Mercer and Weiss, 2002, Tamaki *et al.*, 1980). In *E. coli*, FtsW may also be required for Z ring stabilization (Boyle DS, 1997, Mercer and Weiss, 2002). An interaction between FtsW and FtsZ is mediated via C-terminal extensions of FtsW and FtsZ, but this interaction has only been proven for the proteins of *M. tuberculosis* (Datta *et al.*, 2002, Datta *et al.*, 2006, Rajagopalan *et al.*, 2006). In *M. tuberculosis*, FtsW also interacts with FtsI (Datta *et al.*, 2006).

In *E. coli*, PBP2 and RodA proteins are encoded by the *pbpA-rodA* operon and the mutation in *rodA* or inactivation of PBP2 leads to spherical growth, supporting the expected role of these two proteins in cell elongation (Spratt, 1975, Tamaki *et al.*, 1980). In addition, the depletion of

RodA in *B. subtilis* results in the formation of spherical cells and ultimately the destruction of the cell (Henriques *et al.*, 2002). Meanwhile, inactivation of FtsW or FtsI protein blocks cell division and lead to a filamentous morphology. This indicates the involvement of these proteins in septation-specific cell wall synthesis (Boyle DS, 1997). The structures of FtsW and SpoVE proteins from several bacterial species have been studied indicating around 10 transmembrane (TM) segments and a large extracytoplasmic loop (Fig. 1.7 D) (this component has not been determined in tuberculosis FtsW); N- and C- termini are other components that are located in the cytoplasm (Datta *et al.*, 2006, Gérard *et al.*, 2002, Lara and Ayala, 2006, Real *et al.*, 2008).

SpoVE is another SEDS protein homologue found in *B. subtilis* along with its cognate class B PBP, SpoVD which are required in the sporulation process (Ikeda *et al.*, 1989, Joris *et al.*, 2006). Spore cortex synthesis was completely blocked in spoVE mutants with no impact on vegetative growth. The genes encoding both SpoVE and SpoVD are located in the *dcw* cluster of the bacteria that form spores; spoVE occupies the position of the *ftsW* in the cluster which indicates the importance of the coordinated expression of *dcw* genes in the synthesis of the spore cortex (Henriques *et al.*, 1992, Real *et al.*, 2008).

SEDS proteins have a role in localizing their related PBPs, as well as having a role in Z ring stabilization, integrating the signals between the cytoplasmic and the periplasmic components of division, and translocation of the lipid-linked peptidoglycan precursors via the cytoplasmic membrane (Boyle DS, 1997, Datta *et al.*, 2006, Margolin, 2006, Pastoret *et al.*, 2004, Real *et al.*, 2008). The mechanism for localization of the SEDS proteins has yet to be determined.

PBPs are acyl serine transferase proteins and include HMW PBPs, low molecular weight (LMW) PBPs and β-lactamases. The first, HMW PBPs, are multidomain proteins that consist of a cytoplasmic tail, a transmembrane anchor and two domains linked together by a β- rich linker located on the outer surface of the cytoplasmic membrane where peptidoglycan synthesis takes place (Goffin and Ghuysen, 1998, Macheboeuf *et al.*, 2006, Sauvage *et al.*, 2008, Scheffers and Pinho, 2005). HMW PBPs can be divided according to their primary structure and the catalytic activity of the N terminal domain into two classes (A, B); class A proteins are bifunctional with transpeptidase activity provided by the N terminal domain and glycosyltransferase activities (Goffin and Ghuysen, 1998, Sauvage *et al.*, 2008, Scheffers and Pinho, 2005). Class B HMW PBPs have the activity of being morphogenetic determinants by folding and stabilizing the penicillin-binding domain as well as interacting with other proteins (Den Blaauwen *et al.*, 2008).

On the other hand the LMW proteins are monofunctional DD-peptidases since the majority of them behave like a DD-carboxypeptidase, although some proteins of this group have transpeptidase or endopeptidase activity (Sauvage *et al.*, 2008, Scheffers and Pinho, 2005). Generally the LMW PBPs are described as class C PBPs and are not involved in the synthesis of peptidoglycan but help in modifying the peptidoglycan by hydrolysing the carboxy-terminal D-alanyl-D-alanine peptide bond of pentapeptide sidechains (DD-carboxypeptidases) by which they make the peptide side chains inaccessible for cross linking or breaking the peptide cross-links that hold the glycan chains together (endopeptidases) (Den Blaauwen *et al.*, 2008, Scheffers and Pinho, 2005).

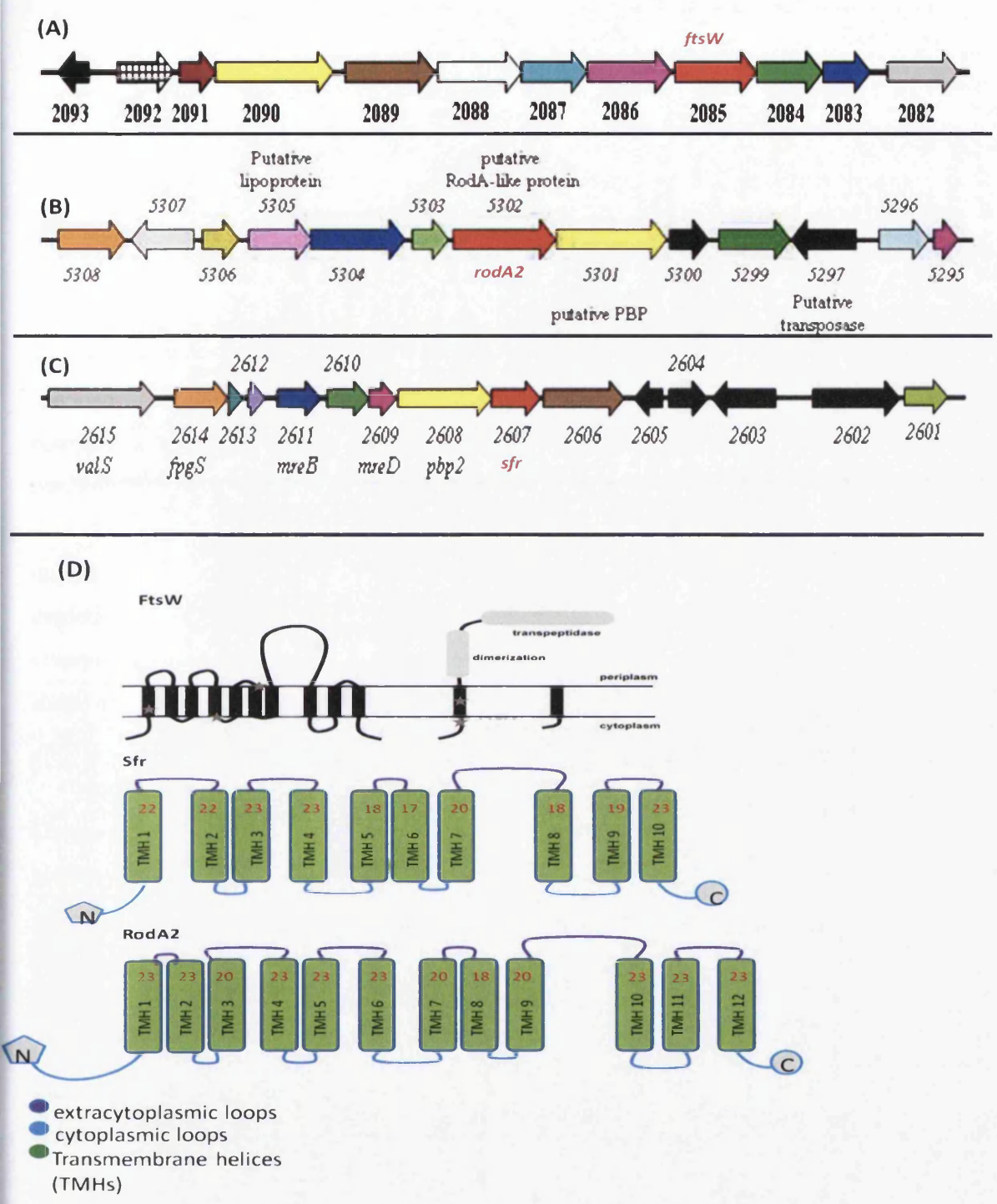


Figure 1.7: (A, B and C) Genetic organization of three of the four SEDS protein genes of *S. coelicolor*. For each locus, the SEDS protein gene is indicated by red colour, and the cognate PBP gene is indicated by yellow colour. *SCO* gene numbers and, where appropriate, gene names are shown above or below each diagram. (D) Diagrammatic presentation of membrane topologes of FtsW, Sfr, and RodA2 proteins of *S. coelicolor*. Numbers in bars with red colour indicate number of amino acids per TMH and numbers with black colour represents number of TMH.

1.9 Growth and Division in *S. coelicolor*

Streptomycete bacteria have their own aspects of growth and division compared to the other bacterial groups. *S. coelicolor* is considered the model organism of the streptomycetes in most studies. They grow asymmetrically via the incorporation of specific peptidoglycan material at the tip and sub-apical branching of hyphae to form a branching mycelium (Flärdh, 2003a, Flärdh, 2003b, Gray *et al.*, 1990, Schwedock *et al.*, 2004). Infrequent cross-wall formation in the vegetative mycelium of *Streptomyces* forms multigenomic compartments. . Cross-wall formation can lead to the growth of a new lateral branch by disconnecting the sub-apical cell from the apical compartment. DivIVA is one of the most important components required for the tip extension in *S. coelicolor* (Flärdh, 2003a, Flärdh, 2003b). (Table 1.7).

Localization studies of a DivIVA-EGFP (Enhanced Green Fluorescent Protein) indicated a unique localization of the protein at the tips of the hyphae and the lateral branches. Partial depletion of the protein leads to abnormal polar growth with curly hyphae and apical branching. Overexpression of the protein can lead to the induction of multiple branching and impacts on the shape of the hyphae (Flärdh, 2003a).

Homologues of DivIVA were also founded in other actinomycetes including *M. tuberculosis*, *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*. These actinomycetes also grow by apical extension at the poles which is the place of DivIVA homologue localization that is important for viability (Kang *et al.*, 2008, Letek *et al.*, 2008, Ramos *et al.*, 2003). Interactions between PBP1a, a HMW class A PBP and DivIVA was shown in C. glutamicum, and this interaction suggest that DivIVA can play a role in localizing peptidoglycan biosynthesis at the cell poles (Valbuena *et al.*, 2007). In *B. subtilis*, the DivIVA homologue has a quite different role in the spatial regulation of cell division and in chromosome segregation during the sporulation phase; this role depends on the ability of DivIVA to isolate MinC and MinD at the cell poles (Barak and Wilkinson, 2007, Thomaides *et al.*, 2001)

Two actin homologues, MreB and Mbl present in *S. coelicolor* and in other streptomycetes are nonessential for viability. The abnormal spore phenotypes of null mutants indicate that these cytoskeletal proteins are involved in the synthesis of the spore wall and maintaining the integrity of that wall (Table 1.6) (Mazza *et al.*, 2006, Noens, 2007). Some research have been done on the localization of MreB-EGFP using fluorescence microscopy indicating diffuse fluorescence in

vegetative hyphae, reflecting random distribution in those hyphae. On the other hand, MreB-EGFP localizes to the septa of sporogenic aerial hyphae during aerial growth, and subsequently underneath the cytoplasmic membrane of the spores (Mazza *et al.*, 2006). There are two types of cell division in *Streptomyces*. The first is vegetative septation that lead to the formation of irregular cross-walls in the substrate mycelium without detachment of the daughter cells. The second mode of cell division involves sporulation septation in sporogenic aerial hyphae leading to the formation of unigenomic spore chains (Flärdh, 2003b, Kwak *et al.*, 2001). From the role that most cell division proteins play in streptomycetes, it appears that their function is related to their role in cell division in other bacteria, with the important caveat that they function during sporulation septation but NOT during vegetative cross-wall formation (Mistry *et al.*, 2008). In unicellular bacteria such as *B. subtilis*, *E. coli* and actinobacteria such as *M. tuberculosis* the cell division is essential for growth and viability. In contrast, null mutants of *S. coelicolor ftsZ, ftsQ, ftsK, ftsL, divIVC* and *ftsX* genes are viable (Bennett *et al.*, 2007, McCormick and Losick, 1996, McCormick *et al.*, 2006, Mistry *et al.*, 2008, Noens, 2007, Wang *et al.*, 2007). An *ftsZ* mutant lacks cross walls in the vegetative hyphae and sporluation septa in aerial hyphae, indicating that both division processes may involve the same basic cell division machinery (McCormick *et al.*, 2006). In contrast *ftsL, ftsQ, ftsW, ftsI* and *divIVC* mutants are uniquely defective in sporulation septation (Bennett *et al.*, 2007, Mistry *et al.*, 2008). As these mutants have normal cross-walls in vegetative hyphae, this indicates that the two types of cell division are distinct, with sporulation septation being mechanistically similar to cell division in unicellular bacteria.

During sporulation septation, the FtsK protein has also been shown to be involved in chromosomal segregation. *ftsK* deletion mutants generate aberrant colonies associated with a large deletion from the chromosome (Wang *et al.*, 1997). FtsX and FtsE are also proposed to have roles in the final stage of cell division during the autolytic separation of the spores (Noens, 2007). *ftsX* mutants shows a kind of frequent branching at the base of spore chains. On the other hand, Z ring assembly during the sporulation and vegetative phase require the control of *ftsZ* transcription. ftsZ1p, ftsZ2p and ftsZ3p as putative promoters were detected in the the *ftsQ-ftsZ* intergenic region (Flärdh *et al.*, 1999). FtsZ1p expression occurs in a quite low manner throughout development, whereas the ftsZ2p promoter is up regulated during sporulation. This up regulation require the present of the six early regulatory genes including *whi*A, *whi*B, *whi*G, *whi*H, *whi*I and *whi*J genes. The ftsZ3p promoter was also found to be expressed strongly during early vegetative

growth, and the activity of this promoter decreases gradually during development. (Flärdh *et al.*, 1999). Once sporulation starts, FtsZ begin polymerizing into spirals which then reorganized into a regular Z ring along the sporogenic aerial hyphae (Grantcharova *et al.*, 2005). Z ring assembly can be prevented by nucleoid occlusion; this can happen in the vicinity of chromosome in *E. coli* and *B. subtilis* but its role in *Streptomyces* species is unclear (Flärdh, 2003b, Schwedock *et al.*, 2004). It is worthwhile mentioning that FtsA, ZipA, ZapA, EzrA and SpoIIE have no apparent orthologs in streptomycetes, but they found to be responsible for the stabilization and the anchoring of the Z ring in *E. coli* and *B. subtilis* (Flärdh, 2003b, Harry et al., 2006b). The lack of these orthologs in the *Streptomyces* genome suggest that there may be other proteins of the divisome involved in the the process of attaching and localizing the Z ring to the membrane. CrgA and SsgA proteins have the possibility to act as negative or positive regulators of the Z ring assembly respectively. CrgA overexpression inhibits Z ring assembly in the aerial hyphae, while the overexpression of the SsgA lead to the formation of hyper-septation in the vegetative and aerial hyphae (Del Sol *et al.*, 2006, Noens *et al.*, 2005, van Wezel *et al.*, 2000b). In addition, FtsZ is positively recruited by the membrane-associated divisome protein SsgB, itself localized by interaction with SsgA (Willemse *et al.*, 2011).

1.10-Aims and Objectives

The aim of this investigation is to understand the role of cell division proteins and their cognate PBPs during *S. coelicolor* division processes. In this study *S. coelicolor* M145, a prototrophic derivative of *S. coelicolor* A3(2) strain lacking its two plasmids SCP1 and SCP2, is used as a model organism. This organism is in many ways unique and interesting for studying growth and cell division. As is already discussed above, this mycelial organism grows by tip extension and undergoes two distinct types of cell division that are not essential for viability and take place in two different cell types formed during the course of the developmental cycle. Cell division in this organism is under tight control of and highly coordinated with fairly well understood yet complex regulatory mechanisms underlying morphological differentiation. The genes that have been shown to be involved in the assembly of FtsZ into a stable Z ring, a critical early stage for successful cytokinesis in bacteria like *E. coli* and *B. subtilis*, are absent from *Streptomyces*. They are in fact absent from almost all actinomycetes, including mycobacteria. Another important feature is the lack of Min system, responsible for spatial and temporal regulation of Z ring assembly in *E. coli* and *B. subtilis*, from the *Streptomyces* genome. So far there is no information available about stabilization and regulation of Z rings in space and time during the developmental cycle of *S. coelicolor* or other actinomycetes. Thus, the molecular mechanisms linking the spatial and temporal regulation of cell division with morphological differentiation are also of interest to understand developmental biology of bacteria and should be useful for developing new antibacterial drugs targeting cell division.

1. More analysis and investigation of *ftsW* and ftsI function in *S. coelicolor* by:
   A) Analysis the complementation of *ftsW* and *ftsI* mutants, also overexpression FtsW and FtsI proteins will be studied.
   B) Fluorescence protein (mCherry) will be used to Investigate FtsW localization and co-localization.
   C) Comparison of the activity and function of a related FtsW and FtsI from *Mycobacterium tuberculosis.*
2. As SEDS family proteins play an essentially important role in cell wall synthesis during different aspects of the bacterial life cycle. Construction of disruption mutants of *ftsW*, *sfr* and *rodA2* SEDS genes in one strain of *S. coelicolor* will be tested.
3. To investigate the interactions between cell division proteins using two hybrid thechniqe.

Chapter 2: Materials and Methods

2.1 Bacterial strains

Bacterial strains used during this study are listed in Table 2.1.

Table 2.1: Bacterial strains

Strain	Genotype	Source
Escherichia coli JM109	*F' traD36 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14 gyrA96 recA1 relA1 endA1 thi hsdR17*	Promega Corp., (Yanisch-Perron *et al.*, 1985)
E. coli BW25113/pIJ790	*E. coli K12 derivative: lacI^q rrnB ΔlacZ hsdR514 ΔaraBAD ΔrhaBAD,* containing *λ-RED* recombination plasmid pIJ790	(Datsenko and Wanner, 2000a, Gust et al., 2003a, Datsenko and Wanner, 2000b, Gust et al., 2003b)
E. coli ET12567 (pUZ8002)	*dam13::Tn9 dcm-6 hsdM hsdR recF143 zjj201 ::Tn10 galK2 galT22 ara14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtlI glnV44, containing the non-transmissible oriT mobilizing plasmid pUZ8002*	(Flett *et al.*, 1997, Flett *et al.*, 2006)
Streptomyces coelicolor M145	Prototrophic; SCP1$^-$ SCP2$^-$ Pgl$^+$	(Kieser *et al.*, 2000)
DSCO5302-1/2607	M145 *rodA2*::Tn*5062*, *sfr*::Tn*5062*-hyg	(Mistry *et al.*, 2008)

* Details of each transposon insertion can be found at http://strepdb.streptomyces.org.uk;

NA – Not applicable

Table 2.1: Bacterial strains (Continued)

Strain	Genotype	Source
E. coli THB101	*F-, cya-99, araD139, galE15, galK16, rpsL1 (Str r), hsdR2, mcrA1, mcrB1.*	(Karimova *et al.*, 1998)
XL10-Gold® ultracompetent cells	*TetR Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte* *[F' proAB lacIqZΔM15 Tn10 (TetR) Amy CamR]a*	Stratagene

* Details of each transposon insertion can be found at http://strepdb.streptomyces.org.uk; NA – Not applicable

2.2 Plasmids and Cosmids

Plasmids and cosmids used in this work are listed in Table 2.2. The map and details of construction of respective plasmids originating from this study can be found in the respective results chapters.

Table 2.2: Plasmids and Cosmids

Plasmid/Cosmid	Characteristics	Source
pBluescript II SK(+)	Cloning vector; Ampicillin resistance, lac promoter (*lacZ*), f1, ColE1	Stratagene,(Alting-Mees and Short, 1989b, Alting-Mees and Short, 1989a)
pIJ8600	*tipAp expression vector, aac(3)IV, tsr, ori pUC18, oriT RK2, int ØC31, attP and tipA* inducible promoter.	(Sun *et al.*, 1999)
pIJ8600H	pIJ8600 containing hygromycin resistance marker	This study

Table 2.2: Plasmids and Cosmids (Continued)

Plasmid/Cosmid	Characteristics	Source
pSE66H	pIJ8600H containing *ftsW*	This study
pSE66Hm	pSE66H containing mCherry	This study
pKT25	*E. coli* expression vector allowing fusions to the C terminus of the T25 fragment of *cyaA*; Km^R	(Karimova *et al.*, 1998)
pKT25-*ftsQ*	*ftsQ* cloned in pKT25; Km^R	This study
pKT25-*crgA*	*crgA* cloned in pKT25; Km^R	This study
pKT25-*sfr*	*sfr* cloned in pKT25; Km^R	This study
pKT25-*rodA2*	*rodA2* cloned in pKT25; Km^R	This study
pKT25-*ftsW*	*ftsW* cloned in pKT25; Km^R	This study
pKT25-*pbp1*	*pbp1* cloned in pKT25; Km^R	This study
pKT25-*pbp2*	*pbp2* cloned in pKT25; Km^R	This study
pKT25-*mreB*	*mreB* cloned in pKT25; Km^R	This study
pKT25-*ftsI*	*ftsI* cloned in pKT25; Km^R	This study
pKT25-*ftsZ*	*ftsZ* cloned in pKT25; Km^R	This study
pUT18C	*E. coli* expression vector allowing fusions to the C terminus of the T18 fragment of cyaA; Amp^R	(Karimova *et al.*, 1998)
pUT18C-*ftsW*	*ftsW* cloned in pUT18C; Amp^R	This study

Table 2.2: Plasmids and Cosmids (Continued)

Plasmid/Cosmid	Characteristics	Source
pUT18C-*ftsI*	*ftsI* cloned in pUT18C; Amp^R	This study
pUT18C-*ftsZ*	*ftsZ* cloned in pUT18C; Amp^R	This study
pUT18C-*pbp1*	*pbp1* cloned in pUT18C; Amp^R	This study
pUT18C-*pbp2*	*pbp2* cloned in pUT18C; Amp^R	This study
pUT18C-*pbp3*	*pbp3* cloned in pUT18C; Amp^R	This study
pUT18C-*rodA*	*rodA* cloned in pUT18C; Amp^R	This study
pUT18C-*rodA2*	*rodA2* cloned in pUT18C; Amp^R	This study
pUT18C-*ftsQ*	*ftsQ* cloned in pUT18C; Amp^R	This study
pUT18C-*crgA*	*crgA* cloned in pUT18C; Amp^R	This study
pUT18C-*sfr*	*sfr* cloned in pUT18C; Amp^R	This study
pUT18C-$crgA_W$	*crgA* site directed mtagenesis for one amino acid Tryptophan ($_W$) and cloned in pUT18C; Amp^R	This study
pUT18C-$crgA_{WN}$	*crgA* site directed mtagenesis for two amino acids Tryptophan and Asparagen ($_{WN}$) and cloned in pUT18C; Amp^R	This study
pKT25-$crgA_{NW}$	*crgA* site directed mtagenesis for two amino acids Asparagen and Tryptophan ($_{NW}$) and cloned in pKT25; Km^R	This study
pKT25-$crgA_N$	*crgA* site directed mtagenesis for one amino acid Asparagen ($_N$) and cloned in pKT25; Km^R	This study

Table 2.2: Plasmids and Cosmids (Continued)

Plasmid/Cosmid	Characteristics	Source
pUT18C-zip	A derivative of pUT18C in which the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment	(Karimova *et al.*, 1998)
pKT25-zip	A derivative of pKT25 in which the leucine zipper of GCN4 is genetically fused in frame to the T25 fragment	(Karimova *et al.*, 1998)
pMt*ftsW*H	pIJ6800H containing *M. tuberculosis ftsW*	This study
pMtFtsIH	pIJ6800H containing *M. tuberculosis ftsI*	This study
pBSW12	pBluescript II SK (+) containing *ftsW* and part of *murD*	(Mistry *et al.*, 2008)
pQM5080	streptomycin ^{R}, spectinomycin ^{R}, Tn5080	(Mistry *et al.*, 2008)
pSF152	*E. coli-S. coelicolor* shuttle vector, derivative of pSET152 with streptomycin and spectinomycin resistance marker.	P. Herron
pME221	pBSW12 site directed mutagenesis (sdm) for *Nde*I	This study
pME222	pME221 site directed mutagenesis (sdm) for *Bgl*II	This study
pME222m	Derivative of pME222 with mCherry gene	This study
pME6	Derivative of pCR®2.1-TOPO® digested with *Eco*RI and religated	(Mistry *et al.*, 2008)

Table 2.2: Plasmids and Cosmids (Continued)

Plasmid/Cosmid	Characteristics	Source
pME6fm	pME6 containing mCherry fused to *ftsW*	This study
pFtsIH	pIJ8600H containing *ftsI*	This study
pBSI1	pBluescript II SK (+) containing *ftsI*, ftsL and SCO2092	(Mistry *et al.*, 2008)
pBSI1N	pBSI1 site directed mutagenesis (sdm) for *NdeI*	This study
pET-16K	Kanamycin T7	Novagen
pETFtsI	pET-16K containing *ftsI*	This study
pET-28a(+)	*E. coli-S. coelicolor* shuttle vector with multicloning site and kanamycin marker	Novagen
pJB201	pET-28a(+) containing *M. tuberculosis ftsW*	This study
pIJ2925	Cloning vector for *E. coli*; bla	Novagen
pMt*ftsW*	pIJ2925 containing *M. tuberculosis ftsW*	This study

2.3 Chemical reagents

The chemicals used during this study were purchased from several companies such as SIGMA Chemical Company, Fisher Scientific Ltd., BDH Chemicals Ltd. and Molecular Probes. The chemical solutions and buffers were prepared using de-ionized water (dH_2O), provided by the MILLI-RO (reverse osmosis) water purification system. Solutions requiring ultra-pure de-ionized water (ddH_2O) were prepared using ddH_2O provided from MILLI-Q water purification system. Also the measurements of the pH of the solutions were done at a room temperature. Solutions were routinely autoclaved at 121° C, 15 psi for 15 minutes when required. When sterilization of solutions by autoclaving was not possible, solutions were filter sterilized using 0.2 μm Millipore filter units. The components of all the commonly used reagents and buffers are listed in Tables 2.3.

Table 2.3: Reagents and buffers

Reagent/Buffer Solution	**Composition**	**Quantity in grams per liter of dH_2O (unless otherwise stated)**
10% Glycerol solution (v/v)	100% Glycerol	100.00 ml
	dH_2O	Up to 1.00 l
20% Glycerol solution (v/v)	100% Glycerol	100.00 ml
	dH_2O	Up to 500 ml
10X TBE	Tris	108.00
	Boric Acid	55.00
	EDTA adjust pH to 8 with HCl	9.30
Bromophenol Blue DNA loading dye	Sucrose	40.00
	Bromophenol Blue	60.00 mg
	1 x TBE	10.00 ml
	dH_2O	90.00 ml
TE buffer	Tris	1.21
	EDTA adjust pH to 8.0	372.00 mg
10X concentrated dNTP mix	dATP 10 mM	7.10 µl
	dTTP 10 mM	7.10 µl
	dCTP 10 mM	7.10 µl
	dGTP 10 mM	7.10 µl
	Digoxigenin-11-dUTP	25.00 µl
	dH_2O	20.30 µl
10% SDS	Sodium dodecyl sulphate	10.00
	dH_2O	Up to 100.00 ml

Table 2.3: Reagents and buffers (continued)

Reagent/Buffer Solution	**Composition**	**Quantity in grams per liter of dH_2O (unless otherwise stated)**
Denaturing Buffer	NaOH	20.00
	NaCl	87.75
Neutralizing Buffer	Tris	121.00
	NaCl	88.00
	Adjust pH to 7.5	
20X SSC	NaCl	175.50
	tri- Sodium citrate	88.20
Buffer I	Tris	12.10
	NaCl	8.80
Buffer II / prehybridisation solution	20 x SSC	25.00 ml
	10% N-Lauryl sarcosine (w/v)	1.00 ml
	10% SDS (w/v)	200.00 µl
	Blocking reagent	5.00
	Formamide	50.00 ml
	dH_2O	24.00 ml
Wash solution I	20× SSC	24.00 ml
	10% SDS	2.40 ml
	dH_2O	213.60 ml

Table 2.3: Reagents and buffers (continued)

Reagent/Buffer Solution	Composition	Quantity in grams per liter of dH_2O (unless otherwise stated)
Wash solution II	20× SSC	1.20 ml
	10% SDS	2.40 ml
	dH_2O	236.40 ml
Colour solution	NBT/ BCIP Tablets (Roche)	1.00 tablet
	ddH_2O	10.00 ml
Antibody solution	Anti-digoxigenin AP conjugate	3.00 µl
	Buffer I	15.00 ml
PBS (Phosphate buffer saline)	NaCl	8.00
	KCl	0.20
	Na_2HPO_4	1.44
	KH_2PO_4	0.24
	Adjust pH to 7.5	
Cell Fixing solution	25% glutaraldehyde	2.00 µl
	40% Formaldehyde	700.00 µl
	PBS	10.00 ml
Lysozyme solution	Lysozyme	20.00 mg
	1M Tris	200.00 µl
	0.5M EDTA	400.00 µl
	50% Glucose solution	180.00 µl
	dH_2O	9.20 ml

Table 2.3: Reagents and buffers (continued)

Reagent/Buffer Solution	Composition	Quantity in grams per liter of dH_2O (unless otherwise stated)
Fluo–WGA / PI solution	FITC-coupled Wheat Germ Agglutinin solution Propidium Iodide solution 2% BSA in PBS	20.00 µl 4.00 µl 10.00 ml
PI wash solution	PI solution PBS	4.00 µl 10.00 ml
PI solution	Propidium Iodide dH_2O	25.00 mg 1.00 ml
Stacking gel (x2)	Acrylamide dH_2O 1M Tris pH6.8 10% SDS 10% TEMED 10% APS	650 µl 3 ml 1.25 ml 50 µl 5 µl 25 µl
12% Resolving gel (x1)	Acrylamide dH_2O 1.5 M Tris pH8.8 10% SDS 10% TEMED 10% APS	3 ml 2.5 ml 1.875 ml 75 µl 7.5 µl 37.5 µl

Table 2.3: Reagents and buffers (continued)

Reagent/Buffer Solution	Composition	Quantity in grams per liter of dH_2O (unless otherwise stated)
5M $CaCl_2.2H_2O$	$CaCl_2.2H_2O$ dH_2O	73.51 Up to 100.00 ml
1N NaOH	NaOH dH_2O	40.00 Up to 1.00 l
0.15M NaOH	NaOH dH_2O	0.60 100 ml
1M $MgSO_4$	$MgSO_4$ dH_2O	12.03 Up to 100 ml
Substrate solution	ONPG PM2 medium	4 g 1 liter
Stop solution	1 M Na_2CO_3	105.99 g
0.1% SDS	Sodium dodecyl sulphate dH_2O	0.1 Up to 100.00 ml

2.4 Culture media

Growth media used during this study are described in (Table 2.4). Ingredients for Bacterial growth media were purchased from various providers such as Fisher Scientific Ltd., DIFCO, Gibco BRL., SIGMA Chemical Company and Oxoid Ltd. De-ionized water dH_2O required to prepare the media was provided by the MILLI-RO water purification system. pH of the solutions and media was measured at room temperature. Media were routinely autoclaved at 121° C, 15 psi for 15 minutes. Solutions required for media preparation were either autoclaved or filter sterilized as applicable.

Table 2.4: Culture media

Medium	Composition	Quantity per litre (unless otherwise stated)
Luria Bertani (LB) Medium (Broth and Solid)	Tryptone	10.00 g
	Yeast Extract	5.00 g
	NaCl	5.00 g
	Glucose adjust pH to 7.0 with NaOH	1.00 g (optional)
	For solid LB add agar	10.00 g
2X YT (Broth)	Tryptone	16.00 g
	Yeast Extract	10.00 g
	NaCl adjust pH to 7.0 with NaOH	5.00 g
Soya Flour Mannitol (SFM) agar	Soy Flour	20.00 g
	Mannitol	20.00 g
	Agar	20.00 g
	Use tap water in place of dH_2O	
PM2 assay medium	70 mM $Na_2HPO_4.12H_2O$	12.4593 g
	30 mM $NaH_2PO_4H_2O$	4.6803 g
	1 mM $MgSO_4$	0.1204 g
	0.2 mM $MnSO_4$, pH 7.0	0.0302 g
	β-mercaptoethanol	35 µl/5 ml PM_2

Table 2.4: Culture media (continued)

Medium	Composition	Quantity per litre (unless otherwise stated)
NZY^+ (Broth)	NZ amine (casein hydrolysate)	10 g
	yeast extract	5 g
	NaCl	5 g
	Add deionized H2O to a final volume	1 liter
	Adjust to pH 7.5 using NaOH	
	Add the following filter-sterilized	
	supplements prior to use:	
	1 M MgCl2	12.5 ml
	1 M MgSO4	12.5 ml
	20% (w/v) glucose	20 ml
	(or 2 M glucose)	10 ml
M63/maltose minimal medium	$(NH_4)_2SO_4$	10 g
	KH_2PO_4	68 g
	$FeSO_4.7H_2O$	2.5 mg
	vitamin B1	5 mg
	Add deionized H2O to a final volume	1 liter
	Adjust to pH 7.0 using KOH	

2.5 Antibiotic and Blue-White selection

The antibiotics were prepared in ddH2O and other appropriate solvent, in order to use it for selection of bacterial transformants. The stock solutions prepared in ddH_2O were filter sterilized. All of these stock solutions were stored at -20° C except Hygromycin solution which was stored at 4° C. Stock concentration and working concentration for respective antibiotic are listed in (Table 2.5).

Table 2.5: Concentration of antibiotics, IPTG and X-gal

Antibiotic	Stock concentration (mg ml^{-1})	*E. coli* working concentration (µg ml^{-1})	*S. coelicolor* working concentration (µg ml^{-1})
Ampicillin	100	50	-
Apramycin	100	100	25
Chloramphenicol[1]	25	25	-
Hygromycin	100	100	50
Kanamycin	25	25	25
Naladixic acid[2]	20	20	-
Thiostrepton[3]	25	-	25
Spectinomycin	200	-	200
Streptomycin	10	-	5
IPTG	25	25	-
X-gal[4]	20	40	-

Solvent used to dissolve: [1]100% Ethanol; [2]0.15M NaOH; [3]DMSO (dimethyl sulfoxide); [4]Dimethylformamide or DMSO

Blue-white selection was used to select clones containing recombinant plasmids carrying an insert disrupting the *lacZ* gene. In the presence of chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and an inducer IPTG (isopropyl β-D-thiogalactopyranoside), *E. coli* transformants containing the recombinant plasmid with a disrupted *lacZ* gene are easily distinguished from the *E. coli* transformants containing the non-recombinat plasmid. The colonies carrying a recombinant plasmid remain white, because the cells containing recombinant plasmid with a disrupted *lacZ* gene are unable to produce β–galactosidase, which is responsible for the conversion of the chromogenic substrate X-gal into a blue pigment, whereas the cells containing plasmid with intact *lacZ* gene are able to produce β–galactosidase that converts X-gal into a blue pigmented compound imparting blue colour to the colonies.

2.6 Culture conditions

2.6.1 Growth and storage of Escherichia coli strains

Firstly, *E. coli* cells were grown on LB media and incubated at 37° C either in a static temperature controlled incubator if being grown on plates or shaken at 220 rpm if liquid culture was used. Then the glycerol stocks of *E. coli* strains were prepared from 5 ml overnight grown cultures. Overnight grown cells were centrifuged and resuspended in 0.5 ml of sterile 20% (v/v) glycerol and stored at -20° C or -70° C. Short term storage of cultures was possible by storing culture broth or plates at 4° C.

2.6.2 Growth and storage of *Streptomyces* coelicolor strains

Cultures of *Streptomyces coelicolor* were grown on a different solid media and incubated at 30° C. And the preparation of aerial mycelium or spore suspensions (as applicable) lawn of the relevant strain was grown on SFM agar, containing appropriate antibiotic (if applicable) for 4-6 days. Spore or aerial mycelium suspensions of different *S. coelicolor* strains were stored at -20° C.

2.6.3 Preparation of spore or aerial hyphae suspensions of *S. coelicolor* strains

For the preparation of the spore suspensions, *S. coelicolor* cells were grown on SFM agar media. After appropriate incubation period, culture plates were flooded with 10 ml of sterile ddH_2O and the surface was gently scraped off with a sterile inoculating loop to release the spores. To remove mycelial fragments, the resulting suspension was poured into a sterile centrifuge tube, vortexed and filtered through a sterile 10 ml syringe containing non–absorbent cotton wool. Then pelleted the spores by centrifugation at 6000 rpm for 5 min and resuspended in 1 ml of sterile 20% glycerol solution. Stocks were stored at -20° C in micorcentrifuge tubes.

To prepare aerial mycelium suspensions of non-sporulating strains, the procedure was similar to that of spore suspension preparation, except that the mycelial suspensions were not filtered through sterile syringe containing non–absorbent cotton wool.

2.7 Transformation

2.7.1 Preparation of electrocompetent cells of *E. coli*

Diluted a 1/100 culture of a fresh *E. coli* JM109 or ET12567 was inoculated overnight into 500 ml LB broth. At an OD_{600} 0.5 – 0.7 the cells were chilled on ice for 20 min then harvested by centrifugation at 4500 rpm for 15 min at 4° C. In all following procedures the cells were kept on ice as much as possible, and all glycerol solutions used were ice cold. The supernatant was decanted and the pelleted cells carefully resuspended in 500 ml ice-cold 10% glycerol. Then pelleted and resuspended in 250 ml ice-cold 10% glycerol. The above procedure repeated using 20 ml ice-cold 10% glycerol. Finally, the cells were resuspended into 2 ml ice-cold 10% glycerol and 40 µl aliquots of the competent cells were dispensed into ice-cold microcentrifuge tubes to store at -70° C. The electrocompetent cells can be kept frozen for many weeks without great loss in the efficiency of its transformation.

2.7.2 Transformation of electrocompetent *E. coli* cells

A 40 µl aliquot of electro-competent cells was thawed slowly on ice, and then 10 ng DNA was added and mixed by gentle pipetting. After 2 min incubation on ice the mixture was transferred into a chilled electroporation cuvette (0.1 cm). The cuvette was then placed in a

MicroPulser™ (BioRad) and electroporated using EC1 program. Immediately 1 ml LB medium or SOC was added and the mixture was incubated in a Bijoux tube for 90 min at 37° C on shaker (225 rpm). The transformation mixture was then plated onto LB agar with appropriate antibiotic selection.

2.7.3 Preparation of calcium chloride competent *E. coli* cells

The calcium chloride (CaCl2) used to prepare the competent cells of *E. coli* JM109 or ET12567. At the step of $CaCl_2$ competent cells preparation, the *E. coli* cultures were grown overnight at 37° C in 10 ml LB broth with appropriate selection antibiotic(s) if applicable. From overnight grown cultures a 5 ml aliquot were used to inoculate 250 ml LB broth supplemented with appropriate antibiotic if applicable. Cultures were then grown at 37° C on shaker (225 rpm) in order to obtain an OD_{600} between 0.4-0.6. Then the cells were transferred to sterile, ice-cold 50 ml universal tubes and incubated on ice for 10 min. During the following steps the cells were kept on ice and all the glassware and equipments were chilled before use. The cells were cedntrifugated at 4500 rpm for 5 min at 4° C. Gently resuspended the pellet in 20 ml ice-cold ice cold 50 mM $CaCl_2$, incubated for 30 min and then pelleted again. Without further incubation, this $CaCl_2$ wash was repeated three times in total. After the final wash the pelleted cells were resuspended in 2 ml $CaCl_2$. Finally, 80 µl aliquots of the competent cells were dispensed into ice-cold microcentrifuge tubes and quickly frozen in liquid nitrogen and stored at -70° C.

2.7.4 Transformation of calcium chloride competent *E. coli* cells

The $CaCl_2$ competent *E. coli* cells were transformed by heat-shock treatment. The aliquots of *E. coli* competent cells were thawed on ice for 10-15 min. Purified DNA (plasmid or ligation mixture) was added and mixed gently. The mixture was incubated on ice for 30 min. The cells were then heat-shocked at 42° C for 90 sec and immediately incubated on ice for 2 min. The mixture was then transferred to a universal tube after mixing with 900 µl of SOC medium and incubated at 37° C on an orbital shaker (225 rpm) for 1 hr. Finally, the heat-shocked culture was then plated on LB agar plate containing appropriate antibiotics and incubated overnight at 37° C.

2.7.5 Intergeneric conjugation

As described in section 2.7.1, electro-competent cells of *E. coli* ET12567 containing pUZ8002 were prepared. The vector of interest containing oriT (for mobilisation into

Streptomyces) was introduced into *E. coli* ET12567 (pUZ8002) by electroporation and transformants were selected by growing the culture at 37° C, on LB agar plate containing 25 μg/ml chloramphenicol, 25 μg/ml kanamycin and other selective antibiotic required for the selection of vector of interest. A transformed colony was inoculated into 10 ml LB containing kanamycin, chloramphenicol and the antibiotic used to select for the oriT containing vector and grown overnight at 37° C, on shaker at speed of 225 rpm. The overnight grown culture was diluted to 1:100 with fresh LB broth plus the antibiotic selection and grown under the same conditions until an OD_{600} of 0.4-0.6 was obtained. Cells were then pelleted and washed twice with an equal volume of LB to remove antibiotics that might inhibit *S. coelicolor* growth. After wash, the cells were resuspended in 1/10 volume of LB broth. While washing the *E. coli* cells, for each conjugation approximately 10^8 *S. coelicolor* spores or mycelial fragments (as applicable) were added to 500 μl 2 x YT broth, heat shocked at 50°C for 10 mins then cooled to room temperature., 500 μl washed *E. coli* were added and mixed to the activated spores or mycelial fragments. The mixture was spin down briefly and the supernatant was decanted off. The pelleted cells were gently resuspended in the residual broth. This conjugation mixture was then plated on SFM agar containing 10 mM $MgCl_2$ and incubated at 30° C for 16–20 hour. After incubation the plates were overlaid with 1 ml sterile ddH_2O containing nalidixic acid (to inhibit *E. coli* growth) and the appropriate antibiotic selecting for the oriT containing vector. Plates were allowed to dry and further incubated at 30°C for 3–5 days until potential exconjugants could be picked and transferred to selective media containing nalidixic acid.

2.7.5.1 Selection of *S. coelicolor* double crossovers

After incubation 3–5 days, single colonies of exconjugants on the overlayed SFM plates could then be screened for successful replacement of transposon disrupted gene with the corresponding wild type gene in the *S. coelicolor* chromosome by double crossover recombination event. Firstly two sets of plates were prepared, one containing the appropriate concentration of the antibiotic whose resistance gene is carried on the plasmid/cosmid (e.g. kanamycin) and the other containing the antibiotic whose resistance gene is carried on the transposon (e.g. apramycin, hygromycin or spectomycin/streptomycin). A large sample of the individual colonies (100–200 colonies) then carried out for replica plating. Any colonies which grew on apramycin, hygromycin or spectomycin/streptomycin containing media plate but not on

kanamycin containing media plates were selected as probable double crossover mutants, and these were confirmed using Southern hybridization.

2.8 DNA isolation and manipulation

2.8.1 Plasmid DNA isolation from *E. coli*

The AccuPrep® Plasmid Mini Extraction Kit, from Bioneer Corp was used for small scale preparation of plasmid DNA. The overall principle of systems is based on the modified alkaline lysis method (Sambrook *et al.*, 1989). *E. coli* cells containing appropriate plasmid DNA were grown overnight in 5 ml LB broth with the presence of suitable antibiotic selection. Cells from an overnight grown culture (1-5 ml) were pelleted at 13000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 250 µl Resuspension solution. Then 250 µl Lysis solution was added to the suspension and mixed gently by inverting the tube 3-4 times. The mixture was incubated for 1-5 min at room temperature. After incubation, 350 µl Neutralisation solution was added, mixed by inversion and the resulting suspension was centrifuged at speed of 13000 rpm for 5 min. The cell lysate was transferred to a spin column containing a DNA binding matrix and centrifuged at 13000 rpm for 1 min to provide a good chance for the DNA to bind to the matrix. The binding matrix was washed twice with 750 and 250 µl Wash buffer, respectively. Finally, the DNA was eluted in 100 µl sterile ddH_2O by centrifugation of the spin column, into a sterile microfuge tube, at 13000 rpm for 2min.

2.8.2 Genomic DNA isolation from *S. coelicolor*

For rapid isolation of genomic DNA, the FastDNA® SPIN Kit for Soil from Q-BIOgene was used, by lysing the cells using ceramic and silica particles. Lysing matrix tubes containing 978 µl Sodium Phosphate Buffer and 122 µl MT Buffer, were filled to 7/8 volume with mycelia from 24 h cultures grown on Nutrient agar plates. The tubes were securely fastened into the FastPrep® Instrument and processed for 30 sec at speed 5.5. After processing, the cell debris and lysis matrix were centrifuged to the bottom at 13000 rpm for 30 sec. The supernatant containing genomic DNA was transferred to a fresh tube and mixed by inversion with 250 µl PPS reagent. It was centrifuged again at 13000 rpm for 1 min and after centrifugation the supernatant was transferred to a 15 ml tube and incubated, then gently shaking, for 2 min with 1 ml Binding Matrix Suspension. Once the DNA has bound to the matrix it is left to settle for 3 min then

transferred into a spin filter, which captures the matrix and the bound DNA. The bound DNA was washed with 500 µl SEWS-M and eluted into a fresh catch tube with 50 µl DNase/ Pyrogen free water. Total DNA from *S. coelicolor* was used for Southern blots and as a PCR template for amplification of specific genes.

2.8.3 DNA Enzymatic reactions

All reactions were carried out according to the manufacturers' instructions. Restriction digestions were performed using restriction endonucleases purchased from New England Biolabs (NEB) or Promega Corp. For blunt ending of 3′ and 5′ DNA overhangs, T4 DNA polymerase from NEB was used. T4 DNA ligase was purchased from Invitrogen or NEB and used in accordance with the manufacturers' instructions. To remove the 5′ phosphate groups from DNA, Calf intestinal alkaline phosphatase (CIP) purchased from NEB was used and reactions were performed as instructed.

2.8.3.1 Ligation reactions

Prior to ligation, using QIAquick PCR Purification Kit from Qiagen, DNA fragments were purified from their respective enzymatic reactions. The DNA fragments, in their enzyme reaction mixtures, were mixed with five volumes of Buffer PB and applied to QIAquick spin columns. The columns were then centrifuged at 13000 rpm for 1 min to allow DNA to bind on the matrix. Each of these columns was then washed twice with 750 µl Buffer PE (wash buffer). After an additional centrifugation step at 13000 rpm for 1 min to dry the column, the DNA was eluted in the final step in 50 µl sterile ddH_2O by centrifugation of the spin column, into a sterile eppendorf, at 13000 rpm for 2min. To purify one particular fragment of DNA from agarose gel, the GFX kit from GE healthcare (formerly Amersham Biosciences) was used according to the manufacturers' instructions. The purified DNA (vector and insert) was analyzed on agarose gel and quantified. The ligation reaction was then set by mixing appropriate digested plasmid vector and insert DNA at a ratio of 1:3, and incubated at 16° C overnight.

2.8.4 Redirect technique to exchange the selective marker

To change the selective marker (from apramycin to spectinomycin/streptomycin) in the Tn*5062* disrupted cosmid of a specific gene the Redirect technique was performed (Guts *et al.*, 2003). The electrocompetent cells of *E. coli* BW25113/pIJ790 were first transformed with a

S.coelicolor cosmid of interest and then grown at 30° C in LB broth containing 25 μg/ml chloramphenicol, 100 μg/ml apramycin and 25 μg/ml kanamycin for the selection of a pIJ790 plasmid and a cosmid of interest. The plasmid pIJ790 contains the resistance marker *cat* (chloramphenicol) and a temperature sensitive origin of replication (requires 30° C for replication). The cosmid contains the resistance markers for kanamycin and apramycin. Electrocompetent cells of *E. coli* BW25113/pIJ790 containing a cosmid of interest were prepared and electro-transformed with the extended spectinomycin/streptomycin resistance cassette. The spectinomycin/streptomycin resistance cassette was purified from pQM5080 plasmid (Fernandez-Martinez *et al.*, 2011) as a 3692 bp *Pvu*II band. The transformants of *E. coli* BW25113/pIJ790 containing a cosmid of interest with spectinomycin/streptomycin resistance marker were selected through the growing of the culture on LB agar containing kanamycin (25 μg/ml) and spectinomycin/streptomycin (100 μg/ml) at a temperature of 37° C. Incubation of the culture at 37° C promotes the loss of pIJ790. The replacement of the apramycin cassette with the spectinomycin/streptomycin cassette was promoted by the λ RED (gam, bet, exo) functions present in the λ RED recombinant plasmid pIJ790.

2.9 Polymerase chain reaction (PCR)

PCR reactions were cycled on a PTC-200 DNA Engine (M.J. Research Inc.) using DyNAzyme EXT™ polymerase (Finnzymes) or Pfu Ultra™ HF DNA polymerase (Stratagene). Primers were designed with Beacon Designer 2 software from Premier Biosoft international, and purchased from MWG-Biotech. Primers used in this study are shown in (Table 2.6).

Table 2.6: Primers used in PCR reactions to amplify *M. tuberculosis ftsW* and *pbps*.

Primer name	**Sequence (Bold letters indicate restriction site for relevant restriction enzyme)**	**Restriction enzyme**
MtftsWF2 (Forward)	CCATATGCTAACCCGGTTGCTGCG	*Nde*I
MtftsWR2 (Reverse)	CTCTAGACGGCGAGGCGGC	*Eco*RI
Mtpbp3F2 (Forward)	AAACCATATGAGCCGCGCCGCCCCCAG (28)	*Nde*I
Mtpbp3R2 (Reverse)	AAAAGATCTCTAGGTGGCCTGCAAGAC (27)	*Bgl*II

Reactions were carried out in thin-walled 0.5 ml microfuge tubes with a total reaction volume of 50µl. A typical reaction scheme is as follows:

Template DNA (approx. 100 µg/ml)	2.0 µl
10X DyNAzyme EXT^{TM} buffer	5.0 µl
Sense primer (10 pmol/µl)	2.0 µl
Antisense primer (10 pmol/µl)	2.0 µl
2.5 mM dNTP mix	4.0 µl
dH_2O	34.0 µl
DyNAzyme EXT^{TM} polymerase	1.0 µl
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Total volume	50.0 µl

PCR cycle program used for the reaction mix was as follows:

Program	Temperature	Time	Cycle number
Initial denaturation	94° C	4 min	1
Denaturation	94° C	30 sec	
Annealing	55° C	30 sec	30
Extension	72° C	1 min	
Final extension	72° C	10 min	1
	4° C	hold	

2.10 Qualitative and quantitative analysis of DNA

2.10.1 Agarose gel electrophoresis

In this step the DNA was separated out according to size by electrophoresis through agarose gels. The percentage of agarose is varied to reflect the size of the DNA of interest. 1% or 0.8% agarose (w/v) gels used to separate restriction digests of plasmid DNA (0.5 to 15 Kb), 0.5% gels were used to visualise large pieces of DNA, for example, *Streptomyces* chromosomal DNA. To separate smaller PCR products 1.5 to 2% agarose gels were used. Standard 100 ml of 1% (w/v) agarose gels containing a final concentration of 0.1 μg/ml ethidium bromide were routinely prepared in 1X TBE buffer. The gel was then immersed in the BIORAD electrophoresis tank filled with 1X TBE buffer and the samples were loaded into wells after mixing with Bromophenol blue DNA loading dye at the ratio of 5:1 (DNA:dye). Gel was run by applying an electric field of 100 V through the gel using BIORAD power supply, for 45 – 60 min. To estimate the size of DNA samples an appropriate DNA marker (Phage λ DNA digested with *Hin*dIII was used in most cases) was run along side of the samples. Once the DNA migrated upto the desired distance, the gel was then exposed to UV light using a BIORAD transilluminator at 254 nm and the DNA samples were visualized through BIORAD gel documentation (GelDoc) system.

2.10.2 DNA quantification

The quality and quantity of DNA was assayed using a NanoDrop® ND-1000 Spectrophotometer. The quantity of DNA was measured by applying 1 µl sample on the pedestal of the instrument after initializing the instrument and setting the blank. The ratio of A_{260} / A_{280} determines the purity of the DNA, where pure DNA in 10 mM Tris-Cl (pH 8.5) has an A_{260}/ A_{280} ratio of 1.8 – 2.0.

2.11 Southern Hybridization

Using Southern hybridization analysis, the successful replacement of wild type gene with transposon disrupted copy of the same gene in *S. coelicolor* mutant chromosomes was confirmed. In brief, a restriction digests of chromosomal DNA from the mutant strain and a positive control (often the transmitted cosmid) were run on an agarose gel. The DNA samples were then transferred to a nitrocellulose membrane and detected immunologically using an appropriate probe (usually Tn*5062*). These steps are described individually in more detail below:

2.11.1 Preparation of Digoxigenin labelled probes

Suitable DNA fragment(s) was selected and labelled randomly with alkali-labile Digoxigenin-11-2′-deoxy-uridine-5′-triphosphate (DIG-11-dUTP) using DIG DNA Labelling Kit from Roche Applied Sciences. For most Southern hybridization analysis, a 3442 bp *Pvu*II fragment of Tn*5062* DNA excised from pQM*5062* was used as a labelled probe. For Southern hybridization analysis of *ftsW* mutants, a 4896 bp *Hin*dIII fragment, containing *ftsW, murG* and *ftsW* genes, excised from pBSW1 was used as a gene specific probe. Phage λ DNA digested using *Hin*dIII was labelled and used as a probe to hybridise with the λ *Hin*dIII marker. Labelling the respective purified DNA fragments done by the use of random priming method, this method based on hybridization of random oligoncleotides to the denatured DNA template. To label the DNA, the DNA fragments were denatured by heating at 95° C for 10 min and cooled quickly on ice. Then, hexanucleotide mix, DIG-11-dUTP/dNTP mixture and Klenow enzyme were added to the denatured DNA. The reaction was mixed properly and incubated at 37° C overnight. The reaction was stopped by adding 2 µl of 0.2 M EDTA, pH 8. A standard reaction scheme is as follows:

Denatured DNA	1-15 µl (10 ng – 3 µg DNA)
ddH_2O (sterile) (to make the final volume 15 µl)	x µl
Final volume	15 µl
Hexanucleotide mix	2 µl
DIG-11-dUTP/dNTP mixture	2 µl
Klenow polymerase	1 µl
Total volume of reaction mix	20 µl

Prior to hybridization, 10µl each of labelled probes (Tn*5062* or gene specific and λ *Hin*dIII) is denatured by boiling for 10 min in boiling water bath and cooled quickly by keeping it on ice for 1-2 min and added to 15-20 ml of prehybridization solution. The solution thus prepared is called as 'probe solution' from here after.

2.11.2 Blotting

Suitable restriction enzyme used to digest chromosomal DNA of interest and the corresponding cosmid DNA control and the DNA fragments of digested DNA (chromosomal and cosmid) along with marker (λ *Hin*dIII) were separated on a 0.8% agarose gel in 1X TBE until the loading dye had progressed close to the end of the gel. The gel was visualized under UV transilluminator to check the digestion. The gel was then immersed in denaturing buffer for 15min with gentle shaking. This step was repeated twice. The gel was then rinsed twice with dH_2O. After rapid rinse, the gel was immersed in neutralisation buffer for 20min with gentle shaking. This step was repeated twice. During the neutralization step, an appropriate size (gel size) of nitrocellulose membrane (Hybond-N, Amersham Pharmacia Biotech) was soaked in dH_2O for 20min and then soaked in 10X SSC solutions for 10 min. Two pieces of Whatman filter paper (1 cm bigger in width and height than the gel) were also soaked in 10X SSC solutions for 10 min.

A Stratagene Posiblot Pressure blotter was used to transfer the DNA from the gel onto the treated nitrocellulose membrane prior to hybridisation and immunological detection. The pressure blotter was assembled with Whatman filter paper, soaked in 10X SSC at the bottom, then the nitrocellulose membrane and on top of this was a plastic mask, containing a hole (0.5 cm smaller than the gel in width and height) in the centre. Once assembled the gel was positioned on top of the mask, sealing the hole. Finally a sponge saturated with 10X SSC was placed over the gel and the apparatus was closed. The pressure of 75 mmHg was applied for 1 hour to transfer the DNA efficiently on to the membrane. Once transfer was completed, the membrane was then baked at 80°C for 60 min to fix the DNA.

2.11.3 Hybridisation

After fixing the DNA to the membrane, it was rinsed in dH_2O and rolled in a piece of nylon mesh soaked in 2X SSC with the DNA facing upwards. The rolled membrane with the mesh was placed into a hybridisation tube (Appligene) and washed with 2X SSC. A 10 ml of prehybridization solution was added in the hybridization tube and incubated at 42° C for 1 h. After the prehybridization step the solution was decanted and a 20 ml denatured labelled probe solution was added to the tube and incubated at 42° C for overnight in the rotating oven. The following day, the probe was collected and stored at -20°C for future use. The membrane was washed twice in wash solution 1 at 42°C for 5 min each and then it was washed twice in wash solution 2 at 68°C for 15 min each.

2.11.4 Immunological detection

After washing with wash solution 2, the membrane was rinsed in Buffer I for 1 min at room temperature and incubated with freshly prepared Buffer II (Prehybridization solution) for 30 min. After this, another brief wash of 1 min with buffer I was given to the membrane. The membrane was then incubated with Anti-digoxigenin antibody solution (15 ml Buffer I with 3 µl Anti-digoxygenin AP) for 30 min in rotating incubator at room temperature. To remove unbound antibody, the membrane was washed twice in Buffer I for 15 min each. After these washes in the hybridisation tube, the membrane was transferred to a plastic bag where 10 ml of colour solution was added and the bag was sealed. To facilitate the colour reaction the membrane was placed flat in the dark, at 37°C. After sufficient band intensity had been reached (30 min to 2 h incubation), the reaction was stopped by washing the membrane in dH_2O.

2.12 Microscopy

2.12.1 Sample preparation

Coverslip impressions:

S. coelicolor strains were grown on the surface of SFM agar medium for 2 to 4 days, as appropriate. After incubation, a clean coverslip was pressed against the surface of the growing culture. The coverslip was placed in a clean Petri plate with the aerial mycelium fragments and/or spores attached to the glass facing up. The attached cells or spores were fixed by flooding the coverslip surface with fixing solution and incubating for 15 min. Alternatively cells were fixed by gently washing the coverslip twice with methanol. The fixing solution or methanol was removed. Samples fixed with fixing solution were washed twice with phosphate buffer saline (PBS) solution. The samples were then left to dry thoroughly and stained for fluorescence microscopic analysis as described in the section 2.12.2

Inserted Coverslips:

S. coelicolor strains were inoculated with 2-5 µl of spore suspension for sporulating strains or 6-10 µl of aerial mycelium suspension for non-sporulating strains, at the acute angle formed by the inserted coverslip in agar plates of the desired medium. After an appropriate incubation time the coverslip was removed and placed in a clean Petri plate with cells facing up after removing any remaining agar piece attached on the surface opposite to the attached cells. The cells were then fixed as described above and stained as follows. For fluorescent labelled vancomycin (FL-vancomycin) staining the cells were stained directly without fixing.

2.12.2 Staining

Fluo-WGA/PI staining:

To stain the cell wall and DNA of *S. coelicolor* the lectin staining protocol described by Schwedock *et al.*, 1997, was used. For cell wall staining, fluorescein-conjugated wheat germ agglutinin (Fluo-WGA) from Molecular Probes was used at the concentration of 2 µg/ml and for DNA staining; propidium iodide (PI) was used at 10 µg/ml concentration. After fixing the cells as described above, the fixed cells were then rehydrated by treating the cells with PBS for 5 min. The PBS was gently removed and the cells were treated by the use of 2% BSA in PBS (w/v) for 5 min. The BSA solution was removed and the cells were incubated with Fluo-WGA/PI solution

for 30 min to 3 h in dark at room temperature. After appropriate incubation period, the cells were washed 4-8 times with PBS containing 10 μg/ml PI. Cells were then washed twice with Slow Fade equilibration buffer (SlowFade light antifade kit; Molecular Probe). The second wash was incubated at room temperature for 2-5 min. The buffer was aspirated off and Slow Fade antifade/glycerol or 40% glycerol in PBS solution was added. The coverslip was then mounted on clean slide and excess solution was removed by aspiration. The borders of coverslip were sealed with nail varnish to avoid desiccation and the slide was identified under the microscope.

FL-Vancomycin staining:

BODIPY® FL vancomycin, commercially available fluorescein conjugated vancomycin, from Invitrogen, was used to stain nascent peptidoglycan in *S. coelicolor*. For reaching the optimal staining the FL-vancomycin was mixed with an equal amount of unlabelled vancomycin and the mixture was added into sterile 2X YT broth at the final concentration of 1 μg/ml. An inserted coverslip with attached cells was removed after appropriate incubation and placed in a clean Petri plate with the cells facing up after removing any remaining agar piece attached on the surface that is opposite to the attached cells. The cells were directly flooded with 2X YT broth containing FL-vanocmycin/vancomycin and incubated for 20 min in the dark at room temperature to allow absorption of the antibiotic. The cells were then gently washed twice with PBS and the coverslip was mounted on a clean slide by adding 40% glycerol in PBS solution. Excess solution was removed by gentle aspiration and the borders of coverslip were sealed with nail varnish to avoid desiccation. Thus the slides prepared were observed under microscope.

2.12.3 Visualization of samples under microscope

The samples prepared for microscopy were observed under a Nikon Eclipse E600 epifluorescent microscope. For phase contrast microscopy, samples were irradiated with visual light. The microscope is equipped with a FITC filter (Excitation 465-495, DM 505, BA 515-555) for visualization of Fluo-WGA attached to the cell wall and a G-2A filter (Excitation 510-560, DM 575, BA 590) for PI stained DNA. Phase contrast and fluorescence images were taken using the camera attached to the microscope. The images obtained were later processed using Adobe Photoshop 6.0.

2.13 Bacterial two-hybrid interaction assays

Detection of in vivo interactions between two proteins of interest with the BACTH system requires the co-expression of these proteins as fusions with the T25 and T18 fragments in bacteria that are lacking endogenous adenylate cyclase activity (*E. coli cya*).To detect protein interactions, the *S. coelicolor* genes were amplified with primers listed in (Table 2.7) containing *Bam*HI, *Bgl*II or *PstI* and *EcoRI* sites respectively. Subsequently, PCR fragments were cloned as *Bam*HI, *Bgl*II or PstI and *EcoRI* fragments into plasmids pKT25 and pUT18c to generate translational fusions with the catalytic domains of the *B. pertussis* adenylate cyclase (Karimova *et al.*, 1998). The *E. coli cya* mutant BTH101 was co-transformed with pUT18C and pKT25 derivatives and streaked on LB-agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl □-D-galactopyranoside 40 μg/ml /IPTG 0.5 mM isopropyl β-D-thiogalactopyranoside) and incubated at 30°C.

Table 2.7: Primers used in bacterial two-hybrid assays.

Primer name	Sequence	Restriction enzyme
ftsW.F1	gaggatccaATGCCCGGTAGTCCCCAGAGC	*Bam*HI
ftsW.R1	aggaattcacTCACCGCTCTCCGGACGAACGC	*Eco*RI
ftsI.F1	tgactgcagcaGTGACGGAAGTGTCCGACAGGG	*Pst*I
ftsI.R1	aggaattcaTCAGGGTTTGAAGGTGACCGGG	*Eco*RI
rodA.F1	gaggatccaATGAGCAGTACTACCAACCCG	*Bam*HI
rodA.R1	aggaattcacTCACGGTCGGACCACCTGGG	*Eco*RI
rodA2.F1	gcagatctaGTGGCACAGGCGGACACCCCCG	*Bgl*II
rodA2.R1	gagaattcaTCACGGCTCCGCCCGCCCGCC	*Eco*RI
PBP1.F1	gcagatctaGTGAACAAGCCACTGCGCCG	*Bgl*II
PBP1.R1	gagaattcaTCACTTCTTGCTGTCGATGACGGCC	*Eco*RI
PBP2.F1	gaggatccaGTGACCAACATCCCCGAGACCGGC	*Bam*HI
PBP2.R1	gagaattcaTCATACGCGTGCCCTCCGGCTTC	*Eco*RI
PBP3.F1	gaggatccaGTGACCCGGAACATCCG	*Bam*HI
PBP3.R1	gagaattcaTCATCCCCCGAGCACCGCCTCC	*Eco*RI

Table 2.7: Primers used in bacterial two-hybrid assays (continued)

Primer name	Sequence	Restriction enzyme
mreB.F1	tgactgcagcaATGGGGAACTCAATGTCGTTC	*Pst*I
mreB.R1	cggaattcaTCATCTACGGGGCGAGGCGTCCAG	*Eco*RI
ftsZ.F1	gaggatccaGTGGCAGCACCGCAGAACTACC	*Bam*HI
ftsZ.R1	cggaattcaTCACTTCAGGAAGTCCGGC	*Eco*RI
ftsQ.F1	gaggatccaGTGGCCGGACCGACCA	*Bam*HI
ftsQ.R1	gagaattcaTCAACTCCCGGATG	*Eco*RI
CrgA.F1	gaggatccaGTGCCGAAGTCACGTATCCGCA	*Bam*HI
CrgA.R1	aggaattcacTTGCGGATACGTGACTTCGGCAC	*Eco*RI

2.13.1 β-galactosidase assays

For a quantification, β-galactosidase assays performed as described (Karimova *et al.*, 2005). Briefly, co-transformants were inoculated in 5 ml LB- broth containing 0.5 mMIPTG and the appropriate antibiotics, and incubated overnight at 30°C. After diluting 1:5 with M63-medium, the optical density was measured at 600 nm. 2.5 ml samples of the cell suspension was permeabilized with 30 µl toluol and 35 µl 0.1% SDS at 37°C in shaker for 30 to 40 min. 100 µl aliquots was added to 900 µl of PM2 buffer containing β-mercaptoethanol and incubated at 28°C for 5 min. 250 µl of 0.4%ONPG (o-nitrophenol-β-galactoside) substrate solution in PM2 buffer without β-mercaptoethanol was added and incubated at room temperature. The reaction was stopped after sufficient yellow colour has developed (10–15 min.) with 500 µl 1 M Na_2CO_3 stop solution and the optical density was determined at 420 nm. Enzymatic activities were calculated as follows: 200*[(OD_{420} OD_{420}control)/incubation time]*dilution. The dry weight of bacteria was

calculated from the OD600 as 1 ml of culture of OD600 = 1 corresponds to 300 µg dry weight bacteria to give the results in units mg^{-1}.

2.14 Site directed mutagenesis

In this study QuikChange XL and QuikChange II XL Site-Directed Mutagenesis kits (Stratagene) were used. Firstly, the QuikChange XL technique allows site specific mutations to be introduced into plasmids. Therefore, to introduce a restriction site to the translational start site of *ftsW*, two complementary oligonucleotides were designed containing the desired mutation flanked by unmodified sequence (Table 2.8).

Secondly, the QuikChange II XL kit is used to make point mutations, replace amino acids, and delete or insert single or multiple adjacent amino acids. In this study, the QuikChange II XL Site-Directed Mutagenesis technique was also used to replace amino acid coding sequences in the *crgA* gene.

Prepare the sample reaction(s) as indicated below:

5 µl of 10× reaction buffer

(10 ng) of dsDNA template

(125 ng) of oligonucleotide primer #1

(125 ng) of oligonucleotide primer #2

1 µl of dNTP mix

3 µl of QuikSolution

ddH2O to a final volume of 50 µl

Then add

1 µl of *Pfu*Turbo DNA polymerase (2.5 U/µl) → for QuikChange XL Site-Directed Mutagenesis.

1 µl of *Pfu*Ultra HF DNA polymerase (2.5 U/µl) →for QuikChange II XL Site-Directed Mutagenesis.

Cycling Parameters for the QuikChange® II XL Method:

95°C	1 minute
95°C	50 seconds
60°C	50 seconds
68°C	1 minute/kb of plasmid length
68°C	7 minutes

After temperature cycling, the reaction was placed on ice for 2 min. 1ul *Dpn*I was added to the reaction mixture and incubated at 37°C for 60 min. to digest the parental (nonmutated supercoiled dsDNA). The newly mutated plasmid was transformed into XL10-Gold Ultracompetant cells. The cells were thawed on ice and mixed with 2 µl β-mercaptoethanol for 10 min. on ice. 2 µl *Dpn*I treated DNA was added to the cells and incubated on ice for 30 min. The cells were shocked at 42°C for 30 sec. and then incubated with 0.5 ml preheated LB or NZY^+ broth at 37°C, 225 rpm. After 60 min. transformed cells were plated onto LB agar with appropriate antibiotic for the plasmid vector.

Table 2.8: Site-directed mutagenesis primers

Primer name	Sequence
Sul1	CGTTCGTCCGGAGATCTGTGAATTTCGGTGC
Sul2	GCACCGAAATTCACAGATCTCCGGACGAACG
$CrgA_{N65}AF$	CGACTCTCTGGGCAACTGGgcCATCGTGGTGGGCTTCGG (39)
$CrgA_{N65}AR$	CCGAAGCCCACCACGATGGCCCAGTTGCCCAGAGAGTCG (39)
$CrgA_{W85}AF$	GGCGTCTCGACGCAGgcGAAGTAGGTGAATTC (32)
$CrgA_{W85}AR$	GAATTCACCTACTTCGCCTGCGTCGAGACGCC (32)

2.15 Bioinformatics techniques

2.15.1 Databases

Gene annotations and protein sequences were obtained from publicly available databases: *Streptomyces coelicolor* genome database ScoDB (http://streptomyces.org.uk/); *S. avermitilis* genome database (http://avermitilis.ls.kitasato-u.ac.jp/); *S. griseus* genome database (http://streptomyces.nih.go.jp/griseus/); Sanger Institute bacterial genomes (http://www.sanger.ac.uk/Projects/Microbes/); NCBI Microbial Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi); GenoList genome browser (http://genolist.pasteur.fr/); Joint Genome Institute Bacterial Genomics Database (http://www.jgi.doe.gov/genome-projects/); *Nocardia farcinia* genome database (http://nocardia.nih.go.jp/). Sequences are referred to by the ordered locus name provided in these databases.

2.15.2 BLAST analyses

Sequence similarity searches were performed by BLASTP against complete microbial genome sequences deposited in the NCBI Microbial Genome Project and other bacterial genome databases mentioned above using the default parameters.

2.15.3 Multiple alignments and phylogenetic analyses

Multiple alignments of amino acid sequences were constructed using CLUSTAL-W2 from EMBL-EBI server (http://www.ebi.ac.uk/Tools/clustalw2/) using the default parameters. Phylogenetic analyses were conducted using the MEGA 4.0.1 version (Tamura *et al.*, 2007, Kumar *et al.*, 1994). All default parameters were used. Unrooted trees were computed by the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap values for the consensus tree were derived from 1000 replicates. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Chapter 3: Analysis of *ftsW* and *ftsI* function in *Streptomyces coelicolor*

3.1 Introduction

In the division cell wall *(dcw)* gene cluster, most genes are considered to be essential for bacterial growth. As the name suggests, the genes of the *dcw* gene cluster are very important for cell division and it is a highly conserved gene cluster among bacteria. Mutagenesis of genes in the *dcw* cluster of *S. coelicolor,* including *ftsZ, ftsQ, ftsI, ftsW* and *ftsL*, has shown that cell division is not essential for the viability of *Streptomyces* (Bennett *et al.*, 2007; McCormick *et al.*, 1994; McCormick and Losick, 1996; Mistry *et al.*, 2008). FtsW is one of four SEDS family proteins encoded in the genome of *S. coelicolor.* In this chapter, the effect of *ftsW* complementation, and its overexpression in *S. coelicolor*, and role of other related proteins will be studied.

3.2 Genetic complementation of an *ftsW* mutant

Previous mutagenesis of *ftsW* had revealed that mutants have a white, non-sporulating phenotype (Mistry *et al.*, 2008). For complementation and overexpression analysis of the *S. coelicolor* FtsW protein, the gene was obtained from plasmid pME221. A 1459 bp *Nde*I/*Bam*HI fragment containing the *ftsW* gene was excised and sub-cloned under the *tipA* promoter in pIJ8600H digested with *Nde*I/*Bgl*II to obtain plasmid pSE66H (Fig. 3.1). The plasmid constructed pSE66H was confirmed by restriction digestion analysis and sequencing.

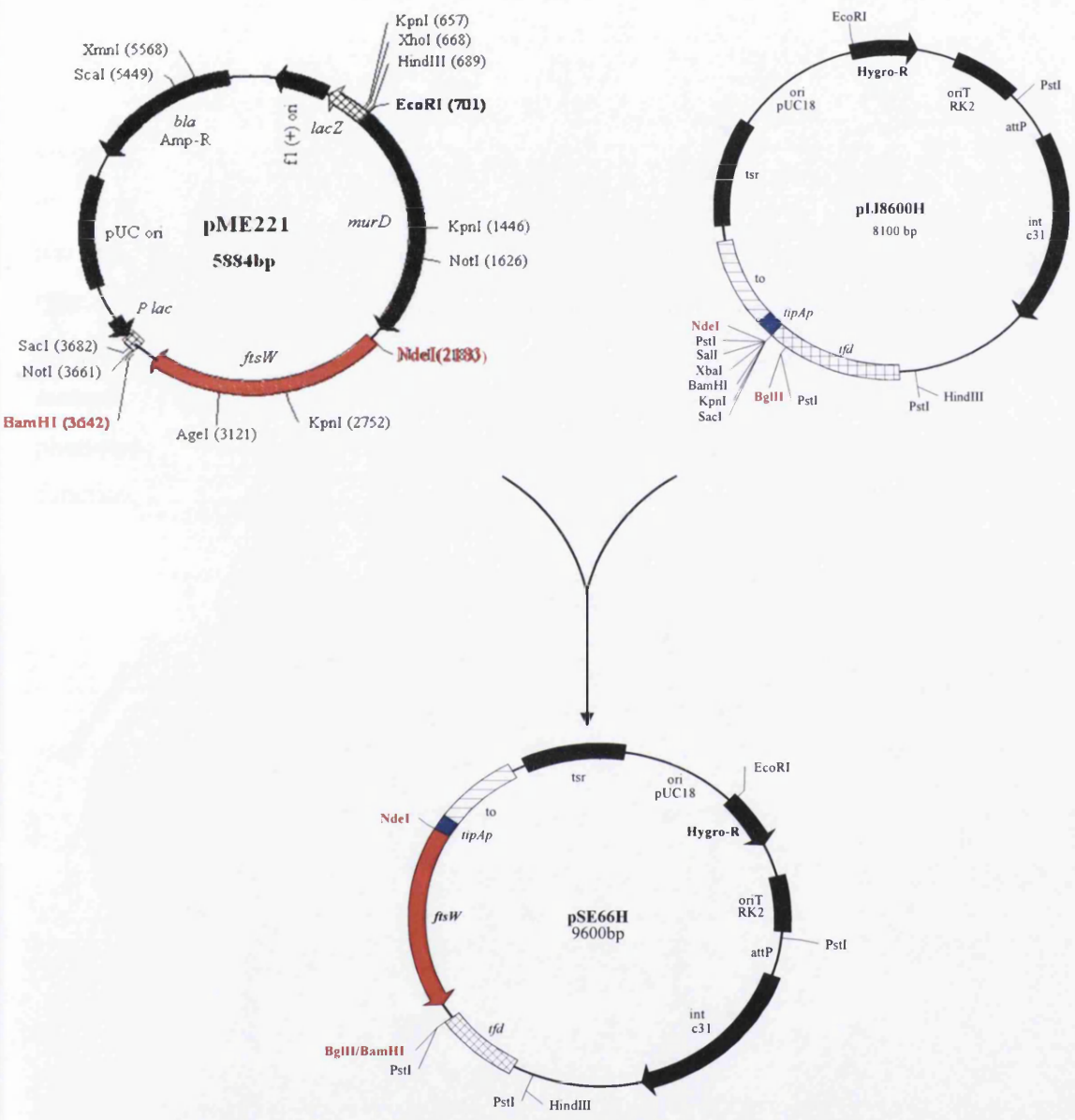


Figure 3.1: Construction of plasmid pSE66H. A *Nde*I/*Bam*HI fragment from pME221 (red), and was sub-cloned into pIJ8600H vector at its unique *Nde*I/*Bgl*II site (red), to construct pSE66H plasmid.

The plasmid pSE66H was then transferred into a *ftsW* mutant with a non-sporulating white phenotype by intergeneric conjugation. The ex-conjugants obtained containing the complementing plasmid was screened for hygromycin resistance. The exconjugants obtained with the complementing plasmid were designated as *ftsW*-pSE66H1, 2,3,4,5 and 6. To check that an empty vector does not have any effect on the phenotype of mutant, the pIJ8600H plasmid was introduced into both M145 wild type and *ftsW* mutant strain as described previously. For complementation analysis the strain with a complementing plasmid was plated on SFM agar plates along with wild type with empty vector and the mutant strain with empty vector. Plates were incubated at 30° C for 3 days. The complementation analysis showed that the non-sporulating phenotype of the *ftsW* mutant was restored by pSE66H complementing plasmid (Fig. 3.2). Consequently, the white phenotype is due to *ftsW* gene disruption, and recombinant plasmid construct pSE66H is functional. Expression of *ftsW* is due to basal-level activity of the uninduced *tipA* promoter.

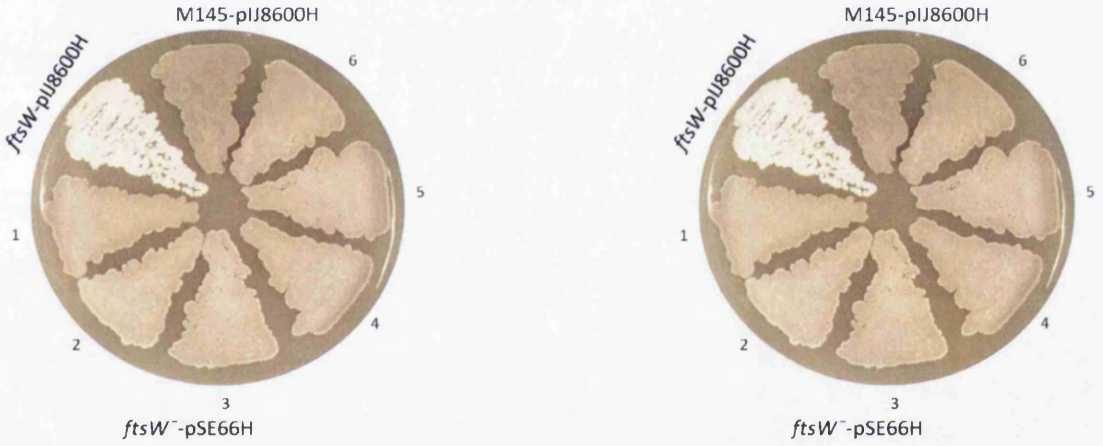


Figure 3.2: Complementation analysis of the non-sporulating phenotype *ftsW* mutant. Strains were grown on SFM sporulation media for 3 days at 30° C. Wild type strain is labelled as M145-pIJ8600H, *ftsW* mutant strain is labelled as *ftsW*-pIJ8600H and complemented strain labelled as *ftsW*-pSE66H1,2,3,4,5 and 6. All the *ftsW* mutant strains containing pSE66H complementing plasmid showed complementation.

3.3 Overexpression of *S. coelicolor* FtsW protein

To analysis the effect of overexpression of *S. coelicolor* FtsW protein in *S. coelicolor*, the strains *ftsW*-pSE66H and, as controls, M145-pIJ8600H and *ftsW*-pIJ8600H were used. The strains were plated on SFM agar plates containing 2.5 μg/ml, 5 μg/ml and 10 μg/ml (final concentrations) of thiostrepton and grown for 3 days at 30° C. No effect of *ftsW* overexpression was observed under the conditions applied, because overexpression of *ftsW* in *S. coelicolor* does not result in any macroscopic difference in phenotype (Fig. 3.3). There was some delay in the development of strains on the thiostrepton-containing medium.

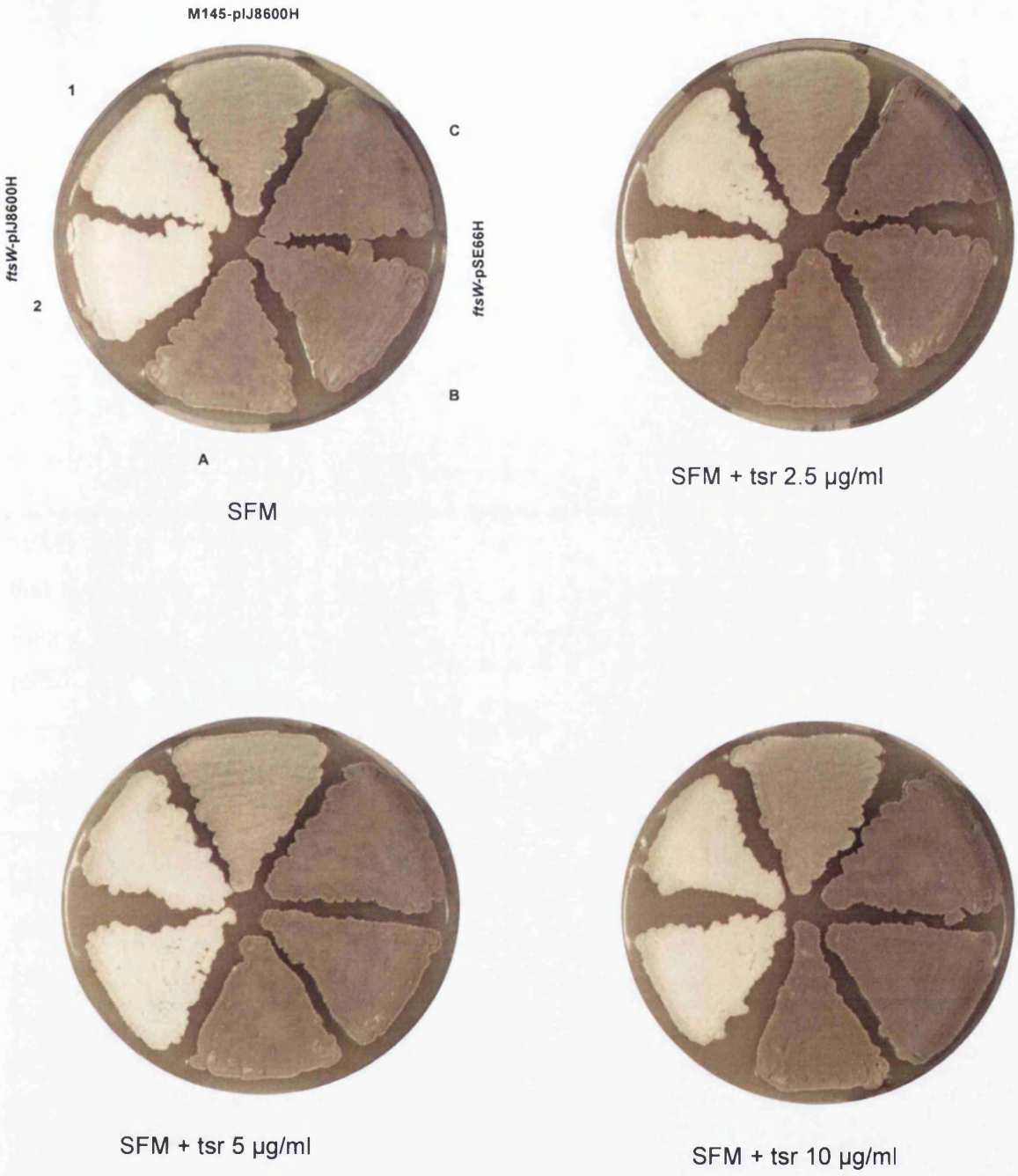


Figure 3.3: Phenotype of the *ftsW* overexpressed strain (*ftsW*-pSE66H A, B and C) along with wild type M145-pIJ8600H strain and mutant *ftsW*-pIJ8600H strain (1 and 2) on SFM and SFM containing 2.5, 5 and 10µg/ml thiostrepton for FtsW protein overexpression. Strains were grown for 72 h at 30° C.

3.4 Microscopic analysis of *ftsW* overexpression strains

Fluorescence microscopy was used to analysis the effects of *ftsW* over- expression on sporulation septation by visualizing the cytological effects on peptidoglycan synthesis and chromosome distribution. Cell wall synthesis during sporulation was examined by staining the aerial hyphae with fluorescein-labelled wheat germ agglutinin (Fluo-WGA). Fluo-WGA stains the cell wall by binding to peptidoglycan precursors that are produced as a result of active peptidoglycan synthesis and breakdown. Chromosomal DNA was stained with propidium iodide (PI). Samples of aerial hyphae were prepared by taking impressions of wild type M145-pIJ8600H and the *ftsW* mutant complemented with *ftsW*-pSE66H grown on the surface of SFM and SFM+thiostrepton agar medium for 38 h and 48h, at 30° C. Regularly spaced sporulation septa that form pre-spore compartments, each containing a single condensed nucleoid stained with red fluorescence dye (Fig. 4), were observed in aerial hyphae of the *ftsW* overexpressed strain *ftsW*-pSE66H, similar to that of the wild type M145-pIJ8600H (Fig.3. 4 g and r).

To check further the effect of *ftsW* overexpression on the dimensions of spores of each strain, the length and width of pre-spore compartments were measured using the Scion Image program. For this analysis, strains of complemented *ftsW*-pSE66H, M145-pSE66H and M145-pIJ8600H were grown on the surface of SFM, while for *ftsW* overexpression these strains were grown on SFM+thiostrepton agar medium. To measure the dimensions of pre-spore compartments Fluo-WGA stained images of the strains were used. The dimensions of 250 pre-spore compartments from each strain were measured randomly. The length of pre-spore compartments of all the strains including the wild type strain M145-pIJ8600H ranges from 0.9 to 1.8 μm, with the majority of pre-spore compartments between 1 to 1.4 μm in length (Fig. 3.5), where the average length of pre-spore compartments of all strains is 1.2 μm (Table 3.1). The microscopic analysis of aerial hyphae of the *ftsW* mutant strain complemented or with overexpressed FtsW showed normal sporulation septa and pre-spore compartment sizes similar to wild type. This indicates that there is no observable effect of *ftsW* overexpression on the sporulation septation of *S. coelicolor.*

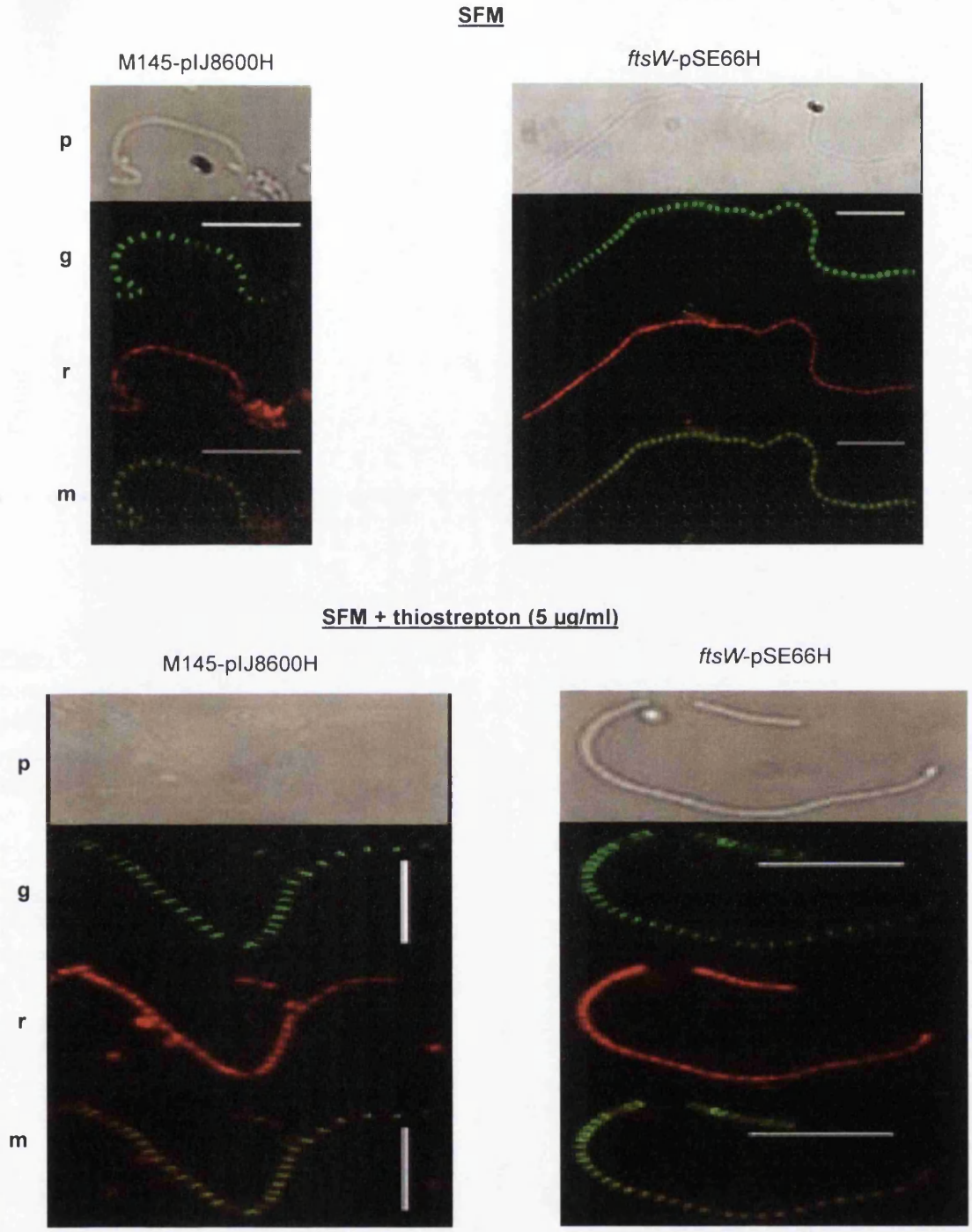


Figure 3.4: Phase contrast and fluorescence microscopy of wild type M145-pIJ8600H and complemented mutant strain *ftsW*-pSE66H. Phase contrast microscopy of aerial hyphae of strains (p), Cell wall staining with Fluo-WGA (g), DNA staining with PI (r), and merged of Fluo-WGA and PI staining of aerial hyphae of respective strains (m). Samples were prepared by taking impressions of each culture grown on SFM agar and SFM + thiostrepton for 48h at 30° C.

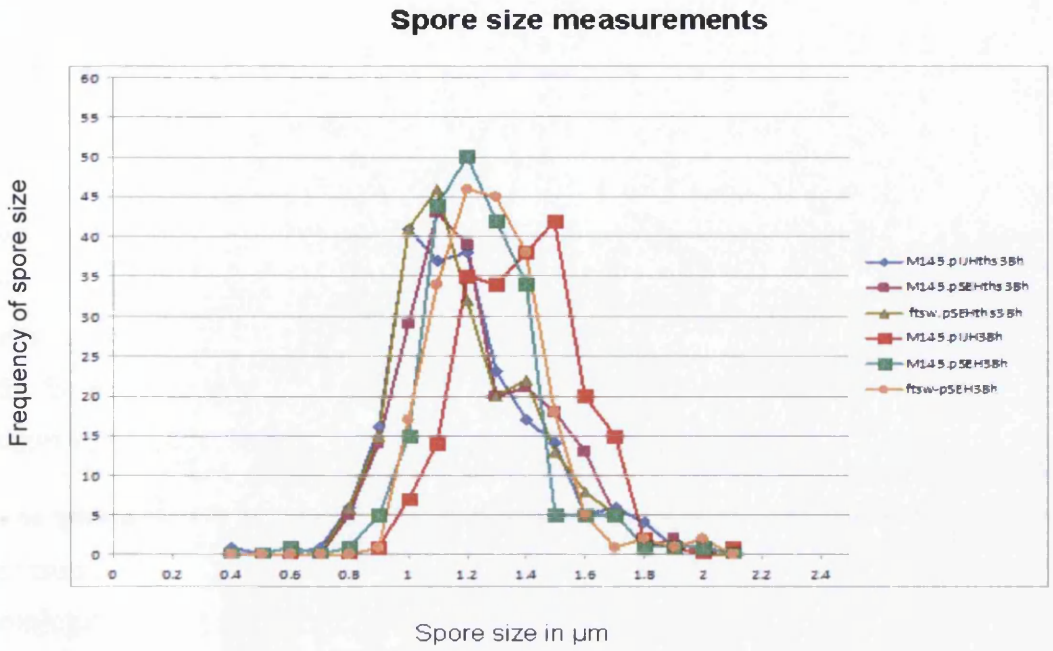


Figure 3.5: Graph of pre-spore compartment length versus frequency of the length of pre-spore compartment in wild type M145-pIJ8600H, complemented *ftsW*-pSE66H and *ftsW* overexpressed (M145-pSE66H, M145-pIJ8600Hthsp., M145-pSE66Hthsp. and *ftsW*-pSE66Hthsp. strains). The length of randomly selected 250 pre-spore compartments of each strain was measured from the Fluo-WGA stained images using Scion Image software.

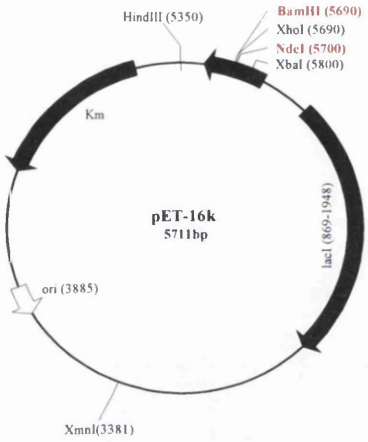
Table 3.1: Average spore size in µm of pre-spore compartments in the sporulation aerial hyphae of M145-pIJ8600H, M145-pSE66H, *ftsW*⁻-pSE66H and M145-pIJ8600H strains which grown on SFM and SFM+ thiostrepton 5µg/ml.

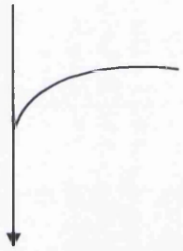
Strain	Medium	Average spore size in µm
M145-pIJ8600H	SFM+thiostrepton 5µg/ml	1.14
M145-pSE66H	SFM+thiostrepton 5µg/ml	1.16
ftsW⁻-pSE66H	SFM+thiostrepton 5µg/ml	1.15
M145-pIJ8600H	SFM	1.33
M145-pSE66H	SFM	1.2
ftsW⁻-pSE66H	SFM	1.24

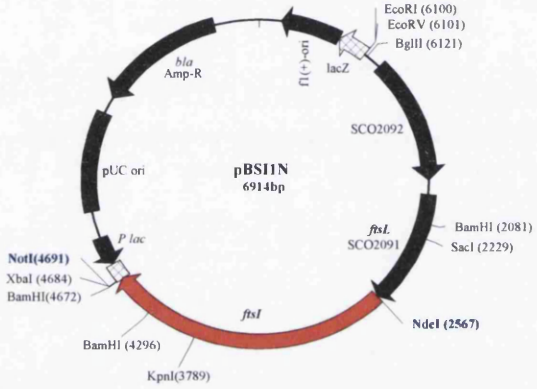
3.5 Overexpression analysis of the FtsI protein

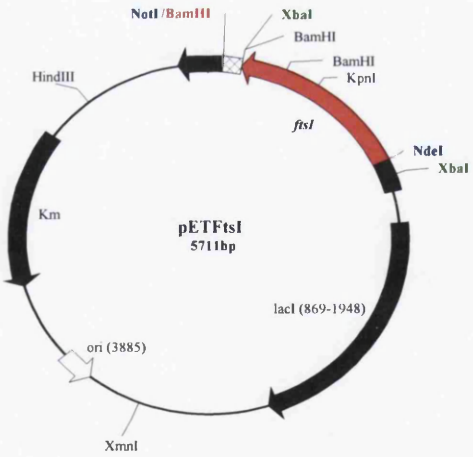
For overexpression analysis of the FtsI protein a plasmid pFtsIH containing the *ftsI* gene was constructed. To construct pFtsIH, a 2124 bp *Not*I blunt-ended/*Nde*I fragment containing *ftsI* was excised from plasmid pBSI1N and cloned into pET-16k digested with *Bam*HI blunt-ended/*Nde*I creating pETFtsI (Fig. 3.6). Then, the *ftsI* fragment from pETFtsI was subsequently subcloned as a 2217 bp *Xba*I fragment into similarly digested pIJ8600H, to create pFtsIH (Fig.3. 6). In pFtsIH plasmid the *ftsI* gene was cloned just downstream and under control of the thiostrepton-inducible *tipA* promoter.

The plasmid construct pFtsIH was confirmed by restriction digestion analysis. The pFtsIH construct was then transferred into *S. coelicolor* M145 and an *ftsI* mutant by intergeneric conjugation to obtain an M145-pFtsIH and *ftsI*-pFtsIH strains respectively. An empty vector pIJ8600H was also introduced into *S. coelicolor* M145 and *ftsI* mutant to obtain M145-pIJ8600H and *ftsI*-pIJ8600H strains as controls.









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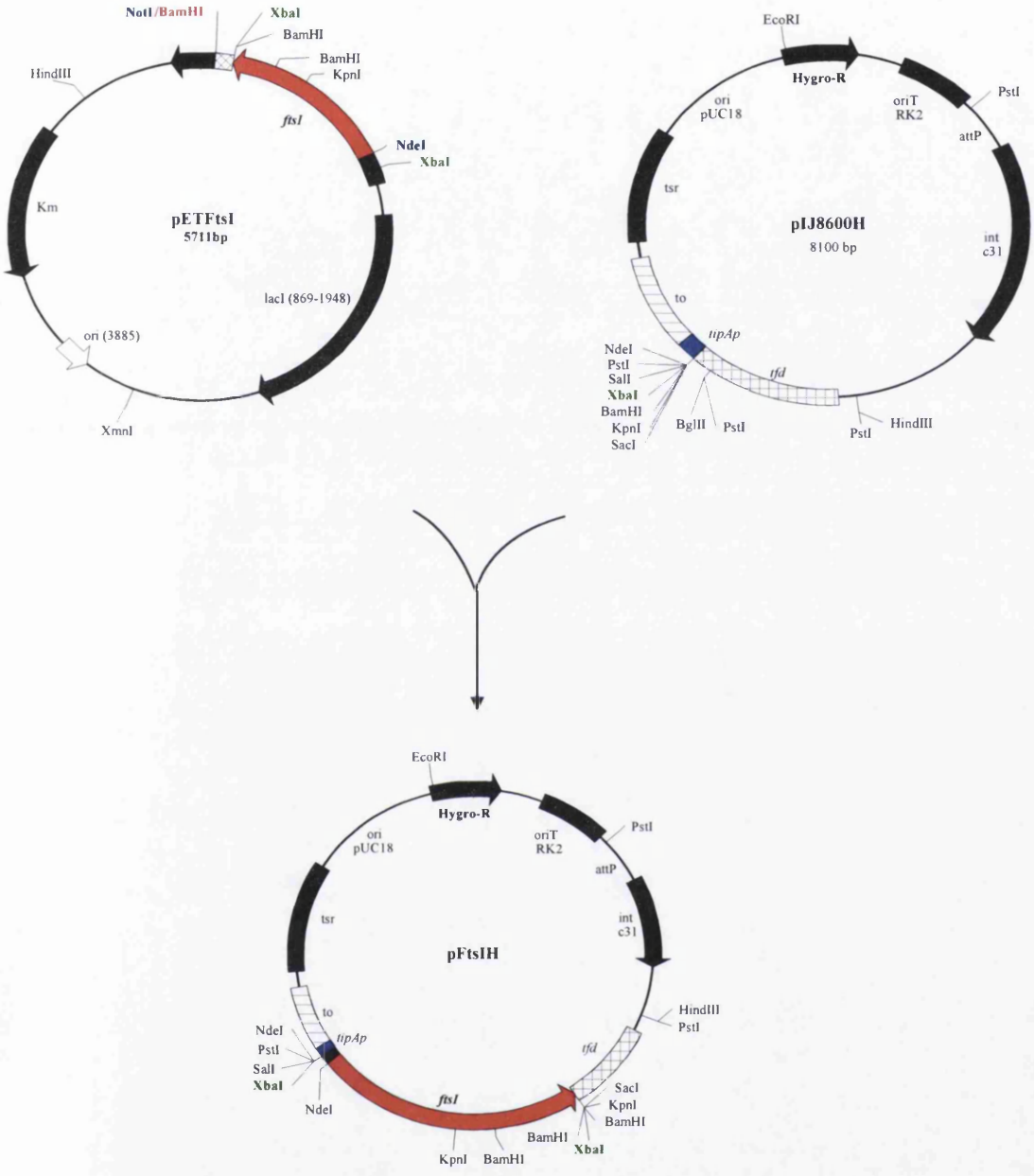


Figure 3.6: Plasmid maps showing the construction of pFtsIH plasmid. The pBSI1N plasmid was digested with *Not*I/*Nde*I (blue) to obtain ftsI gene. The fragment was cloned into pET-16k plasmid digested with *Bam*HI/*Nde*I (red), after *Not*I and *Bam*HI blunt-ending to obtain pETFtsI plasmid. Then an *Xba*I (green) fragment from pETFtsI plasmid was cloned into pIJ8600H at its unique *Xba*I (green) site to construct pFtsIH. Maps are not drawn to the scale.

For phenotypic characterization, and to study the effect of *ftsI* overexpression on the growth and viability of *S. coelicolor*, the strains containing either pFtsIH plasmid or pIJ8600H empty vector were plated on SFM agar plates containing 10 µg/ml (final concentration) of thiostrepton and grown for 3 to 4 days at 30° C. *ftsI* overexpressed strains were sporulating normally, and no effect of *ftsI* overexpression on the growth and viability of *S. coeliolor* was observed (Fig. 3.7). There was some delay in the development of *ftsI*-pIJ8600H mutant strain on the thiostrepton containing medium.

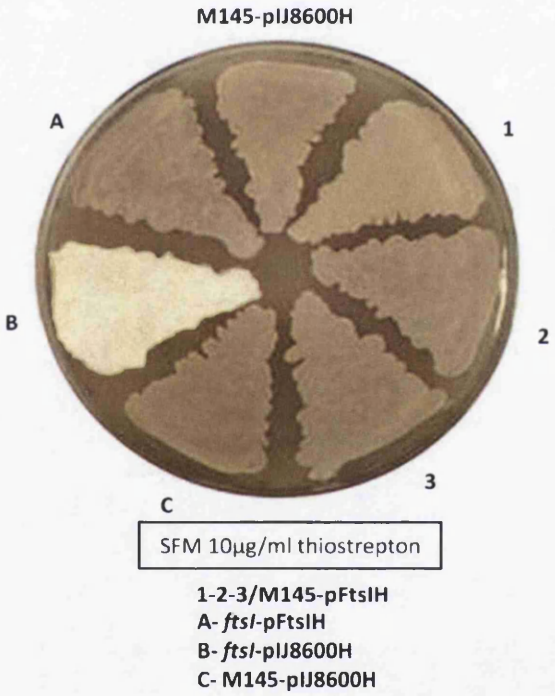


Figure 3.7: Phenotype of the ftsI overexpressed strain *ftsI*-pFtsIH (A), along with mutant strain *ftsI*-pIJ8600H (B) and wild type strain M145-pIJ8600H (C), on SFM containing 10µg/ml thiostrepton for FtsI protein overexpression. Strains were grown for 72 h at 30° C

To check the effect of *ftsI* overexpression on sporulation septation of aerial hyphae, fluorescence microscopy was used. The strains of M145-pFtsIH and *ftsI* pFtsIH including wild type and *ftsI* mutant containing empty vector were grown on the surface of SFM agar plates containing 10 μg/ml of thiostrepton. Cultures were incubated at 30° C for 38 to 48h. Impressions of each culture were taken on coverslips and the samples were observed under the microscope after staining with Fluo-WGA (for cell wall) and propidium iodide (PI) (for chromosomal DNA). Regularly spaced sporulation septa defining pre-spore compartments each containing a single condensed chromosomal DNA were observed in wild type aerial hyphae. Also, fluorescence microscopy of aerial hypha of both M145-pFtsIH and *ftsI* pFtsIH revealed similar morphologies to that of the wild type. On the other hand, the aerial hyphae of the *ftsI* mutant showed no sporulation septa and no apparent chromosome condensation (Fig. 3.8). Consequently, this analysis indicated no abnormal effects on sporulation septation due to *ftsI* overexpression.

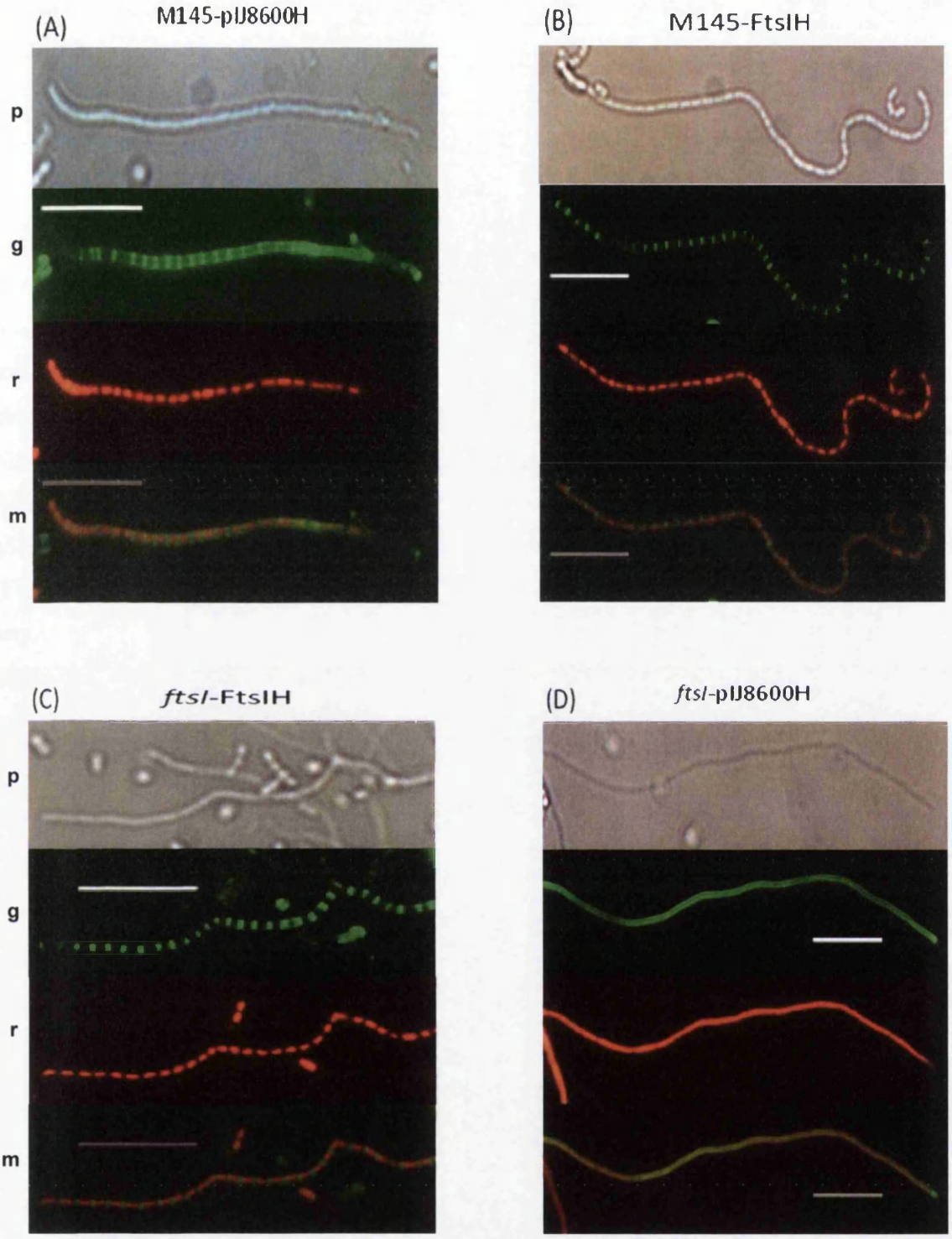


Figure 3.8: Fluorescence microscopy of aerial hyphae of *S. coelicolor* M145 pIJ8600H (A), M145-pFtsIH (B), *ftsI*-pFtsIH (C) and *ftsI*-pIJ8600H (D) strains, using Fluo-WGA (for cell wall) and PI (chromosomal DNA) staining to visualize sporulation septa and chromosome distribution. Samples were prepared by taking impressions of cultures grown on the surface of SFM agar medium containing 10 µg/ml thiostreption for 3 days at 30° C. Each panel shows phase contrast (p), Fluo-WGA staining (g), PI staining (r) and merged (m) images of Fluo- WGA and PI staining of aerial hyphae of respective strains. (Scale bar 10 µm).

3.6 FtsW localization study

3.6.1 Construction of plasmid pSE66Hm

To investigate FtsW localization, a plasmid pSE66Hm containing the *ftsW* gene translationally fused with mCherry (red fluorescent protein) was constructed. To construct pSE66Hm, a plasmid pME222-mCherry was digested with *Sca*I to make it linear, and then a *Not*I fragment containing the *ftsW*-mCherry fusion derived from the linearised plasmid was isolated and sub-cloned into pME6 vector digested with the same enzyme (Fig. 3.9). The vector pME6 with the cloned *ftsW*-mCherry was designated as pME6fm. The pME6fm plasmid was digested with *Nde*I/*Bam*HI to obtain the *ftsW*-mCherry fusion. The fusion was cloned just downstream of the thiostrepton inducible *tipA* promoter in *Nde*I/*Bgl*II-digested pIJ8600H plasmid to obtain pSE66Hm plasmid where the *ftsW*-mCherry fusion gene is under the control of the *tipA* promoter (Fig. 3.9). The plasmid constructed was confirmed by restriction digestion analysis and sequencing. The plasmid pSE66Hm was then introduced into an *ftsW* mutant by intergeneric conjugation. The *ftsW* mutant with pSE66Hm was designated as *ftsW*-pSE66Hm.

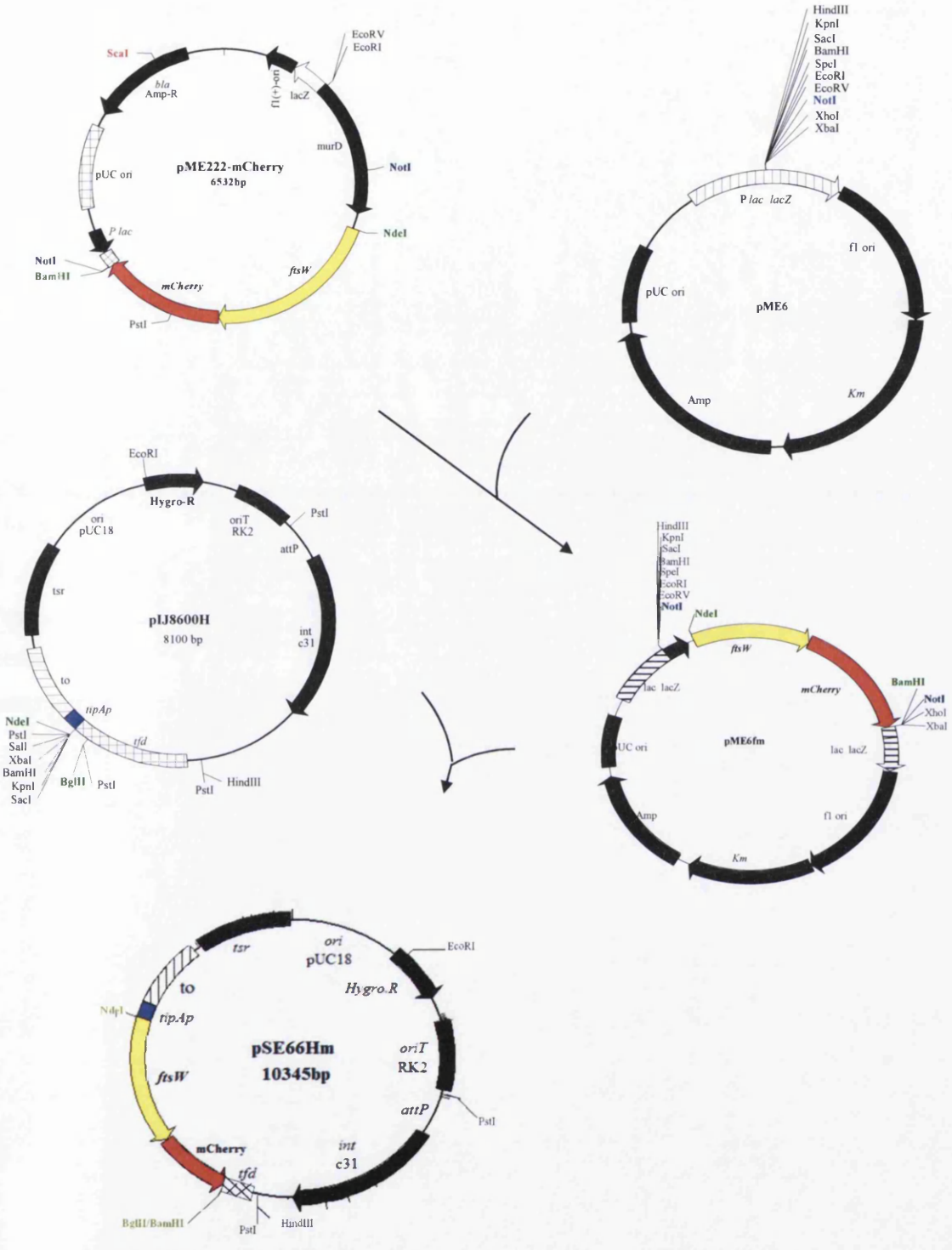


Figure 3.9: Map of the plasmids showing the construction of pSE66Hm. Plasmid pME222-mCherry was digested with *Sca*I (red) to make it linear, then, an *Not*I (blue) fragment isolated and sub-cloned into pME6 vector digested with same enzyme (blue). An *Nde*I/*Bam*HI (green) fragment (*ftsW*-mCherry) from pME6fm was cloned into pIJ8600H vector at its unique *Nde*I/*Bgl*II (green) sites to construct pSE66Hm.

3.6.2 Phenotypic analysis of complemented *ftsW*-pSE66Hm

As a control an empty vector pIJ8600H was also introduced into *S. coelicolor* M145 to obtain the strain M145-pIJ8600H. The mutant strain complemented with pSE66Hm was designated as *ftsW*-pSE66Hm and with the empty vector was designated as *ftsW*-pIJ8600H. To characterize *ftsW*-pSE66Hm macroscopically, the clones of the *ftsW*-pSE66Hm strain were plated on sporulation specific SFM medium along with the wild type strain M145-pIJ8600H and *ftsW* mutant strain *ftsW*-pIJ8600H. The cultures were grown at 30° C for three to four days and assessed for sporulation by visualizing the production of grey pigmented spores on the surface of SFM agar grown culture. Introduction of pSE66Hm into the *ftsW* mutant restored the wild type phenotype. No difference in phenotype of *ftsW*-pSE66Hm strain was observed compare to wild type (Fig. 3.10). This result provides the evidence that the plasmid pSE66Hm containing the *ftsW*-mCherry fusion was functional. Complementation of the Whi phenotype by the *ftsW*-mCherry fusion was observed in the absence of thiostrepton, indicating basal-level transcription from the uninduced promoter was sufficient for this result.

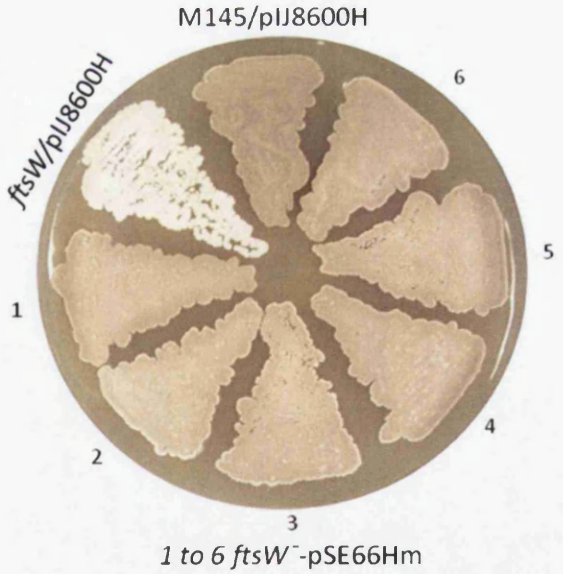
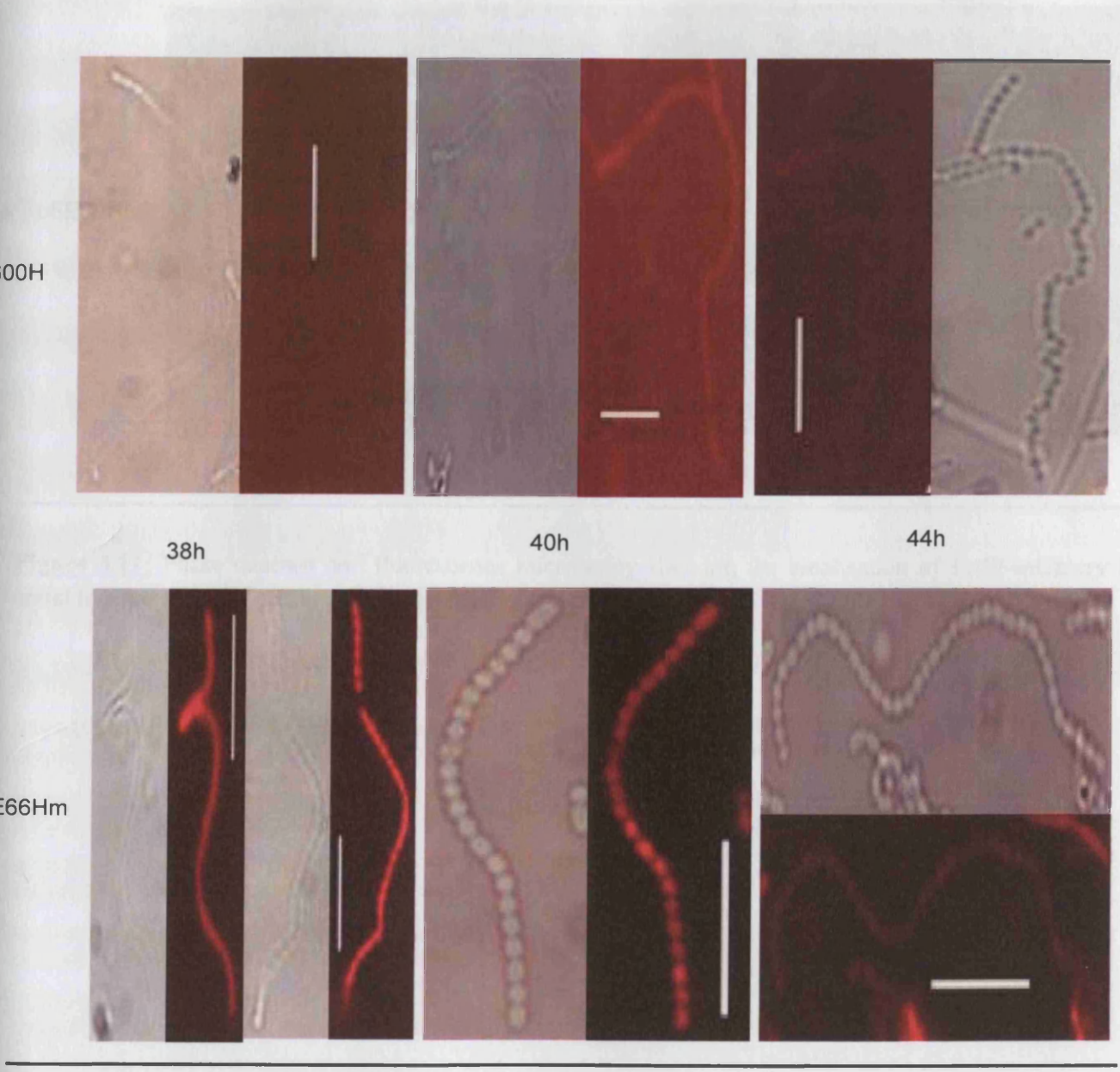


Figure 3.10: Phenotype of *ftsW*-pSE66Hm clones (1, 2, 3, 4, 5 and 6) along with wild type M145-pIJ8600H strain and *ftsW*-pIJ8600H mutant strain on SFM. Strains were grown for 3 to 4 days at 30° C.

3.6.3 Analysis of ftsW-mCherry localization

So far phenotypic and microscopic analysis showed that a typical complemented *ftsW*-pSE66H restores sporulation septa. Therefore to attempt to study the localization of FtsW protein in the aerial hyphae of *S. coelicolor*, the mCherry (fluorescence protein) fused with FtsW (FtsW-mCherry) was expressed in *ftsW* mutant and its distribution was compared to that in wild type. To analyze the localization of FtsW-mCherry, fluorescence microscopy was performed. For the aerial hyphae, cultures of M145-pIJ8600H and *ftsW*-pSE66Hm were grown on SFM agar medium for 24, 30 and 32h at 30° C.

Also to see if the localization of FtsW-mCherry proteins in the aerial hyphae is affected by overexpression, the strain *ftsW*-pSE66Hm was grown along with M145-pIJ8600H (control) on SFM agar containing 5μg/ml thiostrepton for 38, 40 and 44h. Impressions of each sample were taken on coverslips and then directly visualized under the microscope. The aerial hyphae of the complemented strain *ftsW*-pSE66Hm from cultures grown on both SFM and SFM-thiostrepton showed diffuse fluorescence, and no specific localization of FtsW-mCherry was observed (Fig. 3.11). There was no evidence of localization to sporulation septa as one would expect of a divisome protein.



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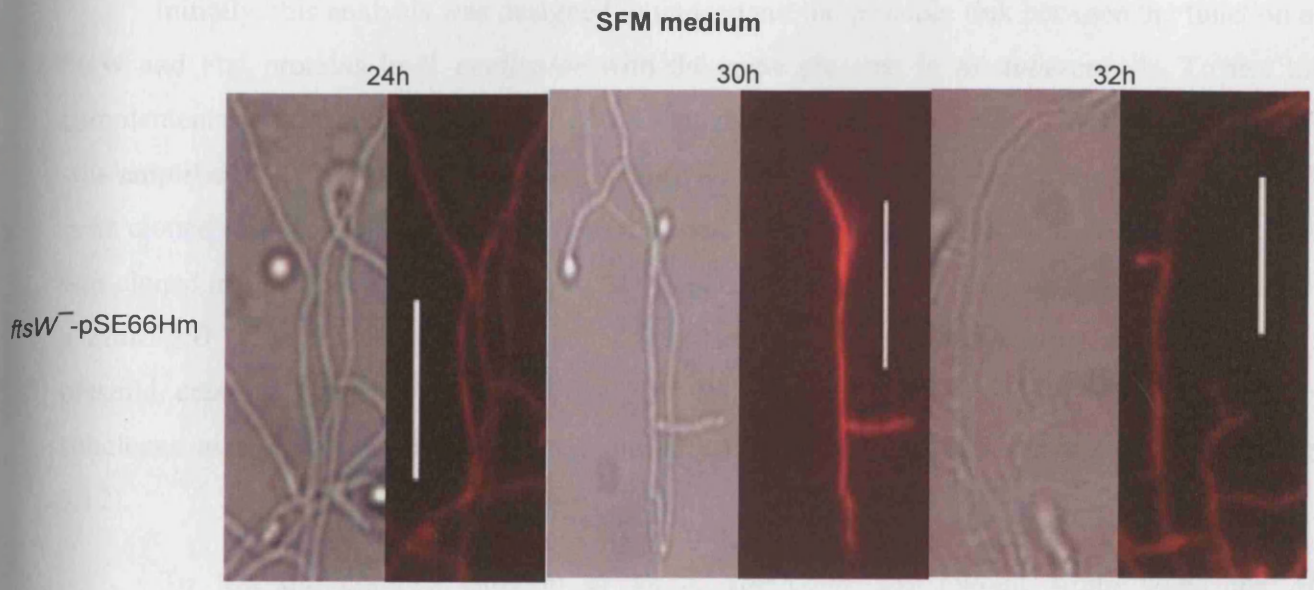

Figure 3.11: Phase contrast and fluorescence microscopy showing the localisation of FtsW-mCherry in aerial hyphae of *ftsW*⁻-pSE66Hm. Diffuse fluorescence was observed.

3.7 Investigation of mycobacterial FtsW and FtsI function in *S. coelicolor*

Initially, this analysis was designed to understand the possible link between the function of FtsW and FtsI proteins in *S. coelicolor* with the same proteins in *M. tuberculosis*. To test the complementation of an *S. coelicolor ftsW* mutant strain, the *M. tuberculosis ftsW* gene (MtftsW) was amplified by PCR with the primers MtftsWF1 and MtftsWR1. The PCR amplified MtftsW gene cloned in pGEM-T easy vector, then digested with *Nde*I and *Eco*RI and purified. The gene was cloned in *Nde*I/*Eco*RI digested pET-28a (+) plasmid to obtain pJB201 plasmid. From pJB201, a *Sal*I/*Bgl*II fragment containing MtftsW gene was excised and sub-cloned into the pIJ2925 plasmid creating pMtftsW plasmid. The MtftsW fragment from pMtftsW was subsequently subcloned as a *Nde*I/*Bgl*II fragment into similarly digested pIJ8600H, to create pMtftsWH (Fig. 3.12).

To test the complementation of an *S. coelicolor ftsI* mutant strain using the *M. tuberculosis ftsI* gene (Mtpbp3), the gene was amplified by PCR with the primers Mtpbp3F and Mtpbp3R. The PCR amplified Mtpbp3 gene was purified and cloned in pGEM-T easy vector. The fragment *Nde*I/*Bgl*II include the Mtpbp3 gene was digested and cloned just downstream of the inducible *tipA* promoter in *Nde*I/*Bgl*II digested pIJ8600H plasmid to obtain pMtftsIH plasmid where the Mtpbp3 gene is under the control of the *tipA* promoter (Fig. 3.13). The plasmids constructed, pMtftsWH and pMtftsIH, were confirmed by restriction digestion analysis and sequencing.

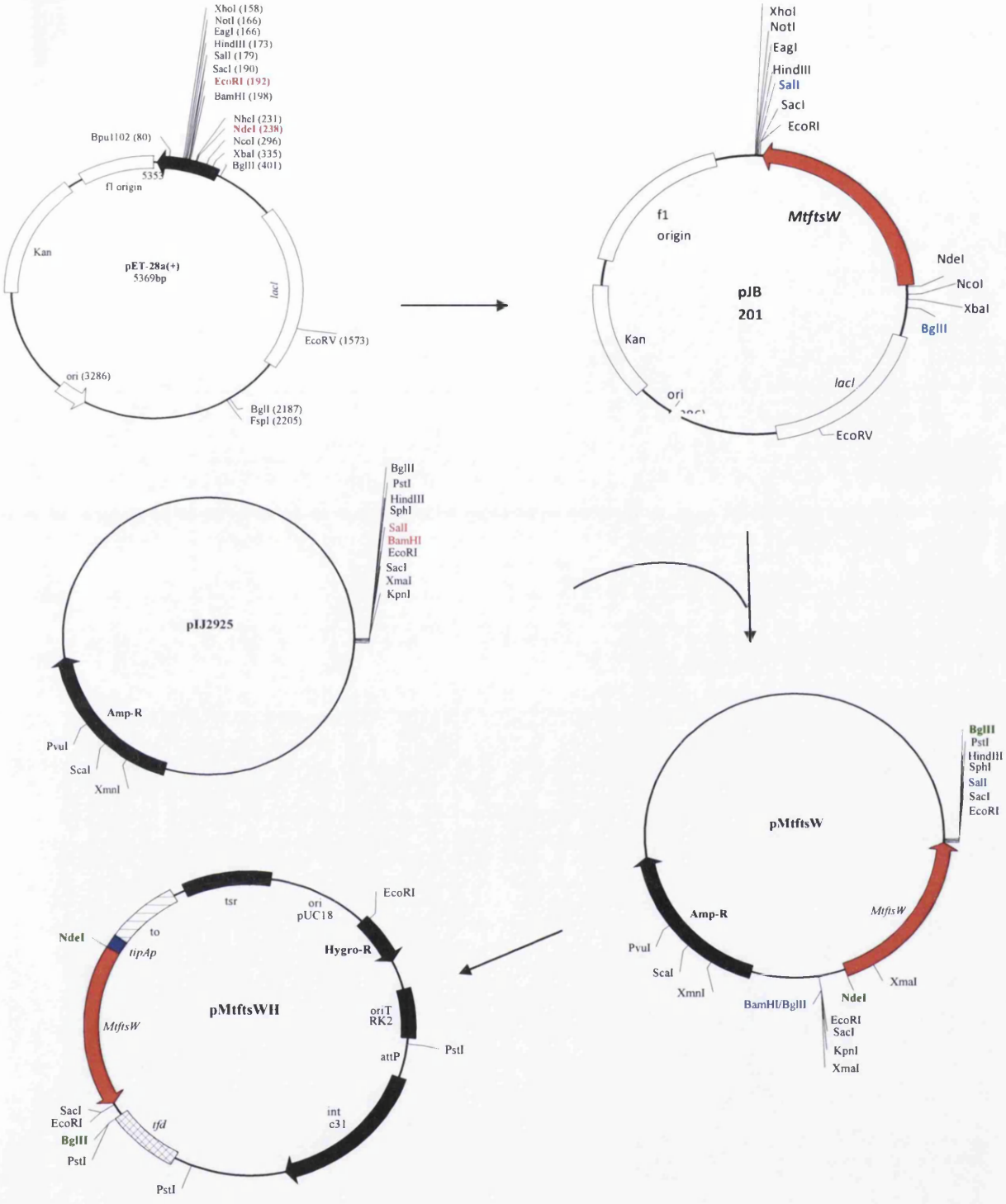

Figure 3.12: Map of the plasmids showing the construction of pMtftsWH. Plasmid pET-28a+ was digested with *Nde*I/*Eco*RI (red) and PCR amplified MtftsW or MtftsI digested with the same enzymes sub-cloned to obtain pJB201. An *Sal*I/*Bgl*II (blue) fragment from pJB201 was isolated and sub-cloned into pIJ2925 vector digested with *Sal*I/*Bam*HI enzymes to obtain pMtftsW plasmid. Fragment *Nde*I/*Bgl*II (green) containing MtftsW gene from pMtftsW was isolated and sub-cloned into pIJ8600H vector digested with the same enzymes to obtain pMtftsWH.

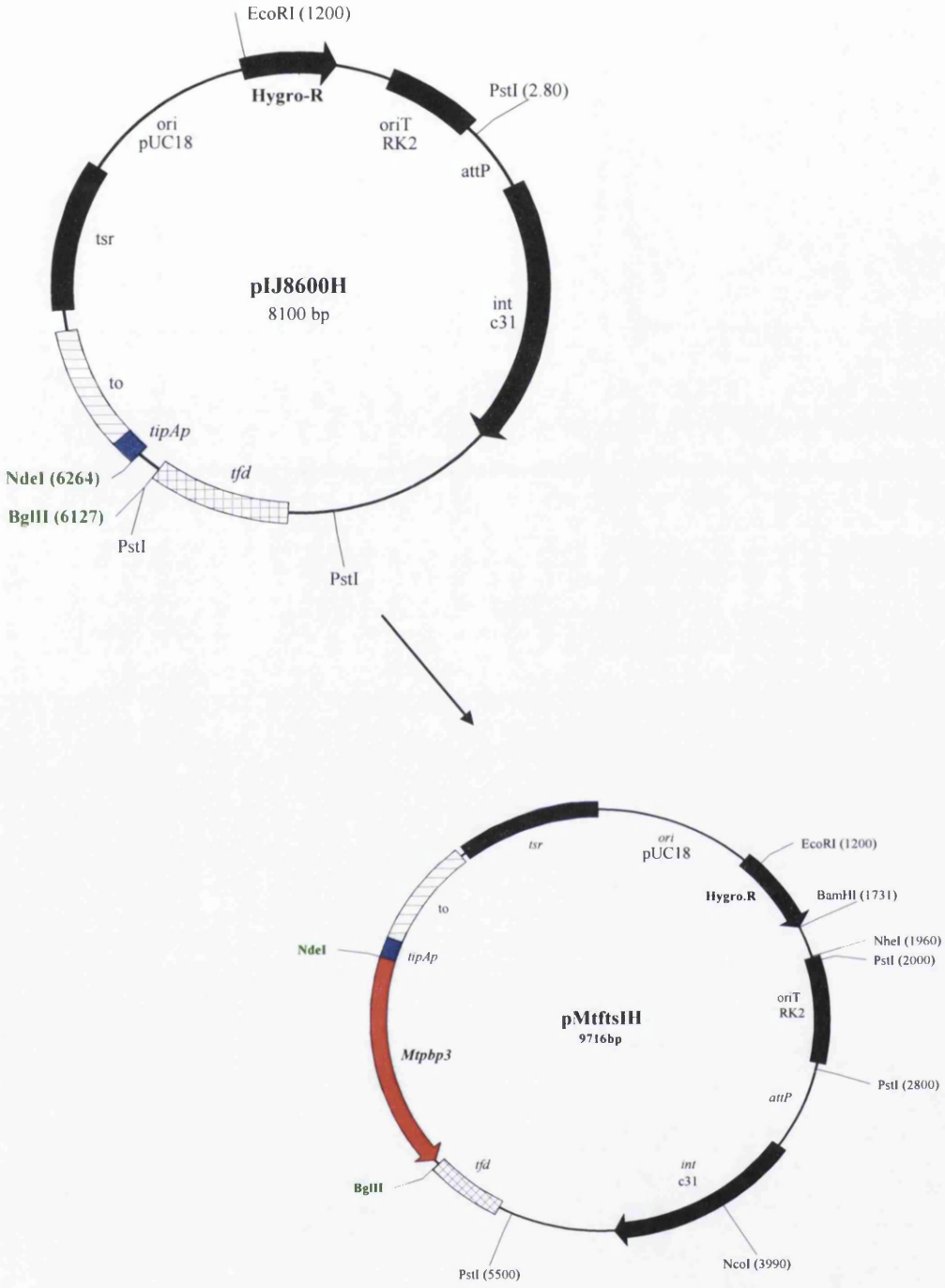

Figure 3.13: Map of plasmids pIJ8600H and pMtftsIH indicating restriction sites. Restriction enzymes used for cloning the Mtpbp3 gene are indicated in green colour. The thiostrepton inducible *tipA* promoter is indicated in blue colour. The Mtpbp3 gene is indicated by a red arrow. The maps are not drawn to the scale.

3.7.1 Complementation analysis of *ftsW* mutant using MtftsW and Mtpbp3

To complement *ftsW* and *ftsI* mutant strains of *S. coelicolor*, pMtftsWH and pMtftsIH were introduced into *ftsW* and *ftsI* mutants respectively by intergeneric conjugal transfer and exconjugants were screened for hygromycin resistance. The resulting strains of *ftsW* and *ftsI* mutants with pMtftsWH and pMtftsIH respectively, were designated as *ftsW*-pMtftsWH (*ftsW* mutant with pMtftsWH) and *ftsI*-pMtftsIH (*ftsI* mutant with pMtftsIH). The strains were characterized for complementation by growing them on SFM agar along with the wild type, for 3 days at 30° C. Introduction of pMtftsWH and pMtftsIH into *ftsW* and *ftsI* mutants of *S. coelicolor* respectively, did not restore the non-sporulating white phenotype of the mutants (Fig. 3.14). That means introduction of recombinant plasmids containing *ftsW* and *ftsI* genes of *M. tuberculosis* in *ftsW* and *ftsI* mutant strains of *S. coelicolor* fails to complement the mutants.

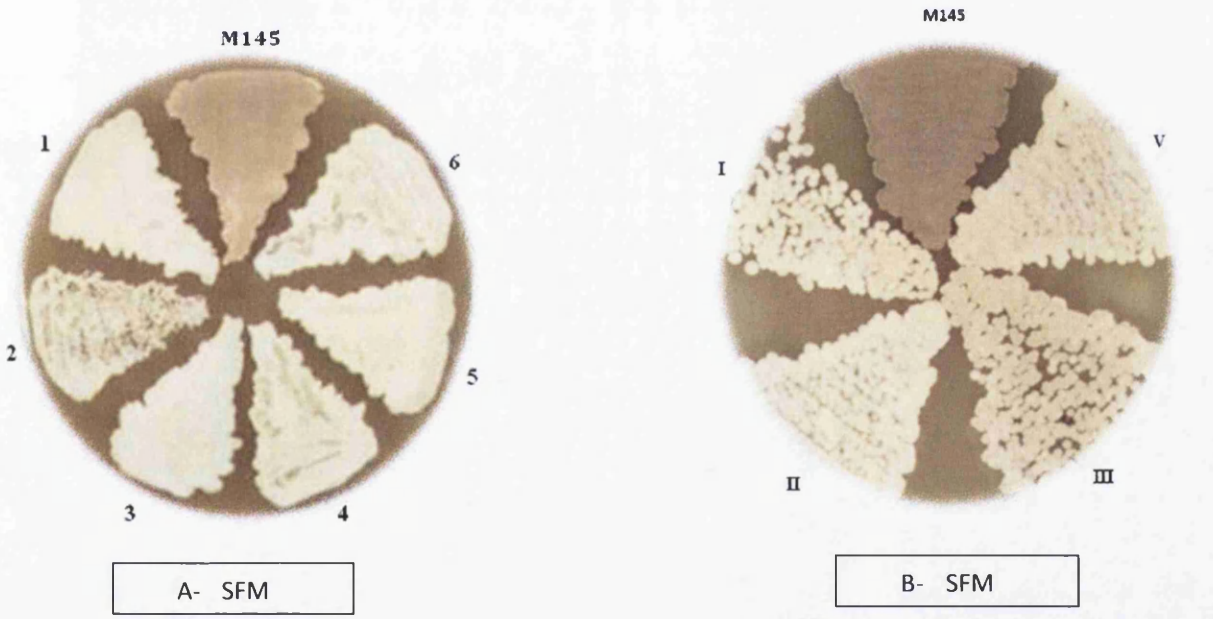

Figure 3.14: Complementation analysis of *ftsW* and ftsI mutant strains of *S. coelicolor* (A) and (B) respectively. Strains were grown on SFM sporulation media for 3 days at 30° C. Wild type strain is labelled as M145; different clones of *ftsW* mutant strain containing pMtftsWH plasmid (A), are labelled as numbers (1 to 6); and clones of *ftsI* mutant strain containing pMtftsIH plasmid (B), are labelled as (I, II, III and V).

3.8 Summary

Complementation of *ftsW* mutant *S. coelicolor* resulted in a sporulating phenotype confirming that the white phenotype was due to *ftsW* disruption, and *ftsW* function was restored by the complementing plasmid. Macroscopically, *ftsW or ftsI* complementation or overexpression in *S. coelicolor* does not show any difference in phenotype. In addition, the microscopic analysis revealed that there is no effect of *ftsW or ftsI* overexpression on the sporulation septation and chromosomal condensation of aerial hyphae under laboratory conditions used. Diffuse distribution of FtsW-mCherry in the aerial hyphae of an *ftsW* complemented strain, and no specific localization was observed. Mycobacterial orthology of FtsW and FtsI cannot replace the function of their related streptomycete proteins.

Chapter 4: Analyses of *ftsW/sfr/rodA2* mutants of *Streptomyces coelicolor*

4.1 Introduction

With access to complete genome sequences it is possible to compare whole genomes to understand genome structure, function and evolution of a variety of bacteria. Analyses of conserved sequence similarities of gene sequences and conserved gene orders among different genomes helps to predict the function of a particular gene and establish orthologous relations of well characterized genes among bacteria and reveal general principles of how functionally coupled genes are physically encoded in the genome and possibly co-regulated at the level of gene expression (Fujibuchi *et al.*, 2000; Kihara and Kanehisa, 2000; Overbeek *et al.*, 1999).

Across the bacterial community some of the SEDS family of proteins that includes FtsW, RodA and SpoVE are highly conserved and have high similarity (Gerard *et al.*, 2002; Ikeda *et al.*, 1989; Lara and Ayala, 2002). In particular, in *E. coli* and *B. subtilis* models of unicellular bacteria, the FtsW, RodA and SpoVE proteins are well studied. Mutational analysis is a good method to reveal the function of a particular gene. To get some idea about the function of the four *ftsW/rodA*-like genes of *S. coelicolor*, independent and combination mutants for each gene have been constructed using transposon disrupted insertions that are obtained by *in vitro* transposon mutagenesis of the ordered cosmid library of *S. coelicolor* genome using transposon Tn*5062* (Bishop *et al.*, 2004; Fernández-Martínez *et al*, 2010).

4.2 Triple mutant *ftsW*, *sfr* and *rodA2* constructions:

To construct this triple mutant and for easy screening of the mutants, the apramycin resistance (Apr^R) cassette of the transposon insertion, 4A10.2.H05, in the *ftsW* gene was replaced by a spectinomycin/streptomycin resistance (*aadA*) marker using the Redirect technique as described in Materials and Methods (Gust *et al.*, 2003). Firstly, and before the Redirect procedure was performed, a plasmid pQM5080 containing the *aadA* gene was constructed. To construct pQM5080, a 2056 bp *Bam*HI fragment from pSF152 plasmid containing the *aadA* gene was blunt-ended and cloned into pQM5066 digested with *Not*I and *Sal*I blunt-ended, to remove the hygromycin resistance (Hyg^R) gene and replace it with the aadA gene (Fig. 4.1). The resulting plasmid pQM5080 was used for the Redirect technique.

The replacement of the marker in the cosmid insertion was confirmed by restriction analysis and sequencing. The 4A10.2.H05 insertion cosmid with the *aadA* cassette was then introduced into the DSCO5302-1/2607 (*sfr*/*rodA2*) double mutant strain, obtained from a previous study (Mistry *et al.*, 2008). Simultaneously, the 4A10.2.H05 *aadA* insertion was also introduced into the wild type strain of *S. coelicolor* to obtain single gene mutants just to check that the marker replacement did not affect any other gene in the cosmid that may result in some false phenotype. The exconjugants obtained by conjugation in both the cases were screened for spectinomycin/streptomycin resistance and kanamycin susceptibility to isolate double crossover mutants of the *ftsW* gene in the *sfr*/*rodA2* disrupted background and wild type background. Several double crossover recombinant mutants of the *ftsW* gene were obtained in both wild type and *sfr*/*rodA2* disrupted backgrounds.

Three clones of the *ftsW*::Tn*5080* single mutant and three clones of the *ftsW*/*sfr*/*rodA2* triple mutant were selected randomly for Southern blot analysis to confirm the mutants. For Southern blot analysis of both single and triple mutants, the chromosomes of the respective mutant clones and the corresponding cosmids containing Tn*5080* insertion in the respective genes were digested with *Xho*I and *Hin*dIII (Fig. 4.2A). Digoxigenin labelled Tn*5062* was used as a hybridization probe. The analysis of both single and triple mutants showed the expected sizes of fragments that corresponded with the sizes of the fragments obtained by *Xho*I and *Hin*dIII digestion of the cosmids with the relevant insertion used for the mutagenesis (Fig. 4.2B)

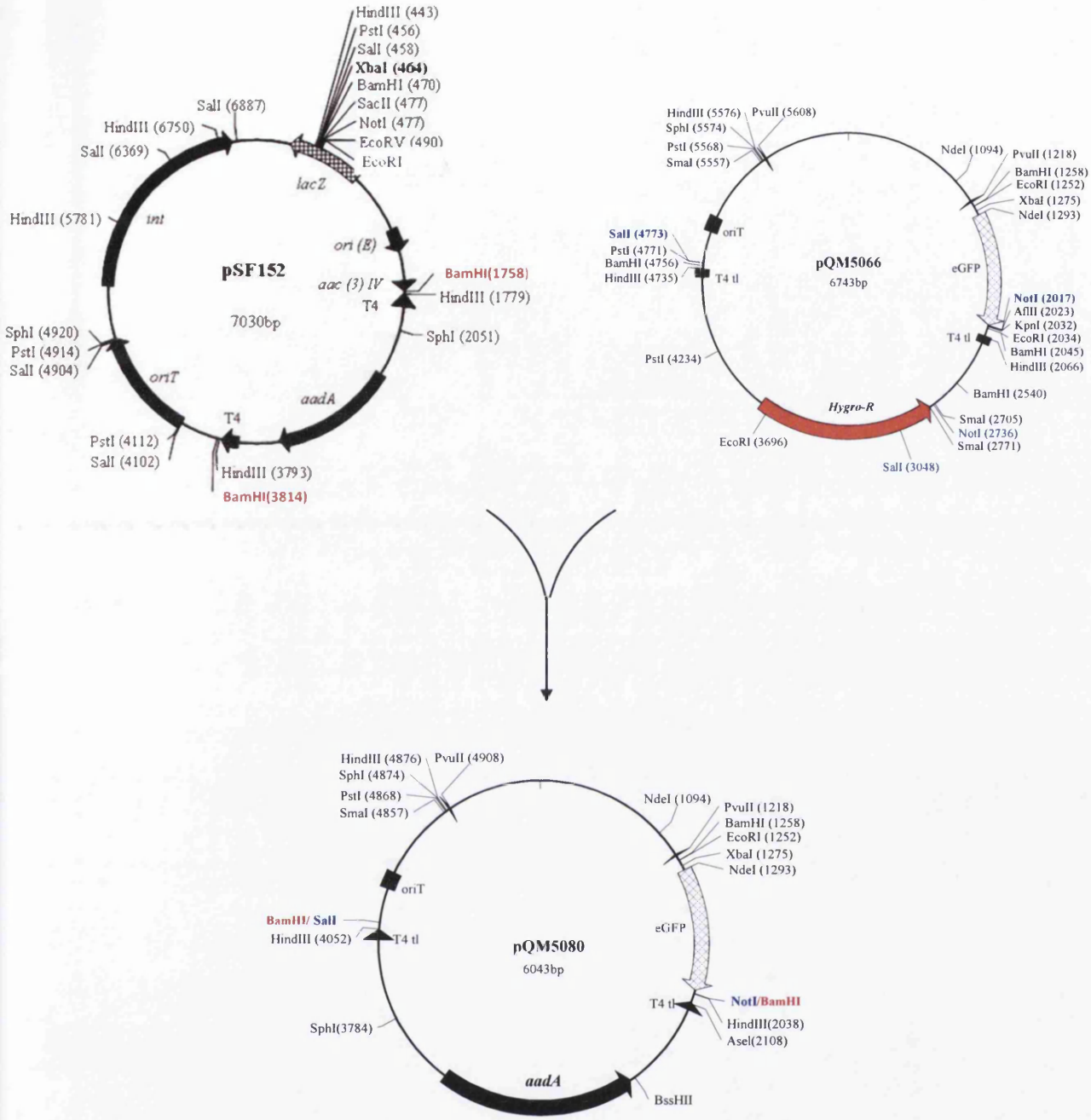

Figure 4.1: Map of the plasmids showing the construction of pQM5080 containing spectinomycin/streptomycin resistance gene, aadA. Plasmid pSF152 was digested with *Bam*HI (red) to obtain a fragment with the spectinomycin/streptomycin resistance gene and blunt ended, and then the fragment was sub-cloned into pQM5066 digested with *Not*I/*Sal*I enzymes and blunt ended.

(A)

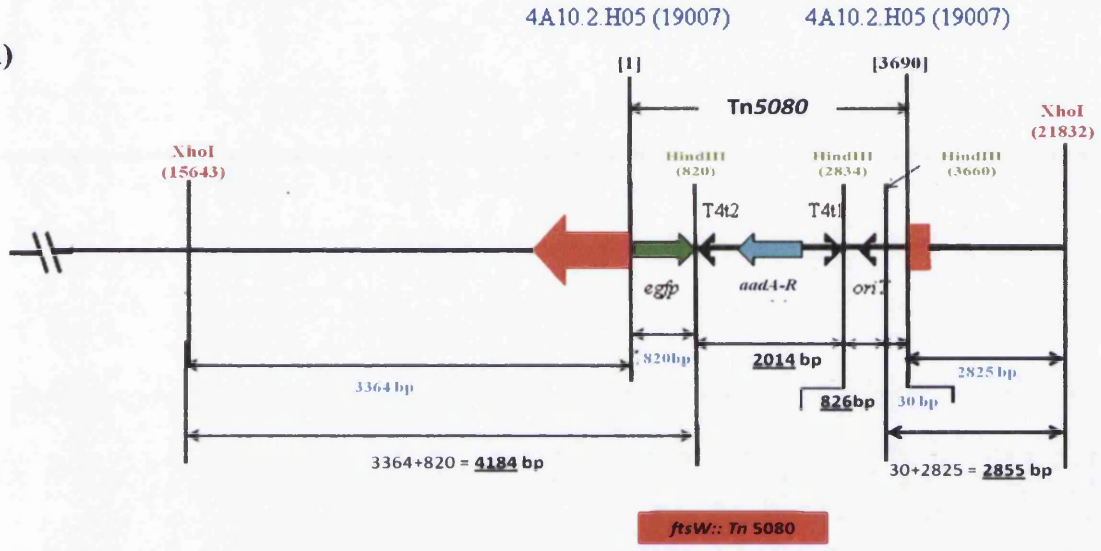

Figure 4.3: Southern blot analysis showing *ftsW* single mutant and *ftsW/sfr/rodA2* triple mutants clones. **(A)** Diagrammatic representation of *ftsW* gene region showing Tn5080 insertion and *Xho*I/*Hind*III restriction sites to calculate the size of bands that should be obtained after Southern hybridization analysis. The cosmid insertion used for *ftsW* mutagenesis is shown in blue colour with the position of insertion in the cosmid presented in brackets. Just under the cosmid name and position, the start and end sites of Tn5080 are written in square brackets. The position of important *Xho*I restriction sites in the *ftsW* region of the cosmid are shown in red colour and *Hind*III in Tn5080 region is shown in green colour. Arrows with different colours represent different genes and their directions. The name of each respective gene is shown just under each arrow. *aadA* – spectinomycin/streptomycin resistance; T4 t1,2 – Transcription terminator t1 & 2; *egfp* – Enhanced green fluorescent protein gene; *oriT* – Origin of transfer. Expected sizes of bands that should be obtained in the Southern blot are shown in bold face with underlining.

(B)

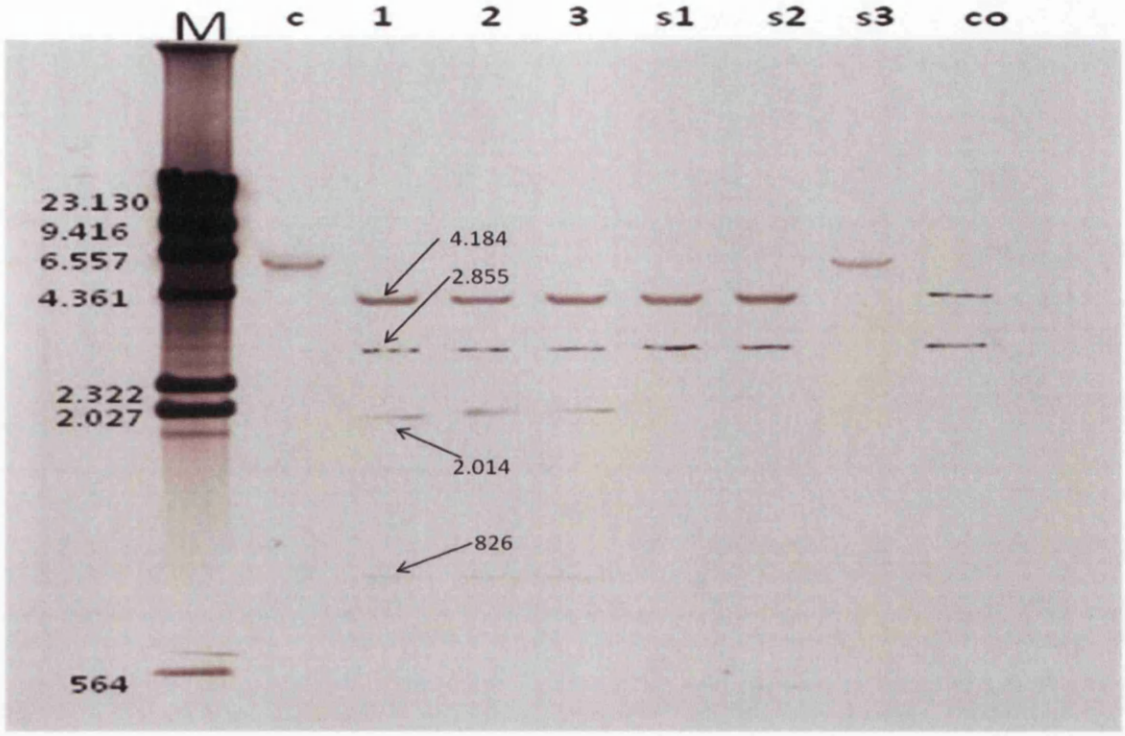

Figure 4.3: (B) Southern blot of three *ftsW/sfr/rodA2* triple mutants clones (1, 2 & 3); M –*Hind*III digested λ DNA marker with the size for each band presented on the left-hand side; C – M145 wild type strain; s1, s2& s3 *ftsW* single mutant clone; co-*ftsW*⁻ cosmid [Tn5080]. The size (in bp) of expected bands is shown on the diagram.

4.3 Phenotypic analysis of *ftsW/sfr/rodA2* triple mutant

For phenotypic characterization, *S. coelicolor* M145 and the triple and double mutant derivatives with insertions in *ftsW/sfr/rodA2* and *sfr/rodA2* genes were plated on some commonly used media. All the mutant strains were visually assessed for the production of grey pigmented spores by plating on soya flour mannitol (SFM). All the strains were incubated at 30° C for three days. As expected, the *ftsW/sfr/rodA2* triple mutant exhibited a white non-sporulating phenotype and has the same macroscopic phenotype as to that of the *ftsW* single mutant strain. On the other hand, the *sfr/rodA2* double mutant strain produced normal grey pigmented spores similar to the wild type on the media tested (Fig.4.3). This result confirms that FtsW is required for formation of sporulation septa.

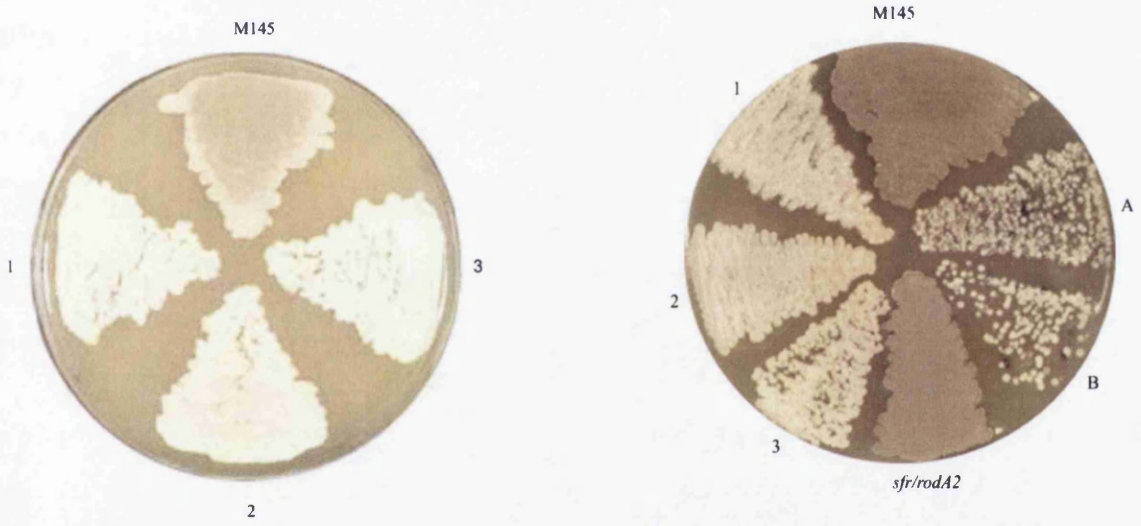

Figure 4.4: Phenotype of *ftsW/sfr/rodA2* triple mutant, *sfr/rodA2* double mutant and their congenic parental strain *S. coelicolor* M145 on SFM media. Strains were grown for 3 days at 30° C on SFM. Numbers surrounding the plates picture represent clones of the triple mutant and (A&B) represent clones of the *ftsW*⁻ single mutant.

4.4 Microscopic Analysis of *ftsW/sfr/rodA2* triple mutant

Both FL vancomycin and Fluo-WGA stain the cell wall by binding to peptidoglycan precursors that are produced as a result of active peptidoglycan synthesis and breakdown. Chromosomal DNA was stained with propidium iodide (PI), which requires fixing the cells and therefore it was only used with Fluo-WGA staining. Active peptidoglycan synthesis during vegetative septation was visualized by using BODIPY FL vancomycin, a fluorescent labelled vancomycin, as described in Materials and Methods.

Fluorescence microscopy was used to visualize the cytological effects of the *ftsW/sfr/rodA2* triple mutation on peptidoglycan synthesis and chromosome distribution and compared with the *S. coelicolor* wild-type, single and double mutants of these genes. To visualize vegetative septa in wild type and the above mentioned mutants, the cultures were prepared by growing each strain on SFM medium with an inserted acute-angled cover slip for 48 h, followed by staining with FL vancomycin. The cultures were then observed under the fluorescence microscope to observe the pattern of fluorescence in the vegetative mycelium. As shown in the Figure. the vegetative mycelium of *S. coelicolor* M145 showed infrequent brightly stained septa. Fluorescence microscopy of the vegetative mycelium of the *ftsW/sfr/rodA2* triple mutant strain also showed similar staining pattern of vegetative septa to the wild type strain, suggesting that these genes are not required for vegetative septation in *S. coelicolor* (Fig. 4.4).

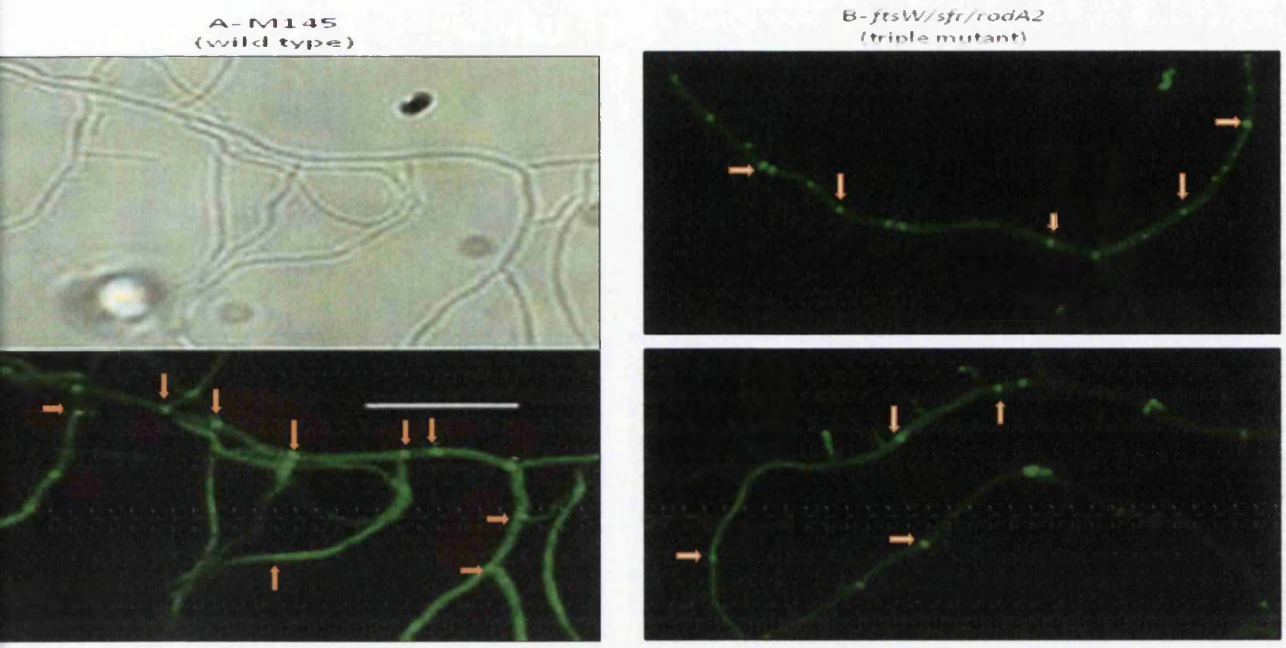

Figure 4.5: Fluorescence microscopy of vegetative mycelium of parental strain *S. coelicolor*, M145 and *ftsW/sfr/rodA2* triple mutant using FL-vancomycin to visualize vegetative septa indicated by arrows. Samples were prepared from cultures grown on SFM agar with inserted coverslips for 48 hours at 30° C. Phase contrast and fluorescence microscopy of wild type M145 (A); *ftsW/sfr/rodA2* triple mutant (B).

Cell wall synthesis during sporulation was examined by staining the aerial hyphae with fluorescein labelled wheat germ agglutinin (Fluo-WGA). Samples for fluorescence microscopy of aerial hyphae were prepared by taking impressions of M145 wild type, *ftsW/sfr/rodA2* triple mutant and the *ftsW* single mutant grown on the surface of SFM agar medium for 38 h, at 30° C. As described in the previous chapter, regularly spaced sporulation septa were observed in aerial hyphae of the wild type as a ladder-like pattern of green Fluo-WGA fluorescence that define pre-spore compartments each containing a single condensed nucleoid stained with red fluorescence dye (Fig. 4.5). In contrast, the aerial hyphae of the triple mutant that showed a typical nonsporulating white phenotype remained unseptated like an elongated continuous tube with no apparent chromosome condensation, even after prolonged incubation (Fig. 4.5).

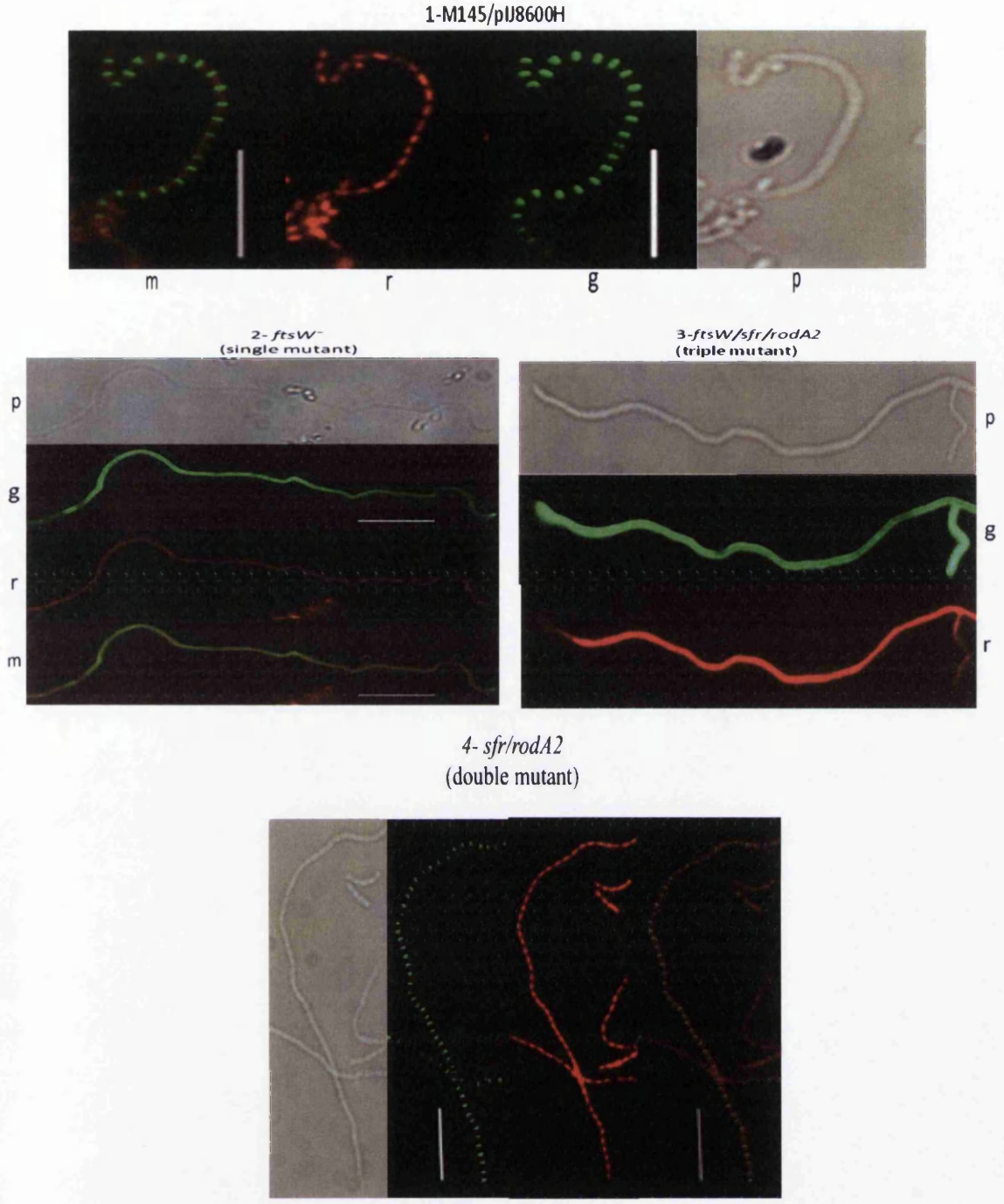

Figure 4.6: Fluorescence microscopy of aerial hyphae of parental strain *S. coelicolor* M145, *ftsW*⁻ single mutant, *ftsW/sfr/rodA2* triple mutant and *sfr/rodA2* double mutant using Fluo-WAG (for cell wall) and PI (chromosomal DNA) staining to visualize sporulation septa and chromosome distribution. Samples were prepared by taking the impression of each culture grown on the surface of SFM agar medium for 38 to 40 hours at 30°C. Panel 1- *S. coelicolor* M145 at sporulation stage; Panel 2- *ftsW*⁻ single mutant; Panel 3- *ftsW/sfr/rodA2* triple mutant; and Panel 4- *sfr/rodA2* double mutant. Each panel shows phase contrast (p), Fluo-WGA staining (g), PI staining (r) and merged (m) images of Fluo-WGA and PI staining of aerial hyphae of respective strains.

4.5 Summary

Construction of disruption mutants of *ftsW*, *sfr* and *rodA2* SEDS genes in one strain of *S. coelicolor* revealed that these genes are dispensable for growth and survival. Comparison of the macroscopic phenotypes showed no difference between the *ftsW/sfr/rodA2* triple mutant strain that exhibited a white non-sporulating phenotype, and the *ftsW*$^-$ single mutant phenotype. In addition, Fluo-WGA staining reveals an absence of sporulation septa in both the triple mutant and *ftsW*$^-$ single mutant aerial hyphae, while an *sfr*$^-$/*rodA2*$^-$ double mutant has normal sporulation septa, confirming that FtsW is required specifically for sporulation septation. In contrast and from the results of fluorescence microscopical analysis of vegetative mycelium of the triple mutant strain and the wild type strain by using FL-vancomycin, similar staining pattern of vegetative septa were observed, suggesting that these genes (*ftsW*, *sfr* and *rodA2*) are not required for vegetative septation in *S. coelicolor*.

Chapter 5: Analysis of *S. coelicolor* cell division proteins using a two-hybrid system.

5.1 Introduction

One of the most fascinating fundamental processes in biology and essential for proliferation of all living species is cell division. To understand the mechanism of cell division, it would be very helpful to arrive at an exact model of the divisome. Which proteins physically interact with each other and when do they interact with each other is essential information to be able to quantitatively describe the synthesis of the new cell poles. Two-hybrid systems (e.g. (Di Lallo *et al.*, 2003, Karimova *et al.*, 2005, Maggi *et al.*, 2008, Wang *et al.*, 1997) have served as the primary genetic tool to discover potential biological interaction partners but they are typically prone to a high frequency of false positives complicating the interpretation of interaction data. To investigate the order of recruitment of divisome proteins during sporulation septation, localization studies of these proteins in wild type as well as in different mutant backgrounds and their interaction analysis using a bacterial two hybrid system was performed. In this work, we attempted to characterize the interactions between the *S. coelicolor* proteins involved in the cell division machinery by using a bacterial adenylate cyclase two-hybrid (BACTH) system, which is based on the interaction-mediated reconstruction of a cyclic AMP (cAMP) signaling cascade. The interaction between the hybrid proteins does not need to take place near the transcription machinery as is the case with yeast or other bacterial two-hybrid systems (Di Lallo *et al.*, 2001, Dove and Hochschild, 1998, Fields and Song, 1989, Hu, 1995, Kolmar *et al.*, 1995). For this reason, the BACTH system seems to be particularly appropriate for studying interactions among membrane proteins (Fig. 5.1).

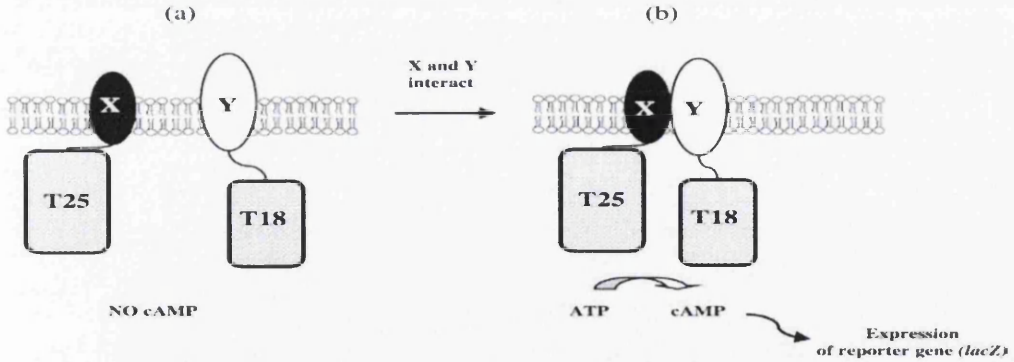

Figure 5.1: Detection of membrane protein associations with the BACTH system. (a) Proteins of interest X and Y are genetically fused to the two complementary fragments, T25 and T18, from the catalytic domain of B. pertussis AC and coexpressed in *E. coli* cya cells. (b) Interaction between the two hybrid proteins results in functional complementation between the T25 and T18 fragments, leading to cAMP synthesis. cAMP, upon binding to the catabolite activator protein (a transcriptional regulator) triggers the expression of *E. coli* catabolic operons, allowing the bacteria to utilize sugars such as lactose and maltose (Karimova *et al.*, 2005).

5.2 Plasmids construction

The **B**acterial **A**denylate **C**yclase **T**wo-**H**ybrid system (BACTH), based on the method of (Karimova et al., 2005), was used to analyse interactions. Genes coding for the different cell division proteins, SEDS proteins and their cognate penicillin binding proteins (PBPs) involved in *S. coelicolor* growth and cell division were analysed by co-transforming the *E. coli cya* strain BTH101 with pairs of recombinant plasmids expressing the T25 and T18 hybrids. To construct the recombinant plasmids used in the BACTH complementation assays, each of these genes was PCR amplified from genomic DNA of *Streptomyces* as a template. Appropriate primer pairs used for PCR amplification contained either *Bam*HI/*Eco*RI restriction sites, *Bgl*II/*Eco*RI restriction sites or *Pst*I/*Eco*RI restriction sites for forward and reverse primers, respectively. Subsequently, amplified DNA fragments were digested with *Bam*HI and *Eco*RI for (*ftsW, ftsZ, ftsQ, sfr, rodA, pbp2* and *pbp3*), with *Bgl*II and *Eco*RI for (*rodA2* and *pbp1*), or with *Pst*I and *Eco*RI for (*ftsI* and *mreB*). The fragments were subcloned in-frame into the corresponding sites of the pKT25 and pUT18C BACTH vectors that have a multicloning site MCS located downstream of the T25 and T18 open reading frame respectively. Therefore, the resulting recombinant plasmids expressed hybrid proteins in which the polypeptides of interest (X and Y) were fused to the C-terminal end of the T25 and T18 fragments of adenylate cyclase (Fig.5.2). DNA sequences of the cloned DNA fragments in all recombinant plasmids were verified by sequencing.

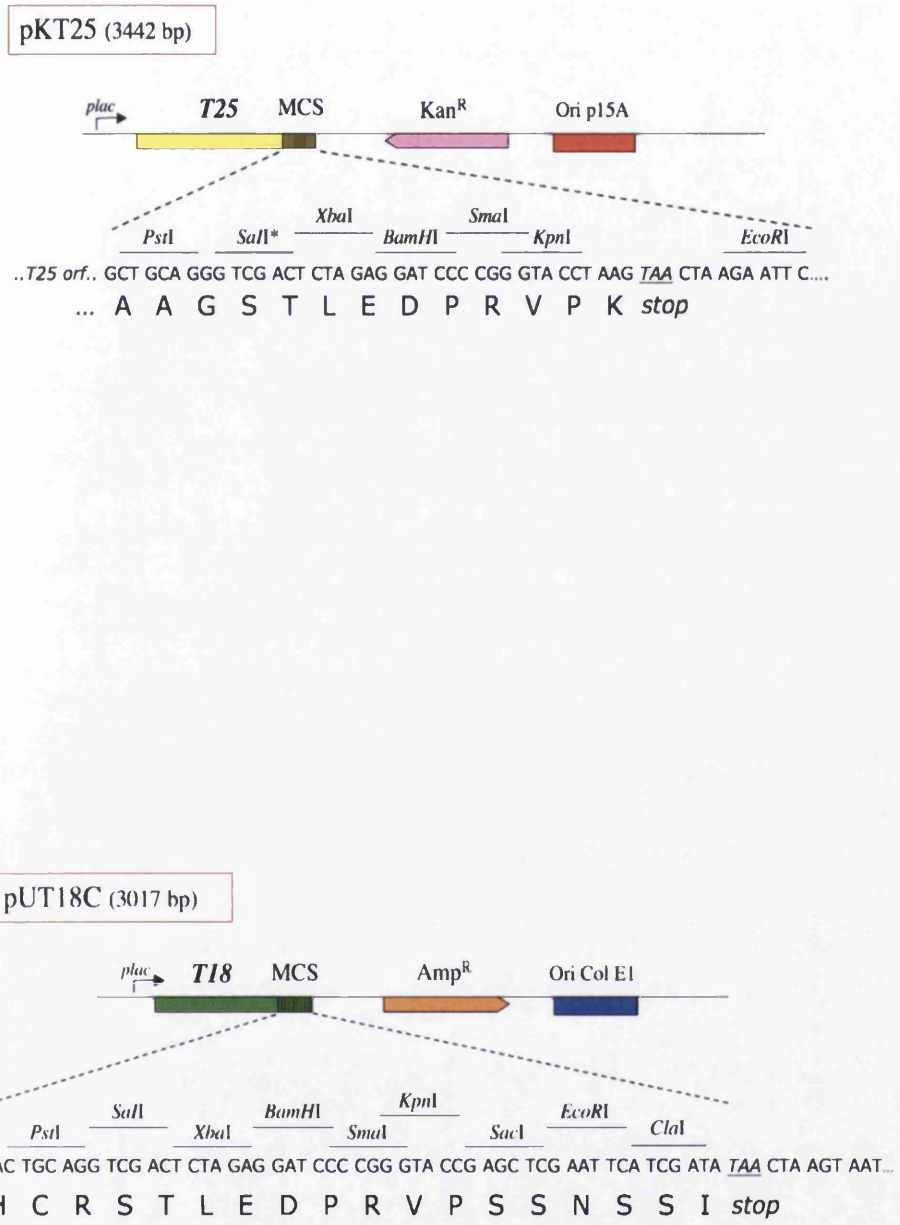

Figure 5.2: BACTH plasmid maps: two different sets of compatible vectors that allow genetic fusions of proteins of interest at the C-termini of the T25 fragment (pKT25) or of the T18 fragment (pUT18C). The multicloning site MCS is located at the 3' end of the T18 or T25 (Karimova et al., 2005).

5.3 BACTH complementation assays

For BACTH complementation assays, recombinant pKT25 and pUT18C carrying *S. coelicolor* cell division genes were used in 110 possible various combinations to co-transform the *E. coli cya* mutant BTH101. The plasmids pKT25-zip/pUT18C-zip and pKT25/pUT18C served as positive and negative controls respectively, for complementation. The transformants were plated onto LB medium containing 40 μg/ml and 0.5 mM of X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside) and IPTG (isopropyl β-D-thiogalactopyranoside) respectively. The plates were incubated at 30°C overnight. The ability of co-transformants to express β-galactosidase, resulting in a blue colour on LB-X-gal plates, is based on a functional adenylate cyclase due to the interaction of the fusion proteins.

In one approach, to confirm the results, at least three independent cultures were performed, by inoculating three colonies from each primary culture and growing in LB broth with appropriate antibiotic, overnight at 37°C in an orbital shaker. 5μl of each culture was spotted on LB agar supplemented with X-Gal and IPTG, with the green-blue colonies indicating a strong interaction (Fig. 5.3).

In a second approach, efficiencies of interactions between different hybrid proteins were quantified by measurement of β-galactosidase activity in liquid cultures, as described in detail in Materials and Methods (Chapter 2). For this measurement at least three independent cultures were prepared by growing three colonies from each primary culture in LB broth containing IPTG and appropriate antibiotics at 30°C. Before the assays, the cultures were diluted 1:5 into M63 medium and the optical density at 600 nm (OD_{600}) was recorded. To permeabilize cells, toluene and a 0.1% sodium dodecyl sulphate solution were added to the bacterial suspension. The tubes were subjected to a vortex and incubated at 37°C for evaporation of toluene. For the enzymatic reaction, aliquots of permeabilized cells were added to buffer PM2 containing β-mercaptoethanol. The tubes were incubated at 28°C in a water bath. The reaction was started by adding 0.4% ONPG in PM2 buffer without β-mercaptoethanol. The reaction was stopped by adding a 1 M Na_2CO_3 solution. The OD_{420} was then recorded. The enzymatic activity, A (in units per milliliter), was calculated according to the following equation: A = 200 x (OD_{420} of the culture x OD_{420} in the control tube)/minutes of incubation) x dilution factor.

Figure 5.3 (A)

	1	2	3	4	5	6	7	8	9	10	11	12	13
T18C	FtsW	FtsZ	Sfi	RodA	RodA2	PBP1	PBP2	PBP3	FtsI	FtsQ	CrgA	CrgAw	CrgAwn
	A	B	C	D	E	F	G	H	I	J	K	L	
T25	FtsQ	FtsI	MreB	RodA2	PBP1	PBP2	FtsZ	Sfi	FtsW	CrgA	CrgAn	CrgAnw	

A

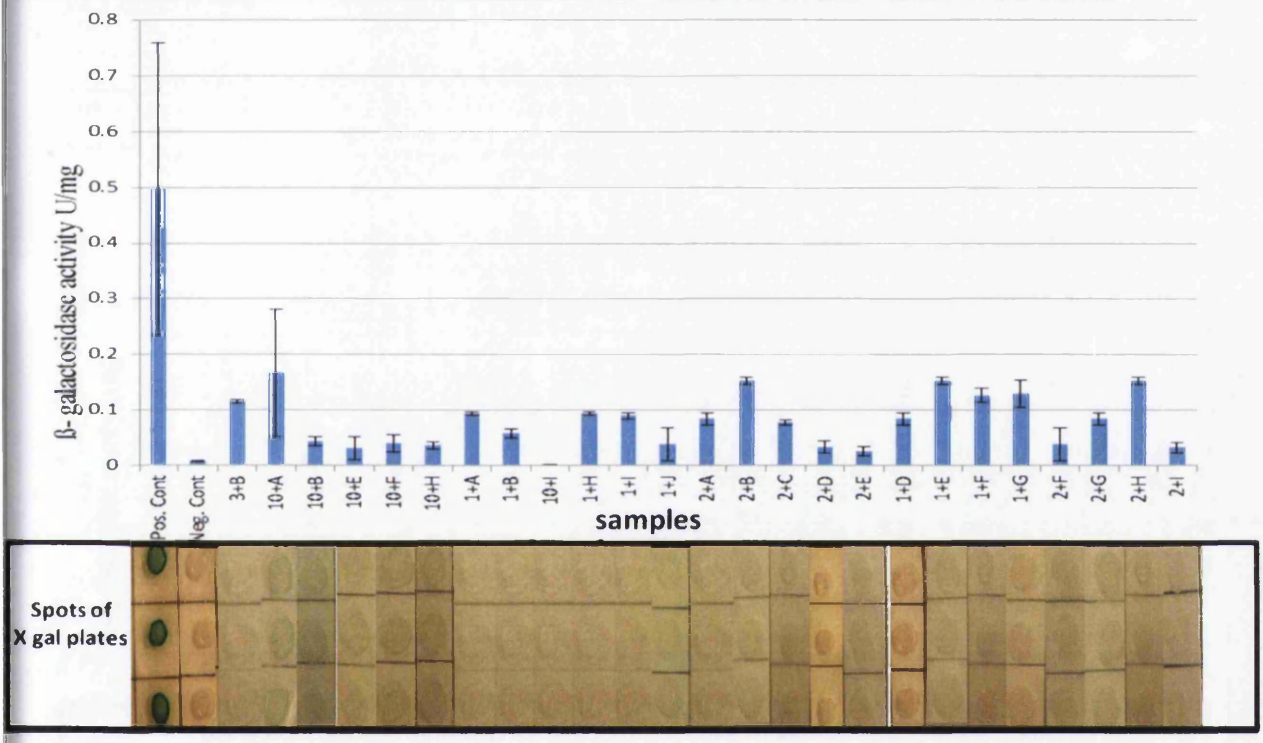

Figure 5. 3: Continued on the next page……..

Figure 5.3 (B)

	1	2	3	4	5	6	7	8	9	10	11	12	13
T18C	FtsW	FtsZ	Sfi	RodA	RodA2	PBP1	PBP2	PBP3	FtsI	FtsQ	CrgA	CrgAw	CrgAwn
	A	B	C	D	E	F	G	H	I	J	K	L	
T25	FtsQ	FtsI	MreB	RodA2	PBP1	PBP2	FtsZ	Sfi	FtsW	CrgA	CrgAn	CrgAnw	

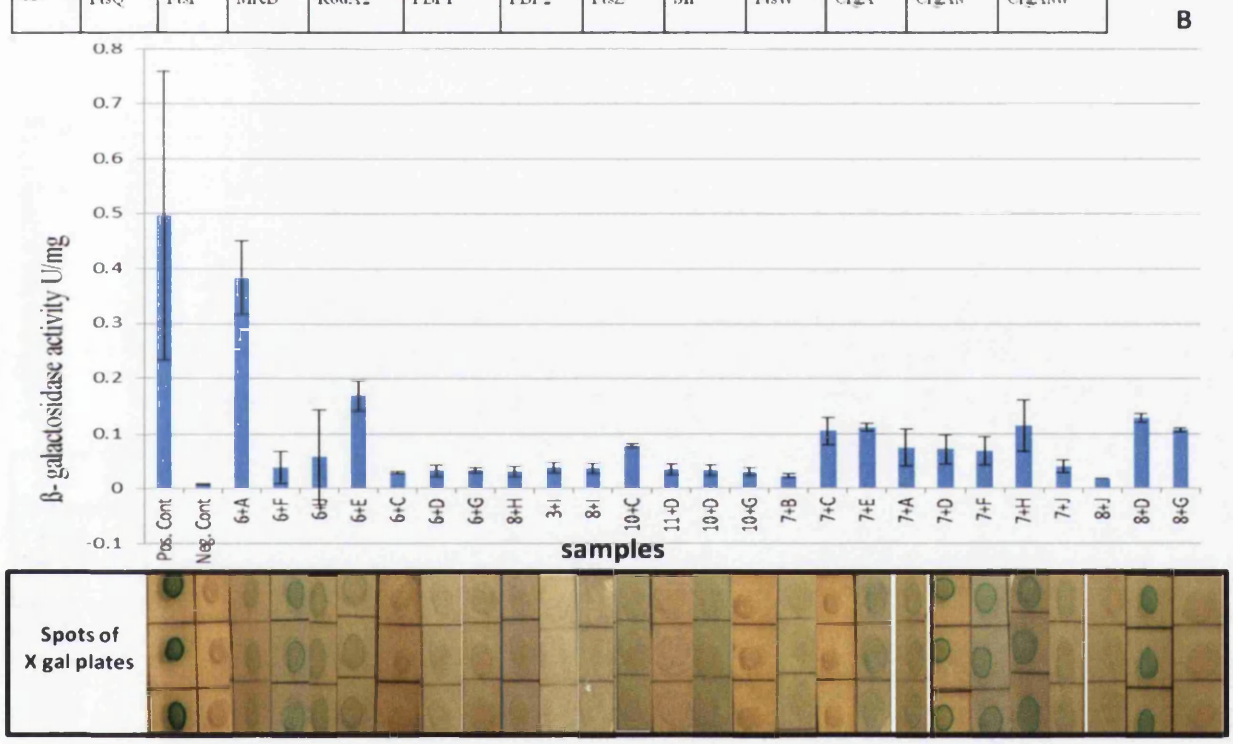

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Figure 5.3 (C)

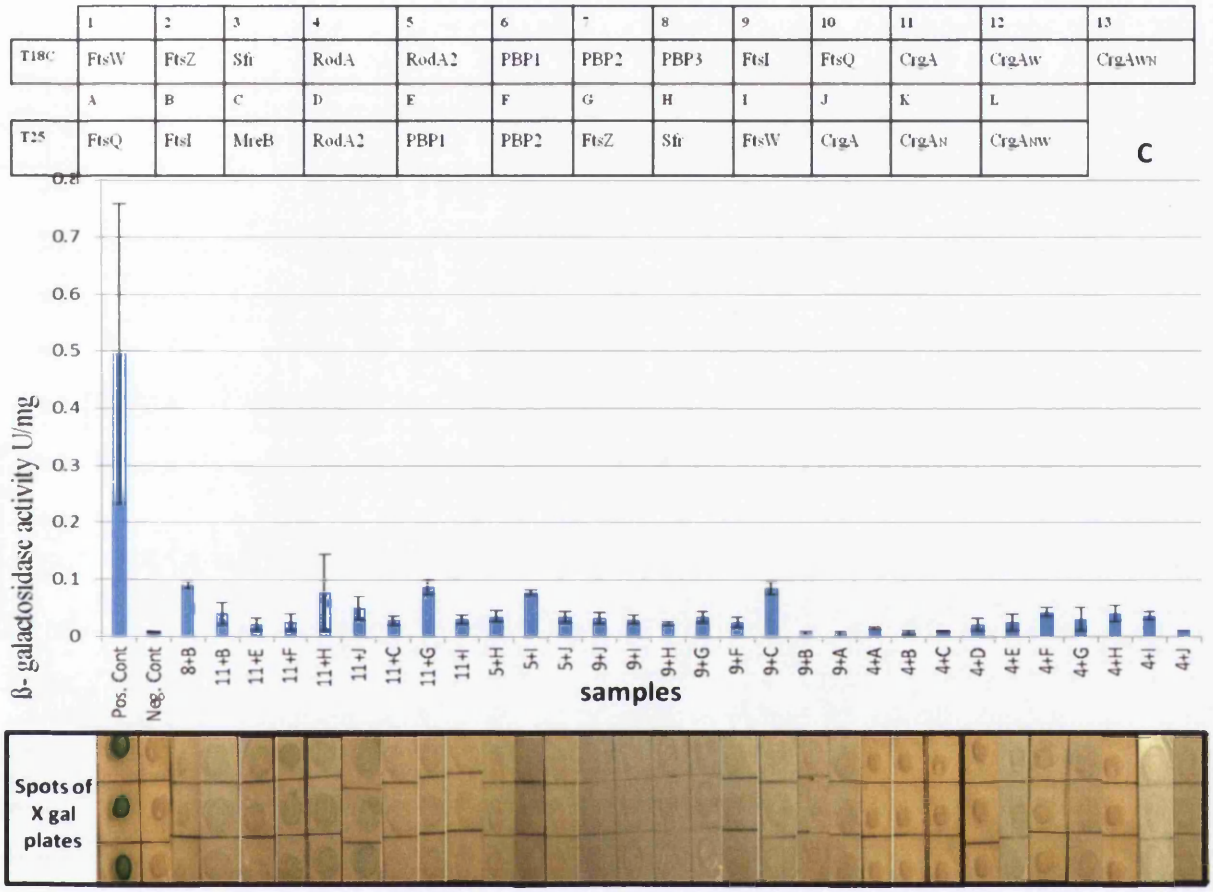

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Figure 5.3 (D)

	1	2	3	4	5	6	7	8	9	10	11	12	13
T18C	FtsW	FtsZ	Sfi	RodA	RodA2	PBP1	PBP2	PBP3	FtsI	FtsQ	CrgA	CrgAw	CrgAwn
	A	B	C	D	E	F	G	H	I	J	K	L	
T25	FtsQ	FtsI	MreB	RodA2	PBP1	PBP2	FtsZ	Sfi	FtsW	CrgA	CrgAn	CrgAnw	

D

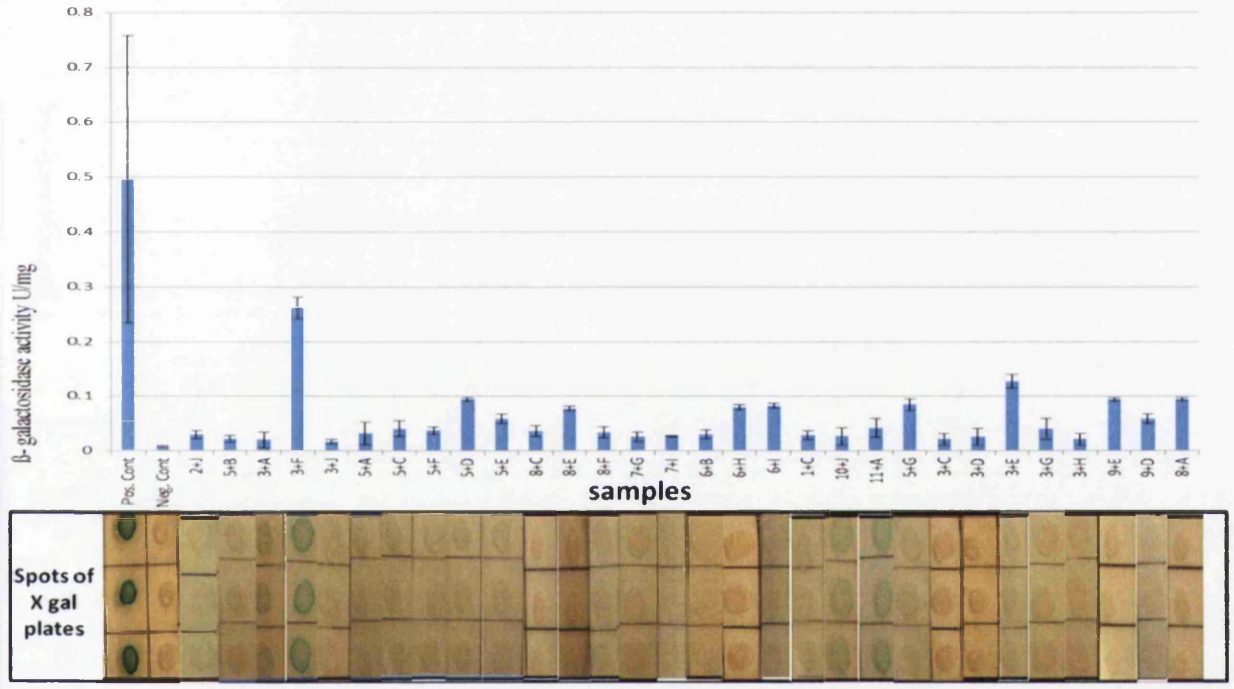

Figure 5. 3: Continued on the next page……..

Figure 5.3 (E)

	1	2	3	4	5	6	7	8	9	10	11	12	13
T18C	FtsW	FtsZ	Sfi	RodA	RodA2	PBP1	PBP2	PBP3	FtsI	FtsQ	CrgA	CrgAw	CrgAwn
	A	B	C	D	E	F	G	H	I	J	K	L	
T25	FtsQ	FtsI	MreB	RodA2	PBP1	PBP2	FtsZ	Sfi	FtsW	CrgA	CrgAn	CrgAnw	

E

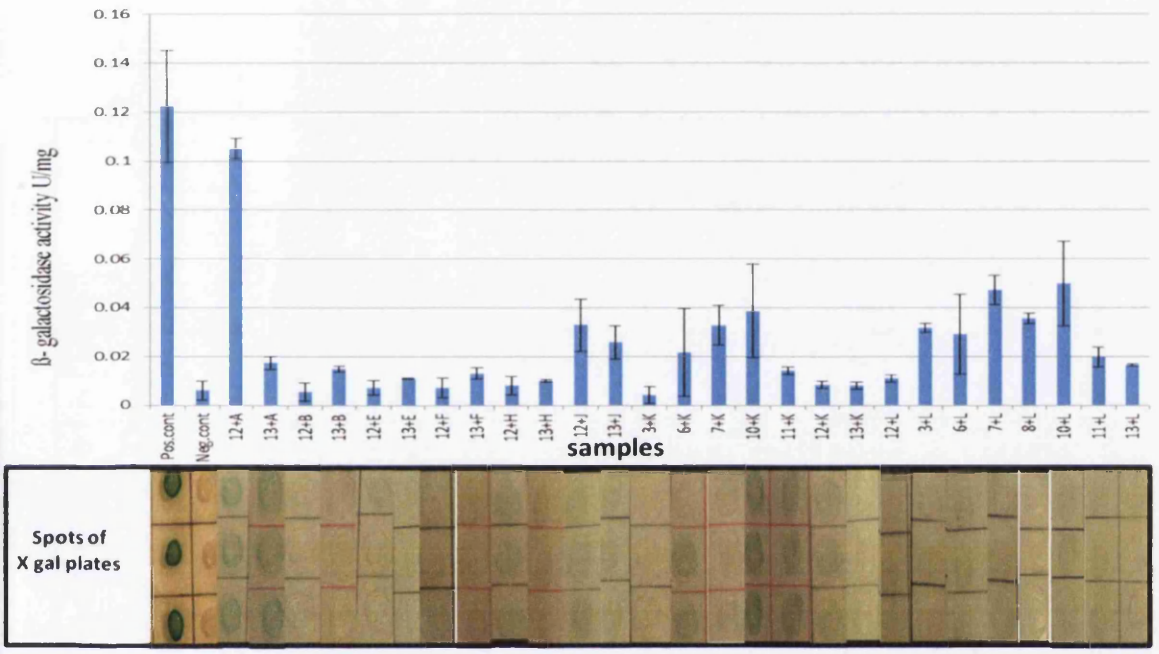

Figure 5.3: Graphs of two-hybrid analysis of interactions of *S. coelicolor* cell division proteins (A, B, C, D and E). The occurrence of interaction between the tested proteins coproduced in *E. coli* BTH101 cells of the BACTH system was ascertained by the production of the ß-galactosidase reporter enzyme. Each bar represents the mean value of three measurements performed on the cellular extracts of three reporter clones originating from three independent transformations, and the error bars represent the standard deviation. The plasmids pKT25-zip/pUT18C-zip and pKT25/pUT18C served as positive and negative controls respectively. Identical inocula 5µl were plated (spotted) on LB agar supplemented with X-Gal and IPTG. Growth of the cotransformed *E. coli* BTH101 on LB medium containing X-Gal and IPTG provided evidence for the occurrence of protein-protein interactions. Green-blue colonies indicate the interaction (figure 5.3: A, B, C, D and E).

Table 5.1: Bacterial two-hybrid interaction assays of *S. coelicolor* cell division proteins. *E. coli* BTH101 was co-transformed with plasmids encoding the indicated T25 and T18 cya fusions and the interaction activity of the co-transformants was determined by growth on LB agar supplemented with X-Gal, IPTG and appropriate antibiotic. (+) indicate the results of at least three independent transformants

		pUT18C												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		FtsW	FtsZ	Sfr	RodA	RodA2	PBP1	PBP2	PBP3	FtsI	FtsQ	CrgA	CrgAw	CrgAwN
pKT25 A	FtsQ	-	-	++	-	-	++	++	-	-	++	++	++	++
B	FtsI	-	-	-	-	-	-	-	-	-	+	+	-	-
C	MreB	-	-	-	-	-	-	-	-	-	-	-		
D	RodA2	-	-	-	-	-	-	+	+	-	-	-		
E	PBP1	-	-	-	-	-	++	+	-	-	+	+	+	+
F	PBP2	-	-	++	-	-	++	++	-	-	+	+	+	-
G	FtsZ	-	-	-	-	-	-	-	-	-	-	-		
H	Sfr	-	-	+	-	-	-	++	-	-	+	+	++	+
I	FtsW	-	-	-	-	-	-	-	-	-	-	-		
J	CrgA	-	-	+	-	-	++	++	+	-	++	++	++	+
K	CrgAN			-			++	+			++	++	+	-
L	CrgANW			-			+	-			+	+	-	-

5.4 Significant results of cell division proteins BACTH analysis

Physical interactions between cell division proteins were investigated. Very significant interactions were found for CrgA and FtsQ, suggesting a central role of these proteins in the cell division process. In addition, these proteins display very similar interaction patterns with other cell division proteins (Table 5.1). The results showed that CrgA and FtsQ interact together, and with themselves, and with penicillin binding proteins PBP1, PBP2, FtsI and Sfr. Furthermore, FtsQ strongly interacted with all mutated CrgA proteins (CrgAN, CrgAw and CrgAwN, see below). Under the same conditions, CrgA and FtsQ did not interact with FtsW, FtsZ, RodA2 and MreB (Table 5.1).

Importantly, the BACTH analysis also showed that, penicillin binding proteins PBP1 and PBP2 were also found to interact together, with themselves and with several of the cell division proteins, in particular FtsQ, CrgA, mutated CrgAN and CrgAw. In addition, PBP2 interacted with RodA2 and Sfr (Table 5.1). Unexpected results were obtained with some important cell division proteins like FtsW, FtsI and FtsZ, as no interactions of these proteins were recorded with any of the other cell division proteins used (Table 5.1). With the exception of the interaction detected between RodA2 expressed from pKT25 and PBP2 expressed from pUT18C, all positive interactions were confirmed when the respective genes were switched between the two plasmids.

5.5 CrgA amino acids conserved in all Actinobacteria

In this section, as a consequence of the significant two-hybrid data obtained, more investigations into *S. coelicolor* CrgA were performed. In previous studies, from analysis of CrgA sequences of various *Actinomycetes*, using topology prediction and alignment software, the most highly conserved regions of the protein were determined to be two transmembrane regions. In addition, this alignment revealed a low level of sequence conservation, with only nine residues with conserved identity (Del Sol *et al* 2006). To complement this, I aligned the *S. coelicolor* CrgA sequence with additional CrgA sequences from a variety of *Actinobacteria*, revealing only four amino acids well conserved, G_{40}, W_{45}, N_{65} and W_{83} (Fig. 5.4). The positions of two amino acids G_{40} and W_{45} were in the first transmembrane domain (TM_1). The third conserved amino acid (Asparagine N_{65}) is located in TM_2 and Tryptophan W_{83} is in the carboxyl-terminal sequence of the protein. The amino acid N_{65} is very interesting because it is a hydrophilic amino acid, which is

very unusual in transmembrane domains (TM_2), which normally containing hydrophobic amino acids; therefore it likely to play a substantial role for the function of the CrgA protein (Fig. 5.5).

Figure 5.4: Multiple alignment of CrgA orthologue sequences from all Actinobacteria. Alignments were generated using ClustalW2 program. Conserved Asparagine (N_{65}) and Tryptophan (W_{83}) residues that are required for interaction of *S. coelicolor* CrgA with itself are shown in red colour and red arrow underneath in all Actinobacteria sequences. Underneath the alignment, "*" means that residues are identical in the aligned sequences, ":" means that conserved substitution have been observed, and "." means that semi-conserved substitution are occurred.

5.6 Site-directed Mutagenesis of CrgA protein

Based upon these results, the conserved amino acids (N_{65}) and (W_{83}) at the carboxyl end of the protein in transmembrane domains (TM_2) (Fig. 5.5), seem likely to be involved in CrgA function and, consequently, could be vital for protein interactions. To investigate the functional relevance of these amino acids, several CrgA mutants were obtained with the QuikChange II XL Site-directed Mutagenesis technique as explained in Materials and Methods (Chapter 2). One or both amino acids N_{65} and W_{83} were replaced in the sequence of the CrgA protein. In ($CrgA_N$) Asparagine N at position 65 was changed to alanine and ($CrgA_W$) Tryptophan W at position 83 changed to alanine, while in ($CrgA_{NW}$) N and W at positions 65 and 83, were substituted by alanine, respectively. Each amino acid exchange resulted in a dramatic decrease or complete loss of interaction (Table5.2).

The results of two hybrid tests using mutated CrgA proteins indicated that the amino acids N_{65} and W_{83} of *S. coelicolor* CrgA are required for interaction of CrgA protein with itself. In more detail, substitution of N_{65} impaired interaction with the protein with substitution of W_{65}, whereas substitution of both amino acids N_{65} and W_{83} led to loss of detectable self interaction between the mutated proteins. The double substitution also impaired interactions of $CrgA_{NW}$ with CrgA, PBP1 and PBP2 (Table 5.2).

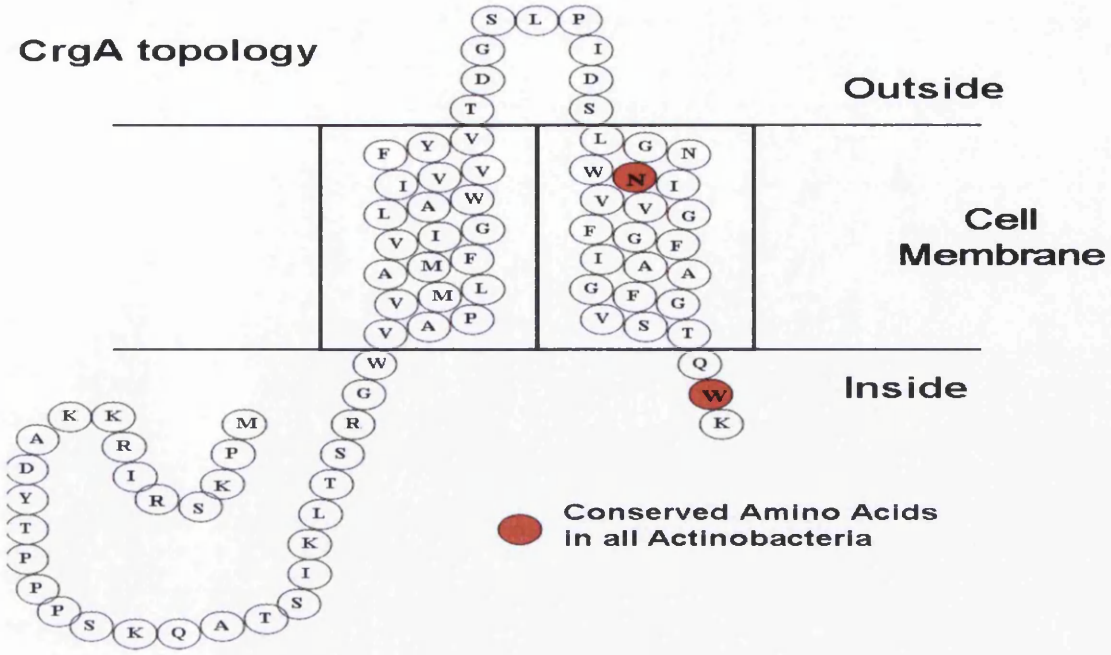

Figure 5.5: The CrgA sequence of various actinomycetes was analyzed using topology prediction and alignment softwares. Amino acids (Asparagine N and Tryptophan W) conserved in all Actinobacteria and used for site directed mutagenesis are shown in red colour.

Table5.2: Results of two hybrid test for CrgA protein modified.

			pUT18C		
			11	12	13
			CrgA	CrgAw	CrgAwn
pKT25	J	CrgA	++	++	+
	K	CrgAn	++	+	-
	L	CrgAnw	+	-	-

5.7 Summary

The ability of each of the *S. coelicolor* division proteins (FtsW, FtsI, FtsZ, FtsQ, CrgA, Sfr, MreB, RodA, RodA2, penicillin binding proteins PBP1, PBP2 and PBP3) to interact with themselves and with each of the remaining proteins was studied in several combinations of protein pairs by the two-hybrid system. Protein–protein interaction assays by the BACTH analysis indicated, in particular, a strong interaction between CrgA and FtsQ, suggesting a central role in the cell division process. The results show another significant point, which was the penicillin binding protein PBP2 interacting with CrgA, and modified $CrgA_N$ or $CrgA_W$ but not with modified $CrgA_{NW}$. Alignment of the *S. coelicolor* CrgA sequence with orthologs from other Actinobacteria, revealed only four amino acids G_{40}, W_{45}, N_{65} and W_{83} were well conserved. Site directed mutagenesis to modify *S. coelicolor* CrgA indicated that the amino acids N_{65} and W_{83} are required for interaction of the protein with itself.

Chapter 6: Discussion and future perspectives

6.1 General statement

In this chapter the role of the cell division proteins in the *S. coelicolor* life cycle will be discussed with regard to the collective results of this thesis and other previous research that consider the cell division process in various bacterial species in order to provide more understanding of the function of the division proteins and introduce some further studies that may improve the understanding the mechanism of the cell division process.

6.2 No effects of *S. coelicolor* FtsW and FtsI overexpression

In *S. coelicolor* the four SEDS genes *SCO2085 (ftsW), SCO2067 (sfr), SCO3846 (rodA)* and *SCO5302 (rodA2)* are located in the central core region of the chromosome. The position of these genes suggests some possibility of their indispensable roles during the life cycle (Mistry *et al.*, 2008). Analysis of these genes revealed that all of these genes are situated near their cognate HMW-PBP genes, and *ftsW* and *rodA* are essential for viability in many bacteria. On the other hand, mutagenesis studies of some genes of the *S. coelicolor dcw* cluster including *ftsZ*, *ftsQ* and *ftsL* have shown that cell division proteins are not essential for viability in *Streptomyces* (Bennett *et al.*, 2007, McCormick and Losick, 1996, McCormick *et al.*, 2006, Mistry *et al.*, 2008).

In *E. coli*, *ftsW* is known as an important component of the *dcw* cluster and is essential for the cell division process. Site-directed mutagenesis experiments combined with fluorescence microscopy studies have revealed that the FtsW protein in *E. coli* is a membrane protein belonging to the SEDS family that is involved in peptidoglycan assembly during cell division. FtsW has 10 trans-membrane segments (TMSs) and it was founded to be recruited later to the division site and is required for subsequent recruitment of the FtsI penicillin binding protein (PBP3) that catalyses cross-linking of newly synthesised peptidoglycan at the division site(Pastoret *et al.*, 2004). Recently, FtsW was also identified as as a transporter of lipid-linked cell wall precursors across the membrane (Mohammadi *et al.*, 2011). *ftsI* is also essential in most bacterial species (Matsuzawa *et al.*, 1989, Tamaki *et al.*, 1980).Two studies indicate that both *ftsI* and *ftsW* are dispensable for growth of the colony, but essential for the sporulation sepatation process (Bennett *et al.*, 2009, Mistry *et al.*, 2008). Both proteins are dispensable for the process of cross-wall formation in substrate hyphae, but FtsW is essential for Z-ring stabilization during

sporulation septation (Mistry *et al.*, 2008). I confirmed the role of FtsW in sporulation septation as a complemented *ftsW* mutant resulted in the grey wild-type phenotype, and microscopy revealed the formation of normal spore compartments in this strain.

Cell division proteins assemble at the ring-like Z-ring structure (Bi and Lutkenhaus, 1991, de Boer *et al.*, 1992, Erickson, 1995). The Z-ring then plays the major role in the division process which is ended by the fomation of the divisome (Erickson *et al.*, 2010, Errington, (2003)). *fts* gene mutations have a variable effect in *S. coelicolor*, ranging from partial formation of sporulation septa in *ftsQ* mutants to a complete absence of septa for many other cell division mutants (McCormick, 2009, Mistry *et al.*, 2008). Although that it was suggested that FtsW may play a role in the stabilization of the Z-ring (Mistry *et al.*, 2008), my data revealed no changes in the frequency or distribution of Z-rings due to over-expression of *ftsW* or *ftsI*. An implication is that placement of Z-rings in the aerial hyphae is due to other factors and consequently the role of FtsW as an early component of the divisome, enabling stabilisation of Z-rings, is secondary. Candidate proteins involved in Z-ring placement are SsgB; that recruits FtsZ to the division site (Willemse *et al.*, 2011) and CrgA, a negative regulator of Z-ring formation (Del Sol *et al.*, 2006).

6.3 FtsW localization and diffuse distribution of FtsW-mCherry in the aerial hyphae

To complement investigations into the effects of FtsW over-expression, I examined if it would be possible to establish where this protein localises in aerial hyphae. If this could be established in the wild-type, we could then examine if FtsW localisation was affected by either SsgB or CrgA (in mutants or over-expression strains). However, although a FtsW-mCherry fusion was functional in restoring the wild type phenotype to an *ftsW* mutant, fluorescence microscopical analysis indicated a diffuse distribution of the red fluorescent protein in the aerial hyphae of the *ftsW* complemented strain. No specific localization was observed. In addition, investigation of the stability of FtsW-mCherry fusion protein using western blot analysis provided evidence for *in vivo* proteolysis of the fusion protein, releasing cytoplasmic mCherry. Consequently, using this approach, it was not possible to address how FtsW is localised in the aerial hyphae.

6.4 Mycobacterial orthologs of FtsW and FtsI cannot replace the function of their related streptomycete proteins

SEDS proteins and their cognate PBPs are known as being essential for peptidoglycan synthesis during cell elongation and division (Den Blaauwen *et al.*, 2008). A study of the four SEDS proteins in *S. coelicolor* revealed that not all the four SEDS are essential for the growth and the viability of this bacterium. Indeed, only RodA is essential, whereas FtsW is exclusively involved in sporulation septation (Mistry *et al.*, 2008) and Sfr is involved in spore maturation (Kleinschnitz *et al.*, 2011). A function of RodA2 has yet to be determined.

Studying the characteristics of the *ftsW* and *ftsI* mutants suggested formation of a complex between FtsW and FtsI as a membrane anchor is necessary to stabilise Z-rings during early stages of sporulation septation (Mistry, 2008). In the *E. coli* paradigm, it is generally believed that FtsW and FtsI are employed later at the divisome in quasi-linear assembly sequence that involves stabilisation of the Z-ring with FtsA and the membrane anchor ZipA, and then employing the FtsK and a trimeric complex that contains FtsQ, FtsL, and FtsB (Buddelmeijer and Beckwith, 2002, Buddelmeijer and Beckwith, 2004, Goehring and Beckwith, 2005, Goehring *et al.*, 2006, Goehring *et al.*, 2005). An interaction between both FtsI and FtsW with FtsQ was supported by a systematic study of protein-proetin interactions using a bacterial two hybrid system (Di Lallo *et al.*, 2003). Several other genetic and biochemical studies support this interaction model.

Just as in *Streptomyces*, in *M. tuberculosis* there are no counterparts of the two membrane anchor proteins ZipA and FtsA. So this raises the possibility that FtsW could be the binding partner for FtsZ in *M. tuberculosis*, making it a likely candidate for linking septum formation to peptidoglycan biosynthesis (Datta *et al.*, 2002). This was the basis for me to introduce and test function of *M. tuberculosis ftsW* and *ftsI* into *ftsW* and *ftsI* mutants of *S. coelicolor* respectively. The results indicated no restoration of the non-sporulating white phenotype of the mutants. This means the introduction of recombinant plasmids containing *ftsW* and *ftsI* genes of *M. tuberculosis* in *ftsW* and *ftsI* mutant strains of *S. coelicolor* fails to complement the mutants. Mycobacterial orthologs of FtsW and FtsI cannot replace the function of their related streptomycete proteins. This indicates that there may be little conservation in the structure and function of key divisome proteins among different actinobacteria.

6.5 *ftsW* is required specifically for sporulation septation in *S. coelicolor*

It was already known that SEDS proteins and their cognate Penicillin Binding Proteins are critical for peptidoglycan synthesis during bacterial growth and cell division (Den Blaauwen *et al.*, 2008). When the *Tn5062* insertion mutants of *ftsW, sfr* and *rodA2* genes were constructed, it showed that these genes in *S. coelicolor* are not considered as essential for growth and viability. It was also shown that *sfr, rodA2, pbp2* and *rodA2/sfr* double mutants have no change in their growth and cell division characteristics compared to the wild-type strain of *S. coelicolor*. On the other hand, *ftsW* mutants along with mutants of the cognate PBP gene, *ftsI*, formed a white phenotype when grown on sporulation specific media similar to the developmental mutants like *whiG, whiI* etc. Because the mutants of these genes are defective in differentiation of aerial hyphae, this indicates that the products of these genes play an important role in sporulation septation, and that the mechanism of cross-wall formation in the substrate hyphae is quite different (Mistry, 2008). More clear evidence was obtained in this study, by disruption of all three *ftsW, sfr* and *rodA2* SEDS genes in one strain. The triple mutant has the white phenotype similar to that of a single *ftsW* mutant of *S. coelicolor*. However, FL-vancomycin staining of vegetative mycelium showed normal cross-wall formation in the vegetative hyphae. No sporulation septa and chromosome condensation were observed in the aerial hyphae of the mutant stained with Fluo-WGA/PI. These observations confirmed that the *ftsW* gene is required specifically for sporulation septation and *ftsW, sfr* and *rodA2* genes are not required for vegetative septation in *S. coelicolor* and there is no redundancy in function of these SEDS proteins in single mutants in terms of vegatative cross-wall formation.

6.6 Significant interactions of *S. coelicolor* cell division proteins

Since cell division in bacterial species leads to the formation of new daughter cells, it is important to know how the Z-ring forms and how the divisome subsequently assembles. Several studies have indicated how different protein networks are formed in the process of cell division (Di Lallo *et al.*, 2003, Goehring and Beckwith, 2005, Karimova *et al.*, 2005, Maggi *et al.*, 2008, Vicente and Rico, 2006). With different analysis techniques, the combined sum of different proteins involved in the divisome was found to be around 15; some of these studies used biochemical methods (Bertsche *et al.*, 2006, Low *et al.*, 2004, Mohammadi *et al.*, 2009, Müller *et al.*, 2007), while others used different versions of technique I adopted, based on two hybrid

systems to investigate protein-protein interactions (Di Lallo *et al.*, 2003, Karimova *et al.*, 2005, Maggi *et al.*, 2008, Wang *et al.*, 1997). In *E. coli*, the divisome assembly was shown to occur in two steps (early and late localizing proteins). Localization studies of these proteins helps in finding some of the possible interactions between these proteins. For example, FtsN was one of the recent proteins that have been discovered to interact directly with others such as the FtsI, FtsW, and ZapA, as well as interacting with itself (Alexeeva *et al.*, 2010). Other research using two hybrid systems has indicated different possible interactions of the FtsN protein with FtsQ, FtsW, FtsI, FtsA and the ability to form homodimers (Di Lallo *et al.*, 2003, Karimova *et al.*, 2005). The interactions between the FtsN and FtsI were supported by other genetic and biochemical studies (Müller *et al.*, 2007, Wissel and Weiss, 2004). The interactions between FtsN and FtsQ were also proved to be positive using two hybrid systems despite the fact that pre-targeted FtsQ failed to target FtsN to the division site (Di Lallo *et al.*, 2003, Goehring *et al.*, 2005, Karimova *et al.*, 2005).

In this work, based on the interaction mediated reconstruction of a cyclic AMP (cAMP) signaling cascade, which does not need to be happen near the transcription machinery, we attempted to characterize the interactions between the *S. coelicolor* proteins involved in the cell division machinery by using a bacterial adenylate cyclase two-hybrid (BACTH) system. Although I obtained unexpected results indicating that some important cell division proteins like FtsW, FtsI and FtsZ, showed no interactions with any of other cell division proteins tested (FtsW, FtsI, FtsZ, FtsQ, CrgA, Sfr, MreB, RodA, RodA2, penicillin binding proteins PBP1, PBP2 and PBP3), another study used the BACTH assay for the *E. coli* FtsZ protein revealed a similar result as we have for *S. coelicolor* FtsZ, in that this protein did not interact with any tested Fts protein except FtsA (Karimova *et al.*, 2005). However, although my data showed no interactions between FtsQ and FtsI proteins, the study of (Karimova *et al.*, 2005) confirmed clearly the interactions between *E. coli* FtsQ and FtsI. In addition, another study used the BACTH analysis system with different lengths of *E.coli* FtsI (short and full length) (Mercer and Weiss, 2002), indicating strong interactions between FtsI and FtsW, in contrast to the results I obtained with *S. coelicolor* FtsI and FtsW. These dissimilarities can be due to differences in the way the genetic constructs were engineered as well as the different sources and consequently amino acid sequences of the cell division proteins. Furthermore there is the possibility that proteins are unable to fold and exist stably in cells retaining their activity as fusion proteins. On

the other hand, in a few cases results (Fig. 5.3 A to E) showed no correlation between assay approachs (β-galactosidase activity and LB X-gal plates) and this could be related to the relative sensitivity of methods.

CrgA has been shown to be a novel member of the cell division complex in mycobacteria and has the possibility to facilitate septum formation (Plocinski *et al.*, 2011). I have shown that CrgA interacts with itself and with FtsQ, and with penicillin binding proteins PBP1, PBP2, FtsI and the SEDS protein Sfr. This is in agreement with results of (Plocinski *et al.*, 2011), that revealed that mycobacterial CrgA interacts with FtsI, Penicillin-binding protein PBPA and FtsQ. However, the latter study also provided evidence of interactions between the CrgA and FtsZ, for which I observed no evidence in the case of the streptomycete orthologs. Taken together, this study and my data both show no interactions between CrgA and RodA. The reason for detecting no interactions between the CrgA and FtsZ could be related to results obtained in the study of (Del Sol *et al.*, 2006) which indicated that CrgA itself does not directly interact with cytoplasmic FtsZ.

More investigations about *S. coelicolor* CrgA were performed in order to analyse the sequence of this protein: the most highly conserved regions of this actinobacterial protein are two transmembrane regions. In addition, the alignment revealed a low level of sequence conservation, with only nine residues with conserved identity (Del Sol *et al.*, 2006). In this study, alignment of the *S. coelicolor* CrgA sequence with other sequenced actinobacterial orthologs, revealed only four amino acids that are well conserved: G_{40}, W_{45}, N_{65} and W_{83}. The positions of two amino acids G_{40} and W_{45} are in the first trans-membrane domain (TM_1), but other two amino acids (Asparagine N_{65}) and (Tryptophan W_{83}) are at the carboxyl end of the protein. The amino acid N_{65} is very interesting because it is a hydrophilic amino acid, which is very unusual in trans-membrane domains (TM_2), which normally containing hydrophobic amino acids; therefore it likely to play a substantial role for the function of the CrgA protein. My data showed although one penicillin binding protein (PBP1) can interact with the mutated $CrgA_N$ and $CrgA_W$ and also with $CrgA_{WN}$.

The results of two hybrid tests using mutated CrgA proteins indicated that the amino acids N_{65} and W_{83} of *S. coelicolor* CrgA are required for interaction of CrgA protein with itself and PBP2. In more detail, substitution of N_{65} impaired interaction with the protein with

substitution of W_{65}, whereas substitution of both amino acids N_{65} and W_{83} led to loss of detectable self interaction between the mutated proteins. The double substitution also impaired interactions of $CrgA_{NW}$ with PBP2. Given time, it would have been interesting to investigate *in vivo* function of the mutated CrgA proteins.

In this study, the investigation of protein-protein interactions based on using of BACTH system which has several features that make it useful for analysis of protein-protein interactions. As the BACTH assay is carried out in *E. coli*, the screening and the characterization of protein-protein interactions are greatly facilitated as established standard molecular biology techniques are employed. *E.coli,* is easily transformed with high efficiency and grows faster than yeast. In addition, the same plasmid constructs used in library screening to identify a putative binding partner to a given "bait", can be employed to express the chimeric proteins in order to characterize their interaction by *in vitro* binding assays. In addition, if an interaction is detected, deletions can be made in the DNA encoding one of the interacting proteins to identify a minimal domain for interaction (Chien *et al.*, 1991) and point mutations can be assayed to identify specific amino acid residues critical for the interaction (Li and Fields, 1993). Further advantages are that interactions are detected within the native environment of the cell and hence no biochemical purification is required. The use of genetic-based organisms like bacteria or yeast cells as the hosts for studying interactions allows both a direct selection for interacting proteins and the screening of a large number of variants to detect those that might interact either more or less strongly (Phizicky and Fields, 1995). On the other hand, one disadvantage of this technique which might have influenced results in this study, is that proteins must be able to fold and exist stably in cells and to retain activity as fusion proteins (Ma and Ptashne, 1987).

There are other techniques that can be used to confirm the results of cell division proteins interactions that were obtained in this study by using two hybrid system, such as: **S**urface **P**lasmon **R**esonance (SPR), **I**sothermal **T**itration **C**alorimetry (ITC). SPR has become one of the most important techniques for studying macromolecular interactions, and is used mainly to study protein-protein interactions. This system has several advantages. First, it requires very little material. Second, the method is very fast. Thirdly, no modifications of the proteins are required, such as labeling or fluorescent tags. Fourth, interactions can be observed even in complex mixtures. Fifth, both the on rate and the off rate are readily obtained. Finally, the system is useful over a wide range of protein concentrations (Alex and Bourrot, 1993).

However associated with SPR measurements there are two problems. Firstly, immobilization of the ligand protein must be of such a nature that it does not impede or artificially enhance interactions (Alex and Bourrot, 1993). Secondly, the sensor chip has to be regenerated under conditions which do not denature the immobilized ligand protein (Herberg *et al.*, 1994).

The ITC technique, is the most quantitative means available for measuring the thermodynamic properties of protein-protein interactions and is becoming a necessary tool for complex structural studies (Ababou and Ladbury, 2005, Ababou and Ladbury, 2006, Chen *et al.*, 2007, Cliff *et al.*, 2004, Jin *et al.*, 2006, Kiel *et al.*, 2004, Knipscheer *et al.*, 2007, Okhrimenko and Jelesarov, 2008, Rainaldi *et al.*, 2007, Siligardi *et al.*, 2004, Yokota *et al.*, 2003, Zhou *et al.*, 2005). In the future the role of ITC as a basic quantitative biochemical tool for characterizing intermolecular interactions should be recognized considering the new directions in the post genomic era for biology and medicine (Velazquez-Campoy *et al.*, 2004).

In conclusion, my research has revealed novel functionalities of SEDS proteins and their cognate PBP's in sporulating *Streptomyces*. As yet it is unclear how these bacteria form vegetative cross-walls, with an implication that RodA, normally involved in cell elongation in other bacteria, is likely involved in the process which apparently functions independently of function of the *fts* genes. In addition, I have compiled an initial data-set indicating the species-specific nature of protein-protein interactions that underly formation of the divisome required for sporulation septation.

6.7 Future prespectives:

The results presented in this thesis provide results relating to the function of *S. coelicolor* cell division proteins. The thesis also sheds light on interesting mutated cell division protein especially CrgA. Two major suggestions for interesting research areas to pursue are:

- To investigate the functional relevance of CrgA conserved amino acids (Tryptophan W_{45} and Guanine G_{40}) identified in Chapter 5.
- To test interaction of CrgA and FtsQ lacking either the N-terminal cytoplasmic domain or the C-terminal extracellular domain using the bacterial two-hybrid system.
- To investigate interaction of CrgA with FtsQ by creating amino acid substitutions in conserved amino acid residues in the transmembrane domain of the latter.
- To test functionality of mutated versions of FtsQ in sporulation septation in an *S. coelicolor ftsQ* mutant.
- To develop a bacterial two-hybrid based screening system for small molecule inhibitors of the interaction between CrgA and FtsQ.
- To further refine the systems developed here on the ability of recombinant strains to undergo sporulation for use to screen for new cell division inhibitors that could be potential new anti-tubercular drugs.

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