Control of sporulation-specific cell division in Streptomyces coelicolor

Noens, E.

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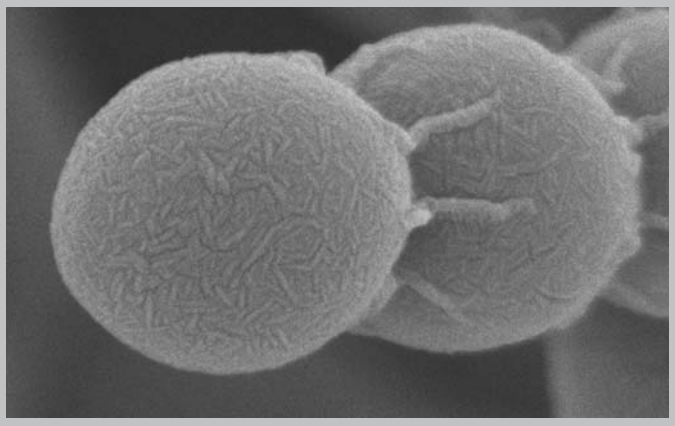
Cell division during growth and development
The cytoskeleton



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Introduction

Actinomycetes are gram-positive soil-dwelling bacteria whose DNA usually has a high GC content. Their natural habitat is very broad ranging from deep-sea deposits to soil and compost and they have even been detected living in symbiosis with ants (Currie *et al.,* 1999; Schultz *et al.,* 1999). They are producers of a long list of secondary metabolites, including the majority of antibiotics used today in medicine, making them extremely relevant for biotechnology. One of the best-identified genera among the actinomycetes is *Streptomyces*, with *Streptomyces coelicolor* as model system for this genus and the main organism of choice for most experiments in this thesis. Recently, the complete genome sequence of *Streptomyces coelicolor* (Bentley *et al.*, 2002) and *Streptomyces avermitilis* (Ikeda *et al.*, 2003) and a part of *Streptomyces scabies* (http://www.sanger.ac.uk/Projects/S_scabies) have become available.

In most prokaryotic species, cell division happens by binary fission; a mother cell will be divided in two equivalent daughter cells by the formation of a division septum at midcell. After completion of chromosome replication and segregation into the two future cells, the division septum will be build at a predetermined site and two progeny cells are created. During the complex life cycle of the gram-positive soil bacterium *S. coelicolor*, cell division consists of two, apparently different events (Fig. 1). *Streptomycetes* produce a mycelium network of branched hyphae, similar to that of filamentous fungi. In these branching vegetative hyphae, cross-walls are occasionally produced to generate multinucleoid compartments (Wildermuth, 1970). Development takes place after an environmental trigger, usually nutrient depletion, and aerial hyphae will grow upwards from the vegetative mycelium and break through the water-air interface. When sporulation starts, a ladder of septa is simultaneously produced at regular intervals (± 1 μm) in the aerial hyphae, dividing the hyphae into prespores, each containing one chromosome. The prespores mature and the mature spores are separated by autolysis.

Streptomycetes are a very good model for the study of bacterial development and cell division. One of the reasons is that cell division is dispensable for growth, which makes this organism an interesting model to study the functional, structural and regulatory aspects of cell division. In this chapter, we focus on all aspects of cell division in relation to growth and development of Streptomyces. The difference in the two types of septa will be presented, as well as the presence or absence of known and new cell division proteins, supported by the genome sequence.

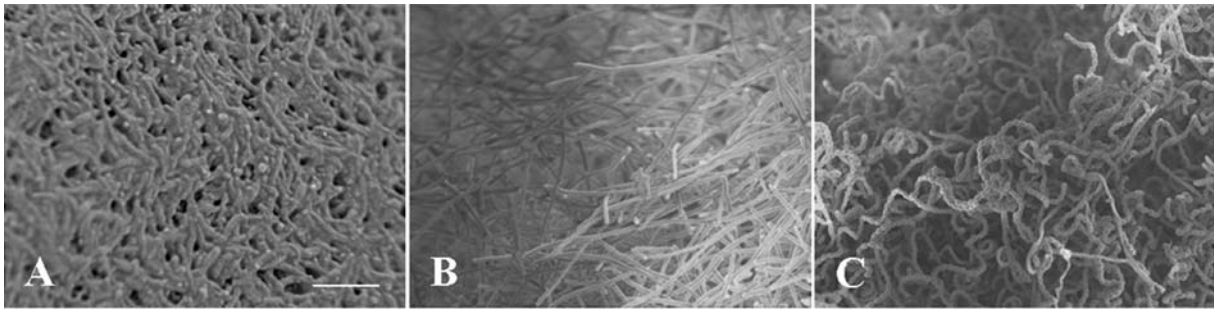

Figure 1*:* Life cycle of *S. coelicolor* grown on solid media. After spore germination, a dense network of branched vegetative mycelium is formed (**A**). During development, aerial hyphae will grow upwards (**B**). Eventually spore septa will divide the hyphae into spores (**C**). Bar = 10 μm.

Growth and vegetative division

Growth of *S. coelicolor* starts with one spore, originated from a place where nutrients are deficient and transported by wind, water or insects as dormant spores to germinate in a more favourable environment. The pleiotropic transcription factor Crp, a cAMP receptor protein, is most likely the key biomolecule responsible for the expression of proteins involved in the shift from dormant to germinating spores (Derouaux *et al.*, 2004; Piette *et al.*, 2005). During germination, one or more germ tubes emerge from the spore, which will grow and branch to form a vegetative or substrate mycelium (Chater and Losick, 1997). Fluorescently labelled vancomycin or radiolabelled N-acetylglucosamine, both incorporated into newly synthesised peptidoglycan, were used to visualise sites of nascent peptidoglycan insertion into the cell wall. In this way, peptidoglycan biosynthetic activity was primarly localised at hyphal tips and branching sites (Daniel and Errington, 2003; Gray *et al.*, 1990; Young, 2003). In *S. coelicolor*, DivIVA is an essential protein for polar growth and morphogenesis and was the first protein to be specifically localised at the tips of growing hyphae and lateral branches (Flärdh, 2003). Partial deletion results in a phenotype with irregular curly vegetative hyphae and apical branching, similar to that of many tip growth mutants in fungi, while overexpression altered cell shape and affected tip extension, causing hyperbranching (Flärdh, 2003). In *B. subtilis*, DivIVA was found to have two functions. Firstly, it is required for the stable polar localisation of MinCD and, therefore, functions as a functional analogue of the MinE protein in *E. coli* (see below) (Edwards and Errington, 1997; Fu *et al.*, 2001). Secondly, DivIVA has a role in localising or attaching the *oriC* region of the chromosome to the cell pole during sporulation (Errington, 2001). Tip growth, hyphal branching and hyphal breakage result in an exponential growth, giving rise to a complex mycelium network (Locci, 1980). In this mycelium, cell division gives rise to the formation of septa or cross-walls, often around

the middle of the growing apical cell when it reaches a certain length (Prosser and Tough, 1991). The two newly created compartments remain attached, although double membranes separate them and, therefore, no physical separation of the cells takes place. Our preliminary data show that these cross walls are more than a physical barrier as pore-like structures are visible, thus communication between the different compartments may be possible. This results in a multicellular mycelium harbouring multinucleoid compartments divided by cross-walls that are infrequently placed between varying numbers of chromosomes in the hyphae. To enlarge the dimensions of the subapical daughter cell, a new lateral branch is created, which usually occurs near a cross-wall and in this way reduces hyphal strength, suggesting some form of coordination between cell division and branching (Wardell *et al.*, 2002). The frequency of branching depends on the growth conditions: when sufficient nutrients are available, the formation of branching is supported to optimally take up the nutrients available, whereas in poor growth conditions, branching is reduced and tip extension is the dominant form of growth, resulting in the formation of "searching hyphae" (Bushell, 1988).

Proteins of the divisome of unicellular bacteria

How mysterious cell division seems to be, it is a very regular and strictly controlled event. In rod-shaped bacteria such as *E. coli*, division involves the invagination of the cell membrane, closely followed by septation, for which a change in direction of peptidoglycan synthesis is necessary. In *E. coli*, these processes involve the assembly of a multiprotein complex at the division site, called the divisome or the septosome (Table 1) (Fig. 2).

The FtsZ-ring

The earliest known component to be targeted to the cell division site is the key cell division protein, FtsZ, a structural homologue of eukaryotic tubulin and well conserved in nearly all bacteria, archaea and some eukaryotic organelles (Erickson *et al.*, 1996). FtsZ is missing in some groups of wall-less bacteria, indicating that cell division has changed in some bacteria during evolution (Vicente *et al.*, 2006). At the division site, FtsZ polymerises into a cytokinetic ring in a GTP-dependent fashion. This so-called Z-ring is located at the inner surface of the cytoplasmic membrane (Bramhill and Thompson, 1994).

The Z-ring acts as a scaffold and recruits other proteins to form a cytokinetic ring, which is also called the septasome or divisome (Fig. 2). At least 15 genes in *E. coli* are known

Table 1: Cell division-related genes in *E. coli, B. subtilis, S. coelicolor* and *S. avermitilis*.

E. coli	*B. subtilis*	*S. coelicolor*		*S. avermitilis*
gene	**gene**	**gene**	**database nr**	**database nr**
ftsA	*ftsA*	NP		NP
ftsB	NP	NP		NP
ftsE	*ftsE*	*ftsE*	SCO2969	SAV6104
ftsI	*ftsI*	*ftsI*	SCO2090	SAV6116
ftsK	*SpoIIIE*	*ftsK*	SCO5750	SAV4542
ftsL	*ftsL*	*ftsL*	SCO2091	SAV6115
ftsN	NP	NP		NP
ftsQ	*divIB*	*ftsQ*	SCO2083	SAV 6123
ftsW	*ftsW*	*ftsW**	SCO2085	SAV 6121
ftsX	*ftsX*	*ftsX*	SCO2968	SAV6105
ftsZ	*ftsZ*	*ftsZ*	SCO2082	SAV6124
zipA	NP	NP		NP
zapA	*zapA*	NP		NP
NP	*ezrA*	NP		NP
NP	*divIC*	*divIC*	SCO3085	SAV3532
minC	*minC*	NP		NP
minD	*minD*	*minD*	SCO5006	SAV3255
			SCO3557	SAV4605
minE	*NP*	NP		NP
NP	*divIVA*	*divIVA*	SCO2077	SAV6129
NP	NP	*ssgA*	SCO3926	SAV4267
		ssgR	SCO3925	SAV4268
NP	NP	*ssgB*	SCO1541	SAV6810
NP	NP	*ssgC*	SCO7289	NP
NP	NP	*ssgD*	SCO7622	SAV1687
NP	NP	*ssgE*	SCO3158	SAV3605
NP	NP	*ssgF*	SCO7175	NP
NP	NP	*ssgG*	SCO2924	NP
mreB	*mreB*	*mreB*	SCO2611	SAV5455
		mbl	SCO2451	SAV5720
NP	*mbl*			
mreC	*mreC*	*mreC*	SCO2610	SAV5456
mreD	*mreD*	mreD	SCO2609	SAV5457
NP	*mreBH*	NP		
parA	*soj*	*parA*	SCO3886	SAV4309
			SCO1772	SAV7508
parB	*spoOJ*	*parB*	SCO3887	SAV4308

NP: not present.

*: other *ftsW*-like genes: SCO2607 (SAV5459), SCO3846 (SAV4340), SCO5302 (SAV2951).

to be involved in septation: *ftsA, -B, -E, -I, -K, -L, -N, -Q, -W, -X, -Z, zipA, zapA, amiC, and envC* (Errington *et al.*, 2003) (Table 1). These division genes are well conserved among bacteria, indicating that most bacterial groups share common division machinery and mechanisms. However, not every protein is present in all the groups, suggesting a flexible cell division machinery, which has adapted to the diversity of bacterial cell envelopes, cell shapes and life cycles (e.g. FtsZ).

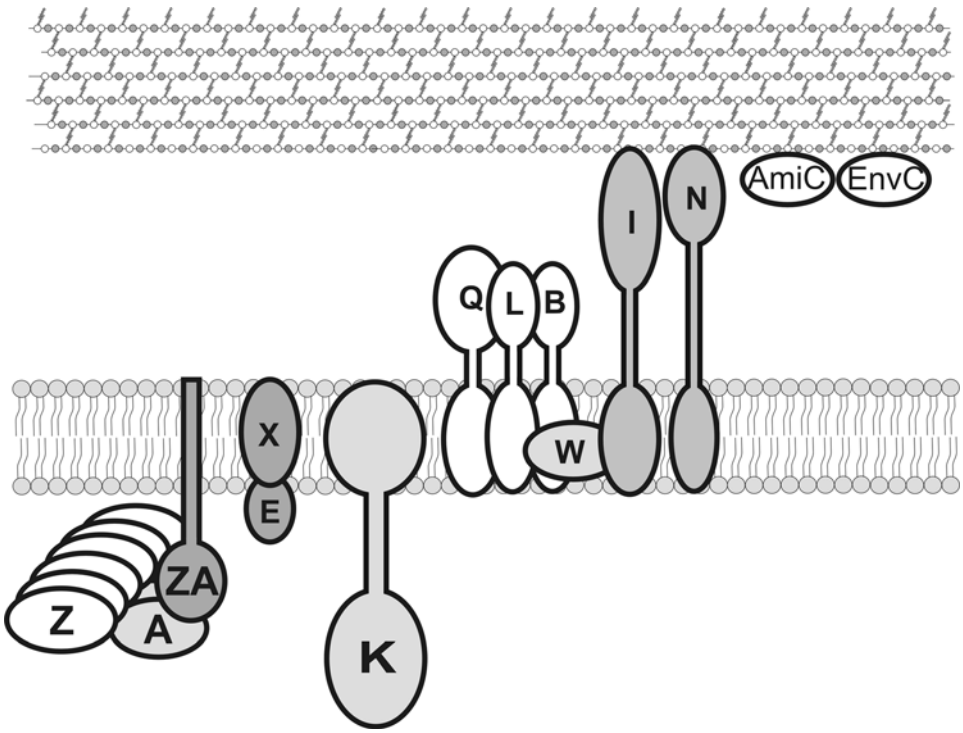

Figure 2: Proteins of the divisome of *E. coli*. Schematic overview of the order of recruitment of the proteins forming the cytokinetic ring. The proteins are ordered from left to right according to the order of assembly, taking the latest model of assembly in a concerted mode (Goehring *et al.*, 2005; Goehring *et al.*, 2006) into consideration. Protein names have been abbreviated by excluding "Fts" from them, except from ZA (ZipA), AmiC and EnvC.

Proteins involved in the early assembly of the cytokinetic ring

ftsA is conserved in most bacterial groups and the gene product belongs to the actin/Hsp70/sugar kinase superfamily and assembles at the Z-ring at an early stage, by directly interacting with FtsZ and stabilising the Z-ring (Bork *et al.*, 1992; Sanchez *et al.*, 1994).

Assembly of FtsZ in *E. coli* depends on either FtsA or ZipA or both. These proteins are bound to the inner cell membrane and are dependent on FtsZ for their localisation. They are most likely involved in linking the Z-ring to the cytoplasmic membrane (Pichoff and Lutkenhaus, 2002). Additionally, the widely conserved, though not essential protein ZapA might positively modulate Z-ring assembly *in vivo* by binding FtsZ polymers (Gueiros-Filho and Losick, 2002). *B. subtilis* harbours a negative assembly regulator, called EzrA, which modulates the frequency and positions of Z-ring formation by destabalising FtsZ polymers (Levin *et al.*, 1999).

Assembly of the downstream proteins

After the assembly of the proteins involved in linking the Z-ring to the membrane, FtsE and FtsX are recruited to the divisome. These two proteins are related to the ABC family of transporters with FtsE resembling the ATP-binding cassette interacting with the membrane component FtsX (de Leeuw *et al*; Schmidt *et al.*, 2004). The exact role of FtsE and FtsX remains unclear, although a role during constriction is suggested (Schmidt *et al.*, 2004). In most bacteria, *ftsEX* are in an operon with *ftsY*, encoding the receptor of the signal recognition particle and responsible for the correct insertion of FtsE, FtsQ, FtsX and ZipA into the *E. coli* inner membrane (de Leeuw *et al.*, 1999; Du and Arvidson, 2003).

Subsequently, FtsK, a large multifunctional membrane protein containing three cytoplasmic domains, will be assembled in the divisome. The essential N-terminal region and the intermediate linker domain have a role in cell division, while the C-terminal domain is an ATP-dependent DNA translocase functioning in chromosome dimer resolution and segregation of the chromosomes into the daughter cells (Bigot *et al.*, 2004). The *B. subtilis* homologue SpoIIIE "pumps" one of the chromosomes into the prespore compartment during the asymmetric cell division, leading to sporulation (Bath *et al.*, 2000). FtsQ assembles with FtsL and FtsB into a trimeric protein complex before localising to the septosome. However, the specific function of FtsQ is not known (Buddelmeijer and Beckwith, 2004). All three proteins harbour a transmembrane domain with the major C-terminal domain oriented on the outside of the membrane. The next proteins assembled into the divisome are FtsI and FtsW, both involved in peptidoglycan synthesis during cell division. FtsW is an integral membrane protein and belongs to FtsW/RodA/SpoVE family of proteins. The genes, encoding for these proteins, are usually paired with a gene, coding for a class B penicillin-binding protein (PBP). FtsW is proposed to act together with FtsI, a PBP3 with transpeptidase activity and responsible for synthesis of septal peptidoglycan, exactly like RodA and PBP2 do in cell elongation (Henriques *et al.*, 1998; Matsuhashi *et al.*, 1990). The last membrane protein involved in the assembly of the septosome of *E. coli*, is FtsN, which spans the periplasma and has a peptidoglycan-binding domain (Addinall *et al.*, 1997). Although the function of this protein is poorly understood, there is limited sequence similarity with cell wall amidases, suggesting a possible role in hydrolysis (Errington *et al.*, 2003). Requirement in both early and late phases of assembly is also hypothesised (Corbin *et al.*, 2004; Goehring *et al.*, 2005). Finally, the first completely periplasmic protein AmiC is recruited to the *E. coli* divisome. Its recruitment is dependent on FtsN. AmiC is an N-acetylmuroamoyl-L-alanine amidase that

specifically cleaves the bond between the peptide moiety and N-acetylmuramic acid in septal peptidoglycan to allow constriction of the septum and separation of the daughter cells (Bernhardt and de Boer, 2003). Another protein to play a direct role in septal peptidoglycan cleavage is EnvC, a lysostaphin-like, metallo-endopeptidase, which has peptidoglycan hydrolytic activity (Bernhardt and de Boer, 2004).

The recruitment of the cell division proteins to the Z-ring in *E. coli* is hypothesised to take place in a hierarchical linear order (Buddelmeijer and Beckwith, 2002) (Fig. 2). However, recent work suggests that assembly of the divisome in *E. coli* involves the formation of complexes, which are assembled in a concerted mode (Goehring *et al.*, 2005; Goehring *et al.*, 2006). In this way, a proto-ring is first formed on the cytoplasmic membrane by interactions between FtsZ, FtsA and ZipA, followed by the addition of FtsK to form the cytoplasmic ring. Later, FtsQ, FtsB and FtsL form the periplasmic connector. Subsequently, FtsW and FtsI, involved in synthesis of septal peptidoglycan are added, followed by FtsN as a ring oriented in the periplasm and connecting with the peptidoglycan (Vicente and Rico, 2006). In *B. subtilis* on the other hand, similar division proteins are cooperative in their recruitment to the division site and they are all completely interdependent for assembly (Errington *et al.*, 2003). Hence, the mode of division ring assembly is quite similar in these two bacteria.

The divisome of S. coelicolor

The genome sequences of *S. coelicolor* (Bentley *et al.*, 2002) and *S. avermitilis* (Ikeda *et al.*, 2003) allowed searching for *Streptomyces* homologues of known cell division proteins identified in other bacteria (Table 1) (Flärdh and van Wezel, 2003).

Not surprisingly, *S. coelicolor* harbours an FtsZ homologue, which is required for cell division, as in other bacteria. However, unlike in most other bacteria, *S. coelicolor* FtsZ is not essential for viability (McCormick *et al.*, 1994). *ftsZ* null mutants of *S. coelicolor* are blocked in septum formation, supporting the central role of FtsZ in cell division (McCormick *et al.*, 1994). As these strains could be sub-cultured, non-septated hyphae could be broken off without loss of viability. *S. coelicolor* is untill now the only FtsZ-containing organism that does not need FtsZ for its growth (McCormick *et al.*, 1994).

The way of recruitment of cell division proteins to the Z-ring in *S. coelicolor* is until now not known. However, certain events of sporulation-specific cell division occur in a

different order as in, for example, *E. coli*, suggesting that the cell division proteins will be differently recruited to the Z-ring in *S. coelicolor*.

Most of the membrane proteins involved in linking the Z-ring with septal peptidoglycan synthesis are present in *Streptomyces*, indicating that the basic mechanism of cell division is similar to that in most other bacteria. On the other hand, the proteins, responsible for the stability (FtsA, ZipA) and bundling of FtsZ protofilaments (ZapA, EzrA) are absent in the genomes of *S. coelicolor* and *S. avermitilis*, raising the important question as to how the Z-ring localises and attaches to the membrane. This suggests that new important division proteins, involved in these processes still need to be found in streptomycetes (Flärdh and van Wezel, 2003).

In *S. coelicolor* and other actinomycetes, *ftsEX* form an operon but *ftsY* is located elsewhere on the chromosome. It is not clear if FtsY is still involved in the membrane topology of these proteins. In chapter 6 of this thesis, the role of FtsE and FtsX is further discussed.

The genome of *S. coelicolor* harbours one clear homologue of FtsK, which has a similar function in chromosome segregation (Wang *et al.*, 2007). Chromosome segregation happens in *S. coelicolor* prior to septum closure during sporulation, while in *E. coli*, this occurs before the start of septum synthesis. This suggests that FtsK will be recruited to the divisome at a different time.

The *ftsQ* homologue of *S. coelicolor*, immediately upstream of *ftsZ*, is not essential and was not absolutely required for septation. Hyphal cross-wall formation was not completely blocked but reduced by 90-95% in an *ftsQ* null mutant, resulting in a phenotype less severe than that observed in an *ftsZ* null mutant (McCormick and Losick, 1996). An *ftsL* homologue, with conserved genomic position is present in the genome of *S. coelicolor*, while FtsB is not present (Flärdh and van Wezel, 2003). Nevertheless, *S. coelicolor* harbours a homologue of DivIC, which interacts with DivIB (FtsQ homologue) and FtsL in *B. subtilis* and most likely has a similar function as FtsB (Daniel *et al.*, 2006).

The *S. coelicolor* genome harbours four genes, whose products belong to the FtsW/RodA/SpoVE family of proteins. All of these genes are genetically paired with a gene, coding for a class B PBP. *ftsW* (SCO2085) is linked with *ftsI* (SCO2090), which act together in septal peptidoglycan synthesis (Bennet *et al.,* 2002) while *sfr* (SCO2607, *rodA* homologue) most likely acts together with PBP2 (SCO2608) in cell elongation (Burger *et al.*, 2000).

Another protein that is not present in *S. coelicolor* and the function of which is until now not clear, is FtsN. This is not surprising, as so far FtsN homologues have only been identified in Gram-negative bacteria.

In *S. coelicolor*, only sporulation-specific cell division results in physical separation of the cells (*i.e.* spores) and the genome harbours several lytic enzymes with a possible role in this process. Our microarray data revealed that for example, SCO5466, encoding a lysozyme-like hydrolase and SCO4132, coding for a lytic secreted transglycosylase (SLT), are transcribed in a developmental way, suggesting a role for these enzymes in spore separation.

Spatial control of the placement of the bacterial division site

One of the most important aspects of cell division is the correct timing and localisation of the septum. DNA must be segregated prior to septum closure to avoid guillotining the chromosome. For this, the correct localisation of FtsZ depends on two inhibitory mechanisms, namely the Min system and nucleoid occlusion (NO).

In *E. coli* as well as in many other bacteria, the Min system consists of the *minCDE* locus (Table 1). MinC is the division inhibitor, interacting with FtsZ to prevent formation of stable FtsZ rings, although it does not show site specificity. MinE is the topological specificity factor and gives, therefore, site specificity to MinC, limiting its activity to sites away from midcell. The membrane association of MinC and MinE is carried out by MinD, member of the large MinD/ParA superfamily of cytoskeletal proteins characterised by altered Walker A-type ATPase motif (Koonin, 1993). The result is a pole-to-pole oscillation, prevented from extending past midcell by the MinE-ring (Hu and Lutkenhaus, 1999; Rothfield *et al.*, 2005). *B. subtilis* and other gram-positive bacteria lack MinE, although DivIVA partly fulfils its role (Errington *et al.*, 2003). *S. coelicolor* does not harbour homologues of MinC and MinE. The function of its two MinD homologues, which lack motifs that are conserved in most other MinDs, is unclear, as *minD* null mutants have no obvious phenotype (McCormick and van Wezel, unpublished data). There is no evidence that *S. coelicolor* DivIVA plays a role in the Min system.

Cells lacking the Min system and cells in which nucleoid replication or segregation is defective have a second mechanism of negative regulation, called nucleoid occlusion (NO), which prevents septation over the nucleoids. Recently, two proteins that have a rol in NO have been identified: SlmA in *E. coli* (Bernhardt and de Boer, 2005) and Noc in *B. subtilis*

(Wu and Errington, 2004) and are essential for cell division in cells where the Min system is non-functional. In the absence of both Min and SlmA or Noc, cells fail to septate.

Two important observations make it very likely that *S. coelicolor* uses a different system for septum site selection. Firstly, the placement of the septa in both vegetative and aerial hyphae is not necessarily at midcell. Secondly, the segregation of the chromosomes into the prespores is carried out prior to septum closure, indicating that the divisome is built over the chromosomes.

The bacterial cytoskeleton

Although the major determinant of the bacterial cell shape is the bacterial cell wall, bacteria possess clear homologues of all three major types of eukaryotic cytoskeletons, which function in the determination of the cell wall architecture and have strong impacts on cell shape. As discussed above, FtsZ is a tubulin homologue and the earliest component of the division machinery to be targeted to the site of cell division site, linking the divisome with septal peptidoglycan synthesis (Erickson *et al.*, 1996). Crescentin, a bacterial equivalent of eukaryotic intermediate filament proteins, produces intermediate filament-like elements in *Caulobacter crescentus*, which maintain its curved shape (Ausmees *et al.*, 2003). The HSP70-actin-sugar kinase superfamily, including MreB, Hsp70, FtsA and ParM, are actin homologues (Bork *et al.*, 1992). Bacterial cells contain another group of cytoskeletal proteins, belonging to the large MinD/ParA superfamily, which have no homology to eukaryotic cytoskeletal elements (Barilla *et al.*, 2005; Shih *et al.*, 2003). They contain unusual Walker A-type ATPase motifs (Koonin, 1993) and are organised in filamentous structures within the cells (Suefuji *et al.*, 2002).

The bacterial actin mreB

MreB is present in Gram-positive and Gram-negative bacteria with nonspherical shapes but is absent from most bacteria displaying coccoid or spherical morphologies (Jones *et al.*, 2001). Gram-negative bacteria usually harbour only one copy of the *mreB* gene, while Gram-positive organisms often have multiple copies (Table 1). For example, *B. subtilis* has three *mreB*-like genes, called *mreB, mbl* and *mreBH*, whereas the genome of *S. coelicolor* contains two copies, *mreB* and *mbl*. MreB appears to be essential in all bacteria studied so far, including *E. coli*, *B. subtilis* and *C. crescentus*. Depletion of MreB in *E. coli, B. subtilis* and *C. crescentus* induced the formation of enlarged cells with extreme morphological defects and, finally,

resulted in cell lysis (Figge *et al.*, 2004; Jones *et al.*, 2001; Kruse and Gerdes, 2005). MreB homologues of *E. coli, B. subtilis* and *C. crescentus* all form helical-like structures underneath the cell envelope (Figge *et al.*, 2004; Jones *et al.*, 2001; Shih *et al.*, 2003; Soufo and Graumann, 2003). The use of a fluorescent derivative of vancomycin that labels nascent PG in gram-positive bacteria, revealed that the insertion of new cell wall material occurred in a helical pattern over the cylindrical part of the cell in *B. subtilis* and that Mbl is required for this lateral wall biosynthesis (Daniel and Errington, 2003). Several PBPs have been shown to display a helical distribution over the lateral wall and the localisation of PBP2 (a PG synthase) (Dye *et al.*, 2005) and LytE (a PG hydrolase) (Carballido-Lopez *et al.*, 2006) were shown to be MreB-dependent, indicating that mreB and its homologues govern cell wall morphogenesis by localisation of PG synthases and hydrolases. Other putative functions of MreB homologues include roles in correct chromosome segregation (Gitai *et al.*, 2005; Kruse *et al.*, 2003; Soufo and Graumann, 2003) and cell polarity (Gitai *et al.*, 2004).

The genome of *S. coelicolor* contains two homologues of *mreB*. One of them is located in a cluster with *mreC* and *mreD*, while the other one is located elsewhere (Burger *et al.*, 2000). In chapter 5 of this thesis, a role of these proteins in *S. coelicolor* is discussed.

The switch to development in *S. coelicolor*

When the time has come to go to a more favourable environment, motile bacteria move using a flagellum, bacterial gliding, twitching motility or changes of buoyancy. The multicellular mycelial streptomycetes are sessile microorganisms that have to go down a different alley.

In nutrient-limiting conditions, vegetative mycelium supports the development of non-branched hydrophobic aerial hyphae, which will break through the water-air surface to serve as a template for spore formation. The nutrients necessary for the production of an aerial mycelium are most likely provided by the lysis of the vegetative mycelium (Mendez *et al.*, 1985). This is one of the reasons why development goes together with antibiotic production, to kill microorganisms that are attracted by the pool of nutrients as a result of cell wall lysis.

DasR, sensing the nutritional state

Nutrient deprivation is an important signal for the onset of development. Recently, it was shown that N-acetylglucosamine (GlcNAc), derivative in nature from the polymer chitin and component of the peptidoglycan layer, is a crucial nutritional signal, whose extracellular concentration determines the choice between vegetative growth and the formation of aerial

mycelium (Rigali *et al.*, 2006). The metabolic regulator DasR, a member of the GntR-family and part of this nutrient-sensing system, controls the GlcNac regulon, including the *pts* genes *ptsH, ptsI* and *crr*, which are necessary for the uptake of GlcNAc (Rigali *et al.*, 2004). A high concentration of GlcNAc prevents the formation of aerial mycelium, while a low concentration of GlcNAc in the presence of glucose results in the phosphorylation by the intracellular components of the sugar phosphotransferase system (PTS) of specific target proteins, including WhiG. This will trigger the switch to development (Rigali *et al.*, 2006).

Mutants blocked in the formation of aerial mycelium (bld)

Mutants, most of them generated by random mutagenesis, that fail to produce the fluffy aerial mycelium are called 'bald' (*bld*) mutants because of their shiny, bald appearance. Several of the *S. coelicolor bld* mutants are often also disturbed in their primary and secondary metabolism and, therefore, lack the characteristic pigmentation of wild type substrate hyphae (Merrick, 1976; Pope *et al.*, 1996). Pope *et al.* (1996) showed that most of the *bld* mutants were affected in the regulation of carbon utilisation, suggesting that these *bld* genes are not involved in morphogenesis *per se*, but instead play a central role in the ability of these organisms to sense and/or signal starvation. Although the precise role of most of the *bld* genes is unclear, several genes encode regulatory proteins (Chater, 2001). Table 2 shows an overview of the *bld* genes in *S. coelicolor* with their possible function.

The best known *bld* gene is *bldA*, encoding a leucyl tRNA, which is necessary for the efficient translation of UUA, the rarest codon in the GC-rich *S. coelicolor* (Leskiw *et al.*, 1991a; Leskiw *et al.*, 1991b). About 150 genes of the *S. coelicolor* genome harbour one or more UUA codon (Bentley *et al.*, 2002). In this way, the translational efficiency of these genes is regulated by the expression of *bldA*. *bldA* mutants are completely defective in sporulation and antibiotic biosynthesis, the last is the result of the presence of a UUA codon in the activators of the undecylprodigiosin (Red) and actinorhodin (Act) biosynthetic clusters (*redZ* and *actII*-ORF4, respectively) (Fernandez-Moreno *et al.*, 1991; White and Bibb, 1997). An important gene that is *bldA*-dependent is *adpA* or *bldH*, the main target through which *bldA* affects differentiation (Nguyen *et al.*, 2003; Takano *et al.*, 2003). AdpA is a pleiotropic regulator belonging to the AraC family. In *S. griseus*, the expression of *adpA* is induced by A-factor, a γ-butyrolactone. This compound binds to the A-factor receptor protein (ArpA), which results in its dissociation from the *adpA* promoter. This causes the induction of the transcription of a number of genes by AdpA. Genes of the AdpA regulon involved in aerial

mycelium formation are *adsA*, the *S. coelicolor bldN* orthologue and encoding a ECF sigma factor (Bibb *et al.*, 2000; Yamazaki *et al.*, 2000), *sgmA*, encoding an extracellular metallopeptidase involved in the lysis of substrate hyphae during aerial hyphae formation (Kato *et al.*, 2002) and *amfR*, the orthologue of *S. coelicolor ramR*, resulting in production of an AmfS derivative, which is similar to SapB (Ueda *et al.*, 2002). Another important gene in *S. griseus*, dependent on AdpA is *ssgA*, a 15-kDa acidic protein involved in spore septum formation in both *S. griseus* (Jiang and Kendrick, 2000) and *S. coelicolor* (van Wezel *et al.*, 2000a). AdpA is also responsible for the regulation of several genes involved in secondary metabolism (Ohnishi *et al.*, 1999). In contract, *S. coelicolor scbA*, which produces the A-factor-like γ-butyrolactone SCB1, has no effect on the expression of *adpA* (Takano *et al.*, 2005). *S. coelicolor ssgA* is fully dependent on SsgR, although the typical upregulation of *ssgA* transcription towards the onset of sporulation was not visible in an *adpA* mutant (Traag *et al.*, 2004). Little is known about other genes present in the AdpA regulon in *S. coelicolor*.

Table 2: the *bld* genes in *S. coelicolor*.

Gene	Gene product	References
bldA	Leucyl tRNA for UUA codon	(Lawlor *et al.*, 1987) (Leskiw *et al.*, 1991b)
bldB	Small DNA-binding protein	(Pope *et al.*, 1998)
bldC	Small DNA-binding protein related to MerR transcriptional activators	(Hunt *et al.*, 2005)
bldD	Small DNA-binding protein repressing *bldN, whiG* and *sigH*	(Eccleston *et al.*, 2002; Elliot *et al.*, 1998; Elliot and Leskiw, 1999; Elliot *et al.*, 2001; Kelemen *et al.*, 2001)
bldG	Anti-anti-sigma factor	(Bignell *et al.*, 2000)
bldH	Pleiotropic regulator of the AraC family	(Nguyen *et al.*, 2003) (Takano *et al.*, 2003)
bldI	Unknown	(Leskiw and Mah, 1995)
bldJ	Unknown	(Nodwell and Losick, 1998)
bldK	Oligopeptide permease	(Nodwell *et al.*, 1996)
bldL	Unknown	(Nodwell *et al.*, 1999)
bldM	Response regulator	(Bibb *et al.*, 2000) (Molle and Buttner, 2000)
bldN	Extracytoplasmic function (ECF) sigma factor, required for the transcription of one of the two promoters of *bldM*	(Bibb *et al.*, 2000)

Although the *bld* genes have an essential role in the formation of aerial mycelium, our microarray data show that most of the *bld* genes are upregulated during sporulation or highly expressed during the whole lifecycle, which suggests that the products of these genes are necessary during more than one stage of development. Some *bldM* and *bldN* mutants result in a white aerial mycelium phenotype, which underlines this theory (Ryding *et al.*, 1999).

Mutants defective in sporulation

The first morphological change during development is the production of white, unbranched aerial hyphae, which will coil, cease to grow and serve as a template for spore formation in later stages of development. Mutants that produce aerial hyphae but fail to produce mature grey-pigmented spores are called 'white' (*whi*) mutants. The early *whi* genes (*whiA-B-G-H-I-J*) are involved in early sporulation events while the late white genes (*whiD-E-L-M-O*) function in septation and spore maturation.

1. Early *whi* genes

From the phenotypes of early *whi* mutants, it can be concluded that they are not blocked at a certain stage during spore differentiation. However, particular growth and/or morphological processes continue after the point at which they are blocked, resulting in mutation-specific terminal phenotypes (Flärdh *et al.*, 1999).

whiG, needed for the earliest stages of spore formation in aerial hyphae, encodes for an RNA polymerase sigma factor, similar to sigma factors involved in motility and chemotaxis (Chater *et al.*, 1989; Tan *et al.*, 1998). A *whiG* null mutant produces long, straight aerial hyphae containing septa with a distance similar to that of vegetative septa. Physicall cell separation was not seen in this mutant (Chater, 1972; Flärdh *et al.*, 1999). Overexpression of WhiG causes hypersporulation of aerial hyphae on solid media and ectopic sporulation of vegetative hyphae on solid and in liquid media (Chater *et al.*, 1989). Although *whiG* expression is repressed by BldD (Elliot *et al.*, 2001), *whiG* transcripts were detected during the whole life-cycle, suggesting post-transcriptional regulation (Kelemen *et al.*, 1996). Interestingly, proteome analysis showed that WhiG depends on the global components PtsH, PtsI and Crr of the PTS, suggesting a link between nutrient utilisation and development (Rigali *et al.*, 2006). The transcription of both *whiH* and *whiI* is regulated by WhiG (Kelemen *et al.*, 1996; Ryding *et al.*, 1998). Mutants of *whiH* display loosely coiled aerial hyphae that are divided into spore-like fragments, harbouring an unequal distribution of condensed DNA into bodies of variable sizes (Flärdh *et al.*, 1999). WhiH is a transcriptional regulator belonging to the GntR family and has autorepressor activity (Kelemen *et al.*, 1996; Ryding *et al.*, 1998). WhiI resembles the response regulators associated with a bacterial two-component system, although there is no histidine sensor kinase present and the phosphorylation domain found in WhiI is not present in most response regulators. This suggests that signals may be sensed through a more complex mechanism and that changes of the active state of WhiI may

depend on an atypical phosphorylation process or other post-translational modifications/activations. *whiI* null mutants produced moderately coiled aerial hyphae with few sporulation septa (Ainsa *et al.*, 1999). *whiA* and *whiB* have unusually long and curly aerial hyphae without any sporulation septa, suggesting WhiA and WhiB are required to stop aerial growth and allow sporulation to occur. *whiA* encodes a protein of unknown function with orthologues in most other Gram-positive bacteria (Ainsa *et al.*, 2000). WhiB belongs to a group of small putative transcription factors containing four conserved cysteines, only occurring in actinomycetes (Davis and Chater, 1992). Several paralogues of WhiB are present in the genome of *S. coelicolor*, including WhiD (Molle *et al.*, 2000). Expression of *parAB*, encoding chromosome partitioning proteins, depends absolutely on WhiA and WhiB (Jakimowicz *et al.*, 2006). *whiJ* mutants produce low numbers of normal spore chains. The product of this gene contains a lambda repressor-like DNA-binding domain at its N-terminus (Ryding *et al.*, 1999).

2. Late *whi* genes

In the final stage of development, the aerial hyphae are divided into unigenomic compartments by spore septa that subsequently develop into grey heat-resistant spores.

The sigma factor encoded by *sigF* is required for the later stages of sporulation. No *sigF* transcripts were detected in the early *whi* mutants, the reason for this is unknown. A *sigF* mutant displays a white phenotype although spores were produced. These spores were smaller than wild type spores with irregular shapes, a thin spore wall and uncondensed DNA. Targets, whose transcription depends on SigF have, until now not been discovered (Kelemen *et al.*, 1996; Potuckova *et al.*, 1995). *whiD* null mutants have a similar phenotype as *sigF* mutants. WhiD belongs to the wbl (WhiB-like) group (Molle *et al.*, 2000). *whiE* consists of a cluster of eight genes, encoding proteins responsible for the production of the grey spore pigment (Davis and Chater, 1992; Kelemen *et al.*, 1998). Mutations in *whiL, whiM* and *whiO* result in a disturbed sporulation-specific cell division but the gene products have not yet been identified (Ryding *et al.*, 1999). *whiK* and *whiN* were later renamed to *bldM* and *bldN*, respectively, after the discovery that null mutants of these genes were bald (Bibb *et al.*, 2000; Molle and Buttner, 2000).

Sporulation septation, Z ring assembly and segregation of the chromosomes

Both vegetative cross-walls and sporulation septa require FtsZ, FtsQ and most other cell division proteins and, therefore, most likely share a basic cell division machinery (Flärdh *et al.*, 2000; Grantcharova *et al.*, 2003; McCormick *et al.*, 1994; McCormick and Losick, 1996). However, there are some crucial differences between the two types of septa (Fig. 3). Sporulation septa are thick and separate into individual spores, while vegetative cross-walls are thinner and form connected compartments. Vegetative cross-walls are laid down with an average distance of 10 μm, often close to the middle of a hyphal cell resulting in multinucleoid compartments, whereas up to one hundred sporulation septa are produced simultaneously in one aerial hypha, at a distance of around 1 μm, creating uninucleoid spore compartments (Wildermuth and Hopwood, 1970).

The first event in sporulation-specific cell division of *S. coelicolor* is the formation of a ladder of regularly spaced FtsZ-rings in sporogenic aerial hyphae. This enormous assembly of Z-rings needs a high number of FtsZ molecules. This is provided by the upregulation of the developmentally regulated promoter of *ftsZ*, ftsZ2p, which is dependent on the early *whi* genes (Flärdh *et al.*, 2000). FtsZ-ring formation and septum synthesis in aerial hyphae occurs over non-segregated chromosomes, which will move to the prespore compartments prior to septum closure (Schwedock *et al.*, 1997). The negative effect of the nucleoid on Z-ring assembly, as in *E. coli* and *B. subtilis*, is obviously not present in *S. coelicolor*. Without a Min system or a system of nucleoid occlusion, it remains unknown how the ladder of FtsZ rings results in uniformly sized prespores, containing one single chromosome. An interesting fact is that *Streptomyces* FtsZ begins by forming spiral-shaped intermediates along the hypha, which will be remodelled into the regularly spaced Z-rings. The positioning of the chromosomes could influence this remodelling or, alternatively, the Z-rings or the synthesised septa could guide the segregation of the chromosomes (Grantcharova *et al.*, 2005). Interestingly, *ftsZΔ2p, ftsZ17* (Spo) and *whiH* mutants fail to make sporulation septa and have condensed but irregularly segregated chromosomes, suggesting a role for septation in the localisation of the nucleoids (Flärdh *et al.*, 1999; Flärdh *et al.*, 2000; Grantcharova *et al.*, 2003). Several proteins have been identified that play a role in chromosome segregation during sporulation-specific cell division in *S. coelicolor*. Developmental control of the second promoter of *parAB*, encoding chromosome-partitioning proteins, is required for the assembly of regularly spaced ParB complexes in the aerial hyphae, which are necessary for efficient chromosome segregation (Jakimowicz *et al.*, 2005; Jakimowicz *et al.*, 2006). *S. coelicolor* has also a

homologue of FtsK, a DNA translocator, which couples the completion of cell division and chromosome segregation in *E. coli* and is localised as part of the divisome (Yu *et al.*, 1998). FtsK helps in the ParB-mediated partitioning of the chromosomes to ensure that the whole moves into the prespore compartment (Wang *et al.*, 2007). The closest homologue of FtsK in *B. subtilis* is SpoIIIE. This protein is essential for sporulation as it translocates the chromosome into the asymmetric prespore complex (Bath *et al.*, 2000).

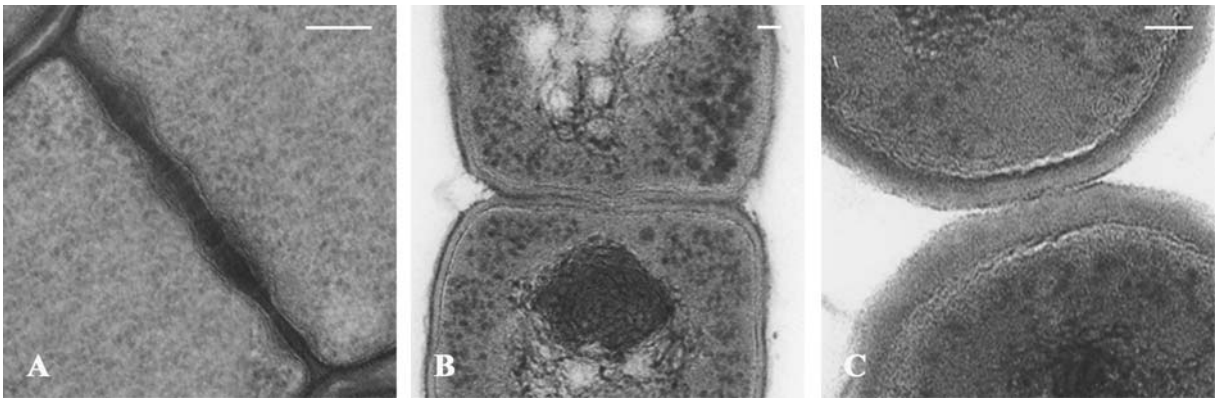

Figure 3: The difference between cross wall and sporulation septa. (**A**) a vegetative septum forming a non-physical separation between the two compartments, while pore-like structures still provide a connection. Immature spore septum (**B**) and mature spore septum (**C**). Bar = 100 μm.

Novel genes involved in development

Chaplins/rodlins

bld mutants that lack aerial hyphae, do not produce and secrete SapB. This small, hydrophobic, lantibiotic-like peptide is derived by posttranslational modification from the product of *rams*, which is part of the *ramCSAB* operon and is regulated by RamR (Keijser *et al.*, 2002). SapB can reduce the water surface tension, helping the hyphae to leave the aqueous environment of the vegetative mycelium and grow into the air. Addition of purified SapB to *S. coelicolor bld* mutants restores the formation of aerial hyphae but not sporulation (Tillotson *et al.*, 1998). After the formation of aerial hyphae, SapB was only detected in the medium and never at the surface of aerial hyphae and spores. SapB was only produced on rich media but not on minimal media containing mannitol (Willey *et al.*, 1991). Therefore, other molecules have to be present to fulfil a similar role to SapB on poor media and be responsible for the modulation of surface characteristics in accordance with environmental conditions.

Recently, two classes of structural proteins, called chaplins (Claessen *et al.*, 2003; Elliot *et al.*, 2003) and rodlins (Claessen *et al.*, 2002) were identified, which are involved in

the formation of aerial hyphae. The interplay between rodlins and chaplins results in the formation of a hydrophobic rodlet layer (Claessen *et al.*, 2004).

Deletion of the rodlin genes *rdlA* and/or *rdlB* resulted in the absence of the typical rodlet layer and the presence of fine fibrils coating the surface of aerial hyphae. Loss of the rodlins does not affect the growth or the hydrophobicity of aerial hyphae (Claessen *et al.*, 2002). The chaplins, a family of hydrophobic proteins consisting of eight members, are inserted into the cell wall of aerial hyphae of cultures grown on any media as a requirement for the aerial hyphae to escape into the air. Deletion of all eight *chp* genes (Δ*chpABCDEFGH*) resulted in a strain where formation of aerial hyphae was strongly affected, lacking both the rodlet layer and the fibrils. Addition of purified chaplins rescued the formation of aerial hyphae by lowering the water surface tension (Claessen *et al.*, 2003; Elliot *et al.*, 2003). Chaplins are assembled into small fibrils that are randomly distributed in the absence of the rodlins. In the presence of both rodlins, these fibrils are aligned into rodlets, containing two rods of each two fibrils, resulting in the hydrophobic layer (Claessen *et al.*, 2004).

SsgA-like proteins

The family of the SsgA-like proteins (SALPs), which are unique to sporulating actinomycetes, consists of seven homologues in *S. coelicolor* (SsgA-G) (Bentley *et al.*, 2002) and six in *S. avermitilis* (Ikeda *et al.*, 2003) (Table 1). All SALPs are small proteins (125-142 aa) with an average amino acid similarity of 30-40% (Keijser *et al.*, 2003).

The highest conservation is found in two sections of the proteins, corresponding to amino acid residues 13-30 and 40-65 of SsgA (Fig. 4). In total, 20 amino acid residues (15 % of the protein) are fully conserved among all 19 SALPs identified so far. Unfortunately, there are no sequences in these proteins that have similarity with known functional motifs (van Wezel and Vijgenboom, 2004).

Figure 4: Alignment of amino acid sequences of the SsgA-like proteins in *S. coelicolor*. Amino acids marked with black or grey boxes indicate sequence identity or similarity, respectively. The dashes indicate the gaps introduced to optimise the alignment.

The best-studied protein is SsgA, which was originally identified as an effector of cell division in *S. griseus* (Kawamoto and Ensign, 1995) and *S. coelicolor* (van Wezel *et al.*, 2000a). *ssgA* mutants produce normal vegetative septa but are defective in sporulation, although some viable spores are produced on mannitol-containing media (Fig 5A), indicating that SsgA only plays a role in sporulation-specific cell division (Jiang and Kendrick, 2000; van Wezel *et al.*, 2000a). SsgA has an activating role in the production of sporulation septa, as its enhanced expression in *S. coelicolor* submerged cultures results in fragmentation of the mycelia and a strong increase in the formation of septa, which were extremely thick and irregular and in this way produced spore-like compartments at high frequency (van Wezel *et al.*, 2000a). In *S.coelicolor*, no *ssgA* transcripts were detected in submerged cultures under normal conditions, while *ssgA* is strongly expressed in liquid cultures of *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b), which may explain why *S. griseus* is able to sporulate in submerged cultures but not *S. coelicolor*. Another difference between the two streptomycetes is the regulation of *ssgA*. While transcription of *ssgA* in *S. griseus* is fully dependent on AdpA (Ohnishi *et al.*, 2002), it is the upstream-located *ssgR*, a member of the family of *iclR*-type regulatory genes, which is responsible for the transcription of *ssgA* in *S. coelicolor* (Traag *et al.*, 2004). Transcription of *ssgA* and *ssgR*, both strongly upregulated during the onset of sporulation, is not dependent on the early *whi* genes in *S. coelicolor*. A possible reason for the *whi* gene-independent expression of *ssgAR* is the involvement of SsgA in the activation of submerged sporulation-specific cell division without the formation of aerial mycelium (Traag *et al.*, 2004).

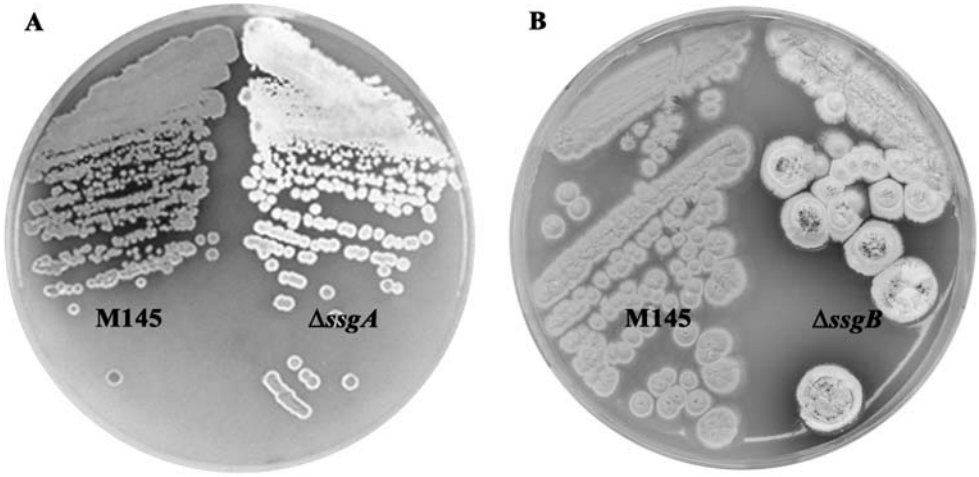

Figure 5: Effect of deletion of *ssgA* and *ssgB* on sporulation. Phenotypes of the *ssgA* mutant (A) and the *ssgB* mutant (B) and their congenic parent *S. coelicolor* M145.

Another member of the family, SsgB, is also identified to be essential for sporulation, as *ssgB* mutants resulted in a strict non-sporulating white phenotype, producing very large white colonies (Fig. 5B) (Keijser *et al.*, 2003; Kormanec and Sevcikova, 2002). *SsgB* is the only *whi* gene that is not a transcriptional regulator (Keijser *et al.*, 2003). Transcription of SsgB corresponds with aerial mycelium formation and depends on the developmental σ^H (Kormanec and Sevcikova, 2002), a sigma factor with a role in stress responses (Kelemen *et al.*, 2001). Chapter 2, 3 and 4 of this thesis go more deeply into this family of proteins.

Outline of this thesis

The study of the two types of cell division and development in *S. coelicolor* is the main focus of this thesis. An important part of the thesis regards the role of the SALPs in these processes.

In **chapter 2**, the mutants for each of the individual SALPs were created and analysed using electron and fluorescence microscopy, revealing various defects in the build-up and the degradation of peptidoglycan during sporulation. This underlines that the SALPs have an important function in the control of the sporulation process, from septum-site selection to spore separation. Using microarray analysis, the expression patterns of PBPs and autolysins present in the genome of *S. coelicolor* were checked to gain insight which one has a possible function during sporulation. In this way, certain PBPs and autolysins could be functionally related to the SALPs.

In **chapter 3**, the possible functions of the SALPs are analysed in more detail. SsgG showed a dynamic localisation, but could be found ultimately at positions resembling the sites for septum synthesis. FtsZ ladders were produced but Z-rings were regularly missing, therefore creating the typical longer spores in an *ssgG* mutant. From these observations, the important role of SsgG in septum-site selection was deduced. The importance of SsgB in the proper onset of sporulation-specific cell division of *S. coelicolor* is shown by the occasional formation of Z-rings in an *ssgB* mutant and the specific localisation of SsgB as an open ring at the sporulation septa. The conditional white phenotype of an *ssgA* mutant is most likely due to the presence of SsgC.

Chapter 4 shows the effect of a deletion of *ssgA* and *ssgR* mutant in global gene expression, using microarray analysis. The array results of the two mutants looked very similar, confirming our earlier data that *ssgA* is most likely the only gene regulated by SsgR. Many changes in gene expression in the *ssgA* mutant compared with the parental strain could be linked to phenotypical defects of an *ssgA* mutant. SsgA could be localised in a dynamically way during development, most likely at places where changes in local cell wall morphogenesis are required.

In **chapter 5,** analysis of null mutants deleted for either *mreB, mreC, mreD* and *mbl*, which encode actin-like cytoskeletal proteins, and for *pbp2*, encoding a PBP involved in lateral cell wall synthesis, were subjected to an intensive study using electron microsocpy. MreB could be localised at the septa of sporulating aerial hyphae, as bipolar foci in young spores, and as a ring- or shell-like pattern inside mature spores. Evidence is provided that all components play an important role in the control of the shape of aerial hyphae and spores.

In **chapter 6**, the function of two cell division proteins FtsE and FtsX, which are recruited to the divisome during sporulation, was studied in detail using mutational analysis and localisation studies. From our observations, we conclude that FtsEX participate in septum constriction, where they are most likely involved in the import of autolytically produced PG subunits for recycling.

In **chapter 7**, the results described in this thesis are summarised and discussed.