Developmental Control of Cell Division in *Streptomyces coelicolor*
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Abstract

Cell division in the Gram-positive bacterium Streptomyces coelicolor starts with the assembly of the tubulin homologue FtsZ into a cytokinetic ring (the Z ring) at the site of septation. In stark contrast to the binary fission of most bacteria, the syncytial hyphal cells of S. coelicolor exploit two types of cell division with strikingly different outcomes depending on the developmental stage.

The main goal of this study has been to identify developmental mechanisms that modulate this differential performance of the basic cell division machinery.

By isolation and characterization of a non-sporulating ftsZ mutant, we demonstrated that the requirements for Z-ring formation differ between the two types of septation. The ftsZ17(Spo) mutation abolished septation without overtly affecting vegetative growth. This mutant was defective in the assembly of FtsZ into regularly spaced Z rings in sporogenic hyphae, suggesting that the assembly of Z rings is developmentally controlled during sporulation.

An FtsZ-EGFP translational fusion was constructed and used to visualize the progression of FtsZ ring assembly in vivo. This revealed that polymerization of FtsZ occurred throughout the sporogenic cell, with no evidence for pre-determined nucleation sites, and that the placement of multiple Z rings is a dynamic process and involves remodeling of spiral-shaped FtsZ intermediates into regularly spaced rings.

The dynamics of the multiple Z-rings assembly during sporulation was perturbed by the action of the protein CrgA, which is important for coordinating growth and cell division in sporogenic hyphae. CrgA was also found to affect the timing of ftsZ expression and the turnover of the FtsZ protein.

S. coelicolor is the main genetic model of the streptomycetes, which are major industrial antibiotic producers. The control of cell division in these organisms differs from that of other bacteria like Escherichia coli. Thus, it is of fundamental importance to clarify how the streptomycetes reproduce themselves.

Keywords: FtsZ, cell division, Streptomyces, GFP, bacterial development

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The important thing is not to stop questioning. Curiosity has its own reason for existing.

Albert Einstein

To Valentina
with my deepest gratitude
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


The author had a minor contribution to the last paper and therefore it is not discussed in the Introduction part.
Contents

Introduction .................................................................................. 7
1. Guided tour of development in Streptomyces coelicolor ...........7
2. Importance of studying cell division in Streptomyces coelicolor ...13
3. Bacterial cell division and the role of FtsZ.................................14
   3.1 Assembly of the Z ring and the cell division machinery....... 15
      3.1.1. Polymerization
      3.1.2. Dynamic assembly of the Z-ring
      3.1.3. Mechanism of constriction
      3.1.4. Assembly of the divisome
   3.2. Regulation of Z-ring assembly.........................................20
      3.2.1. Cell-cycle timing
      3.2.2. Spatial regulation
      3.2.3. Developmental control of cell division
4. FtsZ and cell division in Streptomyces coelicolor......................26
   4.1. Two types of cell division..............................................
   4.2. Cell division machinery .............................................
   4.3. Developmental control of cell division in Streptomyces coelicolor.................................................................

Present study.................................................................32
1. Experimental results..........................................................32
2. The picture...........................................................................47

Reference list...............................................................53

Summary in Swedish..........................................................68

Acknowledgements.........................................................70
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>FtsZ</td>
<td>Filamentous Temperature Sensitive Z</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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1. Guided tour of development in *Streptomyces coelicolor*

The prokaryotic genus *Streptomyces* belongs to the actinomycetes which is a major line of descent among bacteria, often referred to as the high-G+C Gram-positives. Streptomycetes are of immense industrial significance. These bacteria are the source for a vast variety of antibiotics and other “secondary metabolites” with biological activity. About 55% of the bioactive compounds in clinical practise are produced by *Streptomyces* spp. (Challis and Hopwood, 2003; Champness, 2000). Apart from their industrial significance, streptomycetes are fascinating from the point of view of developmental biology because of their complex life cycle.

*S. coelicolor* is by far the genetically most studied member of the genus *Streptomyces*. The spore-to-spore development of *S. coelicolor* involves several stages of differentiation (Fig. 1): spores germinate and grow into branching hyphae that form a substrate mycelium; some hyphae grow away from the substrate and into the air forming a fluffy white aerial mycelium; finally, the apical hyphal cells septate into multiple compartments that mature into grey-pigmented, desiccation resistant spores (Chater, 1998; Kelemen and Buttner, 1998).

One way to grasp the developmental complexity of *S. coelicolor* is to take a guided tour through each level of differentiation (Fig. 1)

1.1. Growth in the substrate:

Unlike most other bacteria, *S. coelicolor* is a truly multicellular organism. Its substrate mycelium is a meshwork of branching hyphae. Growth of hyphae is polar, i.e. incorporation of fresh cell envelope material takes place only at the hyphal tips (Flärdh, 2003c; Prosser and Tough, 1991). Infrequent cross-walls (as well known as vegetative septa) divide the mycelial network into syncytial hyphal cells with multiple copies of the genome. In result, the cellular compartment
enclosed between two cross-walls is capable of further extension only by initiating a new branch. Formation of new growing tips by branching leads to a quasi-exponential increase in mycelial biomass (Chater and Losick, 1997).

Fig. 1. The complex life cycle of *Streptomyces coelicolor* proceeds through several developmental stages (cartoon kindly provided by Nora Ausmees)
Vegetative mycelium is indicated in orange; aerial mycelium is shown in white and the chains of spores – in black and grey.

The hyphal mode of growth of *S. coelicolor* poses several interesting questions about its cellular biology. One concerns the mechanisms that mark the tip and guide the assembly of the peptidoglycan synthetic machinery towards the zone of growth. The first component of a presumably large protein complex associated with the hyphal tip has recently been identified (Flärdh, 2003a). DivIVA protein of *S. coelicolor* affects morphogenesis and is involved in establishment of new tips.

Unlike the polarized extension of the cell wall, DNA synthesis takes place throughout a large portion of the hyphae (Prosser and Tough, 1991). The growing tip and the lateral branches need to be populated by chromosomes. This suggests the existence of a mecha-
nism for transporting DNA along the hyphae (Flärdh, 2003b; Prosser and Tough, 1991).

Another interesting question concerns the link between tip extension and cell division. The frequency and spacing of vegetative septa are subject to considerable variation. Thus, no firm rules for the placement of cross-walls have been formulated although work by Kretschmer on the closely related *S. granaticolor* suggested a model in which septation was initiated around the middle of the apical cell when it reached a critical length (Flärdh and van Wezel, 2003; Kretschmer, 1982). It is clear that tip extension and branching does not require cross-wall formation (McCormick *et al*., 1994), although the frequency and placement of branches is probably affected by the cross-walls. Another unresolved question is whether the hyphal cross-walls are permeable for trafficking of cellular material.

### 1.2. Growth in the air

Following the growth of a substrate mycelium the *S. coelicolor* colony enters a first stationary phase and switches to reproductive growth (Chater, 1993). The surface of the colony is covered by a mat of white aerial mycelium growing away from the substratum and into the air. The aerial mycelium grows parasitically at the expense of nutrients released by degradation of the substrate hyphae (Challis and Hopwood, 2003; Miguelez *et al*., 1999). The formation of aerial mycelium is the result of a complex cascade of intercellular signalling and is concurrent with a transition from primary to secondary metabolism and the production of antibiotics (Champness, 2000; Chater, 1998). Gamma-butyrolactone signalling molecules play important roles in regulation of antibiotics synthesis and differentiation in many streptomycetes (Takano *et al*., 2005).

The formation of aerial mycelium requires the activity of at least three groups of genes, as revealed by genetic analysis: the *ram*, *chp*, and *bld* genes. The *ram* and *chp* genes specify hydrophobic components allowing the hyphae to escape the watery surface of the colony and grow up in the air (Kodani *et al*., 2004; Nguyen *et al*., 2002) (Claessen *et al*., 2003; Elliot *et al*., 2003; Willey *et al*., 2006).

The *bld* genes were identified in genetic screens for *S. coelicolor* mutants defective in aerial mycelium formation (Hopwood *et al*., 1970; Merrick, 1976). All *bld* mutants appear bald on solid media since they lack the characteristic white fuzzy surface of the wild type colonies (Kelemen and Buttner, 1998). Many of the characterized *bld* genes encode regulatory proteins that exert pleiotropic effects on ae-
rial mycelium formation and antibiotic production (Champness, 2000; Chater and Horinouchi, 2003). Interestingly, some bld mutants can restore the aerial mycelium formation of other bld mutants when grown close to one another on rich medium (Willey et al., 1993). Furthermore, the latter extracellular complementation works only between certain pairs of bld mutants placing them in a hierarchy of complementation groups (Nodwell et al., 1999). Taken together, these observations suggest that the bld genes are in some way involved in the production and exchange of signalling substances during the process of differentiation.

1.3. Sporulation in aerial mycelium

At some point of development in the air, the apical parts of the aerial hyphae quit growing and undergo multiple septations, which results in formation of unigenomic spores. The factors that trigger the onset of sporulation are only partially understood.

Genetic studies have identified a group of genes, the whi genes, which are implicated in the regulation of sporulation (Chater, 1972; Hopwood et al., 1970). Mutations of each of these genes resulted in the production of characteristic white colonies because of the inability of the aerial mycelium to form grey spore pigment (Hopwood et al., 1970). The whi mutants form two distinct groups. The early whi mutants fail to produce sporulation septa (Chater, 1972; McVittie, 1974), while the late whi mutants are defective during the subsequent spore maturation (Chater, 1993; Davis and Chater, 1990; Kelemen et al., 1998). The whi genes are involved in an intricate network of morphological and metabolic checkpoints, which need to be met during the sequential stages of spore formation (Chater, 2000, 2001). The family of early whi genes consists of whiG, whiA, whiB, whiH, and whiL. whiG encodes an alternative sigma factor, $\sigma_{\text{WhiG}}$, required for the initiation of the sporulation program (Chater et al., 1989). whiG promoter is repressed by BldD developmental regulator, which shuts off many developmental genes during vegetative growth (Elliot et al., 2001). This dependence of $\text{WhiG}$ expression on BldD is the first known link between the whi and the bld regulatory cascades. The significance of this link is unclear since $\text{WhiG}$ is transcribed both in substrate and aerial hyphae (Elliot et al., 2001; Kelemen et al., 1996). $\sigma_{\text{WhiG}}$ is activated only in aerial hyphae by an unknown mechanism. $\sigma_{\text{WhiG}}$ RNA polymerase holoenzyme transcribes the early sporulation genes whiH (Ryding et al., 1998) and whiI (Ainsa et al., 1999). WhiH encodes a member of the GntR family of transcription factors and
whiI is a response regulator (Ainsa et al., 1999; Ryding et al., 1998). Both whiH and whiI repress their own expression. This repression is released at the time of initiation of septation.

Two other early whi genes, whiA and whiB, are expressed independently of whiG (Ainsa et al., 2000, Soliveri et al. 1992). Inactivation of any of these genes results in very similar and characteristic extremely long and tightly coiled aerial hyphae, which has lead to the suggestion that whiA and whiB are required for the arrest of aerial hyphal growth that precedes the onset of poration septation (Flärdh et al. 1999, Chater, 2000). It has been suggested that growth cessation generates signals that induce WhiH and WhiI to switch from autorepression to activation of later sporulation events (Chater, 2001). The function of these genes is not yet known, but whiA has homologues in most Gram-positive bacteria, and whiB belongs to a gene family that is only found in the actinomycetes.

On the basis of mutant phenotypes, also the whiJ locus may include an early whi gene, but this has not yet been fully characterised (Chater, 2000), and will not be further discussed here.

In summary, the early whi genes are involved in two regulatory pathways (one is dependent on $\sigma^{WhiG}$ and the second involves whiA and whiB) that control the activation of late sporulation events. All of these five early whi genes are needed for sporulation septation, presumably by activation of the sporulation-specific upregulation of ftsZ transcription from the ftsZ2p promoter (Flärdh et al., 2000). The early whi genes are also required for the transcription of sigF, which codes for a the late-acting $\sigma^F$ sigma factor, and for transcription of the genes for the spore pigment biosynthesis within the whiE locus (Kelemen et al., 1996; Kelemen et al., 1998). The similar sporulation phenotypes, including strongly reduced production of the grey pigment, of the whiH deletion mutant and ftsZ mutants (with ftsZ$\Delta2p$ or ftsZ17(Spo) alleles suggest that sporulation septation may be required for the expression of later genes (Flärdh et al., 2000). This could be a way to couple late sporulation events to the completion of sporulation septa.
2. Importance of studying cell division in *S. coelicolor*

The study of cell division in *S. coelicolor* is for several reasons both interesting and highly relevant. Firstly, cellular multiplication is fundamental to life and finding the molecular mechanisms behind this process is a major task in cell biology. Then, why is it of particular relevance in *S. coelicolor*? As will be discussed in detail in later sections, this organism has a typical bacterial cell division machinery, based on the tubulin-homologue FtsZ. However, none of the mechanisms and genes that have been shown to control the critical early stages of the cytokinesis, the assembly of FtsZ into a cytokinetic ring at the division site, in other bacteria like *Escherichia coli* and *Bacillus subtilis* are present in *Streptomyces*. They are in fact absent from most actinomycetes, including the mycobacteria. Thus, no extrapolations can be made, and there is very little insight into how cell division is regulated in this large and important phylogenetic lineage of bacteria. Such insights should be useful for example for developing antimicrobial drugs targeting cell division in the causative agent of tuberculosis, *Mycobacterium tuberculosis*. They should also be complementary to the models for cell division control in other bacteria, and therefore add to our general understanding of bacterial cytokinesis, its diversity and evolution.

As an experimental model organism for studying cell division, *S. coelicolor* is in several ways unique and attractive. This hyphal bacterium makes use of two distinct types of cell division. They both require FtsZ and involve the same basic machinery, but take place in two different cell types which are formed in the course of a complex developmental program. The septation in the substrate mycelium is infrequent and dispensable for growth and viability. Thus, even *ftsZ*-null mutants can be propagated, which simplifies some genetic work. In contrast to that, during sporulation the aerial hyphae are converted into spores by multiple developmentally controlled cell divisions. Those septa are required for spore formation and production of the grey spore pigment. Thus, this organism has a built-in indicator of cell division (grey pigmentation of colonies) that can be employed in genetic screens. The molecular mechanisms implicated in the spatial and temporal regulation of this type of cell division is also of interest in bacterial developmental biology. Septation is under control of and integrated with an intricate regulatory network underlying the mor-
phological differentiation in *S. coelicolor*. These aspects will be developed further in later chapters of this thesis.

Finally, streptomycetes are major antibiotic producers. Therefore, it is of fundamental importance to understand how these organisms grow and proliferate, and *S. coelicolor* is by far the most studied genetic model among the streptomycetes. Knowledge about the control of cell division and how it relates to growth and branching will be useful for solving the technical problems involved in handling mycelial organisms in large-scale fermentations, e.g. inoculum preparations and prevention and control of mycelial clumping and pellet formation. The ability to affect mycelial growth by manipulation of cell division control is therefore desirable goal.

### 3. Bacterial cell division and the role of FtsZ

Binary fission is the paradigm for bacterial cell division. One cell is divided into two progeny cells. This process, known as cytokinesis, is highly regulated in time and space in order to provide faithful partitioning of the genetic information. Bacterial cytokinesis starts with the assembly of the prokaryotic tubulin homologue, FtsZ, in a ring-like structure at the future division site (Bi and Lutkenhaus, 1991; Lowe and Amos, 1998). The Z ring is anchored to the cell membrane and is thought to utilize the energy of GTP hydrolysis (de Boer et al., 1992) for constriction of the cell. In addition, the Z ring recruits a number of proteins required to complete the cytokinesis. Thus, it can be assumed that the spatial and temporal control of cell division would be exerted at the level of assembly of FtsZ into the Z ring.

The process of cell division in *S. coelicolor* is different from the binary fission paradigm. This organism forms two types of hyphal cells which use two different modes of cell division – cross-wall formation without cell separation in substrate hyphae and multiple “true” divisions during sporulation in aerial hyphae. Both processes involve the assembly of FtsZ into cytokinetic rings.

It’s justified to assume that some of the major aspects of FtsZ assembly and function and the regulation of cell division will be valid
for a large variety of species. The following chapter provides a summary of the knowledge about the latter processes in the mostly studied model organisms. This will provide a logical framework for understanding the peculiarities of the cell division in *S. coelicolor*.

### 3.1. Assembly of the Z ring and the cell division machinery

FtsZ is a highly conserved protein. It is the main cell division protein in most bacteria and in the euryarchaeal branch of Archaea. FtsZ is also involved in the division of chloroplasts and some primitive mitochondria (Vaughan *et al.*, 2004).

FtsZ contains four protein regions: a variable N-terminal segment, a highly conserved core region, a variable linker, and a C-terminal tail (Fig. 2) (Margolin, 2005; Vaughan *et al.*, 2004) The functions of the N-terminal segment and the linker are unknown. The core region is responsible for the GTP-binding and hydrolysis which is required for the polymerization of the protein. The core region consists of two independently folding N-terminal and C-terminal domains (Oliva *et al.*, 2004). The C-terminal tail of *E.coli* FtsZ is the binding site for two other division proteins, FtsA and ZipA (Ma and Margolin, 1999; Pichoff and Lutkenhaus, 2002). There is little conservation between the C termini of FtsZ from different organisms (Din *et al.*, 1998; Romberg and Levin, 2003; Vaughan *et al.*, 2004).

![Fig. 2. The structure of FtsZ comprises four regions. (Adapted from Margolin, 2005)](image)
3.1.1. Polymerization of FtsZ

FtsZ is a structural homologue of tubulin. This is evident from the three-dimensional structures of FtsZ of *Methanococcus jannaschii* (Lowe and Amos, 1998) and eukaryotic αβ tubulin (Nogales *et al*., 1998b). FtsZ hydrolyses GTP and assembles into protofilaments similar to the ones formed by tubulin. FtsZ protofilaments consist of head-to-tail linear polymers (Erickson *et al*., 1996; Lowe and Amos, 1999; Mukherjee and Lutkenhaus, 1994). GTP binds at the “plus” end of the FtsZ monomer. Addition of the next monomer completes the GTPase active site; the second monomer interacts with the previous subunit and the nucleotide via the T7 loop at its “minus” end. Thus, GTP hydrolysis requires FtsZ polymerization. The latter is disturbed by the SOS cell division inhibitor SulA which binds to the T7 loop (Cordell *et al*., 2003).

The GTP binding residues are the ones that are most conserved between FtsZ and tubulin. Seven of these amino acids constitute the tubulin signature motif which is found in all tubulins (Lowe and Amos, 1998; Nogales *et al*., 1998a).

Despite those similarities there are notable differences between FtsZ and tubulin concerning the kinetics of the GTPase reaction (Romberg and Mitchison, 2004). GTP hydrolysis takes place immediately upon tubulin polymerization, while nucleotide exchange is very slow. That’s why the polymerized tubulin is mostly GDP-bound, with the GTP-containing subunits forming a cap at the growing end of the polymer. In result, the tubulin protofilaments are susceptible to depolymerisation once the cap is hydrolysed. This leads to the characteristic dynamic instability of microtubules which is in the basis of their mode of function. By contrast, it has been shown that GTP-hydrolysis is the rate-limiting step for FtsZ polymerization (Romberg and Mitchison, 2004) and the protofilaments are mostly GTP-bound (Huecas and Andreu, 2003; Mingorance *et al*., 2001). These data suggest that the existence of dynamic instability in FtsZ polymers is highly unlikely. FtsZ can assemble in the presence of GDP as well forming curved polymers (Lu *et al*., 2000). Their relevance *in vivo* is unclear having in mind that the bound GDP can be easily exchanged for GTP from its large intracellular pool.

Since direct visualization of the Z ring by electron microscopy has proven impossible, insights into the structure of the FtsZ polymer in the Z ring have been gained by studies of FtsZ polymers formed *in vitro* (Romberg and Levin, 2003). It is generally assumed that the FtsZ
protofilaments, observed under a variety of conditions in vitro are the building blocks of the Z ring. The longitudinal interactions between the subunits in these protofilaments are similar to the ones in the tubulin polymers. The lateral arrangement of FtsZ protofilaments which is likely to be very different from microtubules is an open question (Nogales et al., 1998a). In vitro, FtsZ protofilaments associate in higher order structures such as bundles, sheets and tight spirals. Formation of these structures is induced by several factors, such as cations (Lowe and Amos, 1999), macromolecular crowding (Gonzalez et al., 2003) and binding of interacting proteins, ZipA and ZapA (Gueiros-Filho and Losick, 2002; Hale et al., 2000). The isolation of a mutant FtsZ that fails to support division and assembles into protofilaments but not Ca$^{2+}$-induced bundles suggest that higher order structures are essential for FtsZ function in vivo (Koppelman et al., 2004).

FtsZ from M. tuberculosis has recently been crystallized as a tight, laterally oriented dimer (Leung et al., 2004). These structural results combined with previous mutational data, suggest a mechanism by which GTP hydrolysis induces a G-protein like switch in FtsZ conformation and thus leads to lateral assembly of FtsZ monomers into dimers. The latter are proposed to be the building blocks for spiral protofilaments. This model is supported by the ability of FtsZ from M. jannaschii and E. coli to assemble into double-stranded protofilaments (Oliva et al., 2003). These results emphasize the importance of a variety of model systems in elucidating the structure of the Z ring in vivo.

3.1.2. Dynamic assembly of the Z ring

The Z ring is remarkably dynamic. It is able to assemble and disassemble quickly, within 1 min or less (Addinall et al., 1997; Sun and Margolin, 1998). During the cell cycle of E. coli the Z ring goes through phases of assembly, steady state turnover and disassembly. Microscopic studies with rapidly growing cells have revealed that the Z ring forms 1 to 5 min after the previous division and is present for 15 more min, after which constricts to divide the cell (Den Blaauwen et al., 1999; Sun and Margolin, 1998). In addition to this, FRAP (fluorescence recovery after photobleaching) experiments have recently shown that the formed Z ring undergoes continuous and rapid turnover (Stricker et al., 2002). According to the latter study 30% of cellular FtsZ is engaged in the Z ring which readily exchanges subunits with
FtsZ in the cytosol, the half-time for remodelling of the Z ring being 30 sec. An even shorter half-time of about 9 sec in both *E. coli* and *B. subtilis* was suggested by a more recent work (Anderson et al., 2004). Interestingly, ZipA, a division protein which localizes to the Z ring in *E. coli* was also shown to be turning over (Stricker et al., 2002).

The rapid turnover of the Z ring may have interesting implication concerning the mechanism of for generating the force for constriction. It can be imagined that if FtsZ subunits are being removed from the Z ring faster then they are replaced the circumference of the Z ring would shrink thus powering the cytokinesis. Another interesting possibility is that the rapid turnover of the Z ring is implicated in the control of cell division (Romberg and Levin, 2003). The dynamic maintenance of the Z ring might be providing a means to reverse the Z-ring formation if cell division is to be blocked, as in the case of SOS response in *E. coli* (Bi and Lutkenhaus, 1993). Another example of disassembly of a formed Z ring is the switch from medial to polar separation in *B. subtilis* (Ben-Yehuda and Losick, 2002). This will be discussed in detail in the section “Developmental control of cell division”.

### 3.1.3. Mechanism of constriction

Several models for Z-ring constriction have been described (Bramhill, 1997; Errington et al., 2003; Weiss, 2004). The first model proposes sliding of stable FtsZ filaments against each other aided by a hypothetical motor protein. In the second model, FtsZ filaments lose subunits by depolymerization leading to ring constriction. In a third model, constriction is driven by curvature of the polymer upon GTP hydrolysis. More experimental data are required in order to distinguish between these models. Moreover, it has yet to be firmly established whether FtsZ provides the driving force for cytokinesis. An alternative possibility is that the ingrowth of the murein leads to constriction of the cell.

### 3.1.4. Assembly of the divisome

The assembly of the cell division machinery, the divisome, starts with the formation of the Z ring which recruits a set of other proteins to the division site (reviewed in (Errington et al., 2003; Margolin,
The number and identity of the division proteins varies significantly between species. The divisome of *E. coli* comprises at least 12 proteins. The proteins of the divisome have a multitude of functions throughout cell division: modulation of FtsZ assembly (FtsA, ZipA, ZapA), anchoring the Z ring to the membrane (FtsA, ZipA), coordination of division with chromosome segregation (FtsK), synthesis of septal peptidoglycan (FtsI, FtsW), and peptidoglycan hydrolysis during separation of daughter cells (AmiC, EnvC). Some of the proteins of the divisome are of essentially unknown function (FtsEX, FtsQ, FtsB, FtsL, FtsN).

The proteins of the divisome are engaged in multiple interactions which are important for their functions in cytokinesis. It has been shown that FtsA and ZipA in *E.coli* interact directly with the C terminus of FtsZ (Pichoff and Lutkenhaus, 2002) and are required for stabilizing the Z ring. The ZapA protein of *B. subtilis* also binds directly to FtsZ (Gueiros-Filho and Losick 2002). Once FtsA and ZipA are bound to the Z ring the remaining proteins are recruited in a defined and hierarchical order (Buddelmeijer and Beckwith, 2002) as follows: FtsE+FtsX → FtsK → FtsQ → FtsL + FtsB → FtsW → FtsI → FtsN → AmiC. The latter linearity of recruitment may be reflecting the assembly pathway for a multiprotein complex. Interestingly, the recruitment of proteins to the divisome in *B. subtilis* seems to be much more interdependent, pointing towards a cooperative fashion of assembly (Errington et al., 2003).

Recent data from bacterial two-hybrid experiments suggest that the pathway for divisome assembly in *E. coli* is more complex than previously assumed (Di Lallo et al., 2003; Karimova et al., 2005). These results show that most of the Fts proteins interact with multiple partners. These findings suggest a model for the assembly of the divisome in which multiple low-affinity interactions provide stability of the whole complex. Some of the Fts proteins were shown to preassemble in the cell outside of the context of the divisome (Goehring et al., 2005). FtsQ, FtsL, FtsB, FtsW, and FtsI assemble into a subcomplex which is recruited to the septum through the interaction of FtsQ with FtsK. The cooperativity that stems from a network of interactions has important implication for maintaining the divisome as a stable and in the same time, dynamic structure.
3.2. Regulation of Z-ring assembly

The process of cell division is tightly controlled in space and time in order to ensure that the genetic material is identically distributed in the progeny. Cell division starts with the formation of the Z ring which recruits the rest of the division machinery to the septation site. Thus, the assembly of the Z ring is in the spotlight of the molecular mechanisms for control of cell division in bacteria.

3.2.1. Cell-cycle timing

Cell division normally follows each round of chromosome replication in \textit{E. coli} (Donachie, 2001). The onset of the Z-ring formation coincides with the termination of DNA synthesis and takes place between largely segregated nucleoids (Den Blaauwen \textit{et al.}, 1999). The factors that determine the timing of the Z ring are still unknown.

It has been suggested that the assembly of the Z ring is coupled to chromosome replication in \textit{E. coli}, \textit{B. subtilis} and \textit{C. crescentus} (Den Blaauwen \textit{et al.}, 1999; Harry \textit{et al.}, 1999; Quardokus and Brun, 2002; Regamey \textit{et al.}, 2000). However, blocking the initiation of DNA replication does not prevent the Z-ring assembly in either of the three model organisms (Gullbrand and Nordstrom, 2000; Harry \textit{et al.}, 1999; Quardokus and Brun, 2002; Sun and Margolin, 2001). Although DNA replication is dispensable for Z-ring assembly, the correct positioning of the Z ring is dependent on replication initiation in both \textit{B. subtilis} and \textit{C. crescentus} (Harry \textit{et al.}, 1999; Quardokus and Brun, 2002; Sun and Margolin, 2004). Based on these observations a model for the coordination between chromosome replication and cell division has been proposed (Harry \textit{et al.}, 1999; Regamey \textit{et al.}, 2000). This model predicts the existence of a nucleation site for the Z-ring assembly at midcell which is masked by the replication factory during DNA synthesis and unmasked later on, upon segregation of the duplicated nucleoids. The model is consistent with the previously observed stationary placement of the replication machinery at midcell in \textit{B. subtilis} and with the suggested common mechanism of sequential positioning of the origin, the replication factory, the Z ring and the terminus at the future division site in \textit{E. coli} (Lau \textit{et al.}, 2003; Sherratt, 2003). However, recent results have shown that the replication factory in \textit{B. sub-
tilis is highly mobile and more scattered around midcell than the Z ring which is inconsistent with a role in determining the precise placement of the Z ring (Migocki et al., 2004).

An alternative way to ensure the proper timing of cell division is to regulate the levels of production of FtsZ and other division proteins throughout the cell cycle. Accordingly, the expression of several cell division genes was shown to be linked to DNA replication in E. coli (Liu et al., 2001). However, the FtsZ protein levels remain fairly constant throughout the cell cycle during steady state growth of E. coli and B. subtilis (Weart and Levin, 2003). Moreover, altering the FtsZ concentration in the cell is not sufficient to influence the timing of the Z-ring assembly at midcell although overexpression of FtsZ leads to increased frequency of polar Z rings in both E. coli and B. subtilis (Palacios et al., 1996; Ward and Lutkenhaus, 1985; Weart and Levin, 2003). All these observations suggest that the FtsZ activity rather than expression is cell-cycle controlled. The molecular mechanisms of the latter regulation remain to be uncovered.

In contrast to the situation in E. coli and B. subtilis, FtsZ protein levels in C. crescentus vary due to cell-cycle dependent proteolysis and re-synthesis (Kelly et al., 1998). Transcriptional regulation and proteolysis ensure that FtsZ is absent from swarmer cells which do not go through DNA replication and division. FtsZ is highly expressed prior to cytokinesis in the reproductive stalked cells and disappears from those upon septation. However, ectopic expression of FtsZ at the wrong time in the cell cycle does not lead to Z-ring formation (Quardokus et al., 2001; Quardokus and Brun, 2002). Thus, it is not the FtsZ levels per se but rather the state of the cell cycle progression that modulates the initiation of the Z ring.

Another interesting feature of the cell cycle control of septation in E. coli is that FtsZ and FtsA are produced in amounts which can support only a single division event per cell cycle (Begg et al., 1998; Bi and Lutkenhaus, 1990). The molecular mechanisms behind that regulation are unknown (Donachie, 2001).

### 3.2.2. Spatial regulation

The spatial regulation of cell division is exerted by determining the site for the Z-ring placement. Two overlapping mechanisms guide the proper placement of the Z ring in E. coli and B. subtilis. The “nucleoid occlusion” prevents the formation of Z rings over the chro-
mosomes and limits potential division sites to the DNA-free regions at midcell and cell poles (Rothfield et al., 2005; Sun and Margolin, 2004; Woldringh et al., 1991). Another pathway carried out by the Min proteins suppresses the formation of polar Z rings (Bi and Lutkenhaus, 1993; de Boer et al., 1989; Lee and Price, 1993; Rothfield et al., 2005). The nucleoid occlusion and the Min system have partially redundant function and hence it is possible to isolate viable mutants defective in only one of the two processes.

The Min system of *E. coli* consists of three proteins. MinC is a direct inhibitor of FtsZ assembly (Hu et al., 1999). MinD is an ATPase which reversibly binds the cellular membrane. MinD forms a complex with MinC that oscillates from one cell pole to the other causing FtsZ depolymerization on its way (Raskin and de Boer, 1999b). MinE gives a topological specificity to the action of the MinCD complex (Raskin and de Boer, 1999a). MinE is capable of displacing MinC from the membrane-bound MinCD complex (Hu et al., 2003; Ma et al., 2004; Suefuji et al., 2002). MinE binds to MinD and stimulates its ATPase activity which leads to dissociation of the latter from the membrane. MinD-ADP diffuses in the cytosol, exchanges nucleotide to form MinD-ATP and reassembles on the membrane at the opposite pole where the concentration of MinE is lowest (Hu and Lutkenhaus, 2001; Huang et al., 2003; Suefuji et al., 2002). In result of the oscillation behaviour of the MinCD complex, the time-averaged concentration of MinC is highest at the poles and lowest at midcell, thus leading to inhibition of polar Z-ring formation.

Recent results suggest that the pole-to-pole oscillation of the Min proteins follows a membrane-associated helical path (Shih et al., 2003). Interestingly, the FtsZ outside of the Z ring has been shown to move rapidly in a spiral-like pattern (Thanedar and Margolin, 2004). Moreover, FtsZ establishes oscillation waves within that helical pattern, with the period of oscillation comparable to that of the Min proteins (Thanedar and Margolin, 2004). The latter results may be indicative of the presence of a highly mobile FtsZ cytoskeleton in *E. coli* which is able to probe the cell membrane for potential division sites. The rapid movement of FtsZ may be directed by the Min proteins. An interesting question for the future is whether FtsZ travels along a helical path established by the protein itself or predetermined by another factor.

The oscillatory behaviour of MinD has been simulated *in silico* on the basis of the biochemical properties of the Min proteins and FtsZ. These simulations predict that the Min proteins can self-organize
into an oscillatory system thus generating positional information within the cell (Howard and Kruse, 2005; Kruse, 2002). Consistent with this notion MinCDE from the round-shaped *Neisseria gonorrhoeae* are capable of oscillations within the rod-shaped *E. coli* (Ramirez-Arcos et al., 2002).

The MinCD complexes of *B. subtilis* are static and anchored to both poles via the DivIVA protein which is polarly localized (Marston et al., 1998). While the Min system is present in many bacterial species it is not universally conserved. Many species, such as *Caulobacter crescentus* and *S. coelicolor* lack the Min proteins. This leaves space for alternative and largely unidentified systems for spatial control of cell division.

Nucleoid occlusion is another important mechanism for positioning of the division site. Nucleoid occlusion inhibits the assembly of the Z ring in regions occupied by chromosomal DNA (Woldringh et al., 1991). Inactivation of the nucleoid occlusion leads to formation of septa over unsegregated nucleoids and “guillotining” of the chromosome (Sun and Margolin, 2004). On the other hand, in the absence of an active Min system multiple Z rings can assemble in the DNA-free regions. Taken together, these two observations suggest that the whole surface of the cell membrane is equally competent for Z-ring formation (Yu and Margolin, 1999).

Two proteins potentially mediating the nucleoid occlusion have been recently identified in *E. coli* and *B. subtilis* (SlmA and Noc, respectively) (Bernhardt and de Boer, 2005; Wu and Errington, 2004). These proteins were pulled out in genetic screens based on the combined lethal effect of mutations in both the Min system and nucleoid occlusion pathways. Deletions of *slmA* and *noc* in cells lacking the Min system resulted in the frequent formation of FtsZ structures over nucleoids in addition to normal medial Z rings. The excessive non-productive FtsZ formations sequestered the FtsZ protein and blocked cell division. Moreover, artificial block of the DNA replication in cells lacking *slmA* and *noc* led to guillotining of nucleoids. The latter results indicate that SlmA and Noc are involved in checkpoint that prevents guillotining of chromosomes upon DNA replication defects. SlmA and Noc are not related to each other but both are DNA-binding proteins. SlmA was shown to affect FtsZ assembly in vitro suggesting that the two proteins may have a direct effect on Z-ring formation in vivo. The normal appearance of the *slmA* and *noc* cells and the fact that the majority of Z rings still localizes around midcell in the ab-
The presence of SlmA or Noc and MinCD suggest the presence of additional nucleoid occlusion pathways in the cell.

### 3.2.3. Developmental control of cell division

Sporulation in *B. subtilis* and the formation of stalked and swarmer cells by *C. crescentus* are the most studied examples of prokaryotic development. Both organisms use a developmentally programmed asymmetric cell division to generate progeny with different cellular fates.

During vegetative growth *B. subtilis* divides at midcell with remarkable precision (Errington et al., 2003; Harry, 2001). Upon entry into sporulation, the activity of the master regulator SpoOA triggers a switch from medial to polar division (Levin and Losick, 1996). This asymmetric division is central to the establishment of differential gene expression in the forespore and the mother cell (reviewed in (Barak and Wilkinson, 2005; Errington et al., 2003; Hilbert and Piggot, 2004). Recent work has shown that the switch involves redeployment of the FtsZ protein from a medial Z ring into two Z rings at the opposite poles of the cell. During this process a dynamic helical intermediate of the Z ring was formed (Ben-Yehuda and Losick, 2002). The switch to polar Z-ring formation requires the activity of SpoIIE protein and the increased expression of the *ftsAZ* operon from a sporulation-specific σH-dependent promoter (Ben-Yehuda and Losick, 2002).

SpoIIE localizes to the polar septum in a FtsZ – dependent manner (Barak et al., 1996; Levin et al., 1997). This protein is a serine phosphatase which plays a crucial role in sporulation by coupling the asymmetric division to the activation of the first forespore-specific transcription factor, σF (Duncan et al., 1995; Hilbert and Piggot, 2004). SpoIIE interacts with FtsZ *in vitro* and in yeast two-hybrid assays (Lucet et al., 2000). It has been proposed that this interaction affects the formation of the polar Z-ring by anchoring the FtsZ helical intermediate to the cell membrane or by recognizing a polar marker (Ben-Yehuda and Losick, 2002). However, the recent finding that the membrane-spanning domain of SpoIIE has little or no effect on polar septation speaks against the former notion (Carniol et al., 2005).

Prior to asymmetric division in *B. subtilis*, two Z rings are formed near the opposite poles of the cell. However, only one of these potential division sites is used for septation. The block of the second polar division requires three proteins expressed in the mother cell un-
der the control of the $\sigma^E$ transcription factor (Eichenberger et al., 2001). Another interesting question is how the choice of the Z ring that will be utilized for division is made by the cell. FtsA has been implicated in the selection between potential division sites since this protein localizes to only one of the two polar Z rings (Feucht et al., 2001). In addition to that, in the majority of cases spores are being formed at the older pole of the cell. Thus an unknown marker at the cell pole may be another possible determinant of the asymmetric cell division (Hitchins, 1975).

Asymmetric cell division in *C. crescentus* always yields two types of progeny cells with distinct developmental fates: a non-replicating swarmer cell that delays cell division and a stalked cell that immediately enters the next round of DNA replication and subsequent asymmetric cell division (Ausmees and Jacobs-Wagner, 2003; Quadokus and Brun, 2003). The swarmer cell uses a polar flagellum to actively explore the environment. In order to enter the division cycle, the swarmer cell sheds its flagellum and grows a stalk. The new stalked cell initiates DNA replication and grows a new flagellum at the pole opposite of the stalked pole. This cell undergoes asymmetric division which yields a swarmer and a stalked cell.

The asymmetric cell division is developmentally controlled at the level of selective protein synthesis and degradation of FtsZ in the respective cell types. FtsZ is produced only in the stalked cells that are actively forming a Z ring (Kelly et al., 1998; Quadokus et al., 1996; Quadokus et al., 2001). As the Z ring constricts the FtsZ protein is degraded. In result, FtsZ is absent from the newly divided swarmer and stalked cells (Kelly et al., 1998; Quadokus et al., 2001). FtsZ is de novo synthesised upon swarmer-to-stalked cell differentiation and initially localizes at the stalked pole. Later in the cell cycle FtsZ localizes to midcell (Quadokus et al., 2001).

Transcription of FtsZ in the swarmer cells is directly repressed by the activity of the CtrA response regulator. CtrA inhibits chromosome replication (Quon et al., 1998) in the swarmer cells and regulates the transcription of more than 100 genes involved in various aspects of the cell cycle and polar morphogenesis (Laub et al., 2002). CtrA itself is regulated at different levels including transcription control, localization, phosphorylation and programmed proteolysis (Domian et al., 1997; Domian et al., 1999). CtrA is proteolysed during the swarmer-to-stalked cell differentiation which allows chromosome replication (Domian et al., 1997). Later on, CtrA is produced in dividing cells where it is required for the transcription of the cell division
proteins FtsQ and FtsA (Sackett et al., 1998; Wortinger et al., 2000). During the asymmetric cell division CtrA is proteolysed in the stalked compartment and is retained in the swarmer progeny. Thus, CtrA mediates several checkpoint mechanisms coupling development to cell division and DNA replication (Ausmees and Jacobs-Wagner, 2003; Quardokus and Brun, 2003; Ryan and Shapiro, 2003; Wortinger et al., 2000).

4. FtsZ and cell division in S. coelicolor

The mycelial life style and the complex development of S. coelicolor impart unique features to the process of cell division in this organism. S. coelicolor exploits two distinct modes of septation which are confined to different cellular types. Infrequent cross-wall formation takes place in the branched substrate hyphae. Later in development, the apical compartments of the aerial hyphae are synchronously divided into arrays of unigenomic spores. The switch between the two modes of division depends on a variety of signals from developmental pathways and is crucial for the completion of the sporulation program (Chater, 2000).

4.1. Two types of cell division

The two types of cell division in S. coelicolor differ significantly with respect to function, septal morphology, regulation and coupling to chromosome segregation (Flärdh and van Wezel, 2003).

During substrate growth the closure of septa takes place without subsequent detachment of the progeny cells (Wildermuth and Hopwood, 1970). In result, multicellular mycelium is formed with the individual cell compartments being delimited by cross-walls. The hyphal cells contain multiple copies of the genome and cross-wall formation takes place between minorities of the chromosome pairs. Thus, the processes of cell division and DNA replication in the substrate hyphae are coordinated in a fashion, which is very different from the one in E. coli. In the latter each round of replication is followed by cell division at the middle of the cell, between the segregated nucleoids (Donachie, 2001).

The hyphal cells of S. coelicolor grow by tip extension. New peptidoglycan is incorporated at the tips of the apical cells (Flärdh,
Studies in *S. granaticolor* showed that the growing apical cell divides at the middle after reaching a certain critical length (Kretschmer, 1982). However, the frequency and spacing of cross-walls vary between different species of *Streptomyces* and depend on the growth conditions. Thus, the link between tip extension and cross-wall formation during substrate growth of streptomycetes remains unclear.

In contrast to hyphal cross-walls, sporulation septa in *S. coelicolor* lead to separation of individual spores. Sporulation septa are thicker than the hyphal cross-walls (Wildermuth and Hopwood, 1970) and studies in *Streptomyces griseus* showed that the two types of septation involve different sets of penicillin – binding proteins (Hao and Kendrick, 1998). Sporulation septa are placed synchronously and regularly along the length of the sporogenic aerial hyphae. Normally one septum is formed between each pair of chromosomes (Flärdh et al., 1999; Schwedock et al., 1997; Wildermuth and Hopwood, 1970). Thus, cell division during sporulation needs to be co-ordinated with chromosome replication and partitioning in order to insure proper segregation of the genetic material in the spores. Furthermore, the observation that sporulation septa are being placed right up to the hyphal tips suggests that sporulation is preceded by growth arrest in the sporogenic hyphae (Flärdh et al., 1999; Schwedock et al., 1997). The factors that are implicated in the developmental regulation of cell division will be discussed later on.

### 4.2. Cell division machinery

Cell division in *S. coelicolor* starts with the assembly of FtsZ into a cytokinetic ring at division sites (Flärdh and van Wezel, 2003; Schwedock et al., 1997). While being essential for division FtsZ is dispensable for growth and viability of *S. coelicolor* (McCormick et al., 1994). An *ftsZ* deletion mutant is viable and can be propagated in the form of non-septated branching hyphae (McCormick et al., 1994). Thus, *S. coelicolor* is unique among FtsZ-containing species in its ability to survive upon deletion of FtsZ. The latter property of this bacterium makes it a useful model for genetic studies of FtsZ.

Immunofluorescence microscopy revealed that FtsZ assembles into a spectacular ladder of regularly spaced Z rings in the apical compartment of sporogenic aerial hyphae (Schwedock et al., 1997). This process is remarkably synchronous within one sporulating com-
partment and requires increased expression of \( ftsZ \) in the latter. Sporulation specific upregulation of \( ftsZ \) production is achieved by increased activity of one of the three \( ftsZ \) promoters in sporogenic hyphae while the expression of \( ftsZ \) from the main vegetative promoter goes down during development (Flärdh et al., 2000). Accordingly, inactivation of the developmentally regulated \( ftsZ \) promoter was shown to inhibit sporulation septation without affecting vegetative growth (Flärdh et al., 2000).

The genome sequence of \( S. coelicolor \) A3(2) (Bentley et al., 2002) contains several obvious homologues of cell division proteins present in other bacteria. This suggests that the basic design of the cell division machinery in \( S. coelicolor \) may be similar to the one in most other studied bacteria. In the same time the functions of the putative cell division proteins in this organism are largely unknown (Flärdh and van Wezel, 2003).

Several cell division proteins are encoded in the highly conserved \( dcw \) gene cluster (division and cell wall synthesis) in many bacterial species (Mingorance et al., 2004). In \( S. coelicolor \), the \( dcw \) cluster consists of \( ftsL, ftsI, ftsW, ftsQ \) and \( ftsZ \) and lacks the widely conserved \( ftsA \) (Flärdh and van Wezel, 2003). The FtsA protein is an essential ATPase that interacts directly with FtsZ and serves as a membrane anchor of the Z ring (Errington et al., 2003; Goehring and Beckwith, 2005; Pichoff and Lutkenhaus, 2005). \( ftsQ \) is situated immediately upstream of \( ftsZ \) and encodes a small membrane protein with unknown function (Goehring and Beckwith, 2005). FtsQ is essential for cell division in \( E. coli \). An \( ftsQ \) null mutant of \( S. coelicolor \) does not support septation during sporulation but is viable and able to form cross-walls although with a severely reduced frequency (McCormick and Losick, 1996). Two independent mutations that compensate for the division defect of the \( ftsQ \) deletion have been isolated (Bennett and McCormick, 2001). These suppressor mutations map to two uncharacterized loci on the chromosome and therefore appear to identify two novel genes involved in septation in \( S. coelicolor \).

FtsL in \( E. coli \) and DivIC in \( B. subtilis \) are small single-pass transmembrane proteins involved in cell division and essential for survival (Errington et al., 2003; Goehring and Beckwith, 2005). An obvious homologue of each of these proteins is encoded in the genome of \( S. coelicolor \). FtsW is an integral membrane protein of unknown function. FtsW belongs to the FtsW/RodA group of proteins which affect cell division, cell shape and elongation and are encoded
in pair with a penicillin–binding protein (PBP) (Errington et al., 2003; Goehring and Beckwith, 2005). In *S. coelicolor* *ftsW* is located downstream of *ftsI* which encodes a penicillin–binding protein involved in cell division. The genome of *S. coelicolor* contains three more gene pairs coding for an FtsW/RodA type of protein and a PBP. FtsK of *E. coli* is a membrane-associated protein which is required for recruitment of several division proteins and is involved in septal closure (Weiss, 2004). FtsK has a role in resolution of chromosome dimers and DNA-translocation (Aussel et al., 2002; Weiss, 2004). The homologue of FtsK in *B. subtilis*, SpoIIIE, is involved in pumping one of the chromosomes into the prespore during the asymmetric cell division (Bath et al., 2000). The genome of *S. coelicolor* encodes one clear FtsK-homologue and a number of *ftsK*-related genes with unknown functions.

*S. coelicolor* strains containing null mutations for the division genes *ftsI, ftsK, ftsL, ftsW* and *divIC*, have been isolated and characterized (Bennett et al., 2002; McCormick, 2001). The isolated mutants showed variable degrees of sporulation defects and none was completely deficient in cell division. Thus, all five genes are dispensable for growth and viability of *S. coelicolor*, but are required for efficient spore formation. Interestingly, the phenotypes of the listed division mutants differed in severity depending on the culturing medium.

From the latter summary two major points can be made concerning the organisation and function of the cell division machinery in *S. coelicolor*. Firstly, the genome of this organism lacks homologues of proteins known to be involved directly in modulation of FtsZ assembly, such as FtsA, ZipA, ZapA, EzrA or MinC and anchoring the Z ring to the cell membrane (FtsA). Thus, novel proteins with homologous functions are to be found in *S. coelicolor*. Secondly, the facts that none of the studied division genes in *S. coelicolor* is essential for cell division but all are required for efficient sporulation points to the conclusion that the two types of septation are differentially controlled and sporulation septation is more sensitive to perturbations. Other not mutually exclusive explanations are that some of the components of the cell division machinery are functionally redundant and that there may be differences in the constitution of the cell division apparatus during development.
4.3. Developmental control of cell division in *S. coelicolor*

Several groups of genes have been implicated in the developmental control of cell division in *S. coelicolor*. The molecular mechanisms underlying the effect of these genes on septation are unclear and many of them code for proteins of unknown functions.

A set of sporulation regulators, the *whi* genes, have been identified in genetic screens through the inability of strains deleted for these genes to produce the grey spore pigment, which resulted in the characteristic white colour of the colony surface (Chater, 1972; Hopwood *et al*., 1970). The regulatory network specified by the *whi* genes (Chater, 2000, 2001), as well as the functions of their products are discussed in detail in section “Sporulation in aerial mycelium” of this thesis. The early *whi* genes (*whiG, whiA, whiB, whiH, and whiI*) are all required for the sporulation specific upregulation of *ftsZ* expression from the *ftsZ2p* promoter in sporogenic hyphae (Flärdh and van Wezel, 2003; Flärdh *et al*., 2000). This observation possibly explains the failure of the early *whi* mutants to produce sporulation septa. In the same study, the inactivation of the developmental *ftsZ* promoter produced a non-sporulating strain with a phenotype highly reminiscent of the *whiH* deletion (Flärdh *et al*., 2000; Ryding *et al*., 1998). This suggests that sporulation septation is needed for later sporulation processes, such as the synthesis of grey spore pigment. Furthermore, these results imply that *whiH* is primarily involved in the transcriptional control of *ftsZ*, while the other *whi* genes have additional functions during sporulation.

Other genes with a role in the developmental control of cell division in *S. coelicolor* are *ssgA* and *ssgB* (Flärdh and van Wezel, 2003). These belong to a group of paralogues found only in sporulating actinomycetes (van Wezel and Vijgenboom, 2004). SsgA possibly activates the formation of sporulation septa, as its overexpression resulted in fragmentation of the mycelia in liquid-grown cultures (van Wezel *et al*., 2000). *ssgA* mutants of *S. coelicolor* are defective in sporulation but produce normal vegetative septa (van Wezel *et al*., 2000). These observations suggest a direct correlation between SsgA and cell division during sporulation. The molecular mechanisms underlying this connection are unknown. *ssgB* deletion mutant fails in sporulation septation and produces abnormally large colonies (Keijser *et al*., 2003; Sevcikova and Kormanec, 2003). The mode of action of SsgB remains
unclear. Another interesting question relates to the interdependence between \textit{ssgA} and \textit{ssgB} and other developmental regulators. It has been shown that the transcription of \textit{ssgA} is independent of the regulatory network of the \textit{whi} genes (Traag \textit{et al.}, 2004).

Against this background, it is obvious that many genes with functions in regulation of cell division are still to be found in \textit{S. coelicolor}. 
The Question is not what you look at, but what you see.
Henry David Thoreau

The main goal of this work has been to identify the molecular mechanisms involved in the developmental modulation of the cell division machinery during sporulation of *S. coelicolor*.

In an attempt to address this question, genetic analysis of the main cell division protein, FtsZ in *S. coelicolor* was performed. The assembly of FtsZ into regularly spaced Z rings in sporulating aerial hyphae was visualized *in vivo*. The effects of several developmental regulators on this process were also studied.

The following two chapters represent a summary of the main experimental results from this study and discussion about the way in which they fit in the “Big picture” of the cell division control in *S. coelicolor*.

1. Experimental results

1.1. An FtsZ-EGFP translational fusion assembles into Z rings and spiral-shaped intermediates in sporulating aerial hyphae of *S. coelicolor* (Paper I)

We created a translational fusion between FtsZ from *S. coelicolor* and EGFP in order to visualize the assembly of FtsZ in both vegetative and aerial hyphae in this organism. The fusion was introduced on an integrating plasmid (pKF41) into the chromosomal ΦC31 attachment site of the wild type strain M145. The resulting K202 strain, containing both EGFP-tagged and untagged FtsZ, showed unaffected vegetative growth and sporulation. Fluorescence microscopy revealed that FtsZ-EGFP assembled, presumably as mixed polymers with the native FtsZ in this strain, into bands or ring-like structures perpendicular to the hyphal length axis in both vegetative and aerial
hyphae of K202 (Paper I, Fig.1). The appearance and distribution of these Z rings were similar to those observed previously by immunofluorescence microscopy (Schwedock et al., 1997).

In order to study the assembly of FtsZ in sporulating aerial hyphae in detail, the K202 strain was grown on MS agar and living hyphae attached to coverslips were examined microscopically after 28 to 48 h of growth. Many aerial-hyphal cells showed increased levels of fluorescence. This is in accordance with the developmentally controlled up-regulation of \(ftsZ\) during sporulation. Regularly spaced Z rings had formed in some of the sporogenic cells. In addition, characteristic fluorescence patterns different from regular Z ladders were repeatedly observed within the individual hyphae. These patterns appeared to change in relative abundance depending on the time of sampling. In order to facilitate the observation of these FtsZ structures, we applied deconvolution microscopy. This technique reduced the haze in the images caused by out-of-focus light and revealed spiral-shaped FtsZ structures, which extended over variable distances along the sporogenic hyphae (Paper I, Fig. 3). Both the length and the pitch of the spirals were variable. In many cases, helical filaments extended throughout long stretches of the sporogenic cells, sometimes spanning the whole length of the latter. In some hyphae, several individual very short helical structures were observed with just one or a few turns, often with a much shorter pitch than the long spirals. In some cases structures reminiscent of Z rings could be seen, sometimes interrupting the main helical filament. Taken together, these observations pointed towards the possibility of a multistep assembly of the Z rings mediated by remodeling of the FtsZ polymers.

To confirm that the observed helical structures were not artifacts caused by the presence of the EGFP tag on FtsZ, we used immunofluorescence microscopy with anti-FtsZ antibodies to visualize the FtsZ protein in the wild-type strain M145 at different stages of development. We were able to observe spiral-shaped FtsZ structures, which were similar in appearance to the helical filaments formed by the FtsZ-EGFP fusion protein. From this, it was concluded that the assembly of multiple Z rings during sporulation in \(S. coelicolor\) involves extensive helical intermediates and that these were not artifacts caused by the presence of the EGFP tag on FtsZ.
1.2. Dynamics of FtsZ assembly during sporulation (Paper I)

In order to establish the sequence of events taking place during the assembly of FtsZ-EGFP into Z rings in sporogenic hyphae, time course experiments were carried out. We monitored the localization of FtsZ-EGFP in living aerial hyphae every 1 to 2 h between 28 and 42 h of growth on MS agar. The aerial hyphae that showed increased fluorescence were classified according to the patterns of the fluorescence signal (Paper I, Fig. 2). The hyphae from class 1 had one or two Z rings in their basal parts and showed only low levels of fluorescence. They appeared after around 28 h of incubation with some variation between experiments. Class 2 hyphae contained increased levels of fluorescence and the signal was diffuse except for one or two Z rings, mainly in the basal part of the hyphae, and/or occasional spiral-shaped structures. Class 3 hyphae showed extensive spiral-shaped structures and had a high level of diffuse FtsZ-EGFP fluorescence. Class 4 hyphae contained a mixture of short spirals, irregular or tilted Z-ring-like structures, and even some completed Z rings. These structures were present in hyphae where the level of diffuse fluorescence was lower than in classes 2 and 3. Hyphae from class 5 contained regular ladders of Z rings. Class 6 represents hyphae with visibly invaginating septa. The numbers of hyphae from different classes were scored and plotted as percentages of the total number of sporogenic hyphae counted (Paper I, Fig. 4). The time course experiments revealed a succession in the abundances of the different classes of hyphae over time. Thus, in the sporogenic hyphae diffuse fluorescence from FtsZ-EGFP was replaced by spiral-shaped FtsZ-intermediates and irregular ring-like structures, which later coalesced into regular Z rings. As a result, apparently normal spore chains were generated in the tested cultures. These observations allowed us to conclude that FtsZ assembly into regularly spaced Z rings during sporulation is a dynamic process and includes a protracted phase during which spiral-shaped intermediates of the Z ring are formed.
1.3. Effects of sporulation regulators *whiA, whiB, whiG, whiH* and *whiI* on FtsZ-EGFP (Paper I)

The localization of FtsZ-EGFP in living aerial hyphae of the *whiA, whiB, whiG, whiH* and *whiI* developmental mutants was examined microscopically (Paper I, Fig. 6). Only occasional Z rings were formed in aerial hyphae of *whiG, whiA, whiB* and *whiI* mutants without a visible increase in the overall fluorescence. This is consistent with the inability to induce the sporulation-specific *ftsZ2p* promoter in aerial hyphae of these mutants (Flärdh *et al.*, 2000). A larger number of Z rings (4-12) and occasional short helical structures were observed in many aerial hyphae of the *whiH* mutant. Although the presence of Z rings in this mutant was accompanied by a slight increase of the overall fluorescence signal this was much smaller than that observed in the wild type parent. In summary, our observations can be explained by the lack of up-regulation of *ftsZ* expression in aerial hyphae of the mutants (Flärdh *et al.*, 2000).

1.4. Transcriptional analysis, membrane topology and cellular localization of the developmental regulator CrgA (Paper II)

CrgA belongs to a novel family of small actinomycete-specific proteins. Genetic and cytological evidence has suggested a role for *crgA* in coordinating cell division with growth in *S. coelicolor* (Del Sol *et al.*, 2003). Thus, CrgA was proposed to be involved in preventing premature sporulation septation prior to growth cessation in sporogenic aerial hyphae.

Our collaboration partners used a transcriptional fusion between the promoter region of *crgA* and the *luxAB* operon from *Vibrio harveyi* in order to examine the transcription of *crgA* during development of *S. coelicolor*. The activity of the promoter peaked at 24 h of growth and declined thereafter (Paper II, Fig. 1A). Consistent with this, Western-blot analysis indicated the highest abundance of the His-tagged CrgA at 24 h after inoculation (Paper II, Fig. 2C).

In order to study the subcellular localization of CrgA, cell extracts from a strain expressing a C-terminal His-tagged version of the protein were prepared after 24 h of growth. Ultracentrifugation fol-
allowed by Western-blot analysis revealed that His-tagged CrgA was present only in the total protein and membrane fractions (Paper II, Fig. 2B).

The group in Swansea also expressed His-tagged CrgA protein from its own promoter and visualized it by immunofluorescence microscopy. In samples grown for 24 h, the protein localized to discrete foci along branched substrate hyphae and to foci distal from the growing tips of aerial hyphae (Paper II, Fig. 3B). Such foci were absent in samples from later time-points. Overexpression of His-tagged crgA, under the control of the inducible tipA promoter, caused a block in sporulation. Fluorescence microscopy revealed that the overexpressed CrgA formed bright fluorescent foci in all parts of the mycelium, including the growing apical cells of aerial hyphae (Paper II, Fig. 3C and D).

1.5. CrgA influences the timing of FtsZ expression and the dynamic assembly of Z rings during sporulation (Paper II)

In order to examine the initial stages of septation, we introduced an FtsZ-EGFP translational fusion (described above) into the crgA deletion mutant. The timing and abundance of Z rings formed by the FtsZ-EGFP fusion protein in the crgA mutant and the wild type strain M145 were compared on glucose-containing medium. On such media, sporulation of the crgA mutant is accelerated in comparison to the wild type strain which sporulates poorly in the presence of glucose (Del Sol et al., 2003). In the crgA mutant, sporogenic hyphae containing multiple Z rings were evident much earlier (already at 24 h) than in the wild type strain (48 h). These hyphae were 3 to 4-fold more abundant in the crgA mutant than in the wild type strain. Furthermore, the apical hyphal compartments occupied by ladders of Z rings were 30 to 50 % longer in the mutant. Western analysis indicated that the differences in timing and abundance of the Z rings between the crgA mutant and the wild type reflected significant differences in the expression profile of FtsZ in these strains (Paper II, Fig. 4C). Thus, precocious development of the mutant corresponded to an increased expression of FtsZ at 24 h followed by a sharp decline in FtsZ levels thereafter. In the wild type, the abundance of FtsZ increased at 48 h and declined after 72 h of growth. The observed decrease in FtsZ pro-
tein levels correlated with the later stages of sporulation in both strains.

The localization of FtsZ-EGFP in the aerial hyphae of a *S. coelicolor* strain overexpressing CrgA was investigated. The latter strain was grown on mannitol-containing medium and aerial hyphae attached to coverslips were examined microscopically every 24 h during a 96 h growth cycle. Aerial hyphae containing regularly spaced ladders of Z rings were observed in the wild type strain after 48 h of growth. At the same time-point, the fluorescence signal from FtsZ-EGFP in aerial hyphae overexpressing CrgA was either evenly distributed along the apical compartments or localized in the form of spirals and occasional irregularly spaced Z rings. In this strain, hyphae containing regularly spaced Z rings were infrequent and appeared only after 72 h of growth (Paper II, Fig. 5 A-C). Western analysis revealed that the FtsZ protein levels in the CrgA overexpression strain were lower than the ones in the wild type strain at all time-points (Paper II, Fig. 5D). A putative proteolytic product of FtsZ was observed in the protein samples from the CrgA overexpression strain. Comparison of the relative quantities of *ftsZ* transcript in the wild type and CrgA overexpression strains showed no significant differences at the tested time-points (Paper II, Fig. 5E). Thus, the sporulation defect upon CrgA overexpression correlated with an inhibited formation of productive Z rings and low abundance of FtsZ in the sporogenic hyphae of this strain.

1.6. *ftsZ*17(Spo) mutation differentially affects vegetative and sporulation septation (Paper III)

The synchronous placement of multiple Z rings during sporulation of *S. coelicolor* is controlled by unknown mechanisms that are different from those described for *E. coli* and *B. subtilis* (see Introduction). Assuming that such mechanisms may act through direct modulation of one or more sporulation-specific properties of FtsZ, we set up a screen for *ftsZ* mutations affecting sporulation septation, without interfering with vegetative growth. This genetic screen was based on several unique properties of the cell division in *S. coelicolor*. First, sporulation septation is required for the synthesis of the grey pigment of mature spores (Flärdh *et al.*, 2000). Thus, sporulation defects result in the formation of healthy colonies with white aerial mycelium. Second, cell division is dispensable for growth and viability
An ftsZ-null mutant of S. coelicolor forms colonies with distinctive small size, large blue halo and a sparse aerial mycelium. Thus, it is possible to at least tentatively distinguish between defects in the two different types of cell division on the basis of the appearance and color of the colonies as visual indicators for cell division.

Briefly, we mutagenized ftsZ carried on the pKF32 integrating plasmid and introduced this plasmid into the ftsZ-null mutant HU133 by conjugation. The resulting exconjugants were screened for restored vegetative growth and formation of white aerial mycelium. The majority of the colonies were of normal size and sporulated normally, indicating that they had received a functional ftsZ allele. However, some displayed reduced grey pigmentation of the aerial mycelium. Aerial mycelia from such candidate mutants were examined microscopically to detect those with sporulation defects. Screening of about 5000 exconjugants yielded 10 potential mutants. For one of the tested 10 strains, the sporulation phenotype was linked to the pKF32 plasmid. The mutant strain was designated as O17. The plasmid recovered from this mutant was called pO17 and the ftsZ allele carried on it was designated as ftsZ17(Spo). This allele carried a single basepair substitution that changed ftsZ codon 249 from GCG (alanine) to ACG (threonine).

O17 mutant formed normal colonies and white aerial mycelium (although a grey shade developed upon prolonged incubation). Microscopic examination of aerial mycelium from this strain showed no spores and mostly non-septated aerial hyphae. The same characteristics were retained in the strain K102 where the ftsZ17(Spo) allele was introduced into the chromosomal ftsZ locus by allelic exchange (Paper III, Fig. 1). This phenotype was highly reminiscent of the whiH and ftsZΔ2p phenotypes (Flärdh et al., 1999; Flärdh et al., 2000).

In order to examine the effect of ftsZ17(Spo) on vegetative septation, we used fluorescently labeled vancomycin to stain the hyphal cross-walls in liquid-grown mycelium (Daniel and Errington, 2003). Fluorescence microscopy revealed that K102 (carrying the ftsZ17(Spo)) formed cross-walls although with reduced frequency (two- to threefold) compared with the parental strain J1915 (Paper III, Fig. 2). This reduction was much smaller than the nearly complete absence of sporulation septation in the mutant and did not have a strong impact on growth. As a comparison, an ftsQ null mutant of S. coelicolor had a 10-fold reduction in the numbers of vegetative septa and was defective in colony growth (McCormick and Losick, 1996).
These results allowed us to conclude that the \textit{ftsZ17}(Spo) mutation primarily affects sporulation septation.

1.7. FtsZ17(Spo) protein is present at normal levels but fails to assemble into Z rings during sporulation (Paper III)

One possible explanation for the sporulation defect of the K102 strain is that the \textit{ftsZ17}(Spo) is expressed to lower levels than those required for efficient sporulation septation. Western blotting was used to compare the FtsZ protein content in surface-grown hyphae between mutant and wild-type strains. This showed that K102 produced similar quantities of FtsZ as the parent \textit{ftsZ}+ strain J1915 during sporulation.

In order to determine whether \textit{ftsZ17}(Spo) affected the assembly of FtsZ \textit{in vivo}, we introduced an \textit{ftsZ-egfp} translational fusion on an integrating plasmid into the K102 mutant. The resulting strain was designated as K201. The assembly of FtsZ in living hyphae of K201 was examined by fluorescence microscopy. After 30-45 h of growth on solid medium, many hyphae of K201 showed a marked increase in the expression levels of the \textit{ftsZ-egfp}, but no regular ladders of Z rings could be observed. Aerial hyphae were either evenly fluorescent or filled with aberrant fluorescent filaments or patches (Paper III, Fig. 4). In comparison, ladders of regularly spaced Z rings formed in many aerial hyphae of an isogenic \textit{ftsZ}+ strain (K200) carrying the \textit{ftsZ-egfp} fusion. These results showed that \textit{ftsZ17}(Spo) disturbed the formation of regular Z rings in sporogenic aerial hyphae. This conclusion was confirmed by immunofluorescence microscopy. In this experiment, an anti-FtsZ serum was used to visualize the FtsZ17(Spo) protein directly in aerial hyphae from strain K102 (Paper III, Fig. 5). Many hyphae of the parent strain J1915 contained multiple Z rings. In contrast, no regular Z rings could be observed in aerial hyphae of K102 sampled at different times of development. Many aerial hyphae of K102 displayed strong fluorescence, consistent with strongly upregulated expression of the \textit{ftsZ17}(Spo) mutant during sporulation. In summary, the \textit{ftsZ17}(Spo) allele is normally expressed, but the mutant protein fails to assemble into regular Z rings in sporogenic hyphae. This most likely explains the sporulation deficiency of the \textit{ftsZ17}(Spo) mutant.
1.8. Position of Ala249Thr mutation in homology models of the three-dimensional structure of *S. coelicolor* FtsZ (Paper III and unpublished data)

To evaluate the possible effects of this and other mutations, homology models of the structure of *S. coelicolor* FtsZ were constructed, using the known three-dimensional structure of *Methanococcus jannaschii* FtsZ1 (Paper III), (Lowe and Amos, 1998) and *Mycobacterium tuberculosis* FtsZ (Fig. 3, unpublished data) ((Leung et al., 2004). The ftsZ17(Spo) mutation (Ala249Thr) affects a residue that is equivalent to Val277 of the *M. jannaschii* protein (Paper III, Fig. 3). This side-chain points into the hydrophobic core of the protein in the crystal structure. The introduction of the slightly larger, and polar, threonine side-chain at this position is likely to affect the FtsZ surface locally. This prediction was revised upon modeling of the structure of *S. coelicolor* FtsZ on the basis of the three-dimensional structure *M. tuberculosis* FtsZ. Since the sequence similarity between these two proteins is 85%, the latter model is more likely to represent the arrangement of residues in the real-life structure of *S. coelicolor* FtsZ. In this case, the threonine side-chain was predicted to be exposed at the surface of the molecule.

1.9. A set of novel *ftsZ* mutants specifically affected during sporulation (unpublished data)

In sections 2.6 and 2.7, a non-sporulating *ftsZ* mutant was described, which provided useful insights into the developmental control of the Z-ring assembly in *S. coelicolor*. Assuming that the possibilities for isolating non-sporulating *ftsZ* mutants were far from exhausted, we set up a more efficient strategy for mutagenesis. *ftsZ* was mutagenized by error-prone PCR amplification which gave a higher rate of mutations compared to the chemical mutagenesis employed for paper III. The resulting PCR products were cloned into an integrating
Figure 3. Homology model for *S. coelicolor* FtsZ based on the crystal structure of *M. tuberculosis* FtsZ, and position of the ftsZ17(Spo) mutation. The N-terminal domain, which is expected to have GTPase activity, is shown in yellow; the residues of the GTP binding site are light grey, and a modeled molecule of GDP is slate blue. The C-terminal domain where the Ala249Thr mutation is located is shown in tomato color. The mutant 249Thr residue is indicated in blue. The regions from the C-terminus that lie nearby in the *M. tuberculosis* structure are shown in black; these portions almost certainly have a different structure in the two proteins.

A plasmid and subsequently introduced into the ftsZ-null mutant HU133 by conjugation, using the methods described in paper III. 12,000 exconjugants were screened for recovered vegetative growth, loss of blue halo and formation of white or pale grey aerial mycelium (see section 2.6.). Aerial mycelia from 250 exconjugants were examined by phase-contrast microscopy in order to evaluate their ability to produce normal spores. This yielded 25 potential mutants with variable degrees of sporulation defects. For 22 of these mutants, the sporulation phenotype was linked to the plasmid carrying the mutagenized copy of ftsZ. DNA sequencing of the plasmid recovered from these mutants revealed that 18 of the plasmids carried 12 mutant alleles of ftsZ (some mutant alleles were present in more than one sequenced plasmid). The newly isolated mutant strains were designated
as Spo18 to Spo30 and the mutant $ftsZ$ alleles – as $ftsZ18$(Spo)$-ftsZ30$(Spo). When grown on mannitol-containing medium, these mutants formed colonies of variable sizes. The color of aerial mycelia in the mutants ranged from completely white to almost dark grey. Several of these mutants, listed in Table 1, were further characterized in terms of sporulation defects, vegetative septation, expression levels of the $ftsZ$ mutant alleles, ability to form regular Z rings in sporogenic hyphae and possible defects in DNA segregation during sporulation. The possible effects of the novel $ftsZ$ mutations were evaluated with the help of new homology models of the structure of S. coelicolor FtsZ, based on the crystal structures of $M. jannaschii$ FtsZ1 (Lowe and Amos, 1998; Oliva et al., 2004) and $M. tuberculosis$ FtsZ (Leung et al., 2004)

The newly characterized $ftsZ$ mutations could be clustered into several groups based on their location in different functional parts of the protein (Table 1). The sporulation defects of the studied mutants were evaluated by microscopical observation of aerial mycelia after 4 days of growth on solid medium. Several mutants showed severe sporulation defects. Aerial hyphae of these strains were mostly unseptated and only occasional spore-like compartments could be seen. In contrast, several other mutants formed a plenty of irregularly sized spores. In many cases, the shape of the spores was also affected. Fluorescently labeled vancomycin was used to stain the hyphal cross-walls formed in liquid-grown mycelia of the newly isolated mutants. These experiments showed that the frequency of vegetative septation was affected to a variable degree by the different $ftsZ$ mutations. In all cases, the reduction in the frequency of vegetative septation was either lower or comparable to that observed in the $ftsZ17$(Spo) mutant. These results indicated that the set of novel $ftsZ$ mutations have a primary effect on the developmentally controlled cell division.
Table 1. Summary of the properties of a set of *ftsZ* mutants specifically affected during sporulation.

<table>
<thead>
<tr>
<th>Position of mutation in <em>ftsZ</em> model</th>
<th>Mutation</th>
<th>Strain</th>
<th>Sporulation defect</th>
<th><em>ftsZ</em> expression levels</th>
<th>Vegetative septation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface between C-terminal and N-terminal domains</td>
<td>I126F</td>
<td>Spo18</td>
<td>severe</td>
<td>highest at 24h; later lower than normal</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>S189T</td>
<td>Spo24</td>
<td>severe</td>
<td>lower than normal at all time points</td>
<td>reduced</td>
</tr>
<tr>
<td>Active site</td>
<td>E102V</td>
<td>Spo21</td>
<td>irregular spores</td>
<td>lower than normal at all time points</td>
<td>slightly increased</td>
</tr>
<tr>
<td>C-terminus</td>
<td>E358V</td>
<td>Spo19</td>
<td>irregular spores</td>
<td>lower than normal at all time points</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>D396G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E391V</td>
<td>Spo23</td>
<td>irregular spores</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Lateral interface between subunits</td>
<td>V10D</td>
<td>Spo20</td>
<td>severe /irregular spores</td>
<td>normal</td>
<td>reduced</td>
</tr>
<tr>
<td>Longitudinal interface between subunits</td>
<td>N273I</td>
<td>Spo22</td>
<td>severe</td>
<td>highest at 24h; later lower than normal</td>
<td>normal</td>
</tr>
</tbody>
</table>
The assembly of FtsZ in aerial hyphae of the mutants was visualized directly by immunofluorescence microscopy with anti-FtsZ antiserum. Samples for microscopy were taken at different time-points. At 48h after inoculation, aerial hyphae of the wild-type strain M145 showed increased fluorescence and in many cases ladders of regularly spaced Z rings were observed. These samples also contained many mature spore chains. At the same time-point, ladders of Z rings were absent in aerial hyphae of the mutants with severe sporulation defects, but in some cases irregular spiral-shaped FtsZ structures were observed (data not shown). These results suggest that in the newly isolated mutants with severe deficiency in sporulation, the ftsZ mutations affected the assembly of FtsZ into regular Z rings. Multiple Z rings could be detected in aerial hyphae of the mutants with milder sporulation defects; however, these Z rings were often irregularly spaced. Aberrant or spiral-shaped FtsZ structures were also frequent. Exact estimation of the degree to which the studied ftsZ mutants are defective during Z-ring formation was complicated by the insufficient resolution achieved by immunofluorescence microscopy in S. coelicolor.

The FtsZ protein levels in the different mutants were investigated by Western blotting analysis of surface grown mycelia (see Table 1). These experiments showed that the amount of FtsZ in some of the mutants was perturbed, which may explain their deficiency in Z-ring formation. These perturbed FtsZ protein levels may result from changes in the ftsZ expression levels within the sporogenic hyphae of the ftsZ mutants or decreased protein stability, either as a direct consequence of the mutation or indirectly, due to failure of the mutated FtsZ to polymerize correctly. Another possibility is that there may be variations in the numbers of hyphae undergoing sporulation at the time of sampling due to differences between the developmental stages of the mutants. Additional experiments are required in order to distinguish between these possibilities. Interestingly, the Spo20 mutant produced normal levels of FtsZ, but the mutant protein was unable to support sporulation septation. This indicates that the ftsZ20(Spo) mutations confers a defect in FtsZ function and not expression or protein stability. It needs to be pointed out that data from Western blotting of surface grown mycelia are difficult to interpret because of the presence of hyphae from different developmental stages in each sample.

The ability of the Spo mutants to segregate their multiple genomes correctly during sporulation was investigated by visualization
of the DNA by 7-amino-actinomycinD. These experiments revealed that all of the mutants were defective in DNA-segregation and/or condensation. This is in agreement with the previously observed DNA-segregation phenotypes of a whiH-null mutant, *ftsZΔ2p* and *ftsZ17* (Spo) (Paper III; (Chater, 2001; Flärdh *et al.*, 2000).

To evaluate the possible effects of the novel *ftsZ*(Spo) mutations, homology models of the structure of *S. coelicolor* FtsZ were constructed. The model represented in Fig.4, panel A, was based on the crystal structure of a laterally oriented dimer of the *M. tuberculosis* FtsZ (Leung *et al.* 2004), and *S. coelicolor* FtsZ was modeled on each of the two molecules in this structure. The advantage with this was the close relatedness of the mycobacterial and streptomycete proteins, and the lateral contacts revealed in the structure. In the same way, *S. coelicolor* FtsZ was modelled on both molecules in the crystal structure of a longitudinal dimer from reconstituted *M. jannaschii* FtsZ1 protofilaments (Oliva *et al.* 2004), as shown in Fig.4, panel B. This indicated important longitudinal contacts between FtsZ molecules in a protofilament. The two models in Fig. 4 show the positions of the mutated residues identified in our genetic screen. It is clear from Fig.4 that the newly isolated *ftsZ* mutations affected different parts of FtsZ proteins and, thus, probably influenced different properties of FtsZ. The new *ftsZ* mutations were grouped according to the location of the affected residues in the homology models (Table 1). It is important to underline the presence of two mutations affecting the interface between the C-terminal and the N-terminal domains of FtsZ (I126F and S189T). These are the only *ftsZ* mutations to affect this surface that have been reported so far. It has been shown that the C-terminal and the N-terminal domains of FtsZ can fold separately and independently and then be reconstituted to an active protein (Oliva *et al.*, 2004). It was therefore suggested that these two domains could have been derived from separate proteins with distinct functions in the course of evolution. Analysis of the two mutations affecting the interface between them may shed some light on the interplay between these domains.
Figure 4. Positions of the novel \textit{ftsZ} mutations in homology models of the three-dimensional structure of \textit{S. coelicolor} FtsZ.

(A) \textit{S. coelicolor} FtsZ modeled and arranged as a dimer on \textit{M. tuberculosis} FtsZ structure (PDB entry 1RQ7). GDP is shown in gold docked into the N-terminal domain of the A molecule (subunits not labeled in the figure but referred to as molecules A and B). Mutated residues are shown in maroon and blue in molecules A and B respectively. Changes affecting the extreme C-terminus are not shown.

(B) Position of N273I in a homology model of the structure of \textit{S. coelicolor} FtsZ arranged as a dimer on \textit{M. jannaschii} FtsZ structure. (PDB entry 1W5B). The residues that interact with GDP are shown in light grey. Changes affecting the extreme C-terminus are not shown. \textit{S. coelicolor} FtsZ was modeled using \textit{Mycobacterium tuberculosis} FtsZ structure (PDB entry 1RQ7) and \textit{M. jannaschii} FtsZ structure (PDB entries 1W5E, 1W5F). Mutation N273I is shown in maroon and red in A and B molecules respectively.

C-terminal domains of the two subunits are shown in blue and tomato colours, and N-terminal domains are shown in green and yellow.
2. The picture

In this study, we address the developmental mechanisms that modulate the performance of the cell division machinery during sporulation in *S. coelicolor*. Several general conclusions are emerging from the results of the genetic and cell biological analyses presented in this study.

First, cell division in *S. coelicolor* may be developmentally controlled at the level of assembly of FtsZ into Z rings during sporulation. This was demonstrated by the identification of the *ftsZ17* (Spo) mutation, which rendered the mutant protein unable to form multiple and regularly spaced Z rings during sporulation, but did not significantly affect its ability to support septation in vegetative hyphae.

Second, the assembly of FtsZ in sporogenic hyphal cells of *S. coelicolor* is a dynamic process. This involves the formation of spiral-shaped intermediates, which are gradually replaced by regularly spaced arrays of Z rings. This dynamic behavior of FtsZ is likely to be the target of the developmental control of septation during sporulation.

Third, the CrgA protein appears to inhibit sporulation septation prior to growth arrest of aerial hyphae by directly or indirectly affecting the dynamic assembly of the FtsZ protein *in vivo*.

*ftsZ*(Spo) mutations

The fact that the *ftsZ17* (Spo) mutation primarily affected sporulation demonstrated a difference in the requirements for FtsZ assembly between the two types of cell division used by *S. coelicolor*. The *ftsZ17* (Spo) is expressed to normal levels but is unable to support sporulation because of a defect in FtsZ function. This opens the possibility that a developmental regulator may act by directly or indirectly influencing the Z-ring assembly.

There are a number of alternative possibilities for the molecular basis of the sporulation defect conferred by *ftsZ17* (Spo). One possibility is that this mutation disturbs a sporulation-specific interaction between FtsZ and an unknown factor modulating FtsZ activity or assembly. This notion is supported by the positioning of Ala-249 on the lateral, exposed surface of the FtsZ protein predicted by our new homology model (Fig. 3). Alternatively, the mutation could alter a general property of FtsZ, such as polymer stability, lateral contacts between protofilaments or interactions within the cell division machin-
ery. The much weaker effect on vegetative cross-wall formation would then imply that sporulation septation is more sensitive to such alterations of FtsZ function than what vegetative septation is. These alternative models are not mutually exclusive. Analysis of the biochemical properties of FtsZ17(Spo) and isolation of intra- or extragenic suppressors of this mutation will help in distinguishing between these possibilities.

An important implication of the work described so far is that it is possible to separate the requirements for FtsZ function during the two types of cell division by point mutations in the \textit{ftsZ} gene. This makes the genetic analysis of FtsZ a powerful approach for studying the developmental control of cell division in \textit{S. coelicolor}. Based on this, we carried out a more efficient mutagenesis of \textit{ftsZ}, and screened for additional \textit{ftsZ}(Spo) mutants (unpublished data). This yielded several novel \textit{ftsZ} mutants with sporulation phenotypes. The ongoing characterization of these will provide some useful insights into the function of FtsZ during sporulation. Judging from their location on a homology model of the FtsZ structure, the novel \textit{ftsZ} mutations are likely to affect different properties of the protein. Most of the mutations had an effect on vegetative growth and cross-wall formation, albeit much smaller than the sporulation deficiency. These two notions are in line with the possibility that several of the mutations may affect general properties of FtsZ, but since the requirements for correct assembly of FtsZ are tighter during sporulation, the phenotypic consequences of the mutations are primarily seen during sporulation. This is particularly relevant for mutations predicted to affect the catalytic pocket (E102V), longitudinal interface between monomers in the protofilament (A275T), and the interface between the N- and C-terminal domains within an FtsZ molecule (I126F; S189T). Other mutations affect sites that are likely to be involved in interactions with other proteins, most notably the C-terminal mutations in strains Spo19 and Spo23 (Table 1), and could affect interaction with a sporulation-specific protein. It needs to be pointed out that in some of the mutants the cellular contents of FtsZ were affected. Thus, insufficient FtsZ concentrations could explain the failure of some mutants to sporulate. Changes in the FtsZ content could be caused by degradation of misfolded versions of the protein, an increased protein turnover in result of failure to polymerize, or alterations of \textit{ftsZ} expression. In any case, analysis of the biochemical properties of the described FtsZ mutants is
likely to shed some light on the mechanism of FtsZ polymerization in *S. coelicolor*.

Another important result from the study of the novel *ftsZ*(Spo) mutants is the observed inability of all the mutants to correctly segregate their genomes into the prespore compartments. This is consistent with the DNA segregation phenotypes of sporulation septation mutants *whiH* and *ftsZA2p* (Flärdh *et al.*, 1999; Flärdh *et al.*, 2000), and *ftsZI7*(Spo) (paper III). On the other hand, DNA segregation mutants of *S. coelicolor* have been reported to form misplaced or irregular sporulation septa (Kim *et al.*, 2000; Wenner *et al.*, 2003). These observations point towards a possible interdependence between the regular placement of multiple Z rings and proper positioning of the chromosomes during sporulation (see below).

**Making regularly placed Z rings by remodeling of helical filaments**

The developmental modulation of the FtsZ assembly in *S. coelicolor* involves the dynamic remodeling of spiral-shaped FtsZ intermediates into regularly spaced Z rings during sporulation, revealed by our studies using an FtsZ-EGFP translational fusion. Developmentally induced remodeling of FtsZ polymers is involved in the switch from medial to polar septation during sporulation in *B. subtilis* (Ben-Yehuda and Losick, 2002). The formation of helical FtsZ intermediates during this process depended on an increase in *ftsZ* expression and on the FtsZ-interacting sporulation regulator SpoIIE. More recently, highly dynamic helical structures have been reported as a part of the normal cycle of FtsZ assembly in *E. coli* (Thanedar and Margolin, 2004). In the case of *S. coelicolor*, it is challenging to determine the molecular interactions behind the reshaping of these FtsZ spirals into regular arrays of Z rings during sporulation. Disturbances of such interactions are likely to be crucial for the efficiency of sporulation septation and can even be detected as decreases in the amount of the grey spore pigment. Thus, *S. coelicolor* may provide a powerful system for genetic analysis of the dynamics of FtsZ and the nature of the polymers building the Z ring *in vivo*. The latter remains elusive in all of the studied bacterial species.

The results presented in this thesis raise some possibilities regarding the factors involved in selection of division sites during sporu-
lation of S. coelicolor. These factors are likely to be different from the ones known for other organisms since the Min system and an overt “nucleoid veto” does not operate in S. coelicolor. In many other bacteria, these two negatively acting systems direct formation of the Z rings to the middle of the cell by inhibiting FtsZ assembly at other sites. In contrast, our results from visualization of the FtsZ-EGFP fusion in sporogenic cells revealed that the assembly of FtsZ into filaments is, at least initially, allowed along the whole length of the sporogenic hyphal cell, with no overt signs of zones of inhibition or predetermined nucleation sites.

What is then determining the final placement of the Z rings? It is tempting to speculate that the remodeling of FtsZ polymers into regularly spaced Z rings during sporulation could be influenced by the positioning of the nucleoids. Previous observations that mutants defective in DNA segregation had misplaced sporulation septa support this speculation. On the other hand, the DNA segregation defects of the ftsZ mutants reported here, suggest that the dynamic FtsZ structures might play a role in arranging the nucleoids properly along the sporulating hyphae. Thus, an intricate interplay between dynamic FtsZ structures and the segregating nucleoids during sporulation may act to insure that each spore receives a single copy of the genome.

**Regulators of cell division in S. coelicolor**

We have hypothesized that the sporulation specific remodeling of the FtsZ polymers could be the target for developmental regulators of cell division. This notion was supported by the observed effect of CrgA overexpression on the dynamics of Z-ring formation in sporogenic hyphae of S. coelicolor. In this case, developmental upregulation of ftsZ transcription was evident, but the protein failed to assemble into regular arrays of Z rings. In these cells, CrgA affects either correct remodeling of spirals or the stability of FtsZ polymers. Several lines of evidence have shown that CrgA has an important role in coordinating growth with cytokinesis in S. coelicolor (Del Sol et al., 2003). Results presented in this thesis suggest that this role of CrgA in development is mediated by inhibition of Z-ring formation in sporogenic hyphae. It is unlikely that CrgA exerts its effect by direct interaction with FtsZ. Nevertheless, CrgA is the first case of a modulator of the FtsZ assembly proposed for S. coelicolor.

(Chater, 2000). These early *whi* genes are needed for the increased expression of *ftsZ* from the *ftsZ2p* promoter that precedes sporulation in aerial hyphae (Flärhdh *et al.*, 2000). This is consistent with our observations that only occasional Z rings were formed in aerial hyphae of the *whi* mutants. However, it can not be excluded that the *whi* genes may also affect cell division on a level different from the transcriptional upregulation of *ftsZ*. Such effects could be masked by the low level of FtsZ in the aerial hyphae of the *whi* mutants. Additional experimental approaches are needed in order to investigate this possibility. Other candidates for the developmental control of cell division are *ssgA*, *ssgB* and *samR* (Keijser *et al.*, 2003; Tan *et al.*, 2002; van Wezel *et al.*, 2000). These are genes of unknown functions required for the initial stages of sporulation in *S. coelicolor*. One interesting possibility is that these genes may influence cell division by affecting the expression and/or function of *ftsZ*, but this hypothesis has not been addressed experimentally.

**Future perspectives:**

The work presented here provides an insight into the function of FtsZ during sporulation of *S. coelicolor* and points towards interesting possibilities for the mechanisms of developmental control of cell division in this organism.

This study can be a starting point for future work over several lines:
- Identification of intra- and extragenic suppressors of the *ftsZ* mutations presented here. Screening for genes that at elevated copy number would suppress the *ftsZ*(Spo) alleles should also be applicable.
- Analysis of the biochemical properties of the mutant versions of the FtsZ protein, such as *in vitro* polymerization and GTPase activity.
- Detailed investigation of the interplay between FtsZ assembly and nucleoid segregation during sporulation. This may be done by simultaneous visualization of specific chromosomal loci or whole chromosomes and the FtsZ-EGFP translational fusion in the wild type strain. In addition, the chromosomes and proteins involved in the partitioning machinery may be visualized in the *ftsZ* mutants backgrounds.
- The effect of known developmental regulators on FtsZ assembly may be addressed with the help of the FtsZ-EGFP translational fusion.
- The possible negative or positive effects of known regulators of cell division on the FtsZ assembly can be addressed by transferring the fisZ mutations, described here, into strains deleted for or overexpressing the proteins of interest.
REFERENCE LIST

Addinall, S.G., Cao, C., and Lutkenhaus, J. (1997) Temperature shift experiments with an ftsZ84(Ts) strain reveal rapid dynamics of FtsZ localization and indicate that the Z ring is required throughout septation and cannot reoccupy division sites once constriction has initiated. *J Bacteriol* 179: 4277-4284.


52


Involved in Aerial Mycelium Formation in *Streptomyces coelicolor*. *Genetics* **151**: 569-584.


Shih, Y.-L., Le, T., and Rothfield, L. (2003) Division site selection in *Escherichia coli* involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. PNAS 100: 7865-7870.


Utvecklingsbiologisk reglering av celldelning i *Streptomyces coelicolor*

*Bakteriell celldelning och FtsZ*

*Utvecklingsbiologiskt reglerad celldelning i Streptomyces*

Under denna enkla livscykeln används två olika former av celldelning som ger olika resultat. I det vegetative mycelet ger celldelning upphov till tvärvägar som avgränsar hyfens filamentära celler. När en hyfcell nått tillräcklig längd bildas ett sådant delningsseptum ungefär vid mitten av cellen. I lufthyfer, å andra sidan, anläggs synkront inom varje sporulerande cell upp till 50 eller fler regelbundet placerade celldelningar. Dessa ger upphov till sporuleringssepta som omvandlar...
hyfen till en kedja av presporer. Var och en av presporerna innehåller en kopia av genomet, och differentieras ytterligare innan de frisätts som mogna sporer. Båda formerna av celldelning involverar FtsZ och kräver bildning av Z ringar, men de regleras på helt olika sätt.

**Projektets syfte**
Målet med denna avhandling har varit att identifiera de faktorer och molekylära mekanismer som är inblandade i den utvecklings-biologiska regleringen av celldelning under sporulering av *S. coelicolor*.

**Genetiska analyser**

**Cellbiologiska analyser**

*En regulator av celldelning*

68

\textbf{Signifikans}

Signifikansen av det arbete som presenteras här har flera aspekter.
(i) Celldelning och dess reglering är fundamentala processer för alla former av liv, och att finna de molekylära mekanismerna bakom detta är en viktig utmaning inom cellbiologin.
(ii) \textit{S. coelicolor} är en värdefull modellorganism för studier av prokaryot cellulärdifferentiering och multicellularitet, samt hur reglering av celldelning integreras i dessa processer.
(iv) \textit{S. coelicolor} är den viktigaste genetiska modellen bland streptomyceterna, vilka är ytterst viktiga som industriella produktionsorganiser för ett stort antal antibiotika. Kunskap om hur celldelning regleras, och hur detta förhåller sig till tillväxt och hyfförgrening är av stor vikt för att kunna lösa tekniska problem när det gäller hantering av mycelbildande organismer i storskaliga fermentationer.
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