Structural proteins involved in morphological differentiation of streptomycetes
Claessen, Dennis

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Chapter 1

General introduction
**Life cycle of streptomycetes**

Actinomycetes are a group of Gram-positive bacteria that usually have a high GC content. Most of them are rod-shaped to filamentous, are aerobic, and generally non-motile in the vegetative phase (Madigan et al., 1997). Actinomycetes are abundantly present in virtually all environments, e.g. soils, composts, fresh water basins, and deep sea sediments (Colquhoun et al., 1998). Although invisible to the eye, their abundant presence is percepted through a volatile compound called geosmin, which is responsible for the earthy or musty odor characteristic for forests and moist cellars (Gust et al., 2003). Actinomycetes are well-known for their production of a variety of secondary metabolites among which most of the natural antibiotics used today in medicine (Bentley et al., 2002). For instance, Selman A. Waksman received the Nobel Prize in 1952 for his discovery of the anti-tuberculosis remedy, the antibiotic streptomycin, produced by the actinomycete *Streptomyces griseus* (Smith, 2000).

The best-studied members of the actinomycetes are the streptomycetes with *Streptomyces coelicolor* serving as a model organism. Its genome has recently been sequenced. *S. coelicolor* contains one of the largest number of genes (>7800) identified so far in a bacterium (Bentley et al., 2002). This coding potential may reflect the needs for the complex life style of this bacterium in a highly variable environment like the soil. Unlike most other bacteria streptomycetes mainly exist as multicellular organisms with two distinct growth phases. Initially, a branched vegetative mycelium is formed that colonizes the substrate (Fig. 1). Secreted enzymes degrade organic polymers (e.g. those in plant material) into smaller molecules that serve as nutrients for the growing mycelium. In the second growth phase, hyphae...
grow out of the substrate into the air. This results in a white, fluffy appearance of the colony (Fig. 2). The aerial hyphae further differentiate by coiling, and their growth is arrested. Moreover, they undergo multiple cell divisions which are accompanied by septation. As a result, a string of 50-100 unigenomic spores are produced. These chains of spores give the colony a grey appearance due to a pigment in their cell walls (see below). The spores are dispersed by wind and animals and may give rise to a new colonizing mycelium.

The formation of aerial hyphae and spores is believed to be supported by partial degradation of the mycelium. During formation of aerial hyphae part of the submerged mycelium is degraded, while during sporulation non-sporulating parts of aerial hyphae die (Wildermuth, 1970; Miguélez et al., 1999). Hyphae undergo an orderly process of internal cell dismantling resembling programmed cell death in animals. However, the hyphae do not completely disappear but remain part of the developing colony. They may provide a mechanical support for aerial hyphae as well as acting as ‘arteries’ for transport of solutes and water to the aerial structures (Miguélez et al., 1999).

Mutants of *S. coelicolor* have been isolated that are impaired in the formation of aerial structures. Bald (*bld*) mutants are affected in formation of aerial hyphae, while white (*whi*) mutants do produce aerial hyphae that, however, fail to generate mature, grey-pigmented spores. In the following sections several of these mutants will be described.

**The bld gene products, regulators of aerial hyphae formation**

Since the genetic mapping of the first *S. coelicolor bld* mutant (Hopwood, 1967), more than 10 *bld* genes have been isolated. Although the precise role of most of the genes is unclear, several of them encode regulatory proteins (Chater, 2001). The best characterized *bld* gene, *bldA*, specifies a leucyl-tRNA which is the only tRNA efficiently translating the UUA codon. Although the *bldA* promoter is active
throughout growth, processing of the 5' end of the primary transcript is more efficient in older cultures. The increase in accumulation of the processed form of the bldA tRNA coincides with a more efficient translation of mRNAs containing UUA codons (Leskiw et al., 1993). Within the S. coelicolor genome 145 genes contain one or more TTA codons (Bentley et al., 2002). These genes are therefore most likely dependent on bldA for translation. For instance, the presence of this codon in the pathway-specific activators of the undecylprodigiosin (Red) and actinorhodin (Act) biosynthesis clusters (redZ and actII-ORF4, respectively) prevents bldA strains to produce these two pigmented antibiotics (Fernández-Moreno et al., 1991; White and Bibb, 1997). Until now, no bldA-dependent genes have been identified that account for the absence of aerial hyphae in bld mutants.

Other bld genes that will be described in more detail are bldD, bldK, bldM and bldN. The bldD gene encodes a small protein with DNA-binding activity (Elliot et al., 1998; Elliot and Leskiw, 1999). Several targets have been identified, two of which are the genes for the developmental sigma factors whiG (see below; Fig. 3) and bldN (Elliot et al., 2001). In a bldD background expression of these targets is up-regulated during vegetative growth, suggesting that BldD represses premature expression of these genes.

bldN encodes an extracytoplasmic function (ECF) sigma factor. Its transcription is dependent on the presence of BldG, a putative anti-anti-sigma factor. The only known target of BldN is the promoter region of bldM encoding a response regulator (Bibb et al., 2000; Molle and Buttner, 2000). Single amino acid substitutions in the BldM and BldN proteins affected development of spores but did not abolish aerial growth as was observed in null mutants. These data suggest that BldM and BldN have functions in early as well as late stages of development (Molle and Buttner, 2000).

bldA, bldD, bldM, and bldN mutants encode proteins that have an apparent intracellular regulatory function. The bldK gene cluster, consisting of five adjacent open reading frames, seems to be involved in intercellular regulation. These genes specify homologues of the subunits of the oligopeptide-permease family of ATP binding cassette membrane-spanning transporters (Nodwell et al., 1996). From the fact that bldK mutants are resistant to the toxic tripeptide bialaphos, known to enter bacteria via oligopeptide permeases (Diddens et al., 1976), it was concluded that BldK is an oligopeptide importer. This suggested that the block in aerial hyphae formation is due to the inability to import a signalling peptide. Indeed, a peptide of 655 Da was purified from the culture medium of the bldK mutant that restored

The gene products of other \textit{bld} mutants known today, like \textit{bldB} (Champness, 1988; Pope \textit{et al}., 1996; 1998), \textit{bldC}, \textit{bldG} (Bignell \textit{et al}., 2000) \textit{bldH}, \textit{bldI} (Champness, 2000), \textit{bldJ} (Nodwell \textit{et al}., 1996), and \textit{bldL} (Nodwell \textit{et al}., 1999) have not been characterized in detail and their function in development remains to be established. Table 1 gives an overview of current knowledge on the \textit{bld} genes.

\textbf{Table 1.} \textit{bld} genes identified in \textit{S. coelicolor} (adapted with permission from Chater, 2001)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene product</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bldA}</td>
<td>Leucyl tRNA for the UUA codon</td>
<td>Lawlor \textit{et al}., 1987; Leskiw \textit{et al}., 1991</td>
</tr>
<tr>
<td>\textit{bldB}</td>
<td>DNA-binding protein containing an helix-turn-helix motif that likely forms a homodimer</td>
<td>Merrick, 1976; Pope \textit{et al}., 1998</td>
</tr>
<tr>
<td>\textit{bldC}</td>
<td>Unknown</td>
<td>Merrick, 1976; Champness, 2000</td>
</tr>
<tr>
<td>\textit{bldG}</td>
<td>Putative anti-anti-sigma factor</td>
<td>Champness, 1988; Bignell \textit{et al}., 2000</td>
</tr>
<tr>
<td>\textit{bldH}</td>
<td>Unknown</td>
<td>Champness, 1988</td>
</tr>
<tr>
<td>\textit{bldI}</td>
<td>Unknown</td>
<td>Leskiw and Mah, 1995; Champness, 2000</td>
</tr>
<tr>
<td>\textit{bldJ}</td>
<td>Unknown</td>
<td>Nodwell \textit{et al}., 1996</td>
</tr>
<tr>
<td>\textit{bldK}</td>
<td>Oligopeptide permease importing a signalling peptide of 655 Da encoded (indirectly) by the \textit{bldJ} locus</td>
<td>Nodwell \textit{et al}., 1996; Nodwell and Losick, 1998</td>
</tr>
<tr>
<td>\textit{bldL}</td>
<td>Unknown</td>
<td>Nodwell \textit{et al}., 1999</td>
</tr>
<tr>
<td>\textit{bldM}</td>
<td>Response regulator; probably active in early as well as late stages of development</td>
<td>Bibb \textit{et al}., 2000; Molle and Buttner, 2000</td>
</tr>
<tr>
<td>\textit{bldN}</td>
<td>Extracytoplasmic function (ECF) sigma factor; recognizes promoter region of \textit{bldM}; probably active in early as well as late stages of development</td>
<td>Bibb \textit{et al}., 2000; Elliot \textit{et al}., 2001</td>
</tr>
</tbody>
</table>
The *bld* genes are part of a hierarchical cascade

A common feature of *bld* mutants is their failure to produce and secrete a small hydrophobic peptide, called SapB. It consists of 18 amino acids with a predicted mass of 1928 Da. The observed mass of 2027 Da as well as its reactivity with Schiff’s reagent indicates the presence of a non-proteinaceous moiety (Willey *et al.*, 1991). Until now, no gene has been identified in the *S. coelicolor* genome that encodes a peptide or protein containing the N-terminal sequence of SapB and which is in agreement with the total amino acid composition. This indicates that this peptide is synthesized non-ribosomally. However, disruption of each of the three peptide-synthetase genes in the genome of *S. coelicolor* did not abolish SapB production (J.M. Willey, personal communication).

When certain *bld* mutants were grown near differentiating colonies of a wild-type strain, aerial hyphae formation and subsequent sporulation was restored in the area close to the wild-type strain (Willey *et al.*, 1991). This effect was proposed to be due to diffusion of SapB to the *bld* mutant strain since application of purified SapB on the colony surface of *bld* mutants also rescued aerial hyphae formation (Tillotson *et al.*, 1998). Since SapB was shown to reduce the water surface tension from 72 to 30 mJ m⁻² this peptide likely enables formation of aerial hyphae by breaking the water surface tension (Tillotson *et al.*, 1998). Interestingly, the aerial hyphae that had formed did not differentiate further to form spores. Moreover, the effect of purified SapB in stimulating development was transient. After prolonged incubation, colonies that had produced aerial hyphae in response to the application of SapB lost their white fluffy appearance. Microscopically, the aerial hyphae had collapsed and were lying horizontally on top of the underlying substrate hyphae (Willey *et al.*, 1991). Thus, other molecules are essential to complete the morphological program of development of aerial hyphae.

Complementation was also observed when certain pairs of *bld* mutants were grown adjacent to each other even though all these mutants are individually blocked in SapB production. Extracellular complementation is initially unidirectional with one mutant strain acting as a donor for the acceptor strain (Willey *et al.*, 1993).
General introduction

Experiments with the whole set of \textit{bld} mutants showed that most of these mutants, but not all, fall into the following hierarchical cascade, in which each mutant can rescue morphogenesis in mutants to the left:

\[
[bldJ] < [bldK,L] < [bldA,H] < [bldG] < [bldC] < [bldD,M] \rightarrow \text{SapB}
\]

Thus, \textit{bldJ} can be rescued by all other \textit{bld} mutants, whereas \textit{bldD} and \textit{bldM} can only be rescued by the wild-type strain. Complementation results in restoration of SapB biosynthesis which eventually leads to aerial hyphae formation and sporulation of both \textit{bld} mutants (Wösten and Willey, 2000). This cascade is probably explained by the formation of \textit{bld}-dependent signalling molecules (e.g the one isolated from the \textit{bldK} mutant) and inactivation of metabolites. Why does \textit{S. coelicolor} use such a cascade? It was proposed that it allows the bacterium to integrate different environmental conditions like nutrient availability and stress (Chater, 2001; Pope \textit{et al.}, 1996; Kelemen and Buttner, 1998). This is supported by the observation that most bald mutants are affected in the regulation of carbon utilization. Pope \textit{et al.} (1996) showed that the galactose operon was constitutively expressed in \textit{bldA}, \textit{bldB}, \textit{bldC}, \textit{bldD}, \textit{bldG} and \textit{bldH} mutants grown on glucose. They suggested that the \textit{bld} genes are not involved in morphogenesis \textit{per se}, but instead play a role in the regulation of carbon utilization. Indeed, most of these mutants were rescued in development by growing them on mannitol instead of glucose (Pope \textit{et al.}, 1996; Willey \textit{et al.}, 1991; 1993).

Coupling of morphogenesis and primary metabolism was also shown by Viollier \textit{et al.} (2001) and Süssstrunk \textit{et al.} (1998). During primary growth organic acids are excreted by submerged hyphae, resulting from an imbalance between glycolysis and TCA-cycle activities. This leads to a gradual drop of the pH in the medium. During subsequent growth, the wild-type strain switches to an alternative metabolism that neutralizes the culture medium, probably by recycling the secreted acids. The \textit{cya} mutant of \textit{S. coelicolor} MT1110, lacking the adenylate cyclase activity to synthesize cAMP, was unable to deacidify its culture medium by the inability to enter the alternative metabolic route. As a result, it failed to erect aerial hyphae (Süssstrunk \textit{et al.}, 1998). However, buffering of the medium as well as growth of the MT1110 wild-type strain in close proximity of the \textit{cya} mutant rescued morphogenesis. Similar results as obtained with the \textit{cya} mutant were obtained with a mutant affected in the \textit{citA} gene encoding the citrate synthase (Viollier \textit{et al.}, 2001). These data show that cross-feeding experiments can reflect inactivation of a metabolite such as organic acids in the medium (Süssstrunk \textit{et al.}, 1998).
The link between *bld* genes and SapB: the *ram* locus

Formation of aerial hyphae in all *bld* mutants was not only restored by SapB, but also by overexpressing a gene called *ramR* (Nguyen *et al.*, 2002). This gene is part of the *ram* operon of *S. coelicolor* that accelerates development in *Streptomyces lividans* (Ma and Kendall, 1994). It consists of five genes encoding for a membrane-bound kinase (*RamC*), a small 42 amino acids peptide (*RamS*), components of an ABC transporter (*RamAB*), and a response regulator (*RamR*). Homologues of this gene cluster were also identified in *S. griseus* (called *amf* cluster) and *Streptomyces avermitilis* (Ueda *et al.*, 2002). Deletion of *ramR* blocked SapB formation and delayed morphogenesis in the wild-type (Nguyen *et al.*, 2002). A link of the *ram* cluster with the *bld* genes was suggested from the finding that *ramR* is not transcribed in *bldA*, *bldB*, *bldH*, and *bldD*, but that its transcription is not affected in *whiG*, the earliest *whi* mutant (see below; Keijser *et al.*, 2002). Moreover, overexpression of *ramR* restored formation of SapB in all *bld* mutants, and induced formation of this peptide in wild-type strains on minimal media in which it is normally not produced (Nguyen *et al.*, 2002).

RamR was shown to be involved in transcription of the *ramCSAB* operon (Keijser *et al.*, 2002). Transcripts of this operon were already observed during vegetative growth, but strongly increased during the onset of morphological development (Keijser *et al.*, 2002). The *ramS* gene encodes for a small 42 amino acid peptide that is suggested to be transported via the ABC transporter system encoded by the RamAB proteins (Chater and Horinouchi, 2003). Deletion of *ramS* has a similar phenotype as the *ramR* mutant, i.e. delayed formation of aerial hyphae. Because overexpression of RamR in the *ramS* mutant did not restore morphogenesis, RamS is likely required for the *ramR*-induced growth of aerial hyphae (Nguyen *et al.*, 2002). The precise function of RamS remains to be elucidated.

The *whi* gene products, sporulation-specific proteins

A complex regulatory network accounts for the development of aerial hyphae into spores. To date, 12 *whi* mutants have been identified that are impaired in completing sporulation (Chater, 2001; Ryding *et al.*, 1999). Deleting the genes mutated in *whiN* and *whiM* resulted in a *bld* phenotype. These two mutants were therefore renamed as *bldN* and *bldM*, respectively. Two classes of *whi* genes are distinguished. The early *whi* genes (*whiA, whiB, whiG, whiH, whiI* and *whiJ*) are
involved in the initiation of differentiation of aerial hyphae into spores, while the late white genes (\textit{whi}D, \textit{whi}E, \textit{whi}F, \textit{whi}L, \textit{whi}M and \textit{whi}O) function in septation and spore maturation.

The early \textit{whi}G gene plays a central role in the initiation of differentiation of aerial hyphae into spores (Fig. 3). It encodes a RNA polymerase $\sigma$ factor (Chater \textit{et al.}, 1989; Chater, 2001). The aerial hyphae of \textit{whi}G mutants are long without any signs of coiling or septa formation (Chater, 1972; 1975; Wildermuth and Hopwood, 1970). In contrast, overexpression of \textit{whi}G changes the developmental fate of the substrate mycelium. Vegetative hyphae, either in liquid culture or in the agar, undergo metamorphosis into chains of spores, instead of lysing like wild-type cells (Chater \textit{et al.}, 1989). \textit{whi}G is expressed during all growth phases suggesting that WhiG is post-transcriptionally regulated (Kelemen \textit{et al.}, 1996). In a \textit{bld}D mutant \textit{whi}G mRNA levels are increased compared to the wild-type strain showing that BldD represses transcription of \textit{whi}G (Elliot \textit{et al.}, 2001; Fig. 3). The importance of the repression of \textit{whi}G by BldD remains to be established although it represents the first example of a direct link between \textit{bld} and \textit{whi} genes (Fig. 3).

Two other early \textit{whi} loci, \textit{whi}H and \textit{whi}I are regulated by the activity of $\sigma^{\text{WN}G}$ (Ryding \textit{et al.}, 1998; Aínsa \textit{et al.}, 1999). Aerial hyphae of \textit{whi}H mutants are loosely coiled but no further development towards sporulation occurs (Ryding \textit{et al.}, 1998). WhiH shares homology with members of the GntR family of regulators (Haydon and
Guest, 1991). These proteins consist of a DNA-binding domain and a signal-sensing region usually responding to an acidic carbon metabolite (Ryding et al., 1998).

Table 2. *whi* loci discovered through the analysis of white mutants (adapted with permission from Chater, 2001)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene product</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>whiA</em></td>
<td>Protein possibly involved in growth cessation of aerial hyphae; conserved in Gram-positive bacteria</td>
<td>Aínsa et al., 2000; Chater, 2001</td>
</tr>
<tr>
<td><em>whiB</em></td>
<td>Small putative transcription factor containing four conserved cysteine residues; possibly involved in growth cessation of aerial hyphae; conserved in actinomycetes</td>
<td>Soliveri et al., 2000; Chater, 2001</td>
</tr>
<tr>
<td><em>whiD</em></td>
<td>Homologue of WhiB; regulator of late sporulation processes like spore maturation and pigmentation</td>
<td>Molle et al., 2000</td>
</tr>
<tr>
<td><em>whiE</em></td>
<td>Enzymes responsible for formation of the polyketide spore pigment</td>
<td>Davis and Chater, 1990</td>
</tr>
<tr>
<td><em>whiG</em></td>
<td>Early RNA polymerase sigma factor that initiates sporulation processes</td>
<td>Chater et al., 1989</td>
</tr>
<tr>
<td><em>whiH</em></td>
<td>Homologue of the GntR family of regulators containing a DNA-binding motif; activated form induces late sporulation processes</td>
<td>Ryding et al., 1998; Haydon and Guest, 1991</td>
</tr>
<tr>
<td><em>whiI</em></td>
<td>Putative response regulator; activated form induces late sporulation processes</td>
<td>Aínsa et al., 1999</td>
</tr>
<tr>
<td><em>whiL</em></td>
<td>Unknown</td>
<td>Ryding et al., 1999</td>
</tr>
<tr>
<td><em>whiM</em></td>
<td>BldM (see Table 1)</td>
<td>Ryding et al., 1999; Molle and Buttner, 2000</td>
</tr>
<tr>
<td><em>whiN</em></td>
<td>BldN (see Table 1)</td>
<td>Bibb et al., 2000; Molle and Buttner, 2000; Elliot et al., 2001</td>
</tr>
<tr>
<td><em>whiO</em></td>
<td>Unknown</td>
<td>Ryding et al., 1999</td>
</tr>
</tbody>
</table>

Aerial hyphae of *whii* mutants resemble those of *whiH* mutants. WhII is homologous to members of the response regulator family of transcriptional activators (Hakenbeck and Stock, 1996). However, it lacks two conserved residues in the phosphorylation pocket found in these regulators, making WhII an atypical member of the family. WhII negatively regulates its own production and that of *whiH* (Ryding et al., 1998; Aínsa et al., 1999). Both WhI and WhII are believed to be activated upon cessation of growth of aerial hyphae, thereby releasing their autorepressor activities (Aínsa et al., 1999; 2000; Chater, 2001). The activated
proteins induce late sporulation processes like septation and genome partitioning (Ainsa et al., 1999; Flärdh et al., 1999; Chater, 2001).

*whiD* and *whiE* are late *whi* loci. WhiD shares similarity with WhiB and likely represses abundant septation and activates spore maturation (Molle et al., 2000). The *whiE* locus encodes proteins responsible for production of the grey spore pigment (Davis and Chater, 1990). It consists of at least seven genes showing homology to genes encoding components of type II polyketide synthases (Kelemen et al., 1998). All known *whi* genes are summarized in Table 2.

**Surface layers of aerial hyphae and spores of streptomycetes**

In contrast to substrate hyphae, aerial hyphae and spores of streptomycetes are hydrophobic. These aerial structures are surrounded by a thin fibrous sheet, called the rodlet layer, that consists of a mosaic of parallel rods dubbed rodlets (Hopwood and Glauert, 1961; Wildermuth et al., 1971). The rods are up to 450 nm in length and have a diameter of 8-12 nm (Fig. 4). Although often in pairs, the rods also occur individually, and sometimes a number of them radiate from one point. The rodlet layer remains intact when aerial hyphae differentiate into spores. As they begin to separate, the layer ruptures between adjacent spores thus partially exposing one of the two underlying spore wall layers that have no apparent ultrastructure (Hopwood and Glauert, 1961). Smucker and Pfister (1978) proposed that the exterior face of the rodlet mosaic is covered with two additional layers, a granular matrix of unknown nature and a thin fibrous sheet, suggesting the existence of in total five spore layers outside the plasma membrane. The significance of the rodlet layer and the other surface layers in streptomycetes is unknown. One or more of them are expected to render spores hydrophobic. The water-repellent nature was proposed to prevent aerial hyphae to grow back into the hydrophilic substrate (Wösten and Willey, 2000). Furthermore, it would provide rigidity to the aerial hyphae and facilitates the dispersal of spores through the air.
Rodlets have not only been observed at surfaces of aerial structures of streptomycetes (Hopwood and Glauert, 1961; Bradley and Ritzi, 1968; Wildermuth, 1972; Bradshaw and Williams, 1976), but also at those of other actinomycetes (McVittie et al., 1972; Takeo and Uesaka, 1975; Takeo, 1976), and fungi (Hess et al., 1968; Hess and Stocks, 1969; Beever et al., 1979). Until recently, it remained unknown which components form the bacterial rodlet layer (Claessen et al., 2002; 2003; 2004; Chapters 3, 4, 5). In the past, it was suggested that lipids contribute to formation of the rodlets that disappeared from the spore surface of Streptomyces venezuelae after organic extractions with xylene or benzene (Bradley and Ritzi, 1968). Studies on S. griseus showed that the rodlet layer could be removed from spore surfaces with virtually all solvents. However, this was caused by the general disruptive effect of the treatment. The rodlets themselves were not, or only partially dissolved (Bradshaw and Williams, 1976). Smucker and Pfister (1978) stated that the rodlets were composed of polymers of N-acetylated glucosamine. However, a significant amount of protein was detected in these samples as well, suggesting that rodlets are formed by one or more proteins. SapB was not expected to form the rodlets, because they were observed on aerial structures of cultures of S. coelicolor grown on minimal medium (Wildermuth et al., 1971). Under this condition SapB is not formed (Willey et al., 1991). Furthermore, using immunogold labelling SapB could only be localized in the culture medium but not within or on the surface of aerial hyphae (Wösten and Willey, 2000).

Fungal rodlets and the similarity in formation of aerial structures by filamentous fungi and filamentous bacteria

The rodlet layer of filamentous fungi is the result of self-assembly of class I hydrophobins at hydrophilic-hydrophobic interfaces (Wösten et al., 1993; 1994a; 1995). Class I hydrophobins are small secreted proteins (~100 amino acids) that are characterized by eight conserved cysteine residues and a typical hydropathy pattern (Wessels, 1997; Wösten, 2001). The rodlet layer is an amphipathic film of about 10 nm thin (Wösten et al., 1993; 1994a; 1995), which can only be dissociated by agents like trifluoroacetic acid or formic acid (de Vries et al., 1993; Wösten et al., 1993; 1994a; 1995).
The best-studied hydrophobin is SC3 of the basidiomycete *Schizophyllum commune*. SC3 has several functions in development of *S. commune*. SC3 secreted by submerged hyphae assembles at the medium-air interface. Self-assembly is accompanied by a dramatic decrease of the water surface tension from 72 to as low as 24 mJ m⁻². This decrease in surface tension enables hyphae to breach the medium-air interface to grow into the air (Wösten et al., 1999a; Fig. 5). In a strain in which the SC3 gene was disrupted (ΔSC3 strain) lowering of the water surface tension was severely affected and correlated with a reduced capacity to form aerial hyphae (van Wetter et al., 1996; Wösten et al., 1999a). Addition of purified SC3 to the medium of the ΔSC3 strain restored the formation of aerial hyphae that, in contrast to those of the wild-type strain, were hydrophilic. Reintroduction of the SC3 gene restored the formation of hydrophobic aerial hyphae. This showed that aerial hyphae have to secrete SC3 to obtain their hydrophobic nature (Wöst en et al., 1999a). Apart from mediating escape of hyphae into the air and rendering aerial hyphae hydrophobic, SC3 also attaches hyphae to a hydrophobic surface by assembling at the interface between the cell wall and the hydrophobic solid (Wösten et al., 1994b).

Formation of aerial hyphae in filamentous fungi and filamentous bacteria have strikingly similar mechanisms. Like SC3 in *S. commune*, SapB is believed to facilitate the emergence of aerial hyphae of *S. coelicolor* by breaking the surface tension at the water-air interface (Fig. 5). Similar to SapB, SC3 complemented the morphological defect of *bld* mutants of *S. coelicolor* when it was applied at the colony surface (Tillotson et al., 1998). On the other hand, streptofactin of...
**Streptomyces tendae**, the supposed orthologue of SapB (Richter et al., 1998), not only restored the capacity of the bld strains of *S. coelicolor* to erect aerial structures but also that of the ΔSC3 strain of *S. commune* (Wösten et al., 1999b). These data show that structurally unrelated molecules found in filamentous prokaryotic and eukaryotic microbes have been functionally converged (Wösten and Willey, 2000). However, as mentioned above, SapB is not expected to render aerial hyphae of *S. coelicolor* hydrophobic. Thus, in contrast to filamentous fungi, streptomycetes use different structural molecules to allow formation of aerial structures. Furthermore, it was not known whether streptomycetes, like filamentous fungi, attach to hydrophobic surfaces and, if so, which molecules mediate this process.

**Outline of this thesis**

Morphological differentiation of the filamentous bacterium *S. coelicolor* has been of interest to many scientists. Analysis of mutants impaired in formation of aerial hyphae on solid growth media led to the discovery of many regulatory proteins involved in differentiation. Until now, only little information was available on structural proteins involved in this process. The following chapters of this thesis describe the process of aerial hyphae formation in liquid standing cultures and the identification and characterization of structural proteins that are involved in formation of aerial hyphae and attachment.

**Chapter 2** describes growth and differentiation of *S. coelicolor* in liquid standing cultures. Initially, this bacterium grows submerged only. Part of the mycelium occurs free in the medium, while the other part is attached to the walls of the plate. At a certain moment, colonies migrate to the air-medium interface, where they become fixed by a rigid light-reflecting film. These colonies form aerial hyphae that differentiate into spores. Standing liquid cultures provide a good system to study morphogenesis.

**Chapter 3** describes the first identified structural proteins that coat aerial structures of *Streptomyces coelicolor*. These proteins, called rodlins A and B, were isolated by adopting the procedure to isolate fungal hydrophobins from aerial structures. Rodlins are secreted by growing aerial hyphae and are necessary for the formation of the rodlet layer. They are also secreted by submerged hyphae that contact a hydrophobic solid. Attachment to this substratum was strongly reduced in strains lacking both rodlin genes.
Chapter 4 describes the identification and characterization of a class of eight hydrophobic secreted proteins, called chaplins. Mature forms of ChpD-H are up to 63 amino acids in length, while those of ChpA-C are larger. The latter proteins consist of two domains similar to those of ChpD-H. *chpA*-*H* are expressed in aerial hyphae while transcripts of *chpE* and *chpH* were also detected during submerged growth. The Δ*chpABCDEH* strain, lacking 6 out of 8 *chp* genes, was severely affected in aerial growth, but could be rescued by the extracellular addition of purified ChpD-H. This mixture assembled into amyloid-like fibrils at the water-air interface coinciding with a high surface activity. The fibrils resembled those present at aerial hyphae of the Δ*rdlAB* strain, thus showing that these proteins enable aerial growth by lowering the water surface tension and rendering aerial structures hydrophobic.

Chapter 5 describes that formation of the rodlet layer is conserved in streptomycetes and results from the interplay between the non-redundant rodlins and chaplins. Strains of *S. coelicolor* in which the rodlin genes *rdlA* and/or *rdlB* were deleted no longer formed rodlets. Instead, fine fibrils were observed. Deletion of all eight chaplins (Δ*chpABCDEFGH* strain) prevented formation of these fibrils as well as the rodlet layer. This suggests that rodlets are composed of these fibrils. The strong reduction in the number of aerial hyphae in the Δ*chpABCDEH* and Δ*chpABCDEFGH* strains was accompanied by a decrease in expression of the *rdl* genes although the level of expression per aerial hypha was identical to that of the wild-type strain. This suggests that expression of the *rdl* genes is initiated after the hypha has sensed that it has left the aqueous environment.

Chapter 6 describes the role of chaplins in attachment and dispersed growth of *S. coelicolor*. Two different mechanisms for attachment were observed. Strong attachment of wild-type hyphae in mMMP standing liquid cultures coincides with the formation of fibrils with a diameter of 30-100 nm that are hardly observed in the Δ*chpABCDEFGH* strain but are present in the Δ*rdlAB* strain. Hyphae that adhere weakly, as in gNMMP standing medium, do not form these fibrils but require both rodlins and chaplins to remain attached. Chaplins can assemble into amyloid fibrils at the interface between water and a hydrophobic solid and even in the absence of a hydrophobic-hydrophilic interface provided that an assembled nucleus is present. These data strongly suggests that attachment in mMMP as well as gNMMP is mediated by amyloid fibrils. Moreover, they appear to function in dispersed growth. Mycelium of the Δ*chpABCDEH* and Δ*chpABCDEFGH* strains grew less dispersed than the wild-type strain.

The results described in this Thesis are summarized and discussed in Chapter 7.