Chapter 7

Summary and general discussion
Within the bacterial domain streptomycetes have an unusual life cycle. These microorganisms colonize dead and living organic material by means of hyphae that grow at their apices. The hyphae are part of an interconnected network, which is called a mycelium. At a certain moment, hyphae grow out of the substrate into the air. The aerial hyphae eventually septate to form chains of exospores that, after dispersal, give rise to new mycelia. Streptomycetes not only grow in moist substrates or in the air but they may also grow over and attach to hydrophobic surfaces such as the leaf of a plant or the skin of an animal. The mechanisms enabling streptomycetes to leave the aqueous environment and to grow into the air or to attach to a hydrophobic solid was the subject of this Thesis. Two classes of proteins, called chaplins and rodlins, were identified that are involved in these processes.

**Formation of aerial structures in *S. coelicolor***

**The role of SapB in formation of aerial hyphae**

The regulatory pathways underlying aerial growth in the model streptomycete *Streptomyces coelicolor* have been studied for many years. A variety of mutants have been characterized that fail to grow into the air. These so called bald (*bld*) mutants have in common their inability to produce and secrete a small hydrophobic peptide called SapB (Willey *et al.*, 1991; 1993). Formation of aerial hyphae could be restored in *bld* mutants by applying this peptide on the colony surface. This suggested that SapB functions as a surfactant allowing hyphae to breach the water-air interface. Indeed, aerial hyphae formation was also restored by the surfactants streptofactin from *Streptomyces tendae* and the SC3 hydrophobin from *Schizophyllum commune* (Tillotson *et al.*, 1998). SapB was not detected in cell walls of substrate hyphae, aerial hyphae as well as spores (Wösten and Willey, 2000), suggesting that SapB only functions in the medium.

The aerial hyphae induced by SapB, streptofactin, and SC3 turned out to be erected vegetative hyphae that were unable to sporulate. True aerial hyphae formation and subsequent sporulation was restored in most *bld* mutants by growing them adjacent to each other (Willey *et al.*, 1993) or by growing them on minimal medium containing mannitol instead of glucose (Willey *et al.*, 1991). Interestingly, in minimal media SapB is not produced. From these data it can be concluded that 1) SapB is not sufficient to initiate full development; 2) that other molecules take over the function of SapB in mannitol containing media; and 3) that other
molecules than SapB change the surface characteristics of hyphae when these hyphae grow into the air.

**The role of rodlin in formation of the rodlet layer in *S. coelicolor***

In contrast to substrate hyphae, aerial hyphae and spores of filamentous bacteria and filamentous fungi are hydrophobic. Hydrophobicity coincides with a surface layer consisting of a mosaic of 10-nm-wide rods. In filamentous fungi this so-called rodlet layer results from self-assembly of hydrophobins (Wösten et al., 1993). The fungal rodlet layer is highly insoluble, resisting boiling in 2% SDS (Wessels et al., 1991a; 1991b). However, these proteins could be selectively extracted from SDS-treated cell walls with trifluoracetic acid (TFA) (de Vries et al., 1993). This extraction method was applied on cell walls of sporulating cultures of *S. coelicolor* and its close relative *Streptomyces lividans* (Chapter 3). In both cases, a mixture of two highly abundant homologous proteins was identified. These proteins were called rodlin RdIA and RdIB. The *rdl* genes were specifically expressed in growing aerial hyphae. Immuno-labelling showed that the rodlin are located at the outer surface of aerial hyphae and spores where they form a highly insoluble layer (Chapter 3). Deletion of either or both *rdl* genes (Chapter 3, 5) did not affect aerial hyphae and spore formation, but prevented formation of the rodlet layer. This showed that RdIA and RdIB are not redundant. Homologues of the *rdl* genes were identified in other streptomycetes that form rodlet decorated aerial structures but were absent in the genome of *Streptomyces avermitilis* that produces spores with a smooth surface (Chapter 5).

Aerial structures of the *S. avermitilis* wild-type strain and the *S. coelicolor* ΔrdIA, ΔrdIB, and the ΔrdIAB strains were still hydrophobic. This indicated that the rodlet layer and rodlin are neither required for formation of aerial hyphae nor for surface hydrophobicity. The function of the rodlet layer is still unknown. Absence of this surface layer did neither affect germination of spores nor resistance to drought. Possibly, rodlets increase resistance to proteases or provide a more efficient dispersal of spores in nature (by wind or insects).

**The role of chaplin in formation of aerial hyphae**

*Surface hydrophobicity, rigidity and prevention of aggregation of aerial hyphae*

Surfaces of spores of the *S. coelicolor* ΔrdIAB strain no longer possessed the rodlet layer. Instead, fine fibrils with a diameter of 4-6 nm were observed resembling
those formed upon drying down aqueous solutions of TFA extracts of SDS-treated cell walls of the wild-type strain or the $\Delta$rdlAB strain (Chapter 4). MALDI-TOF mass spectrometry identified five proteins in these extracts with molecular weights of about 5 kDa. The proteins were called chaplins D-H. Their masses corresponded to the deduced molecular weights of hydrophobic proteins encoded by five ORFs in the genome sequence of *S. coelicolor* (assuming cleavage of their predicted signal sequences). Three additional homologues were found in the genome sequence. The mature forms of these secreted proteins, ChpA-C, consist of two so-called chaplin domains (i.e. sequences similar to those of mature ChpD-H) followed by a hydrophilic region and a cell wall anchoring domain. The latter is a substrate for sortases that covalently attach molecules to the peptidoglycan layer (Mazmanian et al., 1999; Pallen et al., 2001). Anchoring to the cell wall would explain why ChpA-C were not identified in the TFA extracts of SDS-treated cell walls (Chapter 4). Possibly, the large chaplins co-assemble with ChpD-H, thereby attaching the chaplin fibrils to the cell wall (see below).

Deletion of the *chp* genes affected aerial growth progressively. The $\Delta$chpABCDH strain still formed aerial hyphae, albeit delayed. Thus, the large chaplins ChpA-C are not essential for this developmental process. Formation of aerial hyphae was strongly reduced in the $\Delta$chpABCDEH strain (Chapter 4), and almost completely absent in the $\Delta$chpABCDEFGH strain (Chapter 5). The few aerial hyphae produced by the latter strain were hydrophilic and aggregated in bundles. These bundles were not stable but collapsed on top of the underlying substrate hyphae. Thus, the assembled chaplins provide surface hydrophobicity, keep aerial hyphae apart and provide rigidity (Chapter 5). In contrast to previously described *bld* mutants (Willey et al., 1991; 1993), aerial growth of the *chp* mutants was affected regardless of the medium used. This clearly shows the essential role of chaplins in morphogenesis.

Spores of the $\Delta$chpABCDEFGH strain lacked both the rodlet layer and the chaplin fibrils (Chapter 6). This suggested a link between these structures, and thus between rodlins and chaplins. Rodlets consist of two rods that are composed of two fibrils similar in size to those formed by chaplins (Chapter 4). Based on these data it was proposed that rodlets consist of four chaplin fibrils that are aligned by rodlin molecules (Chapter 5). No biochemical data support this model yet. Rodlets could not be reconstituted in vitro by mixing rodlins and chaplins. This failure may be explained by the fact that rodlins isolated from cell walls of aerial hyphae of *S. coelicolor* or heterologously produced in *Escherichia coli* were unfolded. Possibly, rodlins only attain their active conformation with the help of foldases (Chapter 6).
Formation of aerial hyphae was restored in the ΔchpABCDEH strain by applying mixtures of ChpD-H on top of the colony surface (Chapter 4). The erected hyphae possessed the typical rodlet layer showing that these are true aerial hyphae (unpublished). Addition of chaplins also accelerated formation of aerial hyphae in the wild-type strain (Chapter 4). The presence of rodlets at surfaces of the induced aerial hyphae indicates that the chaplins indirectly activate the expression of rdl genes and possibly their own expression as well (Chapter 5). Possibly, S. coelicolor hyphae sense their presence in the air and, as a consequence, activate aerial hyphae specific genes (Fig. 1). This sensing mechanism (the Skyscraper pathway) may play a key role in development of streptomycetes (see below).

The role of chaplins in the medium
Stimulation of aerial hyphae formation by exogenously added chaplins suggested that chaplins not only provide rigidity and hydrophobicity to aerial hyphae but have a function in the aqueous environment as well. Indeed, ChpE and ChpH were already formed before aerial hyphae formation was initiated (Chapter 4; unpublished). ChpD-H were shown to be highly surface-active. These chaplins lowered the water surface tension from 72 to 26 mJ m\(^{-2}\) within minutes, which was accompanied by formation of a rigid membrane at the water-air interface (Chapter 4). Thus, chaplins are likely involved in the escape of aerial hyphae from the liquid environment by lowering the water surface tension. However, in contrast to the surfactants SapB, streptofactin and SC3, chaplins did not induce aerial hyphae formation in bld mutants (Chapter 4). This came to a surprise because if lowering of the surface tension is sufficient to enable hyphae to grow into the air, chaplins should have been able to induce escape of vegetative bld hyphae. Possibly, the chaplin membrane is too rigid to allow hyphae to breach. A mixed membrane, for instance with SapB or with components that form the film at static liquid cultures (see below) may reduce this rigidity and would thus contribute to aerial growth (Fig. 1). At the moment aerial hyphae have formed, the Skyscraper pathway would take over the regulation of development and trigger production of rodlets and the chaplins ChpA-H (Fig. 1). The gene encoding the skyscraper sensor is expected to be under control of the bld genes, otherwise bld aerial hyphae induced by surfactants would have undergone full development. To study the effect of deleting chp genes on gene expression a genome wide analysis is currently being performed.

Apart from reduction of the surface tension, chaplins enable dispersed growth of the mycelium (Chapter 6). In contrast to the wild-type, the
*ΔchpABCDEFGH* strain formed large clumps of mycelium, the *ΔchpABCDEFGH* strain displaying an intermediate phenotype. Thus, like aerial hyphae (Chapter 5), substrate hyphae do not aggregate in the presence of chaplins (Chapter 6). The underlying mechanism is not known yet.

**Fig. 1.** Integrated model for the formation of aerial hyphae in the filamentous bacterium *Streptomyces coelicolor*. Extracellular signalling and environmental signals exert their influence on development via the *bld* cascade. Once environmental conditions are met, repression of *bldN* expression by BldD is relieved leading to the formation of RamR, and the chaplins ChpE and ChpH. The latter proteins are secreted into the medium, while RamR activates the synthesis of RamS, that is converted to SapB. This morphogenetic peptide is secreted (via the RamAB transporter) and functions as a signal to initiate aerial growth. SapB together with ChpE and ChpH are involved in lowering of the water surface tension enabling aerial growth. From that moment, the "Skyscraper pathway" takes over regulation of development. Escape sensors perceive their presence in the air. As a consequence, the rodlin and chaplin genes, and possibly other developmental genes as well, are activated. Rodlins and chaplins are secreted and assemble into a hydrophobic rodlet coat at the outer surface of aerial hyphae.
Other proteins involved in aerial growth

Formation of *Streptomyces* aerial hyphae and spores had solely been studied on solid agar media. However, our studies show that these differentiation processes also occur in standing liquid cultures of *S. coelicolor* (Chapter 2). These cultures may resemble flooded soils, a condition streptomycetes have to cope with in nature. After a period of submerged growth colonies emerged at the water-air interface and formed hydrophobic aerial hyphae and spores. Interestingly, the colonies were fixed at the interface by a light-reflecting film that was present at the water-air interface. Immuno-detection showed that this film does not consist of SapB (Chapter 2), while genetic evidence indicates that chaplins are not the major constituent (Chapter 4). It was suggested that this film enabled hyphae to grow into the air (Chapter 2). ∆chp strains did produce the film but no aerial hyphae were formed. Possibly, in wild-type strains the light-reflecting film consists of chaplins and a component of unknown nature. This may be the film allowing hyphae to grow into the air (see above).

Formation of aerial hyphae in standing liquid media requires migration of the non-motile bacterium to the air-liquid interface. Vertical migration of cyanobacteria and halophilic archaea in the water column is mediated by proteinaceous organelles called gas vesicles (Walsby, 1994). Interestingly, the genome of *S. coelicolor* contains two gene clusters encoding gas vesicle proteins (Chapter 2). Whether these gene clusters are instrumental in migration to the water-air interface is currently under investigation (G. van Keulen, unpublished data).

Attachment of *S. coelicolor* to hydrophobic surfaces

Attachment of microbes to host surfaces is crucial for initiation of infection. Moreover, attachment may be important for effective degradation of a substrate. Whether adherence to surfaces is essential for pathogenic streptomycetes (e.g. the plant pathogen *Streptomyces scabies* and the human pathogen *Streptomyces somaliensis*) or saprotrophic representatives of this group of bacteria is not yet known. However, it is clear that hyphae of *S. coelicolor* and *S. lividans* have the ability to attach to the hydrophobic surface of microtiter plates by two mechanisms. Strong attachment, as observed in mNMMP liquid standing cultures, coincided with the presence of an intercellular matrix consisting of 30- to 100-nm-thin fibrillar structures surrounding the hyphae (Chapter 6). These fibrils were also formed in the absence of RdIA and RdIB showing that the rodlin proteins do not contribute to
their formation. Yet, chaplins are involved. The fibrils were hardly seen in the ΔchpABCDEFGH strain that attached only weakly. Instead, amorphous material was observed at surfaces of this strain. This suggests that the fibrils consist of chaplins and a component(s) of unknown nature. This additional component would also explain the difference in diameter of the fibrils and that of assembled chaplin.

The second mechanism of attachment was observed in gNMMP. This mechanism depends on both rodlin and chaplin (Chapter 2, 6). In the absence of either class of proteins attachment was strongly reduced. This could be explained by assuming that rodlets mediate attachment (Chapter 5). Experimental data suggest that chaplins can assemble into fibrils on the cell wall-solid interface (Chapter 6; see below). Concurrently, the rodlin would align these fibrils at the solid interface into rodlets. This rodlin-dependent chaplin-mediated attachment was weaker than the rodlin-independent attachment. However, this could be an artefact because the wild-type strain grew less dispersed in gNMMP compared to mNMMP.

**Functional amyloid fibrils**

**Assembly of chaplins into amyloid-like fibrils**

Circular dichroism indicated that random coiled monomers of chaplins adopt a β-sheet-rich conformation upon assembly into the typical 4- to 6-nm-wide fibrils (Chapter 4). This conformational change was not only observed when the proteins were dried down but also when a dynamic water-air interface was introduced by vortexing or when a small amount of assembled chaplin was added to a solution of monomers. The latter shows that chaplins assemble in solution once an aggregation nucleus has been formed (Chapter 6). Conversion to the β-sheet-rich structure coincided with an increase in fluorescence of the amyloid specific dye Thioflavin T (ThT). From this it was concluded that chaplins assemble into amyloid-like fibrils.

Affinity of chaplins for hydrophobic solids was shown when the proteins were incubated with colloidal Teflon. Chaplins bound to the surface in an α-helical conformation (Chapter 6). Heating the mixture in diluted detergent induced formation of β-sheet and increased fluorescence of ThT. The α-helical conformation could be an intermediate in the assembly process as was observed for hydrophobins (see below) and other amyloid proteins (Giacomelli and Norde, 2003). Possibly, streptomycetes growing at a hydrophobic solid secrete molecules
that assist in the conversion of the intermediate form into the β-sheet-rich amyloid fibrils. These fibrils would be aligned into rodlets by the rodlin

**Other surface located functional amyloids**

It is generally accepted that any protein has a propensity to form amyloid fibrils. These fibrils result from (partial) unfolded proteins. Their formation generally proceeds via an intermediate (Giacomelli and Norde, 2003; de Vocht et al., 2002). The tendency to form amyloid fibrils can be increased by environmental conditions like low pH (Guijarro et al., 1998), post-translational modifications (Bouma et al., 2003), or mutations (Booth et al., 1997). It was shown that several amyloid-forming proteins harbor an α-helix in the polypeptide chain where a β-strand was predicted (Kallberg et al., 2001). These so-called α-helix/β-strand discordant stretches were proposed to be associated with amyloid fibril formation. Whether functional amyloids like chaplins have discordant helices remains to be established.

For many years amyloid formation was thought to be associated with loss of function and disease (e.g. Alzheimer’s and Huntington’s disease). However, amyloids can also be beneficial. For instance, functional amyloids have been shown within the eggshell of insects and fish, and at surfaces of bacteria and fungi. In case of insects and fish, amyloid fibrils are abundantly present in the chorion, the major component of the eggshell. These amyloid fibrils protect the egg and the developing embryo from mechanical pressure, proteases, bacteria and viruses (Iconomidou et al. 2000). It cannot be excluded that chaplins have similar functions.

Curli of the Gram-negative bacteria *Escherichia* and *Salmonella* spp. were the first example of prokaryotic functional amyloids. These 6- to 12-nm-wide fibrils are found at the cell surface and are involved in formation of biofilms, colonization of surfaces and binding to the host (Chapman et al., 2002). Assembly of CsgA, the major constituent of the fibrils, depends in vivo on a nucleation machinery. Possibly, initial assembly of chaplins in vivo also depends on nucleator proteins, especially when a hydrophobic-hydrophilic interface is absent.

The chaplins of *S. coelicolor* can be considered the functional equivalents of the class I hydrophobins in fungi. Hydrophobins enable fungal aerial growth, confer hydrophobicity to surfaces in contact with air and mediate attachment of hyphae to hydrophobic solids in a way similar to that of chaplins (see Wösten and de Vocht, 2000; Wösten, 2001). Both hydrophobins and chaplins assemble into amyloid fibrils at hyphal surfaces. These fibrils seem to be formed from water soluble forms via an intermediate α-helical state. Assemblages of chaplins and class I hydrophobins are
both highly surface active, insoluble in SDS and soluble in TFA. There are, however, also differences between the fungal and bacterial ‘hydrophobins’. Hydrophobins exclusively assemble at hydrophilic-hydrophobic interfaces. Thus, in contrast to chaplins, assembly cannot be initiated by seeding fibrils that serve as aggregation nuclei. This property is caused by the four disulfide bridges in the hydrophobin; when these bridges were reduced the protein spontaneously assembled in water (de Vocht et al., 2000). This difference in self-assembly may have consequences for the organization of the cell wall. Whereas hydrophobins only assemble at the surface of the cell, assembly of chaplins may occur throughout the wall. Interestingly, in contrast to S. coelicolor and S. lividans, Streptomyces griseus forms spores in liquid cultures. These spores are covered by a rodlet layer, implying assembly of chaplins and a role for rodlin. Indeed, SGrdIA, encoding the RdlA homologue from S. griseus, was expressed in liquid shaken cultures (Chapter 5). How assembly is initiated in submerged cultures (i.e. in the absence of a hydrophilic-hydrophobic interface) remains to be established. Possibly, nucleation proteins are involved like in the case of curli of Escherichia and Salmonella (Chapman et al., 2002). Another difference between chaplins and hydrophobins is that the latter proteins have not yet been reported to be cross-linked to the cell wall. However, hydrophobins do exist that have N-terminal extensions with homology to structural cell wall proteins (de Vries et al., 1999). Also these extensions may aid in integration in the cell wall.

Why does S. coelicolor have so many copies of the chaplin genes? Apparently they did not evolve to fulfil specific functions since they were shown to be partially redundant (Chapter 4; Chapter 6). Possibly, transcription from a single chp gene is not sufficient to supply the amount of chaplin needed to render the aerial mycelium hydrophobic. This would especially be the case if this protein is located throughout the cell wall. This hypothesis is supported by the observation that successive deletion of the chp genes affected aerial growth progressively.