Chapter 6

Chaplins mediate attachment and dispersed growth in *Streptomyces coelicolor*

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This chapter was submitted to the *Journal of Bacteriology*
SUMMARY

Chaplins enable the filamentous bacterium *Streptomyces coelicolor* to escape the aqueous environment to grow into the air and confer hydrophobicity to the aerial hyphae and spores. In this paper we show that chaplins also mediate attachment of hyphae to a hydrophobic surface, for which two mechanisms were observed. Chaplin-mediated attachment depended in gNMMP medium on the rodlins RdlA and RdlB, while in mNMMP attachment was independent of these proteins. In mNMMP, fibrils with a diameter of 30-100 nm were shown at surfaces of attached hyphae of the wild-type and the ∆rdlAB strain. These fibrils were hardly observed in case of the chp-less mutant and in hyphae of the wild-type strain grown on gNMMP. Chaplins assemble into amyloid fibrils at an interface between water and a hydrophobic solid, and even in solution once an assembled nucleus of chaplins is present. We therefore propose that the chaplin fibrils are involved in both attachment mechanisms. Moreover, they appear to be functional in providing dispersed growth of the mycelium. In contrast to a wild-type strain, hyphae of the chp-less mutant formed large clumps of mycelium when they were in contact with a hydrophobic solid or grown in a liquid shaken culture.

INTRODUCTION

Within the bacterial domain streptomycetes are well-known for their characteristic mode of growth by forming filaments that extend at their apices. This feature makes these soil bacteria particularly successful in colonizing and degrading organic material. After a feeding substrate mycelium has been formed, hyphae grow out of the substrate into the air to form chains of hydrophobic spores. These spores are dispersed by wind or insects enabling this organism to colonize a substrate elsewhere.

Formation of aerial hyphae and spores has been studied intensely in the model streptomycete *Streptomyces coelicolor* (Bentley *et al*., 2002). Bald (*bld*) mutants have been isolated that fail to form aerial hyphae under certain growth conditions (Kelemen and Buttner, 1998; Chater, 2001). In most of these *bld* strains genes are mutated that encode proteins with an apparent regulatory function (Kelemen and Buttner, 1998; Chater, 2001). Recently, two classes of structural proteins, called chaplins (Claessen *et al*., 2003; Elliot *et al*., 2003) and rodlins (Claessen *et al*., 2002), were identified that are involved in formation of aerial hyphae.
Eight chaplin genes, called chpA-H, were identified in the genome of *S. coelicolor* encoding hydrophobic secreted proteins (Claessen et al., 2003; Elliot et al., 2003). Mature forms of ChpA-C (± 225 aa) consist of two domains similar to ChpD-H (± 55 aa) followed by a cell wall sorting signal that is expected to covalently anchor the proteins to the peptidoglycan layer (Mazmanian et al., 1999; Mazmanian et al., 2001). Submerged growing hyphae secrete ChpE and ChpH into the culture medium. These chaplins facilitate the escape of hyphae into the air by lowering the water surface tension. ChpA-H are secreted in the cell walls of aerial hyphae where they provide surface hydrophobicity (Claessen et al., 2003). Chaplins enable aerial growth and confer surface hydrophobicity by assembling into small 4- to 6-nm-wide amyloid fibrils at the water-air and water-cell wall interface, respectively. Instead of being deposited randomly, as occurs *in vitro*, the chaplin fibrils are aligned into rodlets at the surface of aerial hyphae (Claessen et al., 2004). This process seems to be mediated by the two homologous rodlins RdlA and RdlB (Claessen et al., 2004), which are produced by aerial hyphae (Claessen et al., 2002). Deletion of either rdl gene resulted in a random deposition of the chaplin fibrils at the hyphal surface. Thus, rodlins are not redundant. Apart from aligning the chaplin fibrils, rodlins attach hyphae to hydrophobic surfaces during growth in liquid standing cultures with glucose as carbon source (Claessen et al., 2002; van Keulen et al., 2003). Hyphae that had attached to the hydrophobic surface of microtiter plates expressed both rodlin genes, while hyphae surrounded by liquid did not. The mechanism by which rodlins attach hyphae is not known yet.

We here show that chaplins mediate attachment of hyphae to hydrophobic surfaces. Two mechanisms are described, one of which being rodlin-dependent the other being rodlin-independent. Since chaplins assemble at a water-hydrophobic solid interface and even in solution when a nucleus of assembled chaplins is present, it is proposed that in both cases attachment is mediated by the amyloid fibrils of chaplins. These fibrils probably also provide dispersed growth of wild-type strains.

**METHODS**

**Strains and plasmids**

The following streptomycete strains were used: *Streptomyces coelicolor* M145 (Kieser et al., 2000), *S. coelicolor* ΔrdlAB (Claessen et al., 2004), *S. coelicolor* ΔchpABCDEFGH (Claessen et al., 2003), and *S. coelicolor* ΔchpABCDEFGH (Claessen et al., 2004). Cloning was done in *Escherichia coli* DH5α, while *E. coli* BL21(DE3) (Stratagene) was
used for heterologous expression of \( rdlA \) and \( rdlB \). Vectors and constructs used in this work are shown in Table 1.

**Table 1.** Vectors and constructs used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II KS+</td>
<td>pUC18 derivative for cloning in ( E. coli )</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pProEX-HTA</td>
<td>( E. coli ) expression vector for translational fusions to an N-terminal polyhistidine (6x His) followed by a TEV protease cleavage site</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pProEX-A</td>
<td>pProEX-HTA containing the coding sequence of mature ( rdlA )</td>
<td>This work</td>
</tr>
<tr>
<td>pProEX-B</td>
<td>pProEX-HTA containing the coding sequence of mature ( rdlB )</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Growth conditions and media**

\( S. \) *coelicolor* strains were grown at 30 °C on gNMMP, mNMMP (van Keulen *et al*., 2003), R5 or MS (Kieser *et al*., 2000) agar medium. Liquid shaken cultures were grown in YEME (Kieser *et al*., 2000), mNMMP or gNMMP (van Keulen *et al*., 2003). For growth in standing liquid cultures (van Keulen *et al*., 2003), mycelium from 20 ml of a 2-day-old YEME liquid shaken culture was washed twice with 0.1 M \( \text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4 \) buffer (pH 6.8) and taken up in 10 ml of the same buffer. 100 µl of this suspension was added to 125 ml gNMMP or mNMMP, which was subsequently used to fill 96-well flat-bottomed microtiter plates with 200 µl per well (Costar, Corning).

\( E. coli \) strains expressing \( rdlA \) or \( rdlB \) were inoculated in 30 ml LB medium (pH 7.0) containing ampicillin (100 µg ml\(^{-1}\)) and grown for 16 h at 37 °C in shaken flasks (250 rpm). These cultures were used to inoculate 3 L fermentors (1:100) containing LB medium with the antibiotic. Expression of \( rdlA \) and \( rdlB \) was induced by adding 0.6 mM IPTG at OD\(_{600}\) of 0.6. Cells were harvested 4 h after induction.

**Construction of rodlin expression vectors**

For expression of \( rdlA \) in \( E. coli \), a 346 bp fragment was generated by PCR using genomic DNA as a template and the oligonucleotides \( rdlAFW \) (ACGTGTACGACCATGGATATCGGGGACGACAACGCGCCGCGCGGT) and \( rdlAREV \) (ACGTGTACGAGTCGACTACGGCCGGCCCTCGGCCTCGCGACGA) as primers. These primers contain restriction sites for \( NcoI \) and \( SalI \) (underlined), respectively. Similarly, a 352 bp \( rdlB \) fragment was amplified using oligonucleotides \( rdlBFW \) (ACGTGTACGAGTCGACTACGGCCGGCCCTCGGCCTCGCGACGA) and \( rdlBREV \) (ACGTGTACGACCATGGATATCGGGGACGACAACGCGCCGCGCGGT) as primers. These primers contain restriction sites for \( NcoI \) and \( SalI \) (underlined), respectively.
(ACGTGTACGACCATGGATATCGCGACGACAGCGGGCCCGTCTCCGCCAA) and rdlBREV (ACGTGTACGAGTCGACTCAGCCCTTGCCCGCCCTCGCGTT). The resulting DNA fragments, encoding the mature forms of RdlA or RdlB, were digested with NcoI and SalI and ligated into the respective sites of pProEX-HTA, resulting in pProEX-A and pProEX-B, respectively. In this way the rodlin sequences were N-terminally fused with a His-tag separated by a spacer containing a Tobacco Etch Virus (TEV) protease cleavage site. Sequences of the PCR fragments were confirmed by sequence analysis.

**Purification of rodlinis and chaplinis from S. coelicolor and E. coli**

Rodlinis and chaplinis were extracted from SDS-treated cell walls of sporulating cultures of the *S. coelicolor* wild-type strain with trifluoroacetic acid (TFA) (Claessen et al., 2002). The solvent was removed by a stream of air and extracts were taken up in water. After rotating samples for 30 min using a test tube rotator at 28 rpm, the mixture was centrifuged for 15 min at 14,000 g. The rodlinis were found in the supernatant, while assembled chaplinis were contained in the pellet. The latter proteins could subsequently be monomerized using TFA. Chaplinis were also isolated from cell walls of the *S. coelicolor* ΔrdlAB strain as described (Claessen et al., 2003). TFA extracts were taken up in water (50-200 µg ml⁻¹), and, if necessary, adjusted to pH 7 with diluted ammonia.

Recombinant rodlinis were purified from cultures of *E. coli* (see above). Cells were harvested by centrifugation at 4,000 g for 10 min, after which they were resuspended at 4 °C in 50 mM Tris-HCl, pH 8.5 containing 10 mM β-mercaptoethanol and 1 mM PMSF (4 Vol buffer g⁻¹ wet weight). The cell suspension was sonicated and debris was removed by centrifugation at 10,000 g for 10 min. The crude extract containing RdIA or RdIB was applied on a Ni-NTA column and purified according to the instructions of the manufacturer (Invitrogen). The His-tag was removed using TEV-protease (leaving the GAM tripeptide of the spacer) followed by separation of the His-tag and the protease from the recombinant rodlin according to instructions of the manufacturer (Invitrogen). Prior to use, the rodlin protein fraction was dialyzed against water.
**Gel electrophoresis and Western blotting**

SDS-PAGE was done in 16% gels as described (Laemmli, 1970). Prestained broad range molecular weight markers of Bio-Rad were used. After separation, proteins were stained with 0.25% Coomassie Brilliant Blue G-250 (CBB) or silver, or blotted onto a polyvinylidene fluoride (PVDF) membrane using semi-dry blotting. PVDF membranes were treated with diluted anti-RdIA/RdIB serum (1:1000) (Claessen et al., 2002) as described (Harlow and Lane, 1988).

**Electron microscopy**

For negative staining, mycelium was placed on Formvar-coated nickel grids. After extensive washing with water, staining was done for 2 sec with 0.2% uranyl acetate. Samples were analysed with a Philips CM10 electron microscope.

**Circular dichroism (CD)**

CD-spectra were recorded over the wavelength region 190-250 nm on an Aviv 62A DS Circular Dichroism spectrometer, using a 5 mm quartz cuvette. The temperature was kept constant at 25 °C and the sample compartment was flushed with a continuous stream of N₂. Spectra are the average of three scans using a band width of 1 nm, a step width of 1 nm and 5 seconds averaging per point. The spectra were corrected by using a reference solution without the protein. Typically, a protein concentration of 50-200 µg ml⁻¹ was used. To determine the secondary structure of the protein interacting with a hydrophobic support, an amount of colloidal Teflon was added such that the protein in solution could cover 10% of the surface of the solid (de Vocht et al., 1998).

**Fluorescence spectroscopy**

To study the interaction of chaplins with the amyloid-specific fluorescent dye Thioflavine T (ThT), 3 µM of ThT was added to the proteins in different conformations. The fluorescence at 482 nm (excitation = 450 nm) was followed on an Aminco-Bowman series 2 luminescence spectrometer (SLM-Aminco®).
Attachment assay

Attachment of hyphae in standing liquid cultures in polystyrene microtiter plates was quantified as follows: 25 µl crystal violet (Acros Organics) was added to each well and left at room temperature for 10 min. For gNMMP cultures, plates were then washed three times with water using a Vaccu-Pette/96 (Sigma), removing all non-adherent cells. For mNMMP cultures, plates were vigorously washed by immersing the plate upside down in a sink with running tap water (tap diameter 1.7 cm; distance to the tap 40 cm; water flow 9 l min⁻¹). In this way, wells that had filled with water during downward movement were sucked empty during upward movement. After drying at 30°C, crystal violet associated with the attached biomass was solubilised in 200 µl 10% SDS by shaking at 900 rpm for 30 min. The OD₅₇₀ of 100 µl aliquots was determined in a microtiter plate reader. If necessary, dilutions were made in 10% SDS.

RESULTS

Chaplins are involved in attachment

We previously showed that rodlin s are involved in attachment of S. coelicolor in gNMMP liquid standing cultures using spores as inoculum (Claessen et al., 2002; van Keulen et al., 2003). Using these conditions, attachment of the wild-type strain was stronger compared to that of the ∆rdlAB strain. To establish whether chaplins are also involved in attachment in this medium, the wild-type and the ∆chpABCDEFGH strain were grown in microtiter plates. In this case, mycelium was used as inoculum since the ∆chpABCDEFGH strain hardly forms spores (Claessen et al., 2004). Biomass formation of the strains was similar and reproducible (data not shown). Yet, reproducibility of attachment of the wild-type strain was rather low: the wild-type strain attached to the walls of the microtiter plate in most, but not all, cases. In case the wild-type strain had attached, most of the mycelium resisted gentle washing but all mycelium was removed by washing vigorously. In contrast, mycelium of the ∆chpABCDEFGH strain consistently was already largely removed by gentle washing (Fig. 1A, C). These data show that apart from rodlin s, also chaplins are involved in attachment of hyphae to a hydrophobic surface when grown in gNMMP medium.

To establish whether attachment depends on the medium used, the wild-type strain as well as the ∆rdlAB and the ∆chpABCDEFGH strain were grown in minimal
medium with mannitol as carbon source (mNMMP medium). No difference in attachment was observed between the strains when microtiter plates were gently washed. However, when plates were washed vigorously, 20-40% of the wild-type and 70-90% of the ΔchpABCDEFGH hyphae detached (Fig. 1B). This was irrespective of the growth stage (Fig. 1D). The ΔrdlAB strain behaved like the wild-type (not shown).

**Fig. 1.** Chaplins are involved in attachment of hyphae to polystyrene. Cultures were grown for 7 days on gNMMP (A, C) or mNMMP (B, D). (A, B) Qualitative assessment of attachment of the wild-type (M145) strain and the ΔchpABCDEFGH strain by gentle and vigorous washing with water. Arrows indicate attached mycelium stained with crystal violet. (C, D) Quantitative assessment of attachment of the wild-type (M145) strain (●) and the ΔchpABCDEFGH strain (○) by gentle (C) and vigorous (D) washing with water.
Attached mycelium of the different strains that resisted vigorous washing was studied by electron microscopy. Interestingly, thin fibrillar structures with a diameter of 30-100 nm were abundantly present at surfaces of wild-type and ΔrdlAB adhering hyphae that had grown in mNMMP (Fig. 2A, B). These fibrils were present throughout culturing (up to 15 days) and were tightly associated with the hyphae, thus forming an intercellular matrix. These fibrillar structures were hardly observed in the ΔchpABCDEFGH strain (Fig. 2C). Instead, amorphous material was shown at and between hyphal surfaces of this mutant strain.

![Fig. 1. Negative staining of attached hyphae of the S. coelicolor wild-type strain (A, D), the ΔrdlAB strain (B, E) and the ΔchpABCDEFGH strain (C, F) grown in mNMMP (A-C) and gNMMP (D-F). Arrows indicate the 30-100 nm-wide fibrillar structures. Bar represents 2.5 µm.]

To study whether the fibrillar structures correlate with strong attachment, occurrence of these structures was also analysed in cultures grown in gNMMP. They appeared to be very low in abundance in case of the wild-type strain and the ΔrdlAB strain, and were completely absent in the ΔchpABCDEFGH strain (Fig. 2D-F). In all three cases, amorphous material was observed. From these data we conclude that formation of the intercellular fibrillar matrix depends on chaplins and is associated with strong attachment.
Chaplins mediate dispersed growth

When strains were grown as standing liquid cultures, a clear difference was observed in the morphology of the mycelium of the wild-type and the \( \Delta chp \) strains. This difference was especially evident on mNMMP medium. The wild-type strain colonized the entire polystyrene surface. In contrast, the \( \Delta chpABCDEFGH \) formed patches of large clumps of mycelium at this surface. The \( \Delta chpABCDEH \) strain had an intermediate phenotype (Fig. 3A). Similar results were obtained in liquid shaken cultures. The \( \Delta chpABCDEFGH \) strain formed larger pellets than the \( \Delta chpABCDEH \) strain, the wild-type strain growing most dispersed (Fig. 3B).

![Image](image_url)

**Fig. 3.** Chaplins mediate dispersed growth of the mycelium in liquid standing (A) and liquid shaken (B) cultures.

Structural changes of rodlins and chaplins

A mixture of rodlins and chaplins was isolated by extracting SDS-treated cell walls of sporulating cultures of the *S. coelicolor* wild-type strain with TFA (see for details Methods). Vortexing aqueous solutions of this extract resulted in a milky solution coinciding with the assembly of the chaplins into large aggregates that could not
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Fig. 4. TFA extracts of SDS-treated cell walls of the S. coelicolor wild-type strain contain a mixture of chaplins (ChpD-H) and rodlins (RdlA + RdlB) as can be visualized by SDS-PAGE (lane 1). Vortexing induces assembly of the chaplins that can no longer enter the separation gel but rodlins remain soluble (lane 2). Rodlins are found in the supernatant after centrifugation (lane 3), while assembled chaplins are contained in the pellet (lane 4). The assemblage can be dissociated into monomers by TFA.

Secondary structure of rodlins and chaplins was studied using circular dichroism (CD). Previously, it was shown that purified chaplins (ChpD-H) are unstructured when dissolved in water. However, at a water-air interface they self-assemble into amyloid fibrils, which is accompanied by the formation of β-sheet structure (Claessen et al., 2003). The structure of water-soluble chaplins (Fig. 5A, dashed line) also changed upon adding excess of colloidal Teflon (Fig. 5A, thin solid line). The CD spectrum indicated formation of α-helix (Chang et al., 1978). The conversion towards the α-helical state did not increase the fluorescence of the amyloid specific dye ThT (Table 2). The chaplins pelleted together with the Teflon spheres upon centrifugation, indicating that the chaplins were bound to the Teflon (not shown). When the chaplins adsorbed to Teflon were treated at 85 °C in the presence of 0.1% Tween-20, the spectrum became indicative for β-sheet structure (Fig. 5A, thick solid line). This treatment increased ThT fluorescence 36-fold (Table 2). This increase was similar to that obtained after vortexing a chaplin solution and showed that all monomers had assembled into amyloid fibrils (Table 2). From these data it is concluded that the amyloid structure of chaplins can be induced at a Teflon surface by treating the adsorbed protein with diluted detergent at elevated temperature.

The spectrum of an aqueous solution of rodlins obtained from cell walls of S. coelicolor was indicative for random coiled proteins (Fig. 5B, dashed line). Spectra changed dramatically within a few seconds after adding an excess of colloidal Teflon to the aqueous solutions of the rodlins (Fig. 5B, solid line). These changes were indicative for formation of α-helix. Teflon particles as well as the
protein pelleted upon centrifugation, showing that the rodlins adsorbed to the solid. The spectra did not change by treating the samples at 85 °C in the absence or presence of 0.1% Tween-20 (results not shown).

To exclude that treatment with TFA (being part of the purification method) affected the structural changes of the rodlins, it was decided to produce RdlA and RdlB in the medium. However, rodlins isolated from the cell walls of aerial hyphae were shown to be degraded in spent media of 3-day-old shaken cultures of *Streptomyces lividans*. Similarly, rodlins were degraded in spent media of *Bacillus subtilis*, *Aspergillus niger*, *Trichoderma reesei*, *Schizophyllum commune* and *Pseudomonas alcaligenes* (not shown). In contrast, rodlins were found to be stable in spent medium of *Escherichia coli*. Therefore, the coding sequences of mature *rdlA* and *rdlB* were cloned in the expression vector pProEX-HTA, resulting in constructs pProEX-A and pProEX-B. In this way the mature rodlin forms were fused with a His-tag separated by a spacer containing a Tobacco Etch Virus (TEV) protease cleavage site. Expression from these vectors in *E. coli* strain BL21(DE3) resulted in 2-5 mg L⁻¹ His-RdlA and His-RdlB. After removal of the His-tag, masses of the rodlins agreed with those predicted from the gene sequences (Claessen et al., 2002) assuming the presence of the GAM tripeptide of the spacer (results not shown). Spectra and ThT fluorescence of recombinant RdlA and RdlB and mixtures thereof were similar to those obtained with the rodlins purified from the cell walls of *S. coelicolor* after the various treatments. Moreover, the spectra of the recombinant rodlins were identical (random coil) before and after vortexing (results not shown).
Assembly of chaplins in an aqueous solution is concentration dependent and is promoted by the presence of the assembled form

The small chaplins, ChpD-H, were assembled at 22 °C for 10 min at a dynamic water-air interface (obtained by vortexing). No increase in ThT fluorescence was observed at a protein concentration below 10.6 µg ml⁻¹. Above this concentration fluorescence increased with increasing protein concentration (Fig. 6A). From these data it is concluded that assembly of ChpD-H into amyloid-like fibrils is concentration dependent.

Table 2. ThT Fluorescence (3 µM) upon interaction with chaplins (14 µg ml⁻¹) in different conformations. Fluorescence of ThT in the absence of protein was set at 1 and data were corrected for autofluorescence of the Teflon spheres.

<table>
<thead>
<tr>
<th>Conformation of chaplin</th>
<th>Relative ThT fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble</td>
<td>4.6 (± 0.47)</td>
</tr>
<tr>
<td>α-helical conformation on Teflon</td>
<td>0.59 (± 0.069)</td>
</tr>
<tr>
<td>β-sheet conformation on Teflon (5 min after addition of 0.1% Tween-20 at 85 °C)</td>
<td>166 (± 2.1)</td>
</tr>
<tr>
<td>β-sheet conformation induced by vortexing</td>
<td>159 (± 5.7)</td>
</tr>
</tbody>
</table>

Fig. 6. Assembly of chaplins occurs above a critical concentration of 10.6 µg ml⁻¹ and is independent from a hydrophilic-hydrophobic interface once a nucleus of assembled chaplin is present. **(A)** ThT fluorescence after vortexing aqueous solutions of chaplins (0-20 µg ml⁻¹) for 10 min. **(B)** Mixtures of chaplins and rodlets were incubated for 60 min with increasing amounts of seeding chaplin fibrils, water serving as a control (lane 2). The specific decrease in chaplin monomers indicates their assembly. The amount of monomeric chaplins prior to the addition of fibrils is shown in lane 1 (T=0).
Amyloid fibrils of the small chaplins (up to 1.5 µg in 25 µl) were added to 50 µl still aqueous solutions of monomeric chaplins, ChpD-H (50 µg ml\(^{-1}\)). SDS-PAGE showed that the amount of monomeric chaplins did not decrease during 1 h incubation when no amyloid fibrils were added (Fig. 6B). In contrast, more and more monomeric chaplin material disappeared from solution upon addition of increasing amounts of the assembled form. This coincided with increase in ThT fluorescence showing that the soluble chaplins assembled into amyloid fibrils (data not shown). Thus, assembly of chaplins becomes independent from a hydrophilic-hydrophobic interface once a nucleus of assembled chaplins is present.

**DISCUSSION**

This work shows that chaplins of *S. coelicolor* not only are involved in escape of hyphae from the aqueous environment into the air and in making aerial structures hydrophobic (Claessen *et al*., 2003) but also function in hyphal attachment and in dispersed growth of a mycelium. It thus shows that these proteins serve multiple functions in growth and development of this filamentous bacterium.

*S. coelicolor* has evolved two chaplin dependent ways to attach itself to hydrophobic solids. These different mechanisms were observed in gNMMP and mNMMP medium, respectively. Strongest attachment was observed in mNMMP. In this medium, adhering hyphae of the wild-type and the \(\Delta rdlAB\) strain had formed a matrix of fibrillar structures of 30-100 nm in diameter. These filaments were hardly observed in the \(\Delta chpABCDEFGH\) strain. Instead, amorphous material was shown at and in between the hyphal surfaces. Absence of the filaments correlated with a severely reduced capacity to attach as was shown for the \(\Delta chpABCDEFGH\) strain in mNMMP medium and the wild-type and \(\Delta rdlAB\) strains in gNMMP. Thus, the attachment in mNMMP depends on the presence of chaplins but not on rodlin. However, other components are expected to be involved. This is not only concluded from the presence of the amorphous material at hyphal surfaces of the \(\Delta chpABCDEFGH\) strain in mNMMP but also from the fact that the fibrils formed by chaplin monomers are smaller than 30-100 nm (i.e. 4-6 nm; Claessen *et al*., 2003). Assembly of chaplins was shown to be independent from a hydrophilic-hydrophobic interface provided that a nucleus of assembled chaplin is present. The observed 30-100 nm fibrils may thus consist of chaplin amyloid as well as material of unknown nature.

The rodlin-independent chaplin-mediated attachment resembles that by which pathogenic bacteria, like *Escherichia* and *Salmonella* spp., adhere to their hosts.
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(Chapman et al., 2002; Vidal et al., 1998). Also in these organisms amyloid fibrils, called curli (Olsén et al., 1989), form an intercellular matrix. The major constituent of curli fibrils is the CsgA protein, although other proteins are involved in the assembly process, e.g. CsgB and CsgF, that guide nucleation of CsgA (Chapman et al., 2002). The nucleation of chaplins in the absence of an hydrophobic-hydrophilic interface remains to be elucidated.

The second mechanism of chaplin-dependent attachment, as observed in gNMMP, is dependent on rodlin and is much weaker than attachment in mNMMP. Deletion of both rodlin genes in *S. coelicolor* and *S. lividans* strongly reduced attachment in gNMMP (Claessen et al., 2002). Similarly, deletion of the chaplin genes affected attachment in this medium. This may be explained by two phenomena. First, chaplins were shown to affect dispersed growth of submerged hyphae in contact with a solid. The increase in bundling of submerged hyphae would result in less contact sites with the solid, thus reducing attachment. Second, attachment of hyphae at the contact sites may be mediated by rodlets. Previously it was shown that these structures are formed at the cell wall-air interface of aerial hyphae as the result of the interplay between rodlins and chaplins (Claessen et al., 2004). Formation of rodlets at a hydrophobic solid would explain why both chaplins and rodlins affected attachment in gNMMP. Experimental data suggest that at least amyloid fibrils, and therefore rodlets as well, can indeed be formed at the cell wall-solid interface. Chaplins adopted a structure rich in α-helix at the surface of Teflon. This structure may well be an intermediate of the assembly process analogous to that observed for other amyloid forming proteins (de Vocht et al., 1998, 2002; Giacomelli and Norde, 2003). The end state of assembly could be induced by treating the solid at elevated temperatures with 0.1% Tween. Possibly, streptomycetes growing at a hydrophobic solid secrete molecules with a mode of action similar to that of Tween that help chaplin molecules to adopt the amyloid end state. These fibrils would then interact with the rodlins to form the rodlets.

How the chaplin fibrils are aligned in rodlets by the rodlins is still unknown. Rodlins isolated from cell walls of aerial hyphae of *S. coelicolor* or heterologously expressed in *Escherichia coli* were unstructured and did not assemble at the water-air interface. However, at the interface between water and a hydrophobic solid α-helix was formed. Whether this is a functional structure remains to be determined. It can not be excluded that *in vivo* other proteins (foldases) fold rodlins into a conformation that functions in assembly of the rodlet layer. It seems very unlikely that rodlins are present in this layer in an unfolded state, especially as they are insoluble in hot SDS when they reside in the cell wall (Claessen et al., 2002).
The rodlin-dependent chaplin-mediated attachment is similar to that by which certain filamentous fungi adhere to surfaces (Wösten et al., 1994b). The hydrophobin SC3 of the fungus *S. commune* was shown to assemble into (amyloid-like) rodlets at the interface between the cell wall and the hydrophobic solid. Strong attachment is realised due to the fact that the rodlets have an amphipathic nature, thereby bridging the hydrophilic cell wall and the hydrophobic solid.

Chaplins were also shown to affect dispersed growth in liquid shaken cultures. Hyphae in the ΔchpABCDEH and ΔchpABCDEFGH strains clumped together suggesting that chaplins somehow prevent aggregation of submerged hyphae. It was previously shown that ChpE and ChpH are secreted into the medium (Claessen et al., 2003). However, these chaplins are not the (only) chaplins that fulfil this task since deletion of chpF and chpG in the ΔchpABCDEH strain further affected dispersed growth. Possibly, chaplins affect the composition of the cell wall thereby preventing hyphal aggregation. Since chaplins were shown to self-assemble irrespective of a hydrophilic-hydrophobic interface once an assembled nucleus is formed, it may well be that assembled chaplins affect the properties of the cell wall.