Structural proteins involved in morphological differentiation of streptomycetes
Claessen, Dennis

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Chapter 5

The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlins and chaplins

Dennis Claessen, Ietse Stokroos, Heine J. Deelstra, Nynke A. Penninga, Christiane Bormann, José A. Salas, Lubbert Dijkhuizen, and Han A.B. Wösten

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SUMMARY

Streptomycetes form hydrophobic aerial hyphae that eventually septate into hydrophobic spores. Both aerial hyphae and spores possess a typical surface layer called the rodlet layer. We present here evidence that rodlet formation is conserved in the streptomycetes. The formation of the rodlet layer is the result of the interplay between rodlin and chaplin proteins. A strain of *Streptomyces coelicolor* in which the rodlin genes *rdlA* and/or *rdlB* were deleted no longer formed the rodlet layer. Instead, these surfaces were decorated with fine fibrils. Deletion of all eight chaplin genes (strain Δ*chpABCDEFGH*) resulted in the absence of the rodlet layer as well as the fibrils at surfaces of aerial hyphae and spores. Apart from coating these surfaces, chaplins are involved in the escape of hyphae into the air, as was shown by the strong reduction in the number of aerial hyphae in the Δ*chpABCDEFGH* strain. The decrease in the number of aerial hyphae correlated with a lower expression of the *rdl* genes in the colony. Yet, expression per aerial hypha was similar to that in the wild-type strain, indicating that expression of the *rdl* genes is initiated after the hypha has sensed that it has grown into the air.

INTRODUCTION

Streptomycetes exhibit a complex life cycle. These Gram-positive soil bacteria form a colonizing mycelium within the moist substrate by multinucleoid hyphae that grow at their apices. After a feeding substrate mycelium has been established, hyphae leave the aqueous environment to grow into the air. These aerial hyphae differentiate by forming chains of uninucleoid cells, which metamorphose into pigmented spores. These spores are dispersed and may give rise to a new mycelium. Growth into the air is accompanied by a change in the surface properties. Surfaces of hyphae in the moist substrate are hydrophilic, whereas those of aerial hyphae and spores are hydrophobic. Hydrophobicity is attributed to several surface layers, one of which is the rodlet layer. This layer consists of a mosaic of 8- to 10-nm-wide rods (Wildermuth *et al.*, 1971; Smucker and Pfister, 1978). Formation of aerial hyphae and spores has been best studied in *Streptomyces coelicolor* (for recent reviews, see Chater, 1998; 2001; Kelemen and Buttner, 1998; Wösten and Willey, 2000). Three types of secreted proteins, namely SapB (Willey *et al.*, 1991; 1993), chaplins (Claessen *et al.*, 2003; Elliot *et al.*, 2005).
Formation of the streptomycetes rodlet layer

2003) and rodlin (Claessen et al., 2002), were shown to be involved in the formation of aerial hyphae and spores.

SapB is a small secreted peptide of 18 amino acids that is produced when S. coelicolor is grown in rich medium (Willey et al., 1991). It lowers the medium surface tension from 72 to 32 mJ m$^{-2}$, thus enabling hyphae to breach the medium–air interface to grow into the air (Tillotson et al., 1998). Strikingly, no sapB gene could be identified in the S. coelicolor genome sequence (Bentley et al., 2002), and it was therefore proposed to be synthesized non-ribosomally. SapB is not expected to aid in the surface hydrophobicity of aerial hyphae and spores as this peptide could not be detected at the surfaces of these structures (Wösten and Willey, 2000). This hypothesis was strengthened by the observation that S. coelicolor does form aerial hyphae in minimal medium despite the absence of SapB under these culture conditions.

Chaplins were identified as a class of hydrophobic proteins involved in the formation of aerial hyphae in S. coelicolor (Claessen et al., 2003; Elliot et al., 2003). This class consists of eight members, ChpA–H, that are inserted into the cell walls of aerial hyphae of cultures grown on rich or minimal medium. Within the cell wall, mature forms of ChpD–H ($\pm$ 55 amino acids) and ChpA–C ($\pm$ 225 amino acids) self-assemble into amyloid-like fibrils (Claessen et al., 2003). ChpA–C contain two domains similar to ChpD–H as well as a cell wall sorting signal. This signal explains why these larger chaplins could not be extracted from cell walls of aerial hyphae. In contrast, the smaller chaplins ChpD–H could be purified from cell walls using trifluoroacetic acid. Assembly of these chaplins at the water–air interface was found to be accompanied by a huge drop in surface tension (from 72 to 26 mJ m$^{-2}$). This suggested that these proteins could be involved in escape of hyphae from the moist environment into the air. Indeed, ChpE and ChpH were found to be secreted in the culture medium as well as in cell walls of aerial hyphae. The involvement of the chaplins in the formation of these structures was confirmed by gene deletion. Formation of aerial hyphae was strongly affected in a strain in which six chp genes were deleted (ΔchpABCDEH). The surface of the aerial hyphae produced by the mutant strain still possessed a rodlet layer.

The rodlin proteins RdIA and RdIB were shown to be present at surfaces of aerial hyphae and spores where they form a highly insoluble layer (Claessen et al., 2002). Disruption of both rdlA and rdlB in S. coelicolor (ΔrdlAB strains) did not affect the formation and differentiation of aerial hyphae. However, the characteristic rodlet layer was absent. We show here that both rodlin and chaplin proteins are involved in the formation of the rodlet layer of streptomycetes. Our
results indicate that the small chaplins, ChpD–H, assemble into fibrils that are aligned into rodlets by the action of the non-redundant RdIA and RdIB rodlets.

**METHODS**

**Strains and plasmids**

The following streptomycete strains were used: *S. coelicolor* M145 (Kieser et al., 2000), *S. coelicolor* ΔrdlAB6 (Claessen et al., 2002), *S. coelicolor* ΔchpABCDEH (Claessen et al., 2003), *S. tendae* Tü901/8c (Richter et al., 1998), *S. griseus* (ATCC 13273), *S. avermitilis* (ATCC 31267) and *S. scabies* ISP5078. Cloning was done in *E. coli* DH5α, SCS110 or BW25113 (Datsenko and Wanner, 2000). *E. coli* ET12567 containing pUZ8002 was used for conjugation to *S. coelicolor* (Kieser et al., 2000). Vectors and constructs are summarized in Table 1.

<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 <em>E. coli</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>C61A</td>
<td>Cosmid 61A of <em>S. coelicolor</em> containing chpF and chpG</td>
<td>Redenbach et al., 1996</td>
</tr>
<tr>
<td>piJ82</td>
<td>pSET152 (Bierman et al., 1992) derivative containing the hyg gene, replacing a 751 bp SacI fragment containing the aac(3)IV apramycin gene</td>
<td>Dr. B. Gust (JIC)</td>
</tr>
<tr>
<td>piJ82-rdlA</td>
<td>piJ82 containing a 1501 bp fragment encompassing the putative promoter and coding sequence of <em>rdlA</em> as well as a 843 bp sequence 3’ of the stop codon of <em>rdlA</em></td>
<td>This work</td>
</tr>
<tr>
<td>piJ82-rdlB</td>
<td>piJ82 containing a 1412 bp fragment encompassing the putative promoter and coding sequence of <em>rdlB</em> as well as a 748 bp sequence 3’ of the stop codon of <em>rdlB</em></td>
<td>This work</td>
</tr>
<tr>
<td>piJ8630a</td>
<td>piJ8630 containing the 262 bp <em>S. coelicolor</em> promoter region of <em>rdlA</em> with an NdeI site at the 3’ end allowing translational fusions</td>
<td>Claessen et al., 2002</td>
</tr>
<tr>
<td>piJ8630b</td>
<td>As piJ8630a but with the promoter region of <em>rdlB</em></td>
<td>Claessen et al., 2002</td>
</tr>
<tr>
<td>StC18</td>
<td>pBluescript II KS+ derivative with a 3.1 kb SalI fragment of cosmid C18 of <em>S. tendae</em> containing STrdlA and STrdlB</td>
<td>This work</td>
</tr>
</tbody>
</table>
Growth conditions and media

*Streptomyces* strains were grown at 30°C on solid MS agar medium or in YEME medium as liquid shaken cultures (Kieser *et al.*, 2000). R5 was used for regenerating protoplasts (Kieser *et al.*, 2000). *S. griseus* was grown in liquid mNMMP (van Keulen *et al.*, 2003) to obtain submerged spores. For GFP studies, *Streptomyces* strains were grown on solid mNMMP medium (van Keulen *et al.*, 2003).

Molecular techniques

Standard molecular techniques followed the methods described by Sambrook *et al.* (1989). Protoplast preparation and transformation were performed as described by Kieser *et al.* (2000). Chromosomal DNA was isolated according to the method of Verhasselt *et al.* (1989) and modified by the method of Nagy *et al.* (1995).

Total RNA of *S. coelicolor* was isolated according to the method of Veenendaal and Wösten (1998) or van Keulen *et al.* (2004). DNA and RNA were blotted on Nylon filters (Boehringer, Mannheim) and hybridized under conditions described by Church and Gilbert (1984) at 62°C. Under these conditions, *rdlA* and *rdlB* do not cross-hybridize (Claessen *et al.*, 2002).

For preparation of the *rdlA* and *rdlB* probes, the respective coding sequences were amplified using PCR with primers *rdlACSFW* and *rdlACSREV* for *rdlA*, and *rdlBCSFW* and *rdlBCSREV* for *rdlB* (Table 2). The PCR products were radioactively

<table>
<thead>
<tr>
<th>Plasmid</th>
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<th>Reference</th>
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<tr>
<td>StC60</td>
<td>pBluescript II KS+ derivative with a 4.2 kb <em>Sall</em> fragment of cosmid C60 of <em>S. tendae</em> containing STrdlA and STrdlB</td>
<td>This work</td>
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<tr>
<td>Sg12A10</td>
<td>pBluescript II KS+ derivative with a 3.6 kb <em>Sall</em> fragment of cosmId 12A10 of <em>S. griseus</em> containing SGrdlA and SGrdlB</td>
<td>This work</td>
</tr>
<tr>
<td>pJ8630-StC18</td>
<td>pJ8630 derivative (Sun <em>et al.</em>, 1999) with a 3.2 kb <em>Kpni/XbaI</em> fragment containing STrdlA and STrdlB</td>
<td>This work</td>
</tr>
<tr>
<td>pSET-Sg12A10</td>
<td>pSET152 derivative (Bierman <em>et al.</em>, 1992) with a 2.6 kb <em>BamHI</em> fragment of Sg12A10 containing SGrdlA and SGrdlB</td>
<td>This work</td>
</tr>
</tbody>
</table>
labelled using the Prime-a-Gene® kit (Promega). For the SGdIA probe, a 618 bp BstXI–EcoRV fragment of plasmid Sg12A10 was labelled.

Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>rdlAsense</td>
<td>AGGACAGTGCGTCGCTACGACGAAAGGAACGCGAAGTGATTCCGGGGATCCGTCGACC</td>
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<tr>
<td>rdlAansense</td>
<td>AAGTCAGCGGGCCGCCCGTACCGGGCTGGGCTCATGTAGGCTGGAGCTGCTTC</td>
</tr>
<tr>
<td>rdlBsense</td>
<td>TTCGTTGGAGGGGTTTTCCCTCAGAAAGGACTGGCCAGTGATTCCGGGGATCCGTCGACC</td>
</tr>
<tr>
<td>rdlBansense</td>
<td>CGGCCGAAGTGCTCGGGCCGCCGGCGATGTCATGTAGGCTGGAGCTGCTTC</td>
</tr>
<tr>
<td>rdlACSFW</td>
<td>CATATGCTCAAGAAGGCAATTG</td>
</tr>
<tr>
<td>rdlACSREV</td>
<td>GGATCCCTAGAGCGCCCTGCAGCC</td>
</tr>
<tr>
<td>rdlBCSFW</td>
<td>CATATGCTCAAGAAGGATAGTTG</td>
</tr>
<tr>
<td>rdlBCSREV</td>
<td>GGATCCCTAGAGCGCCCTGCAGCC</td>
</tr>
<tr>
<td>ΔchpF forward</td>
<td>CCGGGATGTTGTTGTAACACCAACCAAGGAGCAACTTTCATCATGTTCCGGGGATCCGTCGACC</td>
</tr>
<tr>
<td>ΔchpF reverse</td>
<td>GTACGCGAGCCGGGCTGCGAAGTTGATGACGGCGAGTCTGAGCTGAGCTGCTTC</td>
</tr>
<tr>
<td>ΔchpG forward</td>
<td>CTGCCGCCGCCGCTCCCCCGAGTCGAAAGGAGAACTGGATGATTCCGGGGATCCGTCGACC</td>
</tr>
<tr>
<td>ΔchpG reverse</td>
<td>CCGGGAGGGAGGGGCTGCTCAGGACTGCTGAGCTGCTTC</td>
</tr>
</tbody>
</table>

Isolation of rdl homologues from S. tendae and S. griseus

Coding sequences of rdlA and rdlB from S. coelicolor were radioactively labelled and hybridized to cosmid libraries of S. tendae Tü901/8c (Bormann et al., 1996) and S. griseus (Menéndez et al., 2004). Hybridizing SalI fragments of positive clones were cloned in pBluescript II KS+ and sequenced. Accession numbers for SGdIA, SGdIB, STrdlA and STrdlB are AJ630587, AJ630588, AJ630589 and AJ630590 respectively.

Construction of M145ΔrdlA, M145ΔrdlB, M145ΔrdlAB, M145ΔchpFG and M145ΔchpABCDEFGH

The Redirect© technology (Gust et al., 2003) was used to disrupt rdlA and/or rdlB of S. coelicolor M145. For the disruption of rdlA, the aac(3)IV resistance cassette was amplified using primers rdlAsense and rdlAansense (Table 2). Similarly, for the disruption of rdlB, primers rdlBsense and rdlBansense were used. Primers rdlAansense and rdlBansense were used for the disruption of both rdlA and rdlB.

For the construction of the M145ΔchpABCDEFGH strain, lacking all chp genes, the apramycin cassette was removed in the ΔchpABCDEFGH strain enabling the reuse of this cassette to delete chpF and chpG. Primers used are shown in Table 2. chpF and chpG were mutated on cosmid C61A (Redenbach et al., 1996) using the apramycin cassette. The cosmid containing the mutated copies of both genes was
introduced into the ΔchpABCDEH strain, followed by screening for loss of both chpF and chpG. Similarly, chpF and chpG were deleted in the wild-type strain. Gene deletions were confirmed by Southern analysis.

Electron microscopy

For freeze-fracturing and cryo-scanning electron microscopy, spores were frozen in a mixture of solid and liquid nitrogen. Freeze-fracturing was done in a Polaron freeze-etch apparatus equipped with a Balzers EVM 052 unit. Replicas of Pt/C were cleaned for 16 h in 40% chromic acid at room temperature. Cryo-scanning electron microscopy was done with a JEOL microscope type 6301F at 5.0 kV using sputter coating with gold/palladium.

RESULTS

The rdlA and rdlB genes are contained on a conserved gene cluster in the genomes of streptomycetes

Streptomyces coelicolor and Streptomyces lividans contain identical copies of the rdlA and rdlB genes (Claessen et al., 2002). Hybridization of these genes with genomic DNA of various streptomycetes indicated the ubiquitous occurrence of rodlin genes in this genus (Claessen et al., 2002). To isolate the homologues of Streptomyces tendae and Streptomyces griseus, cosmid libraries were hybridized with probes directed against the coding sequences of rdlA and rdlB. Both genes hybridized with the overlapping S. tendae cosmids C18 and C60 and the S. griseus cosmid 12A10 (data not shown). The hybridizing fragments were contained on 3.2, 4.2 and 3.6 kb SalI fragments respectively. These fragments were cloned and sequenced. The overlapping S. tendae cosmids C18 and C60 contained two open reading frames (ORFs) representing the S. tendae homologues of RdlA and RdlB (Fig. 1). The genes, called STRdlA and STRdlB, are highly homologous to their equivalents in S. lividans and S. coelicolor with respect to the deduced amino acid sequence (Fig. 1B) as well as their genetic organization (Fig. 1A).
They are divergently transcribed from the putative promoter region contained in the 245 bp intergenic region. Similarly, two rodlin genes, called SGrdIA and SGrdIB, were identified on cosmid 12A10 of S. griseus (Fig. 1B). In contrast to S. coelicolor and S. tendae, the rodlin genes of S. griseus are not divergently transcribed (Fig. 1A). In between the S. griseus rodlin genes, an ORF was identified that was highly homologous to ORF2 of S. tendae and SCO2717 of S. coelicolor encoding the small chaplin ChpD. In addition, both S. griseus and S. tendae clones contained part of an ORF (ORF1) with high similarity to SCO2716 of S. coelicolor.
Formation of the streptomycetes rodlet layer

Expression of *rdl* genes correlates with the presence of rodlet-decorated surfaces

*S. coelicolor*, *S. tendae*, *Streptomyces scabies* and *Streptomyces avermitilis* form spores when grown on solid media. In contrast, some *S. griseus* strains also form spores in liquid shaken cultures. The resistance properties of *S. griseus* surface-grown and submerged spores are very similar (Kendrick and Ensign, 1983). Surfaces of spores formed on solid media were analysed by scanning electron microscopy.

In contrast to the *S. coelicolor* ∆*rdlAB* strain (Fig. 2F), rodlets were identified on wild-type spores of *S. coelicolor* (Fig. 2A), *S. tendae* (not shown), *S. scabies* (not shown).

(Fig. 1A) encoding ChpA. These data show that the *rdl* genes are located on a conserved gene cluster in streptomycetes encoding proteins involved in the formation of surface layers.

*Fig. 2.* Spores of *S. coelicolor* (A) and *S. griseus* (B and C) grown on solid media are covered with a rodlet layer. Rodlets are also observed on *S. griseus* spores produced by submerged hyphae (D). These rodlets are very similar to those produced by aerial hyphae (C). On the other hand, no rodlets were observed at surfaces of spores of *S. avermitilis* (E) or the ∆*rdlAB* strain of *S. coelicolor* (F). The latter strain could be complemented by the *rdl* genes from *S. tendae* (G) and *S. griseus* (not shown). Deletion of either *rdlA* (not shown) or *rdlB* (H) is sufficient to eradicate rodlets in *S. coelicolor*; Bars indicate 500 nm (C and D) or 100 nm (A, B, E-H). Surfaces were studied by scanning electron microscopy (A, B, E-F-H) and freeze-fracturing (C and D).
and S. griseus (Fig. 2B). The abundance and shape of the rodlets in the different strains were indistinguishable. To assess whether rodlets were restricted to aerial spores, we analysed surfaces of S. griseus spores formed in liquid cultures by freeze-fracturing. Interestingly, like spores produced by aerial hyphae (Fig. 2C), spores produced by submerged cultures of S. griseus were decorated with rodlets (Fig. 2D). This suggested that, in contrast to S. coelicolor (Claessen et al., 2002), S. griseus expresses its rodlin genes not only on solid media but also in liquid shaken cultures (Fig. 3).

Expression of SGrdIA in solid cultures decreased after most of the aerial hyphae had been formed. In contrast, expression of SGrdIA remained high in liquid shaken medium coinciding with an increase in the number of sporulating hyphae. Interestingly, S. avermitilis did not produce rodlet-decorated spores (Fig. 2E). Accordingly, its genome sequence does not contain rodlin genes (see Discussion). These data therefore show that expression of rdl genes correlates with the presence of rodlets.

**Rodlin genes from S. tendae and S. griseus complement the S. coelicolor ΔrdlAB strain**

To analyse whether the rdl homologues from S. tendae and S. griseus functionally complement the rdlAB null mutant of S. coelicolor, strain ΔrdlAB6 was transformed with pIJ8630-StC18 or pSET-Sg12A10. In this way, rdlA and rdlB homologues of S. tendae and S. griseus were introduced in the φC31 attachment site under the control of their own promoters. Scanning electron microscopy showed that formation of the rodlet layer was restored in both types of transformants. Shape
and abundance of the rodlets formed by the rodlines of *S. tendae* (Fig. 2G) and *S. griseus* (not shown) were similar to those observed in the wild-type strain of *S. coelicolor* (Fig. 2A). These data show that rodlines from *S. tendae* and *S. griseus* can functionally complement those of *S. coelicolor*.

**RdlA and RdlB are not redundant**

Disruption of both *rdlA* and *rdlB* in *S. coelicolor* and *S. lividans* resulted in the absence of rodlets on the surface of aerial hyphae and spores (Claessen *et al.*, 2002). To investigate whether these genes are redundant, the entire coding sequence of *rdlA* and/or *rdlB* was replaced by an apramycin resistance cassette in *S. coelicolor* M145 using the polymerase chain reaction (PCR)-targeting disruption system (Gust *et al.*, 2003). This resulted in the ∆rdlA, ∆rdlB and ∆rdlAB strains respectively. Formation and differentiation of aerial hyphae in the ∆rdlA, ∆rdlB and ∆rdlAB strains was unaffected on different media and growth conditions (data not shown). However, scanning electron microscopy revealed that, unlike the wild-type strain, rodlets were absent on aerial hyphae and spores of the single (Fig. 2H) and the double knock-out strains (Fig. 2F).

Expression of *rdlB*, however, was affected by the deletion of *rdlA* and vice versa. Northern analysis revealed that the amount of mRNA of *rdlB* was 5- to 10-fold lower in the ∆rdlA strain (Fig. 4), while a similar reduction was observed for mRNA of *rdlA* in the ∆rdlB strain.

![Fig. 4](image-url)
In contrast to \textit{rdlB}, two hybridizing mRNAs were observed for \textit{rdlA}. The significance of this is not yet known. To exclude the possibility that the absence of rodlets in the single knock-out strains resulted from the decrease in the expression of the intact \textit{rdl} copy, both single knock-outs were transformed with the integrating plasmids pIJ82-\textit{rdlA} and pIJ82-\textit{rdlB}. Northern analysis revealed that the integration of either plasmid restored the mRNA level of the introduced \textit{rdl} gene (Fig. 4). pIJ82-\textit{rdlA} restored the formation of the rodlet layer in the \textit{ΔrdlA} strain but not in the \textit{ΔrdlB} strain (data not shown). Similarly, formation of rodlets was rescued in the \textit{ΔrdlB} strain by the introduction of pIJ82-\textit{rdlB} (data not shown), but not by pIJ82-\textit{rdlA} (Fig. 4). These data show that RdlA and RdlB are not redundant as they are both necessary for formation of the rodlet layer.

\textbf{The small chaplins are necessary for fibril formation and assembly of the rodlet layer}

Previously, we have shown that rodlets were absent at surfaces of aerial hyphae and spores of the \textit{ΔrdlAB} strain (Claessen \textit{et al.}, 2002) but present on those of the \textit{ΔchpABCDEH} strain (Claessen \textit{et al.}, 2003). Instead, surfaces of the \textit{ΔrdlAB} strain were decorated with fine fibrils. To establish whether the formation of rodlets \textit{in vivo} also depends on the presence of chaplins, the remaining \textit{chpF} and \textit{chpG} genes were deleted in the \textit{ΔchpABCDEH} strain. Formation of aerial hyphae was severely affected in the resulting \textit{ΔchpABCDEFGH} strain (Fig. 5B) compared with that in the wild-type strain (Fig. 5A) and the \textit{ΔchpABCDEFGH} strain.

\textbf{Fig. 5.} Aerial growth is strongly affected in the \textit{ΔchpABCDEFGH} strain (B) compared with the wild-type strain (A). The aerial hyphae that are formed by the mutant strain clump together and collapse at the surface of the colony. Arrows indicate spore chains.
The few aerial hyphae formed clumped together and collapsed on top of the submerged hyphae as a result of the binding of water (Fig. 5B). Apparently, these hyphae were hydrophilic. Indeed, when droplets of water were placed on top of sporulating cultures of the ΔchpABCDEFGH strain, they spread rapidly, whereas they remained spherical in the wild-type and ΔchpABCDEH strains (data not shown). In contrast to the ΔchpABCDEFGH strain, the wild-type strain formed abundant aerial hyphae that were hydrophobic and stable for weeks. Surfaces of the few aerial hyphae of the ΔchpABCDEFGH strain possessed neither rodlets nor fibrils (Fig. 6A).

Formation of the rodlet layer and surface hydrophobicity were restored in the ΔchpABCDEFGH strain after the introduction of C61A, containing chpF and chpG (not shown). On the other hand, deletion of chpF and chpG from the wild-type strain did not affect the formation of the hydrophobic rodlet layer at aerial hyphae and spores, showing that ChpF and ChpG are not the only chaplins involved in rodlet formation. From these data, it is concluded that assembly of the rodlet layer is dependent on the presence of both rodlins and the small chaplins, ChpD–H. In addition, we conclude that the formation of the fibrils seen in the ΔrdlAB mutant strain depends on chaplin proteins.

Expression of rdlA and rdlB depends on aerial hyphae formation

The rdl genes were identified previously as being specifically expressed in aerial hyphae. To analyse whether expression of rdlA and rdlB is downregulated in the
\( \Delta \text{chpABCDEH} \) and \( \Delta \text{chpABCDEFGH} \) mutants, total RNA from 3- and 4-day-old cultures grown on solid medium was hybridized with probes directed against \( \text{rdlA} \) or \( \text{rdlB} \). Compared with the wild-type strain, expression of \( \text{rdlA} \) and \( \text{rdlB} \) was severely affected in the \( \Delta \text{chpABCDEH} \) strain, whereas it was even more reduced in the \( \Delta \text{chpABCDEFGH} \) strain (Fig. 7A).

To establish whether the reduced expression levels of the \( \text{rdl} \) genes in the \( \text{chp} \) mutant strains resulted from the reduced number of aerial hyphae, the \( \Delta \text{chpABCDEH} \) strain was transformed with pIJ8630a containing the \( \text{eGFP} \) gene under the control of the \( \text{rdlA} \) promoter. Fluorescence of green fluorescent protein (GFP) was restricted to the few aerial hyphae that had formed (Fig. 7B). Fluorescence per aerial hypha was similar to that of the wild-type strain. We thus conclude that the expression level of \( \text{rdlA} \) is determined by the number of aerial hyphae.

**Fig. 7:** Accumulation of \( \text{rdlA} \) and \( \text{rdlB} \) mRNA in 3- and 4-day-old cultures of the \( S. \text{coelicolor} \) wild-type strain and the \( \Delta \text{chpABCDEH} \) and the chaplin-less \( \Delta \text{chpABCDEFGH} \) strains (A). Northern blots were rehybridized with 16S rDNA as a loading control and to confirm the integrity of the RNA. Expression of \( \text{rdlA} \) is restricted to the few aerial hyphae formed in the \( \Delta \text{chpABCDEH} \) strain as assessed by GFP fluorescence in the \( \Delta \text{chpABCDEH} \)-pIJ8630a strain (B). Bar indicates 25 \( \mu \text{m} \).
DISCUSSION

Recently, it was shown that not only SapB (Tillotson et al., 1998) but also ChpD–H can reduce the water surface tension to allow *S. coelicolor* hyphae to grow into the air (Claessen et al., 2003). A strain in which six out of eight chaplins were deleted was severely affected in the formation of aerial hyphae, but the aerial hyphae that had formed still possessed the characteristic rodlet layer. We have shown here that a strain in which all chaplin genes were deleted was further reduced in its ability to form aerial hyphae. Moreover, the few aerial hyphae that had formed now lacked the rodlet layer. Apparently, chaplin proteins play a role in escape of hyphae from the aqueous environment and in coating the aerial hyphae with a rodlet layer. Another class of proteins involved in the formation of this surface layer is the rodlins (Claessen et al., 2002). These proteins, however, do not play a role in reduction of the water surface tension as they are only formed by aerial hyphae or hyphae in contact with a hydrophobic solid (Claessen et al., 2002).

Deletion of either *rdl* gene was sufficient to prevent rodlet formation, showing that both proteins are essential. The mechanism of rodlet formation seems to be conserved in streptomycetes. *S. tendae* and *S. griseus* also contain two rodlin genes, and these could functionally complement the ΔrdlAB strain of *S. coelicolor*. In addition, the genetic organization of the rodlin genes has been conserved in streptomycetes. Homologues of *chpA* and *chpD* were found to be clustered with the rodlin genes within the genomes of these three streptomycetes. These species represent members that are quite diverged. Interestingly, neither aerial hyphae nor spores of *S. avermitilis* were decorated with rodlets. This correlates with the absence of rodlin genes in the genome of this streptomycete (Ikeda et al., 2003). In fact, the homologues of *chpA* and *chpD* are also absent while homologues encoding for ChpB, ChpC and the variants detected in the liquid medium (ChpE and ChpH) are present (http://avermitilis.ls.kitasato-u.ac.jp/). Apparently, the part of the genome of *S. avermitilis* containing the rodlin gene cluster is lost in evolution without an obvious loss of its ability to differentiate. In agreement, aerial hyphae formation was essentially unaffected in *S. coelicolor* strains lacking four *chp* genes or the *rdl* genes (Claessen et al., 2002; 2003).

Formation of rodlets is not restricted to aerial spores *per se*. In contrast to *S. tendae* and *S. coelicolor*, some strains of *S. griseus* form spores in submerged cultures as well. These spores are decorated with rodlets, like the spores produced by aerial hyphae. This indicates that the rodlet layer can be assembled in the
absence of a hydrophobic-hydrophilic interface as occurs in filamentous fungi (Wösten et al., 1993). Thus, expression seems to determine where rodlets are formed rather than the presence of a hydrophobic-hydrophilic interface.

We showed previously that the rodlet layer was absent at surfaces of spores of the $\Delta rdlAB$ strain (Claessen et al., 2002). Instead, fine fibrils were observed. Interestingly, neither rodlets nor fibrils could be detected at surfaces of the chaplin-less strain $\Delta chpABCDEFGH$. Surfaces of the few aerial hyphae that had formed were smooth. A detailed view of the ultrastructure of the rodlet layer suggests that a rodlet consists of two rods that are themselves composed of two smaller fibrils (Wildermuth et al., 1971; Claessen et al., 2003). These fibrils have a size similar to that of an assembled chaplin fibril (Claessen et al., 2003). Based on these data, we propose that the RdlA and RdlB rodlins do not assemble into rodlets themselves but align chaplin fibrils into a rodlet layer (Fig. 8).

In the absence of either rodlin, the alignment can no longer take place, and fibrils of small chaplins (ChpD–H) are deposited randomly at the hyphal surface. As rodlets identical to the wild-type strain were observed on surfaces of the $\Delta chpABCDEFGH$ strain, ChpF and ChpG are apparently sufficient for the formation of the individual fibrils. However, they can be substituted by the other chaplins because a strain in which the $chpF$ and $chpG$ genes were deleted still formed rodlets.

We have tried to support the presented model with biochemical data. Rodlins can only be isolated from cell walls using trifluoroacetic acid. However, this solvent completely unfolds the proteins (D. Claessen, unpublished). Similarly,
rodlin produced in *Escherichia coli* were also unstructured. We therefore believe that *in vivo* as yet unidentified chaperones are involved in the folding of these proteins.

*A ‘sensing’ mechanism that directs expression of genes encoding proteins involved in aerial hyphae formation*

Previously, genes have been isolated that are blocked in the formation of aerial hyphae by interfering in regulatory pathways (Kelemen and Buttner, 1998; Chater, 2001). For instance, *bldN* encodes a developmental sigma factor (Bibb *et al*., 2000). Elliot *et al*. (2003) showed that expression of *rdlA* (and *rdlB*) was severely reduced in a *bldN* mutant, suggesting that expression of this gene is controlled by this or any earlier *bld* gene. If expression of *rdlA* and *rdlB* was solely dependent on the *bld* genes, one would expect these rodlin genes to be similarly expressed in the wild-type and chaplin-less strain. Interestingly, this was not observed. Expression of *rdlA* and *rdlB* in the colony was strongly reduced in the chaplin-less strain Δ*chpABCDEFGH*. However, expression per hypha was unaffected, as was shown using GFP as a reporter. These data strongly suggest that expression of the rodlin genes is initiated when a sensor has signalled that the hypha has left the aqueous environment. The *rdl* genes are expressed not only in hyphae that grow in the air but also in hyphae contacting hydrophobic solids (Claessen *et al*., 2002) under oxygen-limited conditions (van Keulen *et al*., 2003). This suggests that regulation of these *rdl* genes, and possibly other developmentally regulated genes, is not signalled through oxygen levels for instance. We propose that a molecule(s) accumulates in the cell wall of aerial hyphae, or hyphae in contact with a hydrophobic solid, that would otherwise diffuse into the medium. Accumulation of this molecule would be sensed and trigger rodlin expression. This mechanism would be similar to that proposed for pheromones in filamentous fungi, the so-called autocrine response (Hartmann *et al*., 1996; 1999).

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