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

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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 rodllet layer. We present here evidence that rodl formation is conserved in the streptomycetes. The formation of the rodl layer is the result of the interplay between rodlins and chaplins. A strain of Streptomyces coelicolor in which the rodlin genes rdlA and/or rdlB were deleted no longer formed the rodl layer. Instead, these surfaces were decorated with fine fibrils. Deletion of all eight chaplin genes (strain ΔchpABCDEFGH) resulted in the absence of the rodl 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 ΔchpABCDEFGH 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 rodl layer. This layer consists of a mosaic of 8- to 10-nm-wide rods (Wösten and Willey, 2000). This hypothesis was strengthened by the observation that SapB (Willey et al., 1991; 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., 2003) and rodlins (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 (±55 amino acids) and ChpA–C (±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 (\(\Delta chpABCDEH\)). 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 rdIA and rdIB in S. coelicolor (\(\Delta rdAB\) 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 rodlins.

**Results**

The rdIA and rdIB genes are contained on a conserved gene cluster in the genomes of streptomycetes

*Streptomyces coelicolor* and *Streptomyces lividans* contain identical copies of the rdIA and rdIB 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 rdIA and rdIB. 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 RdIA and RdIB (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 (Supplementary material, Fig. S1) as well as their genetic organization (Fig. 1).

They are divergently transcribed from the putative promoter region contained in the 245 bp intergenic region. Similarly, two rodlin genes, called SGrdlIA and SGrdlIB, were identified on cosmid 12A10 of *S. griseus* (Supplementary material, Fig. S1). In contrast to *S. coelicolor* and *S. tendae*, the rodlin genes of *S. griseus* are not divergently transcribed (Fig. 1). 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* (Fig. 1) 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.

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

*Streptomyces 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* Δ*rdlAB* strain (Fig. 2F), rodlets were identified on wild-type spores of *S. coelicolor* (Fig. 2A), *S. tendae* (not shown), *S. scabies* (not shown) 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 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 Δ*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–H) and freeze-fracturing (C and D).
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 SGrdlA in solid cultures decreased after most of the aerial hyphae had been formed. In contrast, expression of SGrdlA 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-SIC18 or pSET-Sg12A10. In this way, rdlA and rdlB homologues of S. tendae and S. griseus were introduced in the ΔC31 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 rodlins 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 rodlins 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 ΔrdlA, ΔrdlB and ΔrdlAB strains respectively. Formation and differentiation of aerial hyphae in the ΔrdlA, ΔrdlB and ΔrdlAB 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 five- to 10-fold lower in the ΔrdlA strain (Fig. 4), while a similar reduction was observed for mRNA of rdlA in the ΔrdlB strain.

In contrast to rdlB, two hybridizing mRNAs were observed for 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 rdl copy, both single knock-outs were transformed with the integrating plasmids pIJ82-rdlA and pIJ82-rdlB. Northern analysis revealed that the integration of either plasmid restored the mRNA level of the introduced rdl gene (Fig. 4). pIJ82-rdlA restored the formation of the rodlet layer in the ΔrdlA strain but not in the ΔrdlB strain (data not shown). Similarly, formation of rodlets was rescued in the ΔrdlB strain by the introduction of pIJ82-rdlB (data not shown), but not by pIJ82-rdlA (Fig. 4). These data show that RdlA and RdlB are not redundant as they are both necessary for formation of the rodlet layer.

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

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 \( \Delta \text{chpABCDEFGH} \) strain, they spread rapidly, whereas they remained spherical in the wild-type and \( \Delta \text{chpABCDEH} \) strains (data not shown). In contrast to the \( \Delta \text{chpABCDEFGH} \) strain, the wild-type strain formed abundant aerial hyphae that were hydrophobic and stable for weeks. Surfaces of the few aerial hyphae of the \( \Delta \text{chpABCDEFGH} \) strain possessed neither rodlets nor fibrils (Fig. 6A).

Formation of the rodlet layer and surface hydrophobicity were restored in the \( \Delta \text{chpABCDEFGH} \) strain after the introduction of C61A, containing \( \text{chpF} \) and \( \text{chpG} \) (not shown). On the other hand, deletion of \( \text{chpF} \) and \( \text{chpG} \) 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 rodlin and the small chaplins, ChpD–H. In addition, we conclude that the formation of

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**Fig. 4.** The \( rdlA \) and \( rdlB \) genes are not redundant. RNA isolated from cultures that had started to form aerial hyphae was hybridized with probes directed against \( rdlA \) or \( rdlB \) (A). As the deletion of \( rdlA \) affected the expression of \( rdlB \) and vice versa, the single knock-out strains were transformed with pIJ82-\( rdlA \) or pIJ82-\( rdlB \). The presence or absence of rodlets is indicated by + or – respectively. B. The \( \Delta rdlB \) strain transformed with an additional \( rdlA \) gene does not form rodlets.

**Fig. 5.** Aerial growth is strongly affected in the \( \Delta \text{chpABCDEFGH} \) 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 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 ΔchpABCDE and ΔchpABCDEFGH mutants, total RNA from 3- and 4-day-old cultures grown on solid medium was hybridized with probes directed against rdlA or rdlB. Compared with the wild-type strain, expression of rdlA and rdlB was severely affected in the ΔchpABCDEFGH strain, whereas it was even more reduced in the ΔchpABCDEGH strain (Fig. 7A).

To establish whether the reduced expression levels of the rdl genes in the chp mutant strains resulted from the reduced number of aerial hyphae, the ΔchpABCDEFGH strain was transformed with pIJ8630a containing the egFP gene under the control of the 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 rdlA is determined by the number of aerial hyphae.

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

Fig. 6. Freeze-fracturing revealed the absence of rodlets and fibrils at surfaces of the few aerial hyphae in the ΔchpABCDEFGH strain (A). In contrast, rodlets were observed at surfaces of spores of the wild-type strain (B) and the ΔchpABCDE strain (C).
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 ΔrdlAB 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 ΔchpABCDEFGH. 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

**Fig. 7.** Accumulation of *rdlA* and *rdlB* mRNA in 3- and 4-day-old cultures of the *S. coelicolor* wild-type strain and the ΔchpABCDEFH and the chaplin-less ΔchpABCDEFGH strains (A). Northern blots were rehybridized with 16S rDNA as a loading control and to confirm the integrity of the RNA. Expression of *rdlA* is restricted to the few aerial hyphae formed in the ΔchpABCDEF strain as assessed by GFP fluorescence in the ΔchpABCDEFH-pIJ8630a strain (B). Bar indicates 25 μm.
themselves but align chaplin fibrils into a rodlet layer (Fig. 8).

In the absence of either rodlins, the alignment can no longer take place, and fibrils of small chaplins (ChpD–H) are deposited randomly at the hyphal surface. As rodlins identical to the wild-type strain were observed on surfaces of the ΔchpABCDEH 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 rodlins.

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, rodlins 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 rdiA (and rdiB) 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 rdiA and rdiB was solely dependent on the bld genes, one would expect these rodlins genes to be similarly expressed in the wild-type and chaplin-less strain. Interestingly, this was not observed. Expression of rdiA and rdiB in the colony was strongly reduced in the chaplin-less strain ΔchpABCDEFGH. However, expression per hypha was unaffected, as was shown using GFP as a reporter. These data strongly suggest that expression of the rodlins genes is initiated when a sensor has signalled that the hypha has left the aqueous environment. The rdi 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 rdi 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).

**Experimental procedures**

**Strains and plasmids**

The following streptomycete strains were used: S. coelicolor M145 (Kieser et al., 2000), S. coelicolor ΔrdiAB6 (Claessen et al., 2002), S. coelicolor ΔchpABCDEGH (Claessen et al., 2003), S. tendae Tu901/8c (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 1. Vectors and constructs used in this work.

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pBluescript-II KS+ C61A</td>
<td>pUC18 derivative for cloning in E. coli</td>
<td>Stratagene</td>
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<td>pIJ82</td>
<td>Cosmid 61A of S. coelicolor containing chpF and chpG, replacing a 751 bp Sacl fragment containing the aac(3)IV apramycin gene</td>
<td>Redenbach et al. (1996)</td>
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<td>pIJ82-rrdA</td>
<td>pIJ82 containing a 1501 bp fragment encompassing the putative promoter and coding sequence of rdlA as well as a 843 bp sequence 3' of the stop codon of rdlA</td>
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<td>pIJ82-rrdB</td>
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<td>This work</td>
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<tr>
<td>pIJ8630a</td>
<td>pIJ8630 containing the 262 bp S. coelicolor promoter region of rdlA with an NdeI site at the 3' end allowing translational fusions</td>
<td>Claessen et al. (2002)</td>
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<tr>
<td>pIJ8630b</td>
<td>pIJ8630 containing a 1412 bp fragment encompassing the putative promoter and coding sequence of rdlB as well as a 748 bp sequence 3' of the stop codon of rdlB</td>
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<td>Stc18</td>
<td>pBluescript-II KS+ derivative with a 3.1 kb SaI fragment of cosmid C18 of S. tendae containing STrdlA and STrdlB</td>
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<td>pSET-Sg12A10</td>
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</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 Veenaendaal 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 _rdlA_CSFW and _rdlA_CSFREV for _rdlA_, and _rdlB_CSFW and _rdlB_CSFREV for _rdlB_ (Supplementary material, Table S1). The PCR products were radioactively labelled using the Prime-a-Gene® kit (Promega). For the _SGrdlA_ probe, a 618 bp _BstXI–EcorV_ fragment of plasmid Sg12A10 was labelled.

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 Sau3A fragments of positive clones were cloned in pBluescript-II KS+ and sequenced. Accession numbers for _SGrdlA_, _SGrdlB_, _STrdlA_ and _STrdlB_ are AJ630587, AJ630588, AJ630589 and AJ630590 respectively.

Construction of _M145ΔrdlA_, _M145ΔrdlB_, _M145ΔchpF_ 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 _rdlA_sense and _rdlA_antisense (Supplementary material, Table S1). Similarly, for the disruption of _rdlB_, primers _rdlB_sense and _rdlB_antisense were used. Primers _rdlA_antisense and _rdlB_antisense 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 _ΔchpABCDEH_ strain enabling the reuse of this cassette to delete _chpF_ and _chpG_. Primers used are shown in Supplementary material, Table S1. _chpF_ and _chpG_ were mutated on cosmid C61A (Redenbach et al., 1996) using the apramycin cassette. The cosmids containing the mutated copies of both genes were 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 cryoscanning 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. Cryoscanning electron microscopy was done with a Jeol microscope type 6301F at 5.0 kV using sputter coating with gold/palladium.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/ suppmat/mmi/mmi4143/mmi4143sm.htm

Fig. S1. Alignment of the amino acid sequences of the rodlin.

Table S1. Primers used in this study.

References


