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SapB and the rodmins are required for development of *Streptomyces coelicolor* in high osmolarity media

Wouter de Jong¹, Erik Vijgenboom², Lubbert Dijkhuizen¹, Han A.B.Wösten³ & Dennis Claessen^{1,4}

¹Department of Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Groningen, The Netherlands; ²Gorlaeus Laboratories, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands; ³Department of Microbiology, Institute of Biomembranes, University of Utrecht, Utrecht, The Netherlands; and ⁴Department of Molecular and Developmental Genetics, Institute Biology Leiden (IBL), Leiden University, Leiden, The Netherlands

Correspondence: Dennis Claessen, Department of Molecular and Developmental Genetics, Institute Biology Leiden, Leiden University, Sylviusweg 72, 2300 RA, Leiden, The Netherlands. Tel.: +31 71 5275052; fax: +31 71 5274900; e-mail: d.claessen@biology.leidenuniv.nl

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Introduction

Streptomycetes are filamentous bacteria with a complex life cycle. Spore germination and subsequent growth results in the formation of a substrate mycelium, which consists of a network of interconnected hyphae. Following a period of vegetative growth, aerial hyphae are formed that eventually septate into chains of spores (Claessen *et al.*, 2006). The chaplins (Claessen *et al.*, 2003; Elliot *et al.*, 2003) and SapB (Willey *et al.*, 1991; Tillotson *et al.*, 1998; Kodani *et al.*, 2004; Capstick *et al.*, 2007) have been shown to fulfill a role in spore formation. Two of eight of the chaplins, ChpE and ChpH, are secreted into the environment before aerial growth has started (Claessen *et al.*, 2003). They lower the surface tension of the medium thereby enabling hyphae to grow into the air (Claessen *et al.*, 2003; Sawyer *et al.*, 2011). Aerial hyphae secrete all chaplins, ChpA-H, which assemble on the hyphal surface into an amphipathic protein film that consists of amyloid-like fibrils (Claessen *et al.*, 2003, 2004; Capstick *et al.*,

Abstract

Streptomyces coelicolor produces spore-forming aerial hyphae after a period of vegetative growth. These aerial structures are decorated with a hydrophobic coating of rodlets consisting of chaplins and rodmins. Here, we show that rodmins and the surface-active peptide SapB are essential for development during growth in a medium with high osmolarity. To this end, both vegetative and aerial hyphae secrete SapB, whereas rodmins are only secreted by the spore-forming aerial hyphae.

2011; Sawyer *et al.*, 2011). The rodmin proteins organize these chaplin fibrils into so-called rodlets (Claessen *et al.*, 2004). Yet, under the conditions tested, rodmins were not essential for development (Claessen *et al.*, 2002). SapB is a lantibiotic-like peptide of 207 Da (Willey *et al.*, 1991; Kodani *et al.*, 2004). Like ChpE and ChpH, SapB lowers the surface tension and thus allows hyphae to grow into the air (Tillotson *et al.*, 1998; Capstick *et al.*, 2007). Production of SapB is encoded and controlled by the *ramC-SABR* gene cluster. SapB is derived from the 42 amino acid prepeptide encoded by *ramS*, which is probably post-translationally modified by the action of RamC (O'Connor *et al.*, 2002; Kodani *et al.*, 2004; Willey *et al.*, 2006). The ABC-transporter encoded by *ramAB* is generally believed to transport SapB outside of the cell (Kodani *et al.*, 2004; Willey *et al.*, 2006), while RamR is the transcriptional regulator that controls expression of *ramCSAB* (Keijser *et al.*, 2002; O'Connor *et al.*, 2002).

Interestingly, SapB was shown to be required for differentiation on certain complex media, but not on minimal

media with mannitol as the carbon source (Willey *et al.*, 1991). Here, we show that this difference is because of the osmolarity of the medium. We furthermore demonstrate that in addition to SapB, the rodlet layer contributes to efficient aerial growth when hyphae encounter osmotic stress conditions.

Results and discussion

SapB is present on the spore surface independent of medium composition

To identify proteins present on the cell surface, intact spores of *Streptomyces coelicolor* M145 were analyzed using MALDI-TOF mass spectrometry. Therefore, lyophilized spores were mixed with a matrix solution containing either α -cyano-4-hydroxycinnamic acid [10 mg mL⁻¹ in 50% acetonitrile/0.1% trifluoroacetic acid (TFA)] or sinapinic acid (20 mg mL⁻¹ in 40% acetonitrile/0.1% TFA). These mixtures were spotted for analysis with a Shimadzu Biotech MALDI-TOF Mass Spectrometer (Axima Performance).

Spectra of spores isolated from complex R5 medium (Kieser *et al.*, 2000) or complex MS medium (Kieser *et al.*, 2000) showed peaks ranging from 1 to 12 kDa (Fig. 1a). Relative high intensities were observed for peaks with masses of 5070, 5121, 5182, and 5274 Da (Fig. 1b), which fit the predicted masses of ChpD, ChpH, ChpF, and ChpE, respectively (Claessen *et al.*, 2003; Elliot *et al.*, 2003). Analysis of spores of the *S. coelicolor* *chpABCDH* (Claessen *et al.*, 2003), *chpABCDEH* (Claessen *et al.*, 2003), and *chpABCDEFGH* (Claessen *et al.*, 2004) strains (obtained after prolonged incubation on MS medium) confirmed the identity of these peaks, as they were absent in the respective mutants (Supporting Information, Fig. S1a). The rodlin proteins RdlA and RdlB (Claessen *et al.*, 2002, 2004) were also identified at the spore surface as proteins with masses of 10 517 and 10 708 Da, respectively (Fig. S1b). NepA, whose presence on the spore surface has been demonstrated by immuno labeling (de Jong *et al.*, 2009), could not be identified according to its predicted mass of 7725 Da. In contrast, SapB was found on the spore surface represented by a peak at 2027 Da. Interestingly, SapB was not only found on spores obtained from R5 medium (Fig. 1c) but also on those obtained from MS medium (Fig. 1d), a condition in which SapB was formerly shown not to be secreted by the wild-type strain (Capstick *et al.*, 2007). The intensity of the SapB peak on MS medium was about fourfold lower compared to that found with spores from R5 medium. SapB was also identified on spores on defined minimal medium with mannitol as a carbon source (Fig. 1e). Also in this medium, SapB is not secreted into the medium (Willey *et al.*, 1991). As expected, SapB was absent on spores of

the *ramR* (Fig. S1c) and *ramS* mutants (Fig. 1c–e) that had been collected from cultures grown on R5 or minimal mannitol medium. Similar results were obtained when TFA extracts of spores were analyzed by MALDI-TOF MS (data not shown).

Conditional secretion of SapB by vegetative hyphae

The fact that SapB is not secreted in certain media (Willey *et al.*, 1991; Capstick *et al.*, 2007) suggested a difference between SapB secretion by vegetative hyphae and aerial hyphae and spores. To confirm this, culture media were analyzed for the presence of SapB by MALDI-TOF MS. Agar plates overlaid with cellophane disks were inoculated with spores of the wild-type strain or the *ramR* or *ramS* mutant strains. After 5 days of growth at 30 °C, the agar medium underlying the cellophane membrane was collected and melted. Medium proteins were concentrated 50- to 250-fold by precipitation with 10% trichloroacetic acid (TCA). As expected, MALDI-TOF MS showed that SapB was not secreted by *ramR* or *ramS* mutant strains, irrespective of medium composition, whereas the wild-type strain secreted SapB in R5 medium, but not in the case of minimal mannitol medium (Fig. 1f). Taken together, these data show that SapB is unconditionally secreted by aerial hyphae of the wild type, whereas secretion of SapB by vegetative hyphae depends on medium composition. Previously, the existence of a regulatory mechanism called the *sky* pathway was proposed that operates after the *bld* cascade to control expression of aerial hyphae-specific genes such as those encoding the rodlines, chaplins, and NepA (Claessen *et al.*, 2004, 2006; de Jong *et al.*, 2009). We propose that SapB production by vegetative hyphae is under the control of the *bld* cascade, while the *sky* pathway controls production of SapB by aerial structures. The fact that SapB is produced by aerial hyphae after their emergence infers an additional, yet elusive role, during the later stages of morphological differentiation. Perhaps SapB contributes to spore wall assembly providing protection to the spores. Alternatively, it could contribute to providing a hydrated compartment involved in transport of nutrients up into the air, as suggested previously (Chater & Chandra, 2006; Chater *et al.*, 2010).

SapB is required for differentiation in high osmolarity media

Complete media used for growing *S. coelicolor*, such as R2YE or R5 medium, contain 10.3% sucrose, which is absent in minimal mannitol medium. We here addressed whether the presence of this sugar causes the SapB-dependent differentiation. To this end, the wild-type strain and

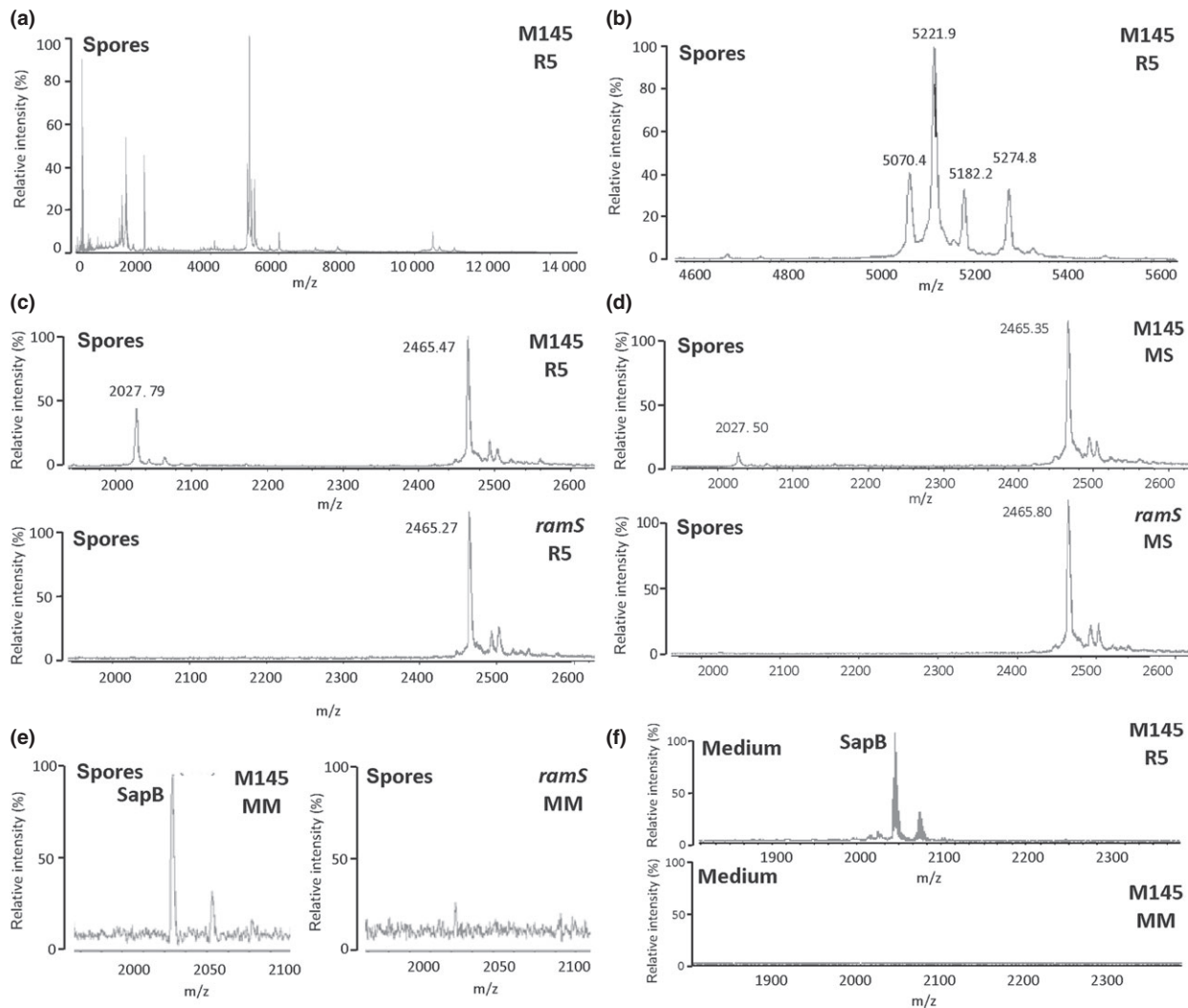


Fig. 1. MALDI-TOF mass spectrometry analysis of spores of *Streptomyces coelicolor* strains. (a) Peaks ranging from 1 to 12 kDa were detected when wild-type spores collected from R5 medium were analyzed. Detailed inspection of these spectra revealed the presence of ChpD, ChpH, ChpF, and ChpE with masses of 5070, 5121, 5182, and 5274 Da, respectively (b). Moreover, SapB (2027 Da) was detected, which was absent in the *ramS* deletion mutant (c). Analysis of spores collected from complex MS medium (d) and minimal medium containing mannitol (MM) (e) showed the presence of SapB on the spore surface in a *ramS*-dependent manner. (f) TCA-precipitated spent culture medium of the wild-type strain showed that SapB was secreted in R5 medium and not in minimal mannitol medium. ACTH-fragment 18–39 (2465 Da) was added to samples to normalize and calibrate MALDI-TOF spectra.

the *ramR* and *ramS* strains were grown on minimal mannitol medium with or without 10.3% sucrose. In the absence of 10.3% sucrose, all mutant strains developed like the wild type (Fig. 2a). In contrast, sucrose strongly delayed development of the *ramR* and *ramS* mutants (Fig. 2b). This indicates that SapB has a direct or indirect role in formation of aerial hyphae under this condition. In agreement, MALDI-TOF MS showed that SapB was present in the culture medium of the wild-type strain when the medium was supplemented with 10.3% sucrose (Fig. 2d).

To study the effect of sucrose on the interfacial surface tension, the pendant droplet technique was used, which is

based on the geometry of a droplet (Thiessen & Man, 1999; Claessen *et al.*, 2003; Sawyer *et al.*, 2011). These data showed that 10.3% sucrose hardly, if at all, reduced the surface tension of R5 medium (values with or without sucrose: 66 ± 1.2 and 64 ± 1.1 mJ m⁻², respectively) and minimal mannitol medium (73 ± 1.8 and 70 ± 1.4 mJ m⁻², respectively). Moreover, sucrose did not alter the capacity of chaplins to assemble at the medium-air interface as was assessed by measuring ThT fluorescence (data not shown). These data indicate that the effect of sucrose is exerted, directly or indirectly, via a reduced turgor pressure in the hyphae. In agreement, the addition

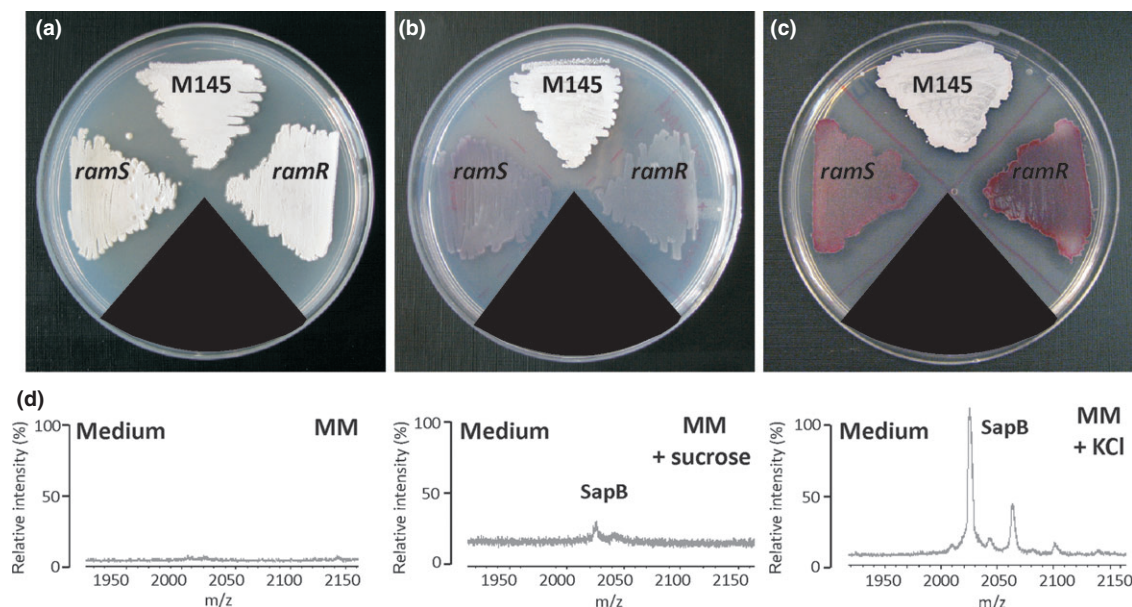


Fig. 2. Aerial growth of wild-type *Streptomyces coelicolor* M145 and *ram* mutants in media with high osmolarity. (a) Development of the *ramR* and *ramS* mutants is similar to that of the wild-type strain on minimal medium, as is indicated by the white fluffy colony surface. In contrast, the addition of 10.3% sucrose (b) or 500 mM KCl (c) strongly delayed development in the *ramR* and *ramS* mutants. (d) The addition of either osmolyte to minimal medium induced the production of SapB in the medium by the wild type, as assessed by MALDI-TOF MS.

of 250 or 500 mM KCl to minimal medium also blocked development of the *ramR* and *ramS* mutants (Fig. 2c), consistent with a critical role for turgor pressure in aerial growth, as previously suggested (Plaskitt & Chater, 1995). In contrast, the wild type did form aerial structures, which, importantly, was accompanied by the secretion of SapB into the medium (Fig. 2d).

The rodlet layer is important for differentiation in high osmolarity media

We previously showed that the rodlin proteins are not essential for aerial growth under normal conditions

(Claessen *et al.*, 2002). Strikingly, development of the *S. coelicolor* strain lacking *rdlA* and *rdlB* was strongly delayed on minimal medium supplemented with sucrose (Fig. 3) or KCl (data not shown). In agreement, increased expression of the rodlin genes was observed in sucrose-containing minimal medium (Fig. S2). Development of the *chpABCDH* mutant strain, lacking five of eight chaplin genes, was also delayed in sucrose-containing medium (Fig. 3). However, the presence of sucrose did not affect the transcript level of *chpH* (Fig. S2). Taken together, these data show that an intact rodlet layer is important for aerial growth under osmotic stress conditions.

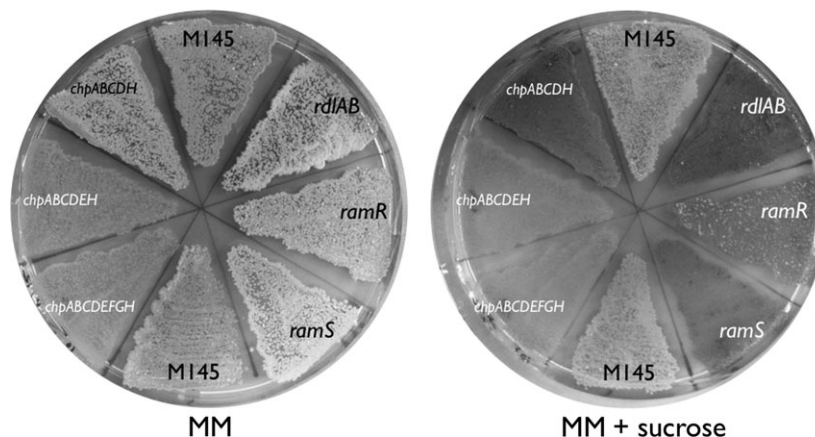


Fig. 3. Aerial growth of 5-day-old colonies of the wild-type strain and developmental mutants of *Streptomyces coelicolor* in media containing 10.3% sucrose. Development of the *rdlAB*, *ramR*, *ramS*, and *chpABCDH* mutant strains is delayed on sucrose-containing medium compared to the M145 wild-type strain, whereas the *chpABCDEH* and the *chpABCDEFGH* strains fail to form aerial structures (regardless of the presence of sucrose).

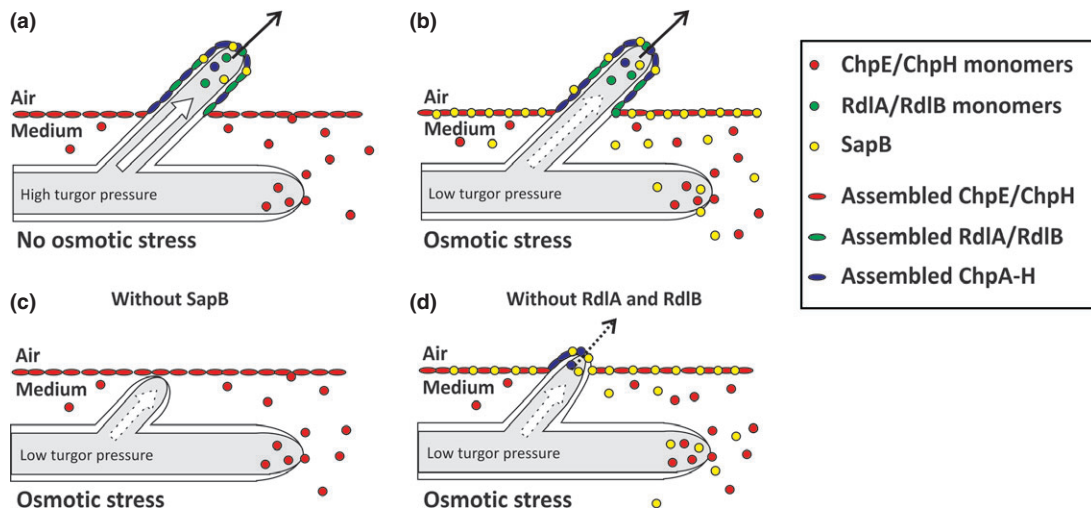


Fig. 4. Proposed model of differentiation in *Streptomyces coelicolor*. (a) The chaplins ChpE and ChpH are secreted into the aqueous environment and assemble at the air-medium interface into an amphipathic film. This is accompanied by a strong reduction in the water surface tension. Under this condition, the turgor pressure within the hyphae enables hyphae to grow into the air. (b) Turgor pressure is low in medium with high osmolarity, hampering hyphae to breach the chaplin film at the medium-air interface. Under this condition, however, SapB is secreted together with ChpE and ChpH. Co-assembly of these surfactants affects rigidity of the chaplin film formed at the air-water interface. This allows hyphae to escape the aqueous environment despite the reduced turgor pressure (c). Elongation of aerial hyphae under osmotic stress conditions requires an intact rodlet layer formed by the rodlines (RdlA and RdlB) and chaplins (ChpA-H) and possibly SapB (d). The rodlines organize the chaplin fibrils into rodlets, thus providing a rigid coat that provides stability to the aerial hyphae.

Model for aerial growth under osmotic stress conditions

On the basis of our data, we propose the following model for aerial growth. At the moment differentiation is initiated, ChpE and ChpH are secreted into the medium. These chaplins assemble into an amphipathic film at the air-water interface. As a result, the water surface tension is dramatically reduced, enabling the growth of hyphae into the air (Wösten *et al.*, 1999). In a low osmolyte aqueous environment, the turgor pressure of hyphae is sufficient to enable hyphae to breach the chaplin film (Fig. 4a). However, in a high osmolyte aqueous environment, the turgor pressure is reduced and insufficient for hyphae to break through the chaplin film to grow into the air. Possibly by intercalation, SapB may change the physical properties of the chaplin film, making it easier to breach. As a consequence, this would enable hyphae to grow into the air, despite their lower turgor pressure (Fig. 4b and c). This model implies that SapB would also affect the properties of the chaplin film at the surface of the aerial hypha. However, rodlines that are secreted by the aerial hyphae align the chaplin fibrils into rodlets resulting in a rigid film. This rigid film may provide stability of the aerial hypha especially when the turgor pressure in the cell is reduced (Fig. 4d).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. MALDI-TOF mass spectrometry analysis of spores of the *S. coelicolor* *chp*ABCDEFGH (a), *rdlAB* mutant (b), and *ramR* mutant strains (c) compared to the M145 wild-type strain, showing the presence of the chaplins (a), rodlines (b) and SapB (c) respectively.

Fig. S2. Semi-quantitative detection of transcripts of *ramS*, *rdlA*, *rdlB*, and *chpH* using RT-PCR analysis of the M145 wild-type strain grown on minimal medium in the absence (left) or presence (right) of 10.3% sucrose.

Table S1. Primers used in this study.

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