A sporulation-specific, sigF-dependent protein, SspA, affects septum positioning in Streptomyces coelicolor

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Introduction

Streptomyces coelicolor is a multicellular, filamentous bacterium that is a model organism for both bacterial development and antibiotic production. Unlike in most bacteria, cell division in S. coelicolor is incomplete during vegetative growth because of the lack of regular divisional septa formation followed by cell–cell separation, which results in long, branched, multigenomic filaments that grow across and into a solid medium. In response to yet unknown signals, aerial filaments break the surface tension and branch away from the network of substrate mycelium (Elliot et al., 2008; McCormick and Flardh, 2012; Świerzcz and Elliot, 2012). Formation of aerial hyphae depends on a set of regulatory genes, called the bld genes, encoding an RNA polymerase sigma factor BldN, transcription factors, such as BldC and BldD or a rare tRNA, bldA (Elliot et al., 2008; Świerzcz and Elliot, 2012). In addition to the regulatory genes, the hyphal emergence into the air is facilitated by hydrophobic, cell surface-associated proteins, such as SapB and the ‘chaplins’, all exhibiting surfactant properties (Claessen et al., 2003; Elliot et al., 2003a; Kodani et al., 2004; Willey et al., 2006).

Initiation of sporulation begins with the cessation of hyphal growth exclusively in the aerial hyphae followed by the separation of the tip-proximal sporogenic compartment and the subapical stem compartment (Kwak et al., 2001; Dalton et al., 2007; Flardh and Buttner, 2009). In the sporogenic hyphae, 50–100 sporulation septa are laid down synchronously at ∼ 1 µm intervals to give rise to the unigenomic pre-spore compartments, while the fate of the subapical stem is cell lysis. Sporulation septation depends on the cell division protein FtsZ, which, unlike in most bacteria, is not essential during vegetative growth in Streptomyces (McCormick et al., 1994). The increased expression of FtsZ in the aerial hyphae is the result of transcription from a second promoter, which is specific to the aerial hyphae and is dependent on the early whi genes (Flardh et al., 2000). Increased levels of FtsZ result in the formation of the initially helical FtsZ filaments followed by FtsZ rings that are spaced evenly along the sporogenic hyphae (Schwedock et al., 1997; Grantcharova et al., 2005). Sporulation septation coincides with chromosome segregation that is governed by the ParA/ParB segregation protein pair (Jakimowicz et al., 2005; 2007) together with

Summary

The RNA polymerase sigma factor SigF controls late development during sporulation in the filamentous bacterium Streptomyces coelicolor. The only known SigF-dependent gene identified so far, SCO5321, is found in the biosynthetic cluster encoding spore pigment synthesis. Here we identify the first direct target for SigF, the gene sspA, encoding a sporulation-specific protein. Bioinformatic analysis suggests that SspA is a secreted lipoprotein with two PepSY signature domains. The sspA deletion mutant exhibits irregular sporulation septation and altered spore shape, suggesting that SspA plays a role in septum formation and spore maturation. The fluorescent translational fusion protein SspA–mCherry localized first to septum sites, then subsequently around the surface of the spores. Both SspA protein and sspA transcription are absent from the sigF null mutant. Moreover, in vitro transcription assay confirmed that RNA polymerase holoenzyme containing SigF is sufficient for initiation of transcription from a single sspA promoter. In addition, in vivo and in vitro experiments showed that sspA is a direct target of BldD, which functions to repress sporulation genes, including whiG, ftsZ and ssgB, during vegetative growth, co-ordinating their expression during sporulation septation.

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Chromosome compaction in the spores relies on the bacterial cytoskeletal proteins, MreB and Mbl (Mazza et al., 2006; Heichlinger et al., 2011). In rod-shaped bacteria, such as *Escherichia coli* or *Bacillus subtilis*, cell wall synthesis of the lateral cell wall is dependent on members of the actin-like MreB family, including MreB, Mbl and MreBH (Cabeen and Jacobs-Wagner, 2010; White and Gober, 2012). In *Streptomyces* the MreB-like proteins are not involved in hyphal growth and their role is exclusive to spore development. Lack of both MreB and Mbl results in deformations in spore shape and the absence of the thick spore wall characteristic of the wild-type, mature spores. This suggests that similar to unicellular rod-shaped bacteria, MreB-like proteins are controlling cell wall synthesis during the transformation of pre-spore compartments to mature spores in *Streptomyces* (Mazza et al., 2006; Heichlinger et al., 2011). We know very little about the enzymes that are involved in the turnover of the spore wall, although as the major component of bacterial cell wall is the three dimensional polymer, peptidoglycan, peptidoglycanases and hydrolases. Recently, a group of cell wall hydrolases, SwlA, SwlB and SwlC, have been implicated in spore development. Lack of both MreB and Mbl results in deformations in spore shape and the absence of the thick spore wall characteristic of the wild-type, mature spores. This suggests that similar to unicellular rod-shaped bacteria, MreB-like proteins are controlling cell wall synthesis during the transformation of pre-spore compartments to mature spores in *Streptomyces* (Mazza et al., 2006; Heichlinger et al., 2011). We know very little about the enzymes that are involved in the turnover of the spore wall, although as the major component of bacterial cell wall is the three dimensional polymer, peptidoglycan, peptidoglycanases and hydrolases. Recently, a group of cell wall hydrolases, SwlA, SwlB and SwlC, have been implicated in spore development (Haisser et al., 2009). Chromosome compaction in the spores relies on nucleoid-associated proteins, such as HupS (Salerno et al., 2009) and the Dps proteins (Facey et al., 2009; 2013; 2011).

Spore maturation in the late stages of differentiation is governed by an RNA polymerase sigma factor, σ^7. Spores of the *sigF* mutant have thinner walls and smaller sizes and the spore chains of the *sigF* mutant do not fragment as easily as those of the wild-type strain, suggesting a deficiency in spore separation (Potuckova et al., 1995; Rezuchova et al., 1997). Within the spores, the chromosome of the *sigF* mutant is not as tightly packed as that of the wild-type strain suggesting that SigF targets will include genes that are involved in spore maturation. Surprisingly, the only SigF target that has been identified to date is the promoter of ORF8 (SCO5321) in the *whiE* biosynthetic cluster for the dark grey spore pigment production (Kelemen et al., 1998; Novakova et al., 2004). While transcription of SCO5321 was dependent on *sigF*, *in vitro* run-off transcription failed to confirm that SigF was directly involved in initiating transcription from this promoter.

Here we identify a novel spore-specific protein, SspA and demonstrate that the single promoter of *sspA* is not only dependent on *sigF*, but its transcription is directed by RNA polymerase holoenzyme containing SigF, which establishes *sspA* as the first direct target identified for the sigma factor, SigF.

**Results**

**2D-PAGE of spore extracts identifies SspA as a potential SigF target**

In order to characterize the role of SigF in spore maturation in *S. coelicolor* we compared protein extracts from spores of wild-type, M145 and *sigF* mutant strains using two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE). Due to the thick spore wall of *Streptomyces* spores, producing protein extracts from spores is not a trivial matter. Sonication, a method that is often reported when presenting proteomics data on spores, only extracted a minority of proteins from the spore wall in our hands and did not release proteins from inside the spores efficiently and reliably. Instead, a mechanical disruption method in which spores were ground in the presence of glass beads under liquid nitrogen, was used to efficiently extract proteins from spores for further analysis. Comparison of protein extracts from spores of the wild-type and *sigF* mutant strains following separation using a narrow pH-range IPG strip of pH 4.5–5.5 and colloidal Coomassie staining revealed a highly abundant protein in the wild-type extract that was clearly absent in the *sigF* mutant (Fig. 1). MALDI-TOF analysis identified this spot as the protein SCO7434, with 9 matched peptides covering 33% of the database protein sequence and producing a probability-based MOWSE score of 94 (expect value 2.2e-05). We therefore designated this protein as SspA (sporulation-specific protein).

**SspA is a lipoprotein with two PepSY domains**

The *sspA* gene (SCO7434) of *S. coelicolor* has been annotated as a gene encoding a putative lipoprotein with a possible N-terminal signal sequence and prokaryotic membrane lipoprotein lipid attachment site. Indeed, SignalP4.1 (Petersen et al., 2011) identified the first 32 amino acids (aa) as a signal sequence. Moreover, the conserved lipobox motif of L_{3-5}[A/S/T]_{1-6}[G/A]_{1-5} [LipoP 1.0; Juncker et al., 2003) is present in SspA (LTAC), predicting a lipoprotein signal peptidase cleavage site between amino acids 28 and 29. This suggests that SspA is secreted through the cell membrane and it is attached to the mem-

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brane, possibly between the lipid bilayer and the peptidoglycan wall. It is consistent with the appearance of SCO7434 in 2D-PAGE gels (Fig. 1) where this protein formed a 'smear' characteristic of lipoproteins, which, during cell lysis, maintain covalent attachment to certain lipids of the cell membrane. In order to gain more information about SspA we performed an iterative BLAST search (Psi-Blast at NCBI; http://www.ncbi.nlm.nih.gov/BLAST.cgi) together with protein domain identification searches using SMART or Pfam (at http://smart.embl-heidelberg.de and at http://www.sanger.ac.uk). According to the SMART and Pfam searches, the 253 aa long SspA contains two PepSY domains between 81 and 167 aa and 188–249 aa. BLAST searches identified homologous domains that are widespread among bacteria, often as the N-terminal pro-peptide domain of the M4 family of peptidases, which contains the thermostable thermolysins and related thermolabile neutral proteases (Yeats et al., 2004). Bioinformatics analysis of the distribution of PepSY domains among bacteria and fungi has indicated three main groups for PepSY containing proteins (Yeats et al., 2004). The first group contains M4 peptidases; members of the second group have normally one to five copies of the PepSY domain and no other domains apart from a signal peptide signature and the third group contains one or two PepSY domains surrounded by transmembrane helices. S. coelicolor possesses eight PepSY domain-containing proteins, none of which has been characterized up to date. Four proteins, SCO1226, SCO2474, SOS446 and SCO5447, belong to group 1, carrying M4 peptidase domains. Two proteins, SspA and SCO5402 belong to group 2 with two PepSY domains. Finally, SCO0863 and SCO0987 are group 3 proteins with additional transmembrane helices.

**The sspA null mutant has irregular spore size**

In order to identify the function of sspA in spores, we generated an sspA null mutant by gene replacement using PCR targetting (Gust et al., 2003). We replaced the sspA gene within the cosmid St6D11 with an apramycin resistance cassette and oriT function using lambda Red recombinase (Datsenko and Wanner, 2000; Gust et al., 2003). The mutant cosmid was then introduced into S. coelicolor M145 using conjugation, and the apramycin-resistant and kanamycin-sensitive colonies were selected and screened for, in order to generate the sspA null mutant, designated K55. The constructed sspA null mutant was confirmed using Southern hybridization (data not shown).

The macroscopic phenotype of the sspA mutant was indistinguishable from the wild-type when grown both as confluent patches and as single colonies on a wide range of media including SFM medium, minimal medium with mannitol as carbon source or R5 medium. We also tested the sensitivity of the sspA mutant to osmotic, heat, cold or oxidative stress. No significant difference was detected when the wild-type or the sspA mutant was grown in the presence of 0.4 M KCl, 0.4 M NaCl or 0.8 M sucrose (osmotic stress), at 37°C (heat shock) or 16°C (cold shock) or using H2O2 disks. Testing the spores of the sspA mutant by treatment with 0.1% (v/v) Triton X-100 established that, unlike the sigF mutant (Potuckova et al., 2004).
1995), spores of the sspA mutant were not sensitive to detergents (data not shown). However, microscopic observation identified subtle, but significant differences between spores of the sspA mutant and the wild-type. The length of 3- to 6-day-old mature spores of the sspA mutant was uneven, generating many spores longer than those of the wild-type, when grown in minimal medium supplemented with mannitol (Fig. 2). In addition, there were differences not only in spore size but also in spore shape between the two strains. While wild-type spores became ovoid following sporulation septation, spores of the sspA mutant were less rounded and rectangular-shaped, suggesting that spore maturation was affected in the sspA mutant (Fig. 2A). Interestingly, the length defects were detectable on several different media, including minimal medium and SFM medium. However, the ‘rectangular’ spore shape of the sspA mutant was most pronounced when the strain was grown in minimal medium (Fig. 2A), but less so in SFM medium (Fig. 2B). Statistical analysis of the length and width of mature spores from 3-day-old cultures grown in SFM medium confirmed that spore length of the sspA mutant varied much more than that of the wild-type (Fig. 2C). The mean length of mature spores was 1.17 μm for the wild-type and 1.18 μm for the sspA mutant with standard deviation (SD) of 0.14 and 0.31 respectively. The spore length varied between 0.69 μm and 2.47 μm for the sspA mutant, compared to the smaller range, between 0.87 μm and 1.75 μm, observed for the
wild-type strain. The higher variance was specific to the length of the sspA spores as the spore-width of both strains was similar with a mean of 0.83 μm, SD = 0.08 (Fig. 2C). Consistent with these measurements, the wild-type spores typically exhibited ovoid shape in SFM medium. In contrast, the wider size range of spore-length of the sspA mutant resulted in a variety of shapes including spherical, ovoid and elongated rods (Fig. 2B and D). Interestingly, the sigF mutant produces smaller, spherical spores with thinner cell wall and less condensed chromosomes than that of the wild-type (Potuckova et al., 1995). However, the spore wall and the condensed chromosomes of the sspA mutant were very similar to those of the wild-type, when assessed using transmission electron microscopy (Fig. 2D). This suggests that apart from the spherical spore shape, the characteristic sigF phenotype is not due to the lack of SspA.

To test whether the differences in spore length of the mature spores were the result of inaccurate septum placement or altered spore maturation, we tested spores at earlier stages, after 48 h growth, when sporulation septa were formed. Septa were stained with Alexa488 conjugate of Wheat Germ Agglutinin (WGA-Alexa488) and propidium iodide staining was used to visualize chromosomal DNA (Fig. 3). Measuring ∼500 pre-spore compartments for each strain, the mean length of pre-spores was slightly shorter, 1.26 μm, for the sspA mutant compared to 1.28 μm for the wild-type strain. While the mode was the same for both strains, the distance between septa of the sspA mutant had a much higher variance (0.08 versus 0.02) and higher standard deviation (0.29 versus 0.16). Pre-spore length varied over a wider range between 0.34 μm and 2.38 μm for the sspA mutant and between 0.81 μm and 1.99 μm for the wild-type strain.
suggesting that lack of SspA affected both early septum placement and later spore maturation. There was no difference in the width of the hyphae at the time of septation (Fig. 3B) with a mean of 0.63 μm and SD = 0.05. The sspA mutant phenotype was fully complemented by the introduction of the sspA gene in trans using the single copy, integrative plasmid pMS82 (Gregory et al., 2003), which confirms that the observed phenotype was due to lack of SspA.

The sspA gene is transcribed from a single, sigF-dependent promoter

SspA was identified from the 2D-PAGE analysis as a protein missing from the sigF mutant. To confirm that expression of sspA was indeed sigF dependent, we monitored sspA transcription using S1 mapping. mRNA was extracted from both wild-type, M145 and sigF mutant strains and samples were taken at various time points during morphogenesis including vegetative growth, aerial growth and sporulation. Using S1 mapping we were able to show that sspA was transcribed from a single, developmentally regulated promoter. The onset of sspA transcription coincides with that of sigF transcription. We also showed that sspA transcription was dependent upon sigF since sspA transcription was absent from the sigF mutant (Fig. 4).

The single transcription start point for sspA was determined using high-resolution S1 mapping (Fig. 4). Interestingly, the transcription start point for sspA is identical to its translational start point. Initiation of translation in bacteria involves binding of the 16S ribosome to the target mRNA, at a sequence of up to 6 ribonucleotides, which precedes the translational start codon. This sequence, called the Shine–Dalgarno sequence, base pairs with a region of the 16S RNA of the small ribosomal subunit. However, leaderless translational initiation, where both transcription and translation are initiated at the same position, is widely represented among bacteria and archaea (Zheng et al., 2011). Leaderless mRNA has been documented for 13 S. coelicolor genes (Zheng et al., 2011), including absA (Anderson et al., 2001), whiH (Ryding et al., 1998) or vanH, vanK and vanR (Hong et al., 2004), just to name a few. It is not clear what the significance of the leaderless transcripts is in bacteria in general but the faithful translation of leaderless mRNAs in heterologous systems suggests that translation of leaderless mRNA is conserved among all kingdom of life (Moll et al., 2002).

SigF directs transcription from the sspA promoter of S. coelicolor in vitro

Beside sspA, the only other sigF-dependent promoter identified is whiEP2, which drives transcription of SCO5321, encoding an aromatic hydro-lase involved in the biosynthesis of the spore-specific, grey pigment of S. coelicolor (Yu and Hopwood, 1995; Kelemen et al., 1998). The sigF-dependent transcription from whiEP2 was proposed to require a transcriptional activator, because RNA polymerase holoenzyme containing SigF alone failed to initiate transcription from whiEP2, in vitro (Kelemen et al., 1998). In order to test whether SigF was sufficient for sspA transcription, an in vitro transcription assay was performed. Previously we have used non-tagged SigF that was over-expressed and purified from E. coli (Kelemen et al., 1998). Here, for easy purification, we overexpressed SigF with an N-terminal His extension in E. coli using the vector pET28a (Novagen). During the purification trials His-SigF was found in the insoluble fraction and was purified using denaturing conditions of the Ni-NTA kit (Qiagen). In B. subtilis ctc transcription is initiated by SigB (Igo and Losick, 1986) and the fact that ctc promoter activity does not require any additional transcription factors has made this promoter a commonly used template when testing the activities of SigB-like sigma factors, such as SigF (Kelemen et al., 1998) or SigH (Viollier et al., 2003) of S. coelicolor. In an in vitro run-off transcription assay, core RNA polymerase from E. coli together with His-SigF was sufficient to produce a specific transcript from a ctc template and also from an sspA template (Fig. 4C). Thus sspA is the first confirmed direct target for the RNA polymerase sigma factor SigF.

SspA is expressed in the spores

The S1 nuclease assays confirmed that sspA transcription temporally coincided with sporulation and SspA was purified from spore extract. To confirm the spatial location of sspA transcription and to monitor SspA production we generated both transcriptional and translational fluorescent protein fusion constructs. The transcriptional fusion was generated using the plasmid pIJ8660 carrying the egfp gene downstream of a DNA fragment containing the sspA promoter, sspAP, but not the translated DNA region. This sspAP–egfp fusion was then introduced into S. coelicolor M145 and the green fluorescence was monitored throughout development. The sspAP-dependent fluorescence was detected in the spore chains, but there was no detectable fluorescence in the vegetative hyphae or the non-sporogenic part of the aerial hyphae, the subapical stem compartment (Fig. 5A).

To establish localization of the produced protein, we introduced an SspA–mCherry fusion into S. coelicolor M145 using plasmid pK39, a derivative of pIJ8668 (Sun et al., 1999). Integration of pK39 into the chromosomal sspA site via homologous recombination replaced the native sspA gene with an sspA–mCherry allele in such a way that no wild-type SspA was produced and the fusion protein was...
Fig. 4. Transcription of \textit{sspA} is \textit{sigF} dependent.

A. S1 nuclease analysis was performed using RNA samples collected at distinct developmental stages of \textit{S. coelicolor} wild-type M145 (left) or \textit{sigF} mutant (right) strains. Three different probes specific to \textit{sspA}, \textit{sigF} and \textit{sigNP2} were used, this latter as a control to assess RNA integrity in the samples. \textit{sp} indicated sporulating samples as was assessed by microscopy.

B. Forty micrograms of RNA from 72-h-old wild-type (M145) samples, grown on SFM medium, were used together with a radioactively labelled probe containing the presumed transcriptional start for \textit{sspA} together with a sequencing ladder to identify the exact transcriptional start point.

C. \textit{In vitro} run-off transcription was performed by RNA polymerase core enzymes (lanes 1 and 3) and RNA polymerase holoenzyme containing His-SigF (lanes 2 and 4) using the templates \textit{ctc} (lanes 1 and 2) or \textit{sspA} (lanes 3 and 4). The 155 nt \textit{ctc}-specific transcript and the 54 nt long \textit{sspA} transcripts are marked with arrows. The sizes of the marker DNA fragments generated using pUC19 digested with Sau3AI (M) are shown in basepairs.

D. Sequence of the probe used for S1 nuclease analysis is shown. The starts of horizontal single arrows mark the 5\' ends of the oligonucleotides used. An asterisk marks the transcription initiation site that coincides with the translational start site. Translated sequences are italicized. The double headed arrows mark the regions protected by cell extracts (below the sequence; see also Fig. 6) or by purified BldD (above the sequence; see also Fig. 7) in DNase I footprinting assays. Grey highlighting indicates the most highly conserved nucleotides of the consensus BldD target sequence (den Hengst \textit{et al}., 2010).

E. Putative promoter sequence of \textit{sspA}. The promoter of \textit{sspA} is compared to the SigF-dependent promoter, \textit{whiEP2} together with the predicted consensus for SigB of \textit{S. coelicolor} (Lee \textit{et al}., 2004) and the established consensus for SigB of \textit{B. subtilis} (Petersohn \textit{et al}., 1999). Asterisks mark the transcription start points. The −10 and −35 promoter sequences are underlined and the BldD box (den Hengst \textit{et al}., 2010) is highlighted in grey.

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expressed from the native sspA promoter. The spore morphology of S. coelicolor M145 carrying pK39 resembled that of the wild-type strain (data not shown), confirming that SspA–mCherry was functional. Monitoring fluorescence from the SspA–mCherry translational fusion confirmed that SspA localized to the spore walls (Fig. 5B). Hence the fluorescence SspA–mCherry signal is ring-like (Fig. 5B) in contrast to the fluorescence from the sspAP–egfp fusion that fills the cytoplasm (Fig. 5A). More interestingly, we could detect SspA–mCherry at an earlier stage, at the developing sporulation septa, which was apparent by the regularly spaced invagination of the hyphal wall (Fig. 5C). To colocalize SspA–mCherry and the developing septa we used WGA-Alexa488 staining (Fig. 4D). Interestingly, at the time when fluorescent WGA-Alexa488 was detectable in sporulation septa,
SspA–mCherry was already found around the spores (Fig. 5D). This suggests that the early, septal SspA–mCherry localization precedes the stage of septation that is detectable by wheat germ agglutinin.

**The promoter of sspA is targeted by BldD**

To test for any potential transcriptional regulators targeting the sspA promoter, we performed electrophoretic mobility shift assays. Cell lysate prepared from the wild-type strain grown for 28 h on SFM medium shifted a radiolabelled DNA fragment that contained the sspA promoter (Fig. 6A), suggesting the presence of a regulator of the sspA promoter. The interaction between the sspA DNA fragment and the putative regulator appeared specific, since the gel shift was detected in the presence of non-specific DNA [poly(dI-dC)]. Moreover, excess cold probe DNA abolished the shift (Fig. 6A). Since the DNA-binding activity was detected in cell extracts made from samples of vegetative mycelium, where sspA is not expressed, it seemed likely that this putative regulator might function as a repressor of sspA transcription.

To determine the binding site of this putative regulator, we carried out DNase I footprinting, using the same sspA DNA fragment used for the S1 mapping and EMSA experiments. The crude protein extract from vegetative mycelium of the wild-type strain weakly protected a region of the sspA promoter (Fig. 6B). A sequence in the protected region resembled the ‘BldD box’ [TnACnnnnnGTnA], the palindromic consensus sequence targeted by the BldD repressor protein (den Hengst et al., 2010), raising the possibility that the sspA promoter was a direct target of BldD. Further, an equivalent cell extract from a bldD null mutant failed to shift the sspA probe in a gel-shift assay (Fig. 6A). Chromatin immunoprecipitation microarray (ChIP-chip) analysis showed that sspA is a direct target of BldD in vivo (Fig. 7A) (den Hengst et al., 2010). To confirm and extend this analysis, the BldD binding site in the sspA promoter was mapped precisely using DNase I footprinting (Fig. 7B). Incubation with purified histidine-tagged BldD protected a ∼30 bp region including the sequences that were protected by the cell extracts from vegetative mycelium of the wild-type strain (Fig. 4D). The overlapping protected region spans the putative BldD box and the −10 promoter site, a location consistent with BldD functioning as a repressor of the sspA promoter (Fig. 4D).

**Discussion**

**SspA controls spore development by affecting septum placement**

The SspA protein has been identified as an abundant protein in wild-type spores and is absent from the spores...
of a sigF null mutant. Bioinformatic analysis identified both an N-terminal signal sequence and a lipoprotein signature suggesting that SspA is exported through the cell membrane and attached to it, presumably from the outside. The rest of the SspA protein sequence comprises of two PepSY domains that are widespread in bacteria and fungi and found often in the propeptide domains of M4 proteases (Yeats et al., 2004). Where examined, the PepSY motif of the propeptide was shown to function as an intramolecular inhibitor preventing premature activation of the protease (Braun et al., 2000; Tang et al., 2003; Gao et al., 2010). While the M4 peptidases are well characterized, no clear biological role was established for those PepSY-family proteins that do not possess additional domains with enzymatic activity. The presence of the PepSY domain in this diverse family of secreted and cell wall-associated proteins suggested a regulatory role for protease activity in the local environment of the cell (Yeats et al., 2004). However, recent studies of PepSY-domain transmembrane proteins suggested that these domains

**Fig. 7.** BldD targets the sspA promoter.

A. BldD ChIP-chip data for the 8 kb region spanning the sspA locus in wild-type S. coelicolor (black circles) and the bldD mutant (grey squares). DNA obtained from immunoprecipitation of BldD was labelled with Cy3 and hybridized to DNA microarrays together with a total DNA control that was labelled with Cy5 (den Hengst et al., 2010). Data generated by den Hengst were retrieved from the Gene Expression Omnibus (GSE23401) and were plotted as Cy3/Cy5 ratios (y-axis), as a function of chromosome location around sspA (x-axis).

B. DNase I footprinting analysis of BldD binding to the promoter region of sspA. 5’ end-labelled probes were incubated in the presence of 0, 0.5, 1.0 or 2.0 μM BldD and subjected to DNase I footprinting analysis as described in den Hengst et al. (2010). Footprints are flanked by Maxam and Gilbert sequence ladders (AG). Protected regions are marked by bars.
can regulate the activity of proteins other than proteases. For example, the PepSY proteins BqsP and BqsQ of Pseudomonas aeruginosa are proposed to regulate the activity of a two-component system that senses extracellular Fe(II) (Kreamer et al., 2012). Similarly, the PepSY protein YycI regulates the YycFG two-component system in B. subtilis (Szurmant et al., 2007). Structural analysis proposed a common structural fold for the PepSY domain, the beta-lactamase inhibitor protein fold (BLIP) and two other protein folds, suggesting that all these protein folds function as inhibitors by binding a partner domain that is located either within the same protein or on a separate protein (Das et al., 2010).

Interestingly, one of the cortex-lytic enzymes in B. subtilis, SleB, is associated with YpeB, a protein with two copies of the PepSY domain (Boland et al., 2000). YpeB has been implicated in the control of a peptidoglycan amidase, SleB, important during spore germination (Atrih and Foster, 2001). YpeB was required for SleB localization (Chirakkal et al., 2002) and it was suggested that YpeB might recruit SleB to the spore cortex. However, recently the inhibition of SleB by YpeB was demonstrated, suggesting that the PepSY protein YpeB controls SleB activity (Li et al., 2013) perhaps by inhibiting the premature activation of the SleB lytic activity in the spores of B. subtilis.

SspA possesses two PepSY domains but lacks any catalytic peptidase domain, so its domain organization is very similar to that of YpeB. However, germination of the sspA mutant spores was not affected (data not shown). Instead, lack of SspA altered septum formation on all media tested and spore maturation when grown on minimal medium. Therefore, we hypothesize that SspA might control the activity of a specific peptidase or peptidoglycan hydrolase involved in either septum formation or spore maturation. Septum positioning depends on the key cell division protein FtsZ that assembles into 50–100 regularly spaced FtsZ rings marking future septum sites (Schwedock et al., 1997). In E. coli or B. subtilis, FtsZ positioning is regulated by FtsZ antagonist proteins. These include the MinCD complex located at cell poles promoting FtsZ ring formation at mid-cell and the chromosome associated SmIA or Noc proteins, which prevent FtsZ ring formation and therefore septum formation over the chromosomes (Adams and Errington, 2009; Lutkenhaus, 2012). In Streptomyces no apparent homologues of either the Min system or nucleoid occlusion proteins have thus far been identified. Instead, formation of the FtsZ rings is under a positive control by SsgB in Streptomyces (Willems et al., 2011; Jakimowicz and van Wezel, 2012) while FtsZ protein levels are negatively affected by CrgA (Del Sol et al., 2006, Del Sol et al., 2003). Following on from the formation of the FtsZ rings, very little is known about the recruitment of specific enzymes for septum synthesis in Streptomyces. Interestingly, the FtsW–FtsI protein pair, which is established in septum formation in E. coli, was proposed for FtsZ ring stabilization in Streptomyces (Mistry et al., 2008; Bennett et al., 2009; McCormick, 2009). In addition, the SsgA-like proteins (SALPs) have been implicated in both septum positioning (SsgA and B) and in septum formation (SsgC-G) (Noens et al., 2005; Willems et al., 2011; Jakimowicz and van Wezel, 2012).

The late sigma factor, SigF is associated with the control of spore maturation, which is a post-septation event. However, the sigF null mutant produces smaller spores than the wild-type (Potuckova et al., 1995). This could be the result of incorrect septum positioning and possibly altered FtsZ placement suggesting that SigF is active when septa are formed. Similarly, the uneven septum positioning in the sspA mutant could arise from altered FtsZ positioning. Alternatively, SspA might influence the recruitment of cell wall lytic or synthetic enzymes after FtsZ ring formation. Interestingly, monitoring SspA localization using an SspA–mCherry fusion confirmed that in addition to its presence in mature spores, SspA accumulated at sporulation septa (Fig. 5B). This suggests that SspA functions as early as septum formation. It will be important to establish the potential link between SspA and the Fts proteins (FtsZ, FtsW and FtsI), CrgA and the SALPs.

After the completion of septation, spore wall synthesis is governed by the cytoskeletal proteins MreB and Mbl (Mazza et al., 2006; Heichlinger et al., 2011). MreB polymers first assemble at sporulation septa, followed by spreading to the entire spore wall (Mazza et al., 2006). This pattern is reminiscent of SspA localization, raising the possibility that SspA positioning might be MreB-dependent. On the other hand, lack of SspA affects septation while MreB only controls post-septational events (Mazza et al., 2006) suggesting that SspA might function prior to MreB assembly during spore development. A recent search for MreB partner proteins established a complex interaction pattern among members of the proposed ‘Streptomyces spore wall synthesizing complex’, SSSC (Kleinschnitz et al., 2011). A knockout mutant of SCO2097, a putative membrane protein identified among the SSSC proteins, produced elongated spores with sensitivity to heat and cell wall-damaging agents (Kleinschnitz et al., 2011). Interestingly, these elongated spores resemble those of the sspA mutant; however, the latter did not exhibit sensitivity to lysozyme or heat (data not shown). In addition, spore wall hydrolytic enzymes have also been shown to affect spore shape (Haiser et al., 2009). It will be of interest to test whether any of the penicillin-binding proteins of the SSSC, including SCO3901, SCO3580 and FtsI (Kleinschnitz et al., 2011) or any of the hydrolytic enzymes, RpfA, SwlA, SwlB and SwIC (Haiser et al., 2009) are targets of or partnered by SspA.
Transcription of sspA is under the control of the sigma factor, SigF and the principal developmental regulator, BldD

Both the production of SspA protein and transcription of sspA are dependent on sigf in vivo, in S. coelicolor (Figs 1 and 4). Moreover, in vitro transcription assays (Fig. 4) confirmed that SigF is sufficient to initiate transcription from the sspA promoter. Hence, in this report we have presented the first example of a SigF target promoter, sspAP where transcription is initiated by RNA polymerase holoenzyme containing the sigma factor, SigF in the absence of any activator. SigF belongs to a group of nine RNA polymerase sigma factors (SigB, F, G, H, I, K, L, M and N) that control response to environmental stresses (SigB, H, I, L and M) or morphological differentiation (SigH, F and N) or in some cases both (SigH and SigB) in S. coelicolor (Potuckova et al., 1995; Cho et al., 2001; Kelemen et al., 2001; Sevcikova et al., 2001; Viollier et al., 2003; Lee et al., 2005). Members of this, so called, SigB-family of S. coelicolor resemble the general stress response sigma factor SigB of B. subtilis (see review, Price, 2000). Predictably, the putative promoter sequence of sspA (GTGT-16N-GGTTAC) resembles the consensus target sequence of SigB both in S. coelicolor and in B. subtilis (Fig. 4E; Petersohn et al., 1999; Price, 2000; Lee et al., 2004). Interestingly, the weak similarity between the two sigF-dependent promoters, sspA and whiEP2, together with the unusually long spacer between the −10 and −35 sequences of whiEP2 might explain why SigF was not sufficient to initiate transcription from whiEP2 in vitro (Kelemen et al., 1998). Identification of further SigF target promoters is paramount in order to establish a consensus SigF target sequence and to address the fundamental question of how members of the SigB-family with potentially overlapping promoter specificity can control distinct sets of genes in vivo. One such mechanism could include specific activators, as it was proposed for the promoters whiEP2 and nepA, which are putative targets for SigF and SigN respectively (Kelemen et al., 1998; Dalton et al., 2007).

Alternatively, transcriptional repressors could restrict expression of target genes both in time and in space, allowing limited access to promoter sites by cognate sigma factors. Interestingly, gel shift assays together with DNase I footprinting demonstrated a DNA binding activity from wild-type cell extracts collected at early stages of development, when sspA was not expressed, suggesting a putative repressor targeting sspA transcription. Both in vivo and in vitro experiments confirmed that this repressor is BldD, a key developmental regulator of Streptomyces morphogenesis. bldD was among the first developmental genes identified in the study of morphological differentiation in Streptomyces (Merrick, 1976). The bldD mutant fails to progress to aerial development and is also blocked in the production of several secondary metabolites (Elliot et al., 1998). BldD, a small DNA-binding protein, has been shown to target, and mainly repress, the transcription of developmental genes, such as the sigma factor genes, whiG, bldN and sigH, during early development while co-ordinating the timing and, in some cases, the location of their expression at later stages of differentiation (Elliot et al., 2001; Kelemen et al., 2001). Recently, the genome-wide BldD regulon has been extensively defined by chromatin immunoprecipitation-microarray analysis identifying ~167 transcription units targeted by BldD (den Hengst et al., 2010). The location of the BldD binding site at the sspA promoter is consistent with BldD functioning as a repressor of sspA, perhaps by blocking access of other SigB-like sigma factors to the sspA promoter during early developmental stages. Surprisingly, microarray analysis showed that sspA transcription was not upregulated but absent in the bldD null mutant (den Hengst et al., 2010). This suggests that either the sspA promoter is recognized exclusively by SigF, which is naturally absent from the bldD mutant, or, more likely, no other SigB-like sigma factors capable of initiating sspA transcription were active under the conditions of the microarray analysis. Interestingly, one of the BldD targets identified in this analysis is the gene encoding SCO4677 (den Hengst et al., 2010), which has been demonstrated to bind SigF, potentially functioning as an anti-sigma factor (Kim et al., 2008). Thus BldD is linked to the sigF regulon via sspA, and perhaps also via SCO4677. Moreover, BldD has also been shown to target cell division genes (den Hengst et al., 2010), such as ftsZ, ssgA and ssgB or the smeA-ssfA operon encoding a DNA translocase required for correct chromosome segregation during sporulation (Ausmees et al., 2007). Hence, BldD appears to co-ordinate the expression of proteins that are required at the time of septum formation, including the expression of SspA.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli DH5α (Hanahan, 1983) was used for routine cloning. E. coli BW25113 carrying pJ790 (Datsenko and Wanner, 2000) was the host for recombination between the extended apramycin resistance cassette and the target gene to generate knockout mutants. E. coli ET12567 (MacNeil et al., 1992) containing pUZ8002 (Kieser et al., 2000) aided the transfer of plasmids or cosmids from E. coli into S. coelicolor by conjugation. The strains of S. coelicolor used in this work are listed in Table 1. Streptomyces strains were grown at 30°C on SFM (soya flour medium containing 1% mannitol), MM (minimal medium) supplemented with mannitol (0.5%) solid media. pJ82 is a hygromycin-resistant derivative of pSET152 (Bierman et al., 1992) in which the apramycin
resistance gene is replaced with the hygromycin resistance gene (Dalton et al., 2007). All plasmids and oligonucleotides used in this work are shown in Tables 2 and 3 respectively.

### Table 1. Bacterial strains used in this work.

| Strain     | Genotype or description                  | Reference or source      |
|------------|-----------------------------------------|-------------------------|
| S. coelicolor M145 | SCP1− SCP2− ΔsspA::apr derivative of M145 | Kieser et al. (2000)   |
|            | ΔsigF: thi derivative of M145            | Kelemen et al. (1998)   |
|            | ΔbldD: apr derivative of M600            | Elliot et al. (2003b)   |
| E. coli    | DH5α                                    | Invitrogen              |
| ET12567/pUZ8002 | Cloning                               | MacNeil et al. (1992), Kieser et al. (2000) |
| BL21 (DE3)pLysS | Protein overproduction                  | Novagen                 |
| BW25113/plJ790 | Knockout generation                     | Datsenko and Wanner (2000) |

### Table 2. Plasmids used in this work.

| Plasmid | Genotype or description                  | Reference or source      |
|---------|-----------------------------------------|-------------------------|
| pET28a  | Vector for protein overproduction       | Novagen                 |
| pJ8660  | This plasmid integrates as a single copy at the ΦC31 attB attachment site on the chromosome of S. coelicolor | Sun et al. (1999) |
| pJ8668  | This plasmid cannot replicate autonomously in S. coelicolor but it can integrate into the S. coelicolor chromosome via homologous recombination through the appropriate inserted sequences. | Sun et al. (1999) |
| pJ82    | This plasmid integrates as a single copy at the at the ΦC31 attB attachment site on the chromosome of S. coelicolor | Dalton et al. (2007) |
| pAT1    | pJ82 carrying the 1.3 kb sspA fragment   | This study               |
| pAT6    | pUC18 carrying a 7.7 kb BamHI fragment of sspA | This study               |
|.pk37    | pJ8660 carrying the sspA–egfp transcriptional fusion| This study               |
| pk39    | pJ8668 derivative for the expression of the SspA–mCherry translational fusion when integrated into the S. coelicolor chromosome | This study               |
| pET28-SigF | pET28a derivative for the overexpression of His-SigF from E. coli | This study               |

### Table 3. Oligonucleotide primers used in this work.

| Primer      | Sequence                  |
|-------------|---------------------------|
| sspA-Bam    | CTGAAGGATCCCGGTCCCGC GCCCA ACGGGGACG |
| sspA-Bam2   | CTGAAGGATCCCGCAGATCATGAGC TCTGACGAGCAGC |
| sspA-Nde    | GATACCATATGCTTGTGACCTTCTGCTGCC |
| sspA-Kpn    | GCAGAGGTATCCGATGCTGTCGTAACCGCTC |
| 6D11.30.1   | GGCCCGTACCGCCCGCCGC CGG |
| 6D11.30.2   | GAGGACGCGGTACTGGAGG |
| 6D11.301    | GTGGTGCTCGTGTAGCAGAGCGGGCTGATCCCCGCACGG |
| 6D11.302    | GGCCCGTACCGCCCGCCGCAGGAGGGGTTGACCTGACGAGCAGTGGCTTG |
| SIGN4       | CTGCTGACCGACCTGCTG |
| SIGN7       | GCAGACGACCCCATCCCC |
| SIGF15      | GAGCGAGGCGGCCACGG |
| SIGF16      | CGGGCGGGCGGTACCGGAGG |
| DIR         | CGCCACGGGGTTTCCCACTGACG |
| EGFPLINKER1 | GATCTTCTACATAGGCGCCGGCCGCG |
| EGFPLINKER2 | TACCAGCCCGCGC CCCCATATGGCATAG |
| 7434_F1     | CTGAGGCTCCGGTCG |
| 7434_R1     | GTGACGCGGTACGACG |

Preparation of protein extracts from spores

Spores were harvested from cultures grown on SFM medium according to Kieser et al. (2000) and were washed with 40 mM Tris pH 9.0; 1 mM EDTA; 1 mM EGTA buffer. Washed spores were transferred to a pestle and mortar submerged in liquid nitrogen (typically a 0.5 ml volume of spore pellet was used), and disrupted by thoroughly grinding in the presence of an equal volume of fine glass beads (106 microns; Sigma G8893). The frozen, ground spore powder was then transferred on ice into the above buffer containing 100 mM DTT and 4 mM Pefabloc SC protease inhibitor and protector solution (Roche). On warming to approximately 4°C, SDS was added to the final concentration of 2% (w/v) and the protein concentration of the sample was determined.
added to a final concentration of 2%, and proteins extracted by boiling for 10 min. Cell debris and glass beads were removed by centrifugation, and the protein extract was precipitated using the Amersham 2D Clean-Up kit (80-6484-51) according to the manufacturer’s instructions. This step was vital for removal of SDS which interferes with subsequent separation by isoelectric focusing. The precipitated protein pellet was finally redissolved in denaturing UTCHAPS isoelectric focusing buffer [7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris, pH 9.0, 1 mM EDTA, 50 mM DTT, 4 mM Pefabloc SC protease inhibitor (Roche)]. Extracts were stored at −80°C until use.

2D gel-electrophoresis and protein identification

Protein extracts were separated by 2D gel-electrophoresis as previously described (Hesketh et al., 2002). Briefly, proteins were separated in the first dimension for 120 000 volt-hours using 18 cm IEF strips pH 4.5–5.5 (Amersham Biosciences) using a Phorise isoelectric focusing unit (Genomic Solutions). Focused IEF strips were separated in the second dimension using in-house fabricated 12.5% SDS-PAGE gels and the Investigator 5000 vertical format system from Genomic Solutions. Protein spots of interest were excised manually from colloidal Coomassie-stained gels, and identified by tryptic digestion and MALDI-TOF mass spectrometry as previously described (Hesketh et al., 2002).

Generation of the sspA knockout mutant

The sspA knockout mutant was generated using PCR targeting (Gust et al., 2003) by replacing the entire sspA gene, excluding the translational start and stop codon, with the apramycin resistance cassette. The apramycin resistance gene and oriT was PCR-amplified using the primers 6D11.301 and 6D11.302 and was introduced into cosmids Scl6D11 in BW25113 (Datseiko and Wanner, 2000) carrying pUJ790 to replace sspA with the apramycin resistance cassette. The cosmid carrying the mutant allele was passaged through the dcm-dam–ET152567 strain (MacNeil et al., 1992) containing pUZ8002 and was introduced into S. coelicolor M145 by conjugation. The double cross-over exconjugants were screened for resistance to apramycin and sensitivity to kanamycin. One of the sspA mutants, which was confirmed by Southern blot hybridization was designated K55.

Complementation of the sspA mutation

A ~ 7.7 kb BamHI DNA fragment containing the entire sspA gene was first moved from cosmid 6D11 into pUC18 and from this subclone, pAT77, a ~ 1.3 kb BamHI–MluI fragment carrying exclusively the sspA gene was introduced, via several steps, into pJL2925 (Janssen and Bibb, 1993) to generate pAT0. The sspA fragment was liberated from pAT0 using BglII, and introduced into the BamHI site of pJL82 (Dalton et al., 2007), to create pAT1. pAT1 was introduced into S. coelicolor M145 and K55 by conjugation and hygromycin-resistant exconjugants, carrying pAT1 integrated to the FCS1 attachment site on the chromosome, were examined for their phenotype.

S1 nuclease mapping

Mycelium grown on solid medium covered with cellophane discs was collected, and initial extracts were generated by grinding in liquid nitrogen. From these extracts RNA was prepared and S1 nuclease protection assays were performed according to Kieser et al. (2000) using 40 µg RNA in each assay. Probes labelled with 32P at their single 5′ end were generated using PCR with two oligonucleotides, one labelled at its 5′ end with [γ-32P]-ATP using T4 polynucleotide kinase. Probes were generated using the following oligonucleotide pairs: sspA probes using 6D11.30.1 and 6D11.30.2; sigF probes using SIGF15 and SIGF16*; sigN probes using SIGN4 and SIGN7* (asterisks mark the labelled oligonucleotides). DNA fragments protected by RNA were separated on 6% sequencing gels. The transcriptional start point of sspA was identified against a dideoxy-sequencing ladder (Amersham Pharmacia biotech; T7 sequencing kit) produced using the same labelled oligonucleotide used to generate the probes for S1 analysis.

Generation of transcriptional and translational fusions

Two appropriate primers, sspA-Bam and sspA-Kpn, were used to generate a PCR product carrying a 100 bp sequence upstream of the sspA transcriptional start. This PCR product was introduced into pJL8660 (Sun et al., 1999) as a BamHI–KpnI fragment generating pK37, which was introduced into S. coelicolor M145 by conjugation. One of the representative apramycin-resistant exconjugants was used to monitor sspA transcription. S. coelicolor M145 carrying pK37 was propagated on medium containing apramycin to maintain selection for the presence of plasmid integrated in a single copy into the FCS1 attB site in the S. coelicolor chromosome.

To monitor the localization of SspA–mCherry we constructed pK39. First a flexible linker encoding Gly4MetAla was inserted upstream of the egfp in pJL8668 (Sun et al., 1999) by cloning the annealed Egfp linker1 and Egfp linker2 primers into the BamHI–Ndel sites of pJL8668. Then the egfp fragment was replaced by an mCherry fragment using Ndel and BsrGI restriction sites. The sspA coding sequences were generated by PCR using the primers sspA-Bam and sspA-Nde, which create BamHI and Ndel sites at the respective ends. The BamHI–Ndel sspA fragment was introduced to the linkeder pJL8668 derivative. This generated a translational sspA–mCherry fusion, which, when introduced to wild-type S. coelicolor M145, integrates via homologous recombination at the sspA site in the chromosome.

Fluorescent protein fusions were monitored using a Leica TCS SP2 laser-scanning confocal microscope with a 63×, 1.4NA oil-immersion objective.

In vitro run-off transcription using His-SigF

In order to produce His-SigF the sigF gene was introduced into pET28a (Novagen) as an Ndel–EcoRI fragment from pJL894 (Kelemen et al., 1998) that was generated previously to overexpress non-tagged SigF. The resulting clone, pK34, was moved into E. coli BL21 (DE3)pLysS (Novagen).
Cultures of BL21(DE3)pLysS/pK34 were grown at 37°C to an OD_{600} 0.4 and, after induction with 1 mM IPTG, for a further 4 h. After harvesting the mycelium, His-SigF was purified under denaturing conditions (8 M urea) using the Ni-NTA Spin Kit (Qiagen) according to the manufacturer’s instructions. Fractions eluted from the Ni-NTA column with a buffer of low pH (4.5) were dialysed against 50 mM NaH_{2}PO_{4} and 50 mM NaCl, pH 8.0 buffer at 4°C. The His-SigF protein was analysed on an 8% SDS-PAGE and stored at −20°C in the presence of 20% glycerol.

In vitro run-off transcription was performed as described by Buttner et al. (1987) using 0.5 μg His-SigF, core RNA polymerase from E. coli (Cambio), [α-32P]-CTP (3000 Ci mmol−1; Amersham Biosciences) and an appropriate DNA template. The 340 bp EcoRI–BamHI fragment of pMI340 (Igo and Losick, 1986) was used to test transcription from the B. subtilis ctc promoter and was expected to produce a 155-nucleotide (nt) ctc transcript. The template carrying the sspa promoter was generated by PCR using the 6D11.30.1, DIR oligonucleotide pair and pTA6 as template. pTA6 carries a 7.7 kb BamHI fragment from cosmid St6D11 in the vector pUC18 and the DIR oligonucleotide anneals to pUC18 sequences. The 312 bp PCR product carried 229 bp Streptomyces sequence that was expected to generate an 54 nt sspa transcript in vitro.

Electrophoretic mobility shift assays and DNase I footprinting

The labelled sspa probe from the S1 nuclease assays were used together with either cell extracts from S. coelicolor strains or BldD protein overexpressed and purified from E. coli (den Hengst et al., 2010). S. coelicolor strains were grown on the surface of cellophane discs positioned onto SFM medium for 24 h producing a lawn of vegetative mycelium. The collected cells were lysed by sonication and the cell debris was removed by centrifugation at 15000 g for 20 min. Samples of the supernatant were incubated with the sspa probe at 30°C for 20 min in the presence of 1 μg Poly(dI-dC)•Poly(dI-dC) (SIGMA) in 25 mM HEPES pH 7.5, 4 mM DTT, 1 mM ATP, 10 mM Mg-acetate, 4% glycerol buffer and the protein:DNA complexes were analysed on a 4% acrylamide gel, followed by autoradiography. For DNase I footprinting using S. coelicolor M145 cell extracts, the binding conditions were identical to that of the gel mobility shift assays, and the DNase I digestion was performed as described previously (Kelemen et al., 2001). The dideoxy-sequencing ladder (Amersham Pharmacia biotech; T7 sequencingTM kit) was produced using the same labelled oligonucleotide used to generate the sspa probe. DNase I footprinting in the presence of BldD purified from E. coli was carried out using single end-labelled sspa probes generated by PCR with the primers 7434_F1 and 7434_R1, as described in den Hengst et al. (2010).

Microscopy

For fluorescence microscopy, spores were inoculated alongside microscope coverslips inserted at 45° angles into SFM medium and incubated at 30°C for 48–96 h. Samples were stained with WGA-Alexa488 (Molecular Probes, 50 μg ml−1) and/or propidium iodide (Sigma, 25 μg ml−1) as described previously (Holmes et al., 2013) and were viewed using Leica TCS SP2 laser-scanning confocal microscope with a 63×, 1.4 NA oil-immersion objective. Scanning electron microscopy and transmission electron microscopy were performed as described previously (Holmes et al., 2013).

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